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Stability of 5,5-dimethyl-1-pyrroline-*N*-oxide as a spin-trap for quantification of hydroxyl radicals in processes based on Fenton reaction

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A B S T R A C T

Fenton reaction was used to produce hydroxyl radicals under conditions similar to AOPs with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap agent in electron paramagnetic resonance (EPR) analysis. A theoretical kinetics model was developed to determine conditions under which the spin-adduct DMPO-OH is not further oxidized by Fe^{3+} and excessive radicals, so that hydroxyl radicals concentration could be accurately inferred. Experiments were designed based upon the model and H_2O_2 and Fe^{2+} concentrations were varied from 1 to 100 mM and from 0.1 to 10 mM, respectively, with a constant H_2O_2: Fe^{2+} ratio of 10:1. Results confirmed that DMPO concentration should be at least 20 times higher than the concentration of H_2O_2 and 200 times higher than iron concentration to produce stable DMPO-OH EPR signal. When DMPO: H_2O_2 ratio varied from 1 to 10, DMPO-OH could generate intermediates and be further oxidized leading to the apparition of an additional triplet. This signal was attributed to a paramagnetic dimer: its structure and a formation mechanism were proposed. Finally, the utilization of sodium sulfite and catalase to terminate Fenton reaction was discussed. Catalase appeared to be compatible with DMPO. However, sodium sulfite should be avoided since it reacted with DMPO-OH to form DMPO-SO$_3$.

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1. Introduction

Over the last two decades, interest in Advanced Oxidation Processes (AOPs) and Electrochemical Advanced Oxidation Processes (EAOPs) for the degradation of various organic pollutants has been growing constantly (Martínez-Huitle and Brillas, 2009; Oturan and Aaron, 2014). Although AOPs and EAOPs include different types of processes such as chemical, photochemical, sonochemical and electrochemical processes, they all aim to produce hydroxyl radicals (•OH). Indeed, •OH is a powerful oxidative agent (2.80 V/SHE) and a highly reactive species able to attack organic pollutants with second order rate constants ranging from 10^7 to 10^{10} L mol^{-1} s^{-1} (Lhomme et al., 2008). No matter which AOP, the generation of hydroxyl radicals is a key parameter to control and optimize in order to improve the efficiency of the process and to elucidate the degradation mechanisms. However, the very short lifetime of •OH

(about 10^{-9} s) makes its detection and quantification difficult, thus leading to the development of indirect detection methods including UV–Vis spectrophotometry (Zhao et al., 2015), luminescence (Tsai et al., 2001), fluorescence (Xiang et al., 2011) and other electrochemical and HPLC methods (Si et al., 2014). However, all of these analytical methods measure neutral products after hydroxyl radical was scavenged. The hydroxyl radical concentration is then inferred from the product concentration instead of measuring radical concentration directly. Although electron paramagnetic resonance (EPR) could not directly measure short-lived species such as •OH, this technique could detect longer-lived radical adducts generated by using spin-trap agents at concentration as low as 10^{-14} M (Haywood, 2013). To date, the very high majority of spin-trap agents used for the detection of •OH are nitrone compounds, mainly because they lead to the formation of relatively stable nitroxide radicals that can be detected by EPR. The current challenges mainly concern the improvement of the reactivity and stability of the spin-traps agents for a more reliable detection. Among them, α-phenyl-*N-tert*-butylnitrone (PBN), 1,1,3-trimethylisoindole *N*-oxide (TMINO), 5-diethoxyphosphoryl-5-

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methyl-1-pyrroline-*N*-oxide (DEPMPO) or 5-methyl-1-pyrroline *N*-oxide (DIPPMPO) have given interesting results, especially under biological conditions (Bottle et al., 2003; Chalier et al., 2014; Luo et al., 2009; Timmins et al., 1999). Nevertheless, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) is still the most commonly used compound. Its reaction with the previously cited species leads to the formation of relatively stable aminoxyl radicals detectable by EPR (Makino et al., 1990). Since the late 80s, the behavior of DMPO for the detection of hydroxyl radicals has been extensively studied, mostly in biological systems. Indeed, the detection of free radicals in living bodies has been of a great interest ever since they were suspected for being responsible of various diseases such as hypertension or cancer. It has been assumed that Fenton reaction is the most important pathway for hydroxyl radical generation in tissues (Yamazaki and Piette, 1990). Most of these studies were carried out with concentrations in hydrogen peroxide and ferrous iron hardly exceeding dozens of μM. To our knowledge, studies under conditions similar to those used in AOPs and EAOPs systems based on Fenton reaction (chemical Fenton, photo-Fenton, electro-Fenton, photoelectron-Fenton, etc.) are rarely reported, despite being of a high interest for these processes. In these systems, concentrations in Fenton reagent are much higher than those reported in biological systems. Indeed, concentrations in H_2O_2 and Fe^{2+} generally could range from hundreds of μM to hundreds of mM, and H_2O_2 concentration can even reach dozens of moles per liter for treatment of wastewaters containing high chemical oxygen demand (COD) loads (Brillas et al., 2000; Gulkaya et al., 2006; Martínez-Huitle and Brillas, 2009). As a result, the stability of DMPO and its •OH spin adduct (DMPO-OH) in presence of high concentrations of iron and hydrogen peroxide become a critical issue in accurate quantification of hydroxyl radical concentration, because of the formation of by-products (paramagnetic or not) affecting the reliability of DMPO-OH (and thus •OH) detection (Buettner, 1993; Li et al., 2007; Makino et al., 1990).

Fenton reaction has some well-known drawbacks such as large amount of sludge formation due to the precipitation of ferric hydroxides, scavenging of hydroxyl radicals by hydrogen peroxide, etc. In fact, AOPs and EAOPs are currently being developed to overcome these limitations, showing excellent potential for the treatment of wastewaters containing dyes (Brillas and Martínez-Huitle, 2015), pharmaceuticals (Antonin et al., 2015), pesticides (Zazou et al., 2015), and also for the treatment of municipal sludge (Fontmorin and Sillanpää, 2015). In order to improve their development in particular by gaining more fundamental understanding about the generation of •OH in these systems and about the degradation mechanisms involved, EPR is the most direct analytical instrument. In this study, we developed a theoretical model to determine under which conditions DMPO should be used to act as a reliable spin-trap for the quantification of •OH generated in AOPs based on Fenton reaction. To our knowledge, this is the first study that quantitatively shows the impact of DMPO concentration, relatively to Fenton's reagent concentration (i.e. H_2O_2 and Fe^{2+}), on the stability of the adduct in AOPs conditions. Therefore, this work could be useful to further understand the behavior of DMPO and other spin-trap agents in such systems, but also to optimize AOPs with a more reliable quantification of hydroxyl radicals.

2. Experimental

2.1. Chemicals

Hydrogen peroxide (H_2O_2, 30%) and iron sulfate ($FeSO_4 \cdot 7H_2O$) were purchased from Merck (Damstadt, Germany). Sodium sulfite (Na_2SO_3) and catalase from bovine liver were acquired by Sigma-Aldrich and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Caymen Chemical Company (Ann Arbor, USA). All chemicals were of analytical grade and used without further purification.

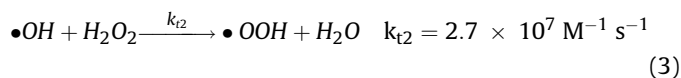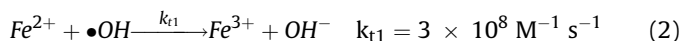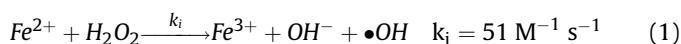### 2.2. Analysis: EPR and UV–Vis measurements

EPR data was obtained using an electron spin (paramagnetic) resonance spectrometer CMS-8400 from ADANI, Minsk, Belarus. The operating conditions were as following: magnetic field 336.5 ± 6.0 mT width, power attenuation 10 dB, field modulation 0.100 mT, sweep time 100 s, microwave frequency 9450 MHz. Computer spectral simulations were carried out by the means of the software EasySpin 5.0 (Stoll and Schweiger, 2006). UV–Vis data was obtained using a UV–visible spectrophotometer evolution 500 from Thermo Electron Corporation.

2.3. Fenton reaction

Experiments were conducted with H_2O_2, Fe^{2+} and DMPO concentrations varying from 1 to 100, 0.1 to 10, and 5–100 mM, respectively. A constant $[H_2O_2]$: $[Fe^{2+}]$ ratio of 10:1 was kept for all experiments as optimal conditions for Fenton reagent (Tang, 2004). For this purpose, stock solutions of each reactant were prepared as follows: 4.5 M H_2O_2, 0.15 M $FeSO_4 \cdot 7H_2O$ and 1 M DMPO. pH of all the solutions was adjusted to 2.8 ± 0.1 using concentrated sulfuric acid. Fenton reactions were performed by mixing reagents at the desired proportions according to the following order: ultra-pure water (pH 2.8), hydrogen peroxide, DMPO and iron sulfate (total volume: 2 mL). Samples were kept under agitation at room temperature during experiment. For quenching experiments, desired amount of either sodium sulfite or catalase was added to the sample by the same procedure after 10 min of Fenton reaction. Catalase was initially dissolved in phosphate buffer pH 7.2. When catalase was added in small amount, pH of the samples naturally increased to 5–6.

2.4. Kinetic model of DMPO-OH EPR signal

Hydroxyl radicals are generated from so-called Fenton reaction (reaction 1 (Tang, 2004)), but can also react with Fe^{2+} and H_2O_2 as described in termination reactions 2 and 3. However, when DMPO is in excess, •OH would quickly attack DMPO (Scheme 1, compound **1**) in reaction 4 (Finkelstein et al., 1980) to form the paramagnetic compound 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxy (DMPO-OH, Scheme 1, compound **2**), thus limiting side-reactions 2 and 3.

$$Fe^{2+} + H_2O_2 \xrightarrow{k_i} Fe^{3+} + OH^- + \bullet OH \quad k_i = 51 \text{ M}^{-1} \text{ s}^{-1} \qquad (1)$$

$$Fe^{2+} + \bullet OH \xrightarrow{k_{t1}} Fe^{3+} + OH^- \quad k_{t1} = 3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \qquad (2)$$

$$\bullet OH + H_2O_2 \xrightarrow{k_{t2}} \bullet OOH + H_2O \quad k_{t2} = 2.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \qquad (3)$$

$$DMPO + \bullet OH \xrightarrow{k_D} DMPO - OH \quad k_D = 3.4 \times 10^9 \text{M}^{-1} \text{ s}^{-1} \qquad (4)$$

where:k_i: Initiation rate constant of •OH generation.$k_{t1,t2}$: Termination rate constants of •OH quenching reaction 2 and 3, respectively.k_D: Formation rate constant of DMPO-OH.

The stability of DMPO depends upon the relative amount of DMPO and Fenton reagent. In the presence of high concentration in Fe^{3+} under acidic conditions, DMPO-OH can lead to the formation of two diamagnetic intermediates (Makino et al., 1992): 1-hydroxy-
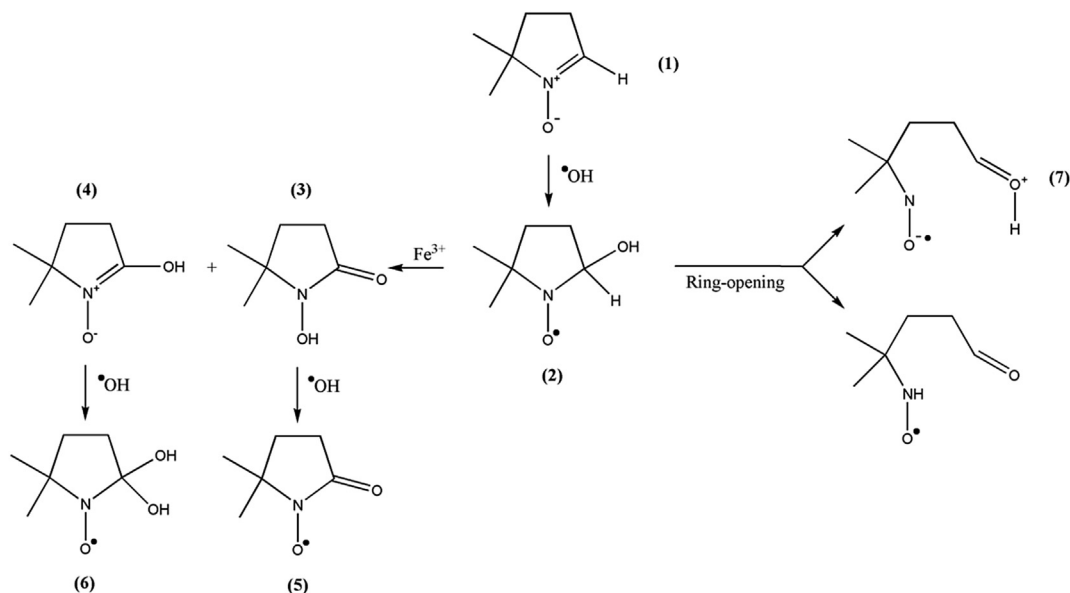
Scheme 1. General oxidation and degradation pathways of DMPO (**1**) and DMPO-OH (**2**). Structures discussed in the text: HDMPN (**3**), HDMPO (**4**), DMPOX (**5**), HDMPO-OH (**6**), End-product from decomposition of DMPO-OH by ring-opening mechanism (**7**).

5,5-dimethyl-1-pyrrolid-2-one (HDMPN, Scheme 1, compound **3**) and its tautomer 2-hydroxy-5,5-dimethyl-1-pyrroline-*N*-oxide (HDMPO, Scheme 1, compound **4**), as described in reaction 5.

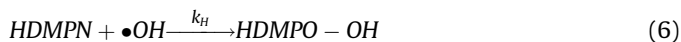$$DMPO - OH + Fe^{3+} \xrightarrow{\ k_I\ } HDMPN \leftrightarrows HDMPO \qquad (5)$$

where k_I is the formation rate constants for diamagnetic tautomers HDMPN and HDMPO. If there is an excess of hydroxyl radicals in the system, these EPR-silent compounds would react with •OH to form the stable paramagnetic radicals 5,5-dimethyl-2-oxopyrroline-*N*-oxyl (DMPOX, Scheme 1, compound **5**) and 2-dihydroxy-5,5-dimethyl-1-pyrrolidinyloxy (HDMPO-OH, Scheme

$$\frac{d[\bullet OH]}{dt} = k_i[H_2O_2]\left[Fe^{2+}\right] - k_D[DMPO][\bullet OH] - k_{t1}[\bullet OH]\left[Fe^{2+}\right]$$
$$- k_{t2}[\bullet OH][H_2O_2] - k_H[HDMPN][\bullet OH]$$
$$- k_X[HDMPO][\bullet OH]$$
$$(8)$$

At steady state concentration of hydroxyl radicals, the above equation should equal to zero and the final form can be expressed as follows:

$$[\bullet OH]_{SS} = \frac{k_i[H_2O_2]\left[Fe^{2+}\right]}{k_D[DMPO] + k_{t1}\left[Fe^{2+}\right] + k_{t2}[H_2O_2] + k_H[HDMPN] + k_X[HDMPO]} \qquad (9)$$

1, compound **6**) (reactions 6 and 7).

$$HDMPN + \bullet OH \xrightarrow{\ k_H\ } HDMPO - OH \qquad (6)$$

$$HDMPO + \bullet OH \xrightarrow{\ k_X\ } DMPOX \qquad (7)$$

where k_H and k_X are respectively the formation rate constants for HDMPO-OH and DMPOX. Therefore, if DMPO is not present in large excess, additional EPR signals might appear, thus preventing from a reliable detection of •OH. The critical question to elucidate is thus at which concentration DMPO could be considered as in excess. For this purpose, a kinetic model was developed based on the previous elementary reactions. With the aid of this model, experiments were designed to answer this critical question by monitoring DMPO-OH and additional signals by using EPR and UV–vis spectrophotometer. The ratio of H_2O_2 and Fe^{2+} was kept at optimal of 10 (Tang, 2004). The conditions under which DMPO is in excess, i.e. traps all the •OH in the system without further reactions 6 and 7, can be found starting from reaction 8:

To accurately quantify DMPO-OH concentration, analytical conditions must be found and defined so that stable quartet EPR signal could be monitored over the analytical time, without further degradation to HDMPO-OH and DMPOX. In terms of elementary reactions, rate of reactions 6 and 7 should approach zero as follows:

$$k_H[HDMPN] = 0 \qquad (10)$$

$$k_X[HDMPO] = 0 \qquad (11)$$

Therefore, the steady state •OH concentration can be simplified as follows:

$$[\bullet OH]_{SS} = \frac{k_i[H_2O_2]\left[Fe^{2+}\right]}{k_D[DMPO] + k_{t1}\left[Fe^{2+}\right] + k_{t2}[H_2O_2]} \qquad (12)$$

Moreover, the reaction rate of DMPO-OH has following expression:

$$\frac{d[DMPO - OH]}{dt} = k_D[DMPO][\bullet OH]_{SS} - k_I[DMPO - OH]\left[Fe^{3+}\right]$$

$$\frac{d[DMPO - OH]}{dt} = k_D[DMPO]\frac{k_i[H_2O_2]\left[Fe^{2+}\right]}{k_D[DMPO] + k_{t1}\left[Fe^{2+}\right] + k_{t2}[H_2O_2]} - k_I[DMPO - OH]\left[Fe^{3+}\right]$$

(13)

The generation of a constant quartet EPR signal relies upon two conditions: the first one is that the rate of reaction (5) approaches zero. Therefore:

$$k_I[DMPO - OH] = 0 \qquad (14)$$

The second condition is to make sure the change rate of d[DMPO-OH]/dt is not function of [DMPO]. The only possible way to satisfy this condition is that the term, $k_D[DMPO]$, is significantly greater than $k_{t1}[Fe^{2+}] + k_{t2}[H_2O_2]$, so that the latter could be negligible. Mathematically, the following conditions must be satisfied:

$$k_D[DMPO] \gg k_{t1}\left[Fe^{2+}\right] + k_{t2}[H_2O_2] \qquad (15)$$

The above conditions can be divided by $k_D[H_2O_2]$ at both sides, and the suitable analytical conditions become:

$$\frac{[DMPO]}{[H_2O_2]} \gg \frac{k_{t1}\left[Fe^{2+}\right]}{k_D[H_2O_2]} + \frac{k_{t2}}{k_D} \qquad (16)$$

The sign of significantly greater means that the right hand ratio of reaction 16 should be negligible. In other words, the left hand ratio should be two to three orders greater than the right hand side
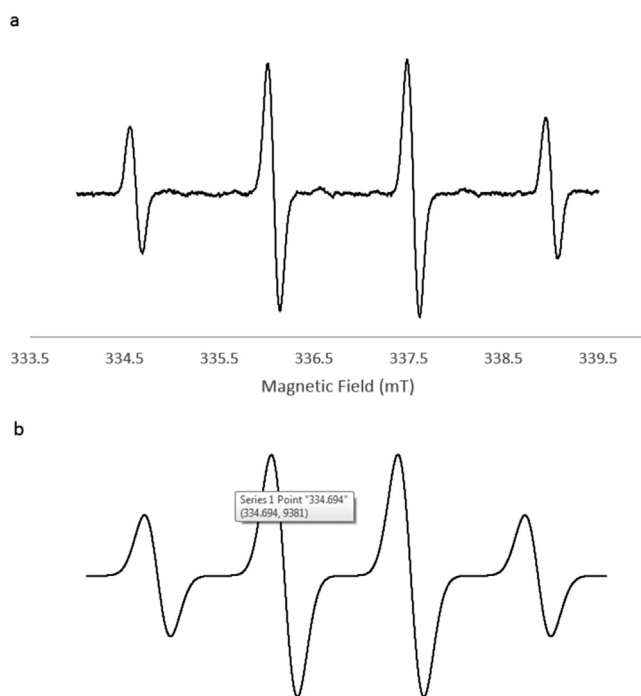
ratio so that Equations (15) and (16) can truly hold. For initial H_2O_2 and Fe^{2+} concentrations of 1 mM and 0.1 mM, respectively, the required [DMPO]: [H_2O_2] ratio should be at minimum 1.68 to 16.8. As a result, the system should generate a stable quartet EPR signal without any additional noise. The experiments were designed based upon these theoretical analyses, and results will help determine the optimal [DMPO]: [H_2O_2] ratio more accurately. In this study, [H_2O_2]: [Fe^{2+}] ratio was kept constant; however, the above model would help predicting optimal concentration of DMPO for any other concentration of Fenton reagent since initial conditions are included in Equation (16).

3. Results and discussion

3.1. Impact of Fenton reagent concentration on EPR background signals

The impact of Fenton reagent concentration was tested with [DMPO]: [H_2O_2] ratio varying from 1 to 100. A typical spectrum of DMPO-OH is presented in Fig. 1a: it is composed by a characteristic 1:2:2:1 quartet with hyperfine couplings $a_N = a_{\beta H} = 1.50$ mT (computer simulation, Fig. 1b). The amplitude of the DMPO-OH signal was followed during the time for different concentrations of Fenton's reagent, and results are presented in Fig. 2. As depicted, the concentration of Fenton reagent had a significant impact on both the intensity and the stability of DMPO-OH signal. Indeed, when H_2O_2 and Fe^{2+} concentrations were 1 and 0.1 mM respectively (i.e. [DMPO]: [H_2O_2] = 100 > 16.8), the peak amplitude of DMPO-OH signal was statistically stable (p = 0.0284 < 0.05), and no clear down trend appears over the time of experiment (60 min). Variations from one point to another could be attributed to the instability of the reaction (no control of pH after the initial adjustment). Nevertheless, the general trend of the system suggested an appropriate concentration of DMPO compared to the concentration of Fenton reagent as predicted by the theoretical
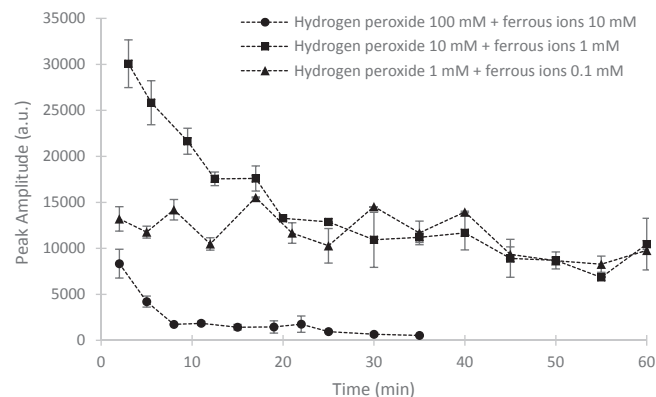

Fig. 1. a) typical DMPO-OH signal obtained with 1 mM H_2O_2, 0.1 mM Fe^{2+} and 100 mM DMPO, b) Simulation of DMPO-OH ($a_N = a_{\beta H} = 1.50$ mT).

Fig. 2. Time-series of DMPO-OH peak amplitude for various concentrations in Fenton reagent and constant 100 mM DMPO concentration.
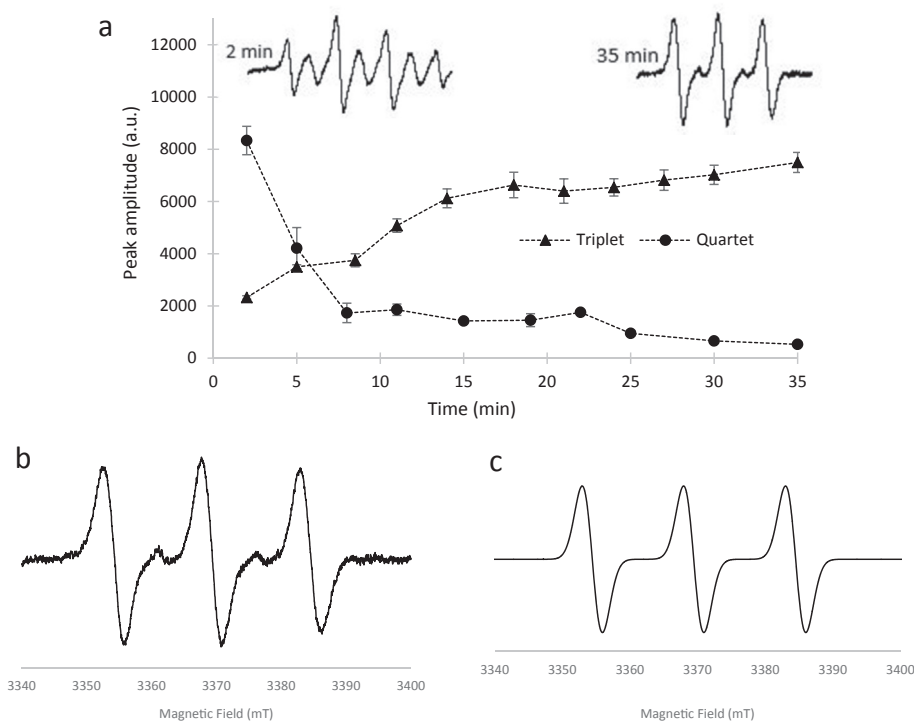
Fig. 3. a) Time-series of quartet (DMPO-OH) and additional triplet signals amplitudes during Fenton reaction with 100 mM H_2O_2, 10 mM Fe^{2+} and 100 mM DMPO, b) Triplet observed after 35 min reaction in these conditions, c) Computer simulation of triplet in b) ($a_N = 1.51$ mT).

model. The increase in Fenton reagent concentration to 10 mM H_2O_2 and 1 mM Fe^{2+} (i.e. [DMPO]: [H_2O_2] = 10 > 1.68) led to a different behavior. The higher intensity measured during the very early minutes suggested the generation of higher concentration of •OH, as expected after the increase of Fenton reagent. However, an important decrease of the DMPO-OH peak amplitude was then observed during the first 20 min, followed by a slower decrease until the end of the experiment. The fast decrease measured during the early stage of the reaction was unlikely related to the lifetime of DMPO-OH, generally reported around 30 min (Buettner, 1993; Makino et al., 1990). Even though the typical DMPO-OH quartet was the only signal detected in EPR spectra without the apparition of additional lines, the fast decrease of the signal intensity suggested the possible quenching or degradation of the spin-adduct. According to the kinetic model developed in Section 2.4, this observation would also suggest that the left hand ratio of Equation (16) should be more than 2 orders greater than the right hand ratio to provide a stable quartet signal. To confirm the possible quenching of DMPO-OH, H_2O_2 and Fe^{2+} concentrations were increased again to 100 mM and 10 mM, respectively ([DMPO]: [H_2O_2] = 1). In this case, the quenching effect of DMPO-OH was very obvious, with a lower signal amplitude from the beginning of the reaction and becoming hardly detectable after 10 min, thus clearly suggesting the oxidation of the spin-adduct due to the excess of •OH. Moreover, the EPR revealed the apparition of additional lines consisting in a 1: 1: 1 triplet. The triplet's intensity increased along with the decrease of DMPO-OH quartet (Fig. 3a). After 35 min reaction, the peak amplitude of the triplet reached to its maximum, whereas DMPO-OH had completely disappeared. The corresponding EPR spectrum is presented in Fig. 3b ($a_N = 1.51$ mT, simulation depicted in Fig. 3c). The apparition of background EPR signals in case of too high initial concentration in Fe^{2+} was already described in the past (Makino et al., 1992). However, these signals previously attributed to HDMPO-OH and DMPOX are different than

the triplet observed in this study, thus implying the generation of a different by-product.

In order to understand the mechanisms leading to the decrease of DMPO-OH and to the formation of the triplet, similar analysis were performed by UV–vis spectrophotometry. The contribution of each reagent (H_2O_2, Fe^{2+} and DMPO) was first analyzed and, as shown in Fig. 4a, the peak measured in visible part ($\lambda_{max} = 520$ nm) only appeared in presence of both Fenton reagent and DMPO. Time series of UV–Vis absorbance were then followed, and the corresponding results are presented in Fig. 4b and 4c. With 10 mM H_2O_2 and 1 mM Fe^{2+}, EPR and UV–Vis signals followed opposite trends. Indeed, the DMPO-OH EPR signal intensity decreased during the first 20 min (Fig. 2), whereas the UV–Vis signal increased during the same period of time (Fig. 4b). This result suggests the generation EPR-silent side products (diamagnetic), which can most likely be explained with the reaction of DMPO-OH with the generated Fe^{3+} to form the tautomers HDMPN and HDMPO (Makino et al., 1992). This result also confirms that a [DMPO]: [H_2O_2] ratio of 1.68 is not high enough to provide a stable EPR signal: under these conditions, reactions 6 and 7 barely took place in the system, whereas reaction 5 is not negligible. Increasing the concentration of Fenton reagent to 100 mM H_2O_2 and 10 mM Fe^{2+} led to the increase of the absorbance in the visible range over the first 20 min (Fig. 4c). It can reasonably be assumed that the higher concentration in Fe^{2+}, and therefore in the generated Fe^{3+}, enhanced the production of the two tautomers. However, since these compounds are EPR-silent, they cannot be directly attributed to the triplet observed. This paramagnetic compound was only observed under specific conditions, with initial hydrogen peroxide and ferrous ions concentrations higher than 50 mM and 5 mM respectively, i.e. with a [DMPO]: [H_2O_2] ratio of 2 (data not shown). In the presence of excessive •OH, the oxidation of HDMPN and HDMPO can lead to the formation of HDMPO-OH and DMPOX (Scheme 1). However, DMPOX generates a 1: 1: 1 triplet ($a_N = 0.72$ mT) of 1: 2: 1 triplet
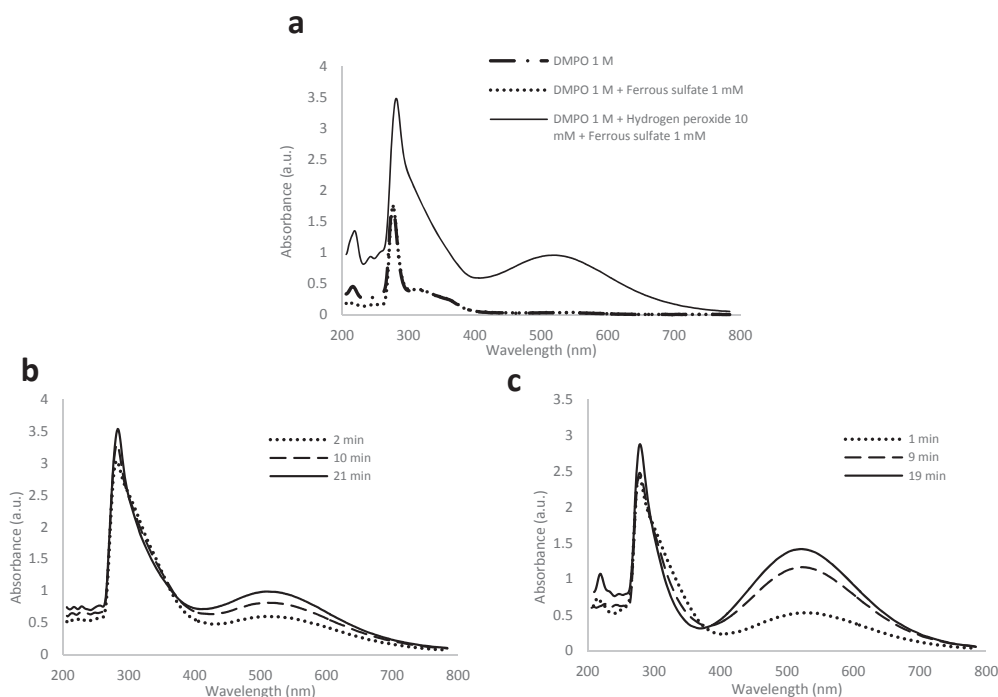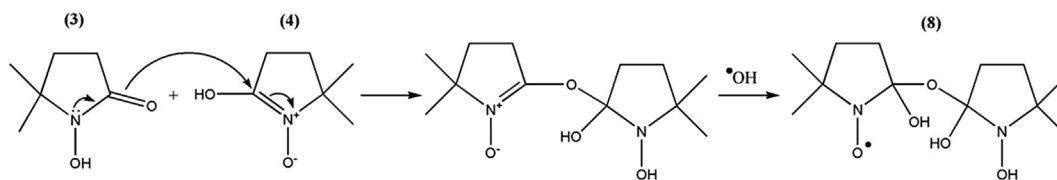
Fig. 4. a) UV–Vis spectra of DMPO, DMPO and ferrous sulfate, Fenton reagent and DMPO, b) UV–Vis spectra during Fenton reaction with 10 mM H_2O_2, 1 mM Fe^{2+} and 100 mM DMPO, c) UV–Vis spectra during Fenton reaction with 100 mM H_2O_2, 10 mM Fe^{2+} and 100 mM DMPO (diluted 10 times).

Scheme 2. Proposed mechanism for the formation of the paramagnetic by-product detected by EPR.

($a_{\gamma H}$ = 0.41 mT, 2H) and HDMPO-OH generates a 1: 1: 1 triplet (a_N = 1.53 mT) of 1: 3: 3: 1 quartet ($a_{\gamma H}$ = 0.12 mT, 3H) (Makino et al., 1992; Nakajima et al., 2005; Verstraeten et al., 2009). Therefore, their presence should be excluded. The computer simulation of the triplet (a_N = 1.51 mT, Fig. 3b) suggests the absence of contribution of hydrogen nuclei in β and γ positions. The possibility of degradation of DMPO-OH spin-adduct *via* C–N bond cleavage and opening of the pyrroline ring was suggested in the past and assigned to a similar triplet signal (Bosnjakovic and Schlick, 2006). However, neither a structure nor a mechanism was proposed. Such a degradation pathway was also discussed in a thermodynamic study stating that this mechanism may play a crucial role in the stability of spin adducts (Villamena et al., 2005). The detailed mechanism related to the DMPO-OH ring opening remains unclear; moreover, the degradation product that could be assigned to the triplet observed (Scheme 1, compound **7**) because of the absence of hydrogen nuclei in α and β positions is thermodynamically highly unstable and is hence unlikely to generate a stable signal over the time. Even though the presence of HDMPO-OH and DMPOX was excluded in this work, UV–Vis analysis are in agreement with the generation of HDMPO and HDMPN tautomers because of the presence of Fe^{3+}. Therefore, and as it was reported in the case of 2,5,5-trimethyl-1-pyrroline-*N*-oxide (M₃PO) tautomers (Barasch et al., 1994), the apparition of the EPR triplet signal could be explained by the dimerization reaction occurring between

HDMPN and HDMPO, thus leading to the formation of a stable diamagnetic dimer, as described in the proposed mechanism presented in Scheme 2. In the presence of excess of hydroxyl radicals compared to the initial concentration of DMPO, the dimer could be further oxidized to generate the corresponding paramagnetic compound **8** associated to the additional triplet. Indeed, compound **8** does not present hydrogen nuclei in β position, and the contribution of hydrogen in γ position is highly unlikely due to steric effect after dimerization. According to the experimental results described in this section, the theoretical model proposed should be adjusted as follows: to ensure a reliable detection of •OH, the left hand ratio of Equation (16) should be 3 orders higher than the right hand ratio. In other words, in these conditions, [DMPO]: [H_2O_2] ratio should be at least 16.8 and [DMPO]: [Fe^{2+}] ratio should be at least 168.

3.2. Role of DMPO concentration for a reliable detection of hydroxyl radicals

The reactions described above suggested that the DMPO concentration should be carefully selected according to the Fenton's reagent concentration and expected magnitude of hydroxyl radical concentration, as demonstrated through the previous theoretical kinetic analysis. The condition illustrated by Equation (16) could be applied to any concentration of Fenton reagent for the
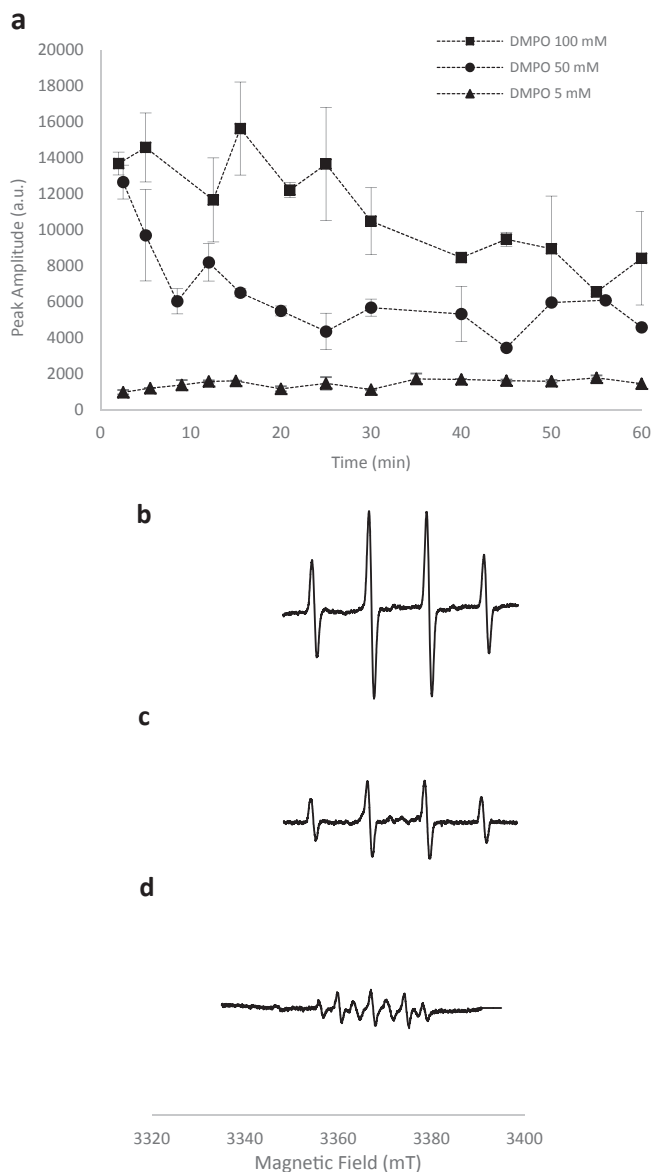
a

b

c

d

Fig. 5. a) Impact of DMPO concentration on DMPO-OH peak area during the time for Fenton reaction with 5 mM H_2O_2, 0.5 mM Fe^{2+}, b) EPR signal obtained with 100 mM DMPO after 9 min reaction, c) EPR signal obtained with 50 mM DMPO after 9 min reaction, d) EPR signal obtained with 5 mM DMPO after 9 min reaction.

Fig. 6. Impact of DMPO concentration on DMPO-OH peak area during the time for Fenton reaction with 100 mM H_2O_2 and 10 mM Fe^{2+}. The three spectra included show the signals recorded after 5, 15 and 60 min reaction with DMPO 500 mM (intensities were normalised to highlight the relative intensities evolution of the quartet and triplet signals).

quantification of •OH concentration by EPR as standard procedure. In this section, the impact of DMPO concentration on EPR signals was validated with a constant Fenton reagent concentration. Initial concentration of H_2O_2 and Fe^{2+} was chosen at 5 mM and 0.5 mM, respectively. These concentrations are commonly used in EAOPs systems such as electro-Fenton and photoelectron-Fenton (Brillas and Martínez-Huitle, 2015). DMPO concentration was varied from 5 to 100 mM ([DMPO]: [H_2O_2] ratio varying from 1 to 20). Time series signal of DMPO-OH are presented in Fig. 5a. As observed, DMPO concentration had a significant impact on DMPO-OH signal intensity. Using DMPO 100 mM ([DMPO]: [H_2O_2] = 20), the signal intensity remained reasonably stable until 25 min (apart from the instability of the system itself). The peak to peak amplitude then decreased slowly until the end of the experiment, which is consistent with the lifetime of DMPO-OH. As shown in Fig. 5b, the corresponding EPR background after 10 min was only consisted of
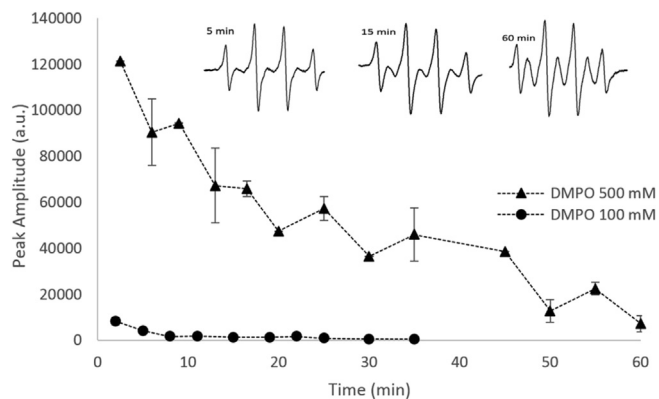
the DMPO-OH quartet, showing that no other by-product was formed. Decreasing DMPO concentration from 100 mM to 50 mM ([DMPO]: [H_2O_2] = 10) also led to a decrease of DMPO-OH signal intensity, even though the 1: 2: 2: 1 quartet was the only signal detected (Fig. 5c). Therefore, under these conditions, it can be assumed that DMPO-OH is partially degraded to HDMPN and HDMPO, but that hydroxyl radicals are not in excess to generate the paramagnetic dimer, which is in agreement with the corrected model explained at the end of Section 3.1. Finally, when DMPO concentration was lowered to 5 mM (DMPO): [H_2O_2] = 1), the EPR background evolved into a combination of quartet and triplet (Fig. 5d). It should also be noted that with the most concentrated system (100 mM H_2O_2 and 10 mM Fe^{2+}), increasing DMPO concentration from 100 mM to 500 mM (i.e. 1.68 < [DMPO]: [H_2O_2] = 5 < 16.8) led to a significant increase of the quartet as well as a decrease of the triplet, as presented in Fig. 6. Indeed, the spectrum recorded at 5 min with DMPO 500 mM shows a barely detectable triplet signal. The relative intensity of the triplet compared to the quartet intensity then increased very slowly along with the decrease of the quartet (see spectra recorded at 15 and 60 min in Fig. 6). The formation of the paramagnetic by-product is negligible during the first stage of the reaction; however, the decrease of DMPO-OH signal intensity during the first 15 min suggests the degradation of the adduct, which is most likely due to the formation of the diamagnetic HDMPO and DMPN, as observed with lower Fenton reagent concentrations and when 1.68 < [DMPO]: [H2O2] < 16.8.

The results presented in Figs. 5 and 6 are consistent with both the theoretical model and the results presented in Section 3.1: to obtain a stable DMPO-OH signal, the left hand ratio of Equation (16) should be at least 3 orders higher than the right hand ratio, in other words, [DMPO]: [H2O2] > 16.8 (and [DMPO]: [Fe^{2+}] > 168 in these conditions). From a more general point of view, this section confirms that in order to achieve a reliable detection of •OH, the concentration of DMPO should be carefully adapted each system, even in the absence of additional lines. Indeed, as explained, the absence of additional lines does not necessarily implies a reliable detection of hydroxyl radicals, since EPR-silent compounds might be formed. Therefore, four conclusions can be reached: 1) [DMPO]: [H2O2]: [Fe^{2+}] ratio holds the key in terms of oxidation kinetics of DMPO-OH: at a fixed ratio, the kinetics are similar. 2) The stability of DMPO-OH depends upon Fe^{3+} and •OH concentrations relatively to DMPO concentration: the higher these concentrations, the shorter the life time of DMPO-OH. 3) To ensure a reliable detection of
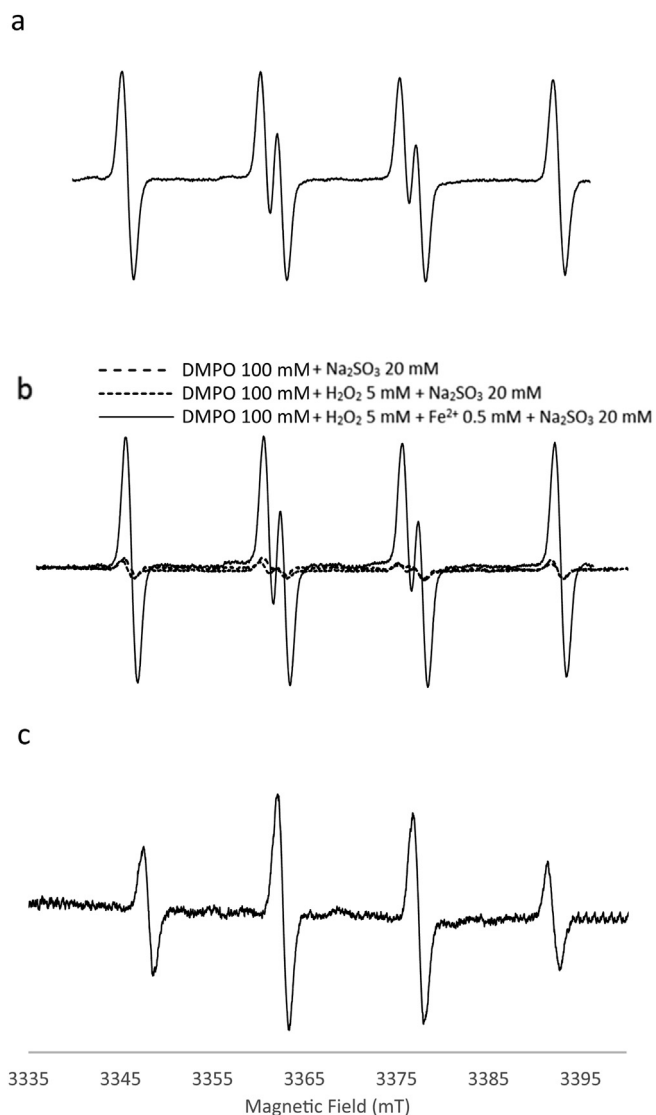
Fig. 7. a) EPR spectrum of Fenton reaction (H$_2$O$_2$ 5 mM, Fe^{2+} 0.5 mM, DMPO 100 mM) quenched after 10 min with 20 mM Na$_2$SO$_3$, b) EPR spectra recorded for DMPO 100 mM and Na$_2$SO$_3$ 20 mM; DMPO 100 mM, H$_2$O$_2$ 5 mM and Na$_2$SO$_3$ 20 mM; DMPO 100 mM, Na$_2$SO$_3$ 20 mM and Fenton reagent (H$_2$O$_2$ 5 mM, Fe^{2+} 0.5 mM), c) EPR spectrum of Fenton reaction (H$_2$O$_2$ 5 mM, Fe^{2+} 0.5 mM, DMPO 100 mM) quenched after 10 min with 0.25 mg L^{-1} catalase (spectrum recorded 8 min after addition of catalase).
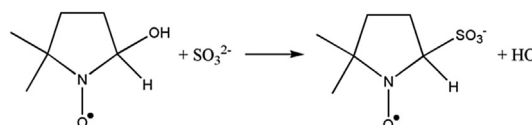
hydroxyl radicals, and under conditions used in this study, DMPO concentration should set so [DMPO]: [H$_2$O$_2$] > 16.8 (and [DMPO]: [Fe^{2+}] > 168 in these conditions). 4) The required DMPO concentration will change depending on [H$_2$O$_2$]: [Fe^{2+}] ratio, but it can be predicted using the theoretical model developed.

3.3. Compatibility of sodium sulfite and catalase as hydrogen peroxide quenchers in presence of DMPO

The reliability of •OH detection is also related to the conditions of experimentation and sensitivity of the equipment. Indeed, in this study, pH was not controlled after the initial adjustment (pH 2.8) and no buffer was used. In order to improve the reliability of the detection for quantification using DMPO as a spin trap, the utilization of a suitable quencher for Fenton reaction must be considered. One way to efficiently terminate Fenton reaction is to quench

H$_2$O$_2$ residuals. However, the quencher should also be carefully chosen to avoid any side reaction with DMPO or DMPO-OH. For example, the impact of sodium sulfite, a commonly used H$_2$O$_2$ quencher (Liu et al., 2003; Trovó et al., 2015), on EPR signals was tested. The Fenton system composed of 5 mM H$_2$O$_2$ and 0.5 mM Fe^{2+} was quenched after 10 min reaction with 20 mM sodium sulfite, in the presence of 100 mM DMPO. The corresponding EPR spectrum is presented in Fig. 7a. The formation of a different quartet was observed, due to the reaction between Fenton reagent, sulfite, and DMPO. A similar quartet has been reported in the past, and attributed to the DMPO-SO$_3^-$ adduct after reaction between DMPO and •SO$_3^-$ (Ranguelova and Mason, 2011). However, •SO$_3^-$ is generated from SO$_3^{2-}$ under specific conditions, generally during a one-electron oxidation enzymatically catalyzed by prostaglandin H synthase or horseradish peroxidase (HRP), which have not been used in the present work. Even though the presence of •SO$_3^-$ was highly unlikely, the apparition of the quartet depicted in Fig. 7a could be attributed to the reaction between SO$_3^{2-}$ itself and DMPO or DMPO-OH. In the first case, residual DMPO would undergo a nucleophilic addition of sulfite and the resulting hydroxylamine would lead to the DMPO-SO$_3^-$ adduct after a mild one-electron oxidation according to the so-called Forrester-Hepburn mechanism (Ranguelova and Mason, 2011). However, as observed in Fig. 7a, the only signal observed corresponds to the DMPO-SO$_3^-$, which suggests the consumption of DMPO-OH after the addition of sodium sulfite. To confirm this hypothesis, Na$_2$SO$_3$ was added to DMPO, DMPO/H$_2$O$_2$, DMPO/H$_2$O$_2$/Fe^{2+} respectively. The corresponding recorded spectra are reported in Fig. 7b. As observed, signals of very low and comparable intensities were recorded when Na$_2$SO$_3$ was added to DMPO and DMPO/H$_2$O$_2$. This suggests that the reaction of DMPO with Na$_2$SO$_3$ to form DMPO-SO$_3^-$ cannot be totally excluded in the conditions studied, even though the contribution of this reaction is very low. However, Fig. 7a and b also shows that the addition of Na$_2$SO$_3$ to DMPO/H$_2$O$_2$/Fe^{2+} (and thus DMPO-OH) leads to the formation of DMPO-SO$_3^-$ signals of much higher intensities, preventing a reliable detection of DMPO-OH. Therefore, a reaction between SO$_3^{2-}$ and DMPO-OH can also be envisaged to form DMPO-SO$_3^-$ *via* a nucleophilic substitution, as proposed in Scheme 3.

Even though sodium sulfite is an efficient quencher for Fenton reaction, it shouldn't be utilized for the detection of •OH with DMPO as a spin trap agent. For this purpose, a different quencher that does not react with either DMPO or DMPO-OH should be considered. Similarly to sulfite anion, catalase is known for being an efficient quencher for H$_2$O$_2$ (Liu et al., 2003). The impact of catalase on EPR signals was tested in the same conditions. Fenton reaction was carried out for 10 min in presence of DMPO and then terminated with the addition of 0.05 mg mol^{-1} H$_2$O$_2$ of catalase in this case 0.25 mg L^{-1} of catalase. The EPR spectrum recorded 8 min after the addition of catalase is given in Fig. 7c. As presented, a typical DMPO-OH was observed which implies that no reaction take place between the quencher and hydroxyl radicals spin adduct. In this study, the residual H$_2$O$_2$ concentration was not measured after addition of catalase, but from an analytical point of view, EPR spectra have shown that the addition of catalase was compatible with the utilization of DMPO as a spin trap agent for the detection of hydroxyl radicals.

Scheme 3. Formation of DMPO-SO$_3^-$ adduct in presence of DMPO-OH and sulfite.

4. Conclusions

In this study, the impact of DMPO concentration for the detection of hydroxyl radicals under conditions similar to AOPs/EAOPs based on Fenton reaction has been investigated. Impact of high concentrations of Fenton reagents (up to 100 mM H_2O_2 and 10 mM Fe^{2+} with a constant ratio of 10: 1) was studied.

- Experiments were based on a theoretical model developed to determine the optimal DMPO concentration for a reliable detection of hydroxyl radicals.
- In our system, for a reliable detection of hydroxyl radicals, DMPO concentration should be respectively 20 and 200 times higher than H_2O_2 and Fe^{2+} concentrations, which is in agreement with the theoretical model.
- When these conditions were not fulfilled, the typical quartet associated to DMPO-OH was unstable and an additional triplet signal was observed by EPR. The formation of a paramagnetic dimer was proposed based upon the observed EPR signal.
- The impact of two commonly used H_2O_2 quenchers on EPR backgrounds was studied. The utilization of sodium sulfite is not suitable because of the reaction of the generated SO_3^{2-} with DMPO and DMPO-OH, therefore leading to an undesirable EPR signal.
- Catalase, also well known for efficiently terminating Fenton reaction, appeared to be a suitable quenching chemical because the typical DMPO-OH quartet remained unchanged after quenching reaction.

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