Chapter

Antioxidant Compounds and Their Antioxidant Mechanism

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Abstract

An antioxidant is a substance that at low concentrations delays or prevents oxidation of a substrate. Antioxidant compounds act through several chemical mechanisms: hydrogen atom transfer (HAT), single electron transfer (SET), and the ability to chelate transition metals. The importance of antioxidant mechanisms is to understand the biological meaning of antioxidants, their possible uses, their production by organic synthesis or biotechnological methods, or for the standardization of the determination of antioxidant activity. In general, antioxidant molecules can react either by multiple mechanisms or by a predominant mechanism. The chemical structure of the antioxidant substance allows understanding of the antioxidant reaction mechanism. This chapter reviews the *in vitro* antioxidant reaction mechanisms of organic compounds polyphenols, carotenoids, and vitamins C against free radicals (FR) and prooxidant compounds under diverse conditions, as well as the most commonly used methods to evaluate the antioxidant activity of these compounds according to the mechanism involved in the reaction with free radicals and the methods of *in vitro* antioxidant evaluation that are used frequently depending on the reaction mechanism of the antioxidant.

Keywords: antioxidants, oxidative stress, reactive oxygen species, free radical, hydrogen atom transfer, single electron transfer

1. Introduction

Oxidative stress in biological systems is a complex process that is characterized by an imbalance between the production of free radicals (FR) and the ability of the body to eliminate these reactive species through the use of endogenous and exogenous antioxidants. During the metabolic processes, a great variety of reactions take place, where the promoters are the reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and the superoxide radical anion ($O_2^{\bullet -}$), among others. A biological system in the presence of an excess of ROS can present different pathologies, from cardiovascular diseases to the promotion of cancer. Biological systems have antioxidant mechanisms to control damage of enzymatic and nonenzymatic natures that allow ROS to be inactivated. The endogenous antioxidants are enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, or non-enzymatic compounds, such as bilirubin and albumin. When an organism is exposed to a high concentration of ROS, the endogenous antioxidant system is compromised and, consequently, it fails to guarantee complete protection of the organism. To compensate this deficit

of antioxidants, the body can use exogenous antioxidants supplied through food, nutritional supplements, or pharmaceuticals. Among the most important exogenous antioxidants are phenolic compounds carotenoids and vitamins C and some minerals such as selenium and zinc.

In the study of antioxidant compounds and the mechanisms involved, it is important to distinguish between the concepts of antioxidant activity and capacity. These terms are often used interchangeably. However, antioxidant activity refers to the rate constant of a reaction between an antioxidant and an oxidant. The antioxidant capacity is a measure of the amount of a certain free radical captured by an antioxidant sample [1]. Therefore, during the selection of a method, the response parameter must be considered to evaluate the antioxidant properties of a sample, which may be a function of the concentration of the substrate or concentration and the time required to inhibit a defined concentration of the ROS.

The reaction mechanisms of the antioxidant compounds are closely related to the reactivity and chemical structure of FR as well as the environment in which these reactive species are found. Therefore, it is very important to describe the ROS and, to a lesser degree, the reactive nitrogen species (RNS), which include both precursors and free radicals.

In the literature, there are many *in vitro* methods to evaluate the effectiveness of antioxidant compounds present in a variety of matrices (plant extracts, blood serum, etc.) using lipophilic, hydrophilic, and amphiphilic media (emulsions). The *in vitro* methods can be divided into two main groups: (1) hydrogen atom transfer (HAT) reactions and (2) transfer reactions of a single electron (SET). These methods are widely used because of their high speed and sensitivity. When carrying out a study related to the antioxidant properties of a sample, more than one method is usually used to evaluate the antioxidant capacity/activity [2]. This chapter describes the methods of *in vitro* antioxidant evaluation that are used frequently depending on the reaction mechanism of the antioxidant.

2. Oxidative stress

Oxygen is associated with aerobic life conditions [3], representing the driving force for the maintenance of cell metabolism and viability and at the same time involving a potential danger due to its paramagnetic characteristics. These characteristics promote the formation of partially oxidized intermediates with a high reactivity. These compounds are known as reactive oxygen species (ROS). ROS are free radicals (FR) or radical precursors. In stable neutral molecules, the electrons are paired in their respective molecular orbitals, known as maximum natural stability. Therefore, if there are unpaired electrons in an orbital, highly reactive, molecular species are generated that tend to trap an electron from any other molecule to compensate for its electron deficiency. The oxygen triplet is the main free radical, since it has two unpaired electrons. The reaction rate of triplet oxygen in biological systems is slow. However, it can become highly toxic because it metabolically transforms into one or more highly reactive intermediates that can react with cellular components. This metabolic activation is favored in biological systems, because the reduction of O_2 to H_2O in the electron transport chain occurs by the transfer of an electron to form FR or ROS [4].

Free radicals in a biological system can be produced by exogenous factors such as solar radiation, due to the presence of ultraviolet rays. Ultraviolet radiation causes the homolytic breakdown of bonds in molecules. FR also occur during the course of a disease. In a heart attack, for example, when the supply of oxygen and glucose to the heart muscle is suspended, many FR are produced. Another exogenous factor is

chemical intoxication, which promotes the formation of FR. The organism, because it requires the conversion of toxic substances to less dangerous substances, promotes the release of FR. The toxicity of many drugs is actually due to their conversion into free radicals or their effect on the formation of FR. The presence of contaminants, additives, pesticides, etc., in food can also become a source of FR.

Inflammatory processes are due to endogenous factors that promote the presence of FR in the system. These FR, present inside the cleansing cells of the immune system, have the function of killing pathogenic microorganisms. Tissue damage is caused when FR are excessive during this process. Phagocytic cells (neutrophils, monocytes, or macrophages) use the NADPH oxidase system directly generating the superoxide ion ($O_2^{\bullet-}$). $O_2^{\bullet-}$ is considered the primary ROS and when reacting with other molecules through enzymatic processes or catalyzed by metals generates secondary ROS. $O_2^{\bullet-}$ is protonated to produce H_2O_2 and $HO_2^{\bullet}$. $O_2^{\bullet-}$ is produced from the irradiation of molecular oxygen with UV rays, photolysis of water, and by exposure of O_2 to organic radicals formed in aerobic cells such as $NAD^{\bullet}$, $FpH^{\bullet}$, semiquinone radicals, cation radical pyridinium or by hemoproteins. Likewise, it is produced by phagocytic leukocytes as the initial product of the respiratory explosion when consuming O_2. The radical $O_2^{\bullet-}$ does not react directly with polypeptides, sugars, or nucleic acids.

As a defense mechanism cells generate $^{\bullet}NO$ by the action of nitric oxide-synthase on intracellular arginine. The combination of O_2 with $^{\bullet}NO$ results in the formation of $ONOO^{\bullet}$, which induces lipid peroxidation in lipoproteins. This happens in a very marked way in autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, primary biliary cirrhosis, type 1 diabetes, celiac disease, Graves' disease, Hashimoto's disease, inflammatory bowel disease, scleroderma, multiple sclerosis, psoriasis, and vitiligo.

FR are necessarily present during metabolic processes because many of the chemical reactions involved require these chemical species. For example, the reactions of polymerization of amino acids to form proteins or the reactions of polymerization of glucose to form glycogen involve the participation of FR. FR are also involved in the catalytic activation of various enzymes of intermediary metabolism, such as hypoxanthine, xanthine oxidase, aldehyde oxidase, monoamine oxidase, cyclooxygenase, and lipoxygenase [5]. Generally, antioxidant enzymes efficiently control these radicals.

Another generating source of ROS is the structural alteration of essential macromolecules of the cell (DNA, protein, and lipids) by irreversible chemical reactions. These reactions generate derivatives, such as malonaldehyde and hydroperoxides that propagate oxidative damage.

Additionally, there are also RNS, such as nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), as well as peroxynitrite ($ONOO^{-}$), nitrosoperoxycarbonate ($ONOOCO_2^{-}$), and nitronium ions (NO_2^{+}), and the neutral species, peroxynitrous acid (ONOOH) and dinitrogen trioxide (N_2O_3). These species are generated in small amounts during normal cellular processes such as cell signaling, neurotransmission, muscle relaxation, peristalsis, platelet aggregation, blood pressure modulation, immune system control, phagocytosis, production of cellular energy, and regulation of cell growth [6]. **Table 1** shows the most representative FR present during the process of energy production in aerobic biological systems.

2.1 Oxidative damage to biomolecules

There are many ROS that act as biological oxidants, but the $O_2^{\bullet-}$ is the largest oxidant; the simple addition of a proton leads to the formation of $HO_2^{\bullet}$, becoming a very active oxidizing agent. These transformations are summarized in **Figure 1**.

Specie	Source	Function
$O_2^{\bullet -}$	Enzymatic process, autoxidation reaction, and nonenzymatic electron transfer reactions	It can act as reducing agent of iron complexes such as cytochrome-c or oxidizing agent to oxidize ascorbic acid and α-tocopherol
$HO_2^{\bullet}$	Protonation of $O_2^{\bullet -}$	$HO_2^{\bullet}$ initiates fatty acid peroxidation
$HO^{\bullet}$	H_2O_2 generates $HO^{\bullet}$ through the metal-catalyzed Fenton reaction	$HO^{\bullet}$ reacts with both organic and inorganic molecules including DNA, proteins, lipids, and carbohydrates
$NO^{\bullet}$	Action of nitric oxide-synthase using arginine as a substrate and NADPH as an electron source	$NO^{\bullet}$ is an intracellular second messenger stimulates guanylate cyclase and protein kinases and helps in smooth muscle relaxation in blood vessels
$NO^{\bullet}{}_2$	Protonation of $ONOO^-$ or homolytic fragmentation of $ONOOCO_2^-$	This radical acts on the antioxidative mechanism decreasing ascorbate and α-tocopherol in plasma
$ONOO^{\bullet}$	Reaction of O_2 with $NO^{\bullet}$	$ONOO^{\bullet}$ is a strong oxidizing and nitrating species of methionine and tyrosine residues in proteins and oxidizes DNA to form nitroguanine
$CO_3^{\bullet -}$	The intermediate of reaction superoxide dismutase (SOD)-Cu^{2+}-$OH^{\bullet}$ react with bicarbonate to generates $CO_3^{\bullet -}$	$CO_3^{\bullet -}$ oxidizes biomolecules such as proteins and nucleic acids
$ONOOCO_2^-$	The peroxynytrite-CO_2 adduct is obtained by reaction of $ONOO^-$ with CO_2	This anion promotes nitration of tyrosine fragments of the oxyhemoglobin via FR

Table 1.
Free radicals (FR) generated in biological systems.

Free radicals produce diverse actions on the metabolism of immediate principles, which can be the origin of cell damage [7]:

1. In the polyunsaturated lipids of membranes, producing loss of fluidity and cell lysis because of lipid peroxidation (**Figure 2**).

2. In the glycosides, altering cellular functions such as those associated with the activity of interleukins and the formation of prostaglandins, hormones, and neurotransmitters (**Figure 3**) [8].

3. In proteins, producing inactivation and denaturation (**Figure 4**) [9].

4. In nucleic acids, by modifying bases (**Figure 5**) [8], producing mutagenesis and carcinogenesis.

2.2 Physiological and physiopathological processes related to free radicals (FR)

The human body responds to oxidative stress with antioxidant defense, but in certain cases, it may be insufficient, triggering different physiological and physiopathological processes. Currently, many processes are identified related to the production of free radicals. Among them are mutagenesis, cell transformation, cancer, arteriosclerosis, myocardial infarction, diabetes, inflammatory diseases, central nervous system disorders, and cell aging [10, 11].

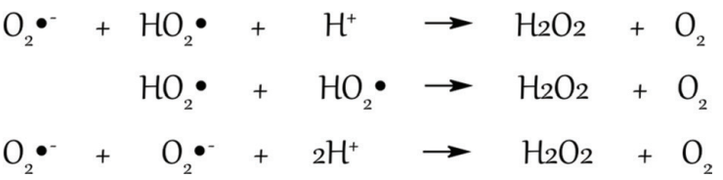

Figure 1.
Reaction mechanism of superoxide radical.

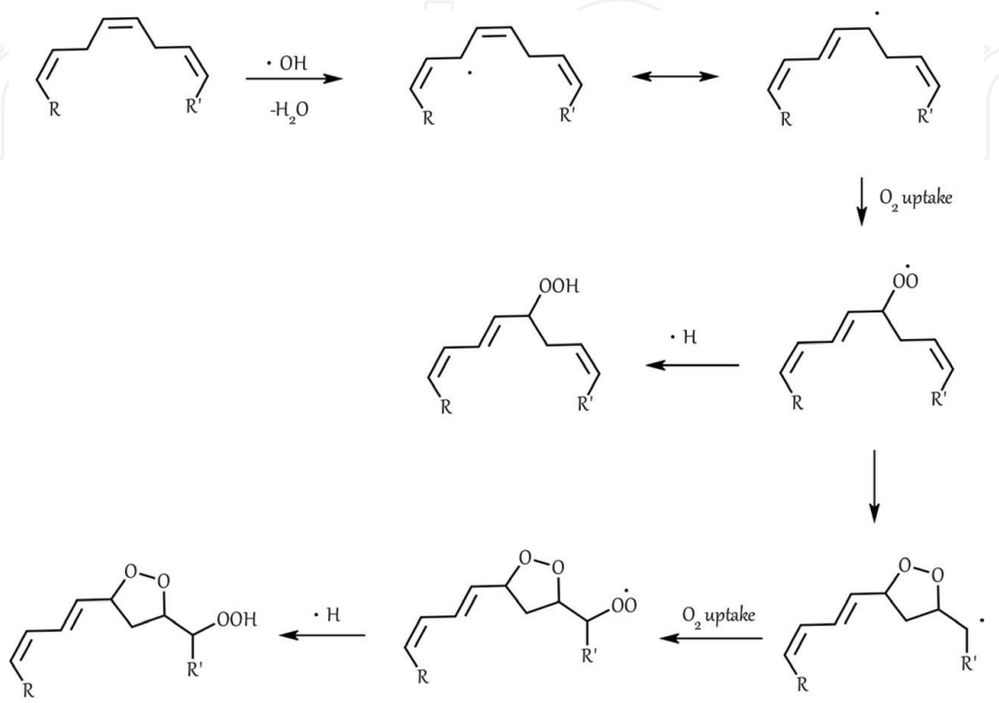

Figure 2.
Reaction of hydroxyl radical with polyunsaturated fatty acids.

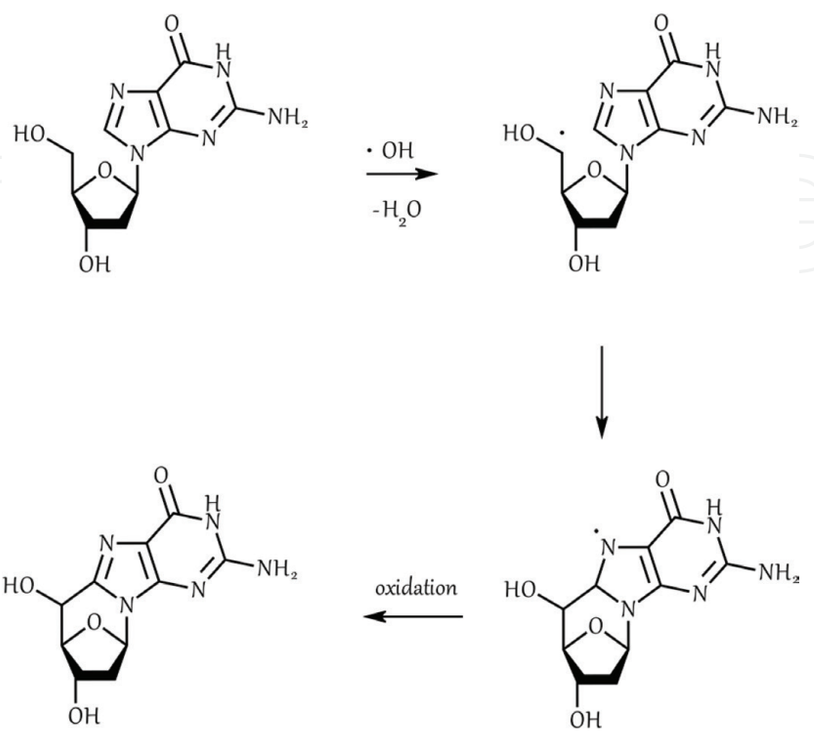

Figure 3.
Reaction of hydroxyl radical with sugar [8].

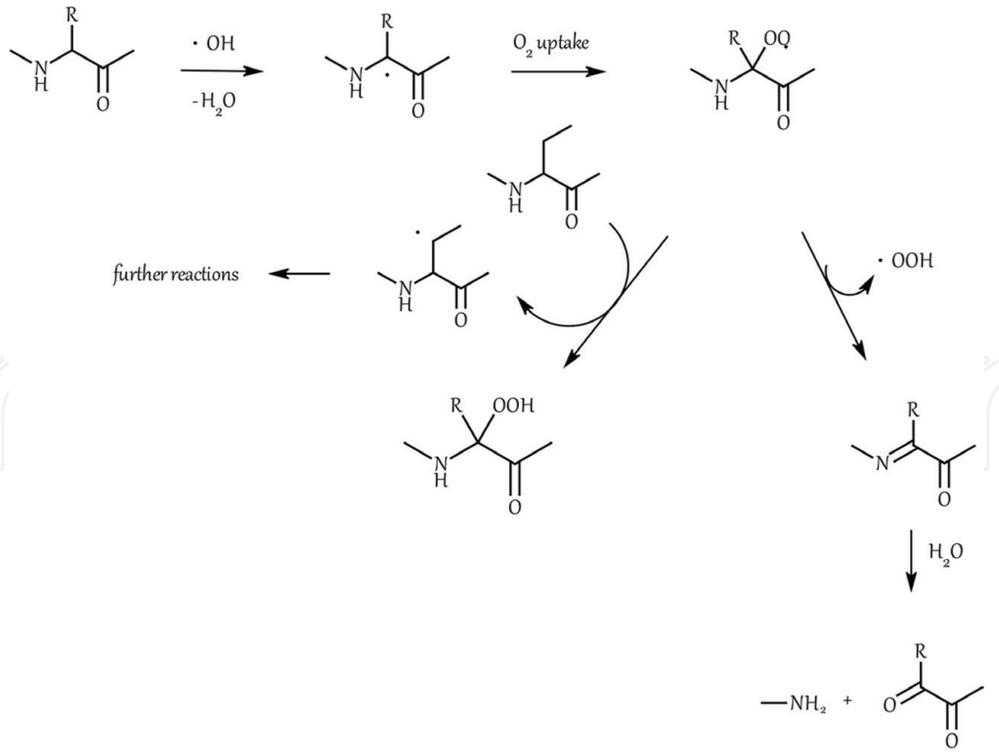

Figure 4.
Reaction of hydroxyl radical with α-aminoacids [9].

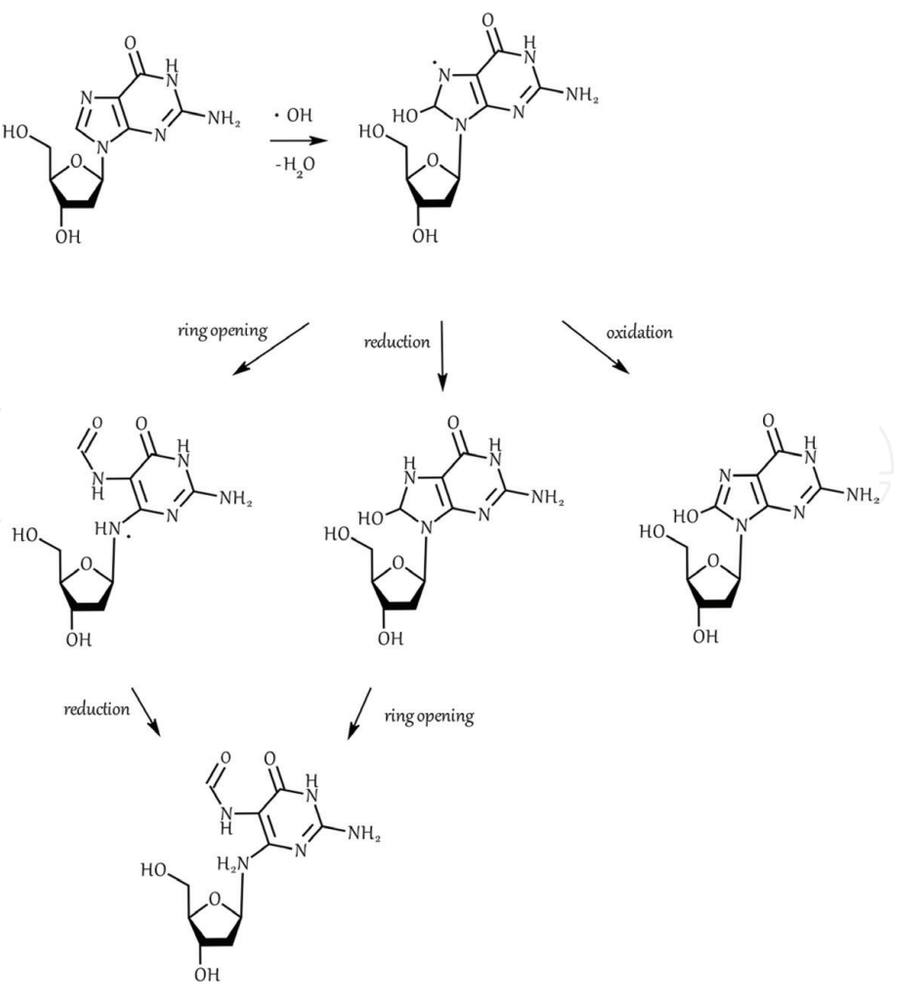

Figure 5.
Reaction of hydroxyl radical with the basepair of DNA guanosine [8].

3. Role of antioxidants

Biological systems in oxygenated environments have developed defense mechanisms, both physiological and biochemical. Among them, at the physiological level, is a microvascular system with the function of maintaining the levels of O_2 in the tissues, and at a biochemical level, the antioxidant defense can be enzymatic or nonenzymatic, as well as being a system for repairing molecules.

3.1 Primary enzymatic system

Aerobic organisms have developed antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and DT-diaphorase. SOD is responsible for the dismutation reaction of O_2 to H_2O_2, which in subsequent reactions, catalyzed by catalase or by GPx, is converted into H_2O and O_2. SOD is the most important and most powerful detoxification enzyme in the cell. SOD is a metalloenzyme and, therefore, requires a metal as a cofactor for its activity. Depending on the type of metal ion required as a cofactor by SOD, there are several forms of the enzyme [12, 13]. CAT uses iron or manganese as a cofactor and catalyzes the degradation or reduction of hydrogen peroxide (H_2O_2) to produce water and molecular oxygen, thus completing the detoxification process initiated by SOD [14, 15]. CAT is highly efficient at breaking down millions of H_2O_2 molecules in a second. CAT is mainly found in peroxisomes, and its main function is to eliminate the H_2O_2 generated during the oxidation of fatty acids. GPx is an important intracellular enzyme that breaks down H_2O_2 in water and lipid peroxides in their corresponding alcohols; this happens mainly in the mitochondria and sometimes in the cytosol [16]. The activity of GPx depends on selenium. In humans, there are at least eight enzymes GPx, GPx1–GPx8 [17]. Among glutathione peroxidases, GPx1 is the most abundant selenoperoxidase and is present in virtually all cells. The enzyme plays an important role in inhibiting the process of lipid peroxidation and, therefore, protects cells from oxidative stress [18]. Low GPx activity leads to oxidative damage of the functional proteins and the fatty acids of the cell membrane. GPx, particularly GPx1, has been implicated in the development and prevention of many diseases, such as cancer and cardiovascular diseases [19]. DT-diaphorase catalyzes the reduction of quinone to quinol and participates in the reduction of drugs of quinone structure [20]. DNA regulates the production of these enzymes in cells.

3.2 Nonenzymatic system

This system of antioxidants consists of antioxidants that trap FR. They capture FR to avoid the radical initiation reaction. Neutralize the radicals or capture them by donating electrons, and during this process, the antioxidants become free radicals, but they are less reactive than the initial FR. FR from antioxidants are easily neutralized by other antioxidants in this group. The cells use a series of antioxidant compounds or free radical scavengers such as vitamin E, vitamin C, carotenes, ferritin, ceruloplasmin, selenium, reduced glutathione (GSH), manganese, ubiquinone, zinc, flavonoids, coenzyme Q, melatonin, bilirubin, taurine, and cysteine. The flavonoids that are extracted from certain foods interact directly with the reactive species to produce stable complexes or complexes with less reactivity, while in other foods, the flavonoids perform the function of co-substrate in the catalytic action of some enzymes.

3.3 Repair system

Enzymes that repair or eliminate the biomolecules that have been damaged by ROS, such as lipids, proteins, and DNA, constitute the repair systems. Common

examples include systems of DNA repair enzymes (polymerases, glycosylases, and nucleases) and proteolytic enzymes (proteinases, proteases, and peptidases) found in both the cytosol and the mitochondria of mammalian cells. Specific examples of these enzymes are GPx, glutathione reductase (GR), and methionine sulfoxide reductase (MSR). These enzymes act as intermediaries in the repair process of the oxidative damage caused by the attack of excess ROS. Any environmental factor that inhibits or modifies a regular biological activity becomes a condition that favors the appearance or reinforcement of oxidative stress.

4. Characteristics of antioxidants

The main characteristic of a compound or antioxidant system is the prevention or detection of a chain of oxidative propagation, by stabilizing the generated radical, thus helping to reduce oxidative damage in the human body [21]. Gordon [22] provided a classification of antioxidants, mentioning that characteristic. There are two main types of antioxidants, the primary (breaking the chain reaction, free radical scavengers) and the secondary or preventive. The secondary antioxidant mechanisms may include the deactivation of metals, inhibition of lipid hydroperoxides by interrupting the production of undesirable volatiles, the regeneration of primary antioxidants, and the elimination of singlet oxygen. Therefore, antioxidants can be defined as "those substances that, in low quantities, act by preventing or greatly retarding the oxidation of easily oxidizable materials such as fats" [23].

5. Mechanisms of action of antioxidants

A compound that reduces *in vitro* radicals does not necessarily behave as an antioxidant in an *in vivo* system. This is because FR diffuse and spread easily. Some have extremely short life spans, on the order of nanoseconds, so it is difficult for the antioxidant to be present at the time and place where oxidative damage is being generated. Additionally, the reactions between antioxidants and FR are second order reactions. Therefore, they not only depend on the concentration of antioxidants and free radicals but are also dependent on factors related to the chemical structure of both reagents, the medium and the reaction conditions.

5.1 Phenolic compounds

The phenolic compounds constitute a wide group of chemical substances, with diverse chemical structures and different biological activities, encompassing more than 8000 different compounds which are a significant part of the human and animal diet [24]. The phenolic compounds are important components in the mechanism of signaling and defense of plants. These compounds combat the stress brought about by pathogenic organisms and predators. The function of these compounds in plants is diverse: they are found as precursors of compounds of greater complexity or the intervention in the processes of regulation and control of plant growth, as well as the defensive medium of plants. Phenolic compounds have the capacity to act as hydrogen donors or to chelate metal ions such as iron and copper, by inhibiting the oxidation of low-density lipoproteins (LDL). These characteristics in the phenolic compounds are associated with a decrease in risks of neurodegenerative diseases, such as cardiovascular diseases [25], gastrointestinal cancers [26], colon [27], breast and ovarian cancers [28], and leukemia [29–31]. Phenolic compounds also have vasorelaxation and anti-allergenic activity [32]. The phenolic compounds inhibit the oxidation of *in vitro* LDL [33].

Phenolic compounds reduce or inhibit free radicals by transfer of a hydrogen atom, from its hydroxyl group. The reaction mechanism of a phenolic compound with a peroxyl radical (ROO•) involves a concerted transfer of the hydrogen cation from the phenol to the radical, forming a transition state of an H-O bond with one electron. The antioxidant capacity of the phenolic compounds is strongly reduced when the reaction medium consists of a solvent prone to the formation of hydrogen bonds with the phenolic compounds. For example, alcohols have a double effect on the reaction rate between the phenol and the peroxyl radical. On the one hand, the alcohols act as acceptors of hydrogen bonds. On the other hand, they favor the ionization of the phenols to anion phenoxides, which can react rapidly with the peroxyl radicals, through an electron transfer. The overall effect of the solvent on the antioxidant activity of the phenolic compounds depends to a great extent on the degree of ionization of the last compounds [34]. Leopoldini et al. [35] conducted a theoretical study to determine the dissociation energy of OH bonds and the adiabatic ionization potentials of phenolic compounds of varied structure and polarity, among them tyrosol, hydroxytyrosol, and gallic and caffeic acids. These studies were performed simulating solvated and vacuum conditions. The results showed a clear difference in the behavior of these phenolic compounds. The compounds most likely to undergo a HAT were tocopherol, followed by hydroxytyrosol, gallic acid, caffeic acid, and epicatechin (**Figure 6**), while the phenolic compounds, which were better able to SET, were kaempferol and resveratrol (**Figure 7**). This undoubtedly gives us an indication that phenolic compounds can suffer both HAT and SET and that this depends mainly on the chemical structure of the phenolic compounds.

The method based on the Folin-Ciocalteu reagent is commonly used to determine and quantify total phenols. This method evaluates the ability of phenols to react with oxidizing agents. The Folin-Ciocalteu reagent contains sodium molybdate and tungstate, which react with any type of phenol [36]. The transfer of electrons at basic pH reduces the sodium molybdate and tungstate in oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}), which have a bright blue color in solution. This color intensity is proportional to the number of hydroxyl groups of the molecule [37].

5.2 Carotenoids

Carotenoids are found in virtually all plants, animals, and microorganisms, and more than 700 carotenoids have been identified and characterized [38]. Most carotenoids have a characteristic symmetrical tetraterpene skeleton. The linear hydrocarbon skeleton is made up of 40 carbons and is susceptible to various structural modifications. These structural characteristics are related to degree of hydrogenation, *cis-trans* isomerization, presence of cycles at one or both ends of the linear skeleton, or the addition of side groups (which often contain oxygen) with their subsequent glycosylation. The most complex changes are related to the shortening or elongation of the resulting tetraterpene skeleton, to form carotenoids with chains of 50 carbons. It is also possible to find carotenoids with tetraterpene skeletons of 30 carbons, from the condensation of two units of farnesyl [39]. These compounds, in addition to conferring pigmentation on biological systems, fulfill other important functions. The most recent studies of these compounds are focused mainly on evaluating their function as antioxidants. The structural base fragment of the carotenoids is a conjugated polyunsaturated chain. This fragment is primarily responsible for the ability of these compounds to inhibit free radicals. Variations in the polyunsaturated chain from one carotenoid to another, together with the presence of hydroxyl groups, substantially modify the reactivity of the carotenoids. The reactivity of these compounds is also affected by the environmental conditions where they are

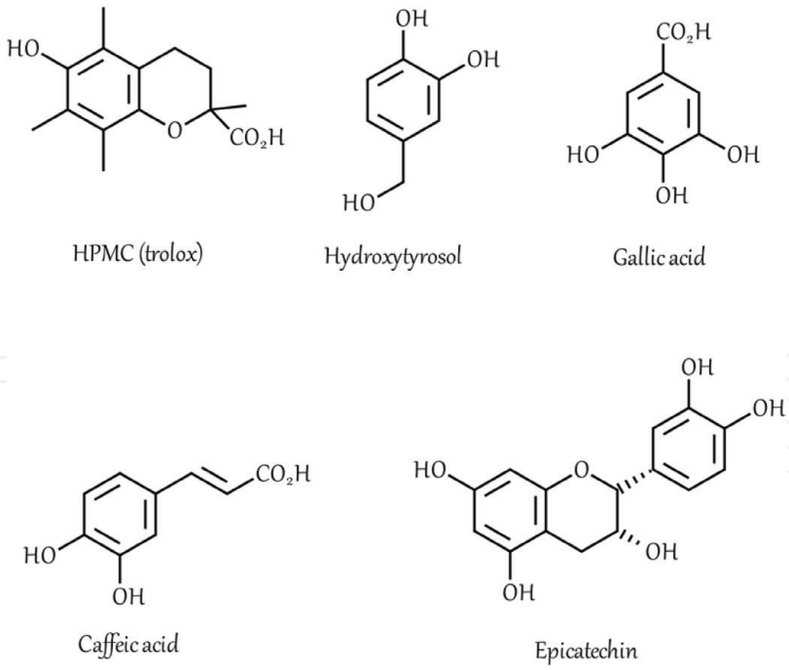

Figure 6.
Phenolic compounds with ability to HAT.

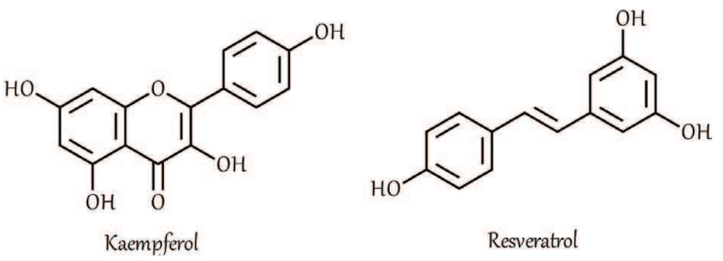

Figure 7.
Phenolic compounds with ability to SET.

found. For example, Edge and Truscott [40] found that carotenoids switch the antioxidant behavior to the prooxidant as a function of oxygen concentration. The study used a system that emulates a cell, to observe the protection effect induced by lycopene when exposing the system to high-energy radiation. Total protection is achieved in the absence of O_2, but in the presence of 100% O_2, protection is completely lost. Carotenoids are characterized as excellent peroxyl radical scavengers. The polyunsaturated chains that make up the base structure of carotenoids give these compounds a lipophilic character. Carotenoids play an important role in the protection of cell membranes and lipoproteins against peroxyl radicals.

The carotenoids react as antioxidant agents through three mechanisms: the first is the SET, the second from the formation of one adduct, and the third by HAT. In general, the antioxidant properties of carotenoids are related to their high capacity for electron donation. Everett et al. [41] found that β-carotene reacts with $NO_2^{\bullet}$ via SET. Carotenoid reactivity studies have also been carried out in the presence of the benzyl peroxyl radical, which has low reactivity, and it was concluded that in this case, the reaction mechanisms involved the formation of an adduct, while reactions by HAT are of little relevance [42].

Other studies have evaluated the effect of the chemical structure of carotenoids on the reactivity toward FR. One of these studies found that carotenoids substituted with electrons are more susceptible to oxidation than carotenoids with withdrawn electron groups. A study of carotenoid reactivity with phenoxy radicals shows the

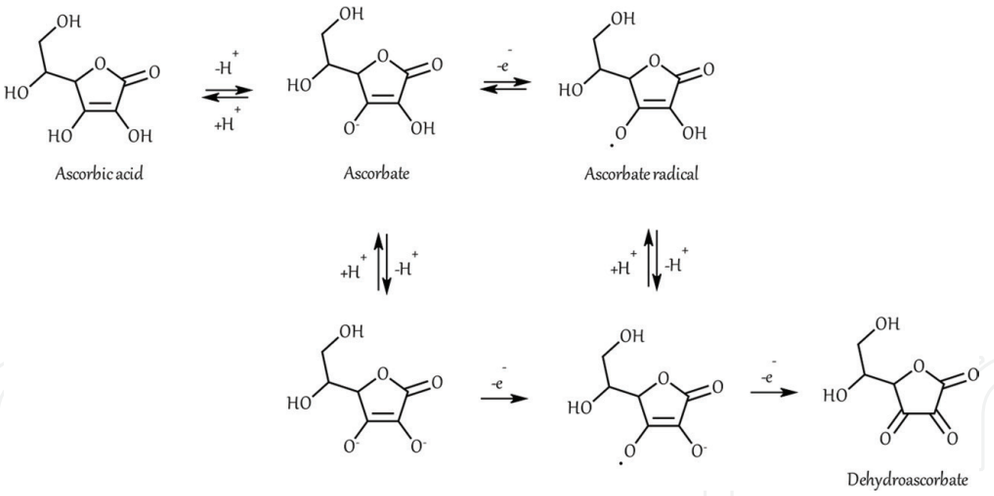

Figure 8.
Chemical species related to vitamin C.

order of reactivity to be lycopene > β-carotene > zeaxanthin > lutein > echinenone > astaxanthin [43].

The effect of the solvent on the reactivity of carotenoids in the presence of FR has also been evaluated, and it was found that in nonpolar solvents, the reactions are promoted via adduct formation; while in polar solvents, the formation of adducts takes place first and then the SET [44].

5.3 Vitamin C

Vitamin C refers to a group of ascorbic acid analogs that can be both synthetic and natural molecules. Ascorbic acid is a water-soluble ketolactone with two ionizable hydroxyl groups. Anion ascorbate is the dominant form at physiological pH (**Figure 8**). Ascorbate is a potent reducing agent and undergoes two subsequent losses of an electron, to form an ascorbate radical and dehydroascorbic acid. The ascorbate radical is relatively stable because the unpaired electron is delocalized by resonance. The ascorbate concentration in plasma of healthy humans is around 10 μg/mL. At these concentrations, the ascorbate is a co-antioxidant with vitamin E to protect LDL from peroxyl radicals [45]. The ascorbate radical is poorly reactive and can be reduced to ascorbate by reductase-dependent NADH and NADPH [46]. The ascorbate radical can alternatively undergo a disproportionation reaction that depends on pH, resulting in the formation of ascorbate and dehydroascorbic acid [47].

Vitamin C is chemically capable of reacting with most of the physiologically important ROS and acts as a hydrosoluble antioxidant. The antioxidant reaction mechanisms of vitamin C are based on the HAT to peroxyl radicals, the inactivation of singlet oxygen, and the elimination of molecular oxygen [48, 49]. For example, ascorbic acid can donate a hydrogen atom to a tocopheroxyl radical at the rate of 2×10^5 mol/s [50]. Also, it has been proven that ascorbate can produce reactions with oxidizing agents through SET [51] or a concerted transfer of electron/protons (SET/HAT) [52].

6. Methods to evaluate antioxidant activity

The antioxidant activity of a compound can be evaluated *in vitro* or *in vivo* by means of simple experiments, and at the same time, the possible prooxidant effect

on different molecules can be evaluated. Antioxidant activity cannot be measured directly but is determined by the effects of the antioxidant to control the degree of oxidation. There are a variety of methods to evaluate antioxidant activity. Some methods involve a different oxidation step followed by the measurement of the response, which depends on the method used to evaluate the activity.

When the antioxidant activity of a sample is studied, it is necessary to consider the source of ROS as well as the target substrate. An antioxidant can protect lipids against oxidative damage, while, on the other hand, it can promote the oxidation of other biological molecules [53].

Most assays of antioxidant activity involve inducing accelerated oxidation in the presence of a promoter and controlling one or more variables in the test system, for example, temperature, antioxidant concentration, pH, etc. However, the oxidation mechanisms can change when modifications are carried out on some of these variables. Therefore, it is important to evaluate the intervals in which the quantification of the antioxidant activity is done to generate reliable results.

Method	Reaction mechanism	Characteristics	Reference
Total radical-trapping antioxidant parameter (TRAP)	HAT	TRAP assay involves the initiation of lipid peroxidation by generating water-soluble $ROO^{\bullet}$ and is sensitive to all known chain-breaking antioxidants	[58]
Total oxyradical scavenging capacity total assay (TOSCA)	HAT	Evaluates inhibition oxidation of α-keto-γ-methiolbutyric acid (KMBA) by ROS. The antioxidant activity is measured through ethylene concentration, generated during decomposition of KMBA, relative to a control reaction monitored by headspace gas chromatography (HS-GC)	[59]
Crocin-bleaching assays (CBAs)	HAT	CBA is based on the abstraction of hydrogen atoms and/or addition of radical to the polyene structure of crocin and results in a disruption of the conjugated system accounting for crocin bleaching	[60]
Oxygen radical absorbance capacity (ORAC)	HAT	ORAC assay is based upon the inhibition of peroxyl radical induced oxidation initiated by thermal decomposition of azo compounds such as AAPH	[61]
Inhibition of 2,2-diphenyl-1-picrylhydracyl radical ($DPPH^{\bullet}$)	SET or HAT	Colorimetric method based on the measurement of the scavenging capacity of antioxidants towards $DPPH^{\bullet}$	[62]
Inhibition of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) cation radical	SET or HAT	Colorimetric method to evaluate the decay of $ABTS^{\bullet+}$ in the presence of an antioxidant agent	[63]
Total phenols assay by Folin-Ciocalteu reagent	SET	A mixture of phosphomolybdate and phosphotungstate in highly basic medium oxidized phenolic compounds	[64]
Ferric-reducing antioxidant power (FRAP)	SET	Colorimetric method that evaluates the reduction of Fe^{3+}-tripyridyltriazine complex (Fe^{3+}-TPTZ) by turning it into a ferrous form (Fe^{2+}-TPTZ)	[65]
Total antioxidant capacity (TAC)	SET	This method is used to measure the peroxide level during the initial stage of lipid oxidation. Peroxides are formed during the linoleic acid oxidation, which reacts with Fe^{2+} to form Fe^{3+} and later these ions form a complex with thiocyanate	[66]

Table 2.
Methods most commonly used to evaluate antioxidant capacity/activity in vitro.

The methods to determine the antioxidant capacity are divided into two general groups. This division is based on the reaction mechanisms involved in the RF reduction process. The first group of methods is based on the SET and the second group is based on the HAT. The result is the same: the inactivation of free radicals; however, the kinetics and secondary reactions involved in the process are different. The methods based on SET detect the capacity of a potential antioxidant for the transmission of a chemical species, including metals, carbonyls, and radicals. SET is shown through a change in color as the oxidant is reduced by antioxidant [54]. The group of methods based on HAT measures the ability of an antioxidant to inactivate FR through the donation of a hydrogen atom. HAT reactions are theoretically independent of solvent nature and pH. These reactions are rapid and occur in no more than a few minutes. The presence of other reducing agents in samples, in addition to the antioxidants under study, makes HAT testing difficult and can lead to significant errors [55]. **Table 2** shows the methods of evaluation of the antioxidant activity *in vitro*.

7. Antioxidant capacity/activity *in vitro* evaluation

The methods of evaluation of antioxidant activity must be fast, reproducible, and require small amounts of the chemical compounds to be analyzed, in addition to not being influenced by the physical properties of said compounds [56]. The results of *in vitro* assays can be used as a direct indicator of antioxidant activity *in vivo*; a compound that is ineffective *in vitro* will not be better *in vivo* [53]. These tests can also serve as warnings of possible harmful effects of chemical compounds. Because many factors can affect oxidation, including temperature, the concentration of oxygen in the reaction medium, and metal catalysts, the results may vary depending on the oxidation conditions employed. Tests that measure substrates or products can also give variable results depending on their specificity [57].

These methods are briefly described below.

7.1 Total radical-trapping antioxidant parameter (TRAP)

The TRAP is used to determine the status of a secondary antioxidant in plasma. The results (TRAP value) are expressed as μmol of $ROO^{\bullet}$ trapped per liter of plasma [58]. The test is based on the measurement of O_2 uptake during a controlled peroxidation reaction, promoted by the thermal decomposition of 2,2′-azobis-(2-amidopropane) (ABAP), which produces $ROO^{\bullet}$ at a constant rate (**Figure 9**). This starts with the addition of ABAP to human plasma; the parameter to be evaluated is the "delay time" of the O_2 absorption in plasma induced by the antioxidant compounds present in the medium. The delay time is measured from the O_2 concentration data in plasma diluted in a buffer solution monitored with an electrode. In addition to ABAP, other free radical initiators have been used, such as the ABTS [67], dichlorofluorescein diacetate [68], phycoerythrin [69], and luminol [70].

One of the main disadvantages of the TRAP method is the possibility of an error in the detection of the end point caused by the instability of the O_2 electrode, because this point can take 2 h to reach. To minimize this problem, the electrochemical detection of O_2 can be performed with a chemiluminescent detection based on the use of luminol and horseradish peroxidase [71].

7.2 Total oxyradical scavenging capacity assay (TOSCA)

This method is based on the evaluation of antioxidant activity in the gas phase, which consists of exposing α-keto-γ-methylthiobutyric acid (KMBA) to powerful

oxidizing agents, such as $^{\bullet}OH$, $ROO^{\bullet}$, and $ONOO^{-}$ [59] (**Figure 10**). These oxidizing agents induce a transformation of KMBA to ethylene. To evaluate the effect of antioxidants, the ethylene formation is evaluated and compared to a control reaction by the use of headspace gas chromatography (HS-GC). The TOSCA assay is based on the inhibition of ethylene formation in the presence of antioxidant compounds that compete with KMBA for ROS.

The TOSCA method is not suitable for a high performance analysis because multiple injections of each sample are required to measure ethylene production [55]. The reaction kinetics of this method do not allow a linear relationship between the percentage of inhibition of KMBA oxidation and the concentration of antioxidants [72], which is a serious limitation.

7.3 Crocin-bleaching assay (CBA)

The crocin bleaching test (CBA) is a method originally proposed to evaluate the inhibition of alkoxyl radicals produced photolytically. This is done by measuring the protective effect exerted by antioxidant compounds on crocin, a carotenoid that presents an intense red color, under the effect of alkoxyl radicals [60] (**Figure 11**). To achieve this, reaction kinetics are carried out in a UV-Vis spectrophotometer, measuring the absorbance at a wavelength of 440 nm to obtain the relative velocity constants. These constants present a good correlation with the known antioxidant activity of reference compounds. The absolute bleaching velocity of crocin depends strongly on the type of radical that attacks the polyene structure of crocin. Crocin exhibits a high selectivity toward the alkoxyl radicals produced during the photolysis of hydroperoxides, as well as peroxyl radicals produced after the thermolysis of azo initiators. Ordoudi and Tsimidou [73] carried out a detailed evaluation of the CBA, and among the factors, they considered the crocin probe, the antioxidant compound to be evaluated, the peroxyl radical generation conditions, and the monitoring of the reaction. As a result of this, they found that any commercial saffron could be used as a source of crocin for the preparation of the probe, because it is possible to eliminate interferences, such as tocopherols. They also found that the concentration of the working solution could be adjusted and that changes in the

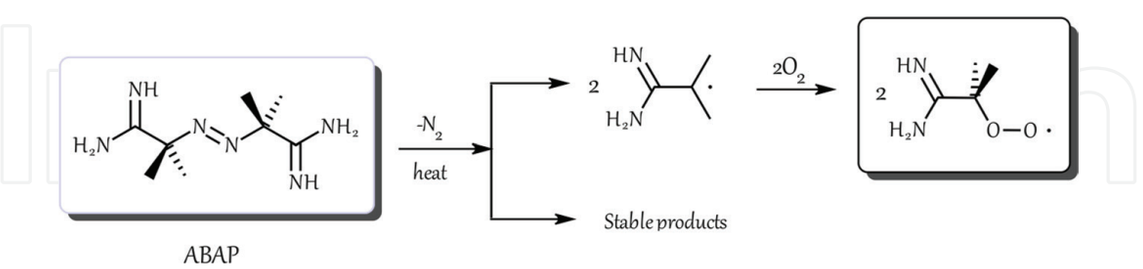

Figure 9.
Formation of peroxyl radical from ABAP.

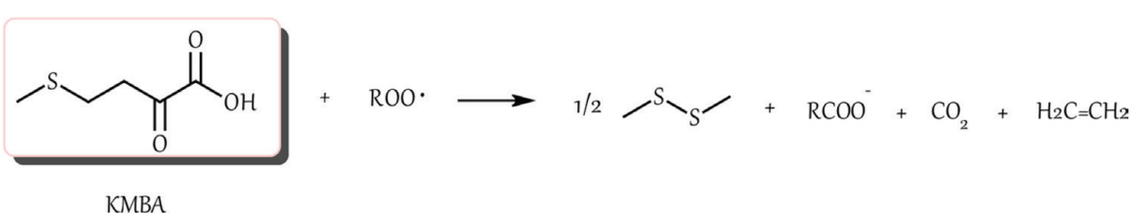

Figure 10.
Reaction between $ROO^{\bullet}$ and KMBA.

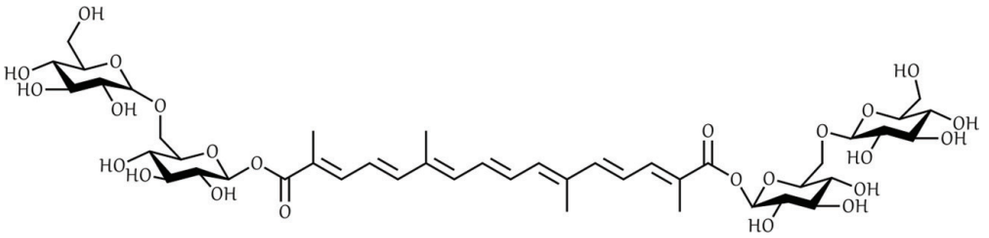

Figure 11.
Chemical structure of crocin.

stock solution of the probe can occur during storage. Ordoudi and Tsimidou [74] also evaluated a group of 39 phenolic compounds of diverse structures, including hydroxybenzoic, hydroxyphenylacetic, hydroxyphenylpropanoic, and hydroxycinnamic acids. The results of that study showed that the activity depends strongly on the position of -COOH groups in relation to the position of the -OH groups. Therefore, the CBA allows evaluation of the effect of the position of functional groups that cause antioxidant activity in a chemical compound.

7.4 Oxygen radical absorbance capacity (ORAC) method

The ORAC method is based on the inhibition of oxidation induced by peroxyl radicals and simultaneously evaluates the time effect and the inhibition degree. The ORAC test is based on hydrogen atom transfer (HAT) and uses a reaction mechanism that competes between antioxidants and a fluorescence probe (fluorescein) for a radical [61]. The test begins with the thermal decomposition of azo compounds, such as [2,2′-azobis-(2-amidino-propane)dihydrochloride (AAPH)], which is the source of free radicals that promotes the degradation of fluorescein. The antioxidant to be evaluated promotes the elimination of the peroxyl radicals, protecting the fluorescein from degradation. The decay in fluorescence due to the attack of the radicals and the protection by the antioxidants results in a curve. The antioxidant capacity is calculated from the area under the fluorescence decrease curve (AUC). This assay uses trolox as a standard; therefore, generally the antioxidant activity in this assay is expressed in terms of trolox equivalents. The ORAC method has been widely used to measure the antioxidant capacity of beverages [75], supplements [55], and vegetables and fruits [55, 76].

There are modifications to this assay that include the use of fluorescein as a probe, adaptation to a high performance format, and the ability to measure the lipophilic, hydrophilic, and total antioxidant capacity of a substance.

The ORAC assay is carried out at pH 7.4, adjusted with a phosphate buffer, in the presence of the antioxidant, AAPH, and fluorescein at a constant temperature of 37°C. Fluorescence is monitored at 1 min intervals for 35 min at an excitation wavelength of 485 nm and an emission wavelength of 520 nm [77].

The ORAC method can also be used for the detection of $^{\bullet}$OH and other radicals by modifying the initiators. In addition, the method has been modified for the detection of lipophilic antioxidants, encapsulating these compounds in β-cyclodextrins [78].

7.5 Radical scavenging capacity DPPH$^{\bullet}$ method

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) (**Figure 12**) is characterized as a stable free radical because pi electrons of the aromatic systems present in the molecule can compensate for the lack of an electron. DPPH$^{\bullet}$ does not dimerize, as most other free radicals do. The delocalization of the electron also gives rise to a

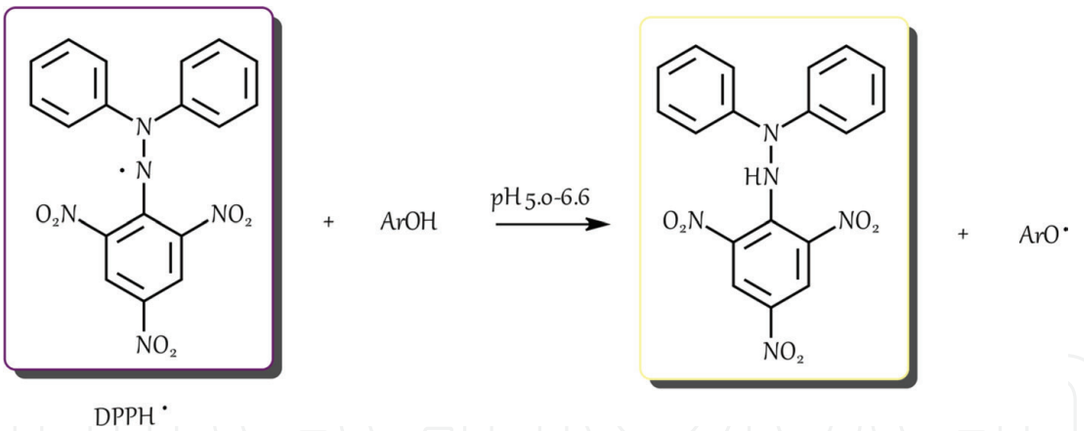

Figure 12.
DPPH• reduction by an antioxidant.

deep violet color, characterized by absorption in solution at around 517 nm. Brand-Williams et al. [62] evaluated the activity of specific compounds or extracts using DPPH• in solution. When a solution of DPPH• is in contact with a substance that can donate a hydrogen atom or with another radical (R•), the reduced form DPPH-H or DPPH-R is produced with the consequent loss of color and therefore the decrease or loss of absorbance (**Figure 8**). Consequently, the reduction of DPPH• provides an index to estimate the ability of the test compound to trap radicals. The alcoholic solutions of 0.5 mM are densely colored, and in this concentration, the law of Lambert-Beer is fulfilled in the useful absorption interval [79].

ArOH is an antioxidant that acts by donating hydrogen atoms, to obtain radicals with stable molecular structures that will stop the chain reaction. The new radical (ArO•) can interact with another radical to form stable molecules (DPPH-OAr, ArO-OAr). The reaction between DPPH• and an antioxidant compound depends on the structural conformation of the same, so quantitative comparisons are not always appropriate.

The basis of this methodology is focused on measuring the reduction of free radicals by antioxidant compounds. Different concentrations and the time of the reaction are measured (30 min or until the steady state is reached). So far, there are no reports about the existence of a mathematical kinetic model that helps to understand the behavior of antioxidants [80].

The experimental models use the percentage of DPPH• remaining to obtain the necessary quantities that are required to reduce the initial concentration to 50% (EC_{50}). In addition, kinetics is performed to determine the amount of time needed for the steady state to reach EC_{50} from the curves. EC_{50} and effective concentration 50 (TEC_{50}) are used to calculate antiradical efficiency (AE). Low values of EC_{50} and TEC_{50} show a high antioxidant strength, and a rapid decrease in absorption is observed during the reaction [81]. The antiradical efficiency can be estimated based on the scale contained in **Table 3**.

It is a fast, simple, inexpensive, and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors. It can also be used to quantify antioxidants in complex biological systems, for solid or liquid samples. The method is applied to measure the overall antioxidant capacity [82] and the activity of eliminating free radicals from fruit and vegetable juices [83]. It has been successfully used to investigate the antioxidant properties of wheat grain and bran, vegetables, oils, and flours in various solvents, including ethanol, aqueous acetone, methanol, and benzene [84–87].

The radical scavenging DPPH• method allows for a reaction with almost any type of antioxidant due to the stability of DPPH•. This means there is sufficient

time for even weak antioxidants to react with DPPH$^{\bullet}$ [82]. This method can be used with both polar and nonpolar organic solvents to evaluate hydrophilic and lipophilic antioxidants [55].

The method has some disadvantages, among which is that DPPH$^{\bullet}$ can react with other radicals and consequently the time to reach the stable state is not linear to the concentration ratio of the antioxidant/DPPH$^{\bullet}$ [62, 80]. The stability of DPPH$^{\bullet}$ can be affected by solvents with properties of a Lewis base, as well as the presence of dissolved oxygen [88]. The absorbance of DPPH$^{\bullet}$ in methanol and acetone is lower than with other solvents [89].

Because the radical scavenging DPPH$^{\bullet}$ method is quite simple and used in various fields of chemistry, automated assays combined with analytical techniques have been developed (**Table 4**).

7.6 Ferric reducing/antioxidant power (FRAP) method

The FRAP analysis was introduced by [65, 96] to measure total antioxidant activity and is based on the ability of samples to reduce ferric ion Fe^{3+} to ferrous ion

Range	Antiradical efficiency classification
$AE = 1 \times 10^{-3}$	Low
$1 \times 10^{-3} < AE = 5 \times 10^{-3}$	Medium
$5 \times 10^{-3} < AE = 10 \times 10^{-3}$	High
$AE \gg 10 \times 10^{-3}$	Very high

Table 3.
Scale of antiradical efficiency (AE) against DPPH$^{\bullet}$ [81].

Automation	Characteristics	References
Flow injection analysis (FIA) by high performance liquid chromatography (HPLC)	Bioassay-guided fractionation of natural products or food samples	[90]
PC-controlled sequential injection analysis (SIA)	SIA is a FIA technique modified by using a pump to continuously draw sample and reagent solutions into different lines of tubing	[91]
Electrochemical selective determination of antioxidant activity based on DPPH$^{\bullet}$/DPPH	Current intensity is proportional to the residual concentration of DPPH$^{\bullet}$ after reaction with the antioxidant	[92]
Relative DPPH radical scavenging capacity (RDSC)	The RDSC uses the area under the curve, expressed as trolox equivalents. These approaches take into account both the kinetic and the thermodynamic measurements of the radical-antioxidant reactions	[93]
High performance thin layer chromatography (TLC)-DPPH$^{\bullet}$	Post-chromatographic derivatization is carried out with DPPH$^{\bullet}$. The plates are scanned before DPPH$^{\bullet}$ and 30 min after DPPH derivatization in absorption-reflection mode at optimized wavelengths	[94]
Hyphenated high speed counter current chromatography (HSCCC)-DPPH$^{\bullet}$	After the HSCCC separation, the effluent is split into two streams by use of an adjustable high-pressure stream splitter. One portion is sent through the detector and the fraction collector, while the second portion is sent to a secondary coil for on-line radical-scavenging detection	[95]

Table 4.
Automated modes to evaluate radical scavenging capacity DPPH$^{\bullet}$.

Fe^{2+}, forming a blue complex. A high absorption at a wavelength of 700 nm indicates a high reduction power of the chemical compound or extract [66]. The value of FRAP has been used to determine the antioxidant activity of red wines [97]. The work of Schleisier et al. [98] was designed to determine the antioxidant activity in tea extracts and juices expressed in Fe^{2+} equivalents. The absolute initial index of the reduction of ferrylmyoglobin determined by spectroscopy in the visible region has been suggested to characterize the antioxidant activity of individual flavonoids [99]. There are several trials to evaluate FRAP; one of them is to evaluate the power of a compound or extract to reduce the complex of 2,4,6-tripyridyl-s-triazine-Fe^{2+} (TPTZ-Fe^{2+}). An antioxidant reduces the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in the TPTZ complex; the latter forms a blue complex (Fe^{2+}/TPTZ), which absorbs at a wavelength of 590 nm (**Figure 13**). The reaction must be carried out under acidic conditions (pH 3.6) to preserve the solubility of Fe. The reducing power is related to the degree of hydroxylation and the conjugation in the phenols [55].

The FRAP assay has an incubation time of 4 min at 37°C for the antioxidant activity of most samples. This is done because the redox reactions, involved in the assay, occur within the incubation period. However, it has been shown that FRAP values can vary significantly, depending on the time scale of analysis [55, 96].

7.7 Method of inhibition of the (2,2′-azinobis-(3-ethylbenozothiazoline-6-sulfonate)) radical cation ($ABTS^{\bullet+}$)

ABTS is a target molecule used to evaluate the reactivity of antioxidant samples in the presence of peroxides. The ABTS initially is subjected to an oxidation reaction with potassium permanganate, potassium persulfate or 2,2′-azo-bis (2-amidinopropane), producing the radical cation of the ABTS ($ABTS^{\bullet+}$) with a blue greenish color that absorbs at wavelengths of 415, 645, 734, and 815 nm [100–102]. The $ABTS^{\bullet+}$ is stable for several minutes. The $ABTS^{\bullet+}$ is subjected to the antioxidant sample causing the reduction of $ABTS^{\bullet+}$ and consequently the discoloration of the reaction mixture (**Figure 14**). Therefore, the degree of discoloration can be expressed as the inhibition percentage of $ABTS^{\bullet+}$, which is determined as a function of antioxidant concentration and time. This method can be used at different pH and is useful to study the effect of pH on antioxidant activity. ABTS is soluble in both aqueous and organic solvents and consequently is useful for evaluating the antioxidant activity of samples in different media and is commonly used in solutions that simulate an ionic serum (pH 7.4) based on a phosphate buffer (PBS) containing 150 mM NaCl. When a medium of PBS is used, the samples react in a time interval of approximately 30 min, while in alcohol, they require longer reaction times [103]. The level of peroxide is determined by the absorbance at some of the above-mentioned wavelengths. The IC50 is calculated by plotting the percentage of inhibition against different

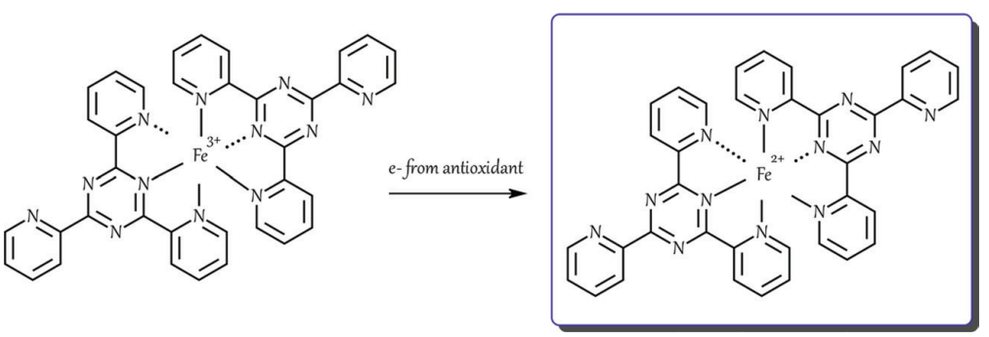

Figure 13.
Reaction mechanism for the FRAP assay in the presence of an antioxidant [55].

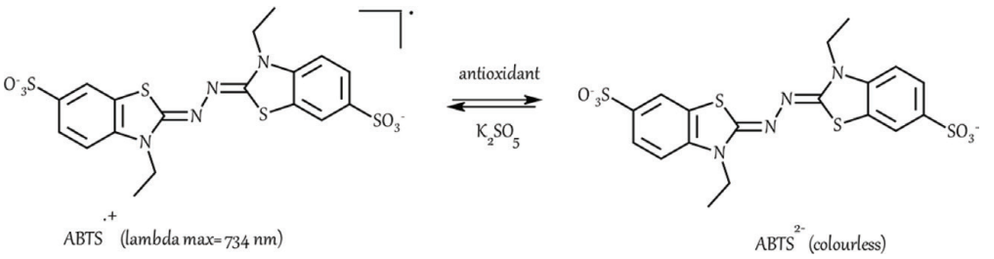

Figure 14.
Reaction of ABTS$^{\bullet+}$ with antioxidant compounds.

concentrations of the antioxidant sample [104]. The IC_{50} values indicate the sample concentration required to eliminate 50% of the ABTS$^{\bullet+}$. Low IC_{50} values indicate high radical uptake activity. The antioxidant activity against ABTS$^{\bullet+}$ can also be evaluated through the unit of antioxidant activity (TAA), which expresses the equivalents of trolox in μmol with respect to each gram of sample extract in dry base.

The inhibition of ABTS$^{\bullet+}$ activity in an antioxidant sample has a strong correlation with the radical scavenging capacity DPPH$^{\bullet}$ because both radicals have the capacity to accept electrons and H$^{\bullet}$ from the antioxidant compounds present in the samples [105, 106].

7.8 Total antioxidant capacity (TAC)

TAC is defined as the ability of a compound to inhibit the oxidative degradation of lipids [66]. Lipid peroxidation involves the oxidative deterioration of lipids with unsaturation. This peroxidation, called the initiation process, begins with the formation of conjugated dienes and trienes, known as primary oxidation products due to the abstraction of a hydrogen atom. Subsequently, a propagation process is carried out that consists of the reaction of the deprotonated species derived from the lipids with O_2, leading to the formation of peroxyl radicals (ROO$^{\bullet}$). The high energy of free radicals promotes the abstraction of hydrogen atoms from neighboring fatty acids. This leads to the formation of hydroperoxides that promotes the formation of new R$^{\bullet}$ radicals. The latter radicals react with each other to produce stable molecules of the R-R and ROOR type [107]. To encourage the antioxidant activity of a chemical compound, it is necessary to inhibit the peroxidation of a fatty acid emulsion; linoleic acid is generally used as a model. The hydroperoxides derived from linoleic acid subsequently react with Fe^{2+}, causing the oxidation of this ion to produce Fe^{3+}. The Fe^{3+} ions form a complex with thiocyanate (SCN^{-}), and this complex has a maximum absorbance at 500 nm [108]. This complex is used to measure the peroxide value.

The ferric thiocyanate method is used to measure the peroxide value in edible oils. To avoid errors in the determination of the peroxide value, it is important to avoid the presence of oxygen in the reaction medium and this can be achieved by bubbling nitrogen [109]. These authors found that the results of the thiocyanate assay also depend on the solvent, reducing agent and type of hydroperoxides present in the sample.

8. Conclusions

The reaction mechanisms involved in the antioxidant activity/capacity of different groups of compounds depend on several factors. Among these factors are the chemical structure of these compounds, the nature of the solvent, the temperature

and pH, as well as the reactivity and chemical structure of free radicals. All these factors can also influence the reaction rate. Consequently, it is very important that, for studies of antioxidant properties, at least three evaluation methods are selected: one to exclusively evaluate the HAT, another the SET, and a combined method, HAT/SET. Also, it is important to perform reaction kinetics. In addition to this, it is essential to consider that in mixtures of antioxidant compounds, possible synergistic effects are present and can enhance the activity/capacity or even modify their reaction mechanisms.

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Conflict of interest

The authors have no conflict of interest to declare and are responsible for the content and writing of the manuscript.

Ethical approval

This chapter does not contain any studies with human participants or animals performed by any of the authors.

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