



Review

A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives

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ABSTRACT

Many studies have been conducted with regard to free radicals, oxidative stress and antioxidant activity of food, giving antioxidants a prominent beneficial role, but, recently many authors have questioned their importance, whilst trying to understand the mechanisms behind oxidative stress. Many scientists defend that regardless of the quantity of ingested antioxidants, the absorption is very limited, and that in some cases prooxidants are beneficial to human health. The detection of antioxidant activity as well as specific antioxidant compounds can be carried out with a large number of different assays, all of them with advantages and disadvantages. The controversy around antioxidant *in vivo* benefits has become intense in the past few decades and the present review tries to shed some light on research on antioxidants (natural and synthetic) and prooxidants, showing the potential benefits and adverse effects of these opposing events, as well as their mechanisms of action and detection methodologies. It also identifies the limitations of antioxidants and provides a perspective on the likely future trends in this field.

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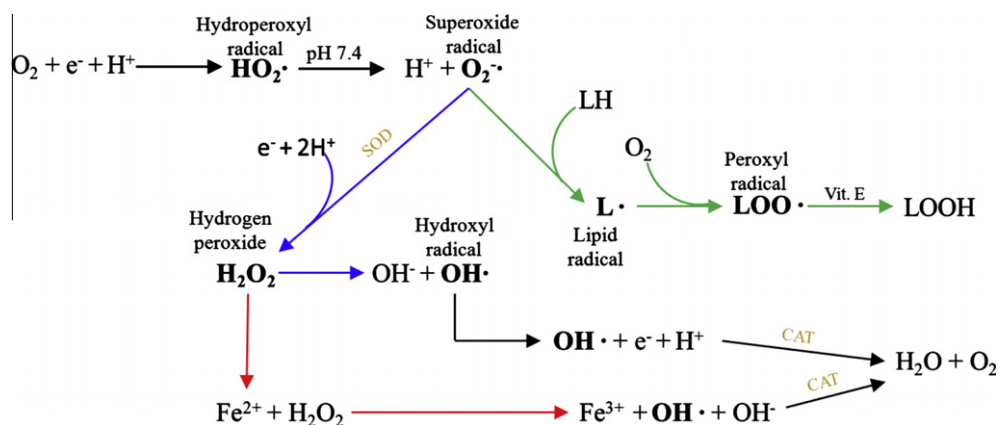


Fig. 1. Overview of the reactions leading to the formation of ROS. Green arrows represent lipid peroxidation. Blue arrows represent the Haber–Weiss reactions and the red arrows represent the Fenton reactions. The bold letters represent radicals or molecules with the same behavior (H_2O_2). SOD refers to the enzyme superoxide dismutase and CAT refers to the enzyme catalase. Adapted from Ferreira et al. (2009) and Flora (2009). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1. Introduction

1.1. From free radicals to oxidative stress

Biochemical reactions that take place in the cells and organelles of our bodies are the driving force that sustains life. The laws of nature dictate that one goes from childhood, to adulthood and finally enters a frail condition that leads to death. Due to the low number of births and increasing life expectancy, in the near future, worldwide population will be composed in a considerable number of elderly. This stage in life is characterized by many cardiovascular, brain and immune system diseases that will translate into high social costs (Rahman, 2007). It is therefore important to control the proliferation of these chronic diseases in order to reduce the suffering of the elderly and to contain these social costs. Free radicals, antioxidants and co-factors are the three main areas that supposedly can contribute to the delay of the aging process (Rahman, 2007). The understanding of these events in the human body can help prevent or reduce the incidence of these and other diseases, thus contributing to a better quality of life.

1.1.1. Free radicals and oxidative stress mechanisms

Free radicals are atoms, molecules or ions with unpaired electrons that are highly unstable and active towards chemical reactions with other molecules. They derive from three elements: oxygen, nitrogen and sulfur, thus creating reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS). ROS include free radicals like the superoxide anion ($\text{O}_2^{\cdot-}$), hydroperoxyl radical (HO_2^{\cdot}), hydroxyl radical (OH^{\cdot}), nitric oxide (NO), and other species like hydrogen peroxide (H_2O_2), singlet oxygen ($^1\text{O}_2$), hypochlorous acid (HOCl) and peroxynitrite (ONOO^-). RNS derive from NO by reacting with $\text{O}_2^{\cdot-}$, and forming ONOO^- . RSS are easily formed by the reaction of ROS with thiols (Lü et al., 2010). Regarding ROS, the reactions leading to the production of reactive species are displayed in Fig. 1. The hydroperoxyl radical (HO_2^{\cdot}) dissociates at pH 7 to form the superoxide anion ($\text{O}_2^{\cdot-}$). This anion is extremely reactive and can interact with a number of molecules to generate ROS either directly or through enzyme or metal-catalyzed processes. Superoxide ion can also be detoxified to hydrogen peroxide through a dismutation reaction with the enzyme superoxide dismutase (SOD) (through the Haber–Weiss

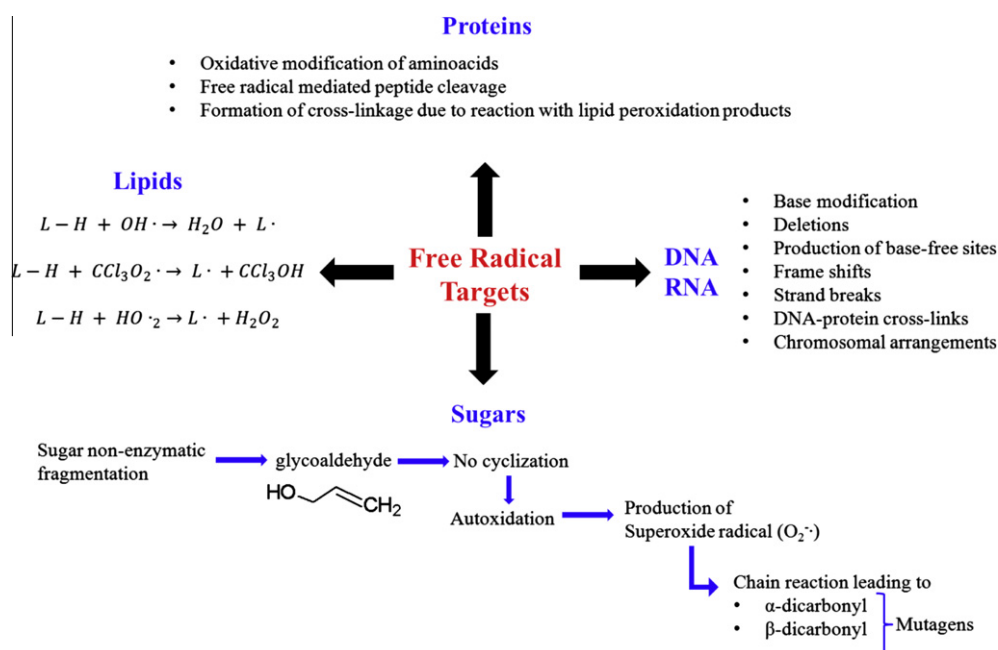


Fig. 2. Targets of free radicals. Adapted from Dizdaroglu et al. (2002), Valko et al. (2004), Benov and Beema (2003), Halliwell and Chirico (1993) and Lobo et al. (2010).

reaction) and finally to water by the enzyme catalase (CAT). If hydrogen peroxide reacts with an iron catalyst like Fe^{2+} , the Fenton reaction can take place ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^-$) forming the hydroxyl radical HO^\cdot (Flora, 2009). With regard to RNS, the mechanism forming ONOO^- is: $\text{NO}^\cdot + \text{O}_2^- \rightarrow \text{ONOO}^-$ (Squadrito and Pryor, 1998). Finally, RSS derive, under oxidative conditions, from thiols to form a disulfide that with further oxidation can result in either disulfide-S-monoxide or disulfide-S-dioxide as an intermediate molecule. Finally, a reaction with a reduced thiol may result in the formation of sulfenic or sulfinic acid (Giles et al., 2001).

1.1.2. Effects of oxidative stress

Internally, free radicals are produced as a normal part of metabolism within the mitochondria, through xanthine oxidase, peroxisomes, inflammation processes, phagocytosis, arachidonate pathways, ischemia, and physical exercise. External factors that help to promote the production of free radicals are smoking, environmental pollutants, radiation, drugs, pesticides, industrial solvents and ozone. It is ironic that these elements, essential to life (especially oxygen) have deleterious effects on the human body through these reactive species (Lobo et al., 2010).

The balance between the production and neutralization of ROS by antioxidants is very delicate, and if this balance tends to the overproduction of ROS, the cells start to suffer the consequences of oxidative stress (Wiernsperger, 2003).

It is estimated that every day a human cell is targeted by the hydroxyl radical and other such species and average of 10^5 times inducing oxidative stress (Valko et al., 2004). The main targets of ROS, RNS and RSS are proteins, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) molecules, sugars and lipids (Lü et al., 2010; Craft et al., 2012) (Fig. 2). Regarding proteins, there are three distinct ways they can be oxidatively modified: (1) oxidative modification of a specific amino acid, (2) free radical-mediated peptide cleavage and (3) formation of protein cross-linkage due to reaction with lipid peroxidation products (Lobo et al., 2010). The damage induced by free radicals to DNA can be described both chemically and structurally having a characteristic pattern of modifications: Production of base-free sites, deletions, modification of all bases, frame shifts, strand breaks, DNA–protein cross-links and chromosomal arrangements. An important reaction involved with DNA damage is the production of the hydroxyl radical through the Fenton reaction. This radical is known to react with all the components of the DNA molecule: the purine and pyrimidine bases as well as the deoxyribose backbone. The peroxy and OH-radicals also intervene in DNA oxidation (Dizdaroglu et al., 2002; Valko et al., 2004).

Regarding sugars, the formation of oxygen free radicals during early glycation could contribute to glycoxidative damage. During the initial stages of non-enzymatic glycosylation, sugar fragmentation produces short chain species like glycoaldehyde whose chain is too short to cyclize and is therefore prone to autooxidation, forming the superoxide radical. The resulting chain reaction propagated by this radical can form α and β -dicarbonyls, which are well known mutagens (Benov and Beema, 2003).

Lipid peroxidation is initiated by an attack towards a fatty acid's side chain by a radical in order to abstract a hydrogen atom from a methylene carbon. The more double bonds present in the fatty acid the easier it is to remove hydrogen atoms and consequently form a radical, making monounsaturated (MUFA) and saturated fatty acids (SFA) more resistant to radicals than polyunsaturated fatty acids (PUFA). After the removal, the carbon centered lipid radical can undergo molecular rearrangement and react with oxygen forming a peroxy radical. These highly reactive molecules can then abstract hydrogen atoms from surrounding molecules and propagate a chain reaction of lipid peroxidation. The hydroxyl radical is the one of the main radicals in lipid peroxidation, it is formed in biological systems, as stated above, by the Fenton reaction as a

result of interaction between hydrogen peroxide and metal ions. This radical acts according to the following generic reaction: $\text{L-H} + \text{OH}^\cdot \rightarrow \text{H}_2\text{O} + \text{L}^\cdot$, where L-H represents a generic lipid and L^\cdot represents a lipid radical. The trichloromethyl radical ($\text{CCl}_3\text{O}_2^\cdot$) which is formed by the addition of carbon tetrachloride (CCl_4) with oxygen also attacks lipids according to this equation: $\text{L-H} + \text{CCl}_3\text{O}_2^\cdot \rightarrow \text{L}^\cdot + \text{CCl}_3\text{OH}$. Isolated PUFA's can suffer damage from the hydroperoxy radical through this equation: $\text{L-H} + \text{HO}_2^\cdot \rightarrow \text{L}^\cdot + \text{H}_2\text{O}_2$. Finally, another way to generate lipid peroxides is through the attack on PUFA's or their side chain by the singlet oxygen which is a very reactive form of oxygen. This pathway does not probably qualify as initiation because the singlet oxygen reacts with the fatty acid instead of abstracting a hydrogen atom to start a chain reaction, making this a minor pathway when compared to the hydroxyl one (Halliwell and Chirico, 1993).

Free radicals have different types of reaction mechanisms, they can react with surrounding molecules by: electron donation, reducing radicals, and electron acceptance, oxidizing radicals (a), hydrogen abstraction (b), addition reactions (c), self-annihilation reactions (d) and by disproportionation (e) (Slater, 1984).

- (a) $\text{OH}^\cdot + \text{RS}^- \rightarrow \text{OH}^- + \text{RS}^\cdot$
- (b) $\text{CCl}_3^\cdot + \text{RH} \rightarrow \text{CHCl}_3 + \text{R}^\cdot$
- (c) $\text{CCl}_3^\cdot + \text{CH}_2=\text{CH}_2 \rightarrow \text{CH}_2(\text{CCl}_3) - \text{CH}_2^\cdot$
- (d) $\text{CCl}_3^\cdot + \text{CCl}_3^\cdot \rightarrow \text{C}_2\text{Cl}_6$
- (e) $\text{CH}_3\text{CH}_2^\cdot + \text{CH}_3\text{CH}_2^\cdot \rightarrow \text{CH}_2=\text{CH}_2 + \text{CH}_3 - \text{CH}_3$

These reactions lead to the production of ROS, RNS and RSS whom have been linked to many severe diseases like cancer, cardiovascular diseases including atherosclerosis and stroke, neurological disorders, renal disorders, liver disorders, hypertension, rheumatoid arthritis, adult respiratory distress syndrome, auto-immune deficiency diseases, inflammation, degenerative disorders associated with aging, diabetes mellitus, diabetic complications, cataracts, obesity, autism, Alzheimer's, Parkinson's and Huntington's diseases, vasculitis, glomerulonephritis, lupus erythematosus, gastric ulcers, hemochromatosis and preeclampsia, among others (Rahman, 2007; Lobo et al., 2010; Lü et al., 2010; Singh et al., 2010).

1.2. Antioxidants and prooxidants

1.2.1. Natural antioxidants

Halliwell and Gutteridge (1995) defined antioxidants as “any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate”, but later defined them as “any substance that delays, prevents or removes oxidative damage to a target molecule” (Halliwell, 2007). In the same year Khlebnikov et al. (2007) defined antioxidants as “any substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defences or inhibit ROS production”. Another property that a compound should have to be considered an antioxidant is the ability, after scavenging the radical, to form a new radical that is stable through intramolecular hydrogen bonding on further oxidation (Halliwell, 1990). During human evolution, endogenous defences have gradually improved to maintain a balance between free radicals and oxidative stress. The antioxidant activity can be effective through various ways: as inhibitors of free radical oxidation reactions (preventive oxidants) by inhibiting formation of free lipid radicals; by interrupting the propagation of the autooxidation chain reaction (chain breaking antioxidants); as singlet oxygen quenchers; through synergism with other antioxidants; as reducing agents which convert hydroperoxides into stable compounds; as metal chelators that convert metal pro-oxidants (iron and copper derivatives) into stable products; and finally as inhibitors of pro-oxidative enzymes (lipooxygenases)

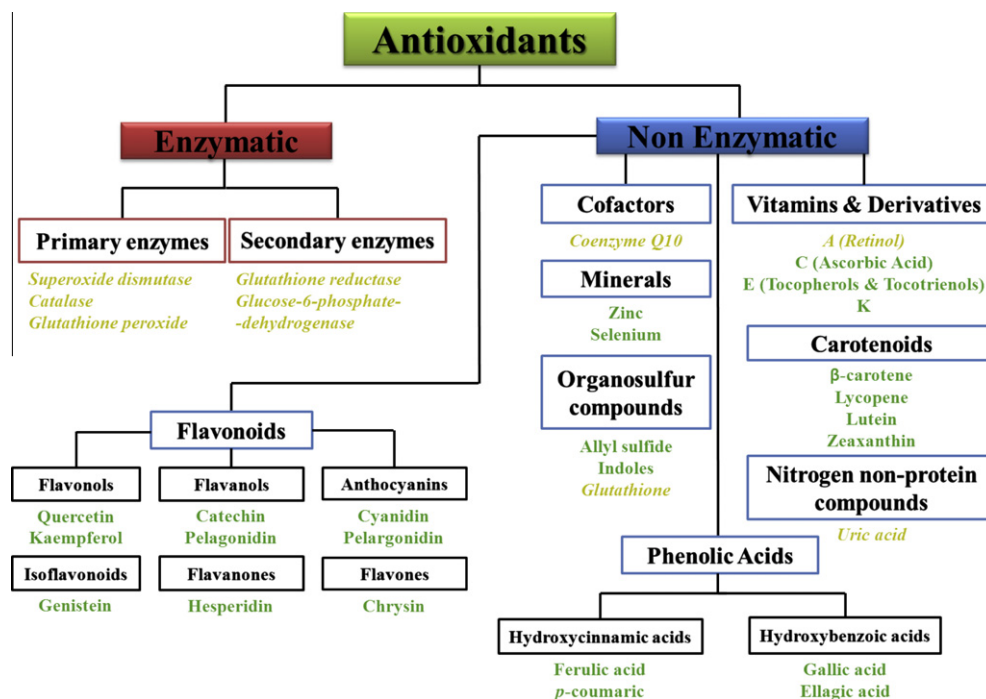


Fig. 3. Natural antioxidants separated in classes. Green words represent exogenous antioxidants, while yellow ones represent endogenous antioxidants. Adapted from Pietta (2000), Ratnam et al. (2006) and Godman et al. (2011). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Darmanyan et al., 1998; Heim et al., 2002; Min and Boff, 2002; Pokorný, 2007; Kancheva, 2009).

The human antioxidant system is divided into two major groups, enzymatic antioxidants and non-enzymatic oxidants (Fig. 3). Regarding enzymatic antioxidants they are divided into primary and secondary enzymatic defences. With regard to the primary defence, it is composed of three important enzymes that prevent the formation or neutralize free radicals: glutathione peroxidase, which donates two electrons to reduce peroxides by forming selenoles and also eliminates peroxides as potential substrate for the Fenton reaction; catalase, that converts hydrogen peroxide into water and molecular oxygen and has one of the biggest turnover rates known to man, allowing just one molecule of catalase to convert 6 billion molecules of hydrogen peroxide; and finally, superoxide dismutase converts superoxide anions into hydrogen peroxide as a substrate for catalase (Rahman, 2007). The secondary enzymatic defense includes glutathione reductase and glucose-6-phosphate dehydrogenase. Glutathione reductase reduces glutathione (antioxidant) from its oxidized to its reduced form, thus recycling it to continue neutralizing more free radicals. Glucose-6-phosphate regenerates NADPH (nicotinamide adenine dinucleotide phosphate – coenzyme used in anabolic reactions) creating a reducing environment (Gamble and Burke, 1984; Ratnam et al., 2006). These two enzymes do not neutralize free radicals directly, but have supporting roles to the other endogenous antioxidants.

Considering the non-enzymatic endogenous antioxidants, there are quite a number of them, namely vitamins (A), enzyme cofactors (Q10), nitrogen compounds (uric acid), and peptides (glutathione).

Vitamin A or retinol is a carotenoid produced in the liver and results from the breakdown of β -carotene. There are about a dozen forms of vitamin A that can be isolated. It is known to have beneficial impact on the skin, eyes and internal organs. What confers the antioxidant activity is the ability to combine with peroxyl radicals before they propagate peroxidation to lipids (Palace et al., 1999; Jee et al., 2006).

Coenzyme Q10 is present in all cells and membranes; it plays an important role in the respiratory chain and in other cellular metabolism. Coenzyme Q10 acts by preventing the formation of lipid peroxyl radicals, although it has been reported that this coenzyme can neutralize these radicals even after their formation. Another important function is the ability to regenerate vitamin E; some authors describe this process to be more likely than regeneration of vitamin E through ascorbate (vitamin C) (Turunen et al., 2004).

Uric acid is the end product of purine nucleotide metabolism in humans and during evolution its concentrations have been rising. After undergoing kidney filtration, 90% of uric acid is reabsorbed by the body, showing that it has important functions within the body. In fact, uric acid is known to prevent the overproduction of oxo-hem oxidants that result from the reaction of hemoglobin with peroxides. On the other hand it also prevents lysis of erythrocytes by peroxidation and is a potent scavenger of singlet oxygen and hydroxyl radicals (Kand'ár et al., 2006).

Glutathione is an endogenous tripeptide which protects the cells against free radicals either by donating a hydrogen atom or an electron. It is also very important in the regeneration of other antioxidants like ascorbate (Steenvoorden and Henegouwen, 1997).

Despite its remarkable efficiency, the endogenous antioxidant system does not suffice, and humans depend on various types of antioxidants that are present in the diet to maintain free radical concentrations at low levels (Pietta, 2000).

Vitamins C and E are generic names for ascorbic acid and tocopherols. Ascorbic acid includes two compounds with antioxidant activity: L-ascorbic acid and L-dehydroascorbic acid which are both absorbed through the gastrointestinal tract and can be interchanged enzymatically *in vivo*. Ascorbic acid is effective in scavenging the superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen and reactive nitrogen oxide (Barros et al., 2011). Vitamin E is composed of eight isoforms, with four tocopherols (α -tocopherol, β -tocopherol, γ -tocopherol and δ -tocopherol) and four tocotrienols (α -tocotrienol, β -tocotrienol, γ -tocotrienol

and δ -tocotrienol), α -tocopherol being the most potent and abundant isoform in biological systems. The chroman head group confers the antioxidant activity to tocopherols, but the phytyl tail has no influence. Vitamin E halts lipid peroxidation by donating its phenolic hydrogen to the peroxy radicals forming tocopheroxyl radicals that, despite also being radicals, are unreactive and unable to continue the oxidative chain reaction. Vitamin E is the only major lipid-soluble, chain breaking antioxidant found in plasma, red cells and tissues, allowing it to protect the integrity of lipid structures, mainly membranes (Burton and Traber, 1990). These two vitamins also display a synergistic behavior with the regeneration of vitamin E through vitamin C from the tocopheroxyl radical to an intermediate form, therefore reinstating its antioxidant potential (Halpner et al., 1998).

Vitamin K is a group of fat-soluble compounds, essential for posttranslational conversion of protein-bound glutamates into γ -carboxyglutamates in various target proteins. The 1,4-naphthoquinone structure of these vitamins confers the antioxidant protective effect. The two natural isoforms of this vitamin are K₁ and K₂ (Vervoort et al., 1997).

Flavonoids are an antioxidant group of compounds composed of flavonols, flavanols, anthocyanins, isoflavonoids, flavanones and flavones. All these sub-groups of compounds share the same diphenylpropane (C₆C₃C₆) skeleton. Flavanones and flavones are usually found in the same fruits and are connected by specific enzymes, while flavones and flavonols do not share this phenomenon and are rarely found together. Anthocyanins are also absent in flavanone-rich plants. The antioxidant properties are conferred on flavonoids by the phenolic hydroxyl groups attached to ring structures and they can act as reducing agents, hydrogen donors, singlet oxygen quenchers, superoxide radical scavengers and even as metal chelators. They also activate antioxidant enzymes, reduce α -tocopherol radicals (tocopheroxyls), inhibit oxidases, mitigate nitrosative stress, and increase levels of uric acid and low molecular weight molecules. Some of the most important flavonoids are catechin, catechin-gallate, quercetin and kaempferol (Rice-Evans et al., 1996; Procházková et al., 2011).

Phenolic acids are composed of hydroxycinnamic and hydroxybenzoic acids. They are ubiquitous to plant material and sometimes present as esters and glycosides. They have antioxidant activity as chelators and free radical scavengers with special impact over hydroxyl and peroxy radicals, superoxide anions and peroxynitrites. One of the most studied and promising compounds in the hydroxybenzoic group is gallic acid which is also the precursor of many tannins, while cinnamic acid is the precursor of all the hydroxycinnamic acids (Krimmel et al., 2010; Terpinc et al., 2011).

Carotenoids are a group of natural pigments that are synthesized by plants and microorganisms but not by animals. They can be separated into two vast groups: the carotenoid hydrocarbons known as the carotenes which contain specific end groups like lycopene and β -carotene; and the oxygenated carotenoids known as xanthophylls, like zeaxanthin and lutein. The main antioxidant property of carotenoids is due to singlet oxygen quenching which results in excited carotenoids that dissipate the newly acquired energy through a series of rotational and vibrational interactions with the solvent, thus returning to the unexcited state and allowing them to quench more radical species. This can occur while the carotenoids have conjugated double bonds within. The only free radicals that completely destroy these pigments are peroxy radicals. Carotenoids are relatively unreactive but may also decay and form non-radical compounds that may terminate free radical attacks by binding to these radicals (Paiva and Russell, 1999).

Minerals are only found in trace quantities in animals and are a small proportion of dietary antioxidants, but play important roles in their metabolism. Regarding antioxidant activity, the most important minerals are selenium and zinc. Selenium can be found

in both organic (selenocysteine and selenomethionine) and inorganic (selenite and selenate) forms in the human body. It does not act directly on free radicals but is an indispensable part of most antioxidant enzymes (metalloenzymes, glutathione peroxidase, thioredoxin reductase) that would have no effect without it (Tabassum et al., 2010). Zinc is a mineral that is essential for various pathways in metabolism. Just like selenium, it does not directly attack free radicals but is quite important in the prevention of their formation. Zinc is also an inhibitor of NADPH oxidases which catalyze the production of the singlet oxygen radical from oxygen by using NADPH as an electron donor. It is present in superoxide dismutase, an important antioxidant enzyme that converts the singlet oxygen radical into hydrogen peroxide. Zinc induces the production of metallothionein that is a scavenger of the hydroxyl radical. Finally zinc also competes with copper for binding to the cell wall, thus decreasing once again the production of hydroxyl radicals (Prasad et al., 2004).

1.2.2. Synthetic antioxidants

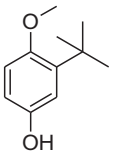
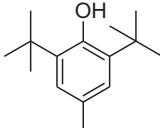
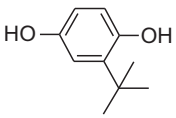
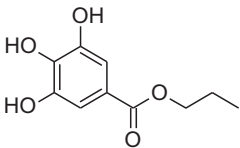
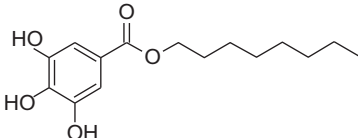
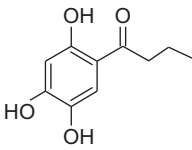
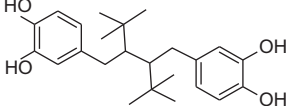
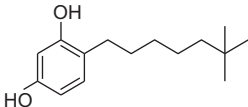
In order to have a standard antioxidant activity measurement system to compare with natural antioxidants and to be incorporated into food, synthetic antioxidants have been developed. These pure compounds are added to food so it can withstand various treatments and conditions as well as to prolong shelf life. Table 1 reports the most important and widely available synthetic antioxidants as well as their uses, showing that the main focus of synthetic antioxidants is the prevention of food oxidation, especially fatty acids. Today, almost all processed foods have synthetic antioxidants incorporated, which are reported to be safe, although some studies indicate otherwise.

BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) are the most widely used chemical antioxidants. Between 2011 and 2012, the European food safety authority (EFSA) re-evaluated all the available information on these two antioxidants, including the apparently contradictory data that have been published. EFSA established revised acceptable daily intakes (ADIs) of 0.25 mg/kg bw/day for BHT and 1.0 mg/kg bw/day for BHA and noted that the exposure of adults and children was unlikely to exceed these intakes (EFSA, 2011; EFSA, 2012). TBHQ (*tert*-Butylhydroquinone) stabilizes and preserves the freshness, nutritive value, flavour and color of animal food products. In 2004 the EFSA published a scientific opinion reviewing the impact of this antioxidant on human health and stated that there was no scientific proof of its carcinogenicity despite previous conflicting data. They pointed out that dogs were the most sensitive species and allocated an ADI of 0–0.7 mg/kg bw/day (EFSA, 2004). Octyl gallate is considered safe to use as a food additive because after consumption it is hydrolysed into gallic acid and octanol, which are found in many plants and do not pose a threat to human health (Joung et al., 2004). NDGA (Nordihydroguaiaretic acid) despite being a food antioxidant is known to cause renal cystic disease in rodents (Evan and Gardner, 1979).

1.2.3. Prooxidants

Singh et al. (2010) wrote that antioxidants have gone from “Miracle Molecules” to “Marvellous Molecules” and finally to “Physiological Molecules”. No doubt these molecules play a vital role in metabolic pathways and protect cells, but recently conflicting evidence has forced the academic community to dig deeper into the role of antioxidants and prooxidants. The latter are defined as chemicals that induce oxidative stress, usually through the formation of reactive species or by inhibiting antioxidant systems (Puglia and Powell, 1984). Free radicals are considered prooxidants, but surprisingly, antioxidants can also have prooxidant behaviour.

Table 1
Chemical structure and applications of the most important synthetic antioxidants.

Compound name		Applications	Reference
BHA (butylated hydroxyanisole)		Food antioxidants	Branen (1975)
BHT (butylated hydroxytoluene)			Botterweck et al. (2000) Aguillar et al. (2011) Aguillar et al. (2012)
TBHQ (<i>tert</i> -butylhydroquinone)		Animal processed food antioxidant	Gharavi and El-Kadi (2005)
PG (propyl gallate)		Food antioxidant	Anton et al. (2004) Soares et al. (2003)
OG (octyl gallate)		Food and cosmetic antioxidant Antifungal properties	Kubo et al. (2001)
2,4,5-Trihydroxy butyrophenone		Food antioxidant	Astill et al. (1959)
NDGA (nordihydroguaiaretic acid)		Food antioxidant	Evan and Gardner (1979)
4-Hexylresorcinol		Prevention of food browning	Chen et al. (2004)

Vitamin C is considered a potent antioxidant and intervenes in many physiological reactions, but it can also become a prooxidant. This happens when it combines with iron and copper reducing Fe^{3+} to Fe^{2+} (or Cu^{3+} to Cu^{2+}), which in turn reduces hydrogen peroxide to hydroxyl radicals (Duarte and Lunec, 2005).

α -Tocopherol is also known to be a useful and powerful antioxidant but in high concentrations it can become a prooxidant due to its antioxidant mechanism. When it reacts with a free radical it becomes a radical itself, and if there is not enough ascorbic acid for its regeneration it will remain in this highly reactive state and promote the autoxidation of linoleic acid (Cillard et al., 1980).

Although not much evidence is found, it is proposed that carotenoids can also display prooxidant effects especially through autoxidation in the presence of high concentrations of oxygen-forming hydroxyl radicals (Young and Lowe, 2001). Even flavonoids can act as prooxidants, although each one responds differently to the environment in which it is inserted. Dietary phenolics can also

act as prooxidants in systems that contain redox-active metals. The presence of O_2 and transition metals like iron and copper catalyze the redox cycling of phenolics and may lead to the formation of ROS and phenoxyl radicals which damage DNA, lipids and other biological molecules (Galati and O'Brien, 2004).

Yordi et al. (2012) published a list of fourteen phenolic acids, considered to be antioxidants but under certain conditions behaved as prooxidants.

2. Methodologies for antioxidant activity screening and antioxidants analysis

2.1. Antioxidant activity screening assays

To date there are various antioxidant activity assays, each one having their specific target within the matrix and all of them with advantages and disadvantages. There is not one method that can

Table 2

List of the most important assays to screen antioxidant activity.

Assay	Mechanism	Reference
ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)	Scavenging activity	Antolovich et al. (2000) Moon and Shibamoto (2009)
DPPH (2,2-diphenyl-1-picrylhydrazyl)	Scavenging activity	Antolovich et al. (2000) Amarowicz et al. (2004) Moon and Shibamoto (2009)
HO [•] scavenging activity	Scavenging activity	Huang et al. (2005)
H ₂ O ₂ scavenging activity	Scavenging activity	Huang et al. (2005)
O ₂ ^{•-} scavenging activity	Scavenging activity	Huang et al. (2005)
Peroxynitrite (ONOO ⁻) scavenging capacity	Scavenging activity	Huang et al. (2005)
ESR (electron spin resonance spectrometry)	Free radicals quantification	Antolovich et al. (2000)
Spin trapping	Alkoxy and peroxy radicals quantification	Gutteridge (1995)
FRAP (ferric reducing antioxidant power)	Reducing power	Antolovich et al. (2000) Huang et al. (2005) Berker et al. (2007)
Conjugated diene	Lipid peroxidation inhibition	Moon and Shibamoto (2009)
FOX (ferrous oxidation-xenolol)	Lipid peroxidation inhibition	Moon and Shibamoto (2009)
FTC (ferric thiocyanate)	Lipid peroxidation inhibition	Moon and Shibamoto (2009)
GSHPx (glutathione peroxidase)	Lipid peroxidation inhibition	Gutteridge (1995)
Heme degradation of peroxides	Lipid peroxidation inhibition	Gutteridge (1995)
Iodine liberation	Lipid peroxidation inhibition	Gutteridge (1995)
TBARS (thiobarbituric reactive substances)	Lipid peroxidation inhibition	Gutteridge (1995) Moon and Shibamoto (2009)
TEAC assay (Trolox equiv. antioxidant capacity)	Antioxidant activity	Huang et al. (2005)
Total oxidant potential using Cu (II) as an oxidant	Antioxidant activity	Huang et al. (2005)
TRAP (total radical-trapping antioxidant parameter)	Antioxidant activity	Antolovich et al. (2000)
ACA (aldehyde/carboxylic acid)	Slow oxidation phenomena	Moon and Shibamoto (2009)

provide unequivocal results and the best solution is to use various methods instead of a one-dimension approach. Some of these procedures use synthetic antioxidants or free radicals, some are specific for lipid peroxidation and tend to need animal or plant cells, some have a broader scope, some require minimum preparation and knowledge, few reagents and are quick to produce outputs. Table 2 provides insights on some of the most important and widely used assays available to determine the antioxidant capacity of synthetic compounds or natural matrixes including food products.

The ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay is a colorimetric assay in which the ABTS radical decolorizes in the presence of antioxidants (carotenoids, phenolic compounds and others) (Moon and Shibamoto, 2009). DPPH (2,2-diphenyl-1-picrylhydrazyl) is based on the premise that a hydrogen donor is an antioxidant. This colorimetric assay uses the DPPH radical, which changes from purple to yellow in the presence of antioxidants, and is widely used as a preliminary study (Moon and Shibamoto, 2009). In the HO[•] scavenging activity, the hydroxyl radical is indirectly confirmed by the hydroxylation of *p*-hydroxybenzoic acid. Fluorescein (FL) is used as a probe and the fluorescence decay curve is monitored in the presence and absence of the antioxidants. The area under curve (AUC) is integrated, and the net AUC is calculated by subtracting the blank from the sample AUC (Huang et al., 2005).

In the H₂O₂ scavenging activity assay, horseradish peroxidases are used to oxidize scopoletin to a nonfluorescent product and antioxidants seem to inhibit this reaction. Its results are ambiguous due to the various pathways that lead to the inhibition (Huang et al., 2005). The O₂^{•-} scavenging capacity assay is optimized for enzymatic antioxidants and relies on the competition kinetics of O₂^{•-} reduction of cytochrome C (probe) and O₂^{•-} scavenger (sample) (Huang et al., 2005). ONOO⁻ and ONOOH cause nitration or hydroxylation in aromatic compounds, particularly tyrosine. Under physiologic conditions, peroxynitrite also forms an adduct with CO₂ dissolved in body fluid. This adduct is believed to be responsible for some damage inflicted to proteins. Therefore, the two methods to measure ONOO⁻ are the inhibition of tyrosine nitration and

inhibition of dihydrorhodamine 123 oxidation (Huang et al., 2005). The ESR (Electron Spin Resonance Spectrometry) assay is the only procedure that allows to specifically detect free radicals involved in autoxidation (Antolovich et al., 2002). Spin traps allow the formation of stable nitroxides which can be examined by electron spin resonance. An advantage of this method is the potential usage *in vivo* (Gutteridge, 1995). The FRAP (ferric reducing antioxidant power) assay was originally applied to plasma but is now commonly used in a vast number of matrixes. It is characterized by the reduction of Fe³⁺ to Fe²⁺ depending on the available reducing species followed by the alteration of color from yellow to blue and analyzed through a spectrophotometer (Antolovich et al., 2002). Lipid peroxidation can be detected through the assay for conjugated dienes, which are formed from a moiety with two double bonds separated by a single methylene group. This usually occurs in polyunsaturated fatty acids by the action of ROS and oxygen. The absorbance of the conjugated diene is around 234 nm which is also the normal absorbance wavelength of biological and natural compounds, making this the major drawback of this assay (Moon and Shibamoto, 2009). In the FOX (ferrous oxidation-xenolol assay) assay, the ferrous ion is oxidized by an oxidant (hydroperoxide) forming a blue-purple compound. The absorbance is then determined with a spectrophotometer (Moon and Shibamoto, 2009). The FTC (ferric thiocyanate assay) assay is very similar to the FOX assay, the only difference between them is the fact that the formed ferric ion is monitored as thiocyanate complex by a spectrophotometer at 500 nm (Moon and Shibamoto, 2009).

The GSHPx (glutathione peroxidase) assay detects lipid peroxides although they must first be cleaved out of their membranes by phospholipases. Then the reaction between GSHPx and H₂O₂ oxidizes GSH (glutathione) to GSSG (oxidized glutathione). Finally the addition of glutathione reductase and NADPH to reduce GSSG to back to GSH consumes NADPH and can be related to the peroxide content (Gutteridge, 1995).

The heme degradation of peroxides relies on the heme moiety of proteins to decompose lipid peroxides with formation of reactive intermediates which can be reacted with isoluminol to produce light (Gutteridge, 1995). Iodine can also be used to detect

Table 3

List of the most important techniques used for antioxidants analysis.

Technique	Compounds	Reference
Antibody techniques	Individual aldehydes (HPLC)	Gutteridge (1995)
Fluorescence assay	Total aldehydes	Gutteridge (1995)
Folin–Ciocalteu spectrophotometric assay	Total phenolics	Huang et al. (2005)
Gas chromatography (GC)	Lipid peroxides	Slover et al. (1983)
	Aldehydes	Gutteridge (1995)
	Tocopherols	Wu et al. (1999)
	Sterols	Moon and Shibamoto (2009)
	Phenolic acids	
	Flavonoids	
High performance liquid chromatography (HPLC)	Flavonoids	Carpenter (1979)
	Tocopherols	Merken and Beecher (2000)
	Aldehydes	Rijke et al. (2006)
	Phenolic acids	Stalinks (2007)
		Moon and Shibamoto (2009)
Light emission	Excited-state carbonyls and singlet O ₂	Gutteridge (1995)

lipid peroxides by oxidizing it from I[−] to I₂ for titration with thio-sulfate. It can be applied to biological samples if other oxidizing agents are absent (Gutteridge, 1995). The widely used TBARS (thio-barbituric acid reactive substances) assay is simple and non-specific and requires rigorous controls. The matrix is mixed with thiobarbituric acid (TBA) resulting in a pink chromogen that can be measured by absorbance at 532 nm or fluorescence at 553 nm (Gutteridge, 1995). The Trolox equivalent antioxidant capacity (TEAC) assay uses the radical ABTS^{•+} which undergoes treatment to gain a dark blue color. It is then diluted in ethanol until its absorbance reaches 0.7 at 734 nm. One milliliter is then mixed with the matrix and the absorbances are read after specific intervals. The difference of the absorbance reading is plotted versus the antioxidant concentration to give a straight line. The concentration of antioxidants giving the same percentage change in absorbance of the ABTS^{•+} as that of 1 mM Trolox is regarded as TEAC (Huang et al., 2005). Cu (II) can also be used as an oxidant in the determination of the antioxidant potential, although this assay is not widely used. The method relies on the reduction of Cu (II) to Cu (I) by the antioxidants present in the sample. Then, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) forms a complex with Cu (I) and has a maximum absorbance at 490 nm (Huang et al., 2005). The total radical-trapping antioxidant parameter (TRAP) assay is used to determine the total antioxidant activity, based on measuring oxygen consumption during a controlled lipid oxidation reaction induced by thermal decomposition of AAPH (2,2'-azobis(2-amidinopropane)hydrochloride) (Antolovich et al., 2002). The ACA (aldehyde/carboxylic acid) assay is used in slow oxidation phenomena (shelf life foods) and converts the alkylaldehyde to alkylcarboxylic acid in the presence of reactive radicals (Moon and Shibamoto, 2009).

2.2. Antioxidant compounds analysis

Some procedures are focused on identifying and determining the exact quantity of a specific compound within complex matrixes. They rely on HPLC, GC and or Mass Spectrometry (MS) and other expensive equipment to detect specific antioxidants. These assays can take quite a long time to provide results and require deep knowledge. Table 3 summarizes some of these assays as well as the compounds they help identify.

The antibody techniques are used to detect proteins modified by lipid peroxidation products: e.g., proteins modified by reaction with unsaturated aldehydes (Gutteridge, 1995). The fluorescence assay is based on the theory that aldehydes when polymerized form fluorescent products in the absence of amino groups (Gutteridge, 1995). Folin–Ciocalteu is a reagent that is known to react with all reducing species in a solution. It is a mixture of tungsten and

molybdenum with a yellow color. Under alkaline conditions it reacts with all the antioxidants in solution, changing its color to blue which can be analyzed with a spectrophotometer. Because it reacts with all reducing species it is not very sensitive and is used as preliminary approach (Huang et al., 2005). GC (gas chromatography) is used to separate and determine the exact quantity of specific antioxidants in various matrixes. It can determine a high number of different compounds, from tocopherols to phenolic acids, among others. HPLC (high performance liquid chromatography) as GC coupled to various detectors are one of the most powerful methods of detecting specific antioxidant compounds (ascorbic acid, tocopherols, flavonoids, phenolic acids, etc.). They require significant investment and knowledge but offer one of the most precise separation and quantification available.

Light emission can be used to detect ROS through chemoluminescence by excited-state carbonyls and singlet O₂ (which emit light when they decay to ground state) reacting with peroxy radicals. The main disadvantage is the emission of light from other sources (Gutteridge, 1995).

3. Controversy and limitations among antioxidants and prooxidants

3.1. Controversy

In recent years, antioxidants and prooxidants have been extensively studied and it seems that most of the dietary antioxidants can behave as prooxidants; it all depends on their concentration and the nature of neighbouring molecules (Villanueva and Kross, 2012). The controversy around dietary antioxidants is because the capacity to display antioxidant and prooxidant behaviour depends on various factors. Numerous studies have shown the beneficial effects of antioxidants, in a few thousand papers, but, many others have demonstrated otherwise. Gilgun-Sherki et al. (2001) published a list with conflicting results of various studies reporting the influence of antioxidants in neurodegenerative diseases. Halliwell (2008) postulated that prooxidant effect can be beneficial, since the imposition of a mild degree of oxidative stress, might raise the levels of antioxidant defences and xenobiotic-metabolising enzymes, leading to overall cytoprotection. Procházková et al. (2011) hypothesizes that prooxidants can have cell signalling properties which are essential to life, once again attributing a useful role to them. Other authors have also shown that the prooxidant effect of flavonoids might mitigate certain types of cancer (Gomes et al., 2003; Galati and O'Brien, 2004; Lambert and Elias, 2010; Perera and Bardeesy, 2011).

As stated above, the research around antioxidants has grown exponentially, but there are still certain limitations that need to

be considered before the real potential of these molecules is unleashed. According to Heim et al. (2002) the daily intake of flavonoids is estimated to be 23 mg in the Netherlands. Although this is quite a high value, the total amount of flavonoids in the blood stream is quite low. Most of them occur in food as *O*-glycosides, glucose being the most common glycosidic unit, followed by glucorhamnose and galactose. Due to their molecular size, the absorption of flavonoids through the intestinal epithelium requires them to be metabolized into smaller, low molecular weight compounds. They undergo *O*-methylations, hydroxylations, cleavages of the heterocycle, deglycolisations, and scission of polymeric compounds into monomeric ones in order to pass through the epithelium (Heim et al., 2002). Lotito and Frei (2006), after reviewing the published research in flavonoid metabolism, related the high antioxidant activity of blood plasma with the intake of flavonoid-rich food and concluded that flavonoids, due to being highly metabolized, may not contribute themselves to this increase, but rather help increase uric acid levels, which could be considered an indirect antioxidant activity. Anthocyanins also tend to bond with sugars, which complicates their absorption. In order to yield satisfactory absorption through the gut they have to be hydrolyzed to anthocyanin aglycones or phenolic acids (Liang et al., 2012). Despite these findings, McDougall et al. (2007) reported that under simulated gastrointestinal conditions, the bulk of anthocyanins were unstable in small intestine conditions and hypothesized that their biological activities were carried out by unidentified breakdown products.

3.2. Limitations

In terms of neuroprotection conferred by antioxidants, it seems that antioxidants failed to deliver satisfying protection, mainly due to the blood brain barrier (Gilgun-Sherki et al., 2001; Fortalezas et al., 2010). Halliwell (2010) goes further reporting that there is still a long road to travel before antioxidants are fully understood, and states that laboratory mice are more sensitive to dietary antioxidants than humans, and when clinical trials are started this fact should be kept in mind. Another limitation regarding antioxidants research are cell cultures, which are altered with time, causing the antioxidants tested *in vitro* to often react with the medium or be neutralized very quickly; thus leading to erroneous results, that are usually overlooked by peer-review. Another significant limitation in antioxidant capacity of food is the vast number of different assays that can be used to determine the same parameters (discussed above). Several antioxidant assays have limitations and interferences which still pose difficulties when comparing results between different procedures and researchers. This, allied to endless different matrixes, raises serious problems that do not help the scientific community to move forward. There is a need to look deeper into all the available antioxidant assays, to improve them and to have a uniform number of procedures that should be mandatory in order to aid comparisons between matrixes and research results. Frankel and Meyer (2000) proposed five simple questions that should not be ignored and promptly answered when analyzing antioxidant activity: (1) What are the true protective properties of antioxidants? What is the antioxidant protecting against? (2) What substrates are oxidized and what products are inhibited? (3) What is the location of the antioxidant in the system? (4) What is the effect of other interacting components? (5) What conditions are relevant to real-life applications? Responding to these questions beforehand could be the first step to narrow down conflicting results.

4. Future perspectives

During the past decades a lot of research has been carried out around antioxidants and their effects on health. There is a lack of

a standard procedure to determine antioxidant activity across the majority of matrixes in order to produce consistent and undoubted results. The published results so far are conflicting and difficult to compare between each other. The antioxidant limitations and metabolism still pose a challenge to future research in this field, and researchers must try and overcome these drawbacks.

The new trends in antioxidant treatments include compounds that behave like the enzyme SOD in order to alleviate acute and chronic pain related to inflammation and reperfusion. Another promising research area are genetics, which aim to breed genetically modified plants that can produce higher quantities of specific compounds, yielding higher quantities of antioxidants (Devasagayam et al., 2004). Suntres (2011) theorizes that antioxidant liposomes will hold an important role in future research on antioxidants. This author reports that they can facilitate antioxidant delivery to specific sites as well as achieving prophylactic and therapeutic action.

Bouayed and Bohn (2010) postulate that the balance between oxidation and antioxidation is critical in maintaining a healthy biologic system. Low doses of antioxidants may be favorable to this system, but high quantities may disrupt the balance.

The main conclusion is that antioxidants do have an impact on our health, but the big question is the method of administration (food vs. supplements) and quantity that might be debatable. The fact that potent antioxidants *in vitro* may not have any effect *in vivo* should not discourage further research but rather stimulate it (Devasagayam et al., 2004).

It is true that antioxidants are beneficial and display a useful role in human homeostasis, but so are prooxidants; the academic community should search deeper into the kinetics and *in vivo* mechanisms of antioxidants to uncover the optimal concentrations or desired functions in order to push forward against cancer, neurodegenerative and cardiovascular diseases.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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