An electrochemical method for the determination of antioxidant capacities applied to components of spices and condiments

by

María Pilar Rivas Romero[a], Rafael Estévez Brito[a], Alberto Palma[b], Mercedes Ruiz Montoya[b], José Miguel Rodríguez Mellado[a]*, Rafael Rodríguez-Amaro[a]

[a]Departamento de Química Física y Termodinámica Aplicada
Facultad de Ciencias
CeiA3, IUIQFN, Campus Universitario Rabanales, edificio Marie Curie
Universidad de Córdoba
E-14014-Córdoba (Spain)

[b]Departamento de Ingeniería Química, Química Física y Ciencias de los Materiales
Facultad de Ciencias Experimentales
Campus de "El Carmen"
Universidad de Huelva
E-21071- Huelva (Spain)

*Corresponding author (Tel: +34 957218647; Fax: +34 957218618: E-mail: jmrodriguez@uco.es)

Abstract

The antioxidant activity of a set of 17 active principles present in foods is studied by using two sets of methods: i) spectrophotometric, DPPH radical scavenging method and CUPRAC assay; ii) electrochemical, using either a mercury electrode or a glassy carbon electrode covered with poly-neutral red and doped with Pt nanoparticles. The modified glassy carbon electrode assesses the scavenging activity of the antioxidants studied, improving the measurement time and easiness respect to the rest of the methods. Compared to Hg electrode, the measurements can be made in conditions close to the physiological conditions (pH 7 and aqueous medium). Using this sensor, antioxidants without activity in the DPPH radical scavenging assay can be studied. Finally, with respect to CUPRAC, the advantages of the modified electrode are the absence of organic solvent, with the consequent closeness to physiological conditions, and the lower measuring time involved. The sensitivity of this sensor is comparable with those shown by DPPH or CUPRAC

Keywords

Antioxidant capacity; DPPH radical scavenging method; CUPRAC assay; nanoparticle-doped sensor; electrochemical methods

Introduction

The antioxidant capacity of natural samples (food extracts as infusions, beverages and biological fluids) has been determined by different methods that can be classified as HAT (hydrogen atom transfer), ET (electron transfer) and mixed HAT-ET methods.[1-3] For example, the TRAP[4] assay (total peroxyl radical trapping antioxidant parameter) and ORAC[5] assay (oxygen radical absorbance capacity) can be classified as HAT methods, based on hydrogen atom transfer mechanism.

Reactive oxygen species (ROS) are radicals generated by organic and inorganic peroxides, including those originated by H_2O_2 or water itself, i.e., $HO^{\bullet}$, $HO^{\bullet}_2$, $O^{\bullet-}_2$, … These species can induce the degradation of biological macromolecules such as lipids, carbohydrates, proteins (including enzymes) and nucleic acids.

HAT-based methods measure the ability of a given antioxidant to scavenge ROS by hydrogen donation. The mechanism includes the transfer of one hydrogen atom from the antioxidant to a ROS to give more stable free radicals.

TAC (total antioxidant capacity methods) are usually non-competitive ET (electron transfer) methods based on single electron transfers. Antioxidants are also good reducing agents able to reduce ROS and, for this reason, these methods are mainly reduction-based assays. The CUPRAC assay[6] used for TAC determination is a collection of various modified methods of antioxidant capacity measurement using Cu(II)−Cu(I) reduction. This assay has been applied to matrices containing both hydrophilic and lipophilic antioxidants.

The DPPH Radical Scavenging Assay[7] is a mixed-mode method (ET plus HAT). It is based on the UV-spectroscopic measurement of the course of the reaction between the antioxidant and the stable radical 2,2-diphenyl-1-picrylhydrazyl. The reaction can be

formulated as a HAT mechanism, but the proton-coupled ET mechanism cannot be excluded. Because its low-cost and relative simplicity, the DPPH assay is widely used in conventional laboratories.

There is not a clear correlation between activities determined by different assays, for the same antioxidant, or by the same assay in different laboratories[8].

H_2O_2 may be formed in tissues through oxidative processes involving electron reduction of O_2 that generates the $O_2^{\bullet -}$ anion, being the end product hydrogen peroxide. This molecule can induce the degradation of lipids, proteins or enzymes, carbohydrates, and nucleic acids through generation of other radicals as $HO^{\bullet}$. Thus, the measurement of the H_2O_2 scavenging activity in foods and biological fluids is relevant in order to evaluate antioxidant capacities.

The antioxidant capacity was also determined electrochemically by monitoring the change in the polarographic oxidation response of hydrogen peroxide originated by the addition of a given amount of antioxidant or extract[9,10]. The method is based on the production of $HO_2^{\bullet}$ and $O_2^{\bullet -}$ on the electrode, which was experimentally verified[11-13] but it presents three problems: first, the use of mercury is restricted by health authorities; second, the measurements must be made at high pH values[10,13] (pH>10) and, third, high amounts of ethanol in the medium are required[13]. This means that the measurements are very far from physiological conditions and this can result in an erroneous comparison of the antioxidant activities of related compounds[14].

Electrodes modified with the conducting polymer poly-neutral red and electrodeposited platinum nanoparticles were also proposed[15]. Radical cations (polarons) localized in the inter-monomer bonds of poly-neutral red are identities stabilized by platinum

nanoparticles, their charge being balanced by the relative slow transfer of anions between the hybrid nanocomposite and solution[16]. The nanocomposite catalyses the H_2O_2 reduction and the polymer acts as a proton reservoir.

The aim of this work was to shown that the use of this modified glassy carbon electrode as sensor for antioxidant activity improves the measurement time and easiness with respect to the spectrophotometric methods, allowing measurements in nearly physiological conditions. This will be assessed by determining the antioxidant activity of a set of active principles of foods by means of different methods, exploring the possible correlation between them.

Materials and methods

All chemicals used as solvents, pH-adjustment and buffer components were of analytical quality. For aqueous solutions, ultrapure water type I (resistivity 18.2 MΩ·cm at 25 ^{O}C) obtained from a MilliporeMilli Q system was used.

Stock solutions were stored in the dark at 277 K to avoid decomposition and H_2O_2 solutions, when required, were freshly prepared just before each experiment.

Neutral Red (>90%) was from Amresco. From Sigma-Aldrich were: 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), free radical (95%), Hexachloroplatinic acid (99.5%), Trolox (97%), $CuCl_2$ (97%), Neocuproine (>98%). All the standards eugenol, sesamol, 3-hydroxycoumarin, cinnamaldehyde, 4-hexylresorcinol, carvacrol, thymol, coumarin, gallic acid and (R)-(+)-limonene, were of chromatographic quality. The structures of these compounds are given in Figure 1.

Figure 1

Spectrophotometric meausurements. DPPH radical scavenging method

DPPH$^{\bullet}$ shows an UV–visible absorption band with maximum wavelength at 515 nm. The action of a given antioxidant causes a decrease of this band, or even provokes its disappearance, due to reactions giving DPPH and AO$^{\bullet}$. The antioxidant capacity is measured from the amount of antioxidant required to decrease the concentration of DPPH$^{\bullet}$ to a value of the 50% of the initial concentration, this value being called "efficient concentration", EC_{50}. Figure 2 illustrates the measurement of EC_{50}. The reverse value, called anti-radical power, $ARP = 1/EC_{50}$, increases as the antioxidant activity increases (see Fig. 2).

Figure 2

UV measurements were made at room temperature on a Genesys 10 UV spectrophotometer from Thermo Electron Corporation with quartz cuvettes of 1.0 cm path-length.

Increasing concentrations of antioxidants were added to a $6 \cdot 10^{-5}$ M DPPH$^{\bullet}$ methanolic solution. The DPPH$^{\bullet}$ concentration in the reaction medium along the reaction time was calculated from the maximum absorbance.

Spectrophotometric meausurements. CUPRAC assay

Samples were prepared by taking, in this order, 1 mL of 0.01 M $CuCl_2$ water solution, 1 mL of 1M NH_4COOCH_3 aqueous buffer at pH 7, 1 mL of $7.5 \cdot 10^{-3}$ M neocuproine ethanolic solution, variable volumes (0, 50, 100, 200 and 500 µL) of stock solution of antioxidant, in most cases at a concentration of $1 \cdot 10^{-3}$ M, and completing the volume to 4.1 mL with water or ethanol, depending on the solubility of the antioxidant tested. In some cases, the concentration of the stock solution of antioxidant was 0.04 M: limonene, cinnamic acid,

salicylaldehyde, cinnamaldehyde, β-pinene and geraniol. Calibration curves for trolox were made by using variable volumes of $2.5 \cdot 10^{-4}$ M stock solutions of this compound in ethanol.

The samples were stored in darkness during 60 min at 25 ^{o}C and their absorbance at 450 nm was measured with a double beam Perkin-Elmer Lambda 750S spectrophotometer. Hanna quartz cuvettes of 1 cm path-length. In all cases the zero-concentration absorbance was substracted, and from the net absorbances so obtained, the corresponding antioxidant capacities were measured in trolox equivalents.

Electrochemical measurements on mercury

The area of the DPV peak corresponding to the hydrogen peroxide oxidation on mercury electrode depends on the ethanol content[11]. For this reason, experiments were made at a constant ethanol content of 30%. Hydrogen peroxide concentration was $5 \cdot 10^{-4}$ M. Solutions were prepared with 6.9 mL of supporting electrolyte, 0.100 mL of $5 \cdot 10^{-2}$ M H_2O_2, variable volumes, V_{AO}, of the stock solution of antioxidant in ethanol and ($3–V_{AO}$) mL of pure ethanol, being the final volume 10 mL. H_2O_2 was added after the solutions were purged with purified nitrogen.

Measurements were made on a CHI650A electrochemical workstation from IJCambria coupled to an EF-1400 controlled growth mercury electrode from BAS instruments, in the HMDE mode. The Hg drop area was 6.70×10^{-3} cm^2. The temperature was kept at 298±0.1 K. All potentials were measured against an Ag|AgCl|KCl_{sat} electrode (BAS MF-2052). A platinum counter electrode BAS MW-1034 was used. The parameters selected in the differential pulse voltammetry (DPV) were: pulse amplitude 0.05 V, pulse width 0.05 s and pulse period 0.2 s.

The reproducibility of the measurements was ensured by repeating the experiments and the standard deviations of the data were less than 5%.

Electrochemical measurements on the glassy carbon electrodes covered with Pt nanoparticles and poly-neutral red

0.1 M PBS buffer at pH 7.0 was used as supporting electrolyte. The *pH* was adjusted with solid NaOH. Poly-neutral red (PNR) deposition was made as reported elsewhere[15]. A solution of 10 mg neutral red in 40 mL of deoxygenated 0.5 M H_2SO_4 was used. The polymerization was performed from 0.4V to –0.7V at the scan rate of 50 mV s^{-1} for 6 cycles.

Platinum nanoparticles deposition was performed at –0.2V for 1 min with a solution containing $5 \cdot 10^{-3}$ M hexachloroplatinic acid and 0.5 M H_2SO_4. After deposition, the electrode was electrochemically conditioned by applying cyclic voltammetry in a 0.1 M PBS solution from –0.4V to 0.7V, at 0.100 V s^{-1} during 2 cycles.

Measurements were made with an Autolab PGSTAT302N potentiostat. A three-electrode cell equipped with a Pt wire counter electrode and a BAS MF-2079 Ag/AgCl 3 M KCl reference electrode was employed. Measurement temperature was 298 K.

A Glassy carbon electrode (GCE) from IJCambria (7.5 mm^2 area) was used as working electrode. The surface of the electrode was regenerated by polishing with a silicon carbide paper, followed by diamond slurry (0.25 μm) and alumina (0.3 and 0.05 μm) slurries. Residual material was removed from the matrix by sonication in a pure water bath for 30 minutes after each polishing.

Results and discussion

Table 1 shows the experimental antioxidant activities obtained by using the four methods described in the preceding section. In this table, the oxidation potentials measured

on mercury electrodes by linear-sweep cyclic voltammetry are also gathered. It has been previously shown that these potentials are related to the antioxidant capacity[17].

Trolox equivalent antioxidant capacity given in the table measures the antioxidant capacity in terms of the standard Trolox. This standard is often used to measure the antioxidant capacity of foods, beverages and nutritional supplements.[18] Trolox solutions of different concentrations are used to develop a standard curve, which the results obtained for the samples are compared.

Table 1

Figure 2 illustrates the determination of ARP by the DPPH radical scavenging method. First, the decrease of the absorbance of DPPH$^{\bullet}$ radical with the reaction time is recorded, at variable concentrations of the antioxidant. In the figure are given two examples. At high times (the threshold varies with each antioxidant) the absorbance remained constant and these data were chosen as the characteristic value for the steady state concentration of the remaining DPPH$^{\bullet}$ radical. The steady state values are plotted versus the normalized antioxidant concentration to obtain the EC_{50} parameter and the ARP is calculated as the reverse of this quantity.

The DPPH radical scavenging ability found was different for each antioxidant. In figure 2 it can be observed that the decrease for ascorbic acid is sharp. The concentrations of reactant needed to decrease the absorbance are low, as well as the time to reach the steady state. On the contrary, high concentrations and reaction times were needed for vanillin. Very low ARP value were found for approximately one half of the compounds tested. In these cases, no significant decrease in absorbance was observed, even at very high concentrations and times. This is due to that DPPH$^{\bullet}$ assay assess the radical scavenging for substances

thermodynamically capable to react with agents having a redox potential below a given value, related to the reduction potential of the probe radical.[17] So, many compounds did not show antioxidant activity in this assay.

Table 1 also shows the results obtained by the CUPRAC assay, referred to trolox. The column Hg of this table corresponds to the evaluation of the antioxidant activity by means of differential pulse voltammetry (DPV) of hydrogen peroxide on mercury electrodes.

As an example, Figure 3 presents the decrease of the peak area of the DPV oxidation peak obtained for H_2O_2 in mercury electrodes after the addition of increasing amounts of $5 \cdot 10^{-3}$ M β-pinene. From the plot of the decrease of the peak area vs. the added volume, the value of concentration at which the oxidation signal of H_2O_2 decreases in a 10 percent is obtained. The reverse of this value, μ_{10}, is a measurement of the antioxidant capacity.[11,14] In the table, the antioxidant capacities measured by this method are also given in trolox equivalents.

Figure 3

The glassy carbon modified electrode was used to study the interaction between the hydrogen peroxide and antioxidants.[15,16] The results obtained for gallic acid and sesamol are shown in Figure 4 as examples. The electrochemical reduction of H_2O_2 produces ROS, and the addition of a radical scavenger interrupts the electrochemical reaction, decreasing the peak intensity, this decrease being related to the antioxidant activity as in the preceding case.

Figure 4

As the antioxidant concentration increases, the peak intensity decreases and, conversely to the oxidation on mercury,[11-13] the peak intensities decreased below the 50% of

the initial value, due to the difference between the mechanisms of the oxidation and reduction of H_2O_2. In the same way as for mercury, the value of μ_{10} is directly related to the antioxidant capacity;[15] the values in terms of trolox equivalents are given in table 1 in the column labeled "PNR". Figure 5 shows a general picture of the antioxidant capacities for those methods that can be referred to trolox.

Figure 5

The results obtained by using the different methods of measurement have been compared by plotting pairs of parameters in two-dimensional representations. The results from each method were first compared with those of CUPRAC assay, as shown in figure 6.

Figure 6

As it follows from the figure, the antioxidant capacities measured by DPV in Hg, DPV in PNR and by oxidation potentials in glassy carbon, vary in a similar manner as CUPRAC measurements, with few exceptions. Thus, for DPV measurements (in both Hg and PNR) the graphs show positive tendencies respect to CUPRAC, whereas for oxidation potentials the tendency is negative, because high oxidation potentials are related to low antioxidant capacity.[17] On the other hand, ARP shows a poor correlation with CUPRAC as was also reported for the comparison between ARP and DPV on Hg.[12]

ARP, DPV in Hg, and oxidation potentials were compared also with the DPV in PNR measurements, as is presented in figure 7, which shows correlations between DPV in Hg and oxidation potentials with DPV in PNR. Again, the ARP values were not fully related to the antioxidant capacity measured by DPV in PNR.

Figure 7

There are antioxidants that cannot be evaluated by the DPPH scavenging assay. This is due to their oxidation potentials, which are higher than the oxidation potential of the $DPPH^{\bullet}$ radical.[12,17]

The above results show that the electrochemical assay based in the carbon electrode modified with PNR and Pt nanoparticles in the presence of hydrogen peroxide can be used for the determination of the antioxidant capacity in a similar way as the rest of techniques (excluded ARP) with some advantages. Moreover, this electrode can be used repeatedly for months from its preparation, if the storage conditions are adequate.[15] The antioxidant capacity of many foods and food components and additives is usually investigated in relation to the action in the organism. So, physiological conditions are very important for the determination of such activities. A method that can assess the antioxidant activity in conditions close to physiological values is more valuable than other methods that cannot be used in these conditions.

Compared to DPV with Hg electrodes, the PNR measurements can be made at pH values close to the physiological pH, whereas Hg measurements must be made at pH>10, because at lower pH values the direct mercury oxidation overlaps with the hydrogen peroxide signal.[11,14] In addition, for compounds with low solubility in water, Hg measurements require a very high non-aqueous content. This implies that the experimental parameters are far from the physiological conditions and that the method becomes more complicated.[11,12]

The main advantage of PNR technique with respect to CUPRAC is the absence of organic solvent and the consequent closeness to physiological conditions. A secondary advantage deals with the lower measuring time involved.

The modified glassy carbon electrode is at least so sensitive as DPPH or CUPRAC both for pure chemicals and food extracts. In the first case, concentrated solutions can be used to explore compounds with very low antioxidant capacity values. For extracts showing poor activities, a pre-concentration step can be used, as is commonly made for CUPRAC.

Conclusions

The different techniques used, DPPH radical scavenging method and CUPRAC (both spectrophotometric), DPV on mercury electrode and DPV on a glassy carbon electrode modified with Pt nanoparticles and poly-neutral red (both electrochemical), assess the antioxidant activity of the active principles of foods here studied. The modified glassy carbon electrode improves the measurement time and easiness with respect to the other methods and the measurements can be made close to the physiological conditions (pH 7 and aqueous medium). In addition, the sensor can study antioxidants or extracts without activity in the DPPH radical scavenging assay. The modified glassy carbon electrode is at least so sensitive as DPPH or CUPRAC both for pure chemicals and food extracts.

Acknowledgements

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Table heading

Table 1. Antioxidant capacities of the studied compounds by different techniques. ARP: DPPH radical scavenging assay; CUPRAC: Cupric Reducing Antioxidant Capacity; Hg: DPV in mercury; PNR: DPV in Pt-doped poly-neutral red; E_p: Oxidation potentials in mercury.

Table 1

Nº	Name	ARP $mmol^{-1}$	CUPRAC eq trolox	Hg eq trolox	PNR eq trolox	E_P* V
1	2,4-dihydroxybenzaldehyde	0	0.41	1.79	0.206	0.841
2	2,5- dihydroxybenzaldehyde	17.5	1.56	1.01	0.488	0.202
3	3-hydroxycoumarin	0	2.41	2.21	0.806	0.763
4	4-Hexylresorcinol	2.3	2.26	1.72	0.425	0.453
5	Ascorbic acid	6.4	1.73	--	1.09	0.079
6	Cinnamic acid	0	0.0005	0.59	0.313	0.552
7	Gallic acid	12.5	2.97	3.05	0.95	0.274
8	Carvacrol	0.1	1.39	1.59	0.569	0.552
9	Cinnamaldehyde	0	0.1	1.47	0.475	0.588
10	Eugenol	5	3.04	2.29	0.738	0.411
11	Geraniol	0	0.01	0.37	0.25	--
12	Limonene	0	0.003	0.21	0.188	--
13	Salicylaldehyde	0	0.05	0.52	0.288	0.86
14	Sesamol	5.5	0.87	0.52	0.675	0.343
15	β-pinene	0	0.04	0.38	0.125	--
16	Thymol	0.8	2.08	0.81	0.681	0.529
17	Vanillin	0.1	0.43	0.79	0.494	0.571

- Extracted from reference 14

Figure headings

Figure 1: Chemical structures and names of the studied compounds

Figure 2: DPPH assay. (up) Dependence of the remaining concentration of DPPH$^{\bullet}$ radical (2,2-diphenyl-1-picrylhydrazyl) with reaction time for two antioxidants, showing the times for the steady state concentration. Numbers in the figures correspond to the ratio mol antioxidant/mol DPPH. (down) Dependence of the DPPH$^{\bullet}$ steady state concentration with the initial concentrations of both antioxidants. EC_{50} values are located in the graphs.

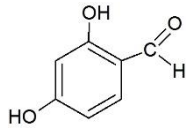
Figure 3: Differential Pulse voltammograms (DPV) in mercury electrode of $5{\cdot}10^{-3}$ M H_2O_2 at pH = 10.50, 30% ethanol, and different amounts (in µL) of $5{\cdot}10^{-3}$ M β-pinene solution in ethanol. Total volume: 10 mL.

Figure 4: Measurements on the glassy carbon electrode modified with poly-neutral red doped with platinum nanoparticles. (up) Linear-sweep voltammograms of $5{\cdot}10^{-3}$ M H_2O_2 oxidation in PBS at pH 7. The amounts (in µL) of 0.1 M gallic acid added to 40 mL of PBS solution are given in the legend. (down) Decrease of the DPV peak area of $5{\cdot}10^{-3}$ M H_2O_2 oxidation in PBS at pH 7 with the added amounts of 0.1 M sesamol solution to 40 mL of PBS solution.

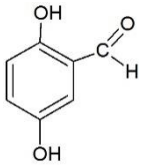
Figure 5: Comparison of antioxidant activities, measured in trolox equivalents, of the tested compounds by three methods: CUPRAC, oxidation of hydrogen peroxide by DPV on mercury (Hg) and reduction of hydrogen peroxide by DPV on the glassy carbon electrode modified with poly-neutral red doped with platinum nanoparticles (PNR).

Figure 6: Plots of antioxidant activities of the tested compounds against those obtained by CUPRAC.

Figure 7: Plots of antioxidant activities of the tested compounds against those obtained by PNR (CUPRAC-PNR comparison is given in figure 6).



2,4-dihydroxybenzaldehyde



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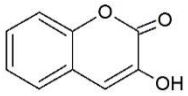
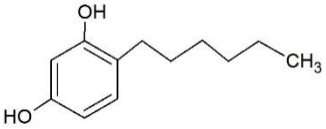
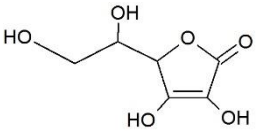


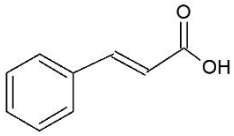
3-hydroxycoumarin



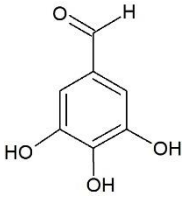
4-Hexylresorcinol



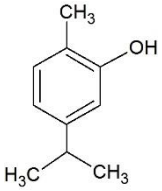
Ascorbic acid



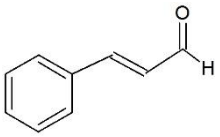
Cinnamic acid



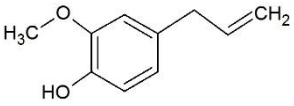
Gallic acid



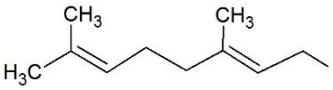
Carvacrol



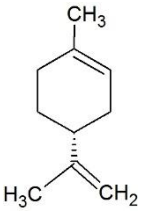
Cinnamaldehyde



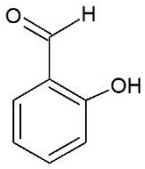
Eugenol



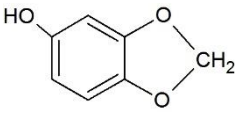
Geraniol



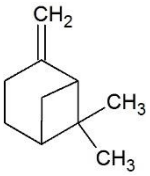
Limonene



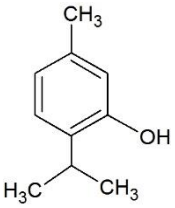
Salicylaldehyde



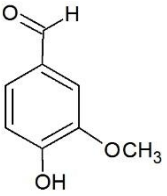
Sesamol



β-pinene



Thymol



Vanillin

Figure 1

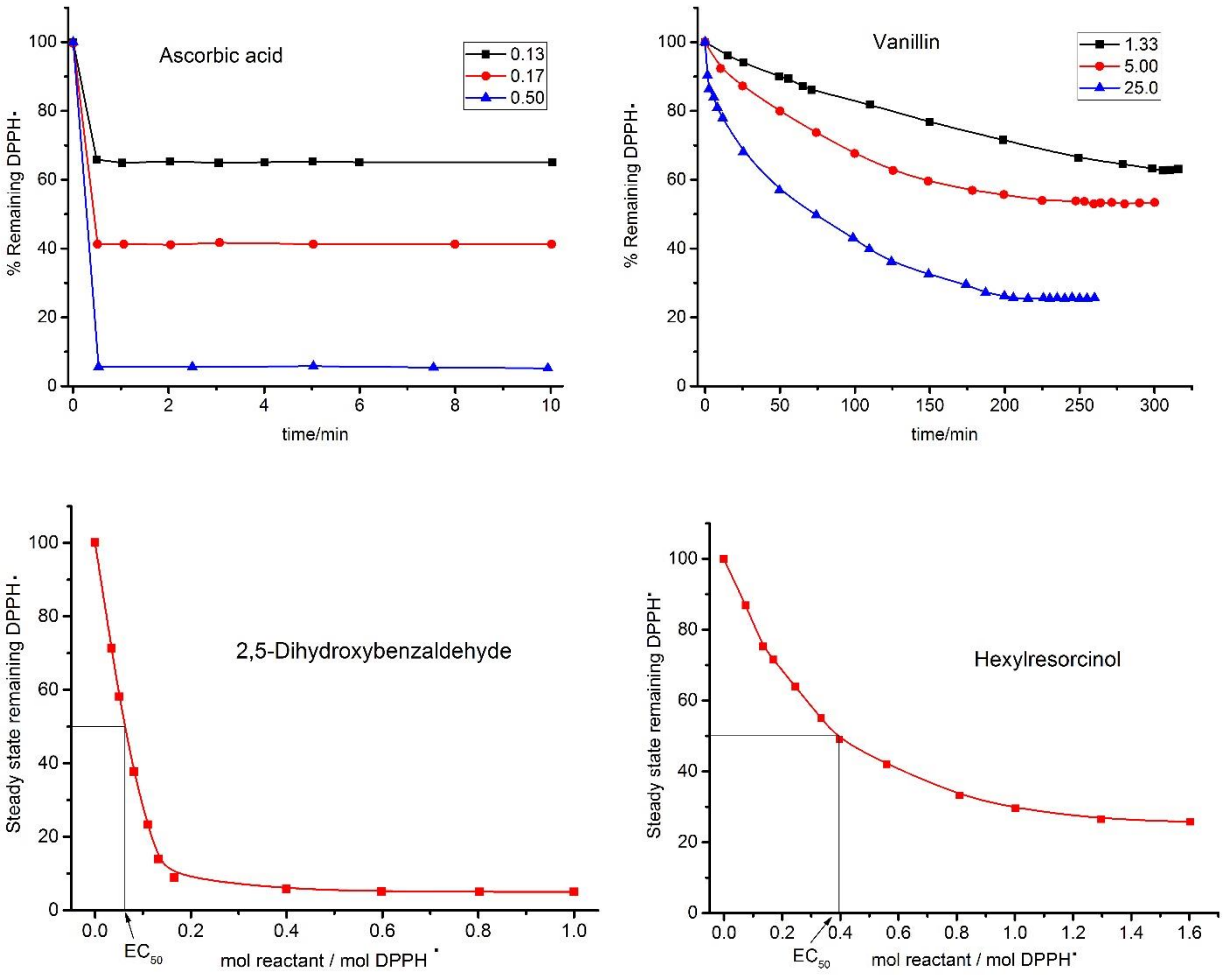

Ascorbic acid
0.13
0.17
0.50
% Remaining DPPH·
time/min
Vanillin
1.33
5.00
25.0
% Remaining DPPH·
time/min
2,5-Dihydroxybenzaldehyde
Steady state remaining DPPH·
EC50
mol reactant / mol DPPH ·
Hexylresorcinol
Steady state remaining DPPH·
EC50
mol reactant / mol DPPH·

Figure 2

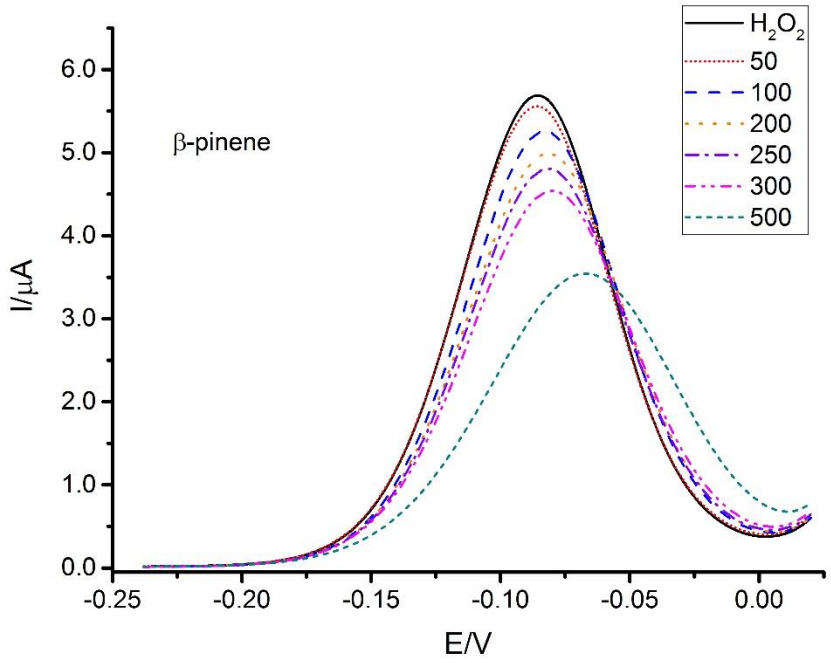

β-pinene
I/μA
E/V
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-0.25
-0.20
-0.15
-0.10
-0.05
0.00
H2O2
50
100
200
250
300
500

Figure 3

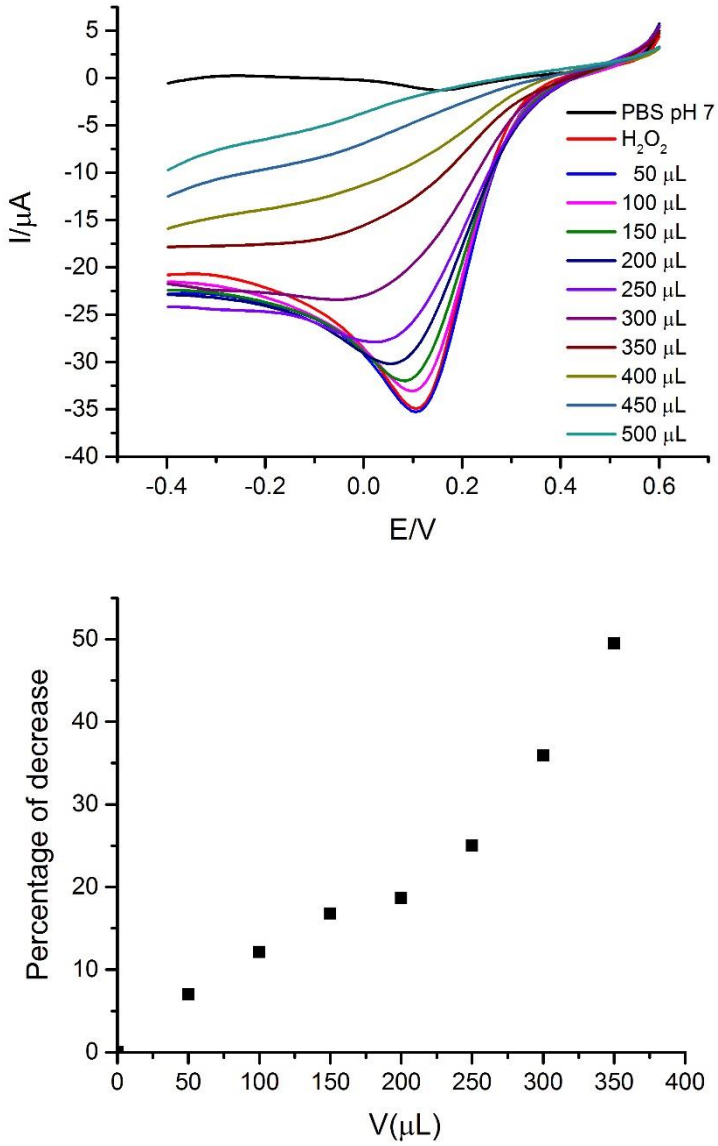

I/μA
E/V
PBS pH 7
H2O2
50 μL
100 μL
150 μL
200 μL
250 μL
300 μL
350 μL
400 μL
450 μL
500 μL
Percentage of decrease
V(μL)

Figure 4

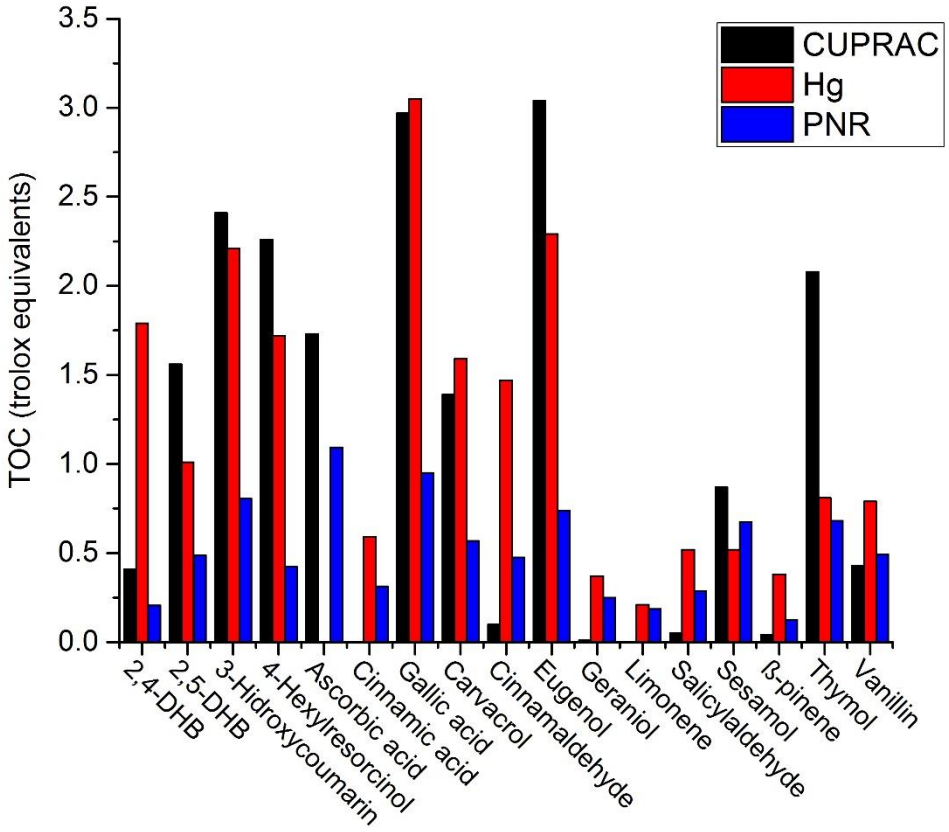

TOC (trolox equivalents)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CUPRAC
Hg
PNR
2,4-DHB
2,5-DHB
3-Hidroxycoumarin
4-Hexylresorcinol
Ascorbic acid
Cinnamic acid
Gallic acid
Carvacrol
Cinnamaldehyde
Eugenol
Geraniol
Limonene
Salicylaldehyde
Sesamol
ß-pinene
Thymol
Vanillin

Figure 5

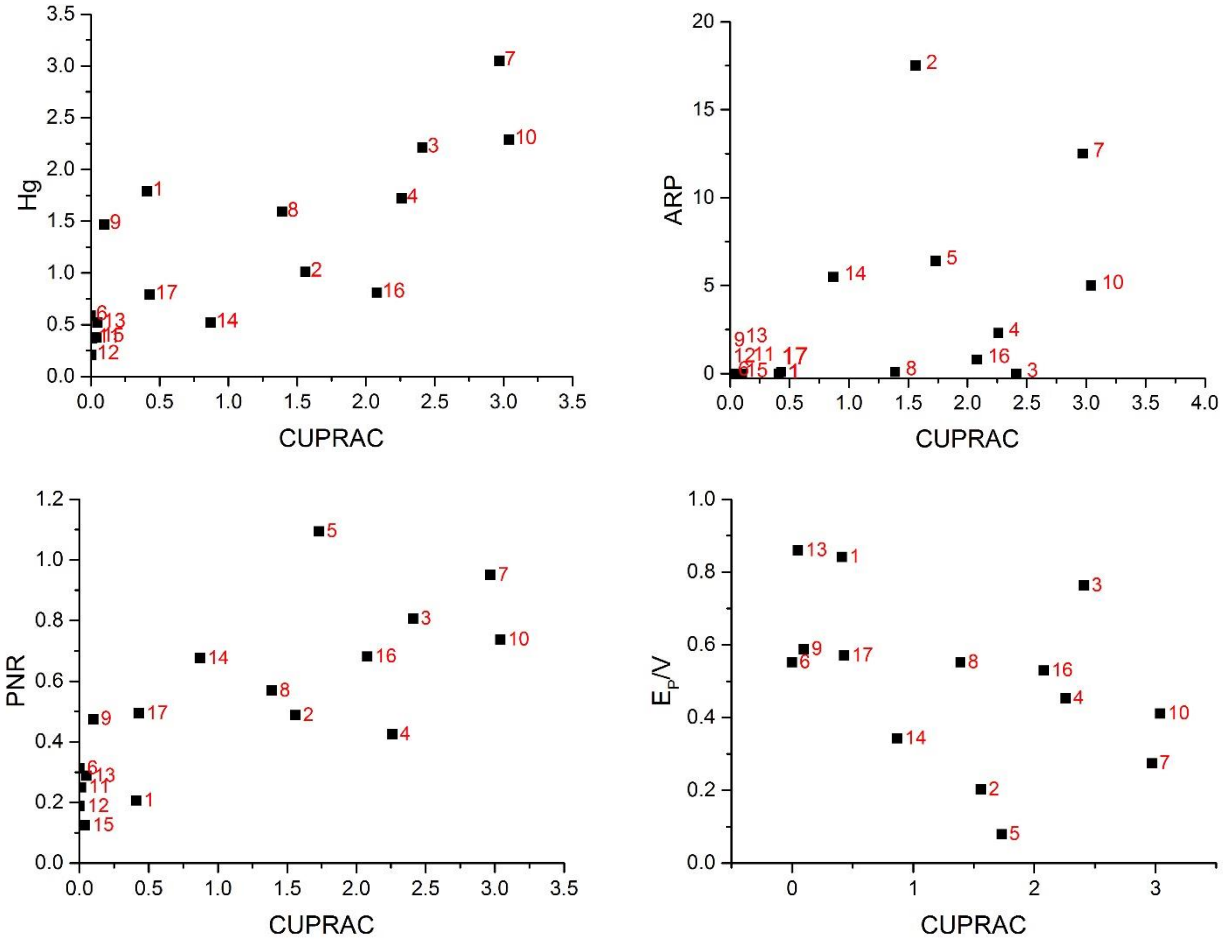


Figure 6

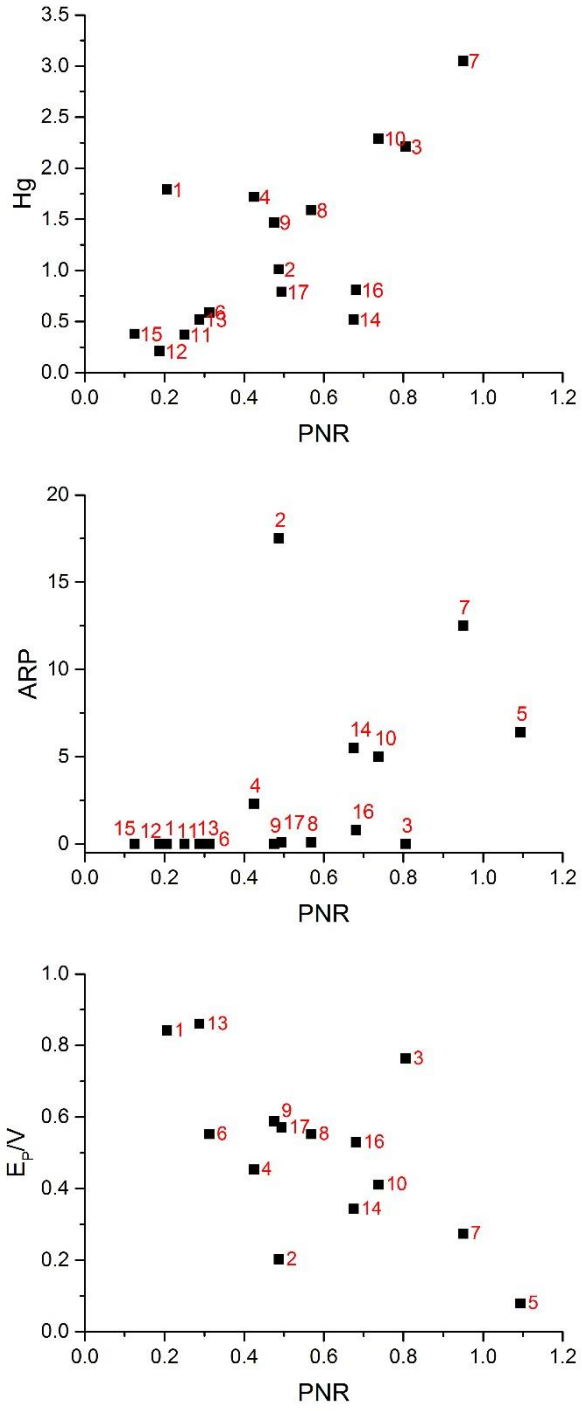


Figure 7

Research Highlights:

- Antioxidant activity of active principles of foods is studied by DPPH, CUPRAC and novel electrochemical methods.

- Pt nanoparticles on poly-neutral red electrode improves the measuring time and easiness with respect to the rest of the methods.

- Other advantage of this electrode is its closeness to physiological conditions: pH close to 7, absence of non-aqueous solvent and high ionic strength.

- The modified glassy carbon electrode is at least so sensitive as DPPH or CUPRAC