Effect of organic co-solvents in the evaluation of the hydroxyl radical scavenging activity by the 2-deoxyribose degradation assay: the paradigmatic case of α-lipoic acid

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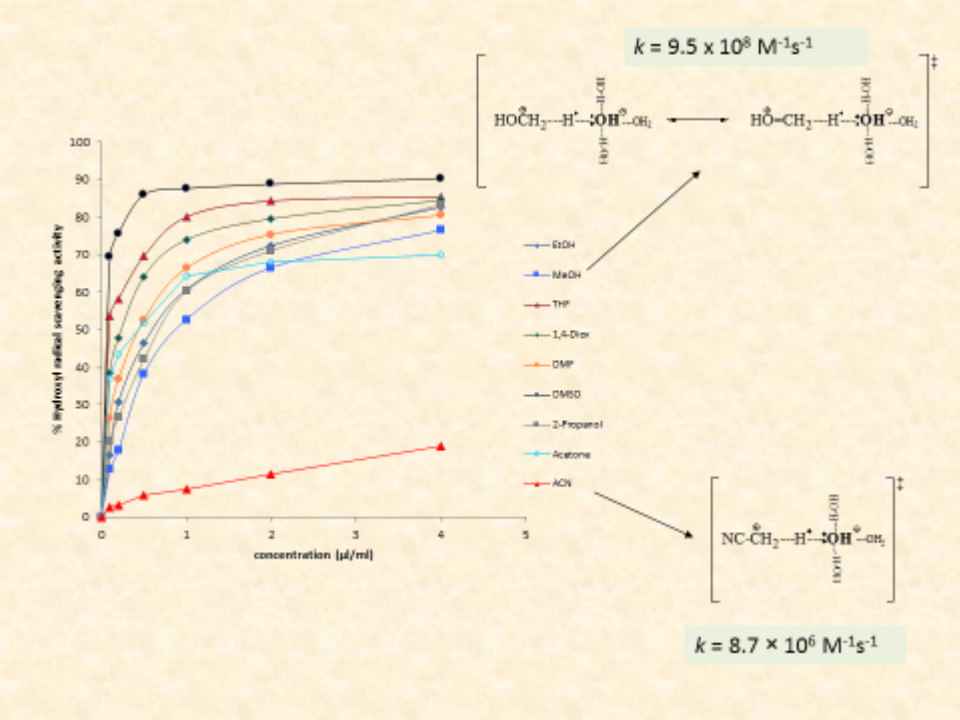
Graphical Abstract



Abstract

The 2-deoxyribose degradation assay (2-DR assay) is an *in vitro* method broadly used for evaluating the scavenging activity against the hydroxyl radical ($HO^{\bullet}$). One of the major drawbacks of the assay, however, is that only water soluble compounds can be tested for their radical-scavenging activity. Lipoic acid (LA) is an excellent scavenger of $HO^{\bullet}$ but it exhibits a low solubility in the aqueous milieu of the 2-DR assay and a high tendency to polymerize under a variety of conditions. We used LA as a paradigmatic substrate to evaluate the effect of several organic co-solvents in increasing its solubility. Most of these solvents, however, demonstrated to be potent scavengers of $HO^{\bullet}$ making their use in the 2-DR assay improper. On the other hand, acetonitrile showed a remarkably low reactivity toward $HO^{\bullet}$ (rate constant ~ 8.7×10^{6} $M^{-1}s^{-1}$) which allowed us to use it as a co-solvent in the preparation of stock solutions of LA ~ 5 mM. We therefore evaluated the radical-scavenging activity of LA by the 2-DR assay in a relatively large range of concentrations, 1 – 200 μM. We found that the rate constant for LA + $HO^{\bullet}$ is diffusion-controlled (~1×10^{10} $M^{-1}s^{-1}$ in water at 25 °C) and uninfluenced by the presence of small quantities of acetonitrile. Therefore, the use of acetonitrile in the 2-DR assay does not interfere with the test and may increase the solubility of the radical scavengers.

Keywords: 2-deoxyribose degradation assay, hydroxyl radical, scavenging activity, lipoic acid, acetonitrile.

List of abbreviations:

2-DR: 2-deoxyribose
DMF: Dimethylformamide
DMSO: Dimethylsulfoxide
EDTA: Ethylenediaminetetraacetic acid
LA: (*R*,*S*)-lipoic acid
MDA: Malondialdehyde
ROS: Reactive oxygen species
TBA: Thiobarbituric acid

1. Introduction

The hydroxyl radical $HO^{\bullet}$ is a short-lived species (half-life in solution ~ 10^{-9} s) [1] that has many important roles in biochemistry as well as in waste-water and atmospheric chemistries. This radical is able to react with essentially all organic molecules with rate constants approaching the diffusional limit. This is particularly the case in water, where the rate constants can reach 10^{9} – 10^{10} $M^{-1}s^{-1}$ making the biologic systems very vulnerable to oxidative damage which can eventually cause cell death. As will be shown later, H-atom abstractions from organic substrates by $HO^{\bullet}$ occur very quickly in water whereas in acetonitrile or other solvents devoid of (abstractable) H-atoms or double bonds (*e.g.*, 1,1,2-trifluoro-1,2,2-trichloroethane, Freon 113) these reactions take place at a lower rate [2]. The hydrophobic regions of biological cells are therefore more resilient to attacks by $HO^{\bullet}$ [2]. On the other hand, this radical contributes to the maintenance of a balanced atmospheric composition through radical-chain oxidations that destroy organic pollutants released in the atmosphere [3,4]. The $HO^{\bullet}$ radical also removes water pollutants including organic and inorganic compounds and bacteria, and thus is important in waste-water treatment processes [4].

In biological systems, reactive oxygen species (ROS), which include $HO^{\bullet}$ and other oxygen radicals, are produced as byproducts of aerobic metabolism [5]. Incomplete transfer of electrons to dioxygen O_2 yields: the superoxide radical anion $O_2^{\bullet-}$ by a one-electron reduction; hydrogen peroxide H_2O_2 by a two-electron reduction; and hydroxyl radical $HO^{\bullet} + HO^{-}$ formed from a three-electron reduction of O_2. Therefore, H_2O_2 can easily form the hydroxyl radical $HO^{\bullet}$ by a one-electron reduction as exemplified in reaction 1. The reductant necessary in reaction 1 can be:

$$H_2O_2 + e^- \rightarrow OH^- + HO^{\bullet} \qquad (1)$$

i) the superoxide radical anion $O_2^{\bullet}$ in the presence of Fe^{3+}/Fe^{2+} (or Cu^{2+}/Cu^{1+} or other transition metal ions) as a catalyst (iron-catalyzed Haber-Weiss reaction) [6]; *ii*) ferrous salts (Fenton reaction) [7]; *iii*) ascorbic acid with or without iron involvement [6,8]. Furthermore, several other reactions that account for the formation of $HO^{\bullet}$ *in vivo* are known [9]. Whatever the origin, all ROS are harmful to human health because they initiate free radical–catalyzed oxidations of biomolecules such as DNA, proteins, lipids of low-density lipoprotein (LDL) and cell membranes, polysaccharides [5] with major consequences for life. Therefore, molecules able to

"capture and deactivate" free radicals (antiradicals and antioxidants) are very important because they have the power to protect the biochemical heritage of the cells. There are several "assays" for measuring the antiradical/antioxidant abilities of compounds according to the radicals involved [10,11]. In the case of the $HO^{\bullet}$ radical, the 2-deoxyribose assay (see below) is largely utilized. This method, however, suffers from a serous limitation because the solvent in the assay is water and many potential anti-$HO^{\bullet}$ compounds are insoluble in this solvent. By employing a well-known (sparingly soluble in water) scavenger of $HO^{\bullet}$, namely, α-lipoic acid (LA, see Figure 1), we explored the effects of several water-soluble organic co-solvents on the 2-DR assay with the aim of improving the solubility of α-lipoic acid. The results of this exploration are presented in this paper.

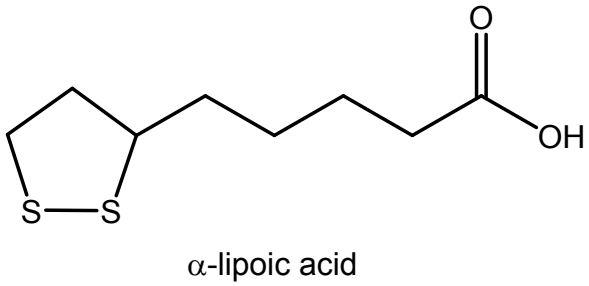


Figure 1. Structure of (*R,S*)-α-lipoic acid.

2. Materials and Methods

2.1. Reagents and solutions

All compounds and solvents were purchased from Sigma-Aldrich Co. (Italy) and were of analytical grade. All solutions were prepared in distilled water. Stock solutions of 2-deoxyribose (25 mM) and phosphate buffer (pH 7.4) were prepared and kept in a refrigerator at 4-6 °C until use. A stock solution of EDTA (10 mM) was prepared at room temperature and pH 7 following the method of Lopes and colleagues [12]. Fresh stock solutions of 10 mM $FeCl_3$, 2 mM H_2O_2 and 0.5 mM ascorbic acid were prepared daily. $FeCl_3$ was dissolved in 0.1 M HCl. 0.5 ml of 10 mM $FeCl_3$ were mixed with 9.5 ml of 10 mM EDTA to obtain Fe^{3+}-EDTA complex (500 μM Fe^{3+}). Fresh solutions of the tested organic compounds were prepared daily. Stock solution of 1% TBA (w/v) was prepared in 50 mM NaOH and was used within one week.

Aqueous solutions of methanol, ethanol, 2-propanol, dimethyl sulfoxide, THF, acetone, 1,4-dioxane, DMF and acetonitrile were prepared at various concentrations in distilled water. Then, 200 µl of these solutions were added to the mixture prepared as described in section 2.3 in place of LA. The final concentration of the solvents ranged from 0.1 $\mu l \times ml^{-1}$ to 4 $\mu l \times ml^{-1}$. The assay procedure was identical to that described in section 2.3.

2.2. Preparation of Stock Solutions of α-Lipoic Acid

Stock solutions of 500 µM LA (5.16 mg of LA in 50 mL of 20 mM phosphate buffer) in phosphate buffer at pH 7.4 were obtained by treating the initial suspension with ultrasound (15 min. at 40 °C). These solutions were then diluted so as to have a final concentration of LA in the assay in the range 1 – 100 µM. At pH 7.4, LA is almost completely dissociated because its pKa is 4.82 [13], and given the amphiphilic structure of the anion it is likely that in these aqueous solutions the LA anion aggregates in micelles [14].

Limpid stock solutions of 5 mM LA in water/acetonitrile 98:2 and 95:5 v/v were prepared by treating the initial suspension of LA with ultrasound for 10 – 15 minutes at room temperature. These solutions were used to prepare the samples for the 2-DR assay at final concentrations of LA in the range 1 – 200 µM. The quantity of acetonitrile present in these final solutions was kept constant at 2µL/mL (for the solutions prepared with the 95% water/acetonitrile solution) and 0.8 µL/mL (for the solutions prepared with the 98% water/acetonitrile solution). Despite the low concentration of acetonitrile, these LA solutions were limpid and stable because the pre-solubilization with larger quantities of acetonitrile (2 – 5%) facilitated the process of solubilization in water.

2.3. The 2-Deoxyribose Assay of α-Lipoic Acid in phosphate buffer

The 2-DR assay was performed following the protocol reported in ref. [15] which is briefly described here for a typical experiment. In a screw capped glass tube, 100 µl of 50 µM Fe^{3+}-EDTA, 100 µl of 200 µM H_2O_2, 200 µl of 5 mM 2-DR and 200 µl of 20 mM phosphate buffer (pH 7.4) were sequentially added. Then, 200 µl of LA solutions (see, Section 2.2), diluted so as to achieve the final concentration range of 1 - 100 µM, were added. The reaction was finally initiated by adding 200 µl of 100 µM ascorbic acid to a final volume of 1 ml at 25 °C. After 40 min, the reaction was stopped by the addition of 1 ml of 4% (v/v) phosphoric acid; then, 1 ml of

1% (w/v) TBA was added. The test-tube was therefore placed for 15 min in a hot water bath (90 – 95 °C) to develop a pink color. Then, the test-tube was cooled down at room temperature and the absorbance was recorded at 532 nm. A reference test was made by replacing the LA solution with 200 μL of buffer. All tests were performed in triplicate. The error was ±10 – 15%.

2.4. The 2-DR Assay of α-Lipoic Acid in water/acetonitrile

Stock water/acetonitrile solutions of LA (5 mM) were daily prepared as described in Section 2.2. These solutions were used to prepare samples for the 2-DR assay at concentrations of LA spanning a larger range (1 – 200 μM) than the one in phosphate buffer.

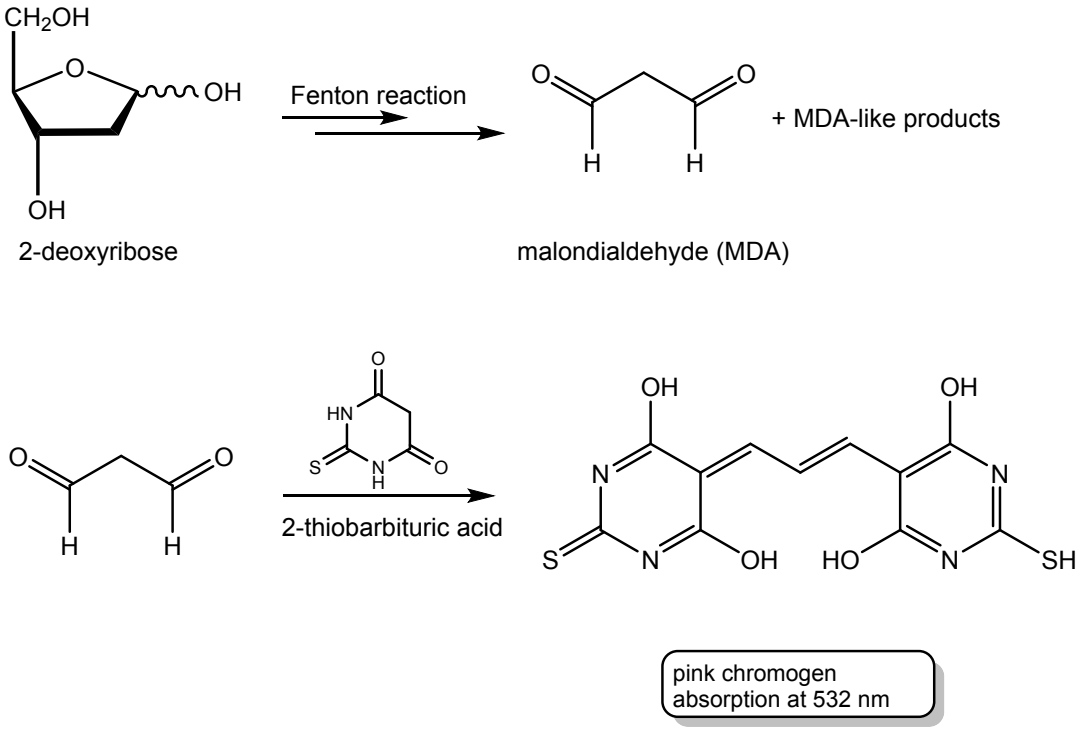
The concentration of acetonitrile in the final solutions of the 2-DR assay was kept constant at 2 and 0.8 μL mL^{-1} (see Section 2.2). A reference test was made by replacing the LA solution with 200 μL of water/acetonitrile. The assay procedure was identical to that described in section 2.3.

3. Results and discussion

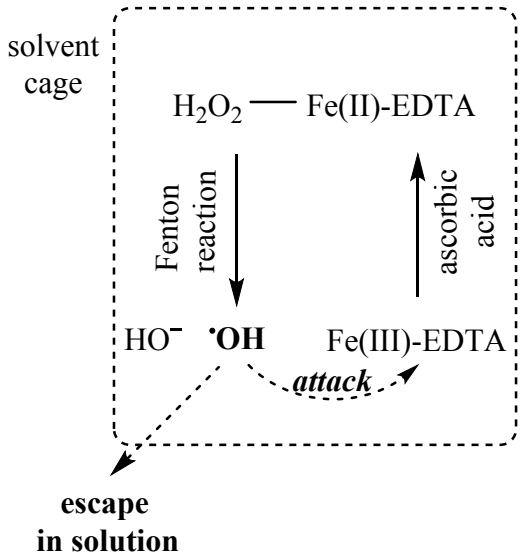
The $HO^{\bullet}$ radical reacts very quickly with the pentose 2-deoxyribose (2-DR) in aqueous solutions (k_{2DR} = 3.1×10^9 $M^{-1}s^{-1}$) to give a mixture of compounds [16]. Some of these compounds, when heated at acidic pHs, transform into malondialdehyde (MDA) and other carbonyl reactive species generally referred to as MDA-like products, which are detected by reaction with 2-thiobarbituric acid (TBA). This reaction produces a pink chromogen with λ_{max} = 532 nm, see Scheme 1. The $HO^{\bullet}$ radicals are generated by dissolving Fe(III)-EDTA, H_2O_2 and ascorbic acid in water. Ascorbic acid reduces Fe^{3+} to Fe^{2+} and the latter produces $HO^{\bullet}$ through the "Fenton reaction" with H_2O_2 [7]. It has been observed that the reaction of Fe^{2+}-EDTA with H_2O_2 is unlikely to occur via an outer-sphere electron-transfer mechanism [7b]. The first step in this process is suggested to be the formation of a transient complex Fe^{2+}-EDTA•••H_2O_2 which decomposes to $HO^{\bullet}$ radical [7b], see Scheme 2.

The large reactivity of $HO^{\bullet}$ toward EDTA (k = 2.76×10^9 $M^{-1}s^{-1}$ [17]) and the fact that the generation of the $HO^{\bullet}$ radicals takes place in the vicinity of Fe(III)-EDTA, make it possible that some fraction of $HO^{\bullet}$ reacts *in cage* with the iron complex (Scheme 2). The remaining fraction of

HO• escapes in solution where can be scavenged by 2-DR. In the presence of LA (or any other HO• scavenger), a *competition* kinetics is established since LA competes with the sugar for HO• preventing therefore the formation of MDA and MDA-like compounds. The absorbance of the solution at 532 nm after treatment with TBA allows therefore to obtain the rate constant for the reaction of HO• with LA.



Scheme 1. Reaction scheme from 2-deoxyribose to the pink chromogen $(TBA)_2$-MDA.



Scheme 2. Generation of HO• radicals *via* the Fenton reaction in H_2O_2 + Fe^{3+}-EDTA + ascorbic acid mix. Some fraction of the HO• radicals attacks *in cage* the complex Fe^{3+}-EDTA (dotted curved arrow) because of the high rate of HO• + EDTA and the proximity of the complex; the remaining (probably small) fraction diffuses freely in solution.

The kinetic treatment of the reaction gives the equation,

$$\frac{A^0_{532}}{A_{532}} = 1 + \frac{k_{\mathrm{LA}}[LA]}{k_{\mathrm{2DR}}[2\text{ - DR}]} \qquad (2)$$

where A_{532} and A^0_{532} are the absorbances at 532 nm in the presence and in the absence of LA, respectively (see Section 2.3); k_{LA} and k_{2DR} the rate constants of LA and 2-DR for the reaction with HO•. This equation is valid because the formation of HO• is slow [17] and its stationary concentration is small in comparison with the concentrations of LA.

Equation 2 shows that a plot A^0_{532} / A_{532} against [LA] should be a straight line of slope k_{LA} / (k_{2DR}×[2-DR]) from which the rate constant k_{LA} is obtained (k_{2DR} = 3.1×10^9 $M^{-1}s^{-1}$ [16] and [2-DR] = 1 mM, see Section 2.3). Figure 2 shows the straight-line obtained with our data acquired in the range of LA concentration 1 – 100 µM (higher concentrations were achieved upon previous solubilisation in water/acetonitrile, see below). The slope is 3200 M^{-1} from which we calculate that k_{LA} is equal to $3200 \times 3.1\times10^9 \times 1\times10^{-3} \approx 1\times10^{10}$ $M^{-1}s^{-1}$ (in water at 25 °C). Our

rate constant appears to be in good agreement with several previously published values of this rate constant, *e.g*. 4.7×10^{10} $M^{-1}s^{-1}$ [18]; 1.92×10^{10} $M^{-1}s^{-1}$ [19]; 3.0×10^{10} $M^{-1}s^{-1}$ [20].

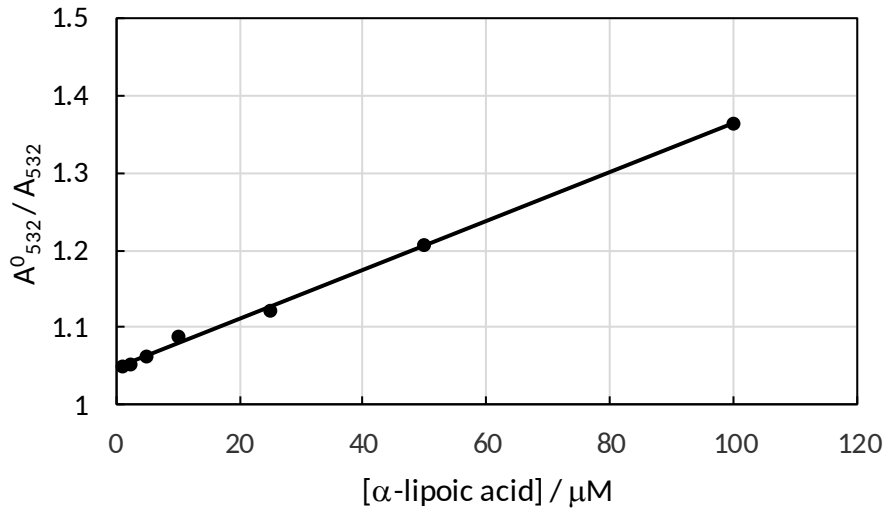


Figure 2. Plot of A^0_{532} /A_{532} *versus* [LA]. The slope of the straight line is equal to 3.2×10^3 M^{-1} and the *R*-squared regression coefficient is 0.998. The rate constant for the HO• + LA reaction is calculated from the slope (see text) to be *ca*. 1×10^{10} $M^{-1}s^{-1}$ (in water at 25 °C). The error is ca. ±10%.

Several controls must be employed before using the 2-DR assay [9]. Here, we want to recall the reader's attention on one issue: the solubility of the scavengers in the assay milieu, a problem well recognized and stated in a leading methodological paper [20] but generally overlooked by most of the assay users. In fact, many possible scavengers of HO• are poorly soluble in water and hence they cannot be assayed. This makes the use of a water-soluble organic co-solvent largely desirable, though, organic solvents, like most organic molecules, react quickly with HO•, therefore interfering with the test.

However, we surprisingly observed (as others, [2,21-24]) that acetonitrile was "poorly" reactive toward the HO• radical while other solvents (acetone, DMSO, DMF, THF, EtOH, MeOH, 2-propanol, 1,4-diox) reacted very rapidly. In Figure 3, the HO• scavenging activity (equal to $(1 - A_{532} / A^0_{532})\times100$) of the solvents is shown. Under our experimental conditions, acetonitrile was able at a concentration of 4 μL/mL to capture only 19% of the HO• radicals while the other solvents scavenged 70 – 90% of the radicals, see Figure 3. By representing again A^0_{532} / A_{532} against [acetonitrile], see Figure 4, we found that the rate constant for the reaction

$HO^{\bullet}$ + CH_3CN in water was only 8.7×10^6 $M^{-1}s^{-1}$ *i.e.* several orders of magnitude lower than those of 2-DR, LA and the other solvents. This value agrees well with the value of 2.2×10^7 $M^{-1}s^{-1}$ reported by Neta and Schuler [21]. Other highly reactive oxygen-centred radicals show little reactivity toward acetonitrile [22], *which is often used as a solvent in the reactions of these species*. The reason for this reduced reactivity seems to be related to the highly polar transition state of these reactions.

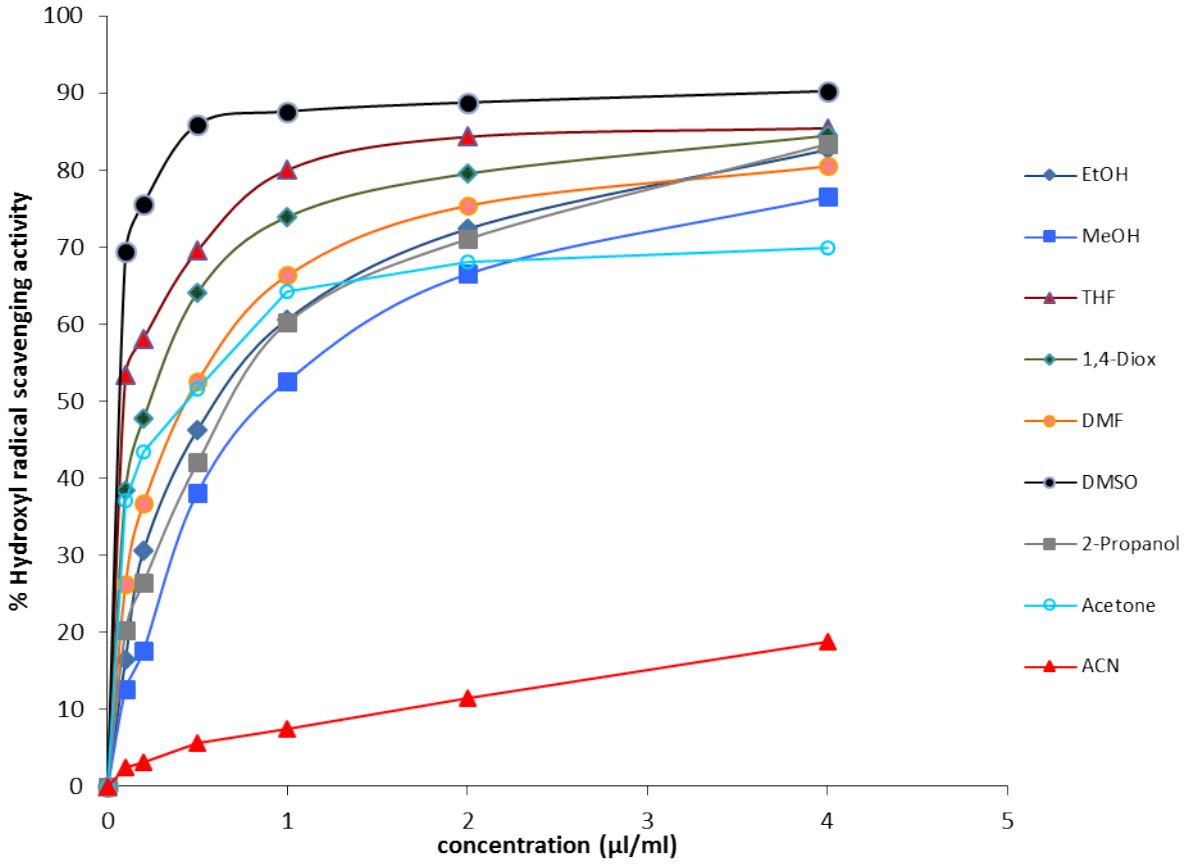


Figure 3. Scavenging ability of several organic solvents toward the $HO^{\bullet}$ radical measured as % of $HO^{\bullet}$ eliminated from the solution in competition with 2-DR, see text. The calculation was done with the equation: % of scavenged $HO^{\bullet}$ = $(1 - A_{532}/A^0_{532}) \times 100$. The concentration of the solvents ranged from 0.1 to 4 µL per mL of solution.

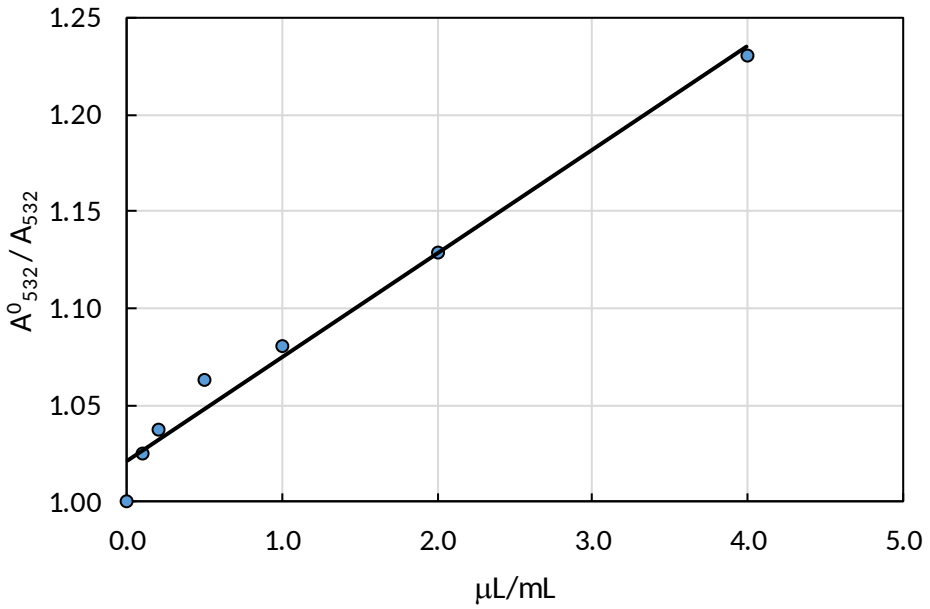


Figure 4. Plot of the ratio A^0_{532} / A_{532} against [acetonitrile] (expressed as µL of acetonitrile per mL of solution). The *R*-squared regression coefficient is 0.98 and the slope (after conversion of µL/mL in mol/L) is 2.794 M^{-1} from which the rate constant for $HO^{\bullet}$ + CH_3CN is calculated to be 8.7×10^6 $M^{-1}s^{-1}$. The error is ca. ±15%.

Tanko et al. [2] reported that the H-atom abstraction from hydrocarbons (R-H) by $HO^{\bullet}$ in water is much faster than in acetonitrile, the rate constants in water being nearly two orders of magnitude greater [2]. The presence of electron withdrawing groups in the hydrocarbon RH decreases its reactivity while electron donating groups increase it, see Figure 5. These kinetic data and solvent effects support the idea that the transition state for R-H + $HO^{\bullet}$ is polar. Computational studies [2, 23] show that the oxygen atom of the hydroxyl radical increases its negative charge in the transition state (a positive charge is therefore formed in R-H). Hence, the reinforcement of the hydrogen bonding with water determines a strong stabilization of the transition state and an acceleration of the H-atom abstraction. Electron-donating groups enhance the stability of the transition state because they support the delocalization of the positive charge. On the other hand, electron-withdrawing groups (*e.g.* the nitrile group in CH_3-C≡N) prevent or reduce the polarization of the transition state, see Figure 5.

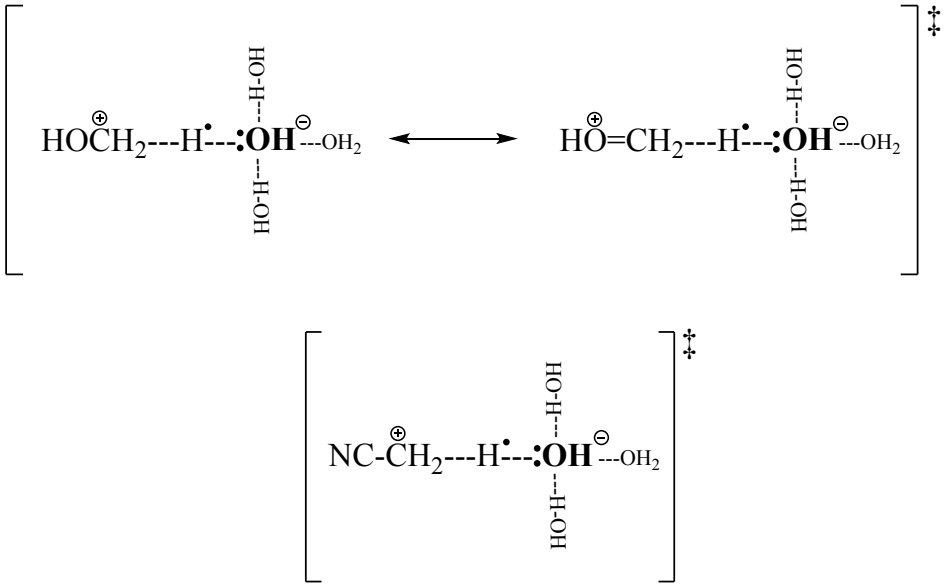


Figure 5. Transition states for H-atom abstraction by HO• from methanol and acetonitrile. The negative charge developed on the HO• radical implies the formation of a partial positive charge on the carbon atom of the C-H bond, which is partially delocalised over the oxygen atom in CH_3OH but for CH_3CN, the -C≡N group is strongly electron-withdrawing and less able to support the delocalization of the positive charge. As a consequence, the rate constant of the reaction with methanol is 95-fold greater than that with acetonitrile (see ref. [2]).

The low reactivity of acetonitrile toward HO• and consequently the low interference with the 2-DR assay allowed us to use it as a co-solvent (2 – 5 % v/v) in the preparation of stock solutions at high concentration of LA (5 mM) (in preliminary experiments, LA was dissolved in a 90:10 water/acetonitrile mixture but the solvent interference was too strong to ensure the reliability of the results). We could therefore explore the reactivity of LA in a larger range of concentrations relative to that reached in the simple phosphate buffer, *i.e.* 1 – 200 μM *vs.* 1 – 100 μM. Figure 6 shows that the reactivity of LA toward HO• is not affected by CH_3CN because the slopes of the three straight-lines (in other terms, the rate constants) are identical and the linearity of the plot within 1 – 200 μM is excellent.

In conclusion, the solubility of LA is significantly improved by CH_3CN. This solvent is characterized by a remarkably low reactivity toward HO•, the rate constant being *only* 8.7×10^6 $M^{-1}s^{-1}$. Calculations show that the transition state of the reactions involving HO• is highly polar and thus the nitrile group of CH_3CN, being electron-withdrawing, does not support the

delocalization of the charge making the reaction relatively slow. We took advantage of this slow reactivity by using CH_3CN as a co-solvent in our 2-DR assay. The reactivity of LA toward $HO^{\bullet}$ was therefore explored in a relatively large range of concentrations. We found that the rate constant of LA + $HO^{\bullet}$ in water is diffusion controlled, being *ca*. 1×10^{10} $M^{-1}s^{-1}$, therefore making LA an excellent scavenger of $HO^{\bullet}$ radicals**.**

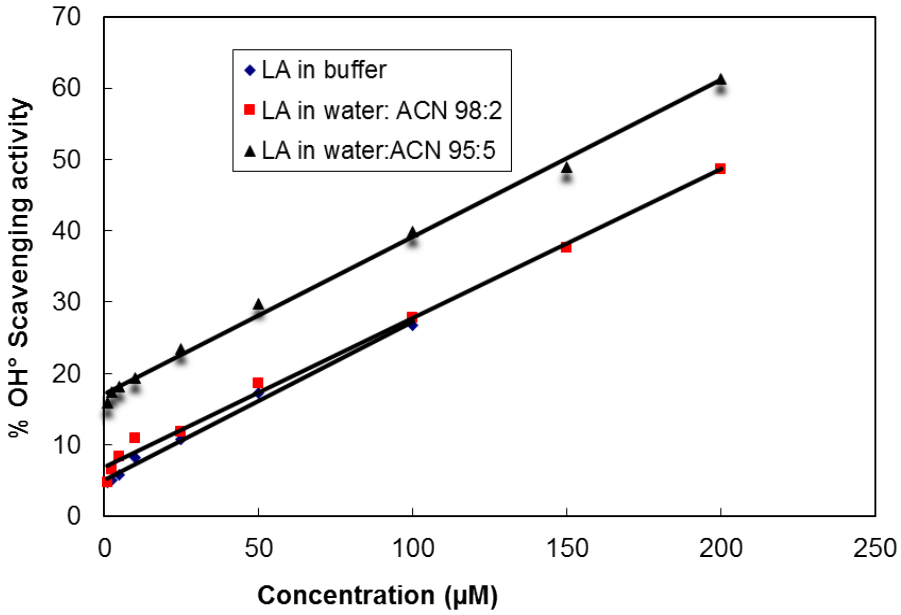


Figure 6. Scavenging activity of LA against $HO^{\bullet}$, $(1 - A_{532}/A^0_{532}) \times 100$, in water and water containing acetonitrile ($\leq$ 2 μL/mL). The slope of the three straightlines is identical, therefore indicating that the rate constant of $HO^{\bullet}$ + LA does not change. The larger intercept of the straightline with solid black triangles is due to the reaction of $HO^{\bullet}$ with CH_3CN which is present at a larger concentration (2 μL/mL) in this data set.

Acknowledgements

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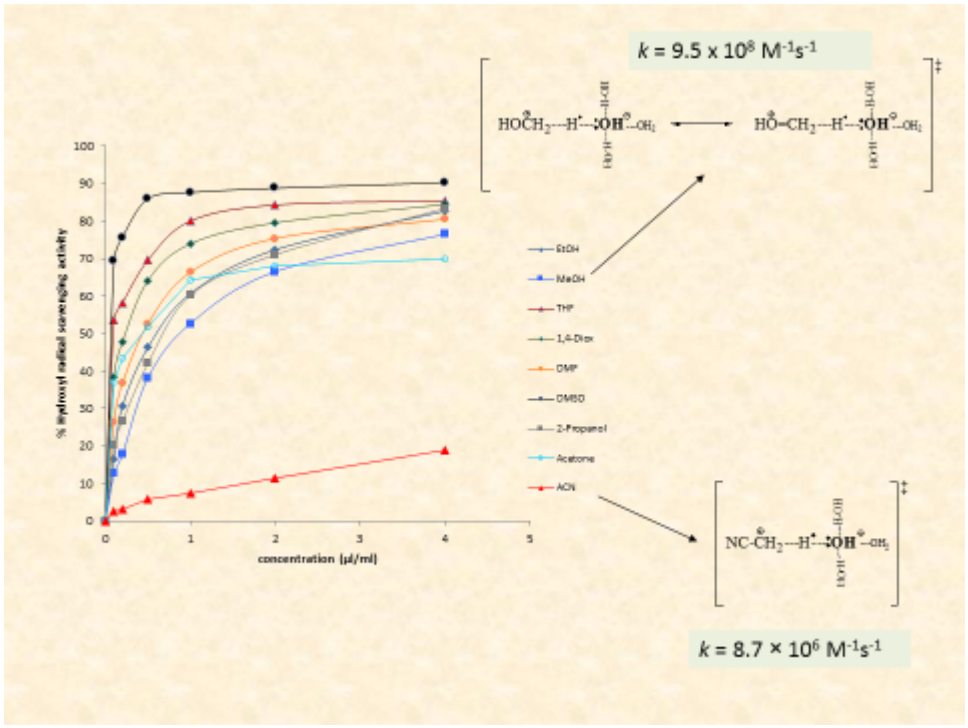
Graphical Abstract



Effect of organic co-solvents in the evaluation of the hydroxyl radical scavenging activity by the 2-deoxyribose degradation assay: the paradigmatic case of α-lipoic acid

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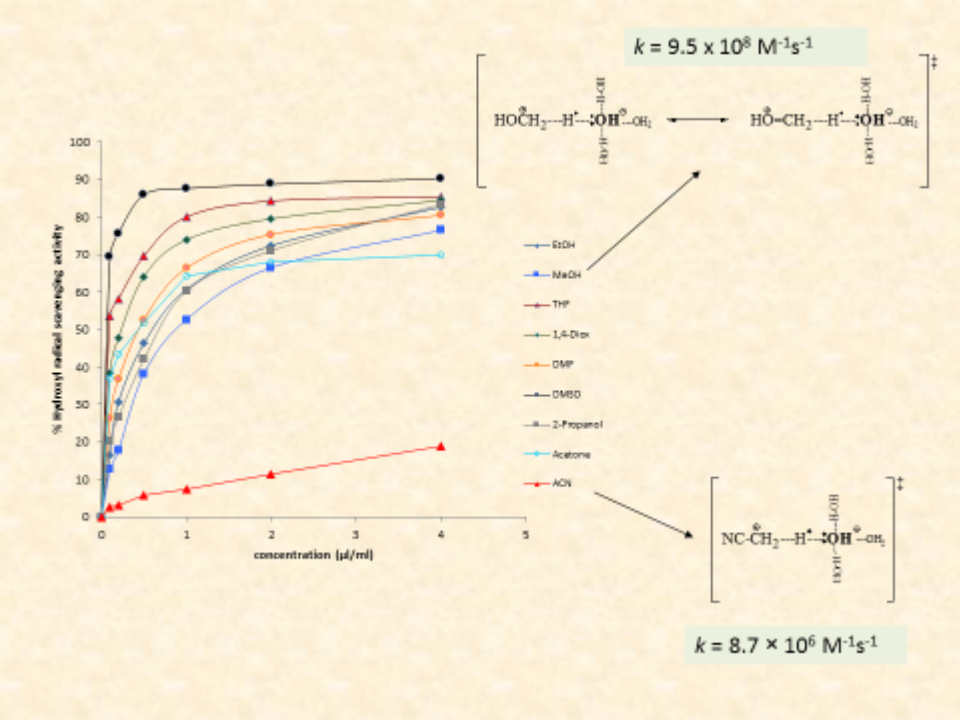
Graphical Abstract



Abstract

The 2-deoxyribose degradation assay (2-DR assay) is an *in vitro* method broadly used for evaluating the scavenging activity against the hydroxyl radical ($HO^{\bullet}$). One of the major drawbacks of the assay, however, is that only water soluble compounds can be tested for their radical-scavenging activity. Lipoic acid (LA) is an excellent scavenger of $HO^{\bullet}$ but it exhibits a low solubility in the aqueous milieu of the 2-DR assay and a high tendency to polymerize under a variety of conditions. We used LA as a paradigmatic substrate to evaluate the effect of several organic co-solvents in increasing its solubility. Most of these solvents, however, demonstrated to be potent scavengers of $HO^{\bullet}$ making their use in the 2-DR assay improper. On the other hand, acetonitrile showed a remarkably low reactivity toward $HO^{\bullet}$ (rate constant ~ 8.7×10^{6} $M^{-1}s^{-1}$) which allowed us to use it as a co-solvent in the preparation of stock solutions of LA ~ 5 mM. We therefore evaluated the radical-scavenging activity of LA by the 2-DR assay in a relatively large range of concentrations, 1 – 200 μM. We found that the rate constant for LA + $HO^{\bullet}$ is diffusion-controlled (~1×10^{10} $M^{-1}s^{-1}$ in water at 25 °C) and uninfluenced by the presence of small quantities of acetonitrile. Therefore, the use of acetonitrile in the 2-DR assay does not interfere with the test and may increase the solubility of the radical scavengers.

Keywords: 2-deoxyribose degradation assay, hydroxyl radical, scavenging activity, lipoic acid, acetonitrile.

List of abbreviations:

2-DR: 2-deoxyribose
DMF: Dimethylformamide
DMSO: Dimethylsulfoxide
EDTA: Ethylenediaminetetraacetic acid
LA: (*R*,*S*)-lipoic acid
MDA: Malondialdehyde
ROS: Reactive oxygen species
TBA: Thiobarbituric acid

1. Introduction

The hydroxyl radical $HO^{\bullet}$ is a short-lived species (half-life in solution ~ 10^{-9} s) [1] that has many important roles in biochemistry as well as in waste-water and atmospheric chemistries. This radical is able to react with essentially all organic molecules with rate constants approaching the diffusional limit. This is particularly the case in water, where the rate constants can reach $10^9 - 10^{10}$ $M^{-1}s^{-1}$ making the biologic systems very vulnerable to oxidative damage which can eventually cause cell death. As will be shown later, H-atom abstractions from organic substrates by $HO^{\bullet}$ occur very quickly in water whereas in acetonitrile or other solvents devoid of (abstractable) H-atoms or double bonds (*e.g.*, 1,1,2-trifluoro-1,2,2-trichloroethane, Freon 113) these reactions take place at a lower rate [2]. The hydrophobic regions of biological cells are therefore more resilient to attacks by $HO^{\bullet}$ [2]. On the other hand, this radical contributes to the maintenance of a balanced atmospheric composition through radical-chain oxidations that destroy organic pollutants released in the atmosphere [3,4]. The $HO^{\bullet}$ radical also removes water pollutants including organic and inorganic compounds and bacteria, and thus is important in waste-water treatment processes [4].

In biological systems, reactive oxygen species (ROS), which include $HO^{\bullet}$ and other oxygen radicals, are produced as byproducts of aerobic metabolism [5]. Incomplete transfer of electrons to dioxygen O_2 yields: the superoxide radical anion $O_2^{\bullet-}$ by a one-electron reduction; hydrogen peroxide H_2O_2 by a two-electron reduction; and hydroxyl radical $HO^{\bullet} + HO^-$ formed from a three-electron reduction of O_2. Therefore, H_2O_2 can easily form the hydroxyl radical $HO^{\bullet}$ by a one-electron reduction as exemplified in reaction 1. The reductant necessary in reaction 1 can be:

$$H_2O_2 + e^- \rightarrow OH^- + HO^{\bullet} \qquad (1)$$

i) the superoxide radical anion $O_2^{\bullet}$☒ in the presence of Fe^{3+}/Fe^{2+} (or Cu^{2+}/Cu^{1+} or other transition metal ions) as a catalyst (iron-catalyzed Haber-Weiss reaction) [6]; *ii*) ferrous salts (Fenton reaction) [7]; *iii*) ascorbic acid with or without iron involvement [6,8]. Furthermore, several other reactions that account for the formation of $HO^{\bullet}$ *in vivo* are known [9]. Whatever the origin, all ROS are harmful to human health because they initiate free radical–catalyzed oxidations of biomolecules such as DNA, proteins, lipids of low-density lipoprotein (LDL) and cell membranes, polysaccharides [5] with major consequences for life. Therefore, molecules able to

"capture and deactivate" free radicals (antiradicals and antioxidants) are very important because they have the power to protect the biochemical heritage of the cells. There are several "assays" for measuring the antiradical/antioxidant abilities of compounds according to the radicals involved [10,11]. In the case of the $HO^{\bullet}$ radical, the 2-deoxyribose assay (see below) is largely utilized. This method, however, suffers from a serous limitation because the solvent in the assay is water and many potential anti-$HO^{\bullet}$ compounds are insoluble in this solvent. By employing a well-known (sparingly soluble in water) scavenger of $HO^{\bullet}$, namely, α-lipoic acid (LA, see Figure 1), we explored the effects of several water-soluble organic co-solvents on the 2-DR assay with the aim of improving the solubility of α-lipoic acid. The results of this exploration are presented in this paper.

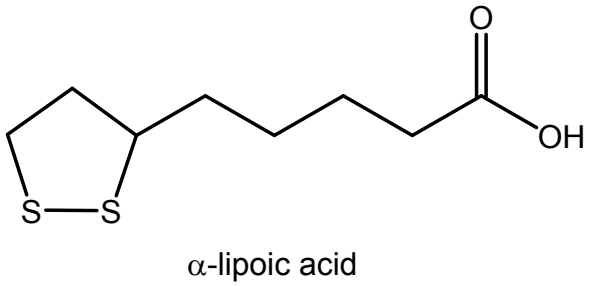


Figure 1. Structure of (*R,S*)-α-lipoic acid.

2. Materials and Methods

2.1. Reagents and solutions

All compounds and solvents were purchased from Sigma-Aldrich Co. (Italy) and were of analytical grade. All solutions were prepared in distilled water. Stock solutions of 2-deoxyribose (25 mM) and phosphate buffer (pH 7.4) were prepared and kept in a refrigerator at 4-6 °C until use. A stock solution of EDTA (10 mM) was prepared at room temperature and pH 7 following the method of Lopes and colleagues [12]. Fresh stock solutions of 10 mM $FeCl_3$, 2 mM H_2O_2 and 0.5 mM ascorbic acid were prepared daily. $FeCl_3$ was dissolved in 0.1 M HCl. 0.5 ml of 10 mM $FeCl_3$ were mixed with 9.5 ml of 10 mM EDTA to obtain Fe^{3+}-EDTA complex (500 μM Fe^{3+}). Fresh solutions of the tested organic compounds were prepared daily. Stock solution of 1% TBA (w/v) was prepared in 50 mM NaOH and was used within one week.

Aqueous solutions of methanol, ethanol, 2-propanol, dimethyl sulfoxide, THF, acetone, 1,4-dioxane, DMF and acetonitrile were prepared at various concentrations in distilled water. Then, 200 µl of these solutions were added to the mixture prepared as described in section 2.3 in place of LA. The final concentration of the solvents ranged from 0.1 $\mu l \times ml^{-1}$ to 4 $\mu l \times ml^{-1}$. The assay procedure was identical to that described in section 2.3.

2.2. Preparation of Stock Solutions of α-Lipoic Acid

Stock solutions of 500 µM LA (5.16 mg of LA in 50 mL of 20 mM phosphate buffer) in phosphate buffer at pH 7.4 were obtained by treating the initial suspension with ultrasound (15 min. at 40 °C). These solutions were then diluted so as to have a final concentration of LA in the assay in the range 1 – 100 µM. At pH 7.4, LA is almost completely dissociated because its pKa is 4.82 [13], and given the amphiphilic structure of the anion it is likely that in these aqueous solutions the LA anion aggregates in micelles [14].

Limpid stock solutions of 5 mM LA in water/acetonitrile 98:2 and 95:5 v/v were prepared by treating the initial suspension of LA with ultrasound for 10 – 15 minutes at room temperature. These solutions were used to prepare the samples for the 2-DR assay at final concentrations of LA in the range 1 – 200 µM. The quantity of acetonitrile present in these final solutions was kept constant at 2µL/mL (for the solutions prepared with the 95% water/acetonitrile solution) and 0.8 µL/mL (for the solutions prepared with the 98% water/acetonitrile solution). Despite the low concentration of acetonitrile, these LA solutions were limpid and stable because the pre-solubilization with larger quantities of acetonitrile (2 – 5%) facilitated the process of solubilization in water.

2.3. The 2-Deoxyribose Assay of α-Lipoic Acid in phosphate buffer

The 2-DR assay was performed following the protocol reported in ref. [15] which is briefly described here for a typical experiment. In a screw capped glass tube, 100 µl of 50 µM Fe^{3+}-EDTA, 100 µl of 200 µM H_2O_2, 200 µl of 5 mM 2-DR and 200 µl of 20 mM phosphate buffer (pH 7.4) were sequentially added. Then, 200 µl of LA solutions (see, Section 2.2), diluted so as to achieve the final concentration range of 1 - 100 µM, were added. The reaction was finally initiated by adding 200 µl of 100 µM ascorbic acid to a final volume of 1 ml at 25 °C. After 40 min, the reaction was stopped by the addition of 1 ml of 4% (v/v) phosphoric acid; then, 1 ml of

1% (w/v) TBA was added. The test-tube was therefore placed for 15 min in a hot water bath (90 – 95 °C) to develop a pink color. Then, the test-tube was cooled down at room temperature and the absorbance was recorded at 532 nm. A reference test was made by replacing the LA solution with 200 μL of buffer. All tests were performed in triplicate. The error was ±10 – 15%.

2.4. The 2-DR Assay of α-Lipoic Acid in water/acetonitrile

Stock water/acetonitrile solutions of LA (5 mM) were daily prepared as described in Section 2.2. These solutions were used to prepare samples for the 2-DR assay at concentrations of LA spanning a larger range (1 – 200 μM) than the one in phosphate buffer.

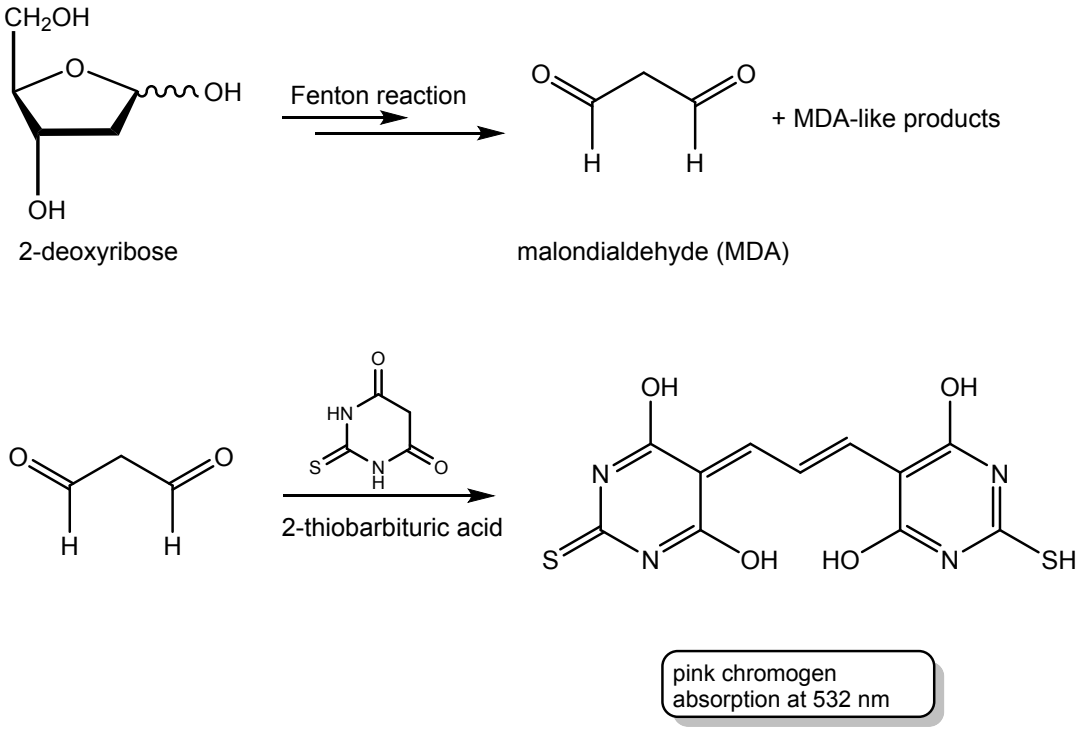
The concentration of acetonitrile in the final solutions of the 2-DR assay was kept constant at 2 and 0.8 μL mL^{-1} (see Section 2.2). A reference test was made by replacing the LA solution with 200 μL of water/acetonitrile. The assay procedure was identical to that described in section 2.3.

3. Results and discussion

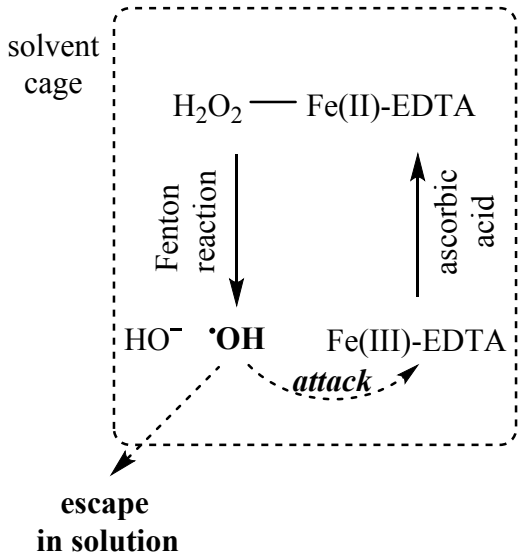
The $HO^{\bullet}$ radical reacts very quickly with the pentose 2-deoxyribose (2-DR) in aqueous solutions (k_{2DR} = 3.1×10^9 $M^{-1}s^{-1}$) to give a mixture of compounds [16]. Some of these compounds, when heated at acidic pHs, transform into malondialdehyde (MDA) and other carbonyl reactive species generally referred to as MDA-like products, which are detected by reaction with 2-thiobarbituric acid (TBA). This reaction produces a pink chromogen with λ_{max} = 532 nm, see Scheme 1. The $HO^{\bullet}$ radicals are generated by dissolving Fe(III)-EDTA, H_2O_2 and ascorbic acid in water. Ascorbic acid reduces Fe^{3+} to Fe^{2+} and the latter produces $HO^{\bullet}$ through the "Fenton reaction" with H_2O_2 [7]. It has been observed that the reaction of Fe^{2+}-EDTA with H_2O_2 is unlikely to occur via an outer-sphere electron-transfer mechanism [7b]. The first step in this process is suggested to be the formation of a transient complex Fe^{2+}-EDTA•••H_2O_2 which decomposes to $HO^{\bullet}$ radical [7b], see Scheme 2.

The large reactivity of $HO^{\bullet}$ toward EDTA (k = 2.76×10^9 $M^{-1}s^{-1}$ [17]) and the fact that the generation of the $HO^{\bullet}$ radicals takes place in the vicinity of Fe(III)-EDTA, make it possible that some fraction of $HO^{\bullet}$ reacts *in cage* with the iron complex (Scheme 2). The remaining fraction of

HO• escapes in solution where can be scavenged by 2-DR. In the presence of LA (or any other HO• scavenger), a *competition* kinetics is established since LA competes with the sugar for HO• preventing therefore the formation of MDA and MDA-like compounds. The absorbance of the solution at 532 nm after treatment with TBA allows therefore to obtain the rate constant for the reaction of HO• with LA.



Scheme 1. Reaction scheme from 2-deoxyribose to the pink chromogen $(TBA)_2$-MDA.



Scheme 2. Generation of HO• radicals *via* the Fenton reaction in H_2O_2 + Fe^{3+}-EDTA + ascorbic acid mix. Some fraction of the HO• radicals attacks *in cage* the complex Fe^{3+}-EDTA (dotted curved arrow) because of the high rate of HO• + EDTA and the proximity of the complex; the remaining (probably small) fraction diffuses freely in solution.

The kinetic treatment of the reaction gives the equation,

$$\frac{A_{532}^{0}}{A_{532}} = 1 + \frac{k_{\mathrm{LA}}[LA]}{k_{\mathrm{2DR}}[2\text{ - DR}]} \qquad (2)$$

where A_{532} and A^0_{532} are the absorbances at 532 nm in the presence and in the absence of LA, respectively (see Section 2.3); k_{LA} and k_{2DR} the rate constants of LA and 2-DR for the reaction with HO•. This equation is valid because the formation of HO• is slow [17] and its stationary concentration is small in comparison with the concentrations of LA.

Equation 2 shows that a plot A^0_{532} / A_{532} against [LA] should be a straight line of slope k_{LA} / (k_{2DR}×[2-DR]) from which the rate constant k_{LA} is obtained (k_{2DR} = 3.1×10^9 $M^{-1}s^{-1}$ [16] and [2-DR] = 1 mM, see Section 2.3). Figure 2 shows the straight-line obtained with our data acquired in the range of LA concentration 1 – 100 μM (higher concentrations were achieved upon previous solubilisation in water/acetonitrile, see below). The slope is 3200 M^{-1} from which we calculate that k_{LA} is equal to $3200 \times 3.1\times10^9 \times 1\times10^{-3} \approx 1\times10^{10}$ $M^{-1}s^{-1}$ (in water at 25 °C). Our

rate constant appears to be in good agreement with several previously published values of this rate constant, *e.g*. 4.7×10^{10} $M^{-1}s^{-1}$ [18]; 1.92×10^{10} $M^{-1}s^{-1}$ [19]; 3.0×10^{10} $M^{-1}s^{-1}$ [20].

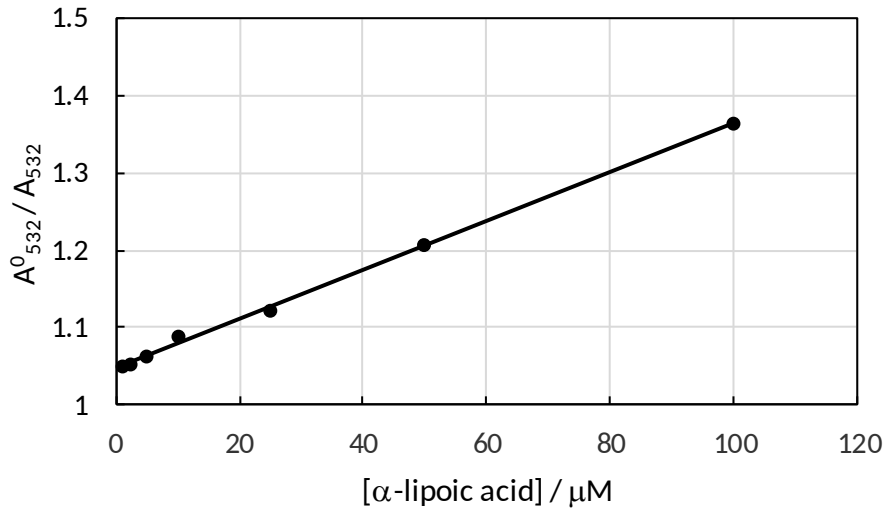


Figure 2. Plot of A^0_{532} /A_{532} *versus* [LA]. The slope of the straight line is equal to 3.2×10^3 M^{-1} and the *R*-squared regression coefficient is 0.998. The rate constant for the HO• + LA reaction is calculated from the slope (see text) to be *ca*. 1×10^{10} $M^{-1}s^{-1}$ (in water at 25 °C). The error is ca. ±10%.

Several controls must be employed before using the 2-DR assay [9]. Here, we want to recall the reader's attention on one issue: the solubility of the scavengers in the assay milieu, a problem well recognized and stated in a leading methodological paper [20] but generally overlooked by most of the assay users. In fact, many possible scavengers of HO• are poorly soluble in water and hence they cannot be assayed. This makes the use of a water-soluble organic co-solvent largely desirable, though, organic solvents, like most organic molecules, react quickly with HO•, therefore interfering with the test.

However, we surprisingly observed (as others, [2,21-24]) that acetonitrile was "poorly" reactive toward the HO• radical while other solvents (acetone, DMSO, DMF, THF, EtOH, MeOH, 2-propanol, 1,4-diox) reacted very rapidly. In Figure 3, the HO• scavenging activity (equal to $(1 - A_{532} / A^0_{532})\times100$) of the solvents is shown. Under our experimental conditions, acetonitrile was able at a concentration of 4 μL/mL to capture only 19% of the HO• radicals while the other solvents scavenged 70 – 90% of the radicals, see Figure 3. By representing again A^0_{532} / A_{532} against [acetonitrile], see Figure 4, we found that the rate constant for the reaction

$HO^{\bullet}$ + CH_3CN in water was only 8.7×10^6 $M^{-1}s^{-1}$ *i.e.* several orders of magnitude lower than those of 2-DR, LA and the other solvents. This value agrees well with the value of 2.2×10^7 $M^{-1}s^{-1}$ reported by Neta and Schuler [21]. Other highly reactive oxygen-centred radicals show little reactivity toward acetonitrile [22], *which is often used as a solvent in the reactions of these species*. The reason for this reduced reactivity seems to be related to the highly polar transition state of these reactions.

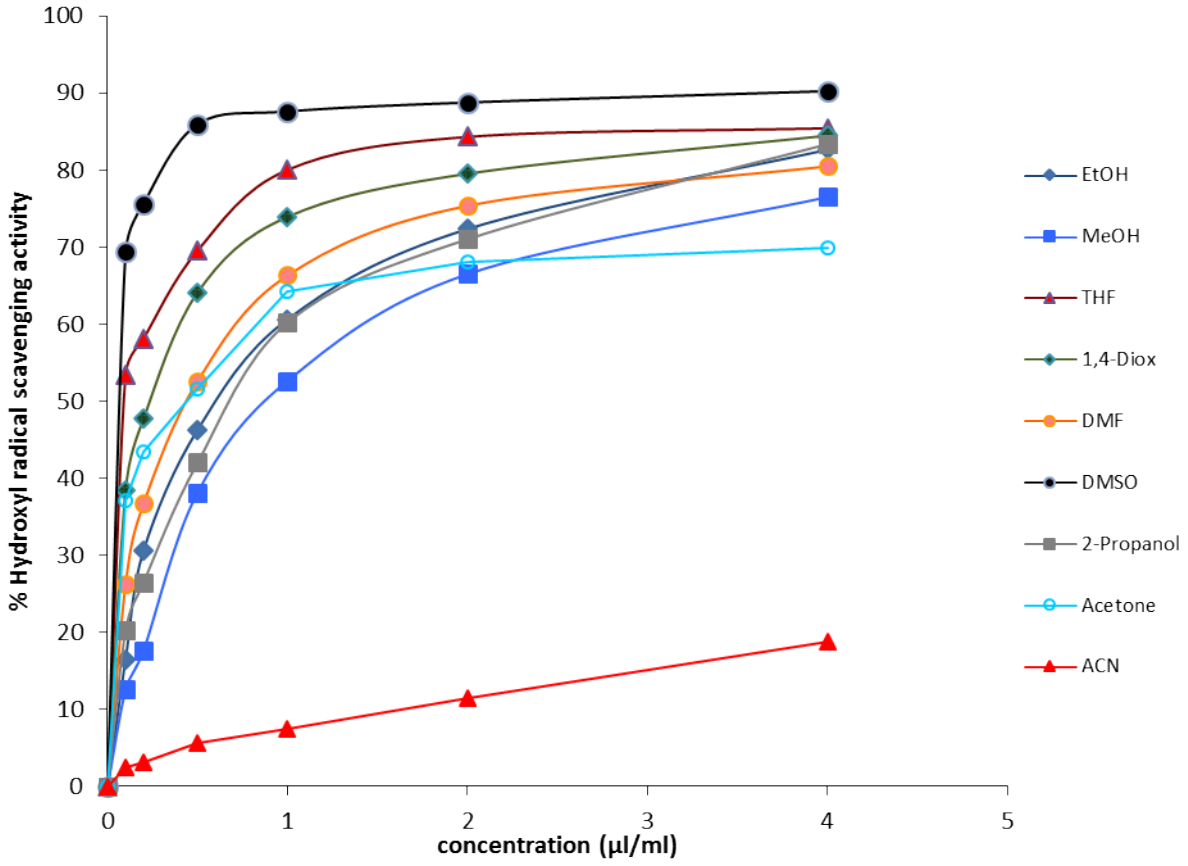


Figure 3. Scavenging ability of several organic solvents toward the $HO^{\bullet}$ radical measured as % of $HO^{\bullet}$ eliminated from the solution in competition with 2-DR, see text. The calculation was done with the equation: % of scavenged $HO^{\bullet}$ = $(1 - A_{532}/A^0_{532}) \times 100$. The concentration of the solvents ranged from 0.1 to 4 µL per mL of solution.

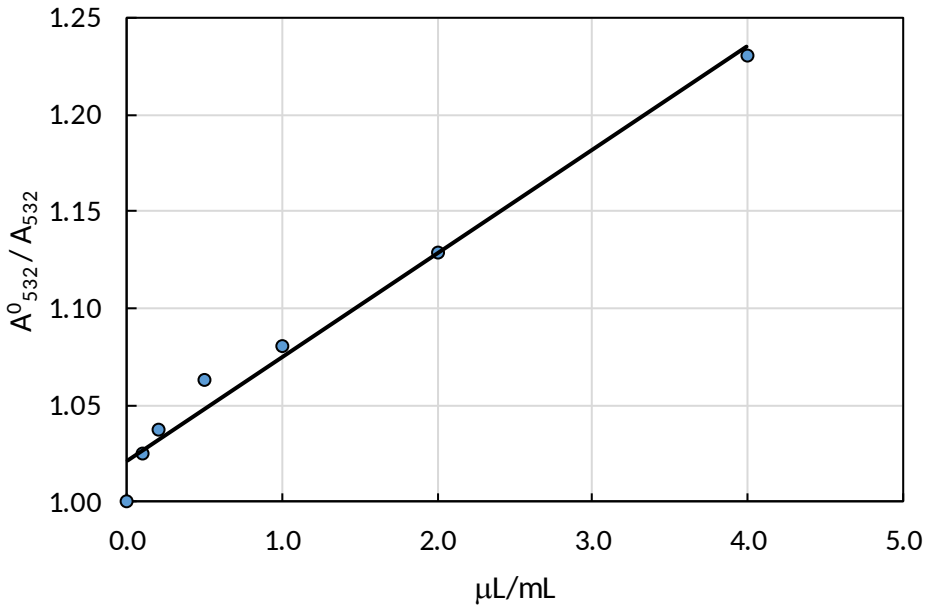


Figure 4. Plot of the ratio A^0_{532} / A_{532} against [acetonitrile] (expressed as µL of acetonitrile per mL of solution). The *R*-squared regression coefficient is 0.98 and the slope (after conversion of µL/mL in mol/L) is 2.794 M^{-1} from which the rate constant for $HO^{\bullet}$ + CH_3CN is calculated to be 8.7×10^6 $M^{-1}s^{-1}$. The error is ca. ±15%.

Tanko et al. [2] reported that the H-atom abstraction from hydrocarbons (R-H) by $HO^{\bullet}$ in water is much faster than in acetonitrile, the rate constants in water being nearly two orders of magnitude greater [2]. The presence of electron withdrawing groups in the hydrocarbon RH decreases its reactivity while electron donating groups increase it, see Figure 5. These kinetic data and solvent effects support the idea that the transition state for R-H + $HO^{\bullet}$ is polar. Computational studies [2, 23] show that the oxygen atom of the hydroxyl radical increases its negative charge in the transition state (a positive charge is therefore formed in R-H). Hence, the reinforcement of the hydrogen bonding with water determines a strong stabilization of the transition state and an acceleration of the H-atom abstraction. Electron-donating groups enhance the stability of the transition state because they support the delocalization of the positive charge. On the other hand, electron-withdrawing groups (*e.g.* the nitrile group in CH_3-C≡N) prevent or reduce the polarization of the transition state, see Figure 5.

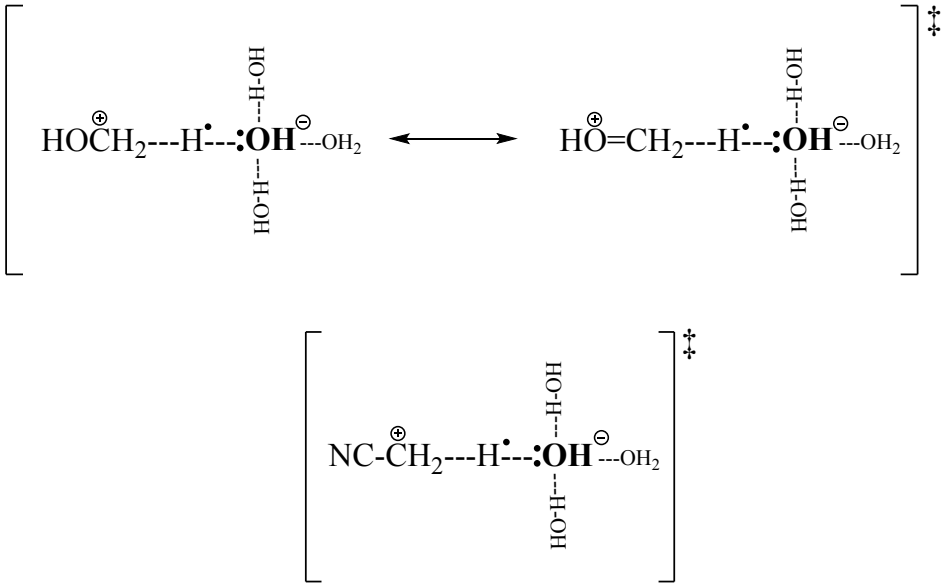


Figure 5. Transition states for H-atom abstraction by HO• from methanol and acetonitrile. The negative charge developed on the HO• radical implies the formation of a partial positive charge on the carbon atom of the C-H bond, which is partially delocalised over the oxygen atom in CH_3OH but for CH_3CN, the -C≡N group is strongly electron-withdrawing and less able to support the delocalization of the positive charge. As a consequence, the rate constant of the reaction with methanol is 95-fold greater than that with acetonitrile (see ref. [2]).

The low reactivity of acetonitrile toward HO• and consequently the low interference with the 2-DR assay allowed us to use it as a co-solvent (2 – 5 % v/v) in the preparation of stock solutions at high concentration of LA (5 mM) (in preliminary experiments, LA was dissolved in a 90:10 water/acetonitrile mixture but the solvent interference was too strong to ensure the reliability of the results). We could therefore explore the reactivity of LA in a larger range of concentrations relative to that reached in the simple phosphate buffer, *i.e.* 1 – 200 μM *vs.* 1 – 100 μM. Figure 6 shows that the reactivity of LA toward HO• is not affected by CH_3CN because the slopes of the three straight-lines (in other terms, the rate constants) are identical and the linearity of the plot within 1 – 200 μM is excellent.

In conclusion, the solubility of LA is significantly improved by CH_3CN. This solvent is characterized by a remarkably low reactivity toward HO•, the rate constant being *only* 8.7×10^6 $M^{-1}s^{-1}$. Calculations show that the transition state of the reactions involving HO• is highly polar and thus the nitrile group of CH_3CN, being electron-withdrawing, does not support the

delocalization of the charge making the reaction relatively slow. We took advantage of this slow reactivity by using CH_3CN as a co-solvent in our 2-DR assay. The reactivity of LA toward $HO^{\bullet}$ was therefore explored in a relatively large range of concentrations. We found that the rate constant of LA + $HO^{\bullet}$ in water is diffusion controlled, being *ca*. 1×10^{10} $M^{-1}s^{-1}$, therefore making LA an excellent scavenger of $HO^{\bullet}$ radicals**.**

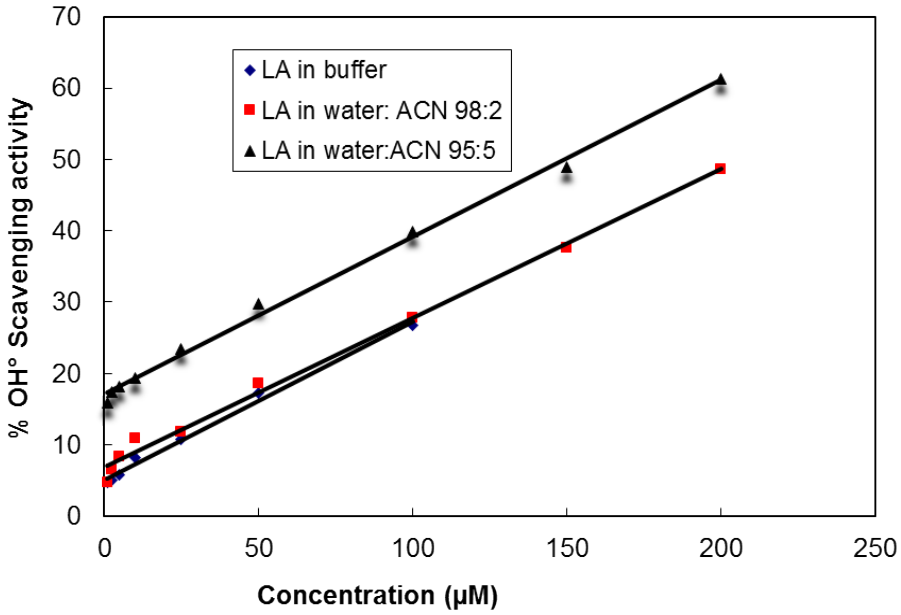


Figure 6. Scavenging activity of LA against $HO^{\bullet}$, $(1 - A_{532}/A^0_{532}) \times 100$, in water and water containing acetonitrile ($\leq$ 2 μL/mL). The slope of the three straightlines is identical, therefore indicating that the rate constant of $HO^{\bullet}$ + LA does not change. The larger intercept of the straightline with solid black triangles is due to the reaction of $HO^{\bullet}$ with CH_3CN which is present at a larger concentration (2 μL/mL) in this data set.

Acknowledgements

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Highlights

► Use of the 2-deoxyribose assay with molecules poorly soluble in water;

►Surprisingly low reactivity of acetonitrile toward the $HO^{\bullet}$ radical;

►Polar transition state for the H-atom abstraction from hydrocarbons by $HO^{\bullet}$;