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Review

Biosensors as a tool for the antioxidant status evaluation

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Abstract

Antioxidants are one of the main ingredients that protect food attributes by preventing oxidation that occurs during processing, distribution and end preparation of food. Physiological antioxidant protection involves a variety of chemical system of endogenous and exogenous origin in a multiplicity of pathways. Associate to this, researches have been directed in the development of methods as biosensors that can characterize antioxidants capable of removing harmful radicals in living organisms in an adequate way. Biosensors have represented a broad area of technology useful for environmental, food monitoring, clinical applications and can represent a good alternative method to evaluate the antioxidant status.

The demonstration of the highlighted current application of biosensor as a potential tool to evaluate the antioxidant status is the main aim of this review.

Keywords: Antioxidant; Biosensors; Oxidative stress

Contents

Abbreviations: AA, antioxidant activity; AOD, ascorbate oxidase enzyme; Aox, antioxidant; AscH, ascorbic acid; CA, caffeic acid; CAT, catalase enzyme; CFME, carbon fiber microelectrode; CGA, chlorogenic acid; CL, chemiluminescence; CNT, carbon nanotube; Conduct., conductometric; CPE, carbon paste electrode; Cys, cysteine; Cyt. *c*, cytochrome *c*; D.L., detection limit; DHA, dehydroascorbic acid; ECL, electrochemiluminescent; Fluoresc., fluorescence transducer; GAO, galactose oxidase enzyme; GCE, glassy carbon electrode; GSH-Px, glutathione peroxidase enzyme; GSHR, glutathione reductase enzyme; GSH-SOx, glutathione sulfhydryl oxidase enzyme; GTE, graphite-teflon electrode; HRP, horseradish peroxidase; MB, methylene blue; ME, modified electrode; PGE, pyrolytic graphite electrode; POD, peroxidase enzyme; SAM, self assembly monolayer; SGE, spectrographic graphite electrode; SPE, screen-printed electrode; SOD, superoxide dismutase enzyme; tyr, tirosinase enzyme; UOD, urate oxidase enzyme; UR, uricase enzyme; Voltam., voltammetric; XOD, xanthine oxidase enzyme

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1. Introduction

The study of free radicals and antioxidants in biology and food technology has been widely recognized. The prevention of the oxidative reactions in foods, pharmaceutical and cosmetics as well the role of reactive oxygen species (ROS) in chronic degenerative diseases are questions that continue to be investigated due to the difficulties associated to the methods used in the evaluation of antioxidant status in these systems.

Methods for assessing antioxidant properties can be classified basically into two broad categories reflecting the idea on activity in foodstuffs and the antioxidant behavior in human body. In case of food systems, the necessity is the evaluation of the efficacy of an antioxidant in providing protection for the product or the necessity to characterize possible antioxidant compounds. Another category involves the contribution of endogenous antioxidants or nutrients for the modulation of the pathological consequences of ROS *in vivo* activity. In this sense, antioxidants tests can be basically classified in two groups: those assays based on the inhibition of the human low-density lipoprotein oxidation and those assays used to measure oxygen free radical scavenging ability. Several procedures are reported in the literature for measuring the total antioxidant capacity of a biological sample or plant extracts. The principles and the information that these methods provide have been extensively reviewed [1–3].

The evaluation of antioxidant properties is not an easy task. Many methods can be used to determine this activity, however several factors can affect the estimated activity, such as conditions and analytical methods [4,5]. Thus, *in vitro* systems are easier, faster and more cost-effective compared to the traditional bioassays *in vivo*. Therefore, test of the direct antioxidant capacity *in vitro* is useful, because if a substance that is poorly effective *in vitro* will not be better *in vivo*. It can also be evaluated about some possibility of damaging effects [6]. In this context is adequate to define the terms *antioxidant activity* and *antioxidant capacity* whose meanings are quite distinct. The antioxidant activity corresponds to the rate constant of a single antioxidant against a given free radical. The antioxidant capacity is the measurement of moles of a given free radical scavenged by a test solution, independently of the antioxidant present in the mixture [7].

Researches have been directed to the development of methods as biosensors that can characterize antioxidants capable of removing harmful radicals in living organisms in an adequate way. Since the pioneering work of Clark, biosensors have represented a broad area of emerging technology useful for environmental and food monitoring. In clinical applications as diagnostics delivering devices of important diseases and related to the ROS generation in excess such as diabetes. Recently this method has been cited for cancer-related screening testing [8,9]. This progress in potential application of biosensors can be attributed to its inherent characteristics such as rapid and real-time analysis that increase the assay speed and flexibility as well as the possibility of automatic and multi-target analyses with low cost. In this sense, the concept of biosensors also proceeded for a more wide modern definition in comparison to the proposed by Clark and coworkers. Biosensors are a sub-group of chemical sensors and it can be define as a self-contained integrated device, which is able to provide specific quantitative or semi quantitative analytical information using a biological recognition element (biochemical receptor), which is retained in direct and spatial contact with the transduction element [10].

Evaluations of the antioxidant status by means of biosensors have been carried out by measurements of biomarkers. Biomarkers are biological molecules whose chemical structure have been modified as a result of an attack by free radicals or others reactive species and that can be used as indicators to reliably asses oxidative stress status in animals models and in humans.

Developed biosensors employ different strategies ranging since direct analysis of compounds with characteristics antioxidants, measuring the antioxidant enzymes activity and detection of free radicals. Most of them uses immobilized enzymes in combination with electrochemical transducer, in particular, amperometric devices. Enzyme-based biosensors are very suitable since they show excellent selectivity for biological substances and can directly determine and/or monitor antioxidant compound in a complex media such as biological or vegetable samples without needing a prior separation step. Those amperometric transducer combine redox enzymes reactions to measure either consumed oxygen or produced hydrogen peroxide by oxidase enzymes and the reduced form of β-nicotinamide adenine dinucleotide (phosphate) NAD(P)H by dehydrogenase enzymes. During the course of the catalytic reaction on the electroactive substrates the current produced at an applied potential is related to the concentration of the species in solution. The measured concentration is attributed to a specific biomarker, which the biosensor is selective. This species acts like an antioxidant in the normal biological process. Various enzyme systems and simple compound known as endogenous antioxidants are essential to remove (scavenging) reactive oxygen species from the body to prevent their harmful effects. When the antioxidant capacity of a biological system is overwhelmed by the presence of excess free radical reactive species or the concentration of antioxidants are not sufficient to protect against oxidation, the disease processes are accelerated by oxidative damage to cells. In this sense, the biosensor monitoring the biomarker concentration may yield information associated to the degree of disease or disorder state in light of a particular clinical or research interest.

This relation is also applied to the developed biosensors to monitor radical species formed *in vitro* specific system. Oxygen free radicals are very difficult to measure directly because of their highly reactive nature and their very short half-life. The toxic byproducts of these reactions are related to the biomolecules modifications and the concentration of these byproducts as well as some related enzymes are measured by biosensors to reflect the degree of oxidative damage.

An interesting investigation of overall antioxidant status of the biological system is the potential benefit to health caused by the compound with antioxidant action supplied from nutritional supplements or dietary. Epidemiological studies suggest that these nutrients have a positive effect on general health and more specifically on cardiovascular diseases and cancer [11]. Potential antioxidants analyzed by biosensors include among others,

Vitamin C and phenol-derived compounds. The measured concentration of these compounds present in plant-origin samples corresponds to the quality of the dietary used as antioxidant supplement.

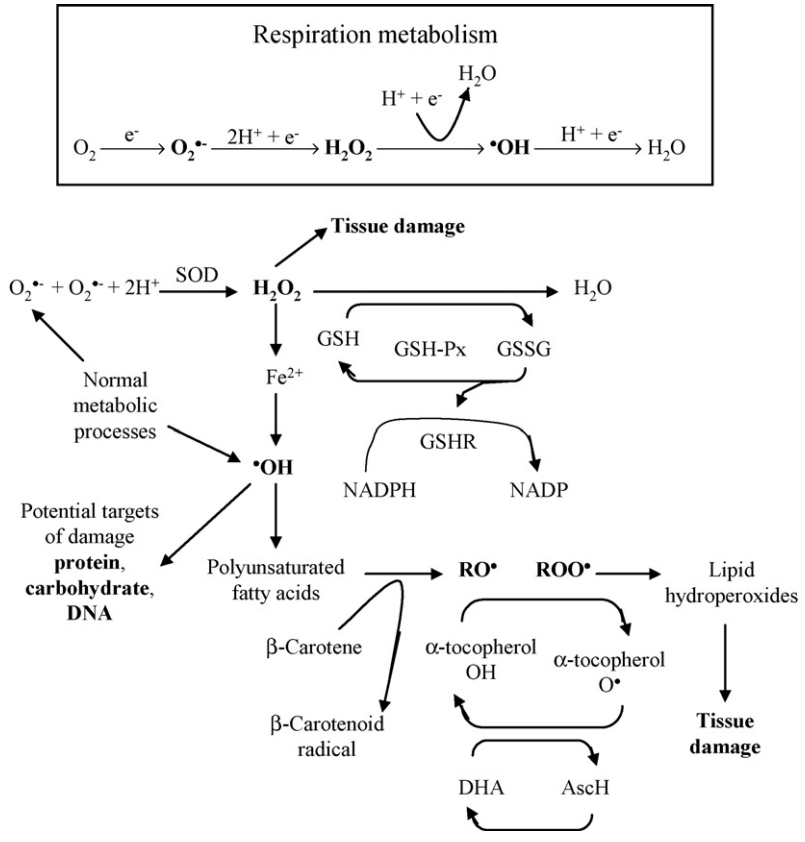
An important indicator in oxidative stress research is the identification of specific oxidized protein in human disease. Damage to DNA is the major endogenous type of pathogenic that induces a variety of diseases including cancer [12], which justify the importance of its early detection. DNA-based biosensors have been used in the identification and quantification of DNA molecules in disease diagnostics; detection of pathogenic organism and detection of toxins [9] and also it has been successful used as a screening method to detect DNA damage. The composition of the developed systems so far consist in a DNA layer immobilized on a surface electrode as oxidation target and using a method of inducing damage such as chemical agent or ionizing radiation. Among the modified substrates, the guanine is the most easily oxidized with the lowest oxidation potential of the nucleic acid bases and its oxidation product 8-oxoguanine is considered a clinical biomarker for oxidative DNA damage [13]. The detection principle reflects the fact that the response of DNA (signal relation to changes of guanine base) is strongly dependent on its structure. Samples containing compounds that can neutralize damaging agents that interact with DNA in an irreversible way and as a result maintain almost constant the DNA signal, can be characterized as antioxidant. The application of these biosensors as screening procedure of antioxidant constitute in a great alternative because the test reduce the time, complexity and cost of the analysis.

In this context, the demonstration of the current application of the biosensors as a method to evaluate the antioxidant status is the main aim to stimulate further investigation in this area.

2. Outline of biology and chemistry of free radicals and antioxidants

Oxidation is a spontaneous process and constitutes in the main deterioration factor in foods, cosmetics and pharmaceutical, causing alteration in its constituents, which result in a key factor in the nutritional and commercial value of these products. These reactions are caused by the atmospheric oxygen or less frequently by ozone, peroxide or oxidant metals and other agents. In biological fluids, the oxidation reactions are involved in important metabolic routes, during the normal metabolism, but an imbalance in the production and/or removal of these primary products induce an oxidative stress condition [14]. The relevant inter-relationships between ROS and antioxidants are shown in Scheme 1.

In case of *in nature* or manufactured products the oxidation reaction usually begins in the lipid fraction, eventhough other components such as proteins, vitamin and pigments are affected in different manner. In living system, the most susceptible targets to the alteration, beyond lipids in cellular membranes are proteins, enzymes and mainly carbohydrates and DNA in tissues [14].



Scheme 1. Relevant relationships between ROS and antioxidants (adapted from [15]).

Table 1
Reactive oxygen species and relevant antioxidants

ROS	Nonenzymatic antioxidants (LMWA)	Enzymatic antioxidants
Singlet O_2	NADPH and NADH	Catalases (CAT)[a]
$O_2^{\bullet -}$ (superoxide radical)	Glutathione (GSH) and thiols	Glutahione peroxidase (GSH-Px)[a]
H_2O_2 (hydrogen peroxide)	Ubiquinol (coenzyme Q)	Superoxide dismutase (SOD)[a]
$^{\bullet}OH$ (hydroxyl radical)	Uric acid	Ceruloplasmin (Cu)[b]
$NO^{\bullet}$ (nitric oxide)	Carotenoids (most commonly β-carotene)[c]	Albumin (Cu)[b]
Lipid peroxide	Vitamin C (ascorbic acid)[c]	Transferin (Fe)[b]
$RO^{\bullet}$ (alkoxyl radical)	Vitamin E (tocopherols)[c]	Ferritin (Fe)[b]
$ROO^{\bullet}$ (peroxyl radical)	Phytochemicals[c]	Myoglobin (Fe)[b]

[a] Free radical scavenging enzymes.
[b] Metal binding proteins.
[c] Dietary antioxidants.

In contrast, the mammalian cells have developed a complex antioxidant defense system to minimize the toxic effect of partially reduced oxygen species. A broader definition of an antioxidant is any substance that when present at low concentration compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate [6] and include two classes, primary and secondary antioxidants (Table 1). The primary or chain-breaking antioxidant, AH, include protection by the synthetic and natural origin low-molecular weight antioxidants (LMWA). When it is present in trace amounts, may delay or inhibit the initiation step by reacting with a lipid radical or inhibit the initiation step by reacting with peroxyl or alkoxyl radical resulting in a lesser reactive radical ($A^{\bullet}$) (reactions (1) and (2)). Besides it can act as chain reaction terminators by forming peroxide antioxidant compounds (reactions (3) and (4)) [1].

$$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet} \quad (1)$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \quad (2)$$

$$RO^{\bullet} + A^{\bullet} \rightarrow ROA \quad (3)$$

$$ROO^{\bullet} + A^{\bullet} \rightarrow ROOA \quad (4)$$

Another form of protection is the preventive or secondary antioxidants that retard the rate of oxidation through the enzymatic activities in different mechanisms such as metal chelating, singlet oxygen quenchers or repair systems of damage resulting in oxidation [1].

3. Potential applications of biosensor for evaluation of antioxidant status

Tables 2–9 present the successful application of biosensors for evaluation of antioxidant status. A brief comment about the main aspects of the biology and chemistry of free radicals and antioxidant in the beginning of each topic are also described.

The review was carried out describing works reported in the literature since 2000. In sequence, tables with biosensors applied for characterization of antioxidants based on radical scavenging property, detection/determination of free radicals, antioxidant compounds and enzymatic activity in blood plasma, urine, whole cells, even tissue or natural origin samples, without sample pretreatment. The study is completed with analysis of phytochemicals and synthetic antioxidants that have not recognized as essential nutrients, but apparently play an important antioxidant role in the body. Among the types of biosensors, it is observed those based on electrochemical transducer have dominated this research field. The main reasons are the chain-breaking activity of both electron-transferring and hydrogen-donating antioxidants, in most cases, showing a redox mechanism that is very suitable for the electrochemical measurements.

3.1. DNA as oxidation target

Studies of DNA damage induced by ROS are of special importance because it is the repository of genetic information and it is also present in single copy. It is characterized by a variety of modifications at DNA level that include base and sugar lesions, strand breaks, DNA-protein cross link and base-free sites. However, DNA of all mammalian cells contains trace amounts of modified bases that are indicative of attack by oxidiz-

Table 2
Biosensors applied to detect the antioxidant activity

Target DNA	Intercalator	Transducer[a]	Sample	Precision (as R.S.D.)	Reference
dsDNA (*Calf thymus*)	$[Co(phen)_3]^{3+}$	Voltam. (SPE)	Plant extracts	19% ($n=8$)	[19]
dsDNA (*Calf thymus*)	$[Co(phen)_3]^{3+}$	Voltam. (SPE)	Yeast	10% ($n=10$)	[20]
dsDNA (*Calf thymus*)	$[Co(phen)_3]^{3+}$	Voltam. (SPE)	Flavonoids		[21]
dsDNA (Salmon)	MB	Voltam. (ITO)	Gallic acid and glutathione		[22]
dsDNA (*Calf thymus*)	–	Voltam. (SPE)	Tea extracts	16% ($n=6$)	[23]

[a] Working electrode.

Table 3
Biosensors applied in the superoxide radical detection

Biocomponent	Immobilization	Transducer[a]	Detection range (D.L.)	Stability	Reference
GAO, SOD, tyrosinase	Physical adsorption	Amp. (O_2)	20–250 μmol L^{-1} (1 μmol L^{-1}) (tyr), 100–1200 μmol L^{-1} (10 μmol L^{-1}) (GAO)	3 days (tyr), 3 days (GAO)	[29]
SOD	Physical adsorption	Amp. (H_2O_2)	20–2000 μmol L^{-1} (10 μmol L^{-1})	≥7 days	[30]
Hemin	Physical adsorption	Amp. (PGE)	–	–	[31]
Cyt. *c*	SAM	Amp. (Au)	–	5 days	[32]
SOD	Physical adsorption	Amp. (H_2O_2)	–	–	[33]
SOD	Physical adsorption	Amp. (O_2)	20–1500 μmol L^{-1} (10 μmol L^{-1})	4 days	[34]
SOD	Physical adsorption	Amp. (ME)	–	–	[35]
Cyt. *c*	SAM	Amp. (Au)	–	–	[36]
Cyt. *c*, AOD, XOD	SAM	Amp. (Au)	–	–	[37]
SOD	Chemical cross-linking	Amp. (Pt)	≥100 μmol L^{-1}	–	[38]
Cyt. *c*	SAM	Amp. (Au)	–	–	[39]
Cyt. *c*	SAM	Amp. (Au)	–		[40]
SOD	SAM	Amp. (Au)	13–130 nmol L^{-1} (6 nmol L^{-1})	7 days	[41]
SOD	SAM	Amp. (Au)	13–130 nmol L^{-1} (6 nmol L^{-1})	–	[42]
SOD	Physical adsorption	Amp. (H_2O_2)	–		[28]
Cyt. *c*	SAM	Amp. (Au)	0.4–1.5 μmol L^{-1}	–	[43]
SOD	Physical adsorption	Amp. (H_2O_2)	20–2000 μmol L^{-1}	–	[44]
SOD	Physical adsorption	Amp. (H_2O_2)	20–2000 μmol L^{-1}	–	[45]
SOD, HRP	Sol–gel encapsulation	Fluoresc. (glass slide)	<0.02 μmol L^{-1}	–	[46]
SOD	Sol–gel encapsulation	Amp. (Au)	0.2–1.6 μmol L^{-1} (0.1 μmol L^{-1})	>20 days	[47]
Cyt. *c*	SAM	Amp. (SPE –Au)	0.3–1.2 μmol L^{-1}	–	[48]
SOD	Physical adsorption	Amp. (H_2O_2)	20–2000 μmol L^{-1}	–	[49]
SOD	Chemical cross-linking	Amp. (Pt)	20–2000 μmol L^{-1} (10 μmol L^{-1})	30 days	[50]
Cyt. *c*	Physical adsorption	Amp. (ME)	0.86–5.93 μmol L^{-1} (0.50 μmol L^{-1})	30 days	[27]
SOD	SAM	Amp. (CFME)	13–105 nmol L^{-1}	7 days	[51]

[a] Working electrode or transducer.

Table 4
Biosensors applied in the detection of nitric oxide

Biocomponent	Immobilization	Transducer[a]	Detection range (D.L.)	Stability	Reference
HRP	Chemical cross-linking	Amp. (GCE)	2.7–11 μmol L^{-1} (2 μmol L^{-1})	–	[59]
Hemoglobin and DNA	Physical adsorption	Voltam. (PGE)	16.3–163 μmol L^{-1}	–	[60]
Hemoglobin	Physical adsorption	Voltam. (PGE)	<5 μmol L^{-1} (20 pmol L^{-1})	–	[61]

[a] Working electrode.

ing species being removed by excision repairing enzymes and they are known to accumulate with age that can be associated with disease processes [12].

One of the most reactive radical species that induce lesions in DNA is the hydroxyl radical (•OH) generated among other systems by the Fenton reaction (reaction (5)). This species cause cell injury when they are generated in excess or the cellular antioxidant defense is impaired.

$$\mathrm{DNA[Fe(II)] + H_2O_2 \rightarrow DNA[Fe(III)] + OH^- + {}^{\bullet}OH} \quad (5)$$

When •OH is generated adjacent to DNA, it attacks both the deoxyribose sugar and the purine and pyrimidine bases resulting intermediates radicals, which are the immediate precursors for DNA base damage [16]. In living systems many

Table 5
Biosensor applied in the analysis of glutathione and enzyme activity

Biocomponent	Immobilization	Transducer[a]	Sample	Detection range (D.L.)	Stability	Reference
GSH-SOx, HRP	Chemical cross-linking	Amp. (CGE)	–	1–200 μmol L^{-1} (0.50 μmol L^{-1} GSH)	6 days	[71]
GSH-Px	Chemical cross-linking	Amp. (PGE)	Serum	19–240 μmol L^{-1}	60 days	[72]
GSHR	Chemical adsorption	Amp. (GCE)	–	–	–	[73]
Tyrosinase	Physical adsorption	Amp. (CPE)	–	1–8 μmol L^{-1} (100 nmol L^{-1})	–	[74]
Cucumber	Physical cross-linking	Amp. (O_2)	Plant	0.1–2 μmol L^{-1}	60 days	[75]
HRP	Physical cross-linking	Amp. (GCE)	–	0.04–90 μmol L^{-1} (0.3 nmol L^{-1})	30 days	[76]
Biosensor for enzyme activity						
GSHR	Physical adsorption	Amp. (Pt)	Erytrocyte hemolysate	0.08–0.74 IU L^{-1}	–	[77]

[a] Working electrode.

Table 6
Biosensors applied in the uric acid determination

Biocomponent	Immobilization	Transducer[a]	Sample	Detection range (D.L.)	Stability	Reference
UR	Physical adsorption	Conduct. (ME)	Serum	500–1250 mg L^{-1}	–	[88]
UR	Sol–gel encapsulation	CL (sol–gel matrix)	–	1–100 mg L^{-1} (0.1 mg L^{-1})	–	[89]
UOD, HRP	Physical cross-linking	Amp. (O_2)	Urine	0.1–0.5 μmol L^{-1}	30 days	[90]
UOD	Sol–gel encapsulation	ECL (sol–gel matrix)	Human serum	1–25 μmol L^{-1}	–	[91]
UR, HRP	Sol–gel encapsulation	Fluoresc. (sol–gel matrix)	Body fluids	<1 μmol L^{-1}	>90 days	[92]
UR	Physical adsorption	Amp. (GCE)	Urine	5–1000 μmol L^{-1} (2 μmol L^{-1})	20 days	[93]
UR	Physical adsorption	Amp. (CPE)	Serum	1–100 μmol L^{-1} (0.19 μmol L^{-1})	–	[94]
UR	Physical adsoprtion	Voltam. (CNT)	Animal tissue	0.1–500 μmol L^{-1} (0.05 μmol L^{-1})	20 days	[95]

[a] Working electrode or transducer.

of the hydroxyl radicals are generated from the metal (M) ion-dependent breakdown of hydrogen peroxide [17,18]. In the presence of ferrous or cupric ions, hydrogen peroxide is converted into the hydroxyl radical by Fenton's reaction. The Fenton-type system is important because it has been implicated as an important mediator of oxidative damage *in vivo* and it is of great interest in terms of reducing the possibility of mutation and consequently the cancer [18].

Mechanisms involving the Fenton system and other way of radical formation like ionizing radiation and nuclease activation have been suggested to evaluate the level of DNA damage that occurs in biological systems and the efficacy of the antioxidant compounds. Procedures based on incubation of calf-thymus DNA with a system producing $^{\bullet}OH$ radicals gives rise the extension of the chemical modifications in the DNA bases evaluating the scavenging property of dietary antioxidants by means of its action as modulators of this degradation process. It is the basic principle used in DNA-based biosensors for characterizing the antioxidants. In many cases are used the Fenton-type reaction as the inducing method to damage the biomolecule. The composition of the system generally consists in a double or single strand DNA layer immobilized on a transducer. Considering that hydroxyl radicals can interact with DNA bases as well as with deoxyribose residues, the antioxidant to be tested is evaluated by changes that occur into DNA layer by means of variation of purine base signal, usually guanine. Table 2 shows screening systems based on DNA-biosensor developed for measuring the antioxidant activity *in vitro* of several types of sample in an adequate and easy way.

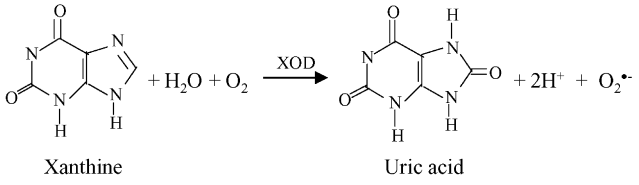
3.2. Monitoring superoxide radical ($O_2^{\bullet -}$)

Superoxide anion ($O_2^{\bullet -}$) a single-electron reduction product of oxygen is one the most abundant radical produced in biological systems. This cytotoxic species is quite reactive and is easily attacked by other active biomolecules (as nitric oxide), scavenged by enzymes (as superoxide dismutase) and antioxidants agents (as ascorbate). Under physiological conditions, superoxide radical is generated in significant quantities, in the nanomolar range, but the variation in its activity occurs in response to neurodegenerative disorders, diabetes, hypertension and others diseases [24,25].

In order to understand the pathological role of superoxides in cellular damage, researches have been leading on the determination of superoxide radical [26].

Electrochemical methods based on biosensors are particularly suitable to determine superoxide anion in a real-time and sensitive way (Table 3) and include two main types. The first group has as mechanism of reduction of cytochrome *c* immobilized on electrode surface by superoxide radical enzymatically produced in solution (reactions (6) and (7)). The superoxide measurement is by means of reaction between superoxide and cytochrome *c* followed by the further oxidation of the later at the electrode surface. The oxidation current is proportional to the superoxide concentration in solution. In this sense, several electrodes configurations have been reported showing the direct or mediated electron transfer between redox protein and the electrode [27].

In solution: Production of superoxide



Xanthine Uric acid (6)

At the electrode: Superoxide detection

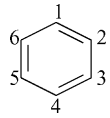
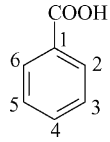
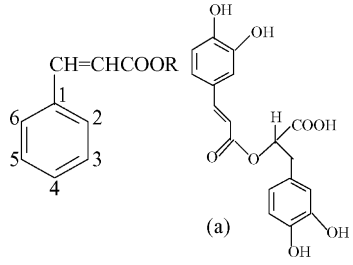
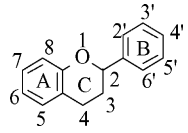
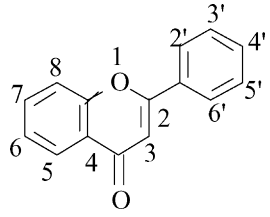
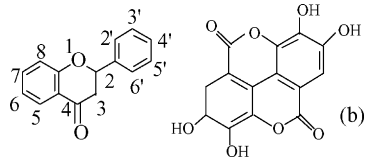
$$\text{Cyt. } c\,[\text{Heme}(Fe^{3+})] + O_2^{\bullet -} \rightarrow \text{Cyt. } c\,[\text{Heme}(Fe^{2+})] + O_2 \quad (7)$$

Table 7
Biosensors applied in the ascorbic acid determination

Biocomponent	Immobilization	Transducer[a]	Sample	Detection range (D.L.)	Stability	Reference
POD	Physical adsorption	Amp. (CPE)	Pharmaceutical formulations	200–500 μmol L^{-1} (22 μmol L^{-1})	–	[101]
AOD	Physical adsorption	Amp. (Pt)	Juice fruit	≥3000 μmol L^{-1} (5 μmol L^{-1})	–	[102]
AOD	Physical adsorption	Amp. (SPE)	Fruit	≥7000 μmol L^{-1}	–	[103]
AOD	Physical cross-linking	Amp. (O_2)	Commercial pharmaceutical	120–1000 μmol L^{-1}	25 days	[104]

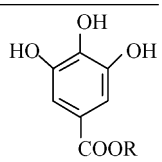
[a] Working electrode.

Table 8
Structures of relevant antioxidants in the dietary (adapted from [106])

Compound	Substituent	Structure
Benzenes		
Catechol	1,2-OH	
Resorcinol	1,3-OH	
p-Hydroquinone	1,4-OH	
Pyrogallol	1,2,3-OH	
Benzoic acid		
p-Hydroxybenzoic acid	4-OH	
Vanillic acid	3-OCH_3; 4-OH	
Syringic acid	3,5-OCH_3; 4-OH	
Gallic acid	3,4,5-OH	
Cinnamic acids		
p-Coumaric acid	4-OH; R = H	
Ferulic acid	3-OCH_3; 4-OH; R = H	
Caffeic acid	3,4-OH; R = H	
Chlorogenic acid	3,4-OH; R = quinic acid	
Rosmarinic acid (a)		
Flavans		
(+) - Catechin	3,5,7,3′,4′-OH	
(−) - Epicatechin	3,5,7,3′,4′-OH	
Flavonols		
Quercitin	3,5,7,3′,4′-OH	
Morin	3,5,7,2′,4′-OH	
Rutin	5,7,3′,4′-OH; 3-rutinose	
Kaempferol	3,5,7,4′-OH	
Flavones		
Apigenin	5,7,4′-OH	
Luteolin	5,7,3′,4′-OH	
Flavonones		
Naringenin	5,7,4′-OH	
Hesperetin	5,7,3′-OH; 4′-OCH_3	
Tannin		
Ellagic acid (b)		

In the second type of sensors, the superoxide radical is produced by the oxidation in aqueous solution of the xanthine to uric acid by the action of xanthine oxidase. In further step the superoxide dismutase immobilized on electrode surface, catalyses the specific dismutation of superoxide radical producing molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (reactions (8) and (9)). Usually, the electrooxidation of H_2O_2 at the electrode surface generates the detection signal. This oxidation reaction occurs in a high potential (>0.5 V versus Ag/AgCl), which result in interference problems, limiting the practical applications of superoxide based-biosensors. Strategies to improve the selectivity of the developed sensors include the using H_2O_2-

Table 9
Naturally derived antioxidant—gallates derivates

Compound	Substituent	Structure
Gallic acid	R = H	
Gallic acid methyl ester (methyl gallate)	R = CH_3	
Gallic acid propyl ester (propyl gallate)	R = $CH_2CH_2CH_3$	
Gallic acid lauryl ester (dodecyl gallate)	R = $CH_2(CH_2)_3CH_3$	

impermeable Teflon membrane or two-channel sensors. The later method allows the simultaneous determination of $O_2^{\bullet -}$ and H_2O_2 [28]. In both mechanisms the effect of the antioxidant substances added in reaction medium is observed by the decomposition of the radical (reaction (10)) resulting in diminished current levels.

Or *at electrode*: Enzymatic dismutation

$$SOD(Cu^{2+}) + O_2^{\bullet -} \rightarrow SOD(Cu^{+}) + O_2 \quad (8)$$

$$SOD(Cu^{+}) + O_2^{\bullet -} + 2H^{+} \rightarrow SOD(Cu^{2+}) + H_2O_2 \quad (9)$$

$$\text{Superoxide decomposition}: \quad O_2^{\bullet -} + \text{Aox} \rightarrow \text{Products} \quad (10)$$

3.3. Monitoring nitric oxide (NO)

Nitric oxide (NO, nitrogen monoxide) was characterized in 1987 as an endothelial-derived relaxing factor [52]. Since then, its biological function as an immune-modulator, neurotransmitter, vasodilator and a potential antioxidant have been studied. NO is a ubiquitous signal transduction molecule, which freely diffuses through cell membranes. Thus, it once formed from cells in different tissues can act as intervention in important biochemistry reactions such as: a mediator in a wide range of both antimicrobial and antitumor activities, an endothelial cell derived vasodilator and anticoagulant, a neurotransmitter with a crucial role in neural communications in the brain and peripheral nervous system [53]. It can also be a toxin in the destruction of pathogens and a precursor to oxidizing and nitrating species mainly at high concentrations or when it reacts with superoxide ($O_2^{\bullet -}$) forming the highly reactive peroxynitrite ($ONOO^-$). On the other hand, some reports carried out in lipid system showed that $NO^{\bullet}$ can inhibit oxidation, as chain-terminating antioxidant by react with chain-carrying peroxyl radicals during lipid peroxidation [54].

Endogenous NO is produced in a nonenzymatic or enzymatic way. Under specific physiological conditions, it is formed by reduction of NO_2^- promoted by ascorbic acid [55].

$$2NO_2^- + AscH^- + 3H^+ \rightarrow 2NO^{\bullet} + DHA + 2H_2O \quad (11)$$

Enzymatic generation of $NO^{\bullet}$ occurs by action of one of threes isoforms of nitric oxide synthase (NOS). This enzyme mediates the oxidation of guanidine nitrogen of L-arginine to L-citrulline producing $NO^{\bullet}$. Neuronal NOS_1 and endothelial NOS_3 are constitutively expressed and are estimulated to produce local $NO^{\bullet}$ concentration in the nanomolar range, whereas NOS_2 activity is inducible and is estimulated to produce concentration of $NO^{\bullet}$ in the micromolar range, for example in the response to inflammatory stimuli in hepatocytes [56].

Moreover the level of NO production can be varied as well as its functional role between cell types. The interaction of NO and reactive oxygen species (ROS) have assumed greater importance due to the simultaneous generation of both in various pathophysiological conditions [57]. Such interactions follow different pathways resulting either in protection or production of more toxic end products. Thus, the NO determination predicts the cellular state, considering that its abnormal production has been associated to a number of serious clinical conditions.

Measuring NO with high precision *in vitro* and/or biological media is very difficult due to its low concentration, short lifetime and difficulty to prepare standard solution [58]. Therefore, in this sense the recent literature concerning to the developed biosensors present few works and all using electrochemical transducer which present an inherent sensitivity and potential for real time determination of NO [26] as can be seen in Table 4. These biosensors use the property of high affinity of NO to heme group, in proteins such as peroxidase and hemoglobin combined with the redox behavior of NO. In a first approach the signal of the amperometric biosensor is related to the activity of Horseradish peroxidase in the presence of nitric oxide. The decrease in the current intensity associated to the electrocatalytic reduction of H_2O_2 is correlated to the concentration of NO in solution [59]. In the second approach, the biosensor response is due to direct electron transfer between hemoglobin and carbon-based electrodes, which allow the direct detection of NO in solution [60,61].

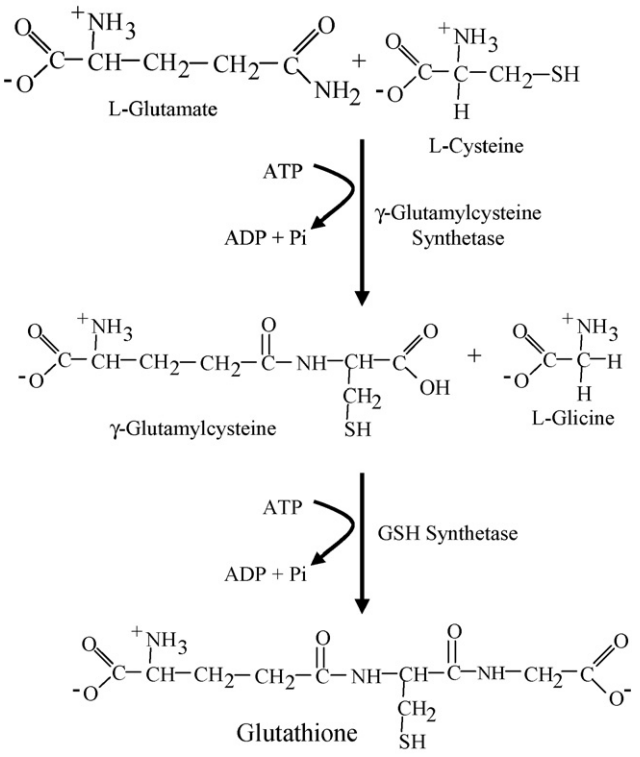
3.4. Monitoring glutathione (GSH)

Reduced glutathione (GSH) (IUPAC name is 2-amino-5-(2-carboxymethyl)-1-amino) is a low molecular weight tri-peptide found in abundance in all cells compartments along numerous other antioxidants such as urate, ascorbate and α-tocopherol [62]. GSH is the main nonprotein intracellular thiol-containing compound. The term total cellular glutathione refers to the fraction of other disulfides compounds that contain thiol groups such as cysteine, coenzyme A and cysteine residues in proteins [63].

GSH is synthesized from amino acids precursors: L-glutamate, L-cysteine and L-glycine by the consecutive action of γ-glutamylcysteine synthetase and GSH synthetase in two ATP-dependent reactions as shown in Scheme 2. First reaction is the limiting step due to the availability of cysteine maintaining a tight regulation of GSH pool [64]. Once GSH is formed, it could function itself or be degraded to participate in many detoxification and transport processes or metabolic pathways including the synthesis of certain leukotrienes and the reduction of disulfide bonds in proteins and DNA precursors [65].

The antioxidants functions of GSH are associated with its central nucleophilic cysteine residue, which is responsible for its high reductive potential. It is able to directly inactivate the reactive oxygen species $O_2^{\bullet -}$ or $^{\bullet}OH$ through donation of its hydrogen atom resulting in the formation of glutathione disulfide (GSSG) [66]. The concentration of GSH in mammalian cells is usually in the millimolar concentration range (0.5–10 mmol L^{-1}). The depletion in its concentration has been speculated to be an important contributing factor to some serious human diseases directly or indirectly associated with oxidative stress [67,68]. Thus, cited concentration in the organism can be relevant to the clinical diagnosis.

Methods based on enzymatic approaches that use oxidoreductase glutathione enzyme immobilized onto electrode surface or in-column reactors have shown to be very suitable for the determination of reduced thiols-derived intracellular compounds in a sensitive way. Biosensors for glutathione



Scheme 2. Biosynthesis of glutathione (GHS).

determination generally use glutathione reductase (GSHR) or glutathione peroxidase (GSH-Px) (Table 5). This later enzyme is specific for GSH as a co-substrate in the reduction of cytotoxic H_2O_2 and other hydroperoxides. GSH-Px enzyme contains four protein sub units, which contains one atom of selenium at its active site and GSH reduces the selenium atom resulting in the formation of GSSG (reactions (12) and (13)). Another biosensor response mechanism is based on formation of glutathione as a product in glutathione reductase (GSHR)-catalyzed reactions. In this case, the oxidized form of glutathione (GSSG) (Fig. 1) is reduced to GSH by glutathione reductase enzyme in the presence of NADPH as a cofactor (reaction (14)). The latter reaction is the great importance when occur in biological system because the reduced form of glutathione, GSH, maintains a redox balance in the cellular compartments. It allows fine-tuning of the cellular redox environment under normal conditions and upon the onset

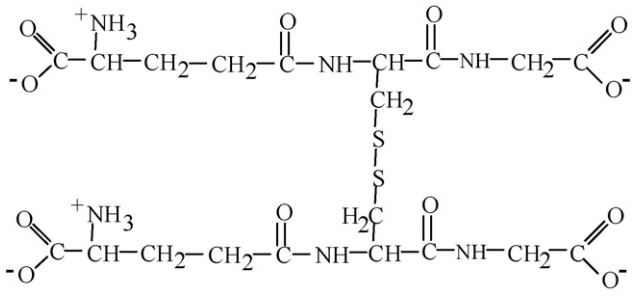


Fig. 1. Structure of oxidized glutathione (GSSG).

of stress and provides the basis for GSH stress signaling [69]. Indeed, the ratio of GSH to GSSG has been used as a marker of oxidative stress and associate to this the measuring of enzyme activity of glutathione reductase is also the great importance in pathological and clinical application to maintain the GSH level [70].

$$2GSH + H_2O_2 \xrightarrow{GSH\text{-}Px} GSSG + 2H_2O \quad (12)$$

$$2GSH + ROOH \xrightarrow{GSH\text{-}Px} GSSG + ROH + H_2O \quad (13)$$

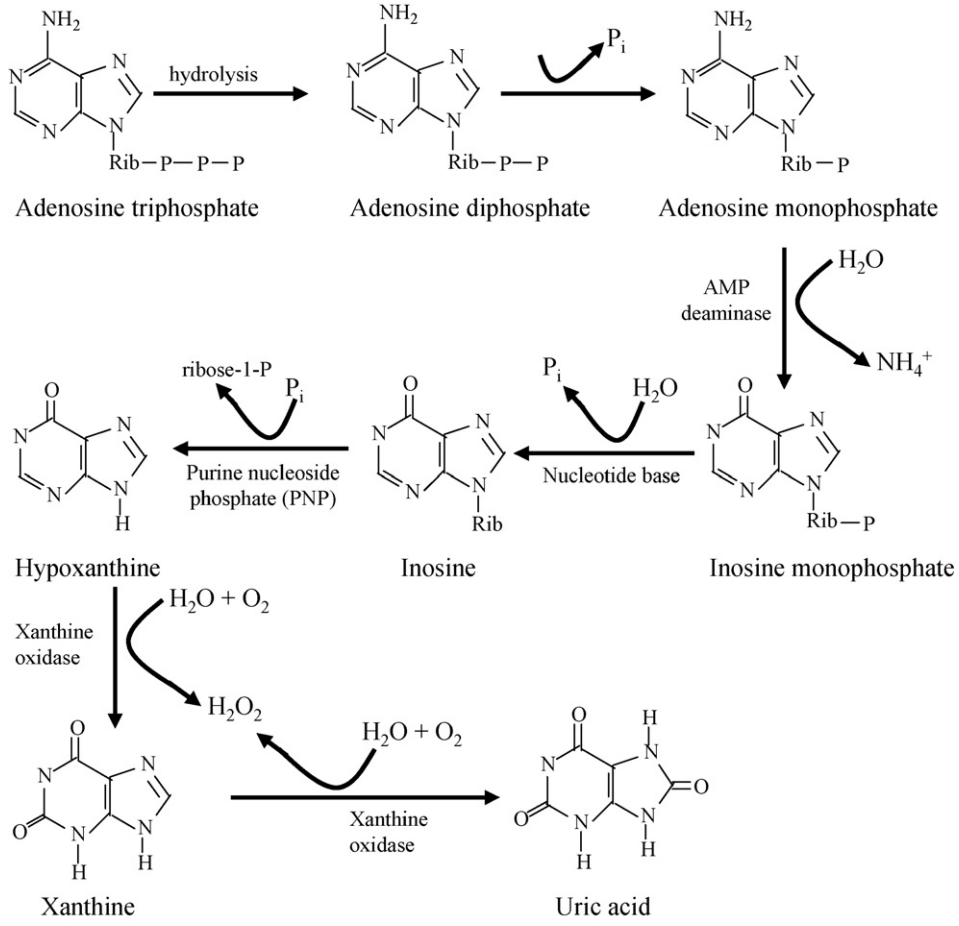
$$GSSG + NADPH + H^+ \xrightarrow{GSHR} 2GSH + NADP^+ \quad (14)$$

3.5. Monitoring uric acid

Uric acid, 2,6,8-trihydroxypurine is the final product of purine metabolism in humans. It is a weak acid and its dissociated form; the monosodium urate is the compound present in human plasma and extra-cellular liquid [78]. Uric acid precursors as well as the intermediates in purine biosynthesis include xanthine and hypoxanthine. As shown in Scheme 3, the product of the adenosine triphosphate (ATP) decomposition reaction, hypoxanthine (Hx) is converted to xanthine (X) and further undergoes in uric acid through xanthine oxidase (XOD) together with H_2O and O_2. This catabolic reaction was found to be the rate determining step in the overall reaction sequence [80,81].

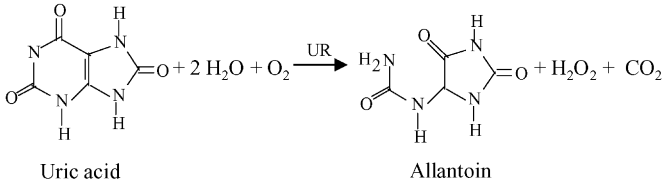
Uric acid is a physiological antioxidant effective inhibitor of the formation of superoxide radical and hydrogen peroxide that are oxygen reactive species formed by the action of xanthine oxidase during the catalysis of xanthine and hypoxanthine [82]. In the past, some studies estimated that uric acid contributes to 35–65% of the total plasma antioxidant capacity [83,84].

Uric acid levels higher than the normal is related to several diseases, being the most common the alteration of the plasma urate concentration known as hyperuricemia (or gout) [85]. Other medical conditions such as muscle damage, leukemia, pneumonia and hypertension have been associated with increased urate levels [86]. Additionally, increasing in purine excretion in urine can also monitor the activity of chemotherapeutic drugs as a result of the nucleoprotein degradation [81]. Thus, monitoring uric acid in human physiological fluids is very important in the diagnosis and therapy of patients suffering from a range of disorders associated with altered purine metabolism [87].Biosensors for uric acid determination are developed involving one (uricase) or two enzymes (uricase and peroxidase) according to Table 6, based on two approaches. In the first approach the use of uricase specifically catalyses the oxidation of urate resulting in the production of allantoin, in a similar mechanism that occurs in animals. The O_2 consumption or the H_2O_2 formed is usually monitored (reaction (15)). In the second method the H_2O_2 produced in the enzymatic oxidation of uric acid is consumed by the peroxidase. This indirect determination of uric acid is commonly observed in a HRP mediated



Scheme 3. Pathways of the formation of uric acid in human system based on purine degradation mechanism (adapted from [79]), Rib: ribose, P: phosphate group.

electrochemical system (reactions (16)–(18)).



(15)

$$HRP_{(reduced)} + H_2O_2 \rightarrow HRP_{(oxidized)} + H_2O \quad (16)$$

$$HRP_{(oxidized)} + Mediator_{(reduced)} \rightarrow HRP_{(reduced)} + Mediator_{(oxidized)} \quad (17)$$

$$Mediator_{(oxidized)} + H^+ + e^-_{(electrode)} \rightarrow Mediator_{(reduced)} \quad (18)$$

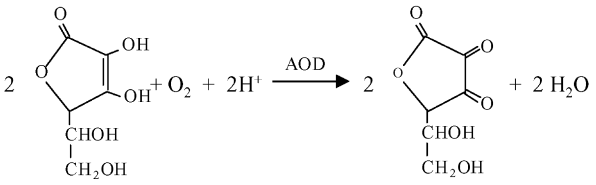
3.6. Monitoring ascorbic acid

L-Ascorbic acid (Vitamin C) is a γ-lactone synthesized by plants and many animals (except primates). It is a water-soluble vitamin usually used as an dietary supplement in pharmaceutical preparations and in manufacture food and beverages as an antioxidant, preventing color changes and alterations of aroma and flavor as well as extending the storage time of the products [96]. This vitamin is among the compound of major biological importance, it plays a key role in the protection against biological oxidation processes participating in many metabolic reactions. It has also been used in the treatment and prevention of common cold, mental illness and infertility. Traditionally, ascorbic acid has been used in clinical in the treatment and prevention of scurvy, but it is also important in regulation of the immunological system and in the tissue reconstruction, which assist the formation of collagen. However, an excess of ascorbic acid can cause gastric irritation and diarrhea and it is metabolized to oxalic acid, which can cause renal problems [97]. On the other hand, the ascorbic acid ingestion is associated to increase absorption of metal transition (as inorganic iron and copper), metabolism of folic acid, amino acid and in biosynthesis of suprarenal hormone, minimizing the stress effect [96].

There is a frequent citation about ascorbic acid as an exogenous important antioxidant. Its chemoprotective action is attributed to two mechanisms of action. It is indeed a good scavenger of free radicals and several reactive oxygen species produced during the metabolic pathways of detoxification. It justifies its association to protection against cancer agents by the prevention of formation of carcinogens precursors' compound [98].

Another property is its ability to act as a reducing agent (electron donor). Ascorbic acid (AA) is a reducing agent by donation of one electron giving the semi-dehydroascorbate radical (DHA). It also regenerates tocopherol from tocopheroxyl radical providing membrane protection [99,100]. Thus, Vitamin C determination is very important for biological and food industry.

Ascorbic acid has been measured by biosensors based on ascorbate oxidase enzyme that catalyze the direct oxidation of L-ascorbic acid in the presence of oxygen (reaction (19)) (Table 7). The monitoring of signal can be related to oxygen depletion using a Clark-type electrode. Other option can use a bienzymatic electrode coupled with peroxidase enzyme. In this later method the generated signal is related to the oxidation of ascorbic acid that acts as a mediator compound in the peroxidase catalytic mechanism.



L – Ascorbic acid Dehydro-L – ascorbic acid (19)

3.7. Monitoring phenol compounds

The active components usually originated from plant-based materials are naturally occurring inhibitors of oxidation. These antioxidants are primary phenolic-derived compounds widely distributed in the vegetable kingdom, mainly in the form of bioproducts generated from secondary plant metabolism. Its basic features are the presence of one or more hydroxylated benzene rings and it can be included in three large groups: phenolic acids (hydroxybenzoic acid and hydroxycinnamic acid), flavonoids (anthocyanidins, flavonols, flavononas, flavonas, isoflavonas and chalconas) and tannins (hydrolysable tannins and condensed tannins) [105] (Table 8). As a result, natural sources of phenolics constitutes in integral part of the diet, which are found as a complex mixture of compounds that provide many functional components present in the free, esterified, glycosylated and bound forms. Thus, with few exceptions such as carotene, this fraction is responsible by the majority of the antioxidant capacity of these foods [107].

The protection action mode of phenol may involve multiple mechanisms, depending on the source material and possible presence of synergists and antagonists. In general, the antioxidant activity of the phenolics-derived compounds is determined by its ideal chemical structure in terms of some properties such as: Free-radical scavengers or chain breakers agents, this property related to reducing properties as hydrogen or electron-donating agents, which is determined to its reduction potential. It also, the fact of the resulting antioxidant-derived radical, namely phenoxy radical is relatively stable due to the resonance delocalization and lack of suitable sites for attack by molecular oxygen. The last property, the transition metal-chelating potential, in special iron and copper supports the role of polyphenols as preventive antioxidants in terms of inhibiting transition metal-catalysed free radical formation [108–110].

It has been observed the worldwide growing trend in the usage of natural antioxidants in food industry justified by their health benefits. Thus, efforts have been made in order to find and to characterize substances of natural origin that exert some antioxidant activity. Commercial sources of antioxidants extracted from plant sources are green tea, spices and rosemary extracts. These later two are used as flavoring but exhibit good antioxidant properties in some cases [111].

Other good examples of effective naturally derived antioxidant that have been applied in food formulations are group of gallates (Table 9). Gallic acid often obtained from alkaline or acid hydrolysis of tannins or from hydrolysis of spent broths from *Penicillium glaucum* or *Aspergillus niger* that are used in the manufacturing of dodecyl gallate and propyl gallate, widely used as food antioxidant additive [112].

Table 10
Biosensors applied in the analysis of phenolic antioxidants

Biocomponent	Immobilization	Transducer[a]	Sample	Detection range (D.L.)	Stability	Reference
Tyrosinase	Physical adsorption	Amp. (O_2)	Olive oil	0.5–6 (0.04) mg L^{-1} (Catechol)	–	[115]
Tyrosinase	Physical adsoprtion	Voltam. (SPE)	Green tea	–	–	[116]
Catalase	Gel encapsulation	Amp. (O_2)	Olive oil	(9.23–5.04) × 10^4 μmol L^{-1} (Cumene hydroperoxide)	16 days	[117]
Tyrosinase	Physical adsorption	FIA (SPE)	Beer	–	–	[118]
Peroxidase	SAM	Amp. (Au)	Wine and tea	<25 (2) μmol L^{-1} (Catechin)	–	[119]
Tyrosinase	Physical adsoprtion	Amp. (CPE)	Wine	1–60 (0.1) mg L^{-1} (Gallic acid)	–	[120]
Peroxidase	Physical adsorption	Amp. (CPE)	Wine and tea	<15 (3) μmol L^{-1} (Catechin)	–	[121]
Laccase	Physical adsorption	Amp. (Pt)	–	10–110 μmol L^{-1} (Catechol)	–	[122]
Laccase	Physical adsorption	Amp. (Pt)	Wine	2.0–14.0 (1) μmol L^{-1} (Catechin and CA)	–	[123]
Peroxidase	Physical cross-linking	Amp. (CPE)	Tea	1–50 (0.7) μmol L^{-1} (CGA)	30 days	[124]
Laccase	Physical assorption	Amp. (SGE)	Plant extract	<10 (0.56) μmol L^{-1} (CA)		[125]
Tyrosinase	Physical cross-linking	Amp. (GCE)	Tea	70–400 (2.52) μmol L^{-1} (CGA)	–	[126]
Tyrosinase	Chemical cross-linking	Amp. (SPE)	Wine	10.6–266.0 (10.5) μmol L^{-1} (CA)	–	[127]
Tyrosinase	Physical adsorption	Amp. (GCE)	Wines	25–900 (7) μmol L^{-1} (Gallic acid)	<18 days	[128]

[a] Working electrode.

Table 11
Biosensors applied in the synthetic antioxidant analysis (propyl gallate)

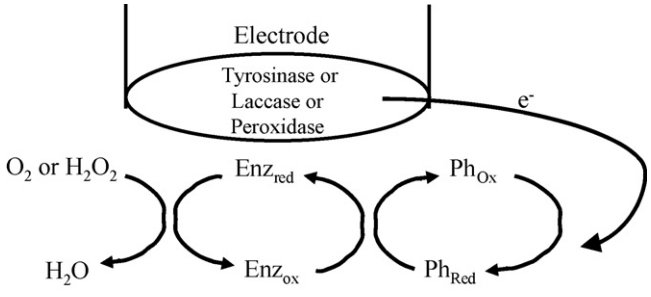
Biocomponent	Immobilization	Transducer[a]	Analyte	Sample	Detection range (D.L.)	Stability	Reference
Hematin	Physical adsorption	CL (fiber-optic)	Propyl gallate	Medicinal plants	100–1×10^5 μmol L^{-1}	–	[129]
Tyrosinase	Physical adsorption	Amp. (GTE)	Propyl gallate	Food samples	2–100 (0.9) μmol L^{-1}	40 days	[130]
Tyrosinase	Physical adsorption	Amp. (GTE)	Propyl gallate	Fatty food samples	8–200 (1.3) μmol L^{-1}	10 days	[131]

[a] Working electrode or transducer.

The detection of phenolic substances in food samples has been performed for many methods among them biosensor approaches. Phenolic-derived compounds are good substrate for oxidases enzymes, then biosensor modified with tyrosinase, laccase, peroxidase and cellobiose dehydrogenase, have been developed for detection of phenolic compounds since phenols can act as electron donor for these enzymes [113]. In many cases, it is more important to measure the total content of polyphenols compounds than to determine each of them individually [114]. The term "total phenol" refers the all phenols that are responsible by the total antioxidant capacity of a specific sample. Tables 10 and 11 present biosensors developed to determine the total polyphenols content and synthetic antioxidant, respectively. Many biosensors are based on electrochemical transducers that use the redox cycling of enzymes and have as main advantage the low interference.

The essential features of the general analytical system using oxidase or peroxidase enzyme based biosensor are shown schematically in Scheme 4. As observed, phenols oxidases and peroxidases have different enzymatic mechanisms in the electrochemical biosensors. The fundamental reaction is the oxidation of enzymes molecules at the surface of the electrode in the presence of substrate, oxygen (for phenol oxidases) or hydrogen peroxide (for peroxidase), the later being reduced to water. The next step reaction is the regeneration of the enzyme to its original oxidation state, which is carried out by the electron transfer from a suitable mediator, such as phenol compounds in its reduced form. The measured reduction current is due to reduction of the mediator to the original form.

The tyrosinase biosensors are restricted to the monitoring of phenolic compounds with at least one free *ortho*-position. On the other hand, the laccase biosensor can detect free *para*- and *meta*-position, but its catalytic cycle is complicated and in its major part is different from tyrosinase. Also, the formed products during the reaction of the laccase still are not well understood.



Scheme 4. Mechanism for the phenolic compounds detection using biosensors based on peroxidases or oxidases enzymes.

4. Concluding remarks

The idea in the improvement of food quality or prevention of antioxidant stress by means of interaction and synergisms of antioxidants demonstrated *in vitro* studies represents an important research field.

A consideration that must be done is the reliability and practicability of the methods; much of the literature in this field reports the use of a very small amount of sample combined with complex methods to easily generate a significant result.

In this direction, the measurement of the total antioxidant capacity or antioxidant power within biological specimens by biosensors is a challenge to be explored. By virtue of its advantages offered such as ready-to-use, miniaturization and reusable properties with the possibility to combine portable equipment, biosensors have been applied in research related to free radicals and antioxidants in several ways. It can monitor the formation of oxidized products in the first step of degradation of cellular targets. Once the stability, sensitivity and specificity of the biosensor have been demonstrated in several systems, it can be explored to detect radicals in the nanomolar range. In this situation, many times, the radical species, can involved in the beginning of inflammatory, apoptosis and /or vascular diseases.

Another option could be incorporated in manufacturing of food as a simple monitor of lipid food spoilage. Besides, the use of biosensor to classify the diet integrators or drug specialties related to antioxidant properties of the active principles that they contain. The direct measurement of the total antioxidant index of plant origin raw materials related to total content of compounds with antioxidant characteristics is another parameters explored by biosensors.

However, in this context, the biosensors developed so far to evaluate the antioxidant status, are based on direct analyze of specific indicators such as biomarkers or monitoring the free radicals in cells or tissue, without caring to obtain a correlation between, the index obtained of these indicators with the overall condition oxidative stress of sample.

The usefulness of biosensors to evaluate the antioxidant status lies in its ability to provide early indication of some disease and or its progression in a non-invasive way to evaluate the effectiveness of the antioxidant therapy. The results obtained by the biosensor just will be efficient if could be interpreted in terms of these cited parameters.

Acknowledgment

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