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# Methods to measure the antioxidant activity of phytochemicals and plant extracts

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## **ABSTRACT**

Measurement of antioxidant properties in plant-derived compounds require appropriate methods that address the mechanism of antioxidant activity and focus on the kinetics of the reactions involving the antioxidants. Methods based on inhibited autoxidations are the most suited for chain-breaking antioxidants and for termination-enhancing antioxidants, while different specific studies are needed for preventive antioxidants. A selection of chemical testing methods is critically reviewed highlighting their advantages and limitations, and discussing their usefulness to investigate both pure molecules and raw extracts. The influence of the reaction medium on antioxidants' performance is also addressed.

## INTRODUCTION

Antioxidants are arguably a family of compounds of major interest, among plant derived molecules.<sup>1-3</sup> This interest is justified by their importance in the protection of any material existing under normal atmospheric conditions, *i.e.* in the presence of oxygen.<sup>4,5</sup> However, the interest is further boosted by the involvement of free radical and red-ox processes both in normal biological functions and in the pathophysiology of several diseases.<sup>6,7</sup> The role of, so called, oxidative stress in human health as well as in the aging process has suggested that natural or dietary antioxidants may have beneficial properties and potential applications as drugs or health-oriented products.<sup>3,7</sup> Clearly, the necessity to test many structurally diverse compounds or even to screen crude plant extracts has given impetus to the development of an impressive variety of testing methods. This, however, creates a rather confusing scenario, as many such methods own their popularity mainly to their simplicity and lack the necessary soundness, leading often to inconsistent or misleading results, because they are applied without consideration of the chemistry behind the antioxidant activity and of what they are actually measuring.<sup>4</sup> Attempts to generate universal or unified “values” of antioxidant activity that have no physical meaning add to the confusion.<sup>4,8</sup>

Antioxidants are an extremely heterogeneous family of compounds, and a distinction needs to be made between *direct antioxidants*, which are able to protect materials from oxidation and can potentially express their activity both in a tube or in a living organism, and *indirect antioxidants*, that are not able to afford any protection to oxidizable materials but will enhance the antioxidant defense in a living organism, *e.g.* by inducing the biosynthesis of antioxidant enzymes.<sup>4</sup> These last might be most valuable for medicinal purposes, but are beyond the scope of this discussion. Although the two activities might co-exist in the same molecule, only the testing of direct antioxidant activity will be addressed in the following. A comprehensive review of the many chemical and cell-based testing methods available in

the literature is outside the scope of this work, and we will focus our discussion on selected chemical testing methods, highlighting strengths and limits, offering the experience of our research group.

## ANTIOXIDANTS AND RADICALS

Antioxidants are sufficiently heterogeneous to escape any comprehensive definition, nonetheless defining them, is necessary to critically assess their performance. Concerning their role in the protection of organic materials, *direct antioxidants* are those compounds or mixtures able to prevent, block or slow down the autoxidation process (Figure 1): a radical-chain reaction that can be initiated by a variety of events (physical, chemical or biochemical) yielding a variety of radical species that react with an oxidizable substrate (*e.g.* unsaturated lipids, or compounds like styrene) either by H-atom abstraction or by addition to a C=C  $\pi$ -system to afford a carbon-centered radical (*e.g.* alkyl, R•) that, in the presence of oxygen, will form a peroxy radical (ROO•).<sup>4</sup> Regardless of the way they have been generated, peroxy radicals are the most important radical species involved in the process, as they are the sole chain-propagating species (with the exception of the hydroperoxy radical, HOO•). Peroxy radicals attack another substrate molecule again by (formal) H-atom abstraction or by addition to a C=C  $\pi$ -system, thereby propagating the chain-reaction as depicted in Figure 1.<sup>4</sup> Termination of the chain occurs by self-reaction of the peroxy radicals or by cross-reaction between radical species and is a statistically unlikely (*i.e.* slow) event, in the absence of antioxidants. Hence (without antioxidants), following any initiation event, several propagation cycles will occur before termination takes place, determining the so called chain-length, the number of substrate molecules transformed into the *primary oxidation products* - *i.e.* hydroperoxides (ROOH) if propagation occurs by H-atom abstraction (*e.g.* in phospholipids) or polyperoxides (-(ROO)<sub>n</sub>-) if it proceeds by C=C addition (*e.g.* in styrene) – for each initiation event.<sup>4</sup> Primary oxidation products can then undergo further reactions to form *secondary oxidation products*

103 such as aldehydes, ketones, carboxylic acids, etc., often referred to as “carbonyl compounds”. Direct  
104 antioxidants can be distinguished in three groups according to their mechanism of interference with the  
105 autoxidation process (Figure 1). *Preventive antioxidants* interfere with the initiation process, *i.e.* they  
106 prevent the on-setting of the autoxidation chain-reaction.<sup>4</sup> Often initiation occurs by Fenton-like redox  
107 reactions where transition metal ions (*e.g.*  $\text{Fe}^{2+}$  and  $\text{Cu}^{+}$ ) will catalytically cleave by electron transfer  
108 peroxides or hydroperoxides ( $\text{ROOR}$ ,  $\text{ROOH}$  or  $\text{HOOH}$ ) into alkoxy or hydroxyl radicals ( $\text{RO}\bullet$ ,  $\text{HO}\bullet$ )  
109 that would rapidly attack any organic substrate and start the autoxidation. Metal-chelating agents like  
110 phytate or curcumin can block the catalytic cycle and prevent initiation;<sup>9</sup> similarly, compounds able to  
111 reduce peroxides, either stoichiometrically like erucin,<sup>3</sup> or catalytically like glutathione peroxidase (GPx)  
112 mimics,<sup>10</sup> would be preventive antioxidants.<sup>1</sup> *Chain-breaking antioxidants* (also called radical-trapping  
113 antioxidants) are those compounds able to react with peroxy radicals (or hydroperoxy radicals) to form  
114 products that do not propagate the radical-chain, thereby impairing the autoxidation.<sup>1-5</sup> In nature they are  
115 typically phenols or polyphenols<sup>1</sup> although non-phenolic compounds like ascorbic acid or urate are also  
116 well known.<sup>11,12</sup> Finally, a third class has recently been formally recognized by our research group:  
117 *termination-enhancing antioxidants*, comprising several non-phenolic terpenoids like citral or gamma-  
118 terpinene: they co-oxidize with the substrate and form peroxy radicals that do propagate the chain, but  
119 have much higher rate of chain-termination, thereby decreasing the chain-length and saving the  
120 oxidizable substrate.<sup>3,13</sup>

121 As it might appear from the foregoing, antioxidants are not simply “those compounds that react with free  
122 radicals”, as every organic molecule would react with some radical and, clearly, not every molecule can  
123 be regarded as an antioxidant! The only radical species that is important to trap is peroxy radicals (or  
124 hydroperoxy) as they are the sole chain-propagating species in the autoxidation: this point should never  
125 be overlooked when developing (or applying) tests for antioxidant activity.<sup>4</sup>

## 126    **HOW MUCH OR HOW FAST?**

127    Although antioxidants are regarded as bioactive compounds, testing their activity requires a substantially  
128    different approach as compared to drugs. Indeed, most drugs, be them natural or synthetic, act by non-  
129    covalent binding to specific cellular receptors, or to specific enzymes, thereby modulating cellular  
130    functions. Therefore, their activity is largely determined by the extent of such binding and measuring it,  
131    from a chemical perspective, actually means measuring an equilibrium constant. Conversely, the activity  
132    of direct antioxidants is ultimately not related to an equilibrium process, but to their influence on the rate  
133    a radical-chain reaction.<sup>1-5</sup> Therefore, measuring their activity should actually imply measuring the rate  
134    of their reaction (*e.g.* with peroxy radicals) or, ultimately, how they affect the rate of autoxidation of the  
135    substrate they are called to protect. For instance, any radical trapping antioxidant, be it modest or  
136    excellent in performance, would react with peroxy radicals to completion as the reaction is typically  
137    exothermic;<sup>5</sup> however, what makes-up for the difference between a modest and an excellent antioxidant  
138    is mainly the rate at which the reaction occurs.<sup>4</sup> Radical trapping antioxidants will save the oxidizable  
139    substrate from attack by peroxy radicals only if their reaction with peroxy radicals is much faster than  
140    that of the substrate. Indeed, the most important and only “universal” parameters to quantify their activity  
141    are, distinctly, the rate constant for peroxy radical trapping and the stoichiometry of such reaction.<sup>1-5, 8-</sup>  
142    <sup>12</sup> Reaction kinetics and stoichiometry should possibly be kept distinct, as mixing them into a single  
143    parameter limits the rational comparison of different antioxidants. A detailed discussion on this point has  
144    been provided.<sup>4</sup>

## 145    **METHODS BASED ON INHIBITED AUTOXIDATION**

146            *Inhibited autoxidation methods* are based on the measurement of the rate of autoxidation of a  
147    reference substrate, both in the presence and in the absence of antioxidants. These methods are the golden  
148    standard because they test antioxidants in close-to-real settings, *i.e.* they challenge their ability to protect



149 a substrate from oxidation.<sup>4</sup> The autoxidation can occur spontaneously at room or at high temperature,  
150 or it can be induced by the addition of specific initiators, such as an azo-compound or the Fenton reagent  
151 ( $\text{H}_2\text{O}_2$  and  $\text{Fe}^{2+}$ ). Compared to other methods of initiation, azo-initiators, such as the lipid-soluble AIBN  
152 (2,2'-azobis-isobutyronitrile), or water-soluble AAPH ((2,2'-azobis(2-amidinopropane)  
153 dihydrochloride), are better suited to perform kinetic studies because their decomposition occurs at a  
154 constant rate at a given temperature, thus providing a constant rate of initiation ( $R_i$ ) throughout the  
155 reaction course. The substrate is usually constituted by purified unsaturated fatty acids or by their  
156 triglycerides;<sup>3,4,8,14</sup> however, also more easy-to-handle synthetic compounds (*e.g.* styrene or cumene)  
157 work just as well.<sup>1-5,10-14</sup> The reference substrate is normally chosen on the basis of its rate of chain  
158 propagation  $k_p$  (*e.g.*  $k_p$  is 0.34, 41, and 62  $\text{M}^{-1}\text{s}^{-1}$  at 30°C in chlorobenzene for cumene, styrene and linoleic  
159 acid, respectively),<sup>15</sup> more effective antioxidants requiring more oxidizable substrates to be studied, so  
160 that the autoxidation is not completely blocked.<sup>4, 14, 15</sup> When performed at constant  $R_i$  in homogenous  
161 solution, with substrates whose rate constants of chain propagation ( $k_p$ ) and chain termination ( $2k_t$ ) are  
162 known, autoxidation studies allow measuring the absolute rate constants ( $k_{\text{inh}}$ ) and the stoichiometry of  
163 reaction ( $n$ ) between peroxy radicals and antioxidants.<sup>1-5,15</sup> Therefore, they are best suited to gain  
164 detailed information about the absolute performance and mechanism underlying the antioxidant  
165 action.<sup>3,4,8,15</sup> These methods can be further classified depending on the method used to follow the kinetics  
166 of the reaction.

167 *Oximetry methods* (Figure 2) measure the consumption of  $\text{O}_2$  in a closed system by using either  
168 a differential pressure transducer,<sup>10-15</sup> a polarographic probe,<sup>15</sup> or a miniaturized fluorescence-quenching  
169 probe. In these experiments, with effective chain-breaking antioxidants the trend of the  $\text{O}_2$  uptake  
170 typically follows a biphasic behavior: a primary period in which the autoxidation is inhibited by the  
171 antioxidant (the induction period,  $\tau$ ), followed by a fast rate of autoxidation, as shown in Figure 2. The  
172 comparison of the inhibited ( $R_{\text{inh1}}$  or  $R_{\text{inh2}}$ ) and uninhibited ( $R_0$ ) oxygen uptake rates affords the rate

constant  $k_{\text{inh}}$ , while  $\tau$  affords the stoichiometry of the tested antioxidant, in case pure molecules. In case that raw extracts are tested for antioxidant activity, the math can be adjusted so that the rate of inhibited oxygen uptake can provide a specific activity of the extract and  $\tau$  will reflect the product of reaction stoichiometry and concentration of the active antioxidant in the extract. Inhibited autoxidations are normally used to test chain-breaking antioxidants,<sup>1,2,5,10-12</sup> but they are also a privileged method to study termination-enhancing antioxidants (*e.g.* many terpenoid essential oil components),<sup>3,13</sup> in which case the inhibition should be tested at different concentrations of the antioxidant so to clearly differentiate the non-monotonic dose/performance behavior from the linear behavior of chain-breaking antioxidants.<sup>13</sup> Oximetry methods have been implemented in homogeneous organic,<sup>1,2,10,11,13</sup> or water solution,<sup>12</sup> as well as in heterogeneous models like micelles,<sup>14</sup> or liposomes.

*Monitoring the substrate.* In principle the autoxidation can also be studied by monitoring the consumption of the oxidizable substrate; however, some practical shortcomings have to be faced. Firstly, the substrate is normally used at molar concentration to maintain a useful rate of propagation,<sup>4,12,15</sup> therefore, in order to consume a measurable amount of oxidizable substrate, the reaction would need to be followed for several days (as compared to several minutes); secondly, monitoring has to be carried on in a discontinuous way, by sampling the reaction mixture at time-intervals and subjecting it to analysis. A very convenient solution to these shortcomings consists of adding a suitable molecular probe to the oxidizable substrate.<sup>16</sup> 1-Phenylbutadiene derivative of well-known fluorescent BODIPY (PBD-BODIPY, Figure 3A) has  $\lambda_{\text{max}}$  at 591 nm and reacts with peroxy radicals in the phenylbutadiene sidechain undergoing autoxidation similarly to styrene, but with much higher rate of propagation (Figure 3). Since its UV-Vis absorption spectrum changes upon oxidation, it can be used as a reporter of the autoxidation progress, upon adding a modest concentration (typically 10  $\mu\text{M}$ ) to styrene as the oxidizable substrate, so that it will co-oxidize with the substrate itself, allowing the reaction to be followed by a standard spectrophotometer.<sup>16</sup> The kinetic plots obtained by this method are similar to those showing the

197 oxygen consumption as a function of time (Figure 2), except the probe consumption is reported in the  
198 vertical axis, and they can be analyzed similarly, providing  $k_{inh}$  and  $n$  of the antioxidant. Since slower  
199 antioxidants need less oxidizable substrates (*e.g.* cumene), PBD-BODIPY might be too reactive for such  
200 compounds and it can be replaced with the alternative probe STY-BODIPY (Figure 3A). STY-BODIPY  
201 has also been successfully used to monitor autoxidations in homogenous water solution, using THF as  
202 the oxidizable substrate.<sup>16</sup>

203 *Monitoring hydroperoxides.* Beside measuring the consumption of the reactants (oxygen or the substrate)  
204 the autoxidation can also be monitored by following the formation of the primary oxidation products:  
205 hydroperoxides. The measurement of hydroperoxides has actually been one of the earliest methods to  
206 assay the oxidation of edible fats. When the oxidizable substrate is a fatty acid (*e.g.* linoleic acid), natural  
207 phospholipids, or a triglyceride carrying non-conjugated unsaturated chains, the reaction can be followed  
208 by the formation of the typical absorption band of conjugated hydroperoxides at 232 nm, which can be  
209 monitored continuously by spectrophotometry<sup>17</sup> or, more accurately but discontinuously, by HPLC-  
210 UV,<sup>18</sup> avoiding the interference of other absorbing species that may be formed during the autoxidation.  
211 Alternatively, or with different substrates, hydroperoxides can be monitored by (time consuming)  
212 iodometric titration, or by colorimetric assays based on  $Fe^{2+}$  oxidation to  $Fe^{3+}$  and formation of colored  
213 iron salts,<sup>19</sup> but these methods experience interferences by several reductants that might be present in the  
214 sample. This limitation was recently overcome by a method based on the reaction of hydroperoxides with  
215 a pre-fluorescent probe, a coumarin–triarylphosphine conjugate with max fluorescence emission at 422  
216 nm upon excitation at 343 nm, which increases its quantum yields of one order of magnitude upon  
217 oxidation to the corresponding phosphineoxide by reaction with hydroperoxides (Figure 3B).<sup>20</sup> Although  
218 hydroperoxides can be monitored only in a discontinuous fashion, these studies can be performed using  
219 a conventional spectrofluorimeter and afford analogous results as the continuous monitoring of the  
220 reaction, *e.g.* by oximetry.<sup>21</sup>

221 *Other methods.* One quite popular method to monitor autoxidation, which instead should be used with  
222 caution, is the  $\beta$ -carotene bleaching assay. It is based on the disappearance of the typical yellow color of  
223  $\beta$ -carotene when it is co-oxidized with linoleic acid in an emulsion. Although this method is based on  
224 autoxidation, the absence of an initiating system combined with the need to prepare an emulsion with  
225 controlled composition and droplet-size reduce the reproducibility of the results.<sup>3,4</sup> The conjugated  
226 autoxidizable triene (CAT) and the apolar radical-initiated conjugated autoxidizable triene (ApoCAT)  
227 assays are recently gaining interest.<sup>19,22,23</sup> They are based on autoxidation of tung oil, rich in conjugated  
228 triene triacylglycerols showing UV absorbtion at 271 nm, which gets lost upon oxidation, allowing the  
229 autoxidation to be followed in a common microplate reader.<sup>22</sup> The two methods differ for the radical  
230 initiator, a water soluble or a lipid soluble azo-compound, respectively. In our opinion the sole critical  
231 point of such interesting methods in the use of the area-under-the-curve (AUC) to analyze results, in  
232 place of kinetic analysis of the autoxidation traces, which would afford distinct information on reaction  
233 rate and stoichiometry.<sup>4</sup>

#### 234 **Monitoring late (secondary) autoxidation products**

235 Most popular methods used to follow the autoxidation are based on the detection of late oxidation  
236 products. Among them, the TBARS (thiobarbituric acid reactive species) assay is a semi-quantitative  
237 colorimetric method used to detect malondialdehyde, which is formed from the breakdown of  
238 hydroperoxides accumulated during the oxidation of polyunsaturated lipids.<sup>4</sup> Other aldehydes formed  
239 during autoxidation of fatty acids are typically propanal, hexanal, and nonanal for omega-3, -6, and -9  
240 fatty acids, respectively) and can be quantified by head-space gas chromatography.<sup>24</sup> Additionally, 4-  
241 hydroxynonenal (4-HNE) a toxic specific oxidation product of unsaturated fatty acids, can be analyzed  
242 by LC-MS or other techniques upon chemical derivatization.<sup>25</sup> Although these methods provide valuable  
243 information on the occurrence of oxidative degradation in lipid matrices like food, they should be used

with caution to test antioxidant activity. One main limit is that they are typically applied in a “single point” fashion, *i.e.* the formation of oxidation products is assayed only after a fixed time, and is compared with a single measurement in a parallel experiment without antioxidant.<sup>4</sup> It would provide proof of principle that some compound is acting as antioxidant, but it cannot afford quantitative evaluation of antioxidant performance, as the amount of carbonyl compounds detected in the reaction mixture at a given time is the results of a variety of subsequent and competing reactions and is influenced by a number of variables that are far beyond the effectiveness of the tested antioxidant. An in-depth discussion on these aspects has recently been provided.<sup>4</sup> *The Rancimat test* is another popular method belonging to this class. A specific apparatus measures the release of volatile acids formed upon the spontaneous oxidation of fats (such as seed oils or lard), under an air stream at 90-120 °C, by a conductometric method.<sup>4</sup> The antioxidant activity is obtained as a function of the induction time observed in the oxidation profiles, which, in turn, provides an estimate of the antioxidant stoichiometry combined with threshold reactivity. It is a useful method for semi-qualitative estimate of the antioxidant performance of extracts, however it should be considered that the high temperature might cause the loss of low-boiling or labile antioxidants, resulting in misestimating their activity.<sup>4</sup>

## ***METHODS BASED ON COMPETITIVE PROBE REACTION AND INDIRECT METHODS***

Many popular methods to assess antioxidant activity are based on the competitive reaction of radicals with the antioxidant or with a probe, whose transformation can be monitored by fluorimetry (*e.g.* the ORAC assay) by spectrophotometry (*e.g.* the crocin bleaching assay), by EPR (*e.g.* spin-trapping methods) or other techniques.<sup>4</sup> All these methods, despite their popularity, do not involve any substrate autoxidation and offer limited information on the actual antioxidant activity, as previously discussed.<sup>3,4</sup> Another very popular family of methods is that of *indirect methods*, which are based on the reaction of

the potential antioxidant with some unnatural colored persistent radical (*e.g.* the DPPH test, the TEAC test, the Galvinoxyl test), or with other oxidizing agents like  $\text{Fe}^{3+}$  ions (*e.g.* the FRAP test), or  $\text{Cu}^{2+}$  ions (*e.g.* the CUPRAC test) or others (*e.g.* the Folin-Ciocalteu test).<sup>4,8</sup> In general, these tests do not provide any measurement of the antioxidant activity, rather they tell, respectively, of the radical-trapping activity or of the reducing ability of a compound or extract, which should never be overlooked when interpreting or presenting their results.<sup>4</sup> Their advantages and limitations have already been discussed in some detail,<sup>4</sup> however one of such methods, in our own experience, can be very useful to study natural antioxidants, and we wish to discuss it further.

DPPH• (2,2-diphenyl-1-picrylhydrazyl radical,  $\lambda_{\text{max}} \approx 520 \text{ nm}$ ), reacts with most antioxidants with similar mechanism as peroxy radicals (due to the similar electronic configuration), albeit at much lower rate, allowing the facile monitoring of the reaction kinetics in a conventional spectrophotometer (Figure 4). The bimolecular rate constant for the reaction between DPPH• and various antioxidants, allows clarify the structure-activity relationship of families of antioxidants such as flavonoids,<sup>26</sup> and their reaction mechanisms.<sup>7</sup> Additionally, a single-point measurement of DPPH• discoloration provides the stoichiometry of radical-trapping,<sup>27</sup> or can be used to titrate the effective content of antioxidants when testing raw extracts of unknown composition. It is recommended that reaction time is set to a short value *e.g.* 1 minute, so to detect real antioxidants (reacting rapidly) and bias from reaction of DPPH• with other molecules is minimized.<sup>3</sup> Conversely, other common single-point uses of DPPH, like measuring the IC<sub>50</sub> – the concentration of an antioxidant able to give 50% discoloration of a solution of DPPH after a fixed time (*e.g.* 30 min) – and reporting it as a quantitative parameter of antioxidant performance, are misleading and void of any chemical or physical meaning and should be discouraged.<sup>4, 27</sup>

## TESTING PREVENTIVE ANTIOXIDANTS

Preventive antioxidants are less frequently investigated than radical-trapping in natural products

290 research. Since preventive activity can be based on several mechanisms, normally specific assays are  
291 required. For instance, Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Glutathione  
292 reductase (GR), thiol peroxidase, Catalase, and other assays can be used to highlight specific enzyme-  
293 like activity.<sup>4,28</sup>

294 *Metal chelating compounds* are perhaps the most common among preventive antioxidants. Their action  
295 relies on blocking the Fenton reaction by chelating transition metal ions with higher affinity for higher  
296 oxidation state, *e.g.*  $\text{Fe}^{3+}$  as compared to  $\text{Fe}^{2+}$ , or  $\text{Cu}^{2+}$  as compared to  $\text{Cu}^{+}$ , which makes their recycling  
297 by reducing agents thermodynamically less favorable and impairs their catalytic redox cycle.<sup>9</sup>  
298 Quantitative metal binding studies (*e.g.* by spectrophotometry) can be very useful to investigate their  
299 activity; however, testing their ability to inhibit autoxidations initiated by the Fenton chemistry (*e.g.* by  
300 the mixture  $\text{Fe}^{2+}$ ,  $\text{H}_2\text{O}_2$ , ascorbate, instead of azo-initiators) would provide most solid evidence that metal  
301 binding translates into antioxidant activity.<sup>4,9</sup>

302 Fenton initiation can be inhibited also by decomposing (*i.e.* depleting)  $\text{H}_2\text{O}_2$  or hydroperoxides, which,  
303 besides catalytic enzyme-like activities, can be accomplished also stoichiometrically, *e.g.* by natural  
304 sulfides like cysteine-derived secondary metabolites. This activity can conveniently be studied by  
305 electrospray mass spectrometry (ESI-MS), *e.g.* by monitoring the kinetics of formation of the  
306 corresponding sulfone.<sup>4</sup>

307

## 308 **THE ROLE OF THE MEDIUM ON THE MEASUREMENT OF ANTIOXIDANT ACTIVITY**

309 In testing the antioxidant activity, it should never be overlooked that the reaction medium has major  
310 importance. It is now well established that the reaction of any radical,<sup>29</sup> including peroxy<sup>30</sup> with typical  
311 antioxidant like phenols or any compound able to act as hydrogen bond donor (HBD) is modulated by

the hydrogen bond accepting (HBA) ability of the solvent, in homogenous solution. Indeed, occurrence of H-bonding of the antioxidants to the solvent impairs their rate of radical trapping. Additionally, H-bonding with the solvent will influence the reactivity of some radicals like the hydroperoxyl,<sup>29</sup> and the two phenomena will combine in autoxidations carried on by hydroperoxyl radicals. Furthermore, the polarity of the solvent might influence the mechanism of reaction of antioxidants (*e.g.* phenols) with radicals, *e.g.* changing from a concerted proton coupled electron transfer (PCET) to a stepwise proton-transfer electron-transfer (PT-ET) or *vice versa*, which would clearly affect the rate of reaction, sometimes the stoichiometry, and, overall, the antioxidant efficacy. For instance, it has been demonstrated that in alcohols the reaction of DPPH• radical with phenols changes from a PCET mechanism to a mechanism named sequential proton-loss electron-transfer (SPLET), accelerating up to thousands-folds.<sup>4,29</sup> The same acceleration is not observed for peroxy radicals in alcohols; therefore, the common approach to test the DPPH• radical-trapping ability in methanol or ethanol to guarantee the solubilization of polar phytochemicals might result in misestimated activity and polar solvents like acetonitrile or dioxane are preferable to parallel the behavior of peroxy radicals. Finally, in water, the influence of pH needs to be considered.<sup>12</sup> Testing antioxidants in heterogeneous media, like emulsions, micelles, and liposomes is even more critical. It is well known that the rate of radical reaction in these systems reflects mainly the rate of reactants exchange among particles,<sup>12,14</sup> additionally the partition of the antioxidant will affect the measured antioxidant activity,<sup>23</sup> which may or may not mimic the real-life-conditions, depending on how closely the experimental model resembles the real material to be protected. Studies in heterogeneous media might be very valuable, but they could be matched to studies in homogeneous solution to gain full rationalization of antioxidant behavior.<sup>14</sup>



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429 **FIGURE LEGENDS**

430

431 **Figure 1.** Autoxidation of an organic substrate and mechanism of interference by direct antioxidants

432 **Figure 2.** Example of oxygen-uptake plot during the autoxidation of a standard substrate RH in the absence of

433 inhibitors (a), in the presence of a modest chain-breaking antioxidant AH (b) or of a good chain-breaking antioxidant

434 AH (c). Corresponding equations allow to obtain the rate constant for peroxy radical trapping ( $k_{inh}$ ), the

435 stoichiometric factor (n) provided the rate of initiation ( $R_i$ ), the rates of chain-propagation ( $k_p$ ) and termination ( $2k_t$ )

436 for the substrate are known.

437 **Figure 3.** (A) Co-oxidizable probes used to monitor by visible spectrophotometry the time course of an inhibited

438 autoxidation. (B) Fluorescent probe that allows monitoring the kinetics of formation of hydroperoxides during

439 inhibited autoxidations.

440 **Figure 4.** Decay of DPPH signal at 517 nm in methanol at  $298 \pm 2$  K after rapid 1:1 mixing of a  $2.0 \times 10^{-5}$  M solution

441 of DPPH with the solvent (spontaneous decay, dotted line) or with a 10 mg/L solution of a standardized Daikon

442 extract (full line). Insert: first order kinetic analysis plot. Reproduced from Ref 7.

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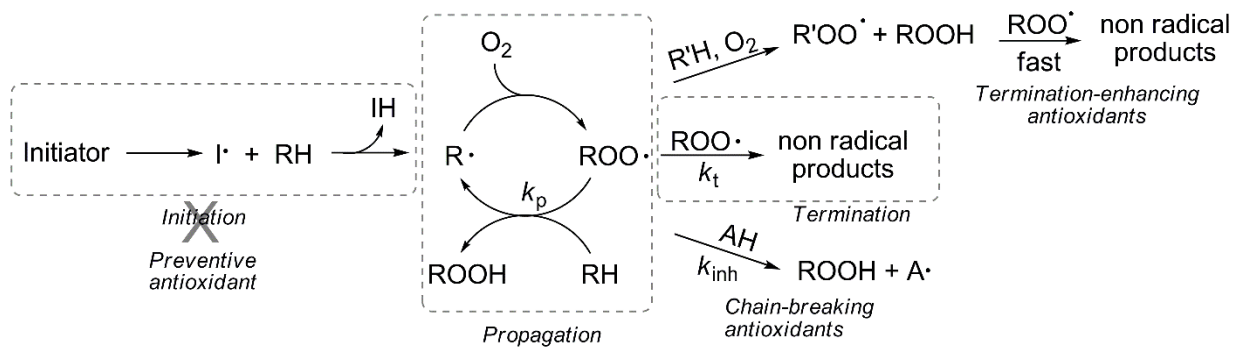
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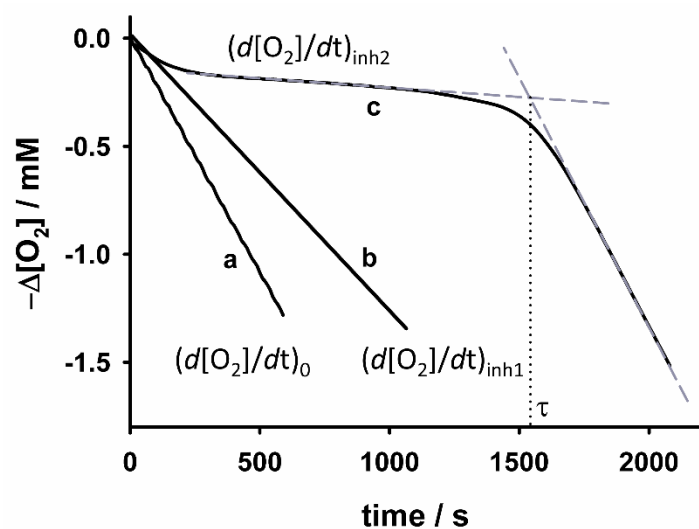
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**Figure 1.**



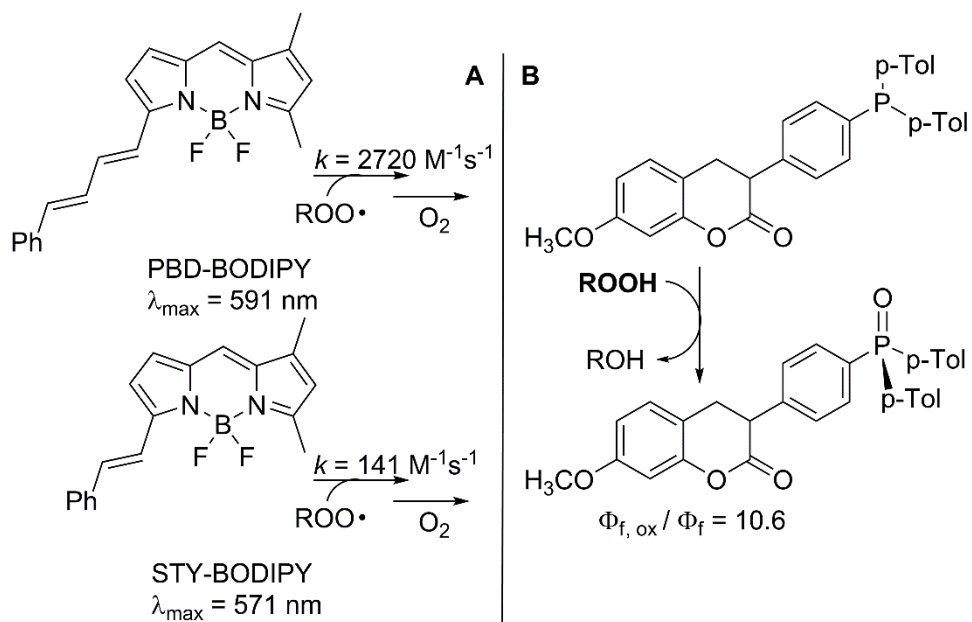
Initiation rate:  $R_i = \frac{n[\text{AH}]}{\tau}$

a)  $-\left(\frac{d[\text{O}_2]}{dt}\right)_0 = \frac{k_p}{\sqrt{2k_t}}[\text{RH}]\sqrt{R_i} + R_i$

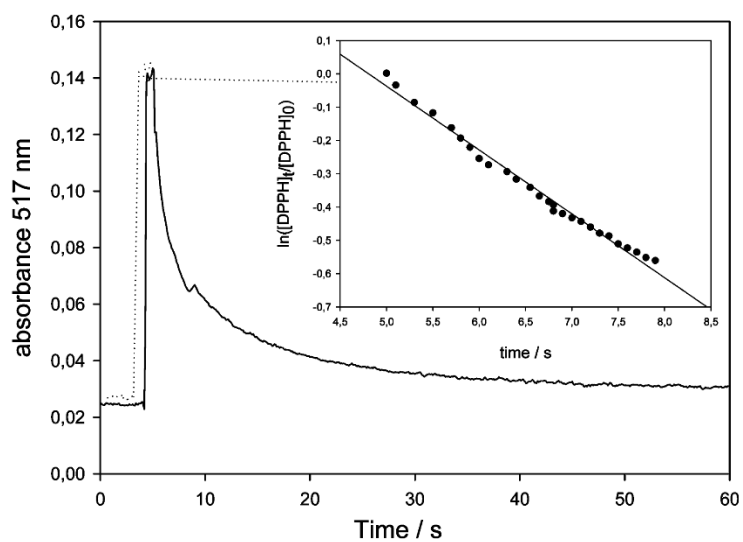
b)  $\frac{-(d[\text{O}_2]/dt)_0}{-(d[\text{O}_2]/dt)_{inh1}} - \frac{-(d[\text{O}_2]/dt)_{inh1}}{-(d[\text{O}_2]/dt)_0} = \frac{nk_{inh1}[\text{AH}]}{\sqrt{2k_t}R_i}$

c)  $-\left(\frac{d[\text{O}_2]}{dt}\right)_{inh2} = \frac{k_p[\text{RH}]R_i}{nk_{inh2}[\text{AH}]} + R_i$

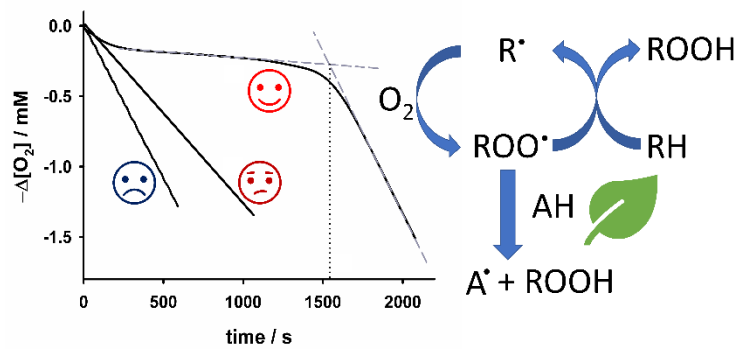
**Figure 2.**



**Figure 3**



**Figure 4**



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467 TOC