

Electrocatalytic evaluation of DNA damage by superoxide radical for antioxidant capacity assessment

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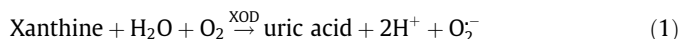
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The integrity of DNA purine bases was herein used to evaluate the antioxidant capacity. Unlike other DNA-based antioxidant sensors reported so far, the damaging agent chosen was the $O_2^{\cdot -}$ radical enzymatically generated by the xanthine/xanthine oxidase system. An adenine-rich oligonucleotide was adsorbed on carbon paste electrodes and subjected to radical damage in the presence/absence of several antioxidant compounds. As a result, partial damage on DNA was observed. A minor product of the radical oxidation was identified by cyclic voltammetry as a diimine adenine derivative also formed during the electrochemical oxidation of adenine/guanine bases. The protective efficiency of several antioxidant compounds was evaluated after electrochemical oxidation of the remaining unoxidized adenine bases, by measuring the electrocatalytic current of NADH mediated by the adsorbed catalyst species generated. A comparison between $O_2^{\cdot -}$ and OH^{\cdot} radicals as a source of DNA lesions and the scavenging efficiency of various antioxidant compounds against both of them is discussed. Finally, the antioxidant capacity of beverages was evaluated and compared with the results obtained with an optical method.

1. Introduction

Deleterious oxidative processes mediated by free radicals, such as ROS, are involved in aging and in a vast array of diseases, including cancer, inflammation, cardiovascular and neurodegenerative diseases [1]. Therefore, overproduction of ROS can be dangerous for cells [2]. The superoxide anion radical ($O_2^{\cdot -}$) is the primary component of ROS and the most abundant radical in biological systems, resulting from the single-electron reduction of oxygen [3]. This cytotoxic species is enzymatically produced by xanthine oxidase (XOD), a metalloenzyme that catalyzes the oxidation of hypoxanthine and xanthine to uric acid generating $O_2^{\cdot -}$ during the respiratory burst of phagocytic cells (Eq. (1)) [1]. Under normal physiological conditions, the highly reactive superoxide radical

undergoes dismutation by non-catalytic and enzymatic reactions, thus the physiological concentration is rather low [4].



The biological effects of highly reactive ROS are controlled *in vivo* by a variety of non-enzymatic and enzymatic antioxidant mechanisms. Superoxide radical is easily attacked by other active biomolecules and scavenged by enzymes and antioxidants [5]. The major scavenger of this radical *in vivo* is the superoxide dismutase enzyme (SOD) that catalyzes its disproportionation to H_2O_2 . Subsequently, catalase detoxifies H_2O_2 , and glutathione peroxidase detoxifies H_2O_2 and converts lipid hydroperoxides into non-toxic alcohols [1]. An additional protection can be provided by exogenous antioxidant compounds, such as low molecular weight molecules, vitamin (A, E, C, β -carotene), and minerals (Se, Zn). This exogenous protective effect can be achieved by the intake of food-stuff and beverages, like vegetables, fruit, whole-grain, tea, juice and wine.

Photometric, chemiluminescent, fluorimetric, chromatographic and electrochemical methods have been proposed for *in vitro* quantification of the antioxidant capacity (AOC) in biological and food samples [6]. Electrochemical biosensors use two main sources of ROS: OH^{\cdot} and $O_2^{\cdot -}$. The former can be generated photocatalytically

[7] or by Fenton reaction in DNA-based antioxidant sensors [8,9], and the latter is mostly enzymatically [2,10,11] but also chemically [3,12,13] or electrochemically [14] formed for the determination of both superoxide radical and AOC. Sensors based on O_2^- commonly rely on the immobilization of cytochrome *c*, which is reduced by superoxide radical, on gold [2–4], carbon [15] or screen printed-Au-electrode [16] surfaces, where it is reoxidized. To enhance the electrical contact between cytochrome *c* and the electrode and to increase the surface coverage of this compound, several immobilization strategies have been proposed mostly based on SAMs of thiols of different length [2–4,15] and hemin modified electrodes [17]. However, these sensors present the interference of H_2O_2 , uric acid and also some electrical communication problems between the protein and the electrode.

Another strategy is the immobilization of SOD by physical adsorption or through SAM [18–20] on the electrode surface in order to follow the disproportionation of superoxide radical by measuring the O_2 and H_2O_2 formed. These biosensors presented interferences derived from the high potential at which the generated H_2O_2 is detected, limiting the practical application of the sensor.

Nonetheless, the protective effect of antioxidants at a cellular level could only be achieved by monitoring the DNA integrity. To the best of our knowledge, all electrochemical DNA-based antioxidant sensors developed so far used the hydroxyl radical as a damaging agent, which caused strand scission or oxidative lesions in nucleobases (guanine or adenine). Superoxide radical has not been used for this purpose probably because the mechanism of O_2^- damage on DNA is not completely understood. It is believed that its participation is limited to promote the production of OH^\bullet radicals [21–23]. However, it is important to develop assays to study other radical sources active in cells and tissues and the way antioxidants eliminate it preventing its deleterious effect. Antioxidants can react by different mechanisms depending on the free radical/oxidant source or by multiple pathways against a single oxidant [24]. This observation implies that there is no a universal assay for the detection of all antioxidants. To obtain a full profile of antioxidant capacity against various ROS, the development of methods specific for each ROS is needed.

In this work, the effectiveness of superoxide radical generated by the enzymatic reaction between XOD and xanthine to induce damage on a DNA-based sensor is studied. Based on previous work on electrochemical oxidation of adenine and guanine derivatives [25–28], a minor product of the radical oxidation was identified. The oxidative lesions were indirectly quantified after electrochemical oxidation of the remaining intact adenine bases to generate a well-known catalyst species that mediates the oxidation of NADH. CV was used to measure the electrocatalytic current after the subsequent immersion of the damaged DNA-modified CPE in a NADH- Ca^{2+} containing solution. A dependence of the electrocatalytic current on the concentration of antioxidant in the damaging solution was found, which allowed the development of a voltammetric method for the determination of AOC in flavored waters.

2. Material and methods

2.1. Chemicals

Deoxyadenylic acid oligonucleotide (dA_{21}) purchased as a desalted product, xanthine oxidase (XOD) and xanthine were from Sigma-Aldrich (Madrid, Spain). Concentrated saline sodium phosphate EDTA ($20 \times$ SSPE; 0.2 M sodium phosphate, 2 M NaCl, 0.02 M EDTA), tris-HCl pH 9.0, phosphate buffer pH 9.0, gallic acid (GA), resveratrol (RES), nicotinamide adenine dinucleotide disodium salt, reduced form (NADH), were also acquired from

Sigma-Aldrich. $\iota(+)$ -ascorbic acid (AA) was from Riedel-de-Haën (Germany). Caffeic acid (CA), and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble derivative of vitamin E) were from Fluka (Madrid, Spain). Other chemicals employed were of analytical grade.

Stock solutions of 1 g L^{-1} dA_{21} were stored at 4°C and diluted with $2 \times$ SSPE buffer solution (prepared by dilution of $20 \times$ SSPE solution) prior to use. All solutions were prepared with water purified with a Direct-Q (Millipore) system.

2.2. Instrumentation

Cyclic voltammetry was performed with a μ Autolab II controlled by GPES software, version 4.8 (EcoChemie, The Netherlands). A conventional three electrode cell was used, which includes a home-made CPE (3 mm in diameter) as a working electrode, a platinum wire counter electrode and a $\text{Ag}|\text{AgCl}||\text{KCl}_{\text{sat}}$ reference electrode to which all potentials are referred. The CPE was prepared by mixing 1.8 g of paraffin oil as pasting liquid with 5 g of spectroscopic grade graphite powder (Ultracarbon, Dicoex, Spain). The unmodified carbon paste was introduced into the well of a Teflon electrode body provided by a stainless steel piston. The surface was smoothed against a plain white paper while a slight manual pressure was applied to the piston. Unless otherwise stated, after each experiment, the CP was discarded and a new electrode surface was freshly prepared.

For temperature-controlled experiments a circulating thermostat HAAKE DC1 (Thermo Electron GmbH, Germany) was used.

2.3. Assay procedure

Unless otherwise mentioned, experiments were structured in four steps: DNA layer preparation, damage of oligonucleotide by immersion of the DNA-CPE on a XOD/xanthine solution in the absence/presence of several antioxidants; electro-oxidation of the remaining unoxidized adenines on the CPE, and detection in a Ca^{2+} -containing NADH solution.

DNA immobilization was performed by dry adsorption placing a 5- μL droplet of dA_{21} (180 mg L^{-1}) in $2 \times$ SSPE solution on the electrode surface and evaporating it to dryness under a stream of warm air.

DNA damage was carried out by immersing the dA_{21} -CPE in a freshly prepared XOD/xanthine mixture (superoxide radical generating solution) in the absence or the presence of antioxidant under controlled temperature ($27.0 \pm 0.1^\circ\text{C}$). The superoxide radical was generated by the addition of XOD (0.1 U mL^{-1}) to oxygen-saturated $2 \times$ SSPE solutions at pH 7.4 containing xanthine ($4.4 \times 10^{-5} \text{ M}$).

After a fixed reaction time, the DNA-CPE was washed with water and immediately immersed in a 0.1 M phosphate buffer (pH 9.0) to carry out the electro-oxidation of the remaining unoxidized adenine bases. 100 potential scans were performed between -0.2 and $+1.4 \text{ V}$ at 500 mV s^{-1} to ensure a complete oxidation [29].

For detection, the DNA-CPE was placed in a NADH solution ($5.0 \times 10^{-4} \text{ M}$ in 0.1 M tris-HCl pH 9.0) containing 0.01 M CaCl_2 . The electrocatalytic current of NADH was obtained by CV sweeping the potential between -0.2 V and 0.5 V at 50 mV s^{-1} .

2.4. Samples and description of alternative methods

Two lemon sparkling flavored water samples corresponding to two different brands were purchased in a supermarket and stored in the dark at $+4^\circ\text{C}$. Sonication was used to eliminate gas from the sample. Label information from brand A indicates the presence of vitamin C, some preservatives, such as sodium benzoate, potassium sorbate and the acidifying regulator citric acid. Label from brand B sample indicates the presence of green tea and citric acid.

A lemon flavor used in the formulation of some water brands was also analyzed. This flavor had no description about its chemical or aroma composition.

For the measurement of AOC in beverages, 200 μL of the flavored water or 10 μL of flavor were diluted in $2\times$ SSPE to a final volume of 500 μL . Then, the DNA-CPE was immersed in the solution and a freshly prepared superoxide radical was added for 10 min. After this period of time the DNA-CPE was washed and immersed in a phosphate buffer to carry out the electro-oxidation of the remaining unoxidized adenine bases. The detection was carried out in a Ca^{2+} -containing NADH solution.

A colorimetric assay, based on a procedure previously reported [30], was used to elucidate the antioxidant profile of the samples, expressed as the total phenolic content (TPC). Folin–Ciocalteu reagent was used, and the reduced phenols produced a stable blue product at 760 nm. The results were expressed as mg of GA L^{-1} .

3. Results and discussion

Oxygen and its reactive species are very important in oxidative metabolism. ROS induce oxidative damage producing a variety of modifications at DNA level including base and sugar lesions, strand breaks, DNA–protein cross-linking and base-free sites [31]. In order to verify that O_2^- generated by a xanthine/XOD reaction is able to oxidize dA_{21} on the electrode surface, the DNA-CPE was placed in a freshly prepared xanthine/XOD solution in $2\times$ SSPE buffer (pH 7.4) for 15 min. After transferring to a phosphate buffer solution (pH 9), a small quasi-reversible redox process was observed at low potentials, $E^{\circ} = 0.041\text{ V}$ (Fig. 1a). The amount of compound generated (surface coverage, Γ) was estimated to be $1.2 \times 10^{-11}\text{ mol cm}^{-2}$ from the integrated charge under the anodic wave. An extended voltammetric scan up to 1.4 V did not show any oxidation peak at 1.2 V (oxidation potential of adenine bases in DNA) but a gradual increase in the magnitude of the redox process at low potentials was observed after several potential scans (Fig. 1b).

Since the only oxidizable species was the oligonucleotide adsorbed on CPE, this behavior is in good agreement with a partial oxidation of adenine bases by the superoxide radical. Therefore, the intact adenines can be further electrochemically oxidized at the electrode surface at 1.2 V leading to the product responsible for the redox couple at low potentials. It is worth mentioning that the oxidation current of the remaining adenines was not observed because it was overlapped by the rising background current at the high potential at which it takes place. Additionally, the E° of redox processes originated from radical attack (Fig. 1a) and electrochemically (Fig. 1b) were virtually identical suggesting that the same compound is formed in both types of oxidation. According to previous studies on the electro-oxidation of adenine derivatives on carbon electrodes in phosphate buffer [28,32,33], the compound

responsible for redox process at +0.041 V is a diimine species strongly adsorbed on the electrode surface. This compound was also identified after oxidation of guanine derivatives [25–27]. Therefore, it can be concluded the existence of a common lesion on DNA generated by O_2^- generated by the xanthine/XOD system and by electrochemical oxidation. However, from Fig. 1 it is apparent that the adsorbed diimine species was a minor product of the radical oxidation and the yield was much lower than in the electrochemical oxidation. This result indicated that the product profile and compound distribution differed, thus, both oxidations are somehow different.

The fact that the O_2^- attack on DNA led to the generation of this adsorbed compound is remarkable because the oxidation of adenine bases through OH^\bullet radicals generated by Fenton-type reaction was recently demonstrated not to occur via the formation of the diimine species, at least, at levels detectable by CV [29]. Given that both radical attacks led to different products, the reported primary OH^\bullet radical promoter role of O_2^- remains uncertain.

The adsorbed species was shown to efficiently catalyze the oxidation of NADH reducing the overpotential by more than 300 mV at pyrolytic graphite electrodes [27,28,32]. This ability can be exploited, in principle, to detect the DNA damage. However, the low yield achieved by radical oxidation did not allow observing an electrocatalytic current sufficiently high to be used as an analytical signal. In fact, no significant current was observed at potentials close to the redox process when NADH was added to the solution after DNA damage by superoxide radicals (data not shown). To solve this problem an indirect method was tested. The unoxidized adenine bases were electrochemically oxidized to generate a larger amount of diimine (catalyst) species. Therefore, the higher the damage, the lower the intact adenine available for further electrocatalytic measurement in the presence of NADH. To electro-oxidize the remaining adenine adsorbed on the CPE, several cyclic scans were carried out up to 1.4 V. After this step, the damaged DNA-CPE was immersed in a NADH– Ca^{2+} solution. The use of calcium ions was reported to greatly improve the electrocatalytic current of NADH [34,35]. An apparent electrocatalytic wave was observed at a potential as low as 0.011 V with a plateau at about 0.14 V (Fig. 2, curve a). Given that the oxidation peak of the uncatalyzed oxidation of NADH at a bare unmodified CPE is 0.70 V [29], a decreased of more than 550 mV is achieved. A low potential is advantageous for analytical purposes because of the diminution of potential oxidizable interferent compounds present in real food samples. Under these conditions, this was the lowest electrocatalytic current possible because it arose from the maximum damage. In the presence of antioxidant compounds a diminution in the damage was expected along with an increase in the electrocatalytic current. When an antioxidant, AA (10 μM), was added to the superoxide radical generating solution, a high augment of the electrocatalytic current was observed (Fig. 2, curve b).

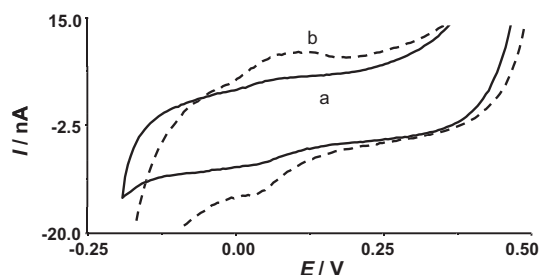


Fig. 1. CVs obtained at 50 mV s^{-1} in tris–HCl pH 9.0 after: (a) immersion of dA_{21} -CPE in a superoxide radical generating solution ($[\text{XOD}] = 0.3\text{ U mL}^{-1}$, $[\text{xanthine}] = 4.4 \times 10^{-5}\text{ M}$) for 15 min and (b) subsequent electrochemical oxidation of the undamaged adenine bases adsorbed on the dA_{21} -CPE.

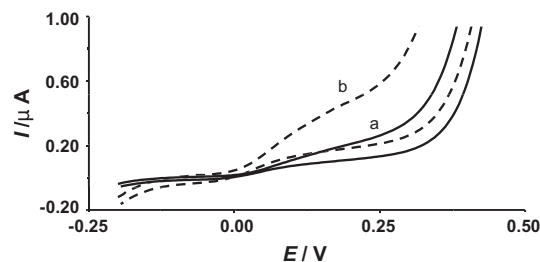


Fig. 2. CVs obtained with a dA_{21} -CPE at 50 mV s^{-1} in tris–HCl pH 9.0 containing 0.5 mM NADH + 0.01 M CaCl_2 after: immersion in O_2^- generating solution ($[\text{XOD}] = 0.1\text{ U mL}^{-1}$, $[\text{xanthine}] = 4.4 \times 10^{-5}\text{ M}$) for 10 min (a) in the absence of antioxidant (b) in the presence of 10 μM of AA; and further complete electrochemical oxidation in both cases.

This anticipated behavior was related to the ability of antioxidant compounds to scavenge or inactivate the ROS and prevent the damage on DNA. As a consequence, the number of lesions diminished, yielding a larger number of adenine available for electrochemical oxidation. A positive correlation between the partial oxidation of DNA by O_2^- and the concentration of antioxidant species in the tested solution would allow the use of the electrocatalytic current of NADH to evaluate the AOC on flavored waters.

3.1. Selection of the experimental conditions for the damaging reaction

In order to determine AOC on beverages, some parameters concerning the damaging reaction (xanthine and XOD concentration, reaction time between superoxide radical and the target molecule) at a fixed concentration of antioxidant compound were varied in order to achieve the highest effect on DNA without a complete damage. For this reason, for each experiment the ratio between the electrocatalytic current obtained after exposing the DNA-CPE to the superoxide radical in the presence of a fixed amount of AA as antioxidant (I_a) and the electrocatalytic current obtained in the absence of AA (I_d , minimum value expected) was estimated. The highest value for this ratio was always selected for further experiments.

The level of DNA damage was evaluated as a function of the amount of radical formed through the variation of the concentration of XOD and xanthine. XOD concentration was studied between 0.05 and 0.20 $U\ mL^{-1}$. Fig. 3A shows the influence of XOD concentration on the electrocatalytic current of NADH.

When increasing the XOD concentration in the absence of antioxidant compound (open circles), the electrocatalytic current decreased until a XOD concentration of 0.10 $U\ mL^{-1}$. At higher concentrations the current remained constant. This behavior suggested that an increase in the enzyme concentration implied a larger number of lesions attributed to the superoxide radical attack. The damage on the dA_{21} layer exhibited a maximum (minimum electrocatalytic current) at a XOD concentration of 0.10 $U\ mL^{-1}$. When the same experiments were carried out in the presence of ascorbic acid (10 μM), the protective effect on the DNA was apparent because the electrocatalytic currents were virtually constant up to 0.10 $U\ mL^{-1}$, within the experimental error (Fig. 3A, filled circles). Only at higher concentrations of enzyme the analytical signal diminished suggesting that the ascorbic acid concentration is not sufficient to compensate the increase in the amount of superoxide radicals generated. In addition to this, it is worth noting that, with the addition of this powerful antioxidant, consistently higher currents were measured at all XOD concentrations (Fig. 3A). The highest value for the I_a/I_d ratio was obtained at a XOD concentration of 0.10 $U\ mL^{-1}$, which was chosen for the next optimization steps.

Xanthine concentration was varied from 4.40×10^{-6} to 4.40×10^{-4} M. The influence of this parameter within the range assayed was very limited. A slight decrease in the electrocatalytic current was observed when increasing the xanthine concentration in the absence of AA, which is not significant within the experimental error (Fig. 3B open circles). In the presence of antioxidant species, all currents were clearly higher and a small but relevant increase was apparent at 4.40×10^{-5} M (filled circles). At higher concentrations a further diminution was observed. This behavior is in good agreement with a scavenging activity of AA. The highest I_a/I_d ratio was observed at a xanthine concentration of 4.40×10^{-5} M, and this value was used for the next experiments.

The reaction time between the superoxide radical and dA_{21} layer depends on the half-time on the generated ROS, so, this parameter is an important feature to select. The reaction time between the free radical, the superoxide, and the DNA adsorbed on the CPE was studied between 5 and 30 min. Increasing the incubation time, the electrocatalytic current of NADH decreased during

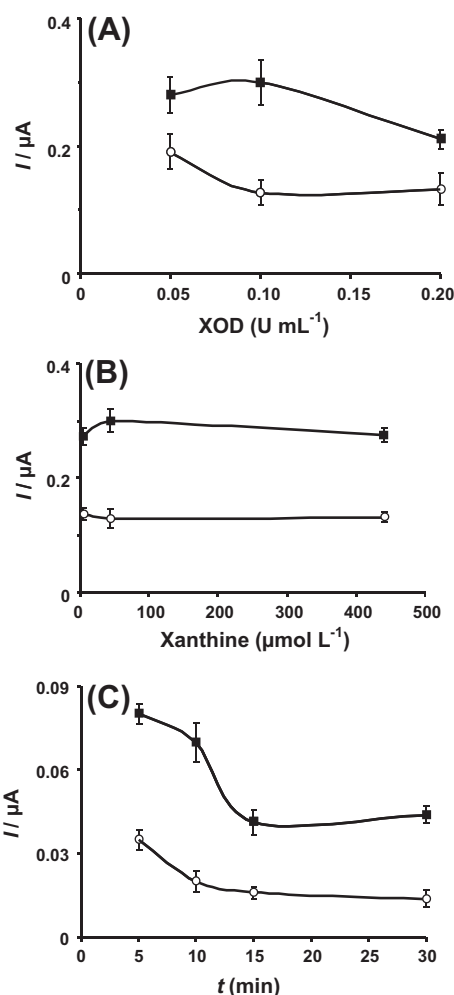


Fig. 3. Influence on the electrocatalytic current of 0.5 mM NADH + 0.01 M $CaCl_2$ in tris-HCl at pH 9.0 of (A): XOD concentration: (B) xanthine concentration: and (C) time reaction: (○) without AA and (■) with 10 μM of AA to the O_2^- .

the first 10–15 min (Fig. 3C, open circles). At longer reaction time the current remained constant. With the introduction of ascorbic acid (10 μM) on the reaction system, the electrocatalytic current measured was higher than in its absence at all reaction times assayed in good agreement with the radical scavenging role (Fig. 3C, filled circles). Nevertheless, a decrease is observed up to 15 min although the remaining electrocatalytic current is significantly higher than in the absence of AA (Fig. 3C). This behavior indicated that, even at very long times, AA was able to partially protect the integrity of DNA from O_2^- radical attack. The highest value of I_a/I_d ratio was found when an incubation time of 10 min was used, so, this value was selected for further studies.

3.2. Determination of AOC

In this work, the antioxidant ascorbic acid was used as a model for the study of the behavior of antioxidants on the protection of DNA against O_2^- radicals generated by XOD/xanthine reaction. The feasibility of measuring the antioxidant concentration was investigated varying the concentration of AA from 10 to 100 μM . A linear range was found for the entire range (I (nA) = (0.85 ± 0.07) [AA (μM)] + (16 ± 5) ; $r = 0.990$ $n = 5$). The limit of detection was estimated using the regression parameters obtaining a value of 10 μM . The reproducibility expressed as RSD was 4.2% at 50 μM . Fig. 4 shows CVs obtained in a Ca^{2+} -containing NADH solution after immersing the DNA-CPE in a superoxide radical solution

with increasing concentrations of AA. The catalytic current of NADH increased up to 100 μM due to the availability of a larger number of undamaged adenines for electrochemical oxidation. At concentrations above this value the electrocatalytic current remained constant indicating the saturation of the ability of AA to counterbalance the radical attack (Fig. 4, inset panel).

Other authors have also used AA in order to study its protective effect on DNA (adsorbed at an electrode surface) against free radicals. However, all these reports only described the scavenging role of AA towards hydroxyl radicals [29,36,37].

As mentioned before, no DNA sensors for antioxidant assessment using other ROS, such as superoxide radical, have been reported so far. Two reports described the use of AA as a standard antioxidant against superoxide radical, but the bilayer on the electrode was formed by cytochrome *c* or SOD [2]; or the electrochemically generated radical was detected directly on a glassy carbon disk electrode [14]. From our previous work on antioxidant activity against OH^\cdot on DNA-CPE, it can be concluded that AA seemed to be less efficient as a scavenger of superoxide radical than hydroxyl radicals. In fact, the minimum AA concentration able to show a protective action is more than two order of magnitude lower in the case of OH^\cdot [29].

In order to compare the efficiency of radical scavenging, several antioxidants (AA, GA, trolox, CA, and RES) were tested at a concentration of 10 μM under the same experimental conditions and the results are shown in Fig. 5.

The efficiency was expressed as the percentage of the electrocatalytic current according to the following expression: % efficiency = $I_a/I_b \times 100$, where I_a is the current intensity measured

after DNA damage in the presence of the antioxidant compound, and I_b is the electrocatalytic current measured when no damage was done (maximum expected value). It was found that the superoxide radical generated 85% of damage on the dA_{21} layer, that is, in the absence of a scavenging molecule. The protective effect of antioxidants ranged from 33% to 63%. The lowest values were found for trolox and CA, 33% respectively. RES presented the highest protective effect (62.5%). AA and GA presented a protective role of 53.8% and 53.2% respectively.

At this point, it is interesting to note that superoxide radicals caused a similar degree of damage on DNA adsorbed on CPE to hydroxyl radical [29]. Although efficiency values were similar or much higher than those obtained with OH^\cdot , the antioxidant concentration employed is much higher, which is in good agreement with the lower scavenging activity above found. This result was not unexpected because it is commonly accepted that not all antioxidants behaves equally against different radicals [24]. It was clear that the efficiency order differed from that obtained against hydroxyl radical. Whereas AA and CA exhibited similar protecting roles against both radicals (about 55% and 33% respectively), the effectiveness of RES dramatically increased from 38% for OH^\cdot to 62.5% for $\text{O}_2^{\cdot-}$. In any case both compounds, AA and RES, were the most effective antioxidants assayed. Similarly, GA was much more active for $\text{O}_2^{\cdot-}$ than for OH^\cdot , shifting from 19.3% (the worst one) to 53.2% virtually identical to AA within the experimental error.

Once the analytical features of the electrocatalytic voltammetric method were characterized in aqueous solution, it was applied to the determination of AOC in real samples. A lemon flavor and two different brands of lemon flavored water samples were chosen because this citrus fruit is used and commercialized all over the world and is rich in antioxidants such as vitamin C and phenolic compounds.

As it is shown in Table 1, all samples presented antioxidant capacity. Lemon flavor exhibited the highest level of AOC expressed in mg L^{-1} of AA. This finding was expected because this flavor is extracted from the fruit along with essential oils, and has several substances at high concentration in its composition. Lemon water from brands A and B had a similar AOC value. However, the composition of both samples was different because brand B had green tea in addition to vitamin C.

Among the methods used for antioxidant capacity assessment, the Folin-Ciocalteu method for the quantification of the phenolic content is widely used because its robustness, simplicity and cost-effectiveness [24]. In general, phenolic compounds content correlates with antioxidant activity and seems to have an important role in stabilizing lipid oxidation. Therefore, the TPC of these samples was evaluated and expressed in mg L^{-1} of GA. As expected, the highest TPC value was found in the lemon flavor

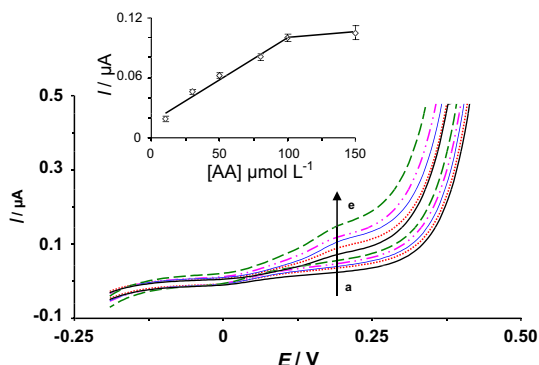


Fig. 4. CVs at 50 mV s^{-1} obtained in tris-HCl at pH 9 containing 0.5 mM NADH + 0.01 M CaCl_2 after immersion of DNA-CPE in $\text{O}_2^{\cdot-}$ generating solution ($[\text{XOD}] = 0.1 \text{ U mL}^{-1}$, $[\text{xanthine}] = 4.4 \times 10^{-5} \text{ M}$) containing a standard solution of AA: (a) 10, (b) 30, (c) 50, (d) 80 and (e) 100 μM for 10 min. Inset panel: relationship between I_a and AA concentration.

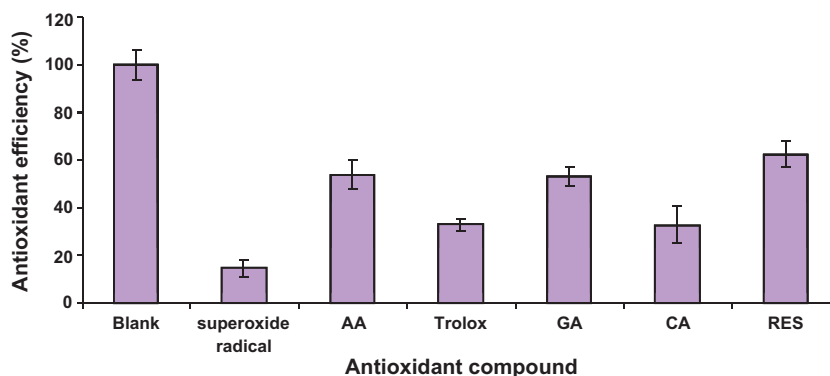


Fig. 5. $\text{O}_2^{\cdot-}$ scavenging efficiency of several antioxidant compounds: AA – ascorbic acid, GA – gallic acid, CA – caffeic acid, RES – resveratrol. Values are expressed as percentage of the electrocatalytic current obtained with an intact (not damaged) dA_{21} layer that remained after exposure to a damaging solution containing 10 μM of antioxidant species.

Table 1

AOC values of flavored waters obtained using the electrochemical and optical methods.

Samples	DNA-CPE (mg L ⁻¹ AA)	TPC (mg L ⁻¹ GA)
Lemon flavor	124 ± 13	380
<i>Lemon flavored water</i>		
Brand A	30.2 ± 0.7	7.2
Brand B	31.0 ± 7.5	39.7

(Table 1). The water sample from brand B exhibited a significantly higher TPC value than water from brand A. This difference on the TPC values was attributed to the presence of green tea in brand B. Green tea contains polyphenols (catechins, epicatechin, epigallocatechin) in addition to vitamins [38,39]. Some phenols react with TPC reagents although they may not necessarily be efficient radical scavengers [40]. The presence of this type of phenolic compounds might explain the discrepancy between values obtained for both voltammetric and TPC methods.

4. Conclusions

A DNA-CPE antioxidant biosensor for the assessment of AOC in beverages was developed. For the first time in this type of devices, the effectiveness against damage of superoxide radical on DNA was evaluated testing different antioxidant compounds. Although the damage in terms of adenine oxidative lesions was similar to that found using hydroxyl radicals, the scavenging activity of the antioxidant tested was lower because a much higher concentration was needed to obtain similar efficiencies. The order of protective efficacy was also different and as follows, RES > AA > GA > trolox ~ CA.

A minor product of the radical oxidation was identified by CV as a diimine compound that did not appear when the oxidant source was the OH[•] radical. This result suggested that the mechanism of O₂^{-•} attack on DNA is more complex than the reported promotion/source of OH[•] radicals.

In spite of the lower efficiency of AA as O₂^{-•} scavenger, the indirect electrocatalytic method described allowed the quantification of ascorbic acid from 10 μM and AOC determination in flavored waters and extracts.

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