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Fe(II)–EDTA chelate-induced aromatic hydroxylation of terephthalate as a new method for the evaluation of hydroxyl radical-scavenging ability

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The investigation of Fe(II)–EDTA chelate-induced aromatic hydroxylation of terephthalate in pH 7.4 phosphate buffer solution and a new method for the evaluation of hydroxyl radical-scavenging ability are reported. The method is based on attack of the hydroxyl radical on the terephthalate to produce highly fluorescent 2-hydroxyterephthalate, which is detected fluorimetrically. The formation of hydroxyl radical is believed to be the result of the reduction of molecular oxygen by Fe(II)–EDTA to form superoxide radical, which in turn dismutates to hydrogen peroxide, and then Fe(II)–EDTA catalyzes the decomposition of hydrogen peroxide to produce hydroxyl radical. The mechanism of the generation of hydroxyl radical in the proposed system was confirmed. This study established a simple and inexpensive method for the evaluation of the scavenging ability of some compounds on hydroxyl radicals.

Introduction

Oxygen-derived free radicals are potent agents causing many pathological effects and aging.[1] Among the various radicals, the hydroxyl radical (•OH), which is formed non-enzymatically from hydrogen peroxide (H_2O_2) in a metal-dependent reaction is the most reactive and toxic oxygen radical known to date.[2] They can initiate radical chain reactions such as lipid peroxidation,[2,3] and have been suggested to play a critical role in many pathological processes. The reactions of •OH with biomolecules have been a subject of particular interest owing to its diverse actions in biological systems which may even lead to lethal damage. It is therefore clearly important to understand the mechanism by which •OH can be efficiently neutralized. Some methods have been developed to study the character of •OH, including electron spin resonance[4,5] and aromatic hydroxylation.[6–9] Chemiluminescence (CL), a highly sensitive method, has also been widely used for monitoring •OH.[10]

Since •OH cannot be detoxified enzymatically, antioxidants have evolved as scavengers to protect against oxidative stress. Many experiments have been conducted on the effects of radical scavengers *in vivo* and *in vitro*. Among them, one of the most commonly used is the CL method.[11,12] The method is based on the monitoring of the luminescence arising from the reaction of luminol with reactive free radicals. Although this method is highly sensitive, it suffers from some drawbacks. First, the CL signal is transient, which is not favorable for investigating the mechanism of scavenging •OH. Second, the specificity of the CL method is not good, as other oxygen-derived species, such as $O_2^{-\bullet}$, singlet oxygen and H_2O_2 will also give rise to the CL signal at the same time,[13] which makes the investigation of the mechanism of •OH scavenging complicated.

In this study, a new method for monitoring •OH was developed. The method employed the reaction of •OH with terephthalate to produce 2-hydroxyterephthalate (HOTP) quantitatively, which then was quantified fluorimetrically. The •OH was generated *via* the reaction of molecular oxygen with Fe(II)–EDTA to produce superoxide radical ($O_2^{-\bullet}$), which in turn dismutated to H_2O_2, and then Fe(II)–EDTA catalyzed the decomposition of H_2O_2 to form •OH (Scheme 1). The •OH produced in the present method was similar to that in the traditional Fenton system, but the present method could produce •OH at physiological pH (pH 7.4) without the addition of H_2O_2. Hence the present method is a simple, fast and efficient way to generate •OH.

The method was then applied to study the •OH scavenging ability of some antioxidants and biological materials in pH 7.4 phosphate buffer solution. A quantitative relationship between •OH scavenger concentration and fluorescence signal was also derived.

$$\text{Fe(II)–EDTA} + O_2 \rightarrow \text{Fe(III)–EDTA} + O_2^{-\bullet} \quad (1)$$

$$2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

$$\text{Fe(II)–EDTA} + O_2^{-\bullet} + 2H^+ \rightarrow \text{Fe(III)–EDTA} + H_2O_2 \quad (3)$$

$$\text{Fe(II)–EDTA} + H_2O_2 \xrightarrow{k_0} \text{Fe(III)–EDTA} + OH^- + {}^\bullet OH \quad (4)$$

Scheme 1

Experimental

Apparatus

The fluorescence spectra and relative fluorescence intensity were measured with a Shimadzu RF-5000 spectrofluorimeter with a 10 mm quartz cuvette. The excitation wavelength was set at 326 nm and the emission wavelength at 432 nm. The excitation and the emission bandpasses were both set at 10 nm.

Reagents

A stock standard solution of 1.0×10^{-3} mol l^{-1} terephthalate was prepared by dissolving 0.0166 g of terephthalic acid (Merk, > 98%) in 100 ml of 0.01 mol l^{-1} NaOH solution. A 1.0×10^{-3} mol l^{-1} ferrous ion solution was prepared by dissolving an

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appropriate amount of ammonium ferrous sulfate in 5.0×10^{-3} mol l^{-1} H_2SO_4. Catalase at 1090 U mg^{-1} was prepared by dissolving an appropriate amount of catalase (Sigma, 10 900 U mg^{-1}) in 0.05 mol l^{-1} phosphate buffer solution (pH 7.0).

The following analytical-reagent grade reagents were used as solutions in distilled, de-ionized water: EDTA (0.01 mol l^{-1}), dimethyl sulfoxide (DMSO) (0.1 mol l^{-1}), citric acid (0.0l mol l^{-1}), glucose (0.01 mol l^{-1}), ethanol (0.01 mol l^{-1}), thiourea (0.01 mol l^{-1}), sodium formate (0.01 mol l^{-1}), L-methionine (0.01 mol l^{-1}), cytosine (5.0×10^{-3} mol l^{-1}) and guanine (1.0×10^{-3} mol l^{-1}).

Measurement procedure

The generation and trapping of ·OH were performed as follows. In a 10 ml calibrated tube containing 1.0 ml of 1.0×10^{-4} mol l^{-1} terephthalate, 0.30 ml of 1.0×10^{-3} mol l^{-1} EDTA, 0.30 ml of 1.0×10^{-3} mol l^{-1} of Fe(II) and 2.0 ml 0.20 mol l^{-1} of pH 7.4 phosphate buffer solution were added in that order and the mixture was diluted to volume with water. Scavengers were added before Fe(II) was introduced into the solution. The mixture was allowed to stand at room temperature for 6 min, then the relative fluorescence intensity was measured at 432 nm with an excitation wavelength of 326 nm.

Results and discussion

Formation of hydroxyl radical in the reaction mixture

Hydroxyl radicals can be produced by a variety of methods. Among them, pulse radiolysis[14] and the Fenton reaction[6,15] are the most commonly used methods. The pulse radiolysis method can generate ·OH specifically and efficiently, but the instrumentation for this method is expensive to set up and operate and is not available to many laboratories interested in free radical research. The Fenton reaction, the reaction between ferrous ion and H_2O_2, is considered to be among the simplest laboratory-scale reactions to produce ·OH. In spite of its wide utilization, this method has some limitations. The optimum conditions for the generation of ·OH by the Fenton reaction are pH 3–4 in H_2SO_4 medium,[16] which are unsuitable for the study of ·OH in some physiological processes. A modified Fenton reaction, the superoxide-driven Fenton reaction, was adopted in some physiological studies;[17] it produced ·OH by the reaction of xanthine with xanthine oxidase to produce O_2^{-}·, which then reacts Fe(III)–EDTA to generate ·OH. Although the method can produce ·OH at physiological pH, an expensive reagent, xanthine oxidase, is needed.

A variety of studies have demonstrated the ability of Fe(II) chelates or complexes to catalyze the formation of reactive oxygen species. Fe(II) is capable of catalyzing the redox reaction between oxygen and biological molecules. Some chelating agents, such as DTPA, histidine, EDTA and citrate, have been shown to facilitate the formation of reactive oxygen species.[18–21] This method produces ·OH in a simple way, and has been applied to study free radicals by using the CL method.[11] Although the CL method is highly sensitive, the transient CL signal makes it unsuitable for the study of ·OH in some respects. To overcome its limitations, an aromatic hydroxylation method based on the Fe(II)–EDTA chelate-induced formation of ·OH is described in this paper.

Some preliminary experiments were performed to prove the mechanism of the formation of ·OH in Scheme 1. First, to confirm that the formation of ·OH was indeed due to the presence of dissolved oxygen in aqueous solution, the reaction mixture was deoxygenated for 15 min with N_2 prior to the addition of Fe(II), and one can see that once the reaction mixture was partly deoxygenated, the fluorescence signal was considerably reduced compared with that under normal conditions, indicating that the generation of ·OH in the proposed system was oxygen mediated. Second, to establish whether H_2O_2 was produced in the reaction mixture, catalase, a specific H_2O_2 scavenger, was introduced, which would decompose H_2O_2 to water and molecular oxygen, and therefore decrease the amount of ·OH generated, thus decreasing the generation of highly fluorescent HOTP. The experimental results proved to be as expected, with a large decrease in fluorescence being observed on addition of catalase to the reaction mixture.

To confirm further that the decrease in fluorescence emission was indeed due to the breakdown of H_2O_2 by catalase and not fluorescence quenching of the system by catalase itself, the same amount of heat-denatured catalase (boiled for 10 min) was added to the solution at the same time, and no fluorescence quenching was observed. This gave strong evidence that H_2O_2 was involved in the formation of ·OH in the present system. Finally, a typical ·OH scavenger, DMSO,[22] was used to prove the formation of ·OH in the reaction mixture. Before the addition of Fe(II) to trigger the reaction, 5.0×10^{-4} mol l^{-1} DMSO was introduced into the reaction mixture and it was observed that the fluorescence of the system was almost fully quenched (as shown in Fig. 1). This was explained by DMSO competing with terephthalate for ·OH, and therefore decreasing the formation rate of HOTP. The same amount of DMSO was added to the solution after the aromatic hydroxylation reaction had finished, and no fluorescence quenching was observed. This showed that the fluorescence decrease was indeed due to the scavenging of ·OH by DMSO and not fluorescence quenching of the system by DMSO. The above experiments provided strong evidence that ·OH was produced in the reaction system.

Order of adding reagents

The order of adding the reagents in the reaction system was of great importance. We found that the optimum sequence of addition of reagents was terephthalate, EDTA, Fe(II) and phosphate buffer solution. If EDTA and Fe(II) were added

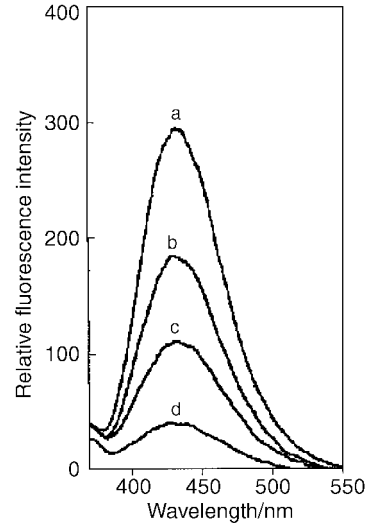

Fig. 1 Fluorescence emission spectra of the different systems. (a) Terephthalate–Fe(II)–EDTA system. (b) As (a) except that the solution was deoxygenated with N_2 for 15 min before the addition of Fe(II). (c) As (a) except for the addition of 20 μl of 1090 U ml^{-1} catalase before Fe(II) was added. (d) As (a) except the addition of 0.5 ml of 1.0×10^{-3} mol l^{-1} DMSO before Fe(II) was added. Terephthalate, 1.0×10^{-5} mol l^{-1}; Fe(II), 3.0×10^{-5} mol l^{-1}; EDTA, 3.0×10^{-5} mol l^{-1}. The reaction was carried out in pH 7.4 phosphate buffer solution for 6 min and then the fluorescence spectra were recorded.

before the terephthalate, a decreased signal was recorded. This could be explained by ·OH, generated by the reaction of Fe(II)–EDTA chelate with molecular oxygen, decaying before the aromatic hydroxylation occurred and therefore decreasing the amount of hydroxylated products formed.

Effect of reaction time

The kinetic behavior of the reaction was studied and the results are shown in Fig. 2. On the addition of Fe(II) to the reaction mixture to stimulate the reaction, the fluorescence signal was recorded as a function of reaction time. As can be seen, the fluorescence signal increased sharply during the first 4 min and then increased slowly with reaction time. As a compromise between high sensitivity and a short analysis time, a 6 min reaction time was selected in subsequent experiment.

Effect of Fe(II)–EDTA

Yildiz and Demiryurek[11] reported that Fe(II) could catalyze the oxidation of luminol by oxygen to give luminescence, and the mechanism was explained similarly to that in Scheme 1. In this experiment we compared the catalytic effect of Fe(II) and Fe(II)–EDTA and the results are shown in Fig. 3. It can be seen that the catalytic activity of Fe(II)–EDTA was much higher than that of Fe(II). This is understandable as the standard electrode potential of Fe(III)–EDTA/Fe(II)–EDTA is much lower than that of Fe(III)/Fe(II) [$E°_{Fe(III)/Fe(II)}$ = 0.77 V, $E°_{Fe(III)–EDTA/Fe(II)–EDTA}$ = 0.14 V]. Hence adding EDTA to Fe(II) solution enhanced its reductive capacity and therefore facilitated the formation of reactive oxygen species *via* the reaction of molecular oxygen with Fe(II), which in turn increased the amount of HOTP generated. Another explanation was that the reaction rate of Fe(II)–EDTA with H_2O_2 was much higher than that of Fe(II), thus increasing the formation rate of ·OH.[23]

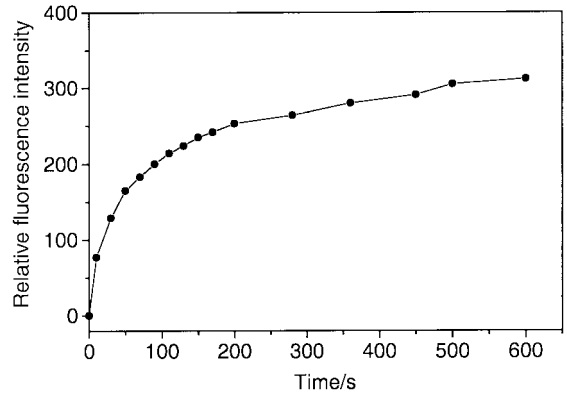

Fig. 2 Kinetic behavior of the system. Terephthalate, 1.0×10^{-5} mol l^{-1}; Fe(II), 3.0×10^{-5} mol l^{-1}; EDTA, 3.0×10^{-5} mol l^{-1}. The fluorescence intensity of the system was recorded as a function of reaction time in pH 7.4 phosphate buffer solution when Fe(II) was introduced.

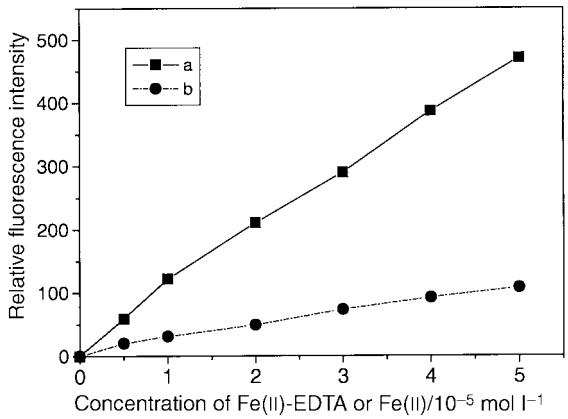

Fig. 3 Effect of the concentration of (a) Fe(II)-EDTA and (b) Fe(II) on the fluorescence intensity. Terephthalate, 1.0×10^{-5} mol l^{-1}. The reaction was carried out with different concentrations of Fe(II)–EDTA or Fe(II) at pH 7.4 phosphate buffer solution for 6 min and then the fluorescence intensity was recorded.

Fig. 3 also shows the effect of Fe(II)–EDTA concentration on the fluorescence signal; it can be seen that the signal was concentration dependent. The fluorescence intensity increased linearly with increase in Fe(II)–EDTA concentration in the range of $(5.0–50) \times 10^{-6}$ mol l^{-1}. A 3.0×10^{-5} mol l^{-1} concentration of Fe(II)–EDTA was selected for the investigation of the ·OH-scavenging ability of some antioxidants in subsequent studies.

Effect of terephthalate

We found that benzoate, phthalate, thiamine and terephthalate could all give strong fluorescence signals once mixed with Fe(II)–EDTA. Terephthalate was selected as the hydroxylation substrate for a number of reasons. First, as its carboxyl groups are *para* to each other, only one isomer of hydroxyterephthalate is formed *via* aromatic hydroxylation and no ·OH is wasted in the formation of hydroxy acids that do not contribute to the fluorescence measured, hence mechanistic interpretation is facilitated. Second, in weak alkaline solution terephthalate does not fluoresce and does not interfere with the fluorescence of HOTP, hence the fluorescence recorded for the reaction mixture represents the true concentration of hydroxylated product. Third, the hydroxylated product, HOTP, is stable and can be detected at physiological pH without much adverse effect on the sensitivity of the method. Fourth, the trapping of ·OH by terephthalate is specific, as other reactive oxygen species such as $O_2^{-\bullet}$, singlet oxygen and H_2O_2 will not react with terephthalate to give rise to the fluorescence signals, so the fluorescence signal is proportional to the amount of ·OH generated in the reaction mixture.

The effect of terephthalate concentration on the fluorescence intensity was studied, and the results showed that fluorescence signal increased with increasing terephthalate concentration. A 1.0×10^{-5} mol l^{-1} concentration of terephthalate was adopted in subsequent experiments.

Scavenging ability of some antioxidants

Any other molecule introduced to the reaction mixture that is capable of reacting with ·OH will compete with terephthalate for ·OH, and therefore decrease the amount of HOTP generated, which in turn will decrease the fluorescence signal. The competing scheme is illustrated in Scheme 2.

$$\text{terephthalate (TP)} + {}^{\bullet}\text{OH} \xrightarrow{k_1} \text{HOTP} \quad (5)$$

$$\text{scavenger (S)} + {}^{\bullet}\text{OH} \xrightarrow{k_2} \text{other products} \quad (6)$$

Scheme 2

In the presence of a scavenger of ·OH, the apparent formation rate of ·OH can be given by the following equations:

$$v_{{}^{\bullet}\text{OH}} = \frac{\mathrm{d}[{}^{\bullet}\text{OH}]}{\mathrm{d}t} = k_0[\text{Fe(II)–EDTA}][\text{H}_2\text{O}_2] - k_1[\text{TP}][{}^{\bullet}\text{OH}] - k_2[\text{S}][{}^{\bullet}\text{OH}] \quad (7)$$

At equilibrium, d[•OH]/dt = 0, yielding

$$[^{\bullet}\mathrm{OH}] = \frac{k_0[\mathrm{Fe(II)–EDTA}][\mathrm{H_2O_2}]}{k_1[\mathrm{TP}] + k_2[\mathrm{S}]} \quad (8)$$

and the formation rate of HOTP is given by

$$v_{\mathrm{HOTP}} = \frac{\mathrm{d[HOTP]}}{\mathrm{d}t} = k_1[\mathrm{TP}][^{\bullet}\mathrm{OH}] \quad (9)$$

Substitution of eqn. (8) into eqn. (9) yields the formation rate of HOTP in the presence of a scavenger:

$$v_{\mathrm{HOTP}} = \frac{\mathrm{d[HOTP]}}{\mathrm{d}t} = \frac{k_1[\mathrm{TP}]k_0[\mathrm{Fe(II)–EDTA}][\mathrm{H_2O_2}]}{k_1[\mathrm{TP}] + k_2[\mathrm{S}]} \quad (10)$$

On the other hand, in the absence of a scavenger, the apparent formation rate of •OH is given by the following equations:

$$v_{^{\bullet}\mathrm{OH}} = \frac{\mathrm{d}[^{\bullet}\mathrm{OH}]_0}{\mathrm{d}t} = k_0[\mathrm{Fe(II)} - \mathrm{EDTA}][\mathrm{H_2O_2}] - k_1[\mathrm{TP}][^{\bullet}\mathrm{OH}] \quad (11)$$

At equilibrium, d[•OH]$_0$/dt = 0, yielding

$$[^{\bullet}\mathrm{OH}]_0 = \frac{k_0[\mathrm{Fe(II)–EDTA}][\mathrm{H_2O_2}]}{k_1[\mathrm{TP}]} \quad (12)$$

and substitution of eqn. (12) into eqn. (9) yields the formation rate of HOTP in the absence of scavenger:

$$v_{\mathrm{HOTP_0}} = \frac{\mathrm{d[HOTP]_0}}{\mathrm{d}t} = k_1[\mathrm{TP}][^{\bullet}\mathrm{OH}] = \mathrm{k_0[Fe(II)–EDTA}][\mathrm{H_2O_2}] \quad (13)$$

By combining eqns. (10) and (13) we obtain

$$\frac{v_{\mathrm{HOTP_0}}}{v_{\mathrm{HOTP}}} = \frac{[\mathrm{HOTP}]_0}{[\mathrm{HOTP}]} = 1 + \frac{k_2[\mathrm{S}]}{k_1[\mathrm{TP}]} \quad (14)$$

Since terephthalate is non-fluorescent in pH 7.4 phosphate buffer solution, all of the measured fluorescence signal is attributed to the aromatic hydroxylation product, HOTP. Hence, the fluorescence signal recorded is proportional to the concentration of HOTP. Hence eqn. (14) can be expressed as

$$\frac{F_0}{F} = 1 + \frac{k_2[\mathrm{S}]}{\mathrm{k_1[TP]}} \quad (15)$$

where F_0 and F are the fluorescence intensities in the absence and presence of a scavenger, respectively, [TP] is the concentration of terephthalate and [S] is the concentration of the scavenger. Rearranging the terms logarithmically yields

$$\log\left(\frac{F_0}{F} - 1\right) = \log[\mathrm{S}] + \log\left(\frac{k_2}{k_1[\mathrm{TP}]}\right) \quad (16)$$

Since k_1, k_2 and the concentration of terephthalate were fixed in the experiment, the second term on the right-hand side of eqn. (16) was constant. The fluorescence signal was measured as a function of scavenger concentration, and a plot of log(F_0/F − 1) *versus* log[S] gave a straight line. The concentration of a scavenger producing half-inhibition of the controlled fluorescence intensity (IC_{50}) was obtained from log(F_0/F − 1) = 0 [when F = 1/2 F_0, log(F_0/F − 1) = 0].

A typical calibration curve for DMSO is given in Fig. 4, from which the IC_{50} of DMSO on •OH was obtained when the term log(F_0/F − 1) = 0, and was 2.57 × 10^{-5} mol l^{-1}. Using the present method, we evaluated the •OH-scavenging abilities of various compounds as IC_{50} values and the results are given in Table 1.

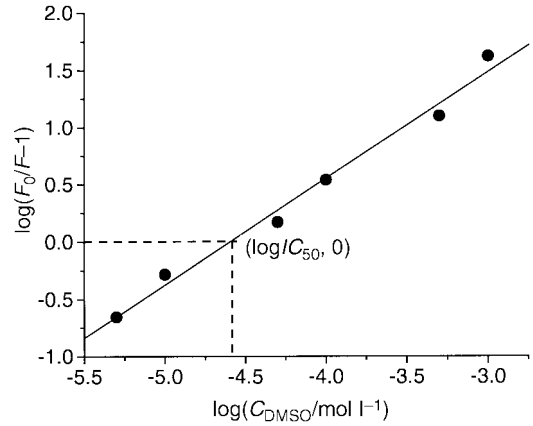

Fig. 4 Scavenging of hydroxyl radicals by DMSO of different concentrations. From the controlled (F_0) and the experimental fluorescence intensity (F), the term log(F_0/F − 1) was calculated and plotted against the logarithm of the concentration of DMSO.

Table 1 •OH-scavenging abilities of various compounds

Compound	IC_{50}/μM
Guanine	12.9
DMSO	25.7
Thiourea	43.9
Sodium formate	52.0
L-Methionine	85.0
Cytosine	86.0
Glucose	147
Ethanol	164

Conclusions

The present method provided a direct determination of •OH formation without the use of expensive instrumentation such as in electron paramagnetic resonance spectroscopy. Hydroxyl radicals were generated from the reaction of Fe(II)–EDTA with molecular oxygen. Compared with the traditional Fenton system used for the generation of •OH, the present method is simple, fast, needs no expensive reagents and can generate •OH at physiological pH. As the terephthalate molecule is symmetrical with respect to ring hydroxylation, only one hydroxylated product is formed, hence the mechanistic interpretation of the hydroxylation reaction was facilitated. The hydroxylated product, HOTP, is stable and can be detected with a standard fluorimeter. Furthermore, the trapping of •OH with terephthalate is specific, as other reactive oxygen species such as $O_2^{-\bullet}$, H_2O_2 and singlet oxygen do not react with terephthalate. Hence this approach could be a simple and convenient method for the evaluation of the scavenging ability of antioxidants.

Acknowledgements

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