The Use and Abuse of the dpph$^{\bullet}$ Radical[1]

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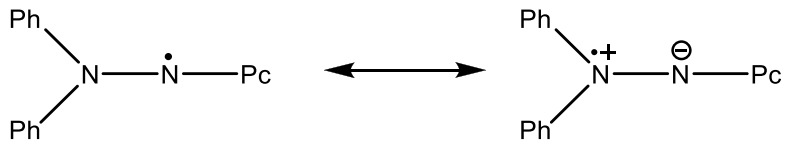
Abstract: The 2,2-diphenyl-1-picrylhydrazyl (**dpph•**) radical is approaching one hundred years from its discovery in 1922 by Goldschmidt and Renn. This radical is colored and remarkably stable, two properties that have made it one of the most popular radical in a wide range of studies. First, the evaluation of the antioxidant abilities of phenols and other natural compounds (**X-H**) through a "test" that – at a closer look – results to be utterly inappropriate to the purpose. In fact, the test-derived EC_{50}, *i.e*. the concentration of **X-H** able to scavenge 50% of the initial **dpph•**, is not a kinetic parameter and hence its purported correlation with the antioxidant properties of chemicals is not justified. Kinetic measurements, like the second-order rate constants for H-atom abstraction from **X-H** by **dpph•**, *in apolar media,* are the only useful parameters to predict the antioxidant ability. Other applications of **dpph•** include kinetic and mechanistic studies, kinetic solvent effects, EPR spectroscopy, polymer chemistry and much more. In this review these applications are evaluated in detail by showing the usefulness of some and the uselessness of others. The chemistry of **dpph•** is also briefly reviewed.

Keywords: **dpph•**, radicals, phenols, antioxidants, mechanisms of reaction, SPLET.

Introduction

The π-radical[1] 2,2-diphenyl-1-picrylhydrazyl (hereafter abbreviated to **dpph•**) is approaching one hundred years from its discovery in 1922 by Goldschmidt and Renn in a more general work on hydrazyl radicals ($R_2NNR^{\bullet}$),[2] radicals that generally are persistent but not stable.[3] The **dpph•** radical is instead remarkably stable and is also intensely coloured, two virtues that have paved the way to an extensive (and often improper) use of this radical in solution. The radical is largely used in polymer chemistry,[4] in EPR spectroscopy[5] and in the evaluation of the antioxidant ability[6,7] of chemicals. This last use was suggested for the very first time by Blois in 1958.[8]

The stability of the **dpph•** radical is primarily due to steric crowding[9,10] around the divalent N-atom (see **Figure 1**) and, to a lesser extent, to the "push-pull" effect[11] exerted by the diphenylamino group (electron-donor) and the picryl group (electron-acceptor) onto the divalent N. This effect stabilizes the canonical structure **B**. EPR measurements show that the spin density on the two hydrazyl N atoms is large and is essentially equal.[9,12] Thus, the two canonical structures **A** and **B** contribute mostly and essentially equally to the spin distribution. Solvent effects on spin distribution are very limited.[12]



A **B**

The UV-vis spectrum of **dpph•** is shown in **Figure 2** (along with those of the reduced form, **dpph-H**, and the anion, **dpph⁻**). The two bands are generated by π–π* transitions with a major contribution of the unpaired electron to the band located in the visible.[13] The λ_{max} of the latter and the extinction coefficient slightly depend on the solvent[14] but on average are ca. 515 nm and ca. 11,700 $M^{-1}cm^{-1}$, respectively. This band is responsible for the deep violet colour of **dpph•** in solution. The reduction of the radical by hydrogen atom transfer from H-donors (antioxidants) with formation of the hydrazine **dpph-H** causes the disappearance of the visible band (see **Fig**. **2**) with a change in the

colour of the solution from violet to pale yellow. This reaction can therefore be easily monitored by UV-vis spectroscopy and often with conventional spectrophotometers. The reaction is known in the literature as "dpph test" (or "dpph assay") and is used for the evaluation of the antioxidant capacity of natural extracts from plants or single compounds (mostly phenols).[6] The potentially good intentions of the users of this test are however blurred by the interpretation of the data pulled out from it. Particularly, the incorrect interpretation of the "Efficient Concentration"[15] (EC_{50}) as a parameter related to the antioxidant properties of chemicals.

The **dpph$^{\bullet}$** radical is poorly soluble in apolar solvents whereas in various polar organic solvents shows a significant solubility. In water, its solubility at ambient temperature is practically nil and this has hampered the study of the effects that this extraordinary solvent might have on the chemistry of the radical. In order to fill this current void of knowledge, nanoparticles of **dpph$^{\bullet}$** soluble in water and β-cyclodextrin incorporating **dpph$^{\bullet}$** were recently prepared.[13b,16] These forms could help in the understanding of the effects of water on the (electron transfer) mechanism of reactions involving biologically relevant water-soluble antioxidants (ascorbic acid, uric acid, bilirubin, glutathione etc.). Indeed, in these last twenty years **dpph$^{\bullet}$** has been very useful in the comprehension of (biologically relevant) solvent effects[17] on the rate of reactions of antioxidants + radicals and in the formulation of new[17b,18] electron transfer (ET) mechanisms involving phenols (*vide infra*).

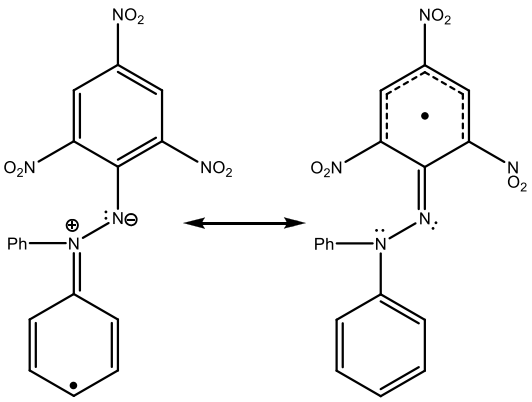
Figure 1 about here

Figure 2 about here

Chemistry of dpph$^{\bullet}$

The **dpph•** radical reacts selectively with radicals and H-atom donors at different reaction sites. While radicals usually attack the phenyl rings (or the picryl ring), see the canonical structures **C** and **D** below, H-atom donors react with the divalent N atom. The limited space around the N atom (see Fig. 1) prevents the addition of cumbersome radicals at this site. H-atom donors can approach the N atom and can release their H-atom therein with formation of the hydrazine **dpph-H**.

Molecular oxygen in its triplet-ground state ,$^3O_2$, despite being a radical, does not react with **dpph•** (or it does very slowly), and solutions of **dpph•** kept *in the dark*[19] are stable for long times. Furthermore, the radical does not dimerize and in solution - as well as in the solid[9] state - is present in the free monomeric form only.



C **D**

Balaban *et al.*[11,20] and Ionita[21] have published several papers concerning the reaction products of **dpph•** with radicals and non-radical oxidant species. Some of these data are gathered in Table 1. Several oxygen radicals afford *para*-substitution products at the phenyl and pycryl rings along with minor quantities of *p*-NO_2-dpph-H and fragmentation products (diphenylamine, Ph_2NH and tetraphenylhydrazine, Ph_2N-NPh_2).[11,20,21] The aryloxyl radicals of syringaldehyde and methyl syringate (two phenolic compounds) react in dichloromethane with excess **dpph•** to yield *p*-substitution products at the picryl ring, **1**, see Fig. 3.[11] Interestingly, the supramolecular complex of the anion of syringaldehyde with crown ethers yields instead a *p*-substitution product at the *phenyl* rings, **2**, see Fig. 3.[11]

Table 1 about here

Figure 3 about here

Reactions of dpph• with H-Atom Donors

Several chemicals react with **dpph•** by H-atom donation. Phenols (ArOH) are probably the most reactive and the most important ones, though non-tertiary aromatic amines[22] and thiols[23] are of interest too. These reactions are often used for probing the antiradical or 'antioxidant' ability of natural compounds (*see below*). The importance of these reactions is clearly witnessed by the numbers. Scopus lists more than 17,000 papers for the decade 2005 – 2015 combining the words "dpph" and "antioxidants". Most of these papers include outcomes of the so-called "dpph test" which consists in the addition of a certain amount of a (potential) antioxidant to a **dpph•** solution and in the evaluation of the % of **dpph•** quenched after an *arbitrary* and often *variable* time[24] (minutes to hours). This test is based on the ability of **dpph•** to abstract H-atoms from X-H bonds, reaction 1. This reaction proceeds relatively quickly only when three constraints are met: *i*) the X-H bond is relatively weak (low bond dissociation enthalpy, BDE); *ii*) the radical X• is stabilized by resonance; and *iii*) X is a heteroatom (O, N, S).

$$\text{X-H} + \mathbf{dpph^{\bullet}} \rightarrow \text{X}^{\bullet} + \mathbf{dpph\text{-}H} \qquad (1)$$

$$\text{X}^{\bullet} + \mathbf{dpph^{\bullet}} \rightarrow \text{closed shell products} \qquad (2)$$

$$2\ \text{X}^{\bullet} \rightarrow \text{closed shell products} \qquad (3)$$

Hydrocarbons (C-H bonds) act as H-donors to **dpph•** very rarely, and when they do the rate constants are small (*see below*). 1,4-Cyclohexadiene, for instance, reacts with **dpph•** in cyclohexane at 298 K with a rate constant of ~ 0.00025 $M^{-1}s^{-1}$ per H-atom.[7,25] On the other hand, phenol whose O-

H is some 13 kcal/mol stronger than the C-H bond of 1,4-cyclohexadiene reacts instead with a bimolecular rate constant of ~ 0.10 $M^{-1}s^{-1}$, *i.e.*, 400 times greater![7]

The reasons as to why **dpph$^{\bullet}$** reacts fast with phenolic O-H bonds and slow with C-H bonds are probably very similar to those formulated for the isoelectronic peroxyl radicals (ROO$^{\bullet}$) by Ingold and Pratt[26] in their recent review on antioxidants. Two interlinked factors have been called for to explain the rate acceleration of H-atom transfers between *heteroatoms*:

1) *the presence of a H-bond between the H-donor and the radical (through one of its lone-pairs) in a pre-reaction complex*; and

2) *a different mechanism for H-abstraction from O-H or N-H vs C-H.*

In the past, it was thought (and still is now for C-H bonds), that the H-*atom* (proton + valence electron) simply moved from X-H toward the SOMO of the radical along the straight line X---H---SOMO.[27] We usually refer to this mechanism with the generic expression of Hydrogen Atom Transfer (HAT) but its domain of definition has now become less clear.[28] The accepted mechanism for the H-atom exchange between two heteroatoms (*e.g.* ArO-H/$^{\bullet}$OOR) is now very different. Formation of a H-bond, in the pre-reaction complex, involving the ArO-H and *a lone-pair* of the radical ROO$^{\bullet}$ (ArO–H···:ȮOR) gives rise to a peculiar exchange of the H-atom, see Fig. 4. In this complex in fact only the nucleus (proton) of the H-atom moves toward the radical leaving behind its bonding electron. The

Figure 4 about here

motion of the proton stimulates a simultaneous electron-transfer from one lone pair of ArÖH toward the SOMO of ROO$^{\bullet}$ yielding the final complex (ArȮ:···H–ÖOR) in one single step. This mechanism

is known as "Proton-Coupled Electron-Transfer" (PCET) because there is a concerted (one single step) delivery of $1e^-$ and $1H^+$ from the donor (phenol) to the acceptor (radical) without formation of ions.[28,29] There are cases however (see below) in which electron and proton are transferred to different acceptors (separated PCET)[30] and for neutral radicals (like **dpph$^\bullet$**) this implies the formation of ion-pairs. H-atom transfer via PCET is faster than HAT because the H-bond reduces the width and height of the reaction barrier.[26] As suggested by calculations, PCET is energetically favoured for the exchange of H between heteroatoms. However, cases of PCET involving H-exchange between C/C[31] and C/N[7] are also known. Alternative mechanisms may include electron-transfer (ET) followed or preceded by proton-transfer (PT) in distinct steps with the formation of high-energy intermediates, see below.

Phenols with electron-rich substituents are excellent H-donors because they respond to all three requisites cited above. They are able to quickly quench ROO$^\bullet$ radicals via PCET (see above).

$$ArOH + ROO^\bullet \rightarrow ArO^\bullet + ROOH \quad (4)$$

$$ArO^\bullet + ROO^\bullet \rightarrow \text{products} \quad (5)$$

The antioxidant abilities of ArOH are quantified by the *rate constant* of reaction 4, k_{ROO}. Typical values of k_{ROO} for excellent phenolic antioxidants, like α-tocopherol, are $\sim 10^6$ $M^{-1}s^{-1}$.[26,33]

Up to now, reactions 4 and 5 have been considered to be of great industrial *and* biological importance since these reactions reduce the rate of oxidation of organic matter exposed to air (including us, of course).[26,33] In the last decade, however, the opinion that the antioxidant abilities can play a marginal role in the health benefits of dietary antioxidants like polyphenols is taking shape.[34] This is because *in vivo* concentration of these compounds is very low and hence other mechanisms are being searched for.[34]

The study of reaction 4 can be difficult because peroxyl radicals require special precursors for their formation; moreover, they generally react rapidly with ArOH and so sophisticated instruments

are required to monitor their reaction.[35] On the contrary, the **dpph**$^{\bullet}$ radical is commercially available, is coloured and is far less reactive than ROO$^{\bullet}$. In apolar solvents, the rate constant of reaction 1 for non-hindered phenols is some three orders of magnitude smaller than k_{ROO}, however the two values are directly related since $k_{\mathrm{ROO}} \approx 4000 \times k_1^{2/3}$.[36] Ordinary spectrophotometers can therefore be used to monitor reaction 1 (for exceptions *see below*) and this has generated an explosion of works aimed at determining the *antioxidant* abilities of phenols in the general belief that the two radicals could simply be swapped. Furthermore, kinetic solvent effects are often erroneously undervalued and reaction 1 is made to occur in protic solvents (methanol and ethanol) because of the limited solubility of phenols. As will be shown in the pertinent section, these omissions and misconceptions make many published results and their interpretations quite improbable. This is because in polar solvents several mechanisms may coexist for ArOH + **dpph**$^{\bullet}$ and the rate constants in these media do not necessarily correlate with the antioxidant ability of ArOH. Moreover, in a great many cases reaction 1 is an equilibrium reaction since the N-H BDE of **dpph-H** is only 78.9 $\pm$ 0.5 kcal/mol.[10] Unreacted **dpph**$^{\bullet}$ remains for instance in the reaction with 2,6-dimethoxyphenol (OH BDE = 82.1 kcal/mol)[7] even with a concentration ratio [ArOH]/[**dpph**$^{\bullet}$] of 52:1.[10] Of course, if the initial [**dpph**$^{\bullet}$] > [ArOH] (typical conditions in the **dpph**$^{\bullet}$ test), then it is likely this time that some 2,6-dimethoxyphenol remains unreacted in solution thereby yielding a wrong EC_{50}. In both cases, slow radical/radical reactions (reactions 2 and 3) shift the equilibrium to the right.[10]

Kinetic Determination of ArO-H BDEs in Apolar Solvents

A useful application of the kinetics of ArOH + **dpph**$^{\bullet}$ was inspired by the observed relationship between the rates of ArOH + **dpph**$^{\bullet}$ in apolar solvents and the phenolic O-H BDEs.[7] This correlation allowed the determination of the ArO-H BDEs of several phenols with great accuracy and simplicity, see Fig. 5.[7]

The experimental measurement of the X-H BDE can generally be done by three broadly applicable techniques which require considerable care: the study of radical kinetics; photoionization

mass spectrometry; and the acidity/electron affinity cycle.[38] In addition, a simple and reliable, though not widely applicable, EPR equilibration technique was quite recently developed by Lucarini and co-workers for the determination of ArO-H BDEs.[39] This technique is based on the EPR determination of the aryloxyl radical's concentration in mixtures of 2,4,6-tri-*tert*-butylphenol (*t*-BuPhOH) + ArOH submitted to continuous photolysis in the presence of di-*tert*-butylperoxide. Equilibration of the aryloxyl radicals following H-atom transfer, *t*-BuPhO$^{\bullet}$ + ArOH ⇆ *t*-BuPhOH + ArO$^{\bullet}$, is usually rapid.[40]

The ΔS^0 for H-atom exchange reactions is usually very small and can be neglected. The equilibrium constant, K_{eq}, therefore, affords directly the value of ArO-H BDE, $\Delta BDE = \Delta H^0 \cong \Delta G^0 = -RTlnK_{eq}$. This technique has however a major disadvantage (and hence the fact that it is not of general applicability) because it cannot be used for phenols that generate short-lived aryloxyls since these species do not survive long enough to rich an equilibrium concentration.

The use of the bimolecular rate constant of ArOH + **dpph**$^{\bullet}$, $k_{\mathbf{dpph}}$, for the determination of the ArO-H BDE has no critical limitations[41] *except* for the solubility of ArOH in apolar solvents. The value of $k_{\mathbf{dpph}}$ is easily obtainable with standard procedures and when there are any kinetic complications it can be obtained from the initial rates. Usually, the observed rate differences are largely due to differences in the activation energy, the Arrhenius pre-exponential factor being essentially constant, range $10^5 - 10^6$ $M^{-1}s^{-1}$.[42] Electronic effects of the substituents and intramolecular H-bonds influence both the ArO-H strengths and the activation energies. The equation that correlates these two last parameters in kcal/mol is:[43]

$$E_{a,1} = 0.918\times(\text{ArO-H BDE}) - 70.27$$

Figure 5 about here

Solvent Effects: the role of the H-Bond

Polar solvents (PS) exert on rates and mechanisms of H-atom abstraction by radicals a strong influence. In the overwhelming majority of cases, they cause a rate reduction[17a] but a few well-documented cases[44] of rate *acceleration* are also known. These kinetic solvent effects (KSEs) were fully rationalized by Ingold and co-workers in their pioneering studies done in these last 20 years.[17] The **dpph•** radical had a pivotal role in these studies[17] in combination with phenols as H-atom donors. Phenols are important antioxidants[33] and the influence that polar environments have on their *in vivo* antioxidant and antiradical properties is of great importance. Phenols protect cellular membranes from peroxidation reducing the concentration of peroxyl radicals present in the membrane; they may also quench radicals in the cytoplasm preventing cell damage. Both reactions are subjected to KSEs.

Phenols form H-bonded complexes with PS, ArOH---PS, in which the H-atom is made it inaccessible by the presence of two bulky heteroatoms around. Because of this steric congestion, free radicals cannot react with these species (at least until a PCET mechanism is involved) but they *do* react with the free fraction of ArOH, see Fig. 6. Since the concentration of free ArOH decreases as the HB-strength increases, the rate of reaction diminishes proportionally to the HB-strength.[17a] These "standard" KSEs are observed in the vast majority of ArOH + **dpph•** reactions.

Nevertheless, a change in the mechanism of reaction from PCET to ET-PT *or* PT-ET can have major effects on the rates because these mechanisms can bypass the steric hindrance in the H-bonded complexes.[32] Solvation of high-energy species such as ArO^- and $ArOH^{\bullet+}$, formed by initial PT or ET, can make the stepwise mechanisms competitive with the e^-/H^+ concerted PCET, see Figure 7. These ET-PT and PT-ET mechanisms usually cause "abnormal" KSEs. In this context, Litwinienko and Ingold[45] and Foti *et al.*[18] discovered independently that in ionizing solvents

Figures 6 and 7 about here

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(methanol, ethanol, water and their mixtures) phenols react with **dpph**$^{\bullet}$ essentially via ET from their anions in a PT-ET process, reactions 5 – 7, that was called Sequential Proton-Loss Electron-Transfer (SPLET) (see the green reaction path in Figure 7). This mechanism was proposed because: *i*) the KSEs in these solvents were "abnormal"[45] in that the rate constants for reaction 1 (X-H = ArOH) were found to be higher than predicted on the basis of the HB-accepting properties of alcohols;[18,17a] *ii*) the rate constants decreased or increased following the addition of acids or bases, respectively; and *iii*) the methyl esters of cinnamic acids, see Figure 8, were 3 – 5 times more reactive than their non-esterified counterparts.[18] The reduced reactivity of cinnamic acids relative to their esters shows that the species responsible for the **dpph**$^{\bullet}$ scavenging are the phenolic anions, reaction 6. Ionization of the COOH group reduces in fact the amount of phenolic anions present in solution.

$$ArOH \leftrightarrows ArO^- + H^+ \quad (5)$$

$$ArO^- + \mathbf{dpph}^{\bullet} \rightarrow ArO^{\bullet} + \mathbf{dpph}^- \quad (6)$$

$$\mathbf{dpph}^- + H^+ \rightarrow \mathbf{dpph}\text{-}H \quad (7)$$

All these kinetic observations led to the formulation of SPLET as a predominant mechanism for ArOH + **dpph**$^{\bullet}$ reactions in protic solvents. Given the usually high oxidation potentials of phenol anions and the high reduction potentials of several biologically relevant radicals,[46] it is likely that in numerous *in vivo* ArOH + radical reactions the formal H-atom transfer occurs via stepwise ET mechanisms (*e.g.* SPLET). The E^0_{red} of **dpph**$^{\bullet}$ is +0.23 V/SCE (+0.47 V/NHE) in acetonitrile[47] and perhaps larger in methanol[48]. Therefore, radicals such as $NO_2^{\bullet}$, $CO_3^{\bullet-}$, $HOO^{\bullet}$ etc. characterized[49] by E^0_{red} > 0.47 V can very likely react with phenols via ET from the anions or the neutral species. It is well-known that strongly oxidizing peroxyl radicals (*e.g.* halogenated peroxyls, peroxyacetyl radical

299 $CH_3(CO)OO^{\bullet}$ etc.) react via ET with several substrates including phenolate, ascorbate and inorganic
300 anions producing the corresponding radicals.[50]
301
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303 **Figure 8 about here**
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305 **Remote H-Bond Effects and Separated PCET**
306 Phenols with pendant piperidine **1 - 3** represent an interesting case of ArOH + **dpph$^{\bullet}$**
307 reactions.[44a] In **1** and **2**, the presence of an intramolecular H-bond influences a lot the process of H-
308 abstraction from both the O-H involved in the HB (compound **1**) and the HB-free *para*-OH
309 (compound **2**). The rate constants measured in *cyclohexane* at 298 K are very revealing of this
310 influence once they are compared with the rate constant of phenol **3**. The values of the three rate
311 constants are in fact very different: 2.5, 7800 and 400 $M^{-1}s^{-1}$, respectively (Table 2). The mechanism
312 involved in these reactions in cyclohexane is most likely PCET/HAT.
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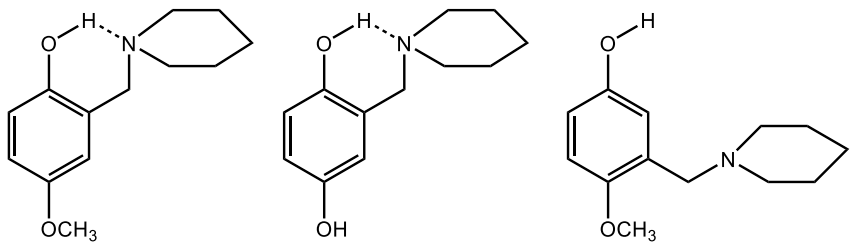

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318 **Table 2 about here**
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The low reactivity of **1** relative to **3** by a factor of about 160 shows the "protective role" that a H-bond may have on phenolic H-atoms. Generally, free radicals react slowly with intramolecularly H-bonded phenols since the HB increases the energy required to pull the H-atom out. In a great many cases, natural polyphenols are intramolecularly H-bonded to methoxy groups which probably serve as a sort of "padlock" to protect phenols from radicals and facilitate their arrival at the final reaction site.

On the other hand, the rate constants show that phenol **2** is much more reactive than both **1** and **3**, Table 2. The higher reactivity relative to **1** suggests immediately that the reaction site in phenol **2** is the H-bond free O-H. Interestingly, phenol **2** is also some 20 times more reactive than **3** for the effects of the "remote H-bond".[51] H-bond effects have been observed in many other dihydroxybenzenes with catechol-, hydroquinone- and resorcinol-like structure and also in 1,8-naphthalenediols.[37,51] The orientation of the remote H-bond determines the sign of the variation: para→meta H-bonds cause an *increase* in the rate of H-abstraction (as in the case of phenol **2**) while meta→para orientations determine a *decrease* in the rate relative to molecules lacking H-bonds.[51b]

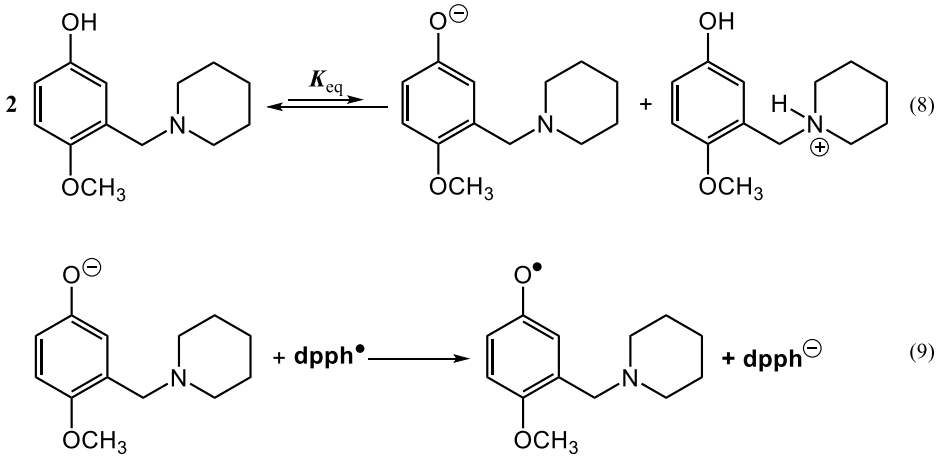
The explanation given for these effects is that the -O$^{\bullet}$ in the aryloxyl radical has strong electron-withdrawing character, see Figure 9. This causes an O-H group in the para position of ArO$^{\bullet}$ to become more acidic than in the parent molecules and hence capable of forming stronger H-bonds with HB-acceptors in the meta position. For the same reasons, HB-acceptors (such as OCH_3) in the para position become more electron-deficient and form therefore weaker HBs in the aryloxyl radicals than in the parent molecules, see Figure 9. These thermochemistries influence the transition states and thus the kinetics of H-abstraction from phenols by free radicals.

Figure 9 about here

On changing the solvent from cyclohexane to acetonitrile, the new rate constants showed clearly that the mechanism of **dpph**$^{\bullet}$ quenching had changed, see Table 2. The rate constant of **1**

increased in acetonitrile by a factor of 480 (k_1 = 1200 $M^{-1}s^{-1}$) while those of **2** and **3** decreased a bit but the decrease was confined within a factor of 1.4 – 3.3 (the expected reduction for a PCET/HAT mechanism in acetonitrile would be by a factor of about 70!).[17a]

An apparently reasonable mechanism for **3** + **dpph**• in acetonitrile, consistent with the above kinetic data, was suggested by the thermodynamically plausible self-ionization of **3**, reaction 8. The equilibrium constant for this autoionization was estimated[44a] to be ~ 10^{-8}, a value much greater than the typical K_a ~10^{-25} – 10^{-20} of phenols in acetonitrile.[32] Thereby, comparatively large quantities of phenol anions[52] were formed in solution by reaction 8 and these most likely reacted via ET to **dpph**•, reaction 9. The overall process represents a special case of SPLET in a non-protic solvent (acetonitrile) stimulated by the presence of the nitrogen base.



On the other hand, it appears highly probable that phenols **1** and **2** reacted both through a *separated*[30] PCET pathway, with e^- and H^+ being transferred to **dpph**• and piperidine nitrogen, respectively, reactions 10 and 11, see also Figure 10. Nevertheless, the kinetic behavior of the two phenols was very different. While **2** reacted with a pseudo first-order process, the kinetics of **1** + **dpph**• showed a biphasic behavior with an initial fast process (first 300 ms) followed by a second slower **dpph**• loss for some tens of seconds. Kinetic evaluations led to attribute the first step to the

equilibrium formation of a metastable distonic radical cation in contact with **dpph**$^-$ whereas the second slow step to the dissolution of the ion-pair, see reaction 10.

The different kinetic behavior of the two phenols was interpreted as due to the fact that the distonic radical cation of **2**, unlike the other case, was able to *rapidly* "shoot" an H^+ from the free OH into the bulk solution since radical cations of phenols are very acidic.[53] This fast process impeded the formation of the equilibrium that was instead attained with **1** and thus the initial step became rate-determining, reaction 11.

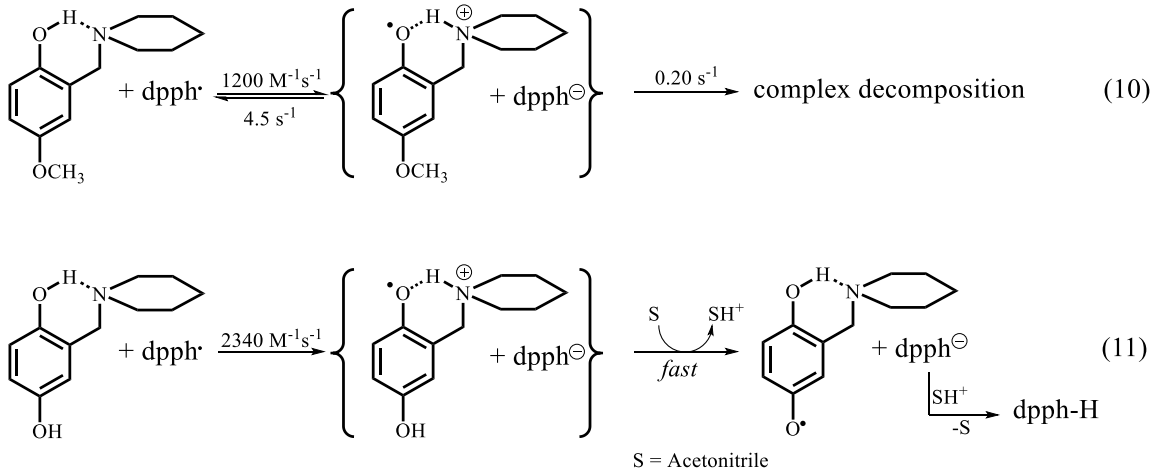


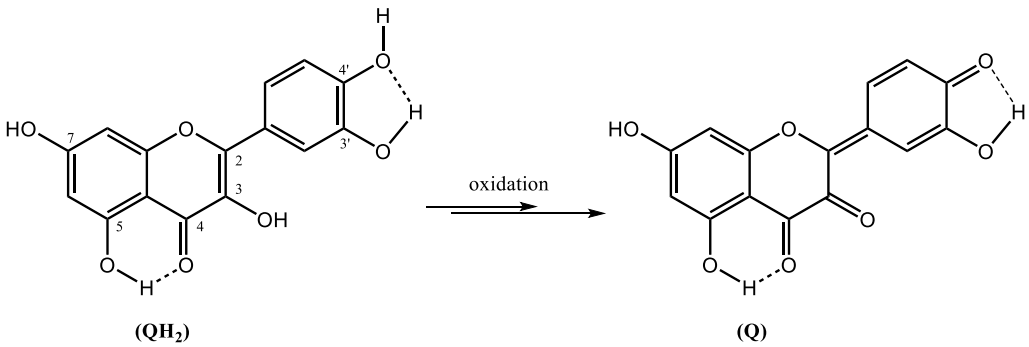
Figure 10 about here

The Remarkable Case of Quercetin + dpph• Reaction

Quercetin (**QH_2**) is a flavonoid largely distributed in the plant kingdom.[54] A balanced daily diet supplies us with a few tens of milligrams of flavonoids chiefly constituted by quercetin.[55] Generally, flavonoids are believed to provide certain health benefits that are usually attributed to their ability to trap free radicals. This ability is actually common to all phenols and not necessarily reflects their "antioxidant properties", see above. As most phenols, **QH_2** is in fact able to quench both **dpph•** and ROO• radicals. This might seem rather trivial in view of the nature of the molecule. The quenching process of **dpph•** in methanol/water deserves however our attention because it shows how

insidious the analysis of the **dpph•** kinetics can be, and how dissimilar these kinetics are from those of the ROO• radical.[56]

The reported values in the literature of the rate constant for **QH_2** + **dpph•** in methanol at room temperature surprisingly span a large range from a low of 264 to a high of 6433 $M^{-1}s^{-1}$.[57] These values were actually measured with conventional spectrophotometers at 519 nm (λ_{max} **dpph•**) in a time frame *long after* the "true" reaction of **dpph•** with **QH_2** had occurred. This was deduced by using stopped-flow spectrophotometers which allowed us to observe that the **QH_2** + **dpph•** reaction in methanol/water 80:20 v/v was over after only 100 – 300 milliseconds![56] So, what was actually being measured when the *apparent* loss of **dpph•** was monitored with conventional spectrophotometers at $t \gg 300$ ms?



(QH_2) **(Q)** (12)

To answer this question it is necessary to re-examine the chemistry of quercetin oxidation.[58] The 2-electron oxidation of **QH_2** yields quinone/quinomethide products, reaction 12, which are intensely coloured.[58] Several tautomers can exist in solution but according to quantum chemical calculations,[58] the tautomer Q shown in reaction 12 is the most stable and the most abundant in solution. The striking aspect of these compounds is that their UV-vis spectrum and colour are fortuitously very similar to those of **dpph•**.[56] This has created a grave problem of interpretation of the 519 nm absorbance decay at long times, since this decay has been interpreted as a relatively slow loss of **dpph•** caused by **QH_2**. Actually, the **QH_2** + **dpph•** reaction is so fast that **dpph•** is consumed long before any measurements in conventional spectrophotometers can be made.[56] This is because

the 2*e* oxidation of **QH2** by **dpph•** proceeds in methanol/water via SPLET in a sequence of reactions shown in Figure 11. The rate law of this process is first-order in **dpph•** but only *ca*. 0.38 order in **QH2** which suggests that the charge-shift occurs in a complex, maybe formed by π-stacking of the reactants, {**QH**$^-$/**dpph•**} → {QH•/**dpph**$^-$}.[56] The semiquinone radical QH• generated is likely to rapidly ionize (the Brønsted base being either the **dpph** anion in the complex or the solvent) since this radical is expected to be more acidic than **QH2** for the electron-withdrawing character of the -O• group (see above).[53] The radical anion **Q•**$^-$ so derived gives rise to a second ET process to **dpph•** with formation of coloured quinones/quinomethides, **Q** (see reaction 12 and Figure 11).

Figure 11 about here

The kinetics at $t > 300$ ms, monitored with ordinary spectrophotometers, are due to the decay of the quinomethide Q by solvent (MeOH, H_2O) and **QH2** addition. Such addition products have been identified by several workers.[56,57a,59] The quinonemethide, Q, can in fact be regarded as a highly stabilized benzylic carbocation,[60] see Figure 12, which can readily undergo a proton-assisted (Michael-type) nucleophilic addition.

Figure 12 about here

"The dpph• Test"

The **dpph•** test for appraising the antioxidant activity of chemicals (XH) was first suggested by Blois in 1958.[8] Then, the test was developed by Brand-Williams et al.[15] in 1995 in the form adopted by the vast majority of the researchers. Briefly, different XH concentrations are used in such a way to determine the concentration of XH that quenches 50% of the initial **dpph•** radical in a specific,

434 though arbitrary, time interval. This concentration is called (efficient concentration) EC_{50}, sometimes
435 (inhibitory concentration) IC_{50}. It seems obvious that the lower the EC_{50} the higher the antiradical
436 ability of XH. However, there are many issues associated with the EC_{50} parameter and its
437 interpretation.

438 First of all, the time interval. EC_{50} is time-dependent and the effect of time can vary from
439 compound to compound.[61] Usually, increasing the reaction time the evaluation of a given compound
440 may improve (lower EC_{50}) and hence the test can be tailored so that a poor antiradical/antioxidant
441 appears to be an excellent antioxidant! It is clear therefore that the interpretation of this test is quite
442 arbitrary and any comparison between results from different labs is not possible if the adopted
443 reaction time is different. In general, short reaction times are better than long times in the questionable
444 evaluation of the antioxidant abilities of chemicals from the **dpph**$^{\bullet}$ test.[61]

445 Secondly, EC_{50} is *not* a kinetic parameter (it is just a concentration) and as such it cannot
446 express the antioxidant or antiradical ability of a compound, because this ability must be represented
447 by a *kinetic* parameter. In order to establish whether XH is or is *not* a good antioxidant, it is necessary
448 in fact to compare the *rate* at which XH quenches peroxyl radicals to the *rate* at which peroxyl
449 radicals attack the substrate. The value of EC_{50} is instead merely related to the stoichiometry, *n*, of
450 reaction 13. Given that, $n = 0.5[\mathrm{dpph}]_0/\mathrm{EC}_{50}$ then $\mathrm{EC}_{50} = [\mathrm{dpph}]_0/2n$.[62] From this last equation it
451 is possible to see that chemicals with large stoichiometric factors, *n*, will be classified as powerful
452 antioxidants despite the fact that the **dpph**$^{\bullet}$ quenching may have occurred in one century! Another
453 alarming aspect is that the stoichiometric factor *n* may depend on the concentration of XH used in the
454 experiments,[6] see Fig. 13. This dependence can be explained via the radical/radical reactions 2 and
455 3, which take place after the formation of the radical X$^{\bullet}$, reaction 1.
456
457 $$\mathrm{XH} + n\,\mathbf{dpph}^{\bullet} \rightarrow \text{products} \qquad (13)$$
458

459 It is clear that if reaction 2 is faster than reaction 3 then $n \rightarrow 2$ per single H-atom donated by XH;
460 whereas in the opposite case $n \rightarrow 1$. Anyhow, as the concentration of XH is increased, reaction 3
461 tends to predominate over reaction 2 and $n \rightarrow 1$, see Fig. 13. Finally, the products formed in reactions
462 2 and 3 may react with **dpph**$^{\bullet}$ increasing the value of *n*.
463
464 **Figure 13 about here**
465
466 Thirdly, the quenching mechanisms of **dpph**$^{\bullet}$ and ROO$^{\bullet}$ may differ. In this case, the evaluation
467 of the antioxidant ability from the **dpph**$^{\bullet}$ test can be deceptive. Usually, the **dpph**$^{\bullet}$ test is done in
468 methanol or ethanol for the ease of solubilisation of many phytochemicals including phenols. In
469 alcoholic solutions, phenols, however, ionize and thus they react with **dpph**$^{\bullet}$ via SPLET (see above),
470 that is, via ET from their anions. In many cases, acidic phenols react rapidly with **dpph**$^{\bullet}$ but *slowly*
471 with ROO$^{\bullet}$ radicals because their O-H bonds are strong and the reaction mechanism with the latter is
472 PCET. The antioxidant ability of phenols in fact correlates in apolar media with the gas-phase ArO-
473 H BDE, see above. One good example displaying this discrepancy is the following. The 4-cyano-2,6-
474 di-*tert*-butylphenol shows in methanol a higher reactivity toward **dpph**$^{\bullet}$ than the 4-methoxy- and 4-
475 methyl-2,6-di-*tert*-butylphenol. The values of the rate constants are: 16, 3.9 and 3.7 $M^{-1}s^{-1}$,
476 respectively.[61,63] On the other hand, the antioxidant abilities follow the order 4-methoxy- > 4-methyl-
477 >> 4-cyano-2,6-di-*tert*-butylphenol.[61]
478 The "three points" exposed above show pretty well that the much-vaunted **dpph**$^{\bullet}$ test is in fact
479 unsuitable to provide the information for which it was created. The antioxidant properties of
480 phytochemicals must be expressed by a kinetic parameter which can be obtained studying the **dpph**$^{\bullet}$
481 decay *vs*. time in appropriate solvents, where the complications of ionic mechanisms do not emerge,
482 and with appropriate instruments (conventional vs. stopped-flow spectrophotometers). The second-
483 order rate constant k_{dpph} of reaction 1 is one of the best surrogate parameters useful to prudently

evaluate the antioxidant properties of chemicals. The **dpph**$^{\bullet}$ test can be used to titrate the content in H-atom donors present in the sample (particularly in natural mixtures).

In conclusion the **dpph**$^{\bullet}$ radical has contributed a lot to the enlargement of scientific knowledge. The long "scientific life" of this radical has allowed the study and understanding of many chemical phenomena like kinetic solvent effects, remote H-bond effects, reaction mechanisms involving free radicals, antioxidant properties of phenols, strength of the ArO-H bond to name but a few. The extreme importance of the antioxidant chemistry, as a major theme of free radical chemistry,[26] can justify the enormous usage of this *artificial* radical in an attempt to discover the "perfect antioxidant".

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42. The *A* value of strongly hindered phenols, like 2,4,6-tri-*tert*-butylphenol, is much lower, ~10^3 $M^{-1}s^{-1}$. These phenols were not included in the group of phenols used to construct the E_a/ArO-H BDE relationship.

43. It is also possible to convert this equation in such a way that the rate constant $k_{\mathbf{dpph}}/M^{-1}\times s^{-1}$ appears in place of $E_{a,1}$. For moderately hindered and non-hindered phenols the equations are: OH BDE $= 84.6 - 0.645 \times \ln k_{dpph}$ and O-H BDE $= 85.65 - 0.645 \times \ln k_{dpph}$, respectively. The energies are in kcal/mol.

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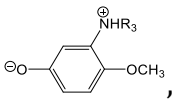
52. Of course, significant quantities of the zwitterionic form,  , could also be present in solution.

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63. At short reaction times, it is likely (see ref. 61) that the EC50 values of these three phenols will follow the same order of reactivity.

Figure Captions

Figure 1. Perspective image of the calculated structure of **dpph•** from ref. 7. Red, blue and gray colours highlight the oxygen, nitrogen and carbon atoms, respectively. The view is approximately perpendicular to the plane defined by the hydrazyl N atoms and the C1 of the picryl ring.

Figure 2. UV-vis spectra of **dpph$^{\bullet}$** (blue, 4.7×10^{-5} M), **dpph-H** (maroon, 4.2×10^{-5} M) and of the anion **dpph^{-}** (green, 7×10^{-5} M) in methanol at ambient temperature.

Figure 3. Structures of the compounds formed in the reaction of **dpph$^{\bullet}$** with the aryloxyl radicals of syringaldehyde and methyl syringate (compounds **1**) and with the anion of syringaldehyde (compound **2**) from ref. 11.

Figure 4. PCET mechanism in the reaction of phenol with an ROO$^{\bullet}$ radical. The nucleus H^{+} moves along the line O--H--O leaving behind its valence electron. An electron is simultaneously transferred from the O lone pair to the ROO SOMO, see ref. 26.

Figure 5. Plot of the activation energy for reaction 1 (X-H = ArOH) *vs* ArO-H BDE from ref. 7. Blue circles are for 16 phenols in the group **1-26**. Phenols **15** and **23** were considered outliers and were excluded from the solid correlation line: $E_{a,1}$ = 0.918(O-H BDE) - 70.27 (r^2 = 0.95). The red square is for 2,4,6-tri-*tert*-butylphenol, **27**.

Figure 6. In polar solvents, free radicals can abstract an H-atom from the free fraction of ArOH. H-bonded ArOHs to PSs are unable to react for steric reasons.

738 **Figure 7**. Reaction of phenols with **dpph$^{\bullet}$** can occur via PCET (or HAT),[31] ET-PT and PT-ET
739 mechanisms. The predominance of one mechanism over the others depends on various factors
740 including the medium polarity. PCET does not form any intermediate (except for the pre-reaction
741 complex) and usually prevails in both apolar and polar solvents (see text). However, for phenols with
742 pendant bases or in a few polar solvents, *e.g.* pyridine, separated PCET (see the corresponding
743 paragraph) and ET-PT can be predominant, see refs. 29, 32 and 41a, despite the formation of a high-
744 energy intermediate, $ArOH^{\bullet+}$. On the other hand, ionizing solvents support the stepwise PT-ET
745 mechanism where the anion ArO^{-} is the high-energy intermediate. Solvation of these polar
746 intermediates can facilitate the intervention of these stepwise mechanisms.
747
748 **Figure 8**. Cinnamic acids react with **dpph$^{\bullet}$** (symbolized as X$^{\bullet}$) in methanol and ethanol via SPLET
749 and their rate constant k_1 decreases as the concentration of cinnamic acid increases. This happens
750 because as the concentration of cinnamic acid rises, ionization of the COOH group limits more and
751 more the formation of phenolic anions (the reactive species), see ref. 18. The rate constants k_1 of the
752 methyl esters are instead large and essentially independent of their concentration.
753
754 **Figure 9**. Canonical structures for aryloxyl radicals with remote H-bonds. In both radicals, the
755 presence of the electron-withdrawing -O$^{\bullet}$ group determines a decrease in the electron density of the
756 O atom in the para position. This causes opposite effects in the two radicals. In the aryloxyl with
757 para→meta oriented H-bond (upper case), the increased acidity of the H-bonded O-H determines a
758 reinforcement of the H-bond. This stabilization is reflected in the transition state of the reaction and
759 hence causes an increase in the reactivity of the parent phenol. Conversely, the strength of the H-
760 bond decreases in the aryloxyl with meta→para orientation and this causes a reduction in the
761 reactivity of the parent phenol since the transition state is destabilized.
762

763 **Figure 10**. The peripheral pathways to the distonic radical cation showed with red arrows include
764 high-energy species and hence may be kinetically disfavored. The diagonal path (PCET) is instead
765 favored because e^- and H^+ are transferred simultaneously to their respective acceptors, *i.e.* **dpph$^\bullet$** and
766 piperidine N, without formation of intermediates.
767
768 **Figure 11**. Time evolution of the reaction **QH$_2$** + **dpph$^\bullet$** in methanol/water 80:20 v/v at room
769 temperature. The pathway in blue shows the conversion of quercetin into quinomethide Q in a time-
770 scale of about 100-300 ms. The pathway in red represents instead the slow (time-scale of seconds)
771 decay of **Q** by solvent and **QH$_2$** addition, see text. The electron donor is the quercetin anion (from 7-
772 OH) since the addition of acetic acid blocks the reaction. On the other hand, the rate law suggests that
773 **QH$^-$** and **dpph$^\bullet$** are associated in a complex before reaction. π-Stacked complexes {**QH$^-$**/**dpph$^\bullet$**} are
774 likely to be present in solution and can convert into the ET-products, QH$^\bullet$ and **dpph$^-$**. Ionization of
775 the semiquinone radical QH$^\bullet$, see text, yields the radical anion Q$^{\bullet-}$ which can give rise to a second ET
776 to **dpph$^\bullet$** with formation of quinones/quinomethides intensely coloured, Q. These compounds can add
777 solvent and **QH$_2$** molecules forming *colourless* compounds.
778
779 **Figure 12**. The 2-position in Q is electrophilic because of the effects of the quinone moiety. Addition
780 of solvent can therefore easily happen with formation of *colourless* compounds.
781
782 **Figure 13**. Stoichiometric coefficients for reaction 13 at various concentrations of the H-atom
783 donors, from ref. 6.
784
785
786
787
788

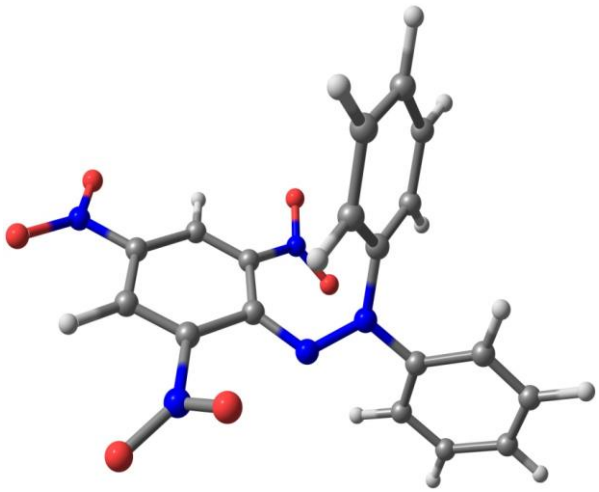
Table 1. Products isolated from the reaction of several radicals and oxidant species with **dpph$^{\bullet}$** (in %) from Ref. 21.

Reactant	**dpph-H**	**NO_2-dpph-H**	**HO-dpph-H**	**Notes**
Hydroxyl Radical	10	5	traces	
TEMPO	---	---	---	no reaction
Galvinoxyl	---	---	---	no reaction
Sodium peroxide	55	33	0	
Potassium superoxide	60	25	Traces	
Potassium hydroxide	25	12	Traces	
Sodium peroxynitrite	30	55	0	
Hydrogen peroxide	100	0	0	
t-Butylperoxide	0	9	0	85% **dpph$^{\bullet}$** recovered
t-Butylhydroperoxide	30	40	traces	

Table 2. Rate constants for the reactions of **1 – 3** with **dpph$^{\bullet}$** at 25 °C from ref. 44a

Phenols	**k_{dpph} / $M^{-1}s^{-1}$**	
	Cyclohexane	**Acetonitrile**
1	2.5 ± 0.5	1200 ± 60
2	7800 ± 30	2340 ± 25
3	400 ± 30	280 ± 60

Figures



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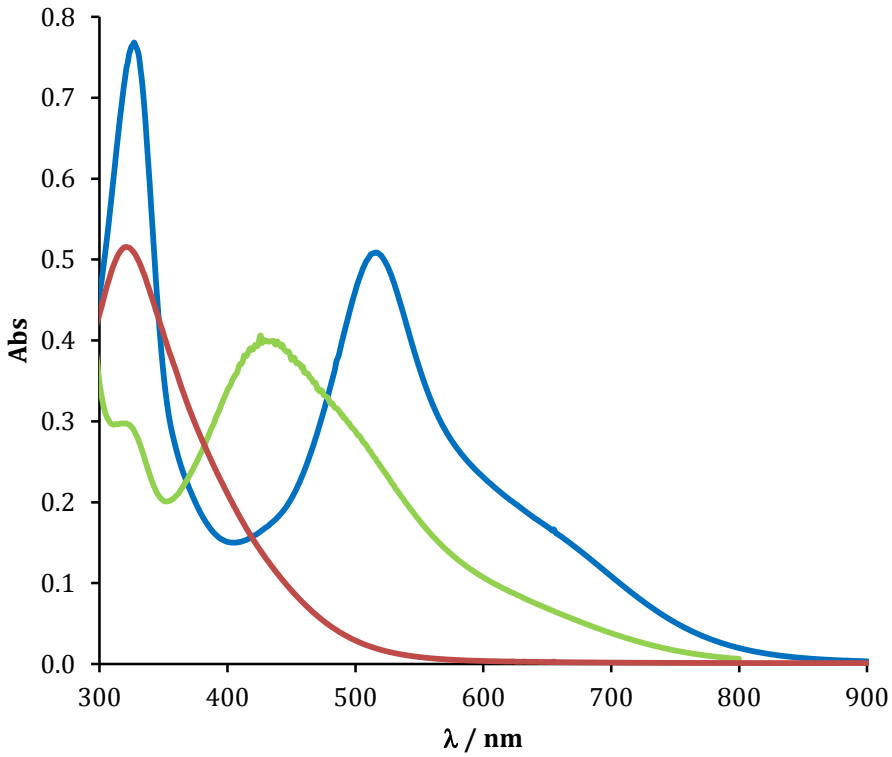

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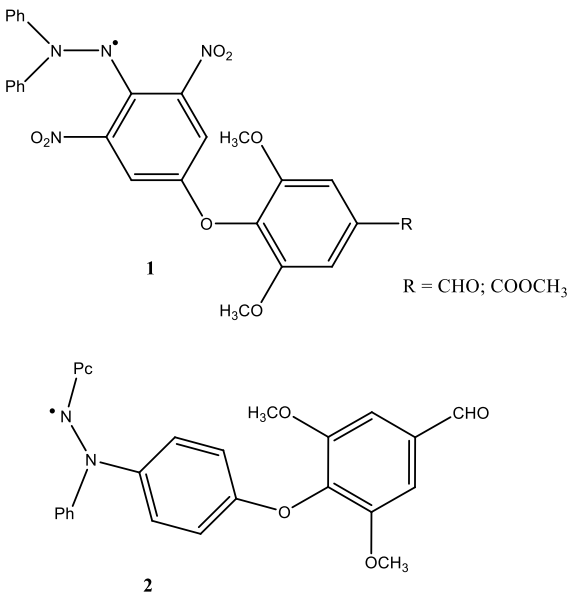

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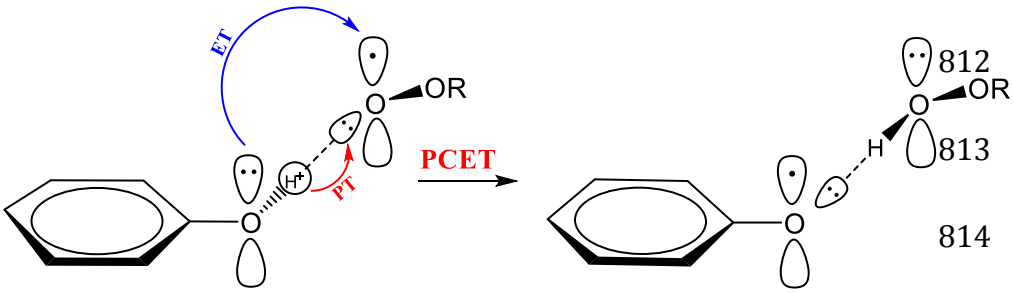

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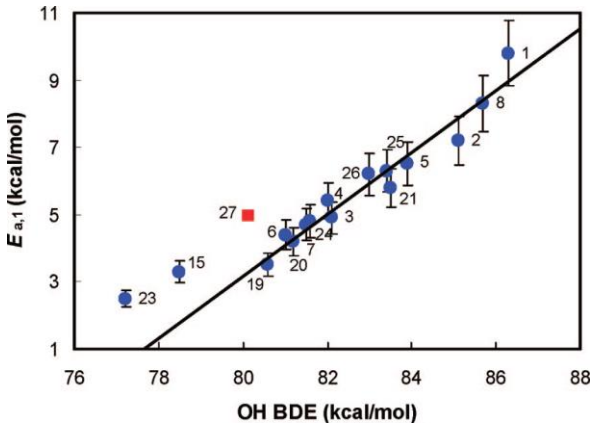

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825 **Figure 5**

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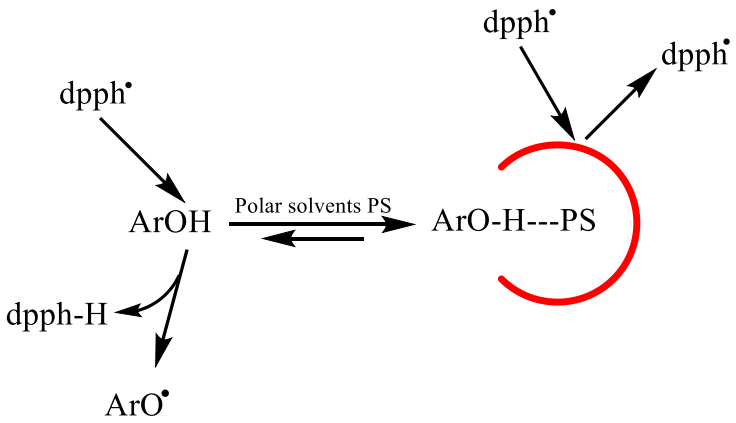

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829 **Figure 6**

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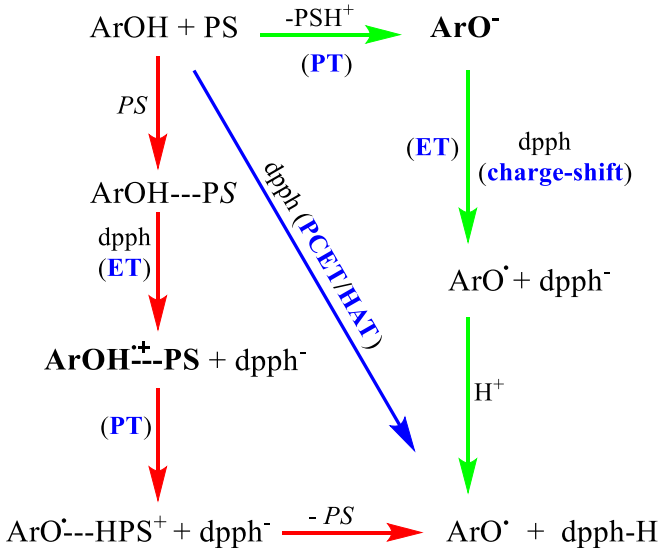

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833 **Figure 7**

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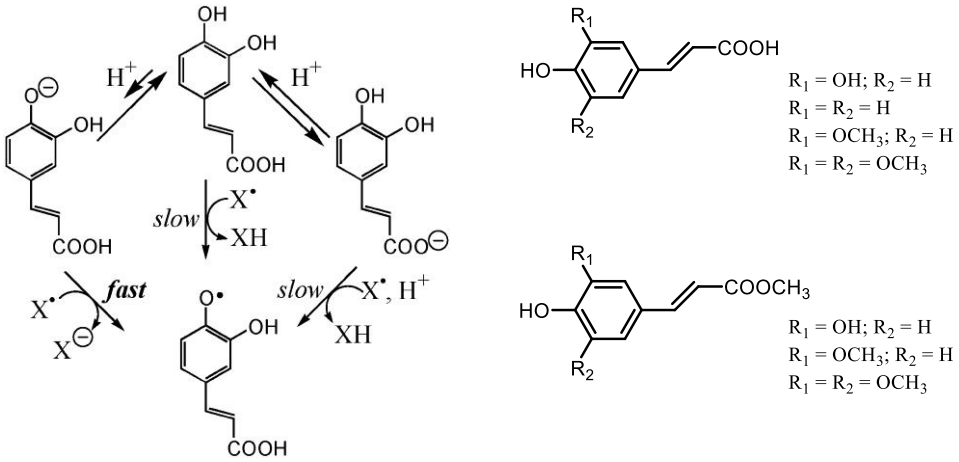

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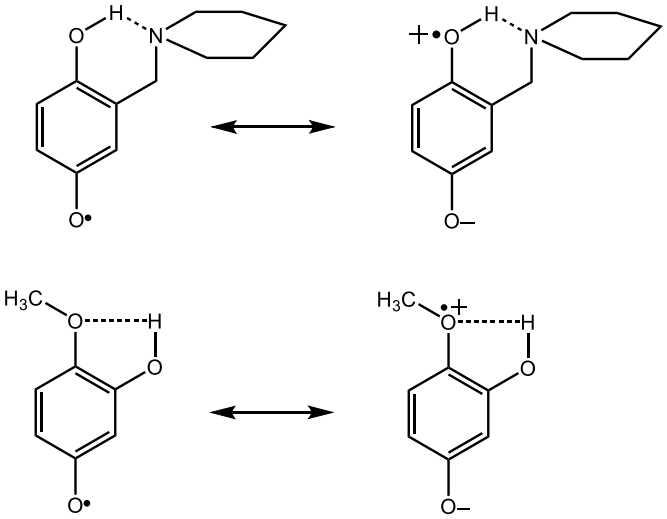

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839 **Figure 9**

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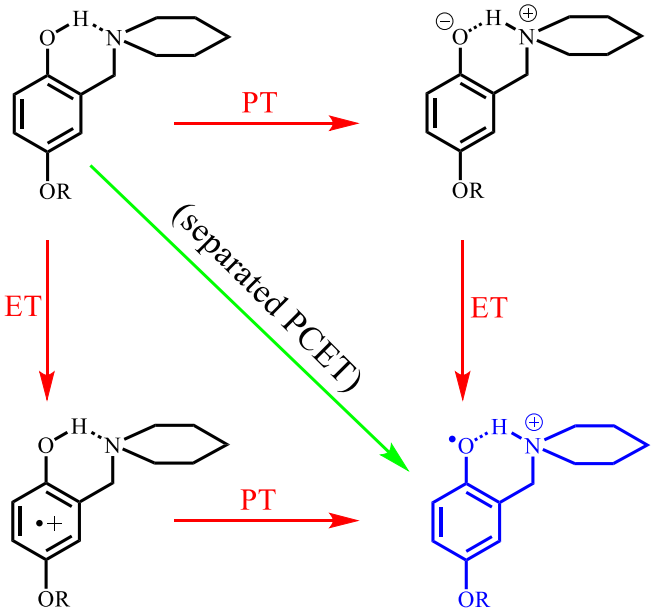

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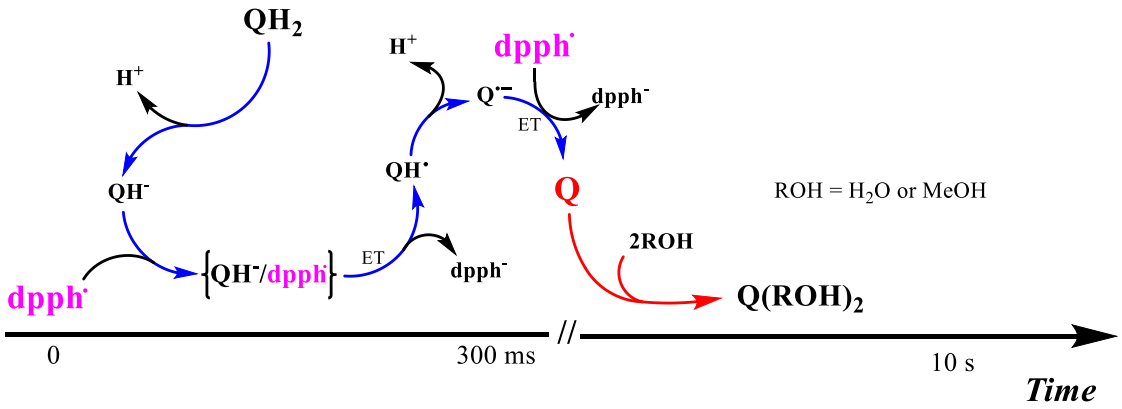

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846 **Figure 11**

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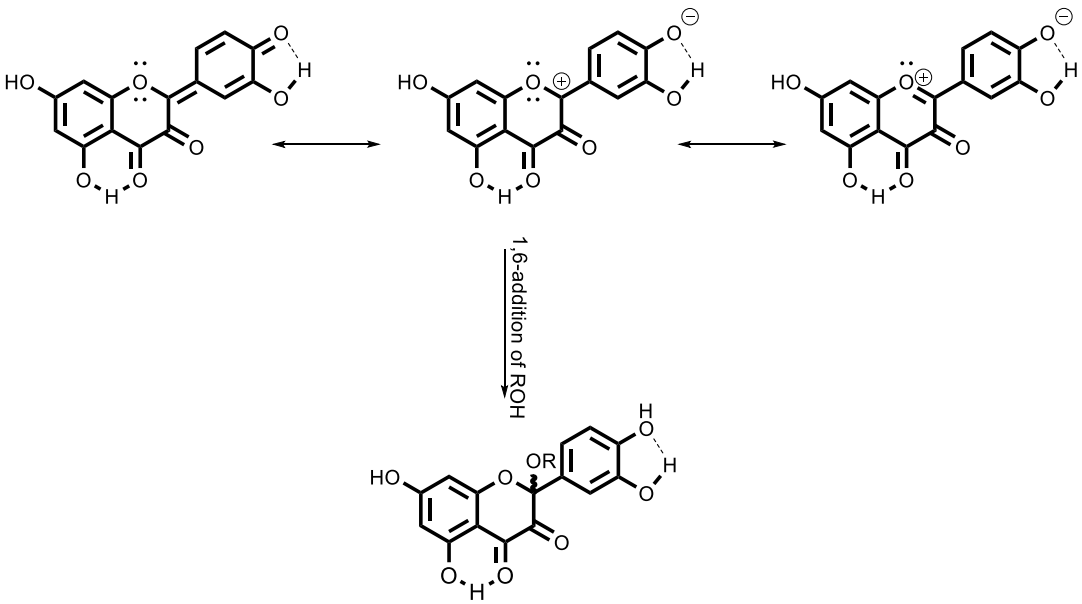

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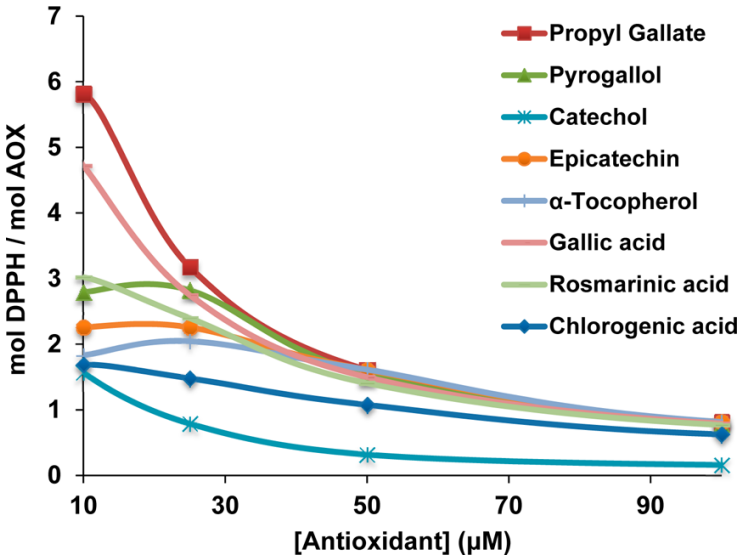
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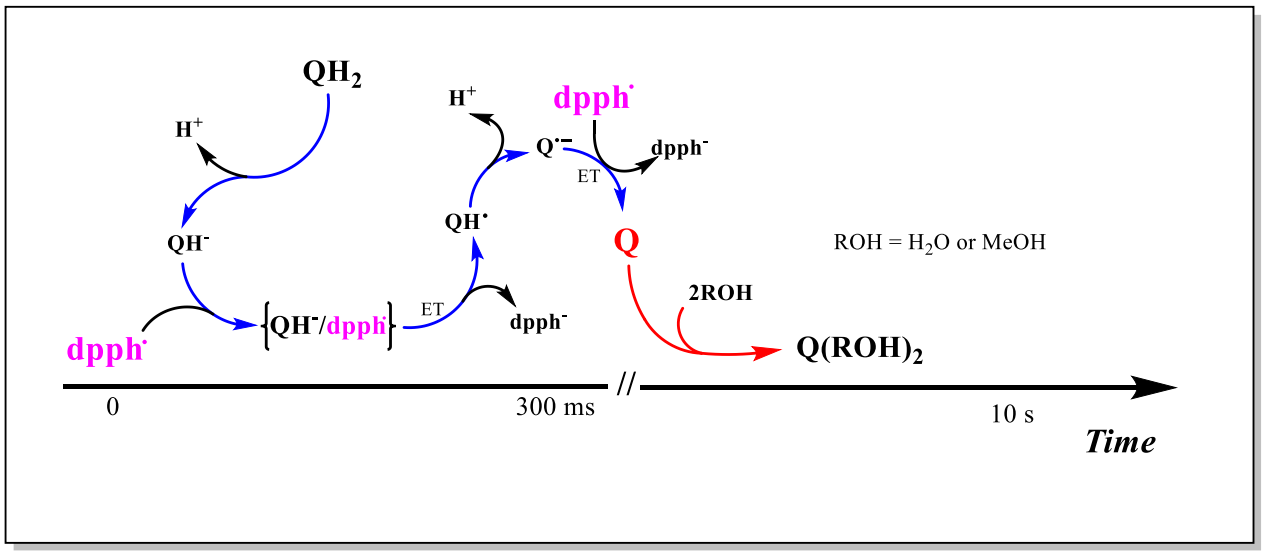
[Antioxidant] (μM)
Propyl Gallate
Pyrogallol
Catechol
Epicatechin
α-Tocopherol
Gallic acid
Rosmarinic acid
Chlorogenic acid
0
1
2
3
4
5
6
7
10
30
50
70
90

Figure 13

868

869 **Graphic for Table of Contents**

870



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