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The potential pitfalls of using 1,1-diphenyl-2-picrylhydrazyl to characterize antioxidants in mixed water solvents

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Abstract
Approaching living systems, aqueous solutions are appropriate to characterize antioxidants, whereas the frequently used standard 1,1-diphenyl-2-picrylhydrazyl (DPPH) is insoluble in water. Therefore, mixed water–ethanol solvents were investigated using the electron paramagnetic resonance (EPR) spectroscopy. Two forms of DPPH were identified: at higher ethanol ratios a quintet spectrum characteristic of solutions, and at lower ratios, a singlet spectrum typical for solid DPPH, were found. Mixed solvents with 0–50% (v/v) water reproduced the same antioxidant equivalent points well and the reaction rate between DPPH and the antioxidant may increase considerably with increasing water ratios, as demonstrated using vitamin E as an antioxidant. But at still higher water ratios (70–90% (v/v)) the antioxidant activities dropped, since a part of the DPPH in the aggregated form does not react sufficiently with the antioxidants. Characteristics of the most common antioxidants were determined in ethanol or its 50% (v/v) aqueous solution.

Keywords: *EPR spectroscopy, DPPH, antioxidant, ethanol, water*

Introduction

One of the most commonly used standards in the characterization of antioxidant properties, and also in the description of many other radical-related systems, is 1,1-diphenylpicrylhydrazyl (DPPH) [1–7]. Its advantages are relatively good stability (frequently assumed) and it is an easily achievable identification, having an absorption maximum in UV/vis at 515 nm, manifested by an intensive violet color [2–7]. The DPPH test for the determination of radical-scavenging activity is frequently applied in the investigation of bioactive compounds (vitamins, flavonoids, phenols [8–16]), as well as food, beverages or plant extracts [17–23]. The static and dynamic variants of the DPPH test are described in the literature [24]. The static approach is oriented on the evaluation of DPPH quantity scavenged by a substrate using EC_{50} value ("Efficient Concentration" defined as that concentration of the substrate, which causes 50% decline of DPPH concentration [2–7,24]). The dynamic version of the DPPH test is focused on the kinetics of the substrate/DPPH reaction, evaluating a rate constant or an initial rate of DPPH elimination [24–26]. The concentration of DPPH in these reaction systems is monitored by UV/vis, electron paramagnetic resonance (EPR) spectroscopy or HPLC [8–26]; automated techniques for DPPH tests have also been published recently [27,28].

Numerous papers report on very variable routes in the reactions of DPPH [29–32]. The DPPH assay is suitable for the investigation of the radical-scavenging activity of hydrogen-donating compounds, especially

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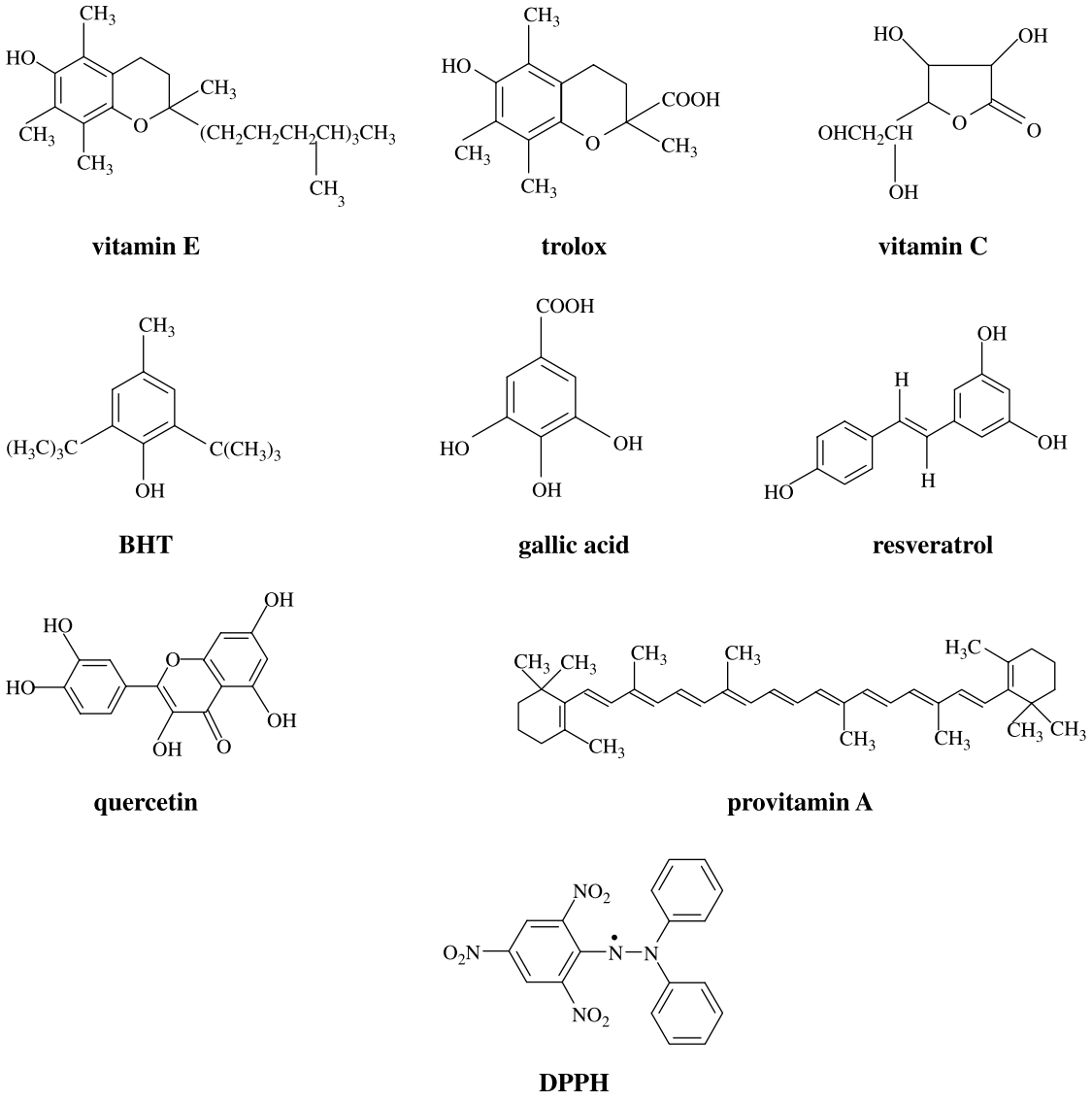
phenolics [2–7]. The interaction of a phenolic compound with DPPH results in the generation of phenoxyl radical and diphenylpicrylhydrazine, and the phenoxyl radical is involved in a variety of consecutive reactions such as coupling, fragmentation or addition [16]. Recently, the reactions of phenols with DPPH were investigated in different solvents, and a significant role of partial phenol ionization in the fast electron transfer from the phenoxide anion to the DPPH radical was suggested in alcohols [30,31].

Yordanov et al. raised the question on the properties of DPPH and its applicability as an EPR standard [33,34]. A limited stability is considered now in most of the papers using freshly prepared DPPH solutions [27,35]. According to our experience, e.g. ethanol DPPH solutions decrease their spin concentrations by 1–2% during the working day.

Focusing on the living systems, to use water as a solvent would be an optimal choice; but, this is contradicted by the low solubility of DPPH in water [36], unless its water-soluble derivatives are used [37,38]. The aim of our contribution is to demarcate suitable conditions, and also limits, for using optimal water ratios as a component of a mixed water–ethanol solvent. EPR spectroscopy was applied as an indication technique. The antioxidants investigated are summarized in the experimental part (Scheme 1). Their characteristics, such as stoichiometric ratios and EC_{50} values, were determined in ethanol or mixed water:ethanol = 1:1 (v/v) solvents. More detailed experiments investigating the influence of water:ethanol ratios on the stoichiometric ratios of antioxidant:DPPH were carried out using four selected antioxidants: namely vitamin C, vitamin E, trolox and gallic acid.



Scheme 1. Structures of antioxidants and DPPH.

A few experiments analogous to those with ethanol were also performed in mixed water–methanol solvents.

Materials and methods

Materials

As antioxidants, the following substances with common and (IUPAC) names from specified producers and with declared purities were used: trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Aldrich 97%; vitamin E (DL-α-tocopherol) Aldrich, 97%; vitamin C (L-ascorbic acid) Sigma 97%; BHT (2,6-di-*tert*-butyl-4-methylphenol) Aldrich 97%; gallic acid monohydrate (3,4,5-*tri*-hydroxybenzoic acid) Sigma-Aldrich 98%; resveratrol (*trans*-3,4′,5-tri-hydroxy-stilbene) Sigma 98%; provitamin A (all-*trans*-β-carotene) Aldrich 95%, along with oxidant DPPH (1,1-diphenyl-2-picrylhydrazyl) 95% Aldrich. Their structural formulas are quoted in Scheme 1. Further antioxidants and chemicals used: ethanol and methanol of spectroscopic grade (Microchem, Slovak Republic), $FeSO_4.7H_2O$, $CuCl_2.2H_2O$, $Fe(NO_3)_3.9H_2O$, $MnCl_2.4H_2O$, $KHSO_3$, of analytical grade were purchased from Lachema (Czech Republic) and TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine *N*-oxyl) Aldrich.

Preparation of solutions

The most frequently used solvent for DPPH in the literature is methanol [2]. Focusing on the human body, ethanol is more likely to be found than methanol, so ethanol was chosen as a co-solvent to water. In addition, a few experiments were also carried out using methanol as a solvent. The results obtained were closely similar to those found in ethanol. As a stock solution 10^{-4} M DPPH was prepared in ethanol. To complete its homogenization, it was kept for 2 min in the low-energy ultrasonic bath, resulting in no significant changes of DPPH concentration. Then, it was diluted with ethanol or mixed water ethanol solvent to 20 μM solutions. DPPH concentrations were determined by UV/vis and also by EPR using TEMPOL as a standard under identical conditions as in the antioxidant experiments. The reproducibility was satisfactory (± 1.5%). Antioxidant stock solutions in ethanol were also prepared similarly to the DPPH and then diluted with water or ethanol to the required concentrations. As will be reported later, at a higher water ratio an equilibrium between two differently solvated forms of DPPH is established. This establishing process interferes with the antioxidant reactions. To avoid it, an equal solvent mixture of water:ethanol was used for DPPH as well as for the antioxidants entering the reaction. The compositions of the mixed water–ethanol solutions are expressed in volume ratios (v/v) throughout the text.

EPR measurements and their evaluations

The EPR measurements were carried out in a flat cell (WG-812, Wilmad-LabGlass, USA) adapted for the flow-technique in a Bruker TM-110 (ER 4103 TM) cylindrical cavity using a Bruker EMX EPR spectrometer working in the X-band. The DPPH and antioxidant solutions were separately prepared and put into two separate syringes and then simultaneously injected via a small mixing chamber flowing into the flat cell. Immediately, after simultaneous injection of DPPH and antioxidant solutions, EPR measurements were commenced, lasting for 10 min, taking 10 spectra. Every spectrum represents an accumulation of three scans. The filling procedure employed for the EPR flat cell resulted in a reproducibility with a standard deviation in the relative EPR intensity of ± 5% for five independent measurements.

An illustration, of such experiments using trolox as the antioxidant is shown in Figure 1. Experiment 1a represents the DPPH reference—one syringe was filled with 20 μM DPPH solution and the second, parallel one, with pure solvent (ethanol) only. In analogous experiments the antioxidants (here trolox) were filled in the second syringe and the antioxidant concentrations were increased until reaching a molar ratio of DPPH:antioxidant = 1:1 (Figure 1(b)–(l)). Starting with the ethanol solvent, such experiments were then expanded to the mixed water–ethanol solvents. A further series of experiments in ethanol or in 50% (v/v) aqueous ethanol solutions focused on the determination of the stoichoimetric ratio of antioxidant:DPPH at the equivalent point (approximation to zero DPPH concentration) were carried out analogously to the procedure described above, but with higher DPPH and antioxidant concentrations (10^{-4} M). The results obtained at these higher DPPH concentrations are similar to those with the lower ones (10^{-5} M) but revealed a higher accuracy.

In most of the cases, the reaction between DPPH and antioxidants is relatively fast, so that after 10 min the final DPPH concentration was established, as illustrated in Figure 1, from the experiment with trolox used as an antioxidant in ethanol solution. Most of the antioxidants investigated showed a still faster reaction, but in few cases, such as vitamin E or industrial antioxidant BHT, especially in the ethanol, the reactions were slower, and in addition the spectra of DPPH overlapped with the EPR signals originating from the antioxidant. In these cases, more complex evaluations were needed, including the simulation of spectra to determine the antioxidant:DPPH ratio. In a set of experiments, we also investigated the initial reaction rate of vitamin E with DPPH at the increasing water ratios evaluated later. The experimental time dependencies were fitted by the nonlinear least-squares method to the second-order kinetic models

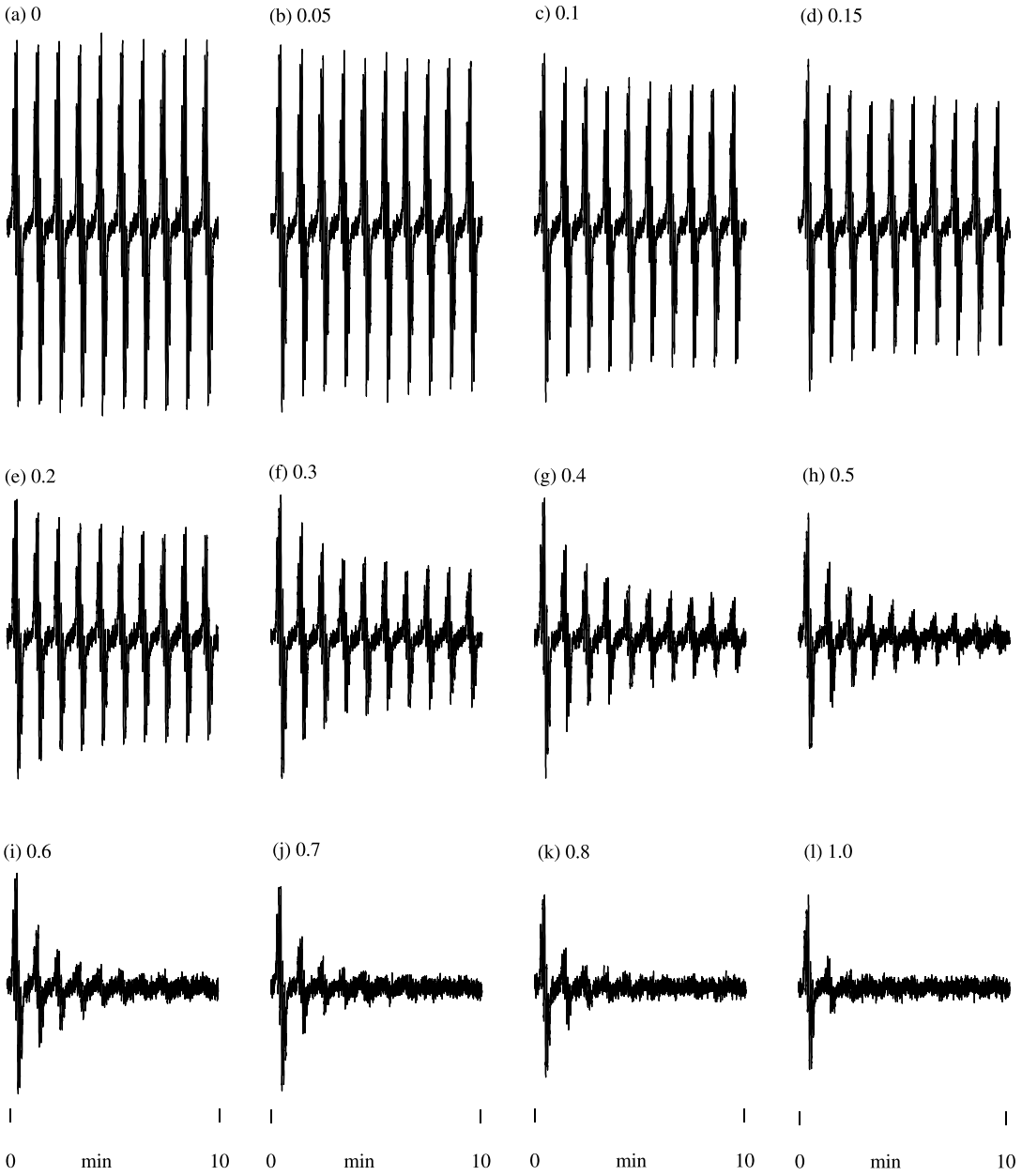


Figure 1. Time course of EPR spectra monitored during 10 min intervals in the series of experiments (a–l) with increasing ratios of trolox:DPPH in ethanol solutions. Figure 1(a) represents the reference with a 10 μM DPPH solution, b–l are experiments with increasing trolox ratios.

(Scientist, MicroMath), and the formal initial rate of DPPH elimination, $R_{in} = (dc_{DPPH}/dt)_{t\to 0}$, was evaluated. The statistical and linear regression analysis was carried out using the Origin (Microcal) program. The parameters were evaluated at the 0.05 significance level.

UV/vis experiments

UV/vis spectra were recorded using a UV/vis spectrometer PC 2000 (Sentronic, Germany) with a DH 2000 lamp. As limited data on the molar absorptivity of DPPH in ethanol are available in literature [34], we determined the radical content of our DPPH probe preparing its methanol solution considering its molar absorptivity of, $\varepsilon_{515} = 12{,}500\ M^{-1}/cm^{-1}$ [2]. Then, in a further calibration procedure the molar absorptivity of DPPH in ethanol with value $\varepsilon_{515} = 15{,}400\ M^{-1}/cm^{-1}$ was determined and used in the quantitative concentration evaluations.

Results and discussion

Solvated and aggregated forms of DPPH

Figure 2 shows EPR spectra of 10^{-5} M DPPH in a mixed water–ethanol solvent arranged by an increasing ratios of water. At the lower water ratios (0–60%

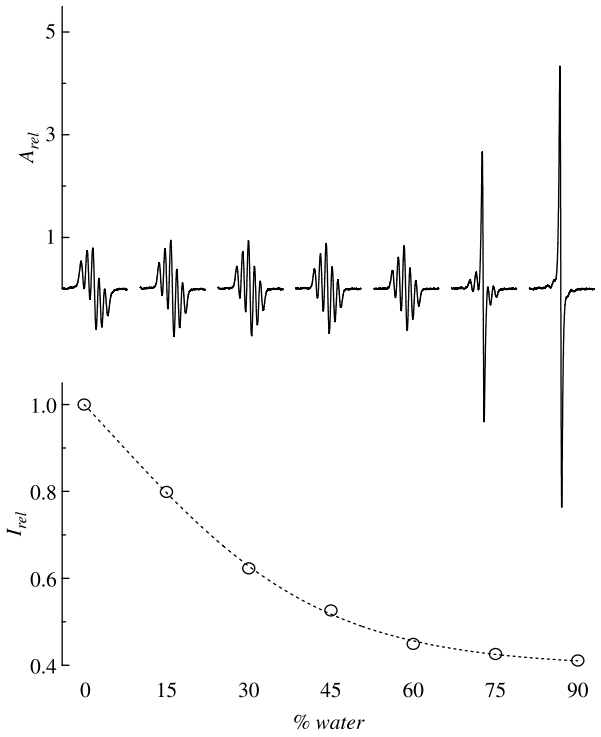


Figure 2. EPR spectra of a 10^{-5} M DPPH solution in ethanol given increasing volume ratios of water. A_{rel} represents the relative amplitudes of the spectra and I_{rel} their relative double integrals. Sweep width was 10 mT.

(v/v)), DPPH shows a quintet EPR spectrum well known from the literature ($a_{N1} = 0.927$ mT, $a_{N2} = 0.846$ mT; $g = 2.0036$ [33,34]), characteristic of DPPH solutions. At higher water ratios (over 60% (v/v)), a further EPR spectrum, a singlet, characteristic of solid DPPH samples, is superimposed and it dominates exclusively at high water ratios (90% (v/v)), evidently reflecting a limited solubility of DPPH in water. There is no precipitation evident and macroscopically the mixture appears to be a homogeneous system. DPPH is probably aggregated to some microscopic particles manifested by an EPR spectrum characteristic of solid DPPH samples.

Considering the mixed water–ethanol solvents microheterogeneity phenomena were reported in the case of water–dioxane and water–acetonitrile [39–41], and also water–ethanol [42–47]. Therefore, it seems probable that at higher water ratios solvated DPPH (quintet spectrum) is incorporated in small ethanol microdomains. The narrow line EPR spectrum of the aggregated DPPH (singlet spectrum) with peak-to-peak width of around 0.2 mT is well compatible with the spectrum assigned to solid state DPPH attributing the narrow EPR line to exchange narrowing in solids [48,49].

The relative amplitude of spectra A_{rel} crosses a moderate maximum at a 30% (v/v) water ratio, and rises again at water ratios over 60% (v/v), whereas the relative double integral I_{rel} decreases continuously (Figure 2). The change of A_{rel} reflects the influence of three parameters summarized in Table I. The first one is the decreasing line width (ΔH_{pp}) of spectrum with increasing water ratios for both DPPH components: $\Delta H_{pp}(l)$ of its dissolved and $\Delta H_{pp}(s)$ of its aggregated (solid) form. The second parameter, contrary to the first one, is the dielectric loss, which increases with the increasing water ratio (increasing relative permittivity, ε_r [50]) resulting in a drop of amplitude. The superposition of both the parameters results in a local maximum of A_{rel} at about 30% (v/v) water ratio. Simultaneously, the ratio of Lorentzian line shape of quintet spectrum decreases with the increasing water ratios. A further increase of A_{rel}, at water ratios over 70% (v/v) reflects the formation of aggregated DPPH species characterized by a relatively sharp singlet line, resulting in a substantial increase of A_{rel}. Below the spectra in Figure 2 are also plotted their relative double integrals I_{rel} continuously decreasing with increasing water ratio. Table I presents two individual parts of I_{rel}, namely, $I(l)$ of its dissolved and $I(s)$ of its aggregated form. It is evident that the aggregated form $I(s)$ dominates at high water ratios. These two forms of DPPH (l) and (s) are reflected in its changing oxidant activity, which is described later.

Table I. The parameters elucidated from the spectra in Figure 2 at various water ratios, where A_{rel} is the relative value of the spectral amplitude, $\Delta H_{pp}(l)$ is the peak-to-peak-widths of the quintet spectrum and $\Delta H_{pp}(s)$ of the singlet spectrum, ε_r is the permitivity of the corresponding solutions, $I(\text{total})_{rel}$ is the relative values of the total integral, and $I(l)$ and $I(s)$ of its quintet and singlet components. Standard deviations of experimental data are 3–5%.

	Water in ethanol (volume %)						
	0	15	30	45	60	75	90
A_{rel}	1	1.18	1.19	1.11	1.05	3.35	5.44
$\Delta H_{pp}(l)$ in mT	0.580	0.520	0.510	0.510	0.490	0.490	0.490
$\Delta H_{pp}(s)$ in mT	–	–	–	0.275	0.275	0.250	0.240
ε_r	24.6	44.2	56.0	63.8	69.3	73.5	76.8
$I(\text{total})_{rel}$	1	0.799	0.623	0.526	0.449	0.426	0.411
$I(l)$ (%)	100	100	100	99.7	99.6	48.2	12.5
$I(s)$ (%)	0	0	0	0.3	0.4	51.8	87.5

Trolox, vitamin E, vitamin C and gallic acid as antioxidants in mixed water–ethanol solvents

To investigate the oxidation properties of DPPH in mixed water–ethanol solvents, the following frequently mentioned antioxidants were chosen: (a) trolox; (b) vitamin E; (c) vitamin C; and (d) gallic acid. The results obtained are summarized in Figure 3. The relative decreases in DPPH concentrations (spectral double integrals) for the individual antioxidants (Figure 3(a)–(d)) are quoted upon the increased molar ratios of antioxidant:DPPH, at various compositions of mixed solvent containing the following volume ratios of water: + 0%, ○ 50%, ⋆ 75% and • 90% (v/v).

As evident from Figure 3, all four antioxidants show similar behavior. At the lower water ratios (e.g. +0% and ○ 50% (v/v) water, Figure 3(a)), the quintet EPR spectrum documents a well-solvated form of DPPH. In this study, the reaction between DPPH and antioxidant is relatively fast and complete. The evaluated stoichiometric ratios of antioxidant:DPPH for the individual antioxidants from Figure 3(a)–(d) in both types of solvents (0 and 50% (v/v) water) are

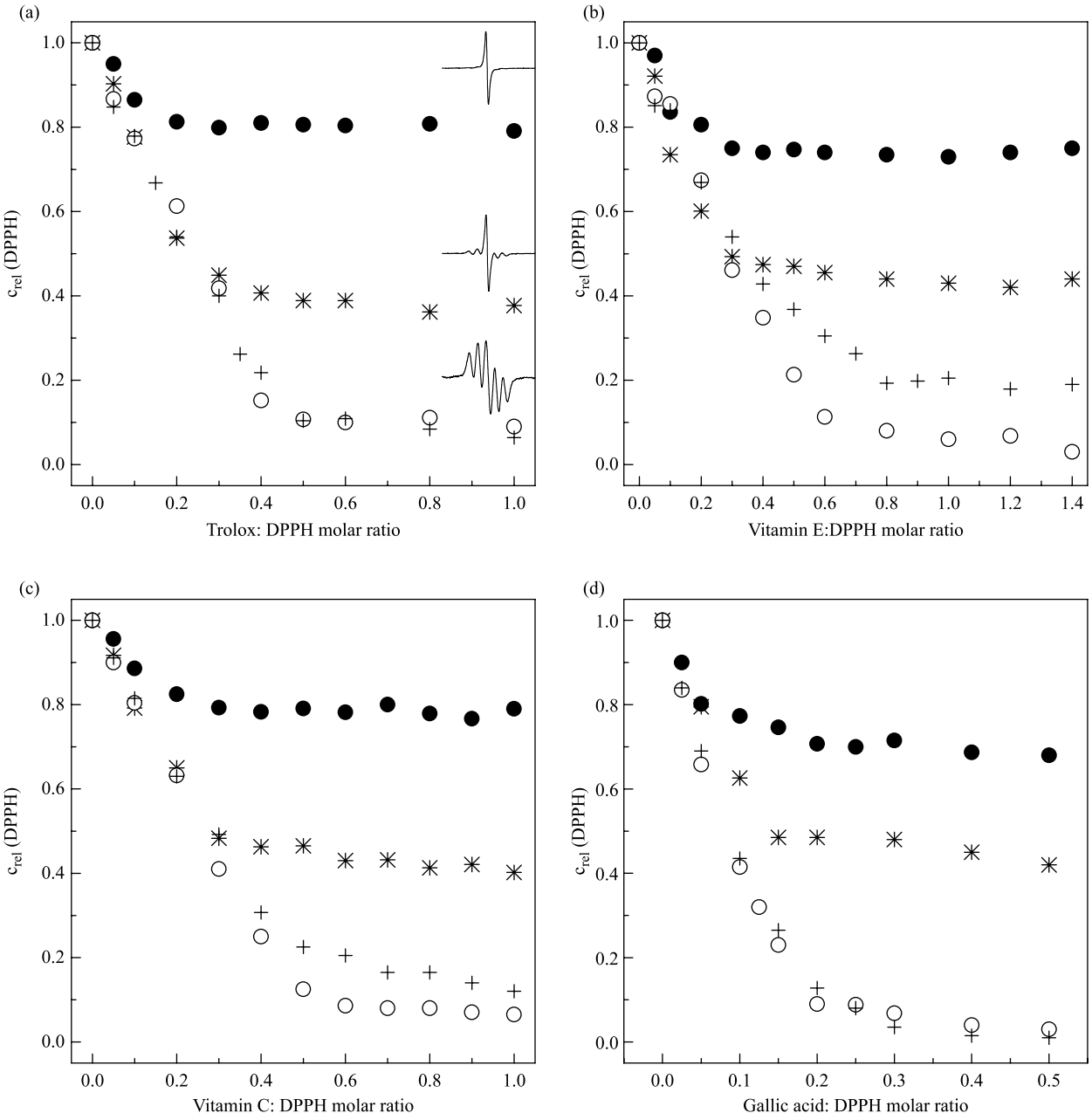


Figure 3. Relative changes of DPPH concentration quoted upon increasing molar ratios of antioxidant:DPPH measured after 10 min reaction time in mixed ethanol–water solvents containing the following volume ratios of water: +0%; ○ 50%; ⋆ 75% and • 90% (v/v) using: (a) trolox; (b) vitamin E; (c) vitamin C; and (d) gallic acid as antioxidants.

Table II. Stoichiometric ratios of antioxidant: DPPH evaluated from Figure 3, considering antioxidants (a) trolox, (b) vitamin E, (c) vitamin C, and (d) gallic acid in ethanol solvent with 0 and 50% (v/v) water.

	Stoichiometric ratios of antioxidants to DPPH			
Ratio of water (% (v/v))	(a) Trolox	(b) Vitamin E	(c) Vitamin C	(d) Gallic acid
0	0.46 ± 0.03	0.56 ± 0.04	0.47 ± 0.03	0.21 ± 0.04
50	0.47 ± 0.04	0.58 ± 0.04	0.50 ± 0.02	0.19 ± 0.05

very similar (Table II). Consequently, using both solvent systems with 0 or 50% (v/v) water, approximately equal stoichiometric ratios can be expected for an antioxidant. However, the dynamic—kinetic values can differ with the changing water ratios as will be demonstrated later in the investigations of the initial reaction rates for vitamin E with DPPH.

The stoichiometric ratios of antioxidant:DPPH = 1:2 were found for trolox (a), vitamin E (b), vitamin C (c) and 1:4 for gallic acid (d). A small divergence to a higher ratio is indicated for vitamin E in 100% ethanol solutions (Figure 3(b)). This pointed to a dependence of the reaction rate on the composition of the mixed solvent. Consequently, we investigated the reaction of vitamin E with DPPH at various ratios of water in more detail. The results obtained are presented in Figure 4(a), where the relative changes of DPPH concentration are quoted upon the reaction time of a 25 μM DPPH with 12.5 μM vitamin E in mixed solvents at increasing volume water ratios (0, 10, 20, 30, 40, 50% (v/v)). The initial reaction rate increases considerably with increasing water ratios as evident from the inset in Figure 4(a), where the initial reaction rate R_{in} is quoted upon the increasing water ratios in ethanol. The second-order rate constants evaluated from the time dependence of reciprocal DPPH concentrations (Figure 4(b)) sensitively reflect the increasing ratio of water in the reaction system (inset in Figure 4(b)). The increased reaction rate given increasing volume ratios of water is in agreement with a recently published paper [32], where the accelerated scavenging of DPPH by vitamin E upon the addition of water into the reaction mixtures was attributed to the enhancing deprotonation of the phenolic group coupled with fast electron transfer from phenoxide anion to DPPH [30–32].

A more complex behavior of DPPH is evident at the higher water ratios (60–90% (v/v)), where two of its paramagnetic forms, namely DPPH(*l*) characterized with a quintet and DPPH(*s*) with a singlet spectrum are overlapped. This can be well explained with the above estimated formation of microdomains containing with ethanol solvated DPPH(*l*), and solid-like state, aggregated DPPH(*s*). On the other hand, (*l*) form reacts relatively quick with the antioxidants, its (*s*) form reacts very slowly. This explains the course of DPPH concentrations quoted in Figure 3 for a solvent mixture containing 75% (v/v) (⋆) and 90% (v/v) (•) water, where noticeable DPPH concentrations are still evident even with an excess of antioxidant. This can be well understood from the set of spectra in Figure 5 taken at increasing trolox ratios in the mixed solvent

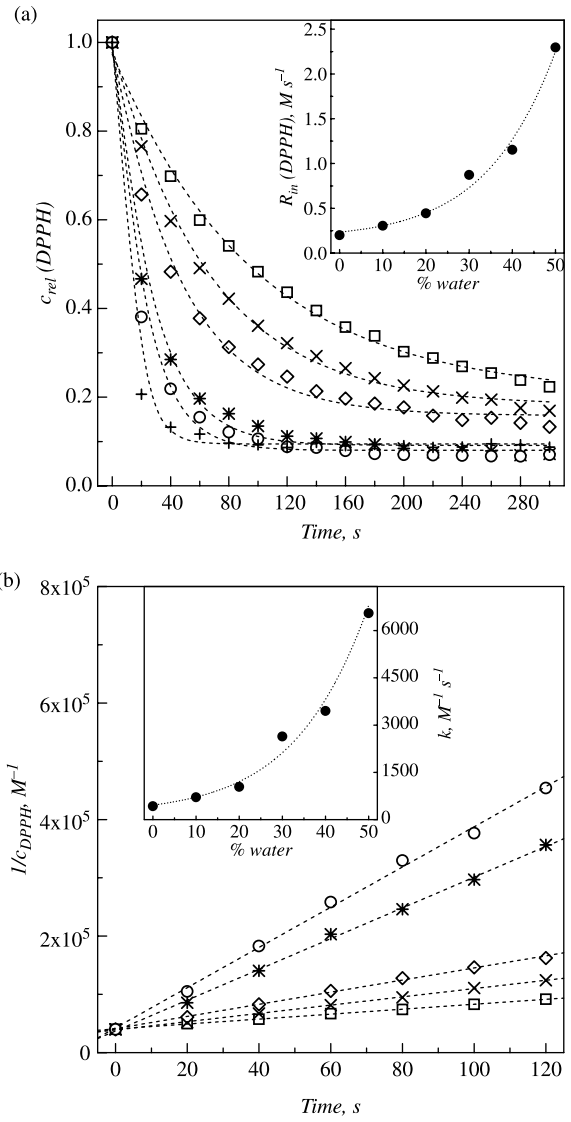


Figure 4. (a) Changes of relative DPPH concentrations c_{rel} over time in the reaction of 25 μM DPPH with 12.5 μM vitamin E in mixed water–ethanol solvent containing □ 0; × 10; ◇ 20; ⋆ 30; ○ 40 and +50% (v/v) water. The inset presents the dependence of the initial reaction rate R_{in} upon the volume ratios of water. (b) Second-order plot ($1/c_{DPPH}$) of DPPH concentrations considering the data extracted from Figure 4(a). The inset presents the dependence of the evaluated rate constant *k* upon volume ratios of water.

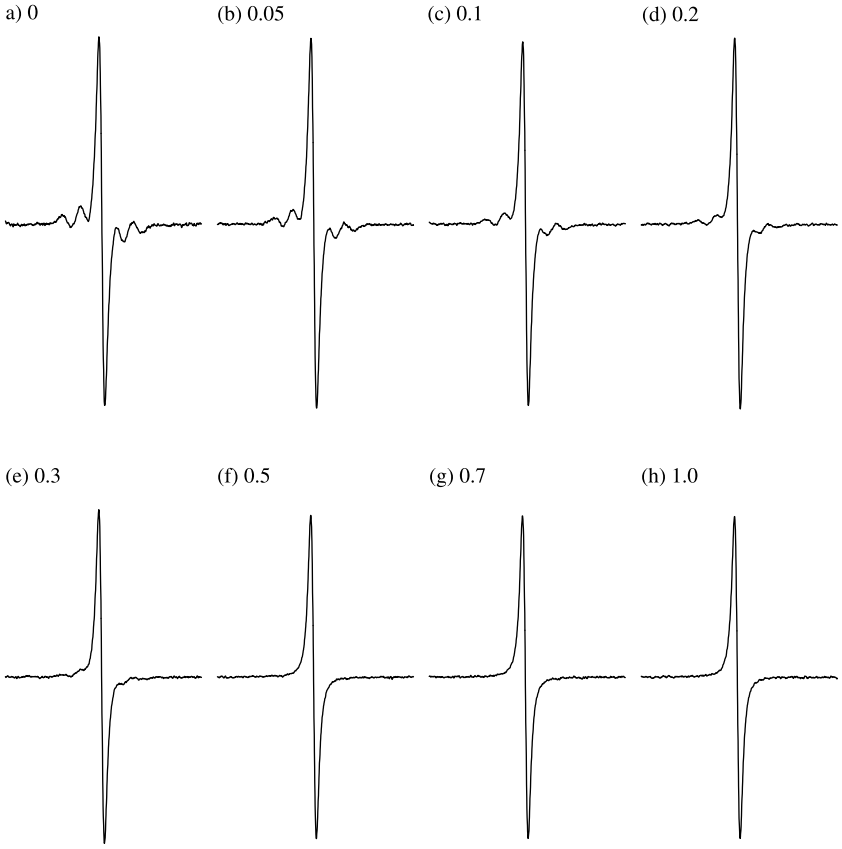


Figure 5. EPR spectra of 10^{-5} M DPPH in mixed water–ethanol solvent (75% (v/v) water) at increasing trolox:DPPH ratios from zero (a) to one (b–h) measured after a 10 min reaction period (sweep width 10 mT).

containing 75% (v/v) water. According to the reference (Figure 5(a)—without antioxidant), a quintet with 48.2% and a singlet with 51.8% are evident. With the increased ratio of antioxidant (Figure 5(b)–(e)), the quintet spectrum is depleted and there remains only the singlet spectrum (e–h), which decreases only very negligibly with the increased antioxidant ratios. A quantitative evaluation of these spectra is presented in Figure 3(a) (trolox, 75% (v/v) water ⋆). Such a phenomenon is still more pronounced in 90% (v/v) aqueous water solvent, where only a very small part of the DPPH enters the reaction. Looking at an analogous data set as shown in Figure 5, but with 90% (v/v) water (not presented), only a negligible ratio (12.5%) of quintet spectrum was evident. Moreover, this rapidly vanishes in the first steps of adding antioxidant and only the DPPH (*s*) form with a singlet spectrum remains, showing

Table III. Stoichiometric ratios of antioxidant:DPPH and values EC_{50} (μM of antioxidant/μM of DPPH) obtained in an EPR study using 43.5 μM DPPH with various antioxidants in ethanol and 50% (v/v) aqueous ethanol solutions after 10 min reaction time.

Antioxidant	Solvent	Stoichiometric ratio antioxidant:DPPH	EC_{50}
Trolox	Ethanol	0.45 ± 0.05 (1:2)	0.22 ± 0.02
Vitamin E	Ethanol	0.53 ± 0.03 (1:2)	0.27 ± 0.01
Vitamin C	Water:ethanol	0.48 ± 0.03 (1:2)	0.26 ± 0.01
BHT	Ethanol	2.85 ± 0.10 (3:1)⋆	2.58 ± 0.05⋆
Gallic acid	Ethanol	0.25 ± 0.02 (1:4)	0.12 ± 0.01
Resveratrol	Ethanol	1.24 ± 0.20 (1:1)	0.64 ± 0.01
Quercetin	Ethanol	0.28 ± 0.05 (1:4)	0.13 ± 0.02
β-carotene	Ethanol	–	–
Fe(II)	Water:ethanol	0.85 ± 0.03 (1:1)	0.43 ± 0.01
HSO_3^-	Water:ethanol	3.0 ± 0.05 (3:1)	1.42 ± 0.03
Fe(III)	Water:ethanol	–	–
Mn(II)	Water:ethanol	–	–
Cu(II)	Water:ethanol	–	–

⋆ Because of slow reaction kinetics the presented value is not compatibly comparable with other antioxidants following rapid kinetics.

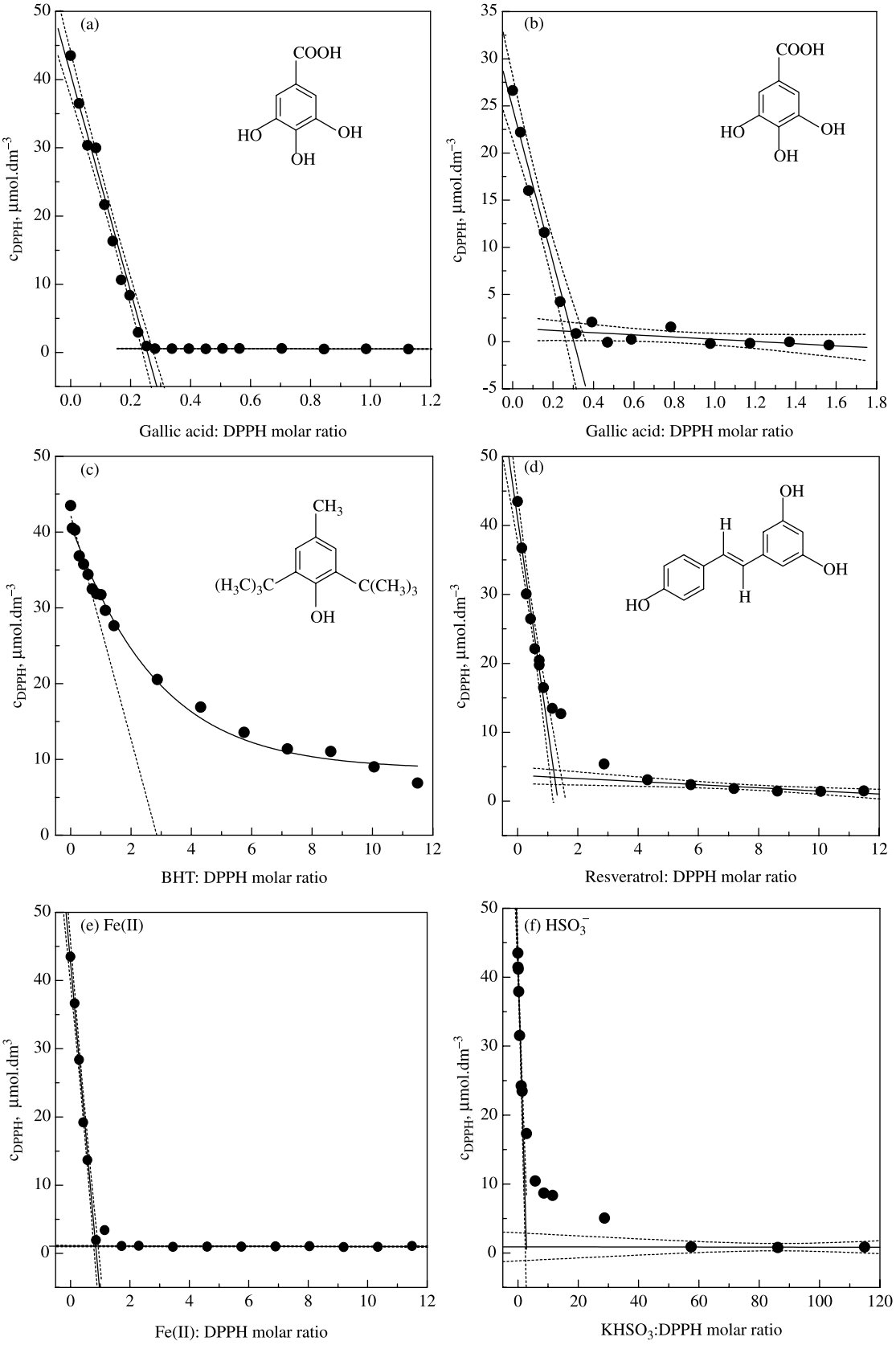


Figure 6. Changes of DPPH concentrations evaluated after a 10 min reaction period quoted upon the increasing molar ratios of antioxidant:DPPH. The concentrations were followed by EPR (a, c–f) or UV/vis (b) in 100% ethanol (a–d) or 50% (v/v) aqueous ethanol solutions (e, f).

a very negligible decrease in concentration with increasing antioxidant concentration. Such a small decrease is evident from Figure 3(a)–(d) for all antioxidants with 90% (v/v) water ratios (•). However, these small changes in DPPH concentrations at high water ratios still reproduce the relative antioxidant activities, such as we found before in the investigations of wine and tea probes [51,52]. But a better choice would be to avoid working with DPPH in mixed solvents at such high water ratios, especially if antioxidant capacities are considered.

Characterization of some further antioxidants

Our investigations applying the earlier-described techniques were also expanded to further frequently considered antioxidants summarized in Table III. Higher DPPH concentrations (43.5 μM compared with the previous 10 μM) were used here to obtain a higher accuracy. Figure 6 presents the data obtained with: (a) gallic acid monitored by EPR; (b) gallic acid monitored by UV/vis; (c) industrial antioxidant BHT; (d) resveratrol; (e) Fe(II); and (f) HS_{O3^-} all (c–f) monitored by EPR.

Most of the investigated substances are characterized by a sharp equivalent point, except BHT, where the reaction is very slow [53–57] and phenoxyl radicals originating from BHT interfere with the spectra of DPPH. The stoichiometric ratio here was extrapolated from the initial decreases in DPPH concentrations (Figure 6(c)). Generally, the highest stoichiometric ratios of antioxidant:DPPH, 1:4, were found for gallic acid and quercetin, then 1:2 for trolox, vitamin E, vitamin C, 1:1 for resveratrol, Fe(II), and 3:1 for BHT and HS_{O3^-}. Further substances, such as β-carotene, Fe(III), Mn(II) and Cu(II) did not show any antioxidant response. In addition to the stoichiometric ratios, we also evaluated the EC_{50} values characterizing the initial concentration of antioxidant needed to decrease the original DPPH concentration to 50% over 10 min, after mixing the reactants. The most effective antioxidants according to EC_{50} values (μM of antioxidant/μM of DPPH) in ethanol solutions appeared to be gallic acid (0.12), followed by quercetin (0.13), trolox (0.22), vitamin C (0.26) and vitamin E (0.27).

Considering the stoichiometric ratios antioxidant:DPPH presented in Table III they are generally in agreement with those from the literature based on detailed mechanistic studies [53,54], where usually the phenolic group is oxidized to carbonyl group eliminating two DPPH radicals. Such ratios were also found here for trolox, vitamin C and vitamin E (Table III). Antioxidants with more phenolic groups (gallic acid, quercetin) revealed a higher number of DPPH radicals scavenged (4). A remarkable deviation from the expected stoichiometric ratio for monophenolic antioxidant was determined for BHT, frequently referred as antioxidant standard (expected BHT:DPPH = 1:2, found here BHT:DPPH = 3:1) as is evident from Figure 6. While the other antioxidants presented show sharp equivalent points, as their reaction is rapidly completed in the 10 min monitoring interval, the reaction of BHT is substantially slower and only a minor part of DPPH reacts with BHT in this interval. According to the monitoring intervals, solvents, as well as different evaluation procedure employed, different stoichiometric ratios BHT:DPPH = 1:2 [55–57], but also 1:2.8 [53,54] were also reported, pointing to more complex behaviour of BHT antioxidant (participation of para-methyl group and dimerization [53,54]).

Conclusions

Although DPPH represents a frequently used standard to characterize antioxidants or other related systems, care should be taken in long-duration experiments due to its limited stability in solutions (in ethanol solution in the course of one day's exposure to light at room temperature, DPPH decreases its concentration by 1–2%). Considering antioxidants with a hydrophilic or hydrophobic character, the 50% (v/v) aqueous ethanol solutions are a suitable choice for both types of antioxidants for obtaining representative stoichiometric ratios of antioxidant:DPPH. These ratios also correspond well to those found in 100% ethanol solvent. However, with increasing water ratios (from 0–50% (v/v)) a higher reaction rate between the antioxidant and DPPH is evident for some antioxidants. A few limits should be considered, especially in quantitative characterizations of antioxidant capacity if DPPH solutions with water ratios over 60% (v/v) are used, because a part of the DPPH coagulates in a solid-like form and is not easily accessible to the reactions with antioxidants.

Acknowledgements

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