Review article

Methodological aspects about in vitro evaluation of antioxidant properties

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A B S T R A C T
Several of the most commonly used methods for in vitro determination of antioxidant capacity are reviewed in the present paper. The chemical principles of methods based either on biological oxidants (peroxyl radical, superoxide radical anion, hydrogen peroxide, hydroxyl radical, hypochlorous acid, singlet oxygen, nitric oxide radical, and peroxynitrite) or on non-biological assays (scavenging of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (TEAC assay), scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH• assay), ferric reducing antioxidant power (FRAP assay), Folin–Ciocalteu reducing capacity (FC assay), electrochemical total reducing capacity) are outlined and critically discussed. The scope of application, the advantages and shortcomings of each method are also highlighted.

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During the past years, the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in the oxidative deterioration of food products as well as in the pathogenesis of several human diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders and certain types of cancer [1–3]. The putative protective effects of antioxidants against these deleterious oxidative-induced reactions have received increasing attention lately, especially within biological, medical, nutritional, and agrochemical fields. In fact, a literature search performed on the ISI Web of Knowledge search engine for articles containing the expression “antioxidant or antioxidants”, revealed that the number of publications has increased about 345% in the past decade (16,080 until 1996, while between 1997 and 2006 around 55,493 papers were published). This situation demands the existence of simple, convenient, and reliable in vitro analytical methodologies for the fast determination of antioxidant capacity of pure compounds or in complex matrices, such as food and biological samples.

In this context, some of the most commonly used methods for in vitro determination of antioxidant capacity are reviewed in the following sections, where the chemical principles, some of its variants, recent applications as well as the advantages and shortcomings are outlined. Special attention will be given to the guidelines proposed by Prior et al. [4] for standardization of the determination of antioxidant capacity. These include the following requirements/criteria: (i) measurement of the chemistry actually occurring in potential applications; (ii) utilization of biological relevant molecules; (iii) technically simple; (iv) with a defined endpoint and chemical mechanism; (v) readily available instrumentation; (vi) good repeatability and reproducibility; (vii) adaptable for assay of both hydrophilic and lipophilic antioxidants; (viii) and adaptable to high-throughput analysis. Considering the second requirement, these assays were roughly divided here into two categories according to the type of the oxidant species: (i) scavenging capacity assays against specific ROS/RNS; (ii) scavenging capacity assays against stable, non-biological radicals and evaluation of total reduction capacity.

While oxidant and reductant are chemical terms, in biological environments they are usually termed as pro-oxidant and antioxidant, respectively [5]. Pro-oxidant is a substance that can induce oxidative damage to various biological targets such as nucleic acids (e.g. base modification, single- and double-strand breaks), lipids (e.g. peroxidation, fatty acid loss), and proteins (e.g. oxidation of specific amino acid residues, formation of carbonyls). An antioxidant is a substance that can efficiently reduce a pro-oxidant with concomitant formation of products having no or low toxicity. Indeed, a broader definition of antioxidant was suggested by Halliwell et al. [6] as “any substance that when present at low concentrations, compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate”. Therefore, according to this definition, not all reductants involved in a chemical reaction are antioxidants; only those compounds which are capable of protecting the biological target meet these criteria. This protection may be based on several mechanisms of action, namely: (i) inhibition of generation and scavenging capacity against ROS/RNS; (ii) reducing capacity; (iii) metal chelating capacity; (iv) activity as antioxidative enzyme; (v) inhibition of oxidative enzymes. The available methodologies for assessing the first two types of action will be discussed here. The determination of the activity of antioxidant enzymes (e.g. superoxide dismutase, catalase, and glutathione peroxidase) or the inhibition of oxidative enzymes (e.g. NOS synthase, xanthine oxidase and cycloxygenase, for instance) is out of the scope of this text. More information about the determination of biological markers of oxidative stress or methods for in vivo assessment of antioxidant properties can be found in recent literature [7–12].

Considering these definitions, knowledge of the chemistry principles of the available methodologies is of utmost importance to select the adequate technique(s). Generally, the in vitro analytical methods for determination of antioxidant capacity rely on two different approaches, named here as competitive or non-competitive scheme (Fig. 1).

[Image: Schematic representation of competitive (a) and non-competitive (b) approaches for in vitro determination of antioxidant capacity.]

Fig. 1 – Schematic representation of competitive (a) and non-competitive (b) approaches for in vitro determination of antioxidant capacity.
<table>
<thead>
<tr>
<th>Assay</th>
<th>Target compound</th>
<th>Detection probe</th>
<th>Principle of measurement</th>
<th>Quantification</th>
<th>Recent applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORAC</td>
<td>β-PE</td>
<td>β-PE</td>
<td>Fluorescence decay along time due to oxidation of probe is delayed/inhibited by antioxidants</td>
<td>Net AUC expressed as Trolox equivalents</td>
<td>Wines [15], spices [16], human plasma [17]</td>
</tr>
<tr>
<td>BODIPY 581/591</td>
<td>BODIPY 581/591</td>
<td>Fluorescence decay along time due to oxidation of probe is delayed/inhibited by antioxidants</td>
<td>Net AUC expressed as Trolox equivalents</td>
<td>Procyanidin dimers [18]</td>
<td></td>
</tr>
<tr>
<td>Fluorescein</td>
<td>Fluorescein</td>
<td>Fluorescence decay along time due to oxidation of probe is delayed/inhibited by antioxidants</td>
<td>Net AUC expressed as Trolox equivalents</td>
<td>Rat brain tissue [19], fruits and vegetables [20]</td>
<td></td>
</tr>
<tr>
<td>TRAP</td>
<td>Human plasma</td>
<td>Oxygen</td>
<td>Oxygen consumption due to oxidation of plasma material is delayed by antioxidants</td>
<td>Lag time expressed as Trolox equivalents</td>
<td>Human plasma [21], umbilical plasma and placental tissues [22]</td>
</tr>
<tr>
<td>β-PE</td>
<td>β-PE</td>
<td>Fluorescence decay along time due to oxidation of β-PE is delayed by antioxidants</td>
<td>Lag time expressed as Trolox equivalents</td>
<td>Human plasma [23], grape seeds and skin [24]</td>
<td></td>
</tr>
<tr>
<td>Crocin bleaching</td>
<td>Crocin</td>
<td>Crocin</td>
<td>Absorbance decrease along time due to oxidation of crocin is inhibited by antioxidants</td>
<td>Ratio of initial crocin bleaching rates in absence and in presence of test sample</td>
<td>Phenolic compounds [25], flavanones [26]</td>
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<tr>
<td></td>
<td>Crocin</td>
<td>Crocin</td>
<td>Absorbance decrease due to oxidation of crocin is inhibited by antioxidants and measured after a fixed time period</td>
<td>Inhibition percentage</td>
<td>Phenolic compounds [27], plasma [28]</td>
</tr>
<tr>
<td>LDL oxidation</td>
<td>LDL</td>
<td>Conjugated dienes</td>
<td>Absorbance increase due to formation of conjugated dienes from oxidation of LDL is inhibited by antioxidants</td>
<td>Lag time</td>
<td>Dehydroascorbate [29], hydroxytyrosol [30], secondary plant metabolites [31]</td>
</tr>
<tr>
<td>TOSC</td>
<td>KMBA</td>
<td>Ethylene</td>
<td>Ethylene, formed due to oxidation of KMBA, is measured along time using GC-FID</td>
<td>Relative AUC</td>
<td>Fish liver homogenate [32], strawberry [33]</td>
</tr>
<tr>
<td>CL-based</td>
<td>Luminol (oxidized)</td>
<td>Luminol (oxidized)</td>
<td>CL emission due to oxidation of luminol is inhibited by antioxidants</td>
<td>Lag time expressed as Trolox equivalents</td>
<td>Human plasma [34], human serum [35]</td>
</tr>
</tbody>
</table>

ORAC: oxygen radical absorbance capacity; β-PE: β-phycoerythrin; AUC: area under the curve that represents the analytical property monitored along time; BODIPY 581/591: 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; TRAP: total radical-trapping antioxidant parameter; LDL: low-density lipoproteins; TOSC: total oxyradical scavenging capacity; KMBA: α-keto-γ-methiolbutyric acid; GC-FID: gas chromatography with flame ionization detector; CL: chemiluminescence.
<table>
<thead>
<tr>
<th>Table 2 – Summary of analytical features of some in vitro methods for determination of superoxide anion radical scavenging capacity</th>
</tr>
</thead>
<tbody>
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<td><strong>Target compound</strong></td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>n.a.</td>
</tr>
<tr>
<td>n.a.</td>
</tr>
<tr>
<td>n.a.</td>
</tr>
<tr>
<td>KMBA</td>
</tr>
<tr>
<td>DMPO</td>
</tr>
<tr>
<td>Luminol</td>
</tr>
<tr>
<td>Lucigenin</td>
</tr>
</tbody>
</table>

n.a.: Not applicable; NBT: nitroblue tetrazolium; KMBA: α-keto-γ-methylbutyric acid; DMPO: 5,5-dimethyl-1-pyrrrole N-oxide; ESR: electron spin resonance; CL: chemiluminescence.

<table>
<thead>
<tr>
<th>Table 3 – Summary of analytical features of some in vitro methods for determination of hydrogen peroxide scavenging capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target compound</strong></td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>n.a.</td>
</tr>
<tr>
<td>HVA</td>
</tr>
<tr>
<td>n.a.</td>
</tr>
<tr>
<td>Aryl oxalate ester</td>
</tr>
<tr>
<td>Lucigenin</td>
</tr>
<tr>
<td>Luminol</td>
</tr>
</tbody>
</table>

n.a.: Not applicable; HVA: homovanillic acid; CL: chemiluminescence; NSAIDs: non-steroidal anti-inflammatory drugs.
**Table 4 – Summary of analytical features of some in vitro methods for determination of hydroxyl radical scavenging capacity**

<table>
<thead>
<tr>
<th>Target compound</th>
<th>Detection probe</th>
<th>Principle of measurement</th>
<th>Quantification</th>
<th>Recent applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyribose</td>
<td>Pink chromogen (TBARS)</td>
<td>Absorbance increase due to reaction of thiobarbituric acid with oxidation products from deoxyribose is inhibited by antioxidants and measured after a fixed time period</td>
<td>Inhibition percentage</td>
<td>Plant extracts [121], brain tissue [122], gonadal tissue [123]</td>
</tr>
<tr>
<td>DMPO</td>
<td>DMPO-OH</td>
<td>Formation of DMPO-OH adduct is measured by ESR and it is inhibited by antioxidants and measured after a fixed time period</td>
<td>Relative inhibition</td>
<td>N-substituted indole-2-carboxylic acid esters [124]</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>2,3-DHBA and 2,5-DHBA</td>
<td>DHBA, formed due to oxidation of salicylic acid, is measured using HPLC-ED and its formation is inhibited by antioxidants</td>
<td>Quantity of DHBA produced</td>
<td>–</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>Fluorescein</td>
<td>Fluorescence decay along time due to oxidation of fluorescein is delayed/inhibited by antioxidants</td>
<td>Net AUC expressed as Trolox equivalents</td>
<td>Barley kernel extract [125]</td>
</tr>
<tr>
<td>Luminol</td>
<td>Luminol (oxidized)</td>
<td>CL emission due to oxidation of luminol is inhibited by antioxidants</td>
<td>Inhibition percentage</td>
<td>Plant extracts [126], pyrazolone derivatives [127]</td>
</tr>
</tbody>
</table>

n.a.: Not applicable; TBARS: thiobarbituric acid-reactive substances; DMPO: 5,5-dimethyl-1-pyrroline N-oxide; DHBA: dihydroxybenzoic acid; HPLC-ED: high performance liquid chromatography with electrochemical detection; AUC: area under the curve that represents the analytical property monitored along time; CL: chemiluminescence.

**Table 5 – Summary of analytical features of some in vitro methods for determination of hypochlorous acid scavenging capacity**

<table>
<thead>
<tr>
<th>Target compound</th>
<th>Detection probe</th>
<th>Principle of measurement</th>
<th>Quantification</th>
<th>Recent applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-AP</td>
<td>Elastase substrate</td>
<td>Absorbance increase due to activity of non-inhibited elastase, using a colour forming substrate</td>
<td>Elastase activity as $\Delta A_{410} \text{ min}^{-1}$</td>
<td>–</td>
</tr>
<tr>
<td>TNB</td>
<td>TNB</td>
<td>Absorbance decrease due to oxidation of TNB to DTNB is inhibited by antioxidants and measured after a fixed time period</td>
<td>Inhibition percentage</td>
<td>S-Allylcysteine [135], plant infusion [136]</td>
</tr>
<tr>
<td>BSA</td>
<td>BSA (carbonyl groups)</td>
<td>The carbonyl content of BSA increases due to oxidation by HOCl and it is inhibited by antioxidants</td>
<td>Inhibition percentage</td>
<td>Onion green leaves extract [137]</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>p-Aminobenzoic acid</td>
<td>Fluorescence decay along time due to oxidation of PABA is delayed/inhibited by antioxidants</td>
<td>Rate constant of reaction between PABA and HOCl</td>
<td>–</td>
</tr>
<tr>
<td>Luminol</td>
<td>Luminol (oxidized)</td>
<td>CL emission due to oxidation of luminol is inhibited by antioxidants</td>
<td>Inhibition percentage</td>
<td>Plant extracts [138], medicinal tincture [139]</td>
</tr>
</tbody>
</table>

α₁-AP: α₁-antiproteinase; TNB: 5-thio-2-nitrobenzoic acid; DTNB: 5,5’-dithiobis(2-nitrobenzoic acid); BSA: bovine serum albumin; PABA: p-aminobenzoic acid; CL: chemiluminescence.
In the competitive scheme, the target species, defined here as a compound that represents a biomolecule which may be attacked in vivo, and the antioxidant compounds compete for the reactive species (radical or non-radicals). The assessment of antioxidant capacity is based on the quantification of a compound that facilitates the analytical measurement, defined here as the probe. In most of the competitive assays, the probe is the target species or its oxidized form. Nevertheless, the probe can also be a compound added after the above-mentioned reaction that allows the quantification of the remaining reactive species or target molecules.

In these assays, the antioxidant capacity of tested compounds is dependent on: (i) the rate of reaction between them and the reactive species, (ii) the rate of reaction between the target molecule and the reactive species, and (iii) the concentration ratio between antioxidants and target. Among the requirements for these type of assays, the following should be highlighted: (i) the target/probe must be reactive with oxidants at low concentration; (ii) there must be a dramatic spectroscopic change between the native and oxidized probe (to maximize the sensitivity), (iii) no radical chain reaction beyond target/probe oxidation should occur, and (iv) the antioxidant should not react with the target species.

In the non-competitive assays, putative antioxidant compounds interact with reactive species without the presence of any other competing target molecule. In this way, these assays involve two components in the initial reaction mixture: the antioxidant compound(s) and the reactive species, which may also be the probe for reaction monitoring. Otherwise, the remaining reactive species may be measured after addition of some derivative reagent. The most common methods will be discussed further considering also this methodological-based division, as highlighted in Tables 1–9.

### 2. Scavenging capacity assays against specific ROS/RNS

#### 2.1. Peroxyl radical (ROO\(^\bullet\)) scavenging capacity assays

Peroxyl radicals (ROO\(^\bullet\)) are commonly found in food and biological samples and they are formed during lipid oxidation chain reactions. They have harmful effects on health and they are also associated to quality deterioration of foods. Their impact on these areas foster the existence of several methods for determining the peroxyl radical (ROO\(^\bullet\)) scavenging capacity (Table 1), which were subject of review recently [13,14].

In general, methods for examination of ROO\(^\bullet\) scavenging capacity measure the ability of an antioxidant to scavenge peroxyl radicals by hydrogen atom transfer (HAT) reactions. In these assays a competitive scheme is applied, where antioxidants or target molecules react with ROO\(^\bullet\). Hence, the assay system has three components: (i) thermolabile azo-compound (R–N=N–R), which yields carbon-centered radicals (R\(^\bullet\)) that react fast with O\(_2\) to give a steady flux of ROO\(^\bullet\) radicals; (ii) oxidizable target (PH); (iii) antioxidant compound(s) (AH), as represented schematically in Fig. 2.

The most frequently applied peroxyl radical generators are the water-soluble 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and the lipid-soluble 2,2′-azobis(2,4-
### Table 7 – Summary of analytical features of some in vitro methods for determination of nitric oxide radical scavenging capacity

<table>
<thead>
<tr>
<th>Target compound</th>
<th>Detection probe</th>
<th>Principle of measurement</th>
<th>Quantification</th>
<th>Recent applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>n.a. NO*</td>
<td>NO*</td>
<td>NO* concentration is measured amperometrically along time and the rate of its disappearance is increased by antioxidants</td>
<td>Second-order rate constant of NO* scavenging</td>
<td>–</td>
</tr>
<tr>
<td>n.a. Nitrite</td>
<td>Absorbance increase after nitrite derivatization using Griess reaction is inhibited by antioxidants</td>
<td>Inhibition percentage</td>
<td>Trypsin inhibitor [157], herbal seed extract [158], NSAIDs [154]</td>
<td></td>
</tr>
<tr>
<td>n.a. Carboxy-PTI spin adduct</td>
<td>Formation of carboxy-PTI spin adduct is measured by ESR and it is inhibited by antioxidants</td>
<td>Inhibition percentage</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>DAF-2 Carboxy-PTI spin adduct</td>
<td>Fluorescence increase due to oxidation of DAF-2 is inhibited by antioxidants</td>
<td>Inhibition percentage</td>
<td>Arylpropionic acid NSAIDs [159], pyrazolone NSAIDs [160]</td>
<td></td>
</tr>
</tbody>
</table>

NSAIDs: non-steroidal anti-inflammatory drugs; Carboxy-PTI: 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl; ESR: electron spin resonance; DAF-2: 4,5-diaminofluorescein; DAF-2T: triazolofluorescein.

### Table 8 – Summary of analytical features of some in vitro methods for determination of peroxynitrite scavenging capacity

<table>
<thead>
<tr>
<th>Target compound</th>
<th>Detection probe</th>
<th>Principle of measurement</th>
<th>Quantification</th>
<th>Recent applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>3-Nitrotyrosine</td>
<td>3-Nitrotyrosine, formed due to oxidation of tyrosine, is measured using HPLC-UV/vis and its formation is inhibited by antioxidants</td>
<td>Inhibition percentage</td>
<td>Flavonoid metabolites [165], anthocyanins [166], lotus seed extracts [167]</td>
</tr>
<tr>
<td>DHR 123</td>
<td>Rhodamine 123</td>
<td>Fluorescence increase due to oxidation of DHR 123 is inhibited by antioxidants</td>
<td>Inhibition percentage</td>
<td>Leaves of Gingko biloba [168], garlic extracts [169], alveolar macrophages conditioned media [170]</td>
</tr>
<tr>
<td>Luminol</td>
<td>Luminol (oxidized)</td>
<td>CL emission due to oxidation of luminol is inhibited by antioxidants</td>
<td>Inhibition percentage</td>
<td>Guanidine compounds [160], rat colon tissue [171], thymol [172]</td>
</tr>
</tbody>
</table>

HPLC-UV/vis: high performance liquid chromatography with ultraviolet/visible spectrophotometric detection; DHR 123: dihydrorhodamine 123; CL: chemiluminescence.
Table 9 – In vitro scavenging capacity assays against stable, non-biological radicals and evaluation of total reducing capacity.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Principle of measurement</th>
<th>Quantification</th>
<th>Recent applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEAC</td>
<td>2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation is reduced by antioxidants, causing absorbance decrease at 414 or 734 nm</td>
<td>Trolox equivalents (μM), ascorbic acid equivalents (mg/100 mL or 100 g)</td>
<td>Plant extracts [182], peaches [183], synthetic oligosaccharides [186]</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl radical is reduced by antioxidants, causing absorbance decrease at 515 nm</td>
<td>EC50, RSE, Trolox equivalents (μM), ascorbic acid equivalents (mg/100 mL or 100 g)</td>
<td>Plant extract [184], l-tyrosine and l-Dopa [185], synthetic oligosaccharides [186]</td>
</tr>
<tr>
<td>FRAP</td>
<td>The ferric 2,4,6-tripyridyl-s-triazine complex is reduced by antioxidants, causing absorbance increase at 593 nm</td>
<td>Ferrous ions equivalents, ascorbic acid equivalents</td>
<td>Food [187], chitosan derivatives [188], tea, wine, fruit juices, beer [189,190]</td>
</tr>
<tr>
<td>Folin–Ciocalteu reducing capacity</td>
<td>Tungstate–molybdate complexes are reduced by antioxidants, causing absorbance increase at 750 nm</td>
<td>Gallic acid equivalents (mg L$^{-1}$)</td>
<td>Tea, wine, fruit juices, beer [189,190], Buckwheat extracts [191], human and horse plasma [192]</td>
</tr>
<tr>
<td>Electrochemical total reducing capacity</td>
<td>The intensity of anodic current is increased due to oxidation of antioxidant compounds at the surface of the electrode</td>
<td>Oxidation potential (E$<em>{1/2}$), intensity of the anodic current (I$</em>{a}$), area under the anodic wave (S)</td>
<td>Buckwheat extracts [191], human and horse plasma [192]</td>
</tr>
</tbody>
</table>

**Fig. 2 – Schematic representation of competitive scheme for determination of ROO$^\cdot$ scavenging capacity. PH: target molecule, AH: antioxidant compound.**

In these competitive assays, the presence of antioxidant compounds inhibits or retards the oxidation of target/probe induced by peroxyl radicals. Therefore, in the beginning of the assay, insignificant spectroscopic changes of the target/probe would be observed (induction period or lag phase). As the reaction proceeds, the antioxidants are consumed by the constant flux of ROO$^\cdot$ and the oxidation of the target/probe would progress at a slower rate when compared with the control (absence of antioxidant compounds/samples). Finally, when the antioxidants are depleted, the reaction rate of oxidation of the target is similar to that obtained for the control.

Although the competitive scheme applied resembles in vivo conditions, the concentration of the target species is usually smaller than the concentration of antioxidants. This is in contradiction with the “definition of antioxidant” [6] and with what is found in real situations, where the antioxidant concentration is much smaller than that of the oxidizable substrate (lipids or proteins, for instance). Moreover, these assays apply a ROO$^\cdot$ reaction without taking into account the essential propagation step in lipid autoxidation, such as the breakdown of hydroperoxides (ROOH) yielding peroxyl and alkoxyl (RO$^\cdot$) radicals [37]. Finally, most of these assays are rather time-consuming and their application requires a significant expertise and experience in chemical kinetics. As a consequence, HAT-assays are commonly not suitable for routine determinations.

The oxygen radical absorbance capacity, or ORAC assay, is one of the most common methods for assessing ROO$^\cdot$ scavenging capacity. The principle of this assay is based on the intensity of fluorescence decrease of the target/probe along time under reproducible and constant flux of peroxyl radicals, generated from the thermal decomposition of AAPH in aqueous buffer. In the presence of a sample that contains chain-breaking antioxidants, the decay of fluorescence is inhibited [38]. Initially, the protein isolated from Porphyridium cruentum, β-phycocerythrin (β-PE), was used as the fluorescent target/probe, which react with ROO$^\cdot$ to form a non-fluorescent product [39]. Nevertheless, some shortcomings were observed such as large lot-to-lot variability, photobleaching of the β-PE after exposure to excitation light, and interaction with polyphenols by non-specific protein binding. To overcome these limitations, the synthetic, non-protein fluorescein has been used as the fluorescent tar-
get/probe, instead of the original β-PE [40]. The application to both lipophilic and hydrophilic chain-breaking antioxidants was carried out using a mixture of acetone/water containing 7% of randomly methylated β-cyclodextrin as a water solubility enhancer [41]. Lipophilic compounds were also quantified by ORAC assay using either organic media or liposomes, AMVN as a lipophilic peroxyl radical generator, and 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY 581/591 C11) as a fluorescent target/probe [42]. To improve the throughput, Huang et al. developed a high-throughput assay using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format [43].

In the ORAC assay, the reaction is monitored for extended periods (≥30 min) and the quantification is based in the area under curve (AUC) that represents the oxidation of the probe along time. The protective effect of antioxidants is evaluated from the net integrated area under the fluorescence decay curves (AUCsample−AUCblank) and results are expressed as μM of Trolox equivalents. The advantage of the AUC approach is that it can be applied for antioxidants that exhibit distinct lag phases and also to those samples that have no lag phases. Moreover, it takes into account the initial reaction rate and the total extent of inhibition, which includes the action of slow-reacting or secondary antioxidant products formed. The principles of the ORAC assay can be adapted to determine the action against other reactive oxygen species [44].

The total radical-trapping antioxidant parameter (TRAP) assay was introduced by Wayner et al. [45], for the determination of the antioxidant status of human plasma. This method was based on the measurement of the time period in which oxygen uptake was inhibited by plasma during a controlled ROO• peroxidation reaction induced by the thermal decomposition of an azo-compound. In this assay, the target was the human plasma while the oxygen consumed in the oxidation of plasma material is the probe molecule used to follow the action of antioxidants. The measurement is based on the “lag time” that corresponds to the time period between the beginning of the assay and the beginning of the oxidation of the target molecules.

One of the major problems with the original TRAP assay lies in the utilization of the oxygen electrode as detector, since it may not maintain its stability over the period of time required [46]. To overcome this limitation, this assay was later improved using β-phycoerythrin (β-PE) as the fluorescent target/probe, and the ability of the plasma to protect β-PE from peroxyl radical oxidation was fluorimetrically monitored [47]. Ghiselli et al. proposed some modifications in order to circumvent interferences from plasma proteins, lipids and metal ions [48]. They also evaluated the contribution and synergistic effects of main antioxidant compounds, and the effect of plasma storage in the TRAP values. Valkonen and Kuusi applied dichlorofluorescein-diacetate (DCFH-DA) as the fluorescent oxidizable substrate [49]. The oxidation of DCFH-DA by peroxyl radicals yields the formation of highly fluorescent dichlorofluorescein (DCF) product. In this case, the presence of antioxidant compounds competitively inhibits the increase of fluorescence signal.

Disregarding the different variations discussed above, the quantification is based on the lag phase duration, in which oxidation is inhibited by the antioxidants, compared to the lag phase of Trolox. The antioxidant capacity expressed as Trolox equivalents (X_{AO}) is calculated as X_{AO} = C_{Trolox} \times T_{AO},

where C_{Trolox} is the Trolox concentration, whilst T_{AO} and T_{AO} are the lag time of the kinetic curve of target oxidation in the presence of Trolox or in the presence of antioxidant/sample, respectively; X_{AO} is then multiplied by 2.0, the stoichiometric factor of Trolox, and by the dilution factor of the sample to give the TRAP value (μM).

The main shortcoming of TRAP assay is the use of the lag phase for quantifying antioxidant capacity, since not every antioxidant possesses an obvious lag phase and also the antioxidant capacity profile after the lag phase is totally ignored. Moreover, the application of different criteria for establishing the endpoint makes difficult the comparison between laboratories. Another important limitation of this assay, and also of the ORAC assay, is that the oxidative deterioration and antioxidant protection of fluorescent target/probe does not necessarily mimic a critical biological substrate [50].

The issues related to lag phase or endpoint definition does not exist in the crocin bleaching assay, where the ability of antioxidant compounds to protect the carotenoid derivative crocin from oxidation by ROO• radicals is measured [51]. The reaction is initiated by the addition of AAPH and the bleaching rate (absorbance decrease/time) of crocin is monitored at 443 nm during 10 min. Antioxidants compete with crocin for ROO•, and the degree of inhibition of crocin oxidation depends on the antioxidant capacity of tested samples. The quantification of antioxidant capacity is based on the ratio of initial crocin bleaching rates in absence (V_{0}) and in presence (V) of antioxidants, and is given by the Stern−Volmer-like relation V_{0}/V = 1 + (k_{AO}/k_{C}) \times ([AO]/[C]), where [AO] and [C] are the concentrations of antioxidant and crocin, respectively, k_{AO} and k_{C} are the rate constants for the reaction of the peroxyl radicals with antioxidant and crocin, respectively. After measuring V_{0}/V value at known ratio of [AO] to [C], k_{AO}/k_{C} is obtained by the slope value and indicates the relative peroxyl radical scavenging capacity. A microplate-adapted crocin bleaching assay based on the inhibition percentage at a fixed time instead of kinetic analysis was also reported [52].

This assay was used to determine the structure−antioxidant activity relationships of flavanones present in citrus fruit [26]. Nevertheless, it has limited applications in food samples since many food pigments, such as carotenoids, absorb at the same wavelength of the determination. Besides this drawback, crocin is a natural food pigment extracted from saffron, which may confer a low reproducibility between assays. In fact, Chatterjee et al. described a more affordable crocin assay using the Indian spice saffron instead of the commercial chemical product and found that the antioxidant capacity values of pure natural compounds, plant extracts, and human plasma from healthy individuals using either approach were similar [53]. Recently, Orduoudi and Tsimidou have examined the crocin bleaching assay performance and validation procedures [54]. The studies were focused on target/probe and test compound characteristics, conditions for peroxyl radical generation, reaction monitoring, and expression of results. They observed that any authentic commercial saffron could be used for target/probe preparation given that: interferences, such as tocopherols, are removed; the concen-
tation of working solution is adequately adjusted; and the changes of the stock target/probe solution during storage are not neglected. Results are expressed as “percent inhibition of crocin bleeding value” instead of the ratio of initial crocin bleeding rates.

The utilization of biologically occurring low-density lipoproteins (LDL) as the oxidizable target/probe has also been proposed [55–57]. The oxidation of LDL, isolated from blood samples, is initiated by thermal decomposition of AAPH, a water-soluble diazo ROO• initiator, or by a transition element such as Cu(II), and assessed through the formation of conjugated dienes, determined spectrophotometrically at 234 nm after HPLC separation [58]. In this assay the use of AAPH as peroxyl radical generator is preferred rather than Cu(II), since it allows a strong resemblance to oxidative reactions that might occur in biological systems. Sanchez-Moreno et al. studied the oxidation of LDL induced by Cu(II) and proposed several oxidizability indexes to measure the antioxidant activity of dietary polyphenols [59]. The concentration of antioxidant that increases the lag time by 50% compared to the control (CLT50) was determined graphically upon the representation of the ratio lag time antioxidant/lag time control as a function of antioxidant concentration. However, the major limitation is that the LDL has to be isolated on a regular basis from different individuals, and therefore, a high inter-batch variation is verified. Moreover, problems with this assay arise because it is difficult to measure the small lag times that occur, and many substances also absorb at the wavelength of the determination.

In the original total oxyradical scavenging capacity (TOSC) assay, peroxyl radicals generated by thermal homolysis of AAPH cause the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene, which is monitored by gas chromatographic analysis of head space from the reaction vessel [60]. The antioxidant capacity of the compounds tested is quantified by their ability to inhibit ethylene formation relatively to a control reaction. In the TOSC assay, the approach for quantification of antioxidant capacity is similar to that applied for the ORAC assay. In this case it is based in the area under the curve that represents the inhibition of ethylene formation as a function of time. The long reaction time (>100 min), the short shelf-life of the assay solutions, and the necessity of verification. Moreover, problems with this assay arise because it is difficult to measure the small lag times that occur, and many substances also absorb at the wavelength of the determination.

Peroxyl radical scavenging capacity assays have also been implemented using chemiluminescence (CL) as detection system. The principles of ROO•-induced luminol-CL assays are described in detail by Alho and Leinonen [62] and a recent outlook on chemiluminescent methods for ROS is given by Lu et al. [63]. Briefly, the addition of the antioxidant/sample induces a CL-lag phase (time during which CL emission was not detected) whose magnitude was directly related to the antioxidant concentration [64,65] or it causes the decrease of CL emission, expressed as inhibition percentage [66]. The application of CL-based methods is frequent but it should be pointed out that it is not selective as other ROS also elicited luminol CL. Luminol-derived radicals may also deplete the antioxidant pool in the sample [63].

### 2.2. Superoxide radical anion (O2•−) scavenging capacity assays

Superoxide radical anion (O2•−) is produced as a result of the donation of one electron to oxygen. This radical arises either from several metabolic processes or following oxygen activation by irradiation [67]. The analytical methods for determination of O2•− scavenging capacity (Table 2) make use of the system XOD/hypoxanthine or xanthine at pH 7.4 to generate superoxide anion radical. To a minor extent, O2•− is also generated using a non-enzymatic reaction of phenazine methosulphate (PMS) in the presence of nicotinamide adenine dinucleotide (NADH). In both generation systems, O2•− may reduce nitroblue tetrazolium (NBT) into formazan, which is spectrophotometrically monitored at 560 nm [68]. Antioxidant compounds compete with NBT for O2•− and decrease the rate of reaction. Another widely used probe for O2•− is cytochrome c. The kinetic analysis of reduction of ferricytochrome c to ferrocytochrome c was monitored at 550 nm [68,69]. In fact, Aruoma et al. observed that inhibition of NBT reduction was generally greater than that of cytochrome c reduction [68]. This is because O2•− reacts much faster with cytochrome c than it does with NBT, so a given concentration of added O2•− scavenger competes less efficiently in the cytochrome c system and exerts less inhibition. This is a clear example of the influence of the nature of the probe in the assessment of antioxidant capacity. Wang and Jiao [70] used hydroxylammonium chloride, with consequent formation of nitrite that was determined spectrophotometrically at 530 nm after addition of sulfanilic acid and α-naphthylamine (Griess reaction) [71]. The scavenging capacity towards O2•−, using XOD/xanthine generating system, has also been measured by reaction with KMBA to produce ethylene, which is measured by gas chromatography [72,73]. The scavenging capacity against this radical can also be measured by using electron spin resonance (ESR) spectrometry [74,75]. Here, the O2•− is trapped by 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and the resultant DMPO-OOH adduct is detected by ESR.

The CL-based determination of O2•− scavenging capacity has also been described. Luminol or lucigenin are frequently applied as target compounds [63], but neither of them is selective towards O2•− [76,77]. CLA (2-methyl-6-phenyl-3,7-dihydridimidazo[1-2-alp]pyrazin-3-one) and MCLA (2-methyl-6-(4-methoxyphenyl)-3,7-dihydridimidazo[1,2-alp]pyrazin-3-one), analogs of coelenterazine, have also been described as more specific targets of O2•− but their application is more focused to in vivo monitoring of superoxide formation [63].

All these methodologies may provide erroneous values if controls are not established for substances that inhibit or interfere with O2•− generation, or if the sample itself directly reduces NBT or ferricytochrome c, for instance. This is an important issue as far as ferricytochrome c is concerned, as it is easily reduced by ascorbic acid. Furthermore, the reduced antioxidant formed by attack of O2•− could also reduce NBT or ferricytochrome c [6]. The non-fluorescent hydroethidine
has also been used as the target/probe for measuring O$_2$•− scavenging capacity [78]. The target/probe is oxidized by O$_2$•− (generated from XOD/xanthine system) to form a species that exhibits a strong fluorescence signal. This approach can circumvent the problem of direct reduction of the target/probe by antioxidants, but possible inhibition of xanthine oxidase by antioxidants/sample remains an issue.

2.3. **Hydrogen peroxide (H$_2$O$_2$) scavenging capacity assays**

Hydrogen peroxide (H$_2$O$_2$) is generated in vivo, under physiological conditions by peroxisomes, by several oxidative enzymes including glucose oxidase and d-amino acid oxidase, and by dismutation of superoxide radical, catalysed by superoxide dismutase. One of the most common methods for assessing the scavenging capacity against this molecule (Table 3) is based on the intrinsic absorption of H$_2$O$_2$ in the UV region [94,95]. As the H$_2$O$_2$ concentration is decreased by scavenger compounds, the absorbance value at 230 nm is also decreased. Nevertheless, it is quite usual that samples also absorb at this wavelength, requiring the performance of a “blank” measurement. This situation can compromise both the precision and the accuracy of this method. First, it may be difficult to distinguish between small changes when there is much larger background absorption. Secondly, the absorption of samples may change after reaction with H$_2$O$_2$ and the blank measurement would not be valid. Other common assay employs horseradish peroxidase (HRP), which uses H$_2$O$_2$ to oxidize scopoletin into a non-fluorescent product [96]. In the presence of putative scavenger compounds, the oxidation of scopoletin is inhibited and the scavenging reaction can be fluorimetrically monitored. Another fluorimetric method widely used is based on homovanillic acid (HVA), whose fluorescent biphenyl dimer is more stable than scopoletin [97]. The presence of substances with H$_2$O$_2$ scavenging capacity prevents the oxidation of homovanillic acid. However, these peroxidase-based approaches do not allow determining whether the antioxidant is reacting directly with H$_2$O$_2$, or reacting with intermediates formed from the enzyme and H$_2$O$_2$ (it is possible that the superoxide radical is produced during enzyme activity). Moreover, antioxidants such as ascorbic acid, quercetin dihydrate, and thiol can be a substrate for the peroxidase enzyme, introducing errors in the determination of scavenging capacity. Therefore, if the compound does interfere with peroxidase-based system, other assays for H$_2$O$_2$ should be used. For instance, the direct reaction of H$_2$O$_2$ and titanium(IV) was applied as it originates a complex (Ti–H$_2$O$_2$) [98] that is dissolved in acidic medium and further measured spectrophotometrically at 410 nm [70]. A valid alternative to these methods was proposed by Arnous et al. [99]. The enzyme-free methodology implemented relied on peroxoxyxalate chemiluminescence (POCL) using 9,10-diphenylanthracene and imidazole as a fluorophore (probe) and catalyst, respectively. Briefly, POCL involves hydrogen peroxide imidazole-catalysed oxidation of an aryl oxalate ester yielding a high-energy intermediate (dioxetanediene) that transfers its energy to the fluorophore. The transition of the excited state of the fluorophore to its ground state causes the emission of light.

Therefore, any compound with capacity to scavenge H$_2$O$_2$ promotes CL inhibition. Due to the non-polar environment employed (ethyl acetate/acetonitrile (9:1)) this method was proposed to evaluate mainly the H$_2$O$_2$ scavenging capacity of lipophilic antioxidants. As a major shortcoming of this assay is the fluorophore employed (9,10-diphenylanthracene), which is suspected to be a carcinogen. The chemilumimetric detection was also the basis for determining H$_2$O$_2$ scavenging capacity using either luminol [100] or lucigenin [101].

2.4. **Hydroxyl radical (HO•) scavenging capacity assays**

Due to the high reactivity of hydroxyl radicals, almost anything in biological systems can be regarded as an HO• scavenger. Hence, this task is not performed by any specific molecule or enzyme. Thus, the evaluation of direct scavenging of HO• may be irrelevant for evaluation of antioxidant action of a compound or matrix, simply because very high concentrations of scavenger are required to compete with adjacent molecules in vivo or in the food matrix for any HO• generated. For these reason, it is more relevant and useful to quantify the capacity of putative antioxidants to scavenge or block the formation of its precursors (O$_2$•−, H$_2$O$_2$, HOCl) and/or to sequester free metal ions related to HO• formation. Scavenger compounds that act in this way would behave as preventive antioxidants.

Despite this remark, several in vitro methodologies for determination of HO• scavenging capacity are available (Table 4), mostly based on Fe$^{3+}$ + EDTA + H$_2$O$_2$ + ascorbic acid system to generate a constant flux of HO• radicals. Those radicals attack the sugar 2-deoxy-d-ribose (used as target), degrading it into a series of fragments, some or all of which react upon heating with thiobarbituric acid at low pH to give a pink chromogen [113]. If a HO• scavenger is added to the reaction mixture, it will compete with deoxyribose for HO• radicals, inhibiting the degradation of the target species. It should be stressed that the substance(s) under test may interfere with the generation system of hydroxyl radicals. Thus, compounds may inhibit the HO• generation by reacting directly with H$_2$O$_2$ or by chelating the metal ion. In this way, the performance of the deoxyribose assay without EDTA allows the identification of compounds which chelate metal ions [114,115]. In this case, iron(III) ions are chelated by deoxyribose causing “site specific” hydroxyl radical damage, and when the test substances are iron-chelating agents the hydroxyl radical damage of deoxyribose is inhibited. On the other hand, compounds (such as ascorbic acid) can reduce Fe$^{3+}$ to Fe$^{2+}$ enhancing the generation of hydroxyl radicals and acting as pro-oxidant agents. Indeed, Hagerman et al. also modified the deoxyribose method by omitting ascorbic acid to evaluate the potential of certain tannins to behave as pro-oxidants [115].

Zhu et al. reported a metal-independent, organic Fenton reaction [116]. The mixture of tetrachlorohydroquinone (TCQH) and H$_2$O$_2$ hydroxylates salicylic acid and this process is inhibited by HO• scavenging agents. The extension of the oxidation of salicylic acid was assessed by HPLC-ED quantifica-
tion of dihydroxybenzoic acid (DHBA). The oxidation reaction was not affected by several iron chelators. Thus, this metal-free TCOH/H_{2}O_{2} system provides the generation of HO• with less redox species involved, which makes it more specific for evaluating the HO• scavenging capacity. Ou et al. developed a fluorimetric assay to evaluate "hydroxyl radical prevention capacity" using fluorescein as the target/probe [44]. In this assay, HO• radical is generated by a Co^{2+}-mediated Fenton-like reaction and the HO• scavenging capacity is mainly due to the metal-chelating capability of the compounds. The quantification approach is the same as that of the ORAC assay except that gallic acid is used as the reference standard compound. Similarly to O_{2}^{•−}, ESR spectrometry using DMPO can also be applied to assess HO• scavenging capacity [74]. In this case, antioxidant compounds inhibit the formation of DMPO-OH adduct.

The CL-based determination of scavenging capacity against HO• using luminol has also been described [117–120]. In these cases, HO• is generated from the reaction between ferrous iron with molecular oxygen, which induces luminol CL. However, other ROS, including O_{2}^{•−}, are generated at the same time, so it is difficult to detect HO• specifically with this detection process.

2.5. Hypochlorous acid (HOCl) scavenging capacity assays

In the analytical methods for in vitro determination of HOCl scavengers (Table 5), this oxidant is obtained from the enzymatic system myeloperoxidase/H_{2}O_{2}/Cl− or by acidifying commercial sodium hypochlorite to pH 6.2 with sulphuric acid [128]. The former approach can be applied if the sample species do not interfere with HOCl generation (e.g. inhibition of myeloperoxidase activity or direct reaction with H_{2}O_{2}). In the second approach, the determination of the concentration of HOCl solution must be performed daily [6].

The elastase assay [129], measures the ability of a compound to protect the α1-antiproteinase (α1-AP), which is the major proteolytic inhibitor in body fluids, against inactivation by HOCl. This is assessed after addition of elastase, which is a proteinase that may be inactivated by any remaining α1-AP. The elastase activity is subsequently measured using an elastase substrate (N-t-BOC-L-alanine p-nitrophenol ester) and monitoring the absorbance increase at 410 nm. In this assay, the different target (α1-AP) and probe (elastase) species occur in vivo. Furthermore, the analytical process also mimics an in vivo situation and therefore, the HOCl scavenging capacity of compounds/samples under study occurs at biologically significant conditions. Nevertheless, this method has been criticized to be time-consuming and compounds which have inhibitory effects on the activities of either enzyme could falsely be interpreted to be HOCl scavengers [6]. Later, the same research group developed a method in which the compound 5-thio-2-nitrobenzoic acid (TNB) was oxidized by HOCl into 5,5-dithiobis(2-nitrobenzoic acid) (DTNB). The absorbance decrease at 412 nm is inhibited by putative HOCl scavenger compounds [130]. However, it was observed that compounds containing free thiol groups, such as dihydrolipoic acid, cysteine, and glutathione, interfered in this method, yielding an excess of TNB. To overcome these limitations, Yan et al. developed a novel application of the protein carbonyl assay [131]. The method is based on the observation that bovine serum albumin carbonyl content is increased upon oxidation by HOCl, and that this increase is inhibited in the presence of HOCl scavengers. In the assay developed by Gatto et al., scavenger compounds inhibit the HOCl-oxidation of human serum albumin [132]. These effects were evaluated by reversed-phase high performance liquid chromatography (HPLC) with spectrophotometric detection at 280 nm. Methods based on luminol-elicited CL have also been proposed, where HOCl scavengers promote the decrease of analytical signal [100,133]. Finally, a fluorimetric competition assay based on para-aminobenzoic acid chlorination was developed to determine the HOCl scavenging rate constants and applied to some non-steroidal anti-inflammatory drugs [134]. The specificity of the system was improved by chromatographic separation of the drugs and the oxidation products.

2.6. Singlet oxygen (1O_{2}) scavenging capacity assays

Singlet oxygen (1O_{2}) is an excited state of molecular oxygen that has no unpaired electrons and it is known to be a powerful oxidizing agent, reacting directly with a wide range of biomolecules [140]. Due to its decay to the lower energy ground state, 1O_{2} emits characteristic phosphorescence at 1270 nm. Therefore, the 1O_{2} scavenging ability of several compounds was measured through the decay rates of the light intensity (Table 6) [141,142]. Nevertheless, the intensity of luminescence based on the self-emission of 1O_{2} is often insufficient to provide reproducible quantitative information, even in aqueous medium. A more sensitivity method based on monitoring the scavenging of singlet oxygen delayed fluorescence of tetramethylrhodamine was developed [143]. The assay was applied to compounds which are well-documented to be singlet oxygen quenchers such as beta-carotene, α-tocopherol, and lauric acid. This method is not widely applied but it is useful for measurement of rate constants for 1O_{2} quenching, requiring commonly available equipment and also applicable to systems where the 1270 nm luminescence is difficult to detect.

Recently, a fluorescence-based microplate screening assay for evaluating 1O_{2} scavenging activity was developed by Costa et al. [144]. The 1O_{2}, selectively generated by the thermal decomposition of the endoperoxide disodium 3,3′-(1,4-naphthalene)bispropionate (NDPO_{2}), oxidize the highly sensitive target/probe dihydrorhodamine 123 (DHR) to the fluorescent form rhodamine 123. The assay was successfully applied for screening scavenging activity of several recognised antioxidant compounds against 1O_{2}.

2.7. Nitric oxide radical (NO•) scavenging capacity assays

Nitric oxide radical (NO•), acclaimed as the “molecule of the year” in 1992 by the Science Magazine [147], has a pivotal role in the regulation of diverse physiological and pathophysiological processes [148]. Vriesman et al. developed a relatively simple method for the quantification of NO• scavenging capacity of sulfur-containing compounds in aqueous solution using...
an amperometric NO• sensor (Table 7) [149]. NO• is added to buffered solutions of the scavenger (glutathione, glutathione disulfide, S-methyl glutathione, N-acetyl cysteine, lipoic acid and dihydrolipoic acid) and its concentration is followed as a function of time. The natural logarithm of the NO• concentration and time are linearly related. After correction for the spontaneous degradation of NO•, second-order rate kinetics of the scavenging reaction was determined. They observed that only those compounds, which contained a thiol group, displayed a considerable NO• scavenging capacity. In this method there is a non-competitive reaction mechanism, since in the reaction medium is only present the reactive species (NO•) and the scavenger molecule(s).

The assessment of NO• scavenging capacity has also been performed using ESR spectrometry [150,151]. The NO• generated from the donor 3-(2-hydroxy-1-methyl-2-nitrosohydrizinio)-N-methyl-1-propanamine (NOC-7) was oxidized to NO2 by the target 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, named as carboxy-PTIO with the formation of carboxy-PTI spin adduct, that was measured by ESR. The method was applied to non-steroidal anti-inflammatory drugs and a semi-competitive scheme was adopted as the drug was incubated with the NO• generator for 30 min, prior to the addition of carboxy-PTIO. The main limitation of this method, considering also the detection technique that is not easily available, is the long total reaction time (120 min).

The Griess reaction is frequently used for assessment of NO• production by whole cells or enzymes [152,153]. Its application to in vitro determination of NO• scavenging capacity is also frequent. In this case, the nitric oxide remaining after reaction with the test sample is measured as nitrite. It is important to emphasize that nitrate may also be formed, thus it should be reduced to nitrite prior to determination. For this, Perez et al. used NADH-dependent nitrate reductase, with elimination of interference of NADH by addition of lactate dehydrogenase and pyruvate [154]. The chromophoric azo derivative formed from nitrite after Griess reaction is then measured spectrophotometrically at 540 nm. Standard curves were generated using sodium nitrite and results were expressed as percentage change from control response. Compared to other methods, this methodology is not straightforward, requiring the addition of several enzymatic reagents.

The fluorescent target/probe 4,5-diaminofluorescein (DAF-2), widely used for in vivo NO• detection and imaging, has also been applied for screening of NO• scavenging capacity [155]. The nitrosation of the weak fluorescent target/probe DAF-2 by derivative NO• species yielded the formation of the strong green-fluorescent triazolofluorescein product (DAF-2T). The NO• scavenging capacity was determined by the ability of compounds to prevent the NO•-induced nitrosation of DAF-2 and results are expressed as the percentage of inhibition of the DAF-2 oxidation as a function of concentration of the scavenger compound. Nevertheless, the results obtained using DAF-2 as a target/probe for NO• quantification should be interpreted with caution, since some antioxidant compounds (dehydroascorbic and ascorbic acid) may react directly with DAF-2 and form fluorescent compounds with emission spectra similar to that of DAF-2T [156].

2.8. Peroxynitrite (ONOO−) scavenging capacity assays

Methodologies for measuring the ONOO− scavenging capacity usually depend either on tyrosine nitration [161,162] or on dihydrolipoic acid 123 (DHR) [163]. The first method is based on the formation of 3-nitrotyrosine that is detected spectrophotometrically along with the unreacted tyrosine after HPLC separation. The concentration of 3-nitrotyrosine is measured after incubation of tyrosine with varying concentrations of putative ONOO− scavengers and the inhibition percentage of 3-nitrotyrosine formation is determined. In the second method, the non-fluorescent DHR is oxidized by peroxynitrite to the fluorescent rhodamine 123. In presence of ONOO− scavengers the fluorescence intensity is lower than that of the control and the inhibition percentage of DHR oxidation is assessed. Both assays have been extensively applied, as depicted by the several examples presented in Table 8. Luminol-enhanced chemiluminescence has also been used to estimate the ONOO− scavenging capacity [100,164].

3. Scavenging capacity assays against stable, non-biological radicals and evaluation of total reduction capacity

3.1. Scavenging of 2,2ʹ-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (ABTS•+) or Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay involves the generation of the long-lived radical cation chromophore 2,2ʹ-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS•+) (Table 9) which has absorption maxima at 414, 645, 734, and 815 nm. The original TEAC assay, developed by Miller et al., was based on the activation of metmyoglobin, acting as peroxidase, with H2O2 to generate ferrylmyoglobin radical, which then reacted with ABTS to form the ABTS•+ radical cation [173]. In this strategy, the sample to be tested is added previously to the formation of the ABTS•+. The test compounds/samples reduce the ABTS•+ radicals formed and the lag phase, which corresponds to the delay time in radical formation, is measured (competitive scheme). This method has been commercialized by Randox Laboratories (San Francisco, USA) as the world’s first kit for the standardization of total antioxidant status measurement in an individual’s serum or plasma. Nevertheless, this commercial kit for the TEAC assay is expensive; the reagent cost per sample estimated in the Randox-TEAC assay is approximately nine times that in the ORAC assay [5].

The order of addition of reagents and sample was then criticized as a major pitfall, because antioxidants (quercetin, for instance) can react with H2O2 and/or with derivated oxidizing species that inhibit the ABTS•+ radical formation and that lead to overestimation of antioxidant capacity [174]. Therefore, a post-addition assay or decolourization strategy was proposed to prevent the interference of antioxidant compounds with radical formation, making the assay more reliable and less susceptible to artifacts. In this case, the sample to be tested was added after generation of a certain amount of ABTS•+.
radical cation and the remaining ABTS\(^{•−}\) concentration after reaction with antioxidant compound/sample was then quantified [175].

In terms of assay conditions, different strategies have been implemented for ABTS\(^{•−}\) generation, reaction time applied, detection wavelength used for monitoring the reaction, and the reference antioxidant chosen. ABTS\(^{•−}\) radical cation can be generated by chemical reaction using manganese dioxide [176], AAPH [177], or potassium persulfate [175], by enzymatic reaction using metmyoglobin [173] or horseradish peroxidase [178], or by electrochemical generation [179]. Reaction times ranging from 1 to 30 min have been adopted throughout the protocols described in the literature. Concerning the wavelength of detection, the determination at 734 nm is preferred because the interference from other absorbing components and from sample turbidity is minimized [180].

In terms of quantification, the absorbance value, proportional to the remaining ABTS\(^{•−}\) concentration, is measured after a fixed reaction time. Results are expressed as Trolox equivalents, that is, the concentration of Trolox solution (mM) with an antioxidant capacity equivalent to that found for 1.0 mM of the substance under investigation. To express the antioxidant capacity in a more familiar and easily understood manner, ascorbic acid was suggested as the reference compound instead of Trolox and results were given as mass of ascorbic acid per 100 g or per 100 mL of test sample, designated as VCEAC—vitamin C equivalent antioxidant capacity [181].

This spectrophotometric assay is technically simple, which accounts for its application for screening and routine determinations. The ABTS\(^{•−}\) scavenging can be evaluated over a wide pH range, which is useful to study the effect of pH on antioxidant mechanisms. Furthermore, the ABTS\(^{•−}\) radical is soluble in water and organic solvents, enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compounds/samples. However, as the results obtained for samples are related to an antioxidant standard compound that shows different kinetic behaviour, the results provided by this assay are dependent of time of analysis. Moreover, this assay has been criticized as the ABTS\(^{•−}\) radical is not representative of biomolecules and not even found in any biological system. Thermodynamically, any compound that has a redox potential accounts for its application for screening and routine determination of antioxidant capacity based on the amperometric reduction of DPPH\(^{•−}\) radical cation and the remaining ABTS\(^{•−}\) concentration after reaction with antioxidant(s). A biamperometric method using DPPH/DPPH\(^{•−}\) redox couple and two identical glassy carbon disc electrodes was also presented [196].

In opposition to what was initially believed, the reaction mechanism is based on an electron transfer (ET) reaction whilst the hydrogen atom abstraction is a marginal reaction pathway, because it occurs slowly in strong hydrogen-bond-accepting solvents, such as methanol and ethanol [197]. As occurs in other ET-based assays, the scavenging capacity against DPPH\(^{•−}\) radical is strongly influenced by the solvent and the pH of reaction [198]. Indeed, Stasko et al. studied the suitable conditions and limits for water as a component of a mixed water–ethanol solvent of DPPH\(^{•−}\) radical assay [199]. They concluded that the 50% (v/v) aqueous/ethanol solutions are a suitable choice for lipophilic and hydrophilic antioxidants and the reaction rate between DPPH\(^{•−}\) and the antioxidant may increase considerably with increasing water ratios. However, at water content over 60% (v/v) the antioxidant capacity decreased, since a part of the DPPH\(^{•−}\) coagulates and it is not easily accessible to the reaction with antioxidant(s).

Generally, the results are reported as the efficient concentration (EC\(_{50}\)), that is the amount of antioxidant necessary to decrease by 50% the initial DPPH\(^{•−}\) concentration [194]. The time needed to reach the steady state with EC\(_{50}\) concentration was calculated from the kinetic curve and defined as T\(_{EC50}\). In recognition of the effect of both parameters the “antiradical efficiency” may be determined by calculating the reciprocal of EC\(_{50}\) × T\(_{EC50}\) [200]. Therefore, the lower EC\(_{50}\) and T\(_{EC50}\), the higher is the “antiradical efficiency”. A conceptually similar parameter designated as radical scavenging efficiency (RSE) was suggested by De Beer et al. [201]. RSE is calculated as the ratio of the reaction rate (obtained during the first minute) and the EC\(_{50}\) value. The main limitation of EC\(_{50}\) determination is that the percentage of radical scavenged is dependent of the initial concentration of DPPH\(^{•−}\) radical [202]. For this reason, it is more accurate to use the absorbance variation (or concentration of DPPH\(^{•−}\) consumed) rather than the percentage of the radical consumed. This absorbance value is further interpolated in a dose–response curve of a standard antioxidant such as ascorbic acid or Trolox and the results are expressed as equivalent concentration.

The steric accessibility of DPPH\(^{•−}\) radical is a major determinant of the reaction, since small molecules that have better access to the radical site have relatively higher antioxidant capacity [37]. On the other hand, many large antioxidant compounds that react quickly with peroxyl radicals may react slowly or may even be inert in this assay. The inexistence of DPPH\(^{•−}\) or similar radicals in biological systems is also a shortcoming. In addition, the spectrophotometric measurements can be affected by compounds, such as carotenoids, that absorb at the wavelength of determination as well as by the turbidity of the sample. In this way, the electrochemical detection proposed by Milardovic et al. [195,196] may be a valid alternative to analyse coloured and/or turbid samples with low content of antioxidant compounds. The DPPH\(^{•−}\) assay is not suitable for measuring the antioxidant capacity of plasma, because proteins are precipitated in the alcoholic reaction medium. Finally, the DPPH\(^{•−}\) scavenging reaction is time-consuming and it may take 20 min up to 6 h [194].

Recently, Magalhães et al. applied a mathematical model to the data collected within the first 3 min of DPPH\(^{•−}\) scaveng-
ing reaction to estimate the total DPPH* consumed [202]. This approach allowed a considerable reduction of the time taken for a single analysis for samples containing or originating slow reacting antioxidant compounds.

Despite the limitations abovementioned, the DPPH* radical is stable, commercially available, and does not have to be generated before assay like ABTS**. Therefore, it is considered an easy and useful spectrophotometric method with regard to screening/measuring the antioxidant capacity of both pure compounds and complex samples.

3.3. Ferric reducing antioxidant power (FRAP assay)

The FRAP assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-s-triazine complex [Fe(III)-(TPTZ)2]3+ to the intensely blue coloured ferrous complex [Fe(II)-(TPTZ)2]2+ in acidic medium (Table 9) [203,204]. FRAP values are calculated by measuring the absorbance increase at 593 nm and relating it to a ferrous ions standard solution or to an antioxidant standard solution (ascorbic acid, for instance). This method has also been adapted to 96-well microplate reader, giving better reproducibility and higher sample throughput [205].

Concerning its limitations, any compound (even without antioxidant properties) with redox potential lower than that of the redox pair Fe(III)/Fe(II), can theoretically reduce Fe(III) to Fe(II), contributing to the FRAP value and inducing falsely high results. On the other hand, not all antioxidants reduce Fe(III) at a rate fast enough to allow its measurement within the observation time (typically 4 min). Indeed, Pulido et al. observed that dietary polyphenols react more slowly and require longer reaction times (>30 min) for total quantification and depending on the analysis time the order of their reactivity is changed [206]. The polyphenols with such behaviour include caffeic acid, ferulic acid, quercetin, and tannic acid.

As the FRAP assay measures the reducing capacity based upon reduction of ferric ion, antioxidants that act by radical quenching (H transfer), particularly thiols and carotenoids, upon reduction of ferric ion, antioxidants that act by radical quenching (H transfer), particularly thiols and carotenoids (Table 9) [206]. The chemistry behind the FRAP assay relies on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue complexes that can be detected spectrophotometrically at 750–765 nm (Table 9) [210]. Generally, gallic acid is used as the reference standard compound and results are expressed as gallic acid equivalents (mg L−1). It should be stressed that, the blue complexes formed are independent of the structure of phenolic compounds, therefore ruling out the possibility of coordination complexes formed between the metal and the phenolic compounds [210].

The FC reagent is non-specific to phenolic compounds as it can be reduced by many non-phenolic compounds (e.g. aromatic amines, sulfur dioxide, ascorbic acid, Cu(I), Fe(II), etc.) and for that reason is not suitable for determination of “total phenolic content”, unless interfering species are considered or removed [4,210]. Therefore, the FC assay was recently proposed for the measurement of total reducing capacity of samples [37]. Excellent linear correlations between FC assay and other ET-based assays (TEAC and DPPH*, for instance) have been established [14]. Indeed, in a recently reported work the FC reducing capacity of a large number of beverages (n = 72) were compared with TEAC assay; a good correlation was found (R > 0.9) for red wines, herbal and tea infusions, and beers [190]. Nevertheless, the contribution from other dietary antioxidant compounds with non-ET mechanism of action (such as β-carotene) may not be assessed by FC assay [189]. Despite that, the relationship between the FC method and ORAC, a HAT-based assay, is usually good [4]. These correlations confirm the value, usefulness of Folin–Ciocalteu reducing capacity for the assessment of antioxidant capacity of food samples. In addition, the FC assay is operationally simple, reproducible and convenient for assessment of dietary antioxidant capacity since the reagent is commercially available, the procedure is rather standardized, and the absorption of the product at a long-wavelength minimizes interferences from the sample matrix. Nevertheless, the original assay is time-consuming (2 h) which makes its implementation for routine analysis difficult. Moreover, it is performed in aqueous phase, thus it is not applicable for lipophilic compounds/matrices.

3.4. Folin–Ciocalteu reducing capacity (FC assay)

The exact chemical nature of the Folin–Ciocalteu reagent is not known, but it is accepted that it contains phosphomolybdic/phosphotungstic acid complexes [209]. The chemistry of the FC assay is based on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue complexes that can be detected spectrophotometrically at 750–765 nm (Table 9) [210]. Generally, gallic acid is used as the reference standard compound and results are expressed as gallic acid equivalents (mg L−1). It should be stressed that, the blue complexes formed are independent of the structure of phenolic compounds, therefore ruling out the possibility of coordination complexes formed between the metal and the phenolic compounds [210].

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3.5. Total reducing capacity estimated by electrochemical methods

Electrochemical properties of pure compounds, foods, and biological samples may be used for the evaluation of their reducing/antioxidant capacity, since the electric oxidation potential has conceptually relation with the expected antioxidant capacity. Among the electrochemical methods, the cyclic
voltammetry (CV) technique has been adapted to the evaluation of the overall reducing capacity of low molecular weight antioxidants in plasma [211], tissue homogenates [212,213], and plant extracts [214] (Table 9). CV tracings are usually recorded from −0.5 to +1.5 V versus the reference electrode at a scan rate of 100 or 400 mV s$^{-1}$ [215].

The reducing capacity of a sample is analysed from three parameters obtained through the CV tracings: (i) the oxidation potential, $E_{1/2}$, which reflects the specific reducing capacity; (ii) the intensity of the anodic current ($I_a$); and (iii) the area under the anodic wave ($S$). Both $I_a$ and $S$ are related to the concentration of the reducing species present in the sample. Nevertheless, as an anodic wave in complex matrices, such as biological and food samples, often represents more than a single component, each of which could donate electron(s) around the same potential, it was proposed the $S$ parameter for estimation of the total reducing capacity rather than the $I_a$ [214,216]. Moreover, changes in $S$ value better reflect a change in a single component within an anodic wave than the corresponding change in $I_a$.

In this context, the CV methodology allows rapid screening of the electrochemical profile of samples and is especially suitable for screening studies. Furthermore, the CV profile can be obtained in aqueous medium as well as in organic solvents like acetonitrile, water/acetonitrile, and acetonitrile/methanol mixtures provided that there are redox-active components and enough electrolytes in the solution to support redox reactions on the electrode surface. This modification allows the quantification of lipid-soluble components [215].

These assays based on the electrochemical properties of the compound/sample do not require the use of reactive compounds, since it is based on electrochemical behaviour and, consequently on their chemico-physical properties. Moreover, turbid and/or intensely coloured samples can be determined without prior sample preparation. The shortcoming of these methodologies is related to the fact that some biologically relevant antioxidants (e.g. glutathione, cysteine and other thiol-containing compounds) show a low response when glass carbon electrodes are applied. In this case, other electrodes such as an Au/Hg electrode are needed for glutathione measurement [213]. Furthermore, an important practical limitation is that the working electrode has to be frequently cleaned to remove residues of sample from its surface and to maintain its sensitivity.

4. Conclusions and perspectives

At the present moment, a large diversity of analytical methods for determination of antioxidant capacity is available as reported here. These assays differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions, and in the form that results are expressed. Even when only one of these assays is considered, different antioxidant standard compounds, solvents, reaction time and pH are frequently applied. Moreover, as the total antioxidant capacity is dependent of a multitude of factors, a “battery” of assays measuring different aspects of the behaviour of antioxidants is strongly recommended to generate a complete antioxidant profile. Considering the broad spectrum of areas where these methods are currently applied (Tables 1–9, “Recent applications”), that includes physiology, pharmacology, nutrition and agrochemistry, it may be difficult to select the most appropriate method(s) to avoid inappropriate applications and misinterpretation of the results. The comparison of data from different studies is also difficult. In this context, a primary factor to consider when selecting a method is the mechanism of reaction and its relationship to what might occur in the envisioned application. It is also advantageous to select methods that are commonly accepted, validated and standardized, with a large body of comparable data available in the literature. Therefore, the efforts that have been made during the last two years to standardize analytical methods and provide valid guidelines are of utmost importance to bring some order to this field, and they should be pursued by future researchers.

In the future, other in vitro analytical assays will be needed as more is learned about the oxidation sources (radical and non-radical) including their concentrations, the interactions with other oxidant species and biological targets, their significance to oxidative stress, and the surrounding environment. The design of novel methods relies definitely on the utilization of oxidant and target/probe species (proteins, triacylglycerols, and cell models) with relevant biological significance and in reaction conditions (concentration, reaction time, pH) as close as possible to those found in vivo. The possibility of developing assays where more than one oxidant species is present in the reaction medium simultaneously should also be considered. In fact, efforts towards this direction can be found in recent literature, where in vitro, biological models [217–219] have been successfully implemented.

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