



ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE
DELLA RICERCA

Alma Mater Studiorum Università di Bologna Archivio istituzionale della ricerca

Methods to Measure the Antioxidant Activity of Phytochemicals and Plant Extracts

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Methods to Measure the Antioxidant Activity of Phytochemicals and Plant Extracts / Amorati, Riccardo*; Valgimigli, Luca. - In: JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY. - ISSN 0021-8561. - STAMPA. - 66:13(2018), pp. 3324-3329. [10.1021/acs.jafc.8b01079]

Availability:

This version is available at: <https://hdl.handle.net/11585/659429> since: 2020-02-25

Published:

DOI: <http://doi.org/10.1021/acs.jafc.8b01079>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

R. Amorati, L. Valgimigli. Methods to measure the antioxidant activity of phytochemicals and plant extracts. *J. Agric. Food Chem.* 2018, 66, 13, 3324–3329.

The final published version is available online at:

<https://doi.org/10.1021/acs.jafc.8b01079>

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

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

2

3 *Riccardo Amorati, * Luca Valgimigli**

4 University of Bologna, Department of Chemistry “G. Ciamician”, Via S. Giacomo 11, 40126-Bologna,
5 Italy.

6

7

8

9 Corresponding Author *

10 LV: Tel: +39 051 2095683; fax: +39 051 2095688; e-mail: luca.valgimigli@unibo.it

11 RA: Tel: +39 051 2095689; fax: +39 051 2095688; e-mail: riccardo.amorati@unibo.it

12

13 ORCID

14 Luca Valgimigli: 0000-0003-2229-1075

15 Riccardo Amorati: 0000-0002-6417-9957

16

17 **Funding:** The work was carried out under funding of the University of Bologna.

18

19 **Notes:** The authors declare no competing financial interest.

20

21

22

23

24

25

26

27

28

29

30 **ABSTRACT**

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

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57 INTRODUCTION

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

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

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

82

83 ANTIOXIDANTS AND RADICALS

84 Antioxidants are sufficiently heterogeneous to escape any comprehensive definition, nonetheless
85 defining them, is necessary to critically assess their performance. Concerning their role in the protection
86 organic materials, *direct antioxidants* are those compounds or mixtures able to prevent, block or slow
87 down the autoxidation process (Figure 1): a radical-chain reaction that can be initiated by a variety of
88 events (physical, chemical or biochemical) yielding a variety of radical species that react with an
89 oxidizable substrate (*e.g.* unsaturated lipids, or compounds like styrene) either by H-atom abstraction or
90 by addition to a C=C π -system to afford a carbon-centered radical (*e.g.* alkyl, R•) that, in the presence of
91 oxygen, will form a peroxy radical (ROO•).⁴ Regardless of the way they have been generated, peroxy
92 radicals are the most important radical species involved in the process, as they are the sole chain-
93 propagating species (with the exception of the hydroperoxy radical, HOO•). Peroxy radicals attack
94 another substrate molecule again by (formal) H-atom abstraction or by addition to a C=C π -system,
95 thereby propagating the chain-reaction as depicted in Figure 1.⁴ Termination of the chain occurs by self-
96 reaction of the peroxy radicals or by cross-reaction between radical species and is a statistically unlikely
97 (*i.e.* slow) event, in the absence of antioxidants. Hence (without antioxidants), following any initiation
98 event, several propagation cycles will occur before termination takes place, determining the so called
99 chain-length, the number substrate molecules transformed into the *primary oxidation products* - *i.e.*
100 hydroperoxides (ROOH) if propagation occurs by H-atom abstraction (*e.g.* in phospholipids) or
101 polyperoxides (-(ROO)_n-) if it proceeds by C=C addition (*e.g.* in styrene) – for each initiation event.⁴
102 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.⁴ Often initiation occurs by Fenton-like redox
107 reactions where transition metal ions (*e.g.* Fe²⁺ and Cu⁺) will catalytically cleave by electron transfer
108 peroxides or hydroperoxides (ROOR, ROOH or HOOH) into alkoxy or hydroxyl radicals (RO•, HO•)
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;⁹ similarly, compounds able to
111 reduce peroxides, either stoichiometrically like erucin,³ or catalytically like glutathione peroxidase (GPx)
112 mimics,¹⁰ would be preventive antioxidants.¹ *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.¹⁻⁵ In nature they are
115 typically phenols or polyphenols¹ although non-phenolic compounds like ascorbic acid or urate are also
116 well known.^{11,12} 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.^{3,13}

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.⁴

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.¹⁻⁵ 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;⁵ however, what makes-up for the difference between a modest and an excellent antioxidant
138 is mainly the rate at which the reaction occurs.⁴ 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.^{1-5, 8-}
142 ¹² 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.⁴

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.⁴ 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 (H_2O_2 and 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;^{3,4,8,14} however, also more easy-to-handle synthetic compounds (*e.g.* styrene or cumene)
157 work just as well.^{1-5,10-14} 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),¹⁵ more effective antioxidants requiring more oxidizable substrates to be studied, so
160 that the autoxidation is not completely blocked.^{4, 14, 15} 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_{inh}) and the stoichiometry of
163 reaction (n) between peroxy radicals and antioxidants.^{1-5,15} Therefore, they are best suited to gain
164 detailed information about the absolute performance and mechanism underlying the antioxidant
165 action.^{3,4,8,15} 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 O_2 in a closed system by using either
168 a differential pressure transducer,¹⁰⁻¹⁵ a polarographic probe,¹⁵ or a miniaturized fluorescence-quenching
169 probe. In these experiments, with effective chain-breaking antioxidants the trend of the 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, τ), followed by a fast rate of autoxidation, as shown in Figure 2. The
172 comparison of the inhibited (R_{inh1} or R_{inh2}) and uninhibited (R_0) oxygen uptake rates affords the rate

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

183 *Monitoring the substrate.* In principle the autoxidation can also be studied by monitoring the
184 consumption of the oxidizable substrate; however, some practical shortcomings have to be faced. Firstly,
185 the substrate is normally used at molar concentration to maintain a useful rate of propagation,^{4,12,15}
186 therefore, in order to consume a measurable amount of oxidizable substrate, the reaction would need to
187 be followed for several days (as compared to several minutes); secondly, monitoring has to be carried on
188 in a discontinuous way, by sampling the reaction mixture at time-intervals and subjecting it to analysis.
189 A very convenient solution to these shortcomings consists of adding a suitable molecular probe to the
190 oxidizable substrate.¹⁶ 1-Phenylbutadiene derivative of well-known fluorescent BODIPY (PBD-
191 BODIPY, Figure 3A) has λ_{max} at 591 nm and reacts with peroxy radicals in the phenylbutadiene
192 sidechain undergoing autoxidation similarly to styrene, but with much higher rate of propagation (Figure
193 3). Since its UV-Vis absorption spectrum changes upon oxidation, it can be used as a reporter of the
194 autoxidation progress, upon adding a modest concentration (typically 10 μM) to styrene as the oxidizable
195 substrate, so that it will co-oxidize with the substrate itself, allowing the reaction to be followed by a
196 standard spectrophotometer.¹⁶ 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.¹⁶

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 spectrophotometry¹⁷ or, more accurately but discontinuously, by HPLC-
210 UV,¹⁸ 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,¹⁹ 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).²⁰ 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.²¹

221 *Other methods.* One quite popular method to monitor autoxidation, which instead should be used with
222 caution, is the β -carotene bleaching assay. It is based on the disappearance of the typical yellow color of
223 β -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.^{3,4} The conjugated
226 autoxidizable triene (CAT) and the apolar radical-initiated conjugated autoxidizable triene (ApoCAT)
227 assays are recently gaining interest.^{19,22,23} They are based on autoxidation of tung oil, rich in conjugated
228 triene triacylglycerols showing UV absorption at 271 nm, which gets lost upon oxidation, allowing the
229 autoxidation to be followed in a common microplate reader.²² 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.⁴

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.⁴ 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.²⁴ 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.²⁵ Although these methods provide valuable
243 information on the occurrence of oxidative degradation in lipid matrices like food, they should be used

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

259

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

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

267 the potential antioxidant with some unnatural colored persistent radical (*e.g.* the DPPH test, the TEAC
268 test, the Galvinoxyl test), or with other oxidizing agents like Fe³⁺ ions (*e.g.* the FRAP test), or Cu²⁺ ions
269 (*e.g.* the CUPRAC test) or others (*e.g.* the Folin-Ciocalteu test).^{4,8} In general, these tests do not provide
270 any measurement of the antioxidant activity, rather they tell, respectively, of the radical-trapping activity
271 or of the reducing ability of a compound or extract, which should never be overlooked when interpreting
272 or presenting their results.⁴ Their advantages and limitations have already been discussed in some detail,⁴
273 however one of such methods, in our own experience, can be very useful to study natural antioxidants,
274 and we wish to discuss it further.

275 DPPH• (2,2-diphenyl-1-picrylhydrazyl radical, λ max \approx 520 nm), reacts with most antioxidants with
276 similar mechanism as peroxy radicals (due to the similar electronic configuration), albeit at much lower
277 rate, allowing the facile monitoring of the reaction kinetics in a conventional spectrophotometer (Figure
278 4). The bimolecular rate constant for the reaction between DPPH• and various antioxidants, allows clarify
279 the structure-activity relationship of families of antioxidants such as flavonoids,²⁶ and their reaction
280 mechanisms.⁷ Additionally, a single-point measurement of DPPH• discoloration provides the
281 stoichiometry of radical-trapping,²⁷ or can be used to titrate the effective content of antioxidants when
282 testing raw extracts of unknown composition. It is recommended that reaction time is set to a short value
283 *e.g.* 1 minute, so to detect real antioxidants (reacting rapidly) and bias from reaction of DPPH• with other
284 molecules is minimized.³ Conversely, other common single-point uses of DPPH, like measuring the IC50
285 – the concentration of an antioxidant able to give 50% discoloration of a solution of DPPH after a fixed
286 time (*e.g.* 30 min) – and reporting it as a quantitative parameter of antioxidant performance, are
287 misleading and void of any chemical or physical meaning and should be discouraged.^{4, 27}

288 **TESTING PREVENTIVE ANTIOXIDANTS**

289 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.^{4,28}

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.* Fe³⁺ as compared to Fe²⁺, or Cu²⁺ as compared to Cu⁺, which makes their recycling
297 by reducing agents thermodynamically less favorable and impairs their catalytic redox cycle.⁹
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 Fe²⁺, H₂O₂, ascorbate, instead of azo-initiators) would provide most solid evidence that metal
301 binding translates into antioxidant activity.^{4,9}

302 Fenton initiation can be inhibited also by decomposing (*i.e.* depleting) H₂O₂ 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.⁴

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,²⁹ including peroxy³⁰ with typical
311 antioxidant like phenols or any compound able to act as hydrogen bond donor (HBD) is modulated by

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

333

334

335 **REFERENCES**

- 336 1) Amorati, R.; Zotova, J.; Baschieri, A.; Valgimigli, L. Antioxidant Activity of Magnolol and
337 Honokiol: Kinetic and Mechanistic Investigations of Their Reaction with Peroxyl Radicals. *J. Org.*
338 *Chem.* **2015**, *80*, 10651–10659.
- 339 2) Amorati, R.; Lynett, P. T.; Valgimigli, L.; Pratt, D. A. The Reaction of Sulfenic Acids with Peroxyl
340 Radicals: Insights into the Radical-Trapping Antioxidant Activity of Plant-Derived Thiosulfinates.
341 *Chem. Eur. J.* **2012**, *18*, 6370 – 6379
- 342 3) Amorati, R.; Foti, M. C.; Valgimigli, L. Antioxidant Activity of Essential Oils. *J. Agric. Food Chem.*
343 **2013**, *61*, 10835–10847
- 344 4) Amorati, R.; Valgimigli, L. Advantages and limitations of common testing methods for antioxidants.
345 *Free Radic. Res.* **2015**, *49*, 633–649.
- 346 5) Hanthorn, J. J.; Valgimigli, L.; Pratt, D. A. Incorporation of Ring Nitrogens into Diphenylamine
347 Antioxidants: Striking a Balance between Reactivity and Stability. *J. Am. Chem. Soc.* **2012**, *134*, 8306–
348 8309.
- 349 6) D'Aleo, V.; Del Guerra, S.; Martano, M.; Bonamassa, B.; Canistro, D.; Soleti, A.; Valgimigli, L.;
350 Paolini, M.; Filipponi, F.; Boggi, U.; Del Prato, S.; Lupi, R. The non-peptidyl low molecular weight
351 radical scavenger IAC protects human pancreatic islets from lipotoxicity. *Mol. Cell. Endocrinol.* **2009**,
352 *309*, 63-66.
- 353 7) Barillari, J.; Iori, R.; Papi, A.; Orlandi, M.; Bartolini, G.; Gabbanini, S.; Pedulli, G. F.; Valgimigli,
354 L. Kaiware Daikon (*Raphanus sativus* L.) Extract: A Naturally Multipotent Chemopreventive Agent. *J.*
355 *Agric. Food Chem.* **2008**, *56*, 7823–7830.
- 356 8) Niki, E. Assessment of Antioxidant Capacity in vitro and in vivo. *Free Radic. Biol. Med.* **2010**, *49*,
357 503–51.

358

- 359 9) Nathan, R. Perron, A. E., Julia, L. Brumaghim. A Review of the Antioxidant Mechanisms of
360 Polyphenol Compounds Related to Iron Binding. *Cell. Biochem. Biophys.* **2009**, *53*, 75–100.
- 361 10) Amorati, R.; Valgimigli, L.; Dinér, P.; Bakhtiari, K.; Saeedi, M.; Engman, L. Multi-faceted
362 Reactivity of Alkyltellurophenols Towards Peroxyl Radicals: Catalytic Antioxidant Versus Thiol-
363 Depletion Effect. *Chem Eur J.* **2013**, *19*, 7510-7522.
- 364 11) Amorati, R.; Pedulli, G. F.; Valgimigli, L. Kinetic and thermodynamic aspects of the chain-breaking
365 antioxidant activity of ascorbic acid derivatives in non-aqueous media. *Org. Biomol. Chem.* **2011**, *9*,
366 3792–3800.
- 367 12) Amorati, R.; Baschieri, A.; Morroni, G.; Gambino, R.; Valgimigli, L. Peroxyl Radical Reactions in
368 Water Solution: A Gym for Proton-Coupled Electron-Transfer Theories. *Chem. Eur. J.* **2016**, *22*, 7924–
369 7934.
- 370 13) Baschieri, A.; Daci Ajvazi, M.; Folifack Tonfack, J. L.; Valgimigli, L.; Amorati, R. Explaining the
371 antioxidant activity of some common non-phenolic components of essential oils. *Food Chem.* **2017**,
372 *232*, 656–663
- 373 14) Matera, R.; Gabbanini, S.; Berretti, S.; Amorati, R.; De Nicola, G. R.; Iori, R.; Valgimigli, L.
374 Acylated anthocyanins from sprouts of raphanus sativus cv . Sango: isolation, structure elucidation and
375 antioxidant activity. *Food Chem.* **2015**, *166*, 397 – 406.
- 376 15) Amorati, R.; Baschieri, A.; Valgimigli, L. Measuring Antioxidant Activity in Bioorganic Samples
377 by the Differential Oxygen Uptake Apparatus: Recent Advances. *J. Chem.* **2017**, *2017*, Article ID
378 6369358.
- 379 16) Haidasz, E. A.; Van Kessel, A. T. M.; Pratt, D. A. A Continuous Visible Light Spectrophotometric
380 Approach To Accurately Determine the Reactivity of Radical-Trapping Antioxidants. *J. Org. Chem.*
381 **2016**, *81*, 737–744.

382

383 17) Foti, M.; Ruberto, G. Kinetic solvent effects on phenolic antioxidants determined by
384 spectrophotometric measurements. *J. Agric. Food Chem.* **2001**, *49*, 342– 348.

385 18) Johansson, H.; Shanks, D.; Engman, L.; Amorati, R.; Pedulli, G. F.; Valgimigli, L. Long-Lasting
386 Antioxidant Protection: A Regenerable BHA Analogue. *J. Org. Chem.* **2010**, *75*, 7535–7541.

387 19) Phonsatta, N.; Deetae, P.; Luangpituksa, P.;Grajeda-Iglesias, C. Maria Cruz Figueroa-Espinoza, M.
388 C.; Le Comte, J.; Villeneuve, P.; Decker, E. A., Visessanguan, W.; Panya, A. Comparison of
389 Antioxidant Evaluation Assays for Investigating Antioxidative Activity of Gallic Acid and Its Alkyl
390 Esters in Different Food Matrices. *J. Agric. Food Chem.* **2017**, *65*, 7509–7518.

391 20) Hanthorn, J. J.; Haidasz, E.; Gebhardt, P.; Pratt, D. A. A versatile fluorescence approach to kinetic
392 studies of hydrocarbon autoxidations and their inhibition by radical-trapping antioxidants. *Chem.*
393 *Commun.* **2012**, *48*, 10141–10143.

394 21) Haidasz, E. A.; Meng, D.; Amorati, R.; Baschieri, A.; Ingold, K. U.; Valgimigli, L.; Pratt, D. A.
395 Acid Is Key to the Radical-Trapping Antioxidant Activity of Nitroxides. *J. Am. Chem. Soc.* **2016**, *138*,
396 5290–5298.

397 22) Panya, A.; Temthawee, W.; Phonsatta, N.; Charoensuk, D.; Deetae P.; Visessanguan, W.; Decker,
398 E. A.; Apolar Radical Initiated Conjugated Autoxidizable Triene (ApoCAT) Assay: Effects of Oxidant
399 Locations on Antioxidant Capacities and Interactions. *J. Agric. Food Chem.* **2015**, *63*, 7546–7555.

400 23) Laguerre, M.; Lûpez Giraldo, L. J.; Lecomte, J.; Figueroa-Espinoza, M.-C. Barla, B.; Weiss, J.;
401 Decker, E. A.; Villeneuve, P.; Chain Length Affects Antioxidant Properties of Chlorogenate
402 Esters in Emulsion: The Cutoff Theory Behind the Polar Paradox. *J. Agric. Food Chem.* **2009**, *57*,
403 11335–11342.

404

- 405 24) Homma, R.; Suzuki, K.; Cui, L.; McClements, D. J.; Decker, E. A. Impact of Association Colloids
406 on Lipid Oxidation in Triacylglycerols and Fatty Acid Ethyl Esters *J. Agric. Food Chem.* **2015**, *63*,
407 10161–10169)
- 408 25) Gabbanini, S.; Matera, R.; Valvassori, A.; Valgimigli, L. Rapid liquid chromatography–tandem
409 mass spectrometry analysis of 4-hydroxynonenal for the assessment of oxidative degradation and safety
410 of vegetable oils. *Anal. Chim. Acta*, **2015**, *869*, 50–58
- 411 26) Musialik, M.; Kuzmicz, R.; Pawłowski, T. S.; Litwinienko, G. Acidity of hydroxyl groups: an
412 overlooked influence on antiradical properties of flavonoids. *J. Org. Chem.* **2009**; *74*, 2699 – 2709.
- 413 27) Foti, M. Use and Abuse of the DPPH• Radical. *J. Agric. Food Chem.* **2015**, *63*, 8765–8776.
- 414 28) Talawar, S. T.; Harohally, N. V.; Ramakrishna, C.; Suresh Kumar, G. Development of Wheat Bran
415 Oil Concentrates Rich in Bioactives with Antioxidant and Hypolipidemic Properties. *J. Agric. Food*
416 *Chem.* **2017**, *65*, 9838-9848
- 417 29) Amorati, R.; Valgimigli, L. Modulation of the antioxidant activity of phenols by non-covalent
418 interactions. *Org. Biomol. Chem.* **2012**, *10*, 4147–4158.
- 419 30) Lucarini, M.; Pedulli, G. F.; Valgimigli, L. Do Peroxyl Radicals Obey the Principle That Kinetic
420 Solvent Effects on H-Atom Abstraction Are Independent of the Nature of the Abstracting Radical? *J.*
421 *Org. Chem.* **1998**, *63*, 4497-4499.

422
423
424
425
426
427
428

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 ± 2 K after rapid 1:1 mixing of a 2.0×10^{-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.

443

444

445

446

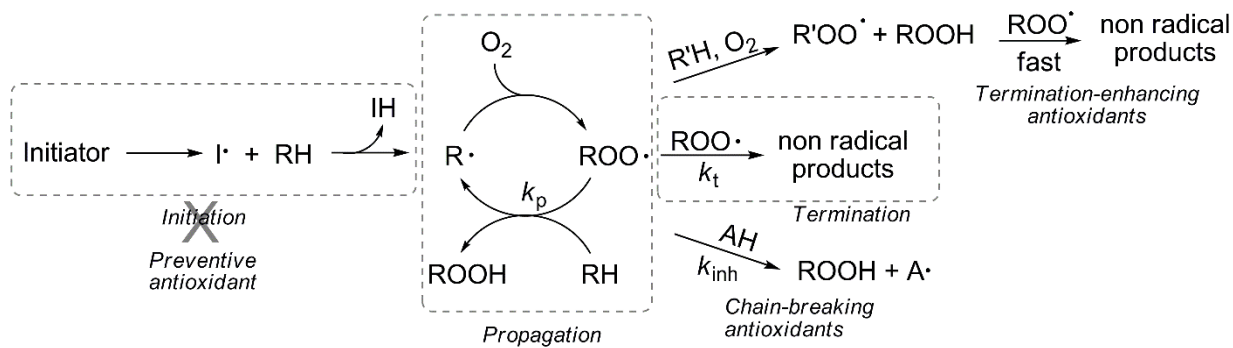
447

448

449

450

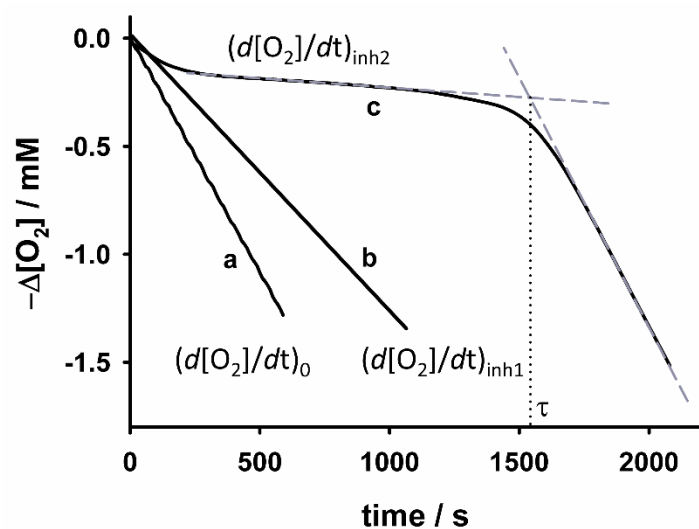
451



452

453 **Figure 1.**

454



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

a) $-\left(\frac{d[O_2]}{dt}\right)_0 = \frac{k_p}{\sqrt{2k_t}}[RH]\sqrt{R_i} + R_i$

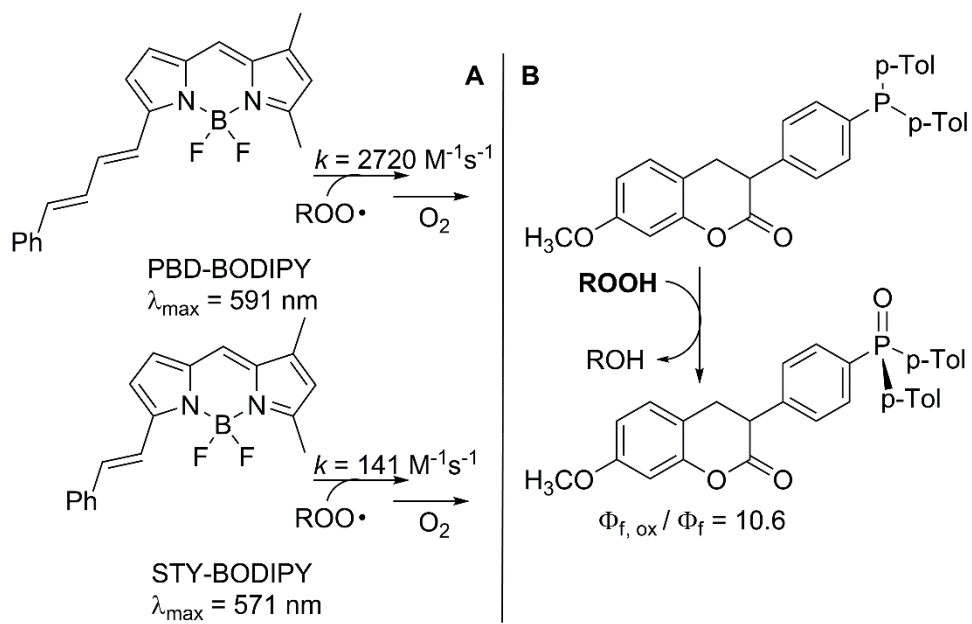
b) $\frac{-(d[O_2]/dt)_0}{-(d[O_2]/dt)_{inh1}} = \frac{-(d[O_2]/dt)_{inh1}}{-(d[O_2]/dt)_0} = \frac{nk_{inh1}[AH]}{\sqrt{2k_t R_i}}$

c) $-\left(\frac{d[O_2]}{dt}\right)_{inh2} = \frac{k_p[RH]R_i}{nk_{inh2}[AH]} + R_i$

455

456 **Figure 2.**

457

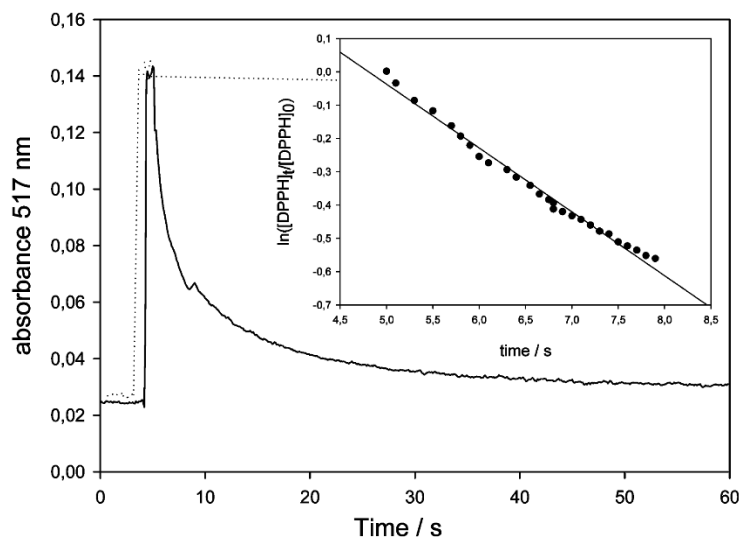


458

459 **Figure 3**

460

461

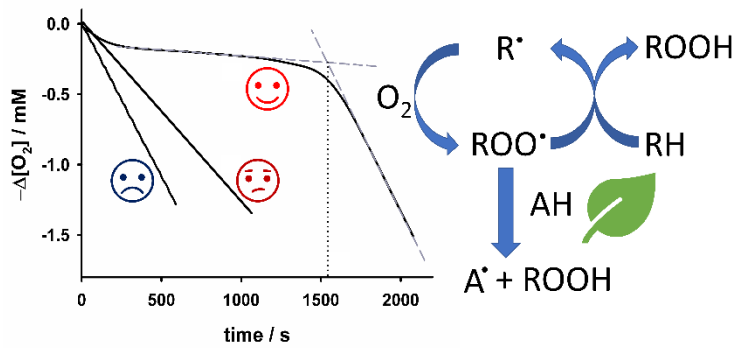


462

463 **Figure 4**

464

465



466

467 **TOC**