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Review

Determination of antiradical and antioxidant activity: basic principles and new insights*

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Although the term "antioxidant" is used very frequently, there are problems with the definition of antioxidants and estimation of antioxidant activity. The distinction between antioxidant and antiradical activities is not always obvious. This minireview discusses critically the principles, advantages and limitations of the most frequently used methods of estimation of antiradical and antioxidant activities.

Keywords: antioxidant, antiradical, DPPH, ABTS, hydroxyl radical

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CURRENT STATE OF THE ART

"It is difficult these days to open a popular science magazine or medical journal without seeing an article about the role of free radicals in human diseases" (Gutteridge & Halliwell, 1994). This sentence written in 1994 by the leading scientists in the field of free radicals and antioxidants, John Gutteridge and Barry Halliwell is true today as well. Another statement of those authors, that "antioxidant is a term widely used but rarely defined" (Halliwell & Gutteridge, 1999), has also remained true. A Google search for "antioxidants definition" brings more than 600 000 entries! Halliwell and Gutteridge propose to define an antioxidant as "any substance that, when present at low concentration compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell & Gutteridge, 1999). This definition covers all oxidation processes, both radical and non-radical ones. But, as noted elsewhere, "a generic definition of an antioxidant is not experimentally constructive unless it is associated with the notion of the oxidant that has to be neutralized" (Azzi *et al.*, 2004). Moreover, the validity of the term "antioxidant" depends on the environment of its action, viz. whether we consider an *in vitro* or *in vivo* action. In this context a precise definition of conditions and processes in which antioxidant action is studied becomes crucial. Outside this context, a statement that some compound is an antioxidant may not bring any biologically meaningful information.

The literature of the last decade concerning free radical reactions *in vivo* shows that our understanding of these processes in the organism, both under normal conditions and in pathological situations, has changed considerably. Free radicals and reactive oxygen species in general are no longer seen only as destructive factors but also (and perhaps first of all) as messengers involved in intracellular and intercellular signalling (Bartosz, 2005; 2009; Halliwell, 2006). The revision of the ideas on the role of free radical reactions in the functioning of cells and organisms has led to a new concept of redox equilibrium. According to this hypothesis, oxidative stress is a modulation of thiol redox reactions, involved mainly in signalling pathways. Therefore, non-radical oxidants (enzymatically generated hydrogen peroxide, other peroxides, quinones, etc.) play a basic role in the oxidation of thiols for the sake of signalling, without the necessity of formation of free radical intermediates (Ghezzi *et al.,* 2005; Jones, 2006; 2008).

Similar changes are taking place with respect to our understanding of the role of vitamin E (α-tocopherol) in living processes. For a long time it was believed that the main function of vitamin E is its antioxidant action in biomembranes. Within the last few years it has become clear that the antioxidant activity of vitamin E is not the only one (and perhaps not the most important) of its physiological functions (Ricciarelli *et al.,* 2001; Atkinson *et al.,* 2008; Jones, 2008; Engin, 2009). The common belief of the beneficial health-improving action of plant phenolics has also been revised (Halliwell, 2007).

In view of the substantial changes in the understanding of the role of reactive oxygen species and antioxidants in living systems, a critical re-evaluation of the methods of determination of the antioxidant activity is also necessary.

ANTIOXIDANT AND ANTIRADICAL ACTIVITY

The general methods of determination of antioxidant activity are summarized in many reviews, including (Sanchez-Moreno, 2002; Huang *et al.,* 2005; Frankel & Finley, 2008). Due to their practical significance much attention is paid to studies of natural products and food supplements (Davalos *et al.,* 2003; Moon & Shinamoto, 2009). Numerous studies have demonstrated that the antioxidant activity measured depends substantially on the test system used (Janaszewska & Bartosz, 2002; Bauzaite *et al.,* 2003) and recommended to base any conclusions on at least two different test systems (Moon & Shinamoto, 2009).

Most of the methods of determination of total antioxidant activity characterize the ability of the tested

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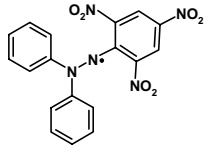
Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid; BHT, butylhydroxytoluene; DPPH, 1,1-diprenyl-2-picrylhydrazyl; TAS, total antioxidant status.

compound or product to scavenge free radicals and/or to complex metal ions driving the oxidation process.

It should be emphasized that there is a great difference between "antiradical" and "antioxidant" activity and that they do not necessarily coincide. According to Burlakova and coworkers (1975) the **antiradical activity** characterizes the ability of compounds to react with free radicals (in a single free radical reaction), but **antioxidant activity** represents the ability to inhibit the process of oxidation (which usually, at least in the case of lipids, involves a set of different reactions). Consequently, all test systems using a stable free radical (for example, DPPH, ABTS, etc) give information on the radical scavenging or antiradical activity, although in many cases this activity does not correspond to the antioxidant activity. In order to obtain information about the **real** antioxidant activity with respect to lipids or food stabilization, it is necessary to carry out the study on the real product (plant oil, lipoproteins, etc.).

DPPH AND GALVINOXYL ANTIRADICAL ACTIVITY TEST SYSTEMS

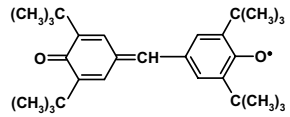
1,1-Diphenyl-2-picrylhydrazyl (DPPH; **I**) is a stable free radical. On accepting hydrogen from a corresponding donor, its solutions lose the characteristic deep purple (λ_{max} 515–517 nm) colour. DPPH is very popular for the study of natural antioxidants (Villano *et al.,* 2007). The PubMed database shows that this radical has been employed in more than 850 studies since 1969.



I

The antiradical activity of tested compounds is expressed as a relative or absolute decrease of concentration of DPPH or as EC_{50} (concentration of a compound decreasing the absorbance of a DPPH solution by 50%). The rate of reaction of various antioxidants with DPPH differs (Janaszewska & Bartosz, 2002). Very often the assay is performed according to the method described in (Bondet *et al.,* 1997). In spite of the wide use of DPPH, this test system in some cases gives incorrect results and recommendations for the proper application of the method have been formulated (Nenadis & Tsimidou, 2002; Molyneux, 2004; Sharma & Bhat, 2009). It is necessary to note that in the DPPH test system BHT, a strong hydrophobic antioxidant, shows low reactivity (Nenadis & Tsimidou, 2002; Musialik & Litwinienko, 2005; Sharma & Bhat, 2009). Some complications could be caused by partial ionization of the tested compounds, which affects the rate of their reaction with DPPH, making it pH-dependent (Musialik & Litwinienko, 2005).

DPPH is a N-centred stable radical. From our experience the best way of measuring free radical scavenging (antiradical) activity would be to use the O-centred stable radical galvinoxyl (**II**) which is more closely related to the physiologically acting oxygen radicals than is DPPH.



II

This stable radical is commercially available; its solutions have the absorbance maximum in the visible region (λ_{max} = 432 nm) and it is recommended for studies with electron and hydrogen donating compounds (Shi *et al.,* 2001). Comparing with DPPH, galvinoxyl is more reactive towards phenolics.

ABTS-BASED TEST SYSTEMS

The peroxidase substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), forming a relatively stable radical (ABTS$^{\bullet}$) upon one-electron oxidation, has become a popular substrate for estimation of total antioxidant capacity. Kinetic assays, including the commercialized TAS assay (Randox), are based on the inhibition of the formation of ABTS$^{\bullet}$ by one-electron oxidants (Bartosz & Bartosz, 1999; Bartosz, 2003). A simpler and more frequently applied approach, is the decolorization of preformed ABTS$^{\bullet}$ (Re *et al.,* 1999). An obvious drawback of ABTS-based assays is the promiscuity of reactions of ABTS$^{\bullet}$ which is a non-physiological free radical.

HYDROXYL RADICAL SCAVENGING ACTIVITY

Generation of hydroxyl radicals is crucial for the irreversible damage inflicted by oxidative stress (Halliwell & Gutteridge, 1999). This generation mainly proceeds *via* Fenton reaction:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO^{\bullet},$$

as well as in reaction between hypochlorous acid and superoxide anion:

$$HOCl + O_2^- \rightarrow O_2 + Cl^- + HO^{\bullet}$$

The rate constant of the latter reaction is greater than that of the reaction of Fe^{2+} with H_2O_2 [2]. Decomposition of peroxynitrous acid also yields HO$^{\bullet}$:

$$HONOO \rightarrow NO_2 + HO^{\bullet}$$

This reaction seems to be responsible for some 20-30% of the decay of peroxynitrite (Ferrer-Sueta & Radi, 2009).

The hydroxyl radical is an extremely reactive species and reacts at a high rate ($k \sim 10^9$–10^{10} $M^{-1} \cdot s^{-1}$) with all surrounding molecules — proteins, lipids, nucleic acids and sugars. Because the hydroxyl radical recombination

$$HO^{\bullet} + {}^{\bullet}OH \rightarrow H_2O_2$$

is also very fast ($k = 5 \times 10^9$ $M^{-1} \cdot s^{-1}$) the steady-state concentration of hydroxyl radical is practically zero (Halliwell & Gutteridge, 1999). Consequently, in spite of their popularity, the methods for determination of reactivity between

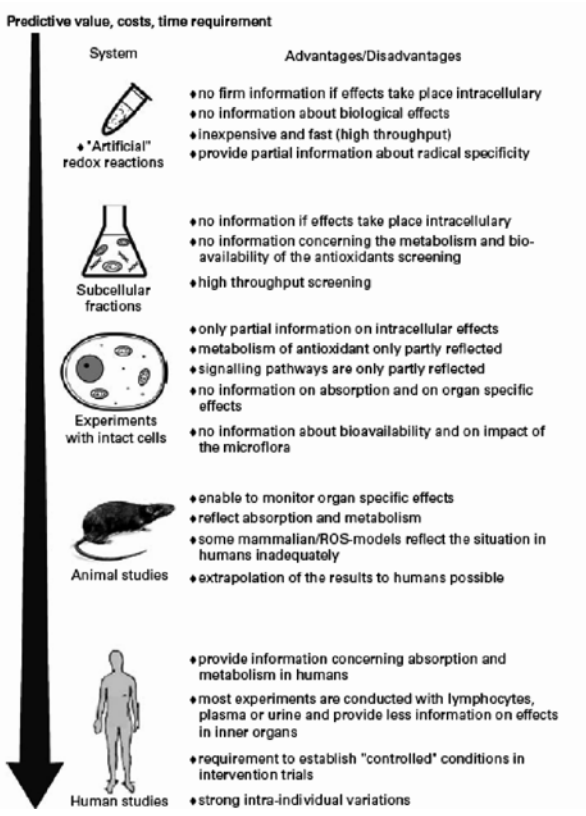


Figure 1. Advantages and disadvantages of different experimental approaches used to investigate ROS–protective effects of phytochemicals.
From (Knasmüllel *et al.,* 2008), with authors' permission.

various compounds and hydroxyl radicals do not possess practical meaning.

INTERPRETATION OF ANTIRADICAL AND ANTIOXIDANT STUDIES

The determination of antioxidant activity for stabilization of lipids and lipid containing products poses no complications. DPPH or other simple test system for screening of a set of compounds or products (for example, plant extracts) can be used and an active compound (extract) chosen for a final test on the real product.

Analysis of clinical samples (usually blood plasma) requires more caution. The results obtained in simple as well as complicated antiradical and antioxidative activity test systems usually correlate poorly with the data on the physiological activity of the compounds. A hot current question is whether or not the radical-scavenging (or antioxidant) activity is responsible for the action of many drugs as well as for the activity of health improving products, or is it only a side effect of these compounds of no relevance to their biological effects? In many cases the latter possibility appears to be true, as demonstrated by large epidemiologic studies (for example, Huang *et al.,* 2006; Bardia *et al.,* 2008). Moreover, the question about the usefulness of the intake of elevated amounts of dietary polyphenols has been a subject of active debate (Halliwell, 2007), leading to a conclusion that antioxidant supplementation does not reduce gastrointestinal cancer (Bjelakovic *et al.,* 2004), and a warning that excessive vitamin E supplements may even be harmful (Miller *et al.,* 2005).

Therefore, it is suggested that the so-called "antioxidant hypothesis" should be considered an intellectual "shortcut" possibly biasing the real understanding of the molecular mechanisms underlying the beneficial effects of various classes of substances including food additives. On the basis of recent work, it is proposed that specific molecules of nutritional interest (in particular polyphenols) may act by their direct interaction with nuclear receptors and by modulation of the signalling pathways of the cell (Virgili & Marino, 2008).

Recently, Knasmüller and co-authors (2008) carefully examined the methods of estimation of antioxidant/antiradical activities at various levels of biological organization and presented conclusions as the "pros and cons" of each method as well as for the suitability of specific methods for the evaluation of dietary antioxidants. The most important facets of this comparison are shown in Fig. 1.

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