Influence of pH, buffers and the role of quinolinic acid, a novel iron chelating agent, in the determination of hydroxyl radical scavenging activity of plant extracts by Electron Paramagnetic Resonance (EPR).

Runnig title: Quinolinic acid ligand for hydroxyl radical scavenging activity of plant extracts.

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ABSTRACT

The Fenton reaction is used to produce hydroxyl radicals for the evaluation of the antioxidant activity of plant extracts. In this paper the parameters affecting the production of hydroxyl radicals and their spin trapping with DMPO were studied. The use of quinolinic acid (Quin) as an Fe(II) ligand was proposed for antioxidant activity determination of Green tea, orange juice and asparagus extracts. Quin, buffers and pH affect the DMPO-OH signal intensity of the EPR spectra. Quin/Fe(II) and low pH enhance the $^{\bullet}OH$ generation. Phosphate and Tris-HCl buffers decrease the signal intensity measured in Fe(II)-sulfate and Fe(II)-Quin systems.

The extracts were analyzed with Fenton systems containing Fe(II)-sulfate and Fe(II)-Quin with and without buffer. The highest activity was shown with Fe(II)-Quin without buffer, this system being less influenced by pH and chelating agents present in the extracts. This paper will help researchers to better design spin trapping experiments for food matrices.

Keywords: food, hydroxyl radical scavenging activity, Electron Paramagnetic Resonance (EPR), spin trapping, buffer effect, quinolinic acid.

1. Introduction

In the last decades there has been an increasing interest in the role of food antioxidants to prevent, or at least slow down, the onset of inflammatory and cardiovascular diseases, carginogenesis and ageing. The importance of fruit and vegetable consumption in the prevention of oxidative stress related diseases has been reviewed (Zhang & Tsao, 2016), and is gaining great attention among consumers which are now aware of the health properties of food.

Fruit and vegetables are rich sources of anthocyans, flavonoids, flavonols and tannins which are involved in the protection of cell macromolecules against oxidative stress caused by Reactive Oxygen Species (ROS) (Shahidi & Ambigaipalan, 2015). ROS are normally produced in cells but living organisms possess both enzymatic and non-enzymatic antioxidant mechanisms to take control of their damaging effects. The hydroxyl radical ($^{\bullet}OH$) is one of the major causes of oxidative damage (Sakai, Imai, Ito, Takagaki, Ui, & Hatta, 2017), due to its high standard redox potential (2.8 V) and its low selectivity, reacting indiscriminately with lipids, proteins and nucleic acids (Salgado, Melin, Contreras, Moreno, & Mansilla, 2013).

Natural antioxidants (or fruit and vegetable antioxidants) have a strong hydroxyl radical scavenging activity (Calliste, Trouillas, Allais, & Duroux, 2005; Staško, Polovka, Brezová, Biskupič, & Malík, 2006; Braga, Lo Scalzo, Dal Sasso, Lattuada, Greco, & Fibiani, 2016). Due to their role against oxidative stress, the measurement of the radical scavenging activity of fruit and vegetable extracts is one of the most popular topics in food research. Amongst the methods developed to determine and quantify the antioxidants' hydroxyl radical scavenging activity, the spin trapping coupled with Electron Paramagnetic Resonance (EPR) spectroscopy is the most specific and reliable, and has been effectively employed to study the antioxidant properties of food, beverages and plant extracts (Calliste, et al., 2005; Staško, et al., 2006; Brezová, Šlebodová, & Staško, 2009; Azman, Peiró, Fajarí, Julià, & Almajano, 2014; Pérez-López, Pinzino, Quartacci, Ranieri, & Sgherri, 2014; Fadda & Sanna, 2015; Fadda, Pace, Angioni, Barberis, & Cefola, 2016).

The spin trapping method is based on the *in vitro* production of hydroxyl radicals, and on their entrapment with diamagnetic spin trap molecules to form relatively stable adducts that have paramagnetic resonance spectra detectable with EPR. In the spin trapping method, applied to food analysis, the DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) is the most frequently used spin trap and hydroxyl radicals are generally produced by the Fenton reaction, even though some other hydroxyl radical generating systems such as Fenton-like reactions using Co(II)/H_2O_2, Cu(I)/H_2O_2 (Moore, Yin, & Yu, 2006), or the thermal decomposition of peroxydisulfate, $K_2S_2O_8$ (Staško, et al., 2006) have been employed.

In the analysis of hydroxyl radical scavenging activity of food based matrices several protocols have been reported differing in the spin trap concentration, the buffer and the ferrous chelating agent used. The effect of the DMPO concentration on the estimation of the radical scavenging activity has seldom been considered. Recently Fontmorin, Burgos Castillo, Tang, and Sillanpää (2016) studied the impact of DMPO concentration on the stability of DMPO-OH adduct in Advanced Oxidation Processes (AOPs) conditions. Regarding plant extracts analyses, the DMPO concentrations used were very different, ranging from 0.78 to 300 mM (Debnath, Park, Deb Nath, Samad, Park, & Lim, 2011; Braga, et al., 2016), and very few studies have been performed to optimize the DMPO concentrations in order to achieve a more reliable determination of hydroxyl radical scavenging activity.

Besides the spin trap concentration, in the analysis of plant extracts other variables, like the presence of buffers, the pH of the solution or the presence of iron chelating agents, may affect the production and the detection of the hydroxyl radical. Their impact on the Fenton reaction have rarely been reported, despite being of great importance in plant extract analysis, due to the presence in these samples of organic acids and natural iron chelating agents. In the literature, most of the reactions are performed at physiological pH, with the aim of mimicking the biological conditions where the food antioxidants should exert their effect, and phosphate is the most commonly used buffer. Despite its broad use, it was demonstrated that the intensity of the EPR signal of the DMPO-

OH adduct in this medium was remarkably lower than in other buffers (Tris-HCl, sodium acetate, sodium trifluoroacetate) (Li, Abe, Mashino, Mochizuki, & Miyata, 2003). For this reason the same authors claimed that the DMPO-OH adduct can be detected, by EPR spectroscopy in phosphate buffer, only when high concentrations of spin trap are used (Li, et al., 2004).

Fe(II) is relatively stable in acidic media (Babuponnusami and Muthukumar, 2014), whereas at physiological pH values it is readily oxidized, making necessary the use of an inert gas to prevent its oxidation, thus complicating the experimental procedure.

On the basis of these considerations, the present study was designed to evaluate the influence of pH, buffers and DMPO concentration in the production, with the Fenton reaction, and determination, by spin trapping with DMPO, of the hydroxyl radical scavenging activity of plant extracts.

Moreover we propose an improvement of the method for hydroxyl radical scavenging activity estimation of plant extracts, based on the use of quinolinic acid as Fe(II) ligand in the Fenton reaction system, with the aim of minimizing the effects of pH, providing easier handling of the solutions and save reactants.

2. Materials and methods

2.1. Chemicals

All reagents and solvents were of analytical grade, unless otherwise specified, and used without further purification. Quinolinic acid (pyridine-2,3-dicarboxylic acid), ferrous sulfate heptahydrate, phosphate, Tris-HCl (2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride) buffers and hydrogen peroxide (30% w/w) were purchased from Sigma Aldrich. DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) was purchased from Enzo Life Sciences. Water was purified with a Milli-Q system from Millipore (Millipore Corporation, Billerica, MA, USA).

112 *2.2. Food extracts preparation*

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114 *2.2.1. Java Green Tea*

115 A commercial green tea was purchased from a retail outlet. According to the producer's instructions
116 the java green tea infusion was prepared by pouring 100 ml of distilled water at 80 °C over a 2 g
117 bag in a beaker. After 5 minutes of infusion the tea was cooled and filtered (Whatman 113). Five
118 independent replicates were carried out.

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120 *2.2.2. Orange juice*

121 Oranges (*Citrus sinensis* L. cv Hamlin) were harvested at the experimental orchard of the Institute
122 of the Sciences of Food Production located in central western Sardinia. The juice was obtained by
123 squeezing 5 fruits per replication (five independent replicates) with a commercial juicer. Before
124 analysis the juice was centrifuged (13,000 rpm for 15 min) and filtered (0.45 μm acetate cellulose
125 filter).

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127 *2.2.3. Purple asparagus extracts*

128 Asparagus spears (*Asparagus officinalis* L. cv Purple passion) were purchased at the local market.
129 The spears were delivered to the laboratory and selected to be free of damages and defects. Sound
130 spears were cut 15 cm from head with a steel knife. Asparagus spears (five independent replicates
131 of 5 g each) were homogenized at 13,000 rpm for 1 minute (Ultra-Turrax, T25 Basic IKA,
132 Germany) in a methanol / water solution (80% MeOH). The homogenates were centrifuged at 6,000
133 rpm for 10 minutes, then the organic extracts were filtered with n. 4 Wathman filter paper.

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138 *2.3. Spin trapping assay of the $^{\bullet}$OH radical*

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140 *2.3.1. Spin trapping assay*

141 The hydroxyl radicals were generated by the Fenton reaction and trapped with the nitrone spin trap
142 DMPO. In the Fenton reaction iron(II) is oxidized by hydrogen peroxide to iron(III) generating an
143 hydroxyl radical and a hydroxide ion. In this work we used Fe(II)-sulfate or Fe(II)-quinolinic acid
144 (Quin) complex as Fe(II) sources. The Fe(II)-Quin complex was prepared by dissolving in water
145 weighed amounts of $FeSO_4$ and quinolinic acid in order to have a concentration of Fe(II) of 0.1 mM
146 and a ligand to metal ratio of 5/1.

147 The DMPO-OH adduct was obtained by mixing a DMPO solution with hydrogen peroxide 0.03%
148 (w/w) (100 μl) and Fe(II)-sulfate or Fe(II)-quinolinic acid complex 0.1 mM (100 μl) to a final
149 volume of 1 ml with water. Fe(II)-sulfate solution was deoxygenated under continuous nitrogen
150 bubbling (in this case the added water was also deoxygenated) in order to keep iron in the ferrous
151 form, whereas for Fe(II)-Quin complex no deoxygenation was needed.

152 The DMPO-OH adduct was detected with a Bruker EMX spectrometer operating at the X-band
153 (9.4 GHz) equipped with an HP 53150A microwave frequency counter using a Bruker AquaX
154 capillary cell. With this cell it is possible to keep constant the value of Q (the quality factor of the
155 resonator) during sample measurements thus allowing quantitative comparisons of the intensity of
156 EPR signals to be made. Spin trapping experiments with DMPO can be used to calculate the
157 concentration of the DMPO-OH adduct, demonstrating the reliability of the method (Eaton, 2010).
158 All the measurements were always compared to a blank sample measured under the same
159 conditions.

160 An EPR instrument was set under the following conditions: modulation frequency, 100 kHz;
161 modulation amplitude, 1 G; receiver gain, 1×10^5; microwave power, 20 mW. This microwave
162 power, using the Bruker ER 4119HS resonator, is below the saturation level. EPR spectra were
163 recorded at room temperature, immediately after the preparation of the reaction mixture. The

intensity of the spin adduct DMPO-OH was estimated from the double integration of spectra. For the experiments without plant extracts three independent replicates were performed and data are reported as mean and standard deviation.

2.3.2. Effect of pH, buffers and DMPO concentration on DMPO-OH signal intensity

To assess the effect of pH on DMPO-OH adduct generation an aliquot of DMPO solution was acidified to pH ~1, using concentrated sulfuric acid, then added to the reaction mixture as described above.

In order to evaluate the effect of DMPO concentration on DMPO-OH signal intensities the following final DMPO concentrations were tested: 0.6, 1, 3, 6 and 10 mM ; whereas the effect of buffers on DMPO-OH yield was evaluated by carrying out the reactions in phosphate or Tris-HCl buffers (both at pH 7.4), at final concentrations ranging from 0 to 70 mM.

2.4. Antioxidant activity of food extracts

On the basis of the literature data and on the results obtained in the previous analyses, the $^{\bullet}OH$ quenching activity of the plant extracts was determined in reaction systems containing: i) Fe(II) sulfate solution in 20 mM phosphate buffer and DMPO at the final concentration of 10 mM; ii) Fe(II)-Quin complex without any buffer and DMPO at the final concentration of 0.6 mM; iii) Fe(II)-Quin complex in 20 mM phosphate buffer solution and DMPO at the final concentration of 0.6 mM.

In order to have a direct comparison of the outcomes provided by the three systems employed, in each of them the same amount of extract was used: 100 µl of diluted orange juice, 200 µl of diluted asparagus extract and 100 µl of diluted Java green tea infusion.

Results are expressed as percentage of inhibition calculated as follows: percent of inhibition = 100 x $(I_0 - I_S)/I_0$, where I_0 is the intensity of the signal of the spin adduct without the extract and I_S is the intensity of the signal of the adduct after the reaction with the extract. Five replications were performed for each extract.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism6 for Windows software (GraphPad software. Inc., La Jolla, CA92037, USA). A one-way ANOVA was carried out to compare DMPO-OH signal intensities obtained with different DMPO or buffer concentrations, using a unifactorial complete randomized block design. Mean separation was calculated by the Fisher's least significance test at $P \leq 0.05$. A student's *t*-test was performed to compare the DMPO-OH signal intensities obtained in systems with phosphate or Tris-HCl buffers at the same final concentration and to compare DMPO-OH signal intensities obtained in systems with Fe(II) sulfate or Fe(II)-Quin complex at the same DMPO concentration.

3. Results and discussion

3.1. Effect of quinolinic acid-iron(II) complex on hydroxyl radical production

In the presence of the spin trap DMPO, the hydroxyl radicals produced in the Fenton reaction are trapped and the signal of the DMPO-OH adduct can be recorded with an EPR spectrometer. The typical EPR signal of the DMPO-OH radical adduct is shown in Fig. 1. The spectrum is a four line signal with the following hyperfine splitting constants $a_N = a_H = 14.9$ G.

The effect on hydroxyl radical formation and EPR spin trapping analysis of Fe(II) and H_2O_2 concentrations, incubation time and light exposure during sample preparation, have been reported in

the literature (Fontmorin, et al., 2016; Jeong, et al., 2016). The effects of these variables were studied with the aim of optimizing the conditions of trials designed for EPR spin trapping applications different from food analysis.

Due to the presence in plant extracts of natural chelating agents the choice of a proper Fe(II) ligand is of great importance. In this paper, with the aim of optimizing the conditions for hydroxyl radical scavenging analysis, we propose the use of quinolinic acid (Quin) as a novel chelating agent in food chemistry applications. Quinolinic acid is a neurotoxin involved in the pathogenesis of several brain disorders (Guillemin, 2012). It forms coordination complexes with Fe(II), coordinating by carboxylate and pyridine nitrogen atom. This coordination pattern gives the complex intermediate characteristics between those with oxygen and nitrogen donors (Pláteník, Stopka, Vejražka, & Štípek, 2001). The stability constants of the iron(II)-complexes formed by quinolinic acid were previously reported in the literature (Pláteník, et al., 2001). In a Fenton reaction system the chelation of iron regulates its reactivity. The presence of iron chelators affects the redox potential of the couple Fe(III)/Fe(II), but the extent and the sign of this change depends on the ligand present in solution. As a general rule ligands with oxygen atoms as donors stabilize iron(III) and decrease the iron reduction potential thus making the oxidation easier. Desferal is an example of such a ligand: its presence in the Fenton reaction system completely inhibited $^{\bullet}OH$ production as shown by the lack of EPR signal (Šnyrychová, Pospíšil, & Nauš, 2006). Conversely, ligands like phenantroline or 2,2'-bipyridine with nitrogen atoms as donors, stabilize iron(II) and increase the iron reduction potential making iron(II) unreactive.

EDTA (ethylenediaminetetraacetic acid), DETAPAC (diethylenetriaminepentaacetic acid), DTPA (Diethylenetriaminepentaacetic acid) citric and oxalic acid are some of the most studied iron ligands. They have been studied to assess their effect on Fe(II) autoxidation (Welch, Davis, & Aust, 2002) and the effect of iron chelation on hydroxyl radical generation (Yamazaki & Piette, 1990; Šnyrychová, et al., 2006). According to Yamazaki, et al. (1990) the production of hydroxyl radical is strongly influenced by the nature of the iron ligand used and increases in the order: phosphate <

ADP (adenosine 5'-diphosphate) < EDTA < DETAPAC. Similarly Li, et al. (2007) demonstrated that chelating agents like EDTA increased the formation of hydroxyl radicals in the Fenton reaction, but they also observed an enhancement of the quenching effect of the DMPO-OH adduct operated by Fe(II) ions in the presence of ligands. The effect of chelators on the rate of hydroxyl radicals generation depends on the chelator/Fe(II) ratio (Yoshimura, Matsuzaki, Watanabe, Uchiyama, Ohsawa, & Imaeda, 1992; Engelmann, Bobier, Hiatt, & Cheng, 2003).

In accordance with previous studies, chelation of iron(II) with quinolinic acid at a ratio of 5:1 significantly enhanced the hydroxyl radical generation (Iwahashi, Kawamori, & Fukushima, 1999; Pláteník, et al., 2001). As shown in Fig. 2 in the Fenton reaction mixture with no buffer, DMPO 10 mM and Fe(II)-Quin complex (Fe(II) 0.01 mM) the DMPO-OH signal intensity was more than twice the intensity measured in reaction mixtures with Fe(II)-sulfate alone (~ 147 % increase). Kubicova, Hadacek, Weckwerth, and Chobot (2015) reported a complicated milieu-dependent effect related to Quin coordination chemistry. With Quin-Fe(II) ratios of 1:1, 1:2, 3:1 and 4:1 these authors described a Quin antioxidant activity, rather than a pro-oxidant one, due to the inhibition of the iron catalytic activity. In chemical systems the antioxidant or prooxidant action of metal chelators seem to depend on the chelator/metal ratio (Engelmann, et al., 2003; Šnyrychová, et al., 2006). In this study we used a high Quin/Fe(II) ratio (5:1) that increases the $^{\bullet}$OH production.

The strong enhancement of DMPO-OH production observed in this study may be the result of the concomitant effect of the ligand, with an appropriate chelator/metal ratio, and pH. In fact, as shown below, Fe(II) is particularly sensitive to pH changes of the reaction mixture, while this effect considerably diminishes in the presence of quinolinic acid. Indeed, the pH values of the solution mixtures containing DMPO and Fe(II)-sulfate are higher than those of Fe(II)-Quin solutions and DMPO because of the buffer capacity of Quin. In particular, the signal intensity of the DMPO-OH adduct, in the system containing Fe(II)-sulfate, decreases as the pH of the reaction mixture increases. In order to distinguish between these two effects the reaction was carried out at pH 7.4 in phosphate buffer 20 mM (Fig.2). In this condition the DMPO-OH signal intensity of the Fe(II)-

Quin complex was still significantly high, but the extent of the increase was reduced (~68 % increase).

This result prompted us to study the effect of buffers on the DMPO-OH adduct generation in Fenton systems with quinolinic acid as iron chelator. The effect of chelators like DETAPAC and EDTA on the generation of hydroxyl radical depends on the buffers used; with no chelating buffers (Hepes and MOPS) the production of hydroxyl radical is strongly reduced, whereas buffers with high affinity for iron (phosphate buffer) foster the generation of $^{\bullet}$OH (Yoshimura, et al., 1992).

3.2. Effect of buffers on DMPO-OH adduct signal intensity

The presence of buffers in the Fenton reaction mixture affects the hydroxyl radical production.

The production of $OH^{\bullet}$ *via* Fenton reaction was markedly reduced in buffer solutions both when Fe(II)-sulfate or Fe(II)-Quin complex were employed (Fig. 2, Fig. 3).

The effect of phosphate and Tris-HCl buffer concentration on DMPO-OH adduct yield in the Fe(II)-Quin system is shown in Fig. 3. The EPR signal of the DMPO-OH adduct was reduced by ~53% and ~88% when the Fenton reaction was carried out in 70 mM phosphate and Tris-HCl buffer solutions, respectively.

The influence of the phosphate buffer solution on the signal intensity of the DMPO-OH adduct was significant up to a concentration of 10 mM, after which an increase of buffer concentration did not cause any further statistically significant decrease in signal intensity. By contrast in Tris-HCl buffer the DMPO-OH signal intensity decreased with increasing buffer concentration. The influence of buffer on the production of DMPO-OH was stronger in Tris-HCl solutions than in phosphate buffer. In the Fenton reaction systems, the presence of chelators and chelating buffers change the reactivity of iron by changing the standard reduction potential of the couple Fe(III)/Fe(II). Moreover buffers can scavenge hydroxyl radicals thus decreasing the amount of the DMPO-OH adduct. In a reaction mixture with no chelating agents Yoshimura, et al. (1992), showed a significant decrease of the

293 production of •OH in phosphate buffer and a slight decrease in Tris-HCl solutions. These authors,
294 ascribed this effect to a weak iron binding capacity of Tris-HCl and to a high rate of Fe(II)
295 autoxidation in phosphate buffer. In the present work the addition of Quin gave opposite results:
296 with the exception of the lowest concentration of buffer tested (2 mM), the generation of DMPO-
297 OH adduct was significantly lower in Tris-HCl than in phosphate solutions. This confirms the
298 hypothesis that the addition of iron ligands to buffer solutions with low affinity (Tris-HCl) for iron
299 reduces or inhibits the generation of •OH, conversely iron chelating buffers (phosphate) enhance the
300 radical production (Yoshimura, et al., 1992). Our results are in agreement with Khosravifarsani,
301 Shabestani-Monfared, Pouramir and Zabihi (2016) who demonstrated that tris-HCl buffer is a
302 stronger •OH radical scavenger than phosphate at pH 7.0.
303 The amount of DMPO-OH adduct detected by EPR may be the result of hydroxyl radical
304 production and of other secondary reactions involving the quencing of DMPO-OH adduct by Fe(II).
305 Li, et al. (2004) demonstrated that the EPR signal of the DMPO-OH adduct disappear rapidly in
306 presence of an excess of Fe(II) ions and this effect was enhanced when phosphate was added to the
307 reaction mixture. At present no data are available on the quenching of DMPO-OH adduct by Tris-
308 HCl buffer however the involvement of secondary reactions based on the reduction of DMPO-OH
309 adduct by Fe(II) could be supposed. Given the sharp decrease of the DMPO-OH signal intensity in
310 Tris-HCl buffer, the following experiments were performed in phosphate buffer solution, which is
311 the most widely adopted medium to simulate biological systems and is frequently used for *in vitro*
312 determination of hydroxyl radical scavenging activity (Khan, et al., 2003; Calliste, et al., 2005;
313 Garcia-Alonso, et al., 2005).
314 The experiments with phosphate buffer only were set up to evaluate the effect of this medium in
315 Fenton reaction systems with chelated and unchelated Fe(II) (Fig. 2). The Fe(II)-Quin complex, in
316 the Fenton reaction, produced more hydroxyl radicals than Fe(II)-sulfate alone both with and
317 without buffer. In the Fenton reaction mixture with no buffer the DMPO-OH signal intensity
318 showed by the Fe(II)-Quin complex was more than twice that of Fe(II) alone. As already discussed

in this paragraph, phosphate buffer 20 mM significantly reduced the DMPO-OH adduct production of Fe(II)-Quin complex, whereas it had no significant effect on the yield of hydroxyl radicals produced by Fe(II) alone. The amount of DMPO-OH adduct detected in Fenton reaction systems with Fe(II)-sulfate strongly depends on the pH of the solution. Without buffer the pH of the reaction mixture was ca.8 as the DMPO solution is alkaline (pH ~ 9); in this situation only the continuous nitrogen bubbling of the stock iron(II) solution (pH ~ 5.3) and water prevents the rapid oxidation of iron.

Phosphate 20 mM buffered the solution at pH 7.4 which is quite close to the pH of the un-buffered solution and it isn't low enough to avoid partial Fe(II) oxidation.

3.3. Effect of pH on hydroxyl radical formation

Fruits and plant extracts are rich in antioxidant compounds and organic acids whose composition and concentration is extremely variable. In the studies of the antioxidant properties of fruit juices or food extracts the presence of organic acids and their effect on the pH of the reaction mixture is seldom considered, however it can affect the determination of the hydroxyl radical scavenging activity.

The effect of pH on DMPO-OH adduct signal intensity was studied by adjusting the pH of the DMPO solution (initially pH ~ 9) to 1.4 and it was compared to solutions with no acidified DMPO. Moreover two concentrations of DMPO were evaluated with the aim to study the effect of the spin trap concentration on the changes of pH in the reaction mixture. As shown in Fig. 4 with no acidified DMPO the pH of the solution is strongly affected by the DMPO concentration. With the highest DMPO concentration used, the pH of the solution was about 8, whereas with the lowest one the pH was reduced to 5. This effect could be explained considering that the DMPO has a pK_a value of 5.99, therefore a DMPO solution 10^{-2} M, that is the highest concentration used, has a pH of about 9. A DMPO solution 1 mM has a pH of about 8 and is much more affected by the acidity of the Fe(II) solution.

The DMPO-OH signal intensity is strongly influenced by the pH of the solution. The signal intensity measured in the reaction mixture with not acidified 10 mM DMPO is 3-fold lower than that measured with acidified DMPO. The same was not found for DMPO solutions 1 mM: in this case un-acidified DMPO gave quite similar adduct signal intensity to acidified DMPO, with no statistical differences observed (Fig. 4). It worth noticing that the pH of both solutions is between 4 and 5, that is close to optimal pH for the Fenton reaction (Salgado, et al., 2013). The observed link between $^{\bullet}OH$ production and pH is in line with Yehia, Eshaq, and ElMetwally (2016) who observed a decrease of nitrophenol degradation by $^{\bullet}OH$ radicals with increasing pH values.

The strong relationship between DMPO-OH signal intensity and pH resides on the effect of pH on the Fenton reaction (Babuponnusami and Muthukumar, 2014). In this reaction, Fe^{2+} reacts with H_2O_2 producing hydroxyl radicals. The reaction is usually written in the following form (1):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \qquad (1)$$

however in acidic aqueous solutions Fe^{2+} and Fe^{3+} do not exist as naked ions but as aqua-complexes, thus it is more correct to write (1) in the following way:

$$[Fe(H_2O)_6]^{2+} + H_2O_2 \rightarrow [Fe(H_2O)_6]^{3+} + OH^- + {}^{\bullet}OH \qquad (2)$$

The existence of the hexaaquairon(III) depends on pH; it exists at very acidic pH since its pK_a value is 2.19, but at higher pH values other hydrolytic species are formed. By contrast the pK_a of $[Fe(H_2O)_6]^{2+}$ is much higher (9.5), meaning that it is stable in neutral aqueous solutions. Therefore, considering the hydrolysis of the metal ions only, the Fenton reaction could be carried out in neutral aqueous solutions, however Fe(II) is very sensitive to atmospheric oxygen. Even with very low oxygen concentration Fe(II) is oxidized to Fe(III) and the oxidation is easier at high pH values due

to the pH dependence of the standard reduction potential of the couple Fe(III)/Fe(II) (Babuponnusami and Muthukumar, 2014).

3.4. Effect of DMPO concentration on the production of hydroxyl radicals

The effect of DMPO concentration on adduct signal intensity was studied in reaction systems with chelated and unchelated Fe(II). No signal was detected when Fe(II) sulfate (unchelated Fe) was used with DMPO at a final concentration of 0.6 mM, the lowest used in this test. This could be explained considering that Fe(II) sulfate, in these conditions, produced a relatively low flux of hydroxyl radical. In this situation the spin trap concentration must be quite high to trap the hydroxyl radicals and produce the adduct.

The effect of pH on the production of hydroxyl radicals in solutions of unchelated Fe(II) was discussee above. Since the DMPO solution has a relatively high pH value, it is expected that, when high concentrations of this spin trap is used, the pH value of the reaction mixture becomes high disfavouring the production of hydroxyl radicals. To check the effect of DMPO concentration and to eliminate the pH effect, the DMPO solution was acidified (pH 1.4).

The effect of DMPO concentration on DMPO-OH signal intensity is reported in Fig. 5. The intensity of the DMPO-OH adduct increased with increasing concentrations of DMPO. These results agree with those of Fontmorin, et al. (2016) who found an increment of the peak to peak amplitude of the DMPO-OH adduct signal with increasing DMPO concentrations. According to these authors to ensure a reliable hydroxyl radical detection, the proper DMPO concentration should be set on the basis of Fe(II) and H_2O_2 concentrations (Fontmorin, et al., 2016).

Under the conditions used in the present study, the DMPO-OH adduct signal consisted only in a four lines spectrum as in Fig. 1. No other EPR detectable by-products could be identified. By contrast Fontmorin, et al. (2016), depending on the DMPO concentration tested, observed a combination of quartet and triplet spectra. According to these authors the presence of triplet lines

was clearly evident when the DMPO concentration was lowered to 5 mM and the DMPO/Fe(II) was 10. In the present work, with very similar DMPO concentrations (3-6 mM) but higher DMPO ratio (300-600), the triplet was not detected thus confirming the key influencece of the DMPO/Fe(II) ratio on the reliability of DMPO-OH detection.

In reaction systems with Fe(II) sulfate, the highest signal intensity was detected with DMPO/Fe ratio between 300 and 600, which corresponds to a final DMPO concentration of 3 and 6 mM respectively. A further increase of DMPO/Fe ratio caused a significant reduction of the adduct signal intensity, that was comparable to that obtained with a DMPO concentration 10 times lower (Fig. 5).

In the Fenton reaction with Fe(II)-Quin complex as a source of Fe(II), no DMPO acidification was necessary. The highest DMPO-OH signal intensity was detected at a DMPO/Fe ratio of 100 and no significant differences were observed with concentrations 3 and 6 fold higher (Fig. 5). These results demonstrate that the DMPO concentration should be set not only on the basis of DMPO/Fe(II) ratio as previously reported, but also on the presence of Fe(II) ligands in the reaction system.

Even with Fe(II)-Quin an increase of DMPO concentration (final concentration 10 mM) caused a significant decrease of the adduct signal intensity. The significant decrease of the adduct intensity signal, observed at DMPO concentration higher than 6 mM, could be explained considering a quenching effect of DMPO itself on DMPO-OH.

The outcomes presented in this section demonstrate that the efficiency of the DMPO-OH adduct formation depends not only on the effectiveness of the hydroxyl radical generation but also on the DMPO concentration.

3.5. Hydroxyl radical scavenging activity of plant extracts.

In the analysis of food antioxidant properties, the use of EPR and the spin trapping technique for the determination of the hydroxyl radical scavenging activity is gaining great attention (Pérez-López, et al., 2014; Braga, et al., 2016). In this manuscript the radical scavenging activity of *Asparagus officinalis* extract, Hamlin orange juice and Java Green Tea infusion were evaluated. The choice of these extracts was based on their different chemical compositions. As reported in our previous work, epigallocatechin gallate (EGCG) was the most abundant compound in java green tea together with other catechins, purine alkaloids and flavonol glysosides (Fadda, Serra, Molinu, Azara, Barberis, & Sanna, 2014). Hamlin Juice was rich in L-ascorbic acid (79 mg/100 ml) followed by Hesperidin (40 mg/100 ml) (Barberis, et al., 2014), whereas, in Purple Passion asparagus spears, rutin was the most abundant phenolic compound (Maeda, et al., 2005).

The hydroxyl radical scavenging activity of plant extracts was determined using: i) Fe(II) sulfate solution in phosphate buffer, ii) Fe(II)-Quin complex without any buffer, iii) Fe(II)-Quin complex in phosphate buffer solution (Fig. 6). With the aim to provide a direct comparison among the experimental conditions applied, the same amount of extract was used (see experimental). As reported in previous paragraphs the three systems generated different amounts of $^{\bullet}OH$ radical which resulted, in the case of Asparagus extract and Orange juice, in different radical scavenging activities. Asparagus extracts had a $^{\bullet}OH$ radical scavenging activity ranging from 20.3% to 49.7% determined in reaction systems containing buffered Fe(II)-sulfate and no buffered Fe(II)-Quin complex solutions, respectively. No statistical differences were observed between scavenging activities obtained in buffered solutions of Fe(II) sulfate and Fe(II)-Quin complex. Even with orange juice the highest activity was shown in reaction systems with Fe(II)-Quin complex with no buffer. No differences were observed between radical scavenging activities measured in Fe(II)-Quin and buffered Fe(II)-sulfate reaction systems, whereas the reaction carried out in buffered Fe(II)-Quin complex system gave remarkably lower antioxidant values. Conversely, in Java Green Tea no significant differences could be found among the Fenton reaction systems applied. These results

highlight a strong influence of the experimental conditions on the evaluation of the radical scavenging activity of food produce. Orange juice, asparagus extract and Java Green Tea showed the highest radical scavenging activity in reactions with no buffer and with the Fe(II)-Quin complex as Fe(II) source. The presence of phosphate buffer (pH 7.4) in the reaction mixture has a different effect depending on the sample analyzed and whether or not Fe(II) coordinating ligands are present. However, considering the samples analyzed no general rule can be identified and no unequivocal explanation can be provided.

The different behaviour of the plant extracts with the three systems could be explained by hypothesizing the presence in the food matrices of iron coordinating compounds, which can change the reactivity of the metal ion and the potential of the couple Fe(III)/Fe(II), leading to different amounts of generated hydroxyl radicals. In orange juice, for example, the main compounds are L-ascorbic acid and hesperidin which have low iron chelating activity (Mladěnka, et al., 2011; Senol, 2016). By contrast rutin, the main component of asparagus spears, is able to chelate iron thus affecting the hydroxyl radical generation in buffered Fe(II)-sulfate reaction system, but not in Fe(II)-Quin system, where the presence of Quin compensates for the effects of rutin (Lue, Nielsen, Jacobsen, Hellgren, Guo, & Xu, 2010). In EGCG, the main compound of Java Green tea, the presence of two gallate residues makes it a stronger iron complexing agent than Quin and phosphate buffer, thus overcoming their effects in the reaction systems. The Fe coordinating properties of EGCG could probably explain the lack of differences observed in the three experimental conditions tested (Fig. 6B).

As seen before, quinolinic acid can be considered a ligand which stabilizes both oxidation states, Fe(II) and Fe(III), making the Fenton reaction more manageable. However, in plant extract iron coordination compounds, like EGCG, can be present and the resulting complexes could be more stable than those formed with quinolinic acid, therefore changing the reactivity of the metal ion.

4. Conclusions

In this work we examined the variables involved in the generation of the hydroxyl radical with the Fenton reaction and in its detection by spin trapping with DMPO, as well as the effects of buffers on the intensity of the DMPO-OH adduct. The DMPO-OH signal intensity depends on the pH of the measuring system: low pH values enhance the signal intensity, while high values lower the hydroxyl radical generation thus reducing the adduct signal.

The effect of DMPO concentration on DMPO-OH adduct signal intensity has been barely studied, with only one report on this topic in the literature. Our results confirm that the efficiency of the DMPO-OH formation depends not only on the effectiveness of the hydroxyl radical generation but also on the DMPO concentration. In reaction systems with Fe(II) sulfate the highest signal intensity was detected with DMPO/Fe ratio between 300 and 600, while in those with Fe(II)-Quin the highest signal intensity was detected at lower DMPO/Fe ratio (100). The presence of Quin in the reaction system decreases the DMPO concentration necessary to achieve a reliable DMPO-OH detection. These results demonstrate that the presence of Fe(II) ligands should be taken into account when setting the proper DMPO concentration.

Since antioxidants present in foods should exert their effects in physiological conditions, buffers are frequently used to mimic these conditions; however, our results demonstrate that their presence in the measuring system decrease the adduct signal intensity. At the same concentrations Tris-HCl buffer gave significantly lower intensities of the DMPO-OH adduct than phosphate buffer.

The effect of Quin as Fe(II) ligand on hydroxyl radical generation and on radical scavenging activity determination was also studied. The choice of the chelating agent is a critical point due to its effect on iron reactivity. Quinolinic acid seems to be a good compromise stabilizing both oxidation states, Fe(II) and Fe(III). The $^{\bullet}OH$ generating system with Fe(II)-Quin produces more hydroxyl radicals than Fe(II) alone, making, at the same time, the Fenton reaction more manageable by avoiding the bubbling of nitrogen or other inert gas necessary to slow down spontaneous

oxidation of Fe(II) by atmospheric oxygen. Moreover, the Fenton system containing quinolinic acid is less influenced by the pH of the solution. Another advantage of Fe(II)/Quin/H_2O_2 hydroxyl generating system is the use of very low DMPO concentrations allowing for more economic and environmentally friendly analyses. In addition, Fe(II)-Quin complex solution stored at room temperature, has the advantage of being stable for several days as no DMPO-OH signal decay was observed when repeating the experiments one week after the preparation of the solution. Since the Fe(II)-Quin solution can be stored and used in more than one experiment, buffers and inert gases are avoided, the proposed method permits saving of time and resources.

This is the first time that quinolinic acid has been used for the hydroxyl radical determination of plant and food extracts. The food matrices (Java green tea, asparagus extracts, blond orange juice) analyzed showed different radical scavenging activities depending on the system employed to measure it. The Fe(II)-Quin system seems to be less influenced by the presence in the food matrices of compounds with chelating properties which change the amount of hydroxyl radicals generated, affecting the estimation of the radical scavenging activity of plant and food extracts. The hydroxyl radical scavenging activity is also influenced by the presence of phosphate buffer in the reaction system. Even low concentrations of phosphate buffer in the reaction mixture brings about a decrease of the radical scavenging activity of the extracts analyzed.

The spin trapping method for the estimation of the hydroxyl radical scavenging activity of food/plant extracts is gaining great attention, however some aspects, related to the complexity of the Fenton reaction, should be taken into account to provide reliable measures. The present results highlight the necessity to standardize the spin trapping method with the Fenton reaction and DMPO as spin trap and, at the same time, to make it easier for routine experiments. The use of quinolinic acid as an Fe(II) chelating agent is an attempt in this direction, since it minimizes the effects of pH changes and allows for an easier handling of the solutions.

Conflicts of interest

The authors declare no conflict of interest.

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Figure captions

Fig. 1. A typical EPR spectrum of the hydroxyl radical generated in the Fenton reaction and trapped with DMPO.

Fig. 2. Effect of phosphate buffer on the production of hydroxyl radicals in Fenton reaction systems with chelated (Fe(II)-Quin) and unchelated Fe (Fe(II). All reaction mixtures contained DMPO 10 mM, Fe(II) 10 μM and H_2O_2 0.03%. Bars marked by unlike letters differ significantly by Fisher's least significant difference (LSD) ($P \leq 0.05$).

Fig. 3. Effect of phosphate and Tris-HCl buffers at different concentrations on the yield of hydroxyl radicals, measured as DMPO-OH signal intensity (AU Arbitrary Units), produced in a reaction system with Quin-Fe(II) complex as source of iron. All reaction mixtures contained DMPO 10 mM, Fe(II) 10 μM and H_2O_2 0.03%. Capital letters show statistical differences in Fenton systems with increasing phosphate buffer concentrations, whereas lowercase letters refer to statistical differences in Fenton systems with increasing Tris-HCl buffer concentrations. Means comparison was performed with Fisher's least significant difference (LSD) ($P \leq 0.05$). An asterisk above points refer to statistical differences between phosphate and Tris-HCl buffers for the same concentration according to Student's *t*-test ($P \leq 0.05$).

Fig. 4. Effect of pH on DMPO-OH adduct signal intensity measured in reaction systems with DMPO concentrations of 1 and 10 mM. Acidified (white bars) and not acidified (grey bars) DMPO solutions were used. Squares indicate the pH values of Fenton systems with un-acidified DMPO solutions. Circles indicate the pH values of Fenton systems with acidified DMPO solutions. Data are presented as mean ± standard deviation.

Fig. 5. Effect of DMPO concentration on DMPO-OH signal intensity in Fenton reaction systems with chelated (Fe(II)-Quin) and unchelated Fe (Fe(II)). In the case of unchelated Fe(II) the DMPO solution was acidified. Capital letters show statistical differences in "Fenton systems" with unchelated Fe(II), whereas lowercase letters refer to statistical differences in "Fenton systems" with chelated Fe(II). Means comparison was performed with Fisher's least significant difference (LSD) ($P \leq 0.05$). An asterisk above bars refer to statistical differences between chelated and unchelated Fe(II) for the same DMPO/Fe(II) molar ratio according to Student's *t*-test ($P \leq 0.05$).

Fig. 6. Hydroxyl radical scavenging activity of Hamlin Orange juice (A), Java Green Tea infusion (B) and Purple passion asparagus spears (C) measured in reaction systems containing: DMPO 10 μM, $FeSO_4$ 10 μM and phosphate buffer 20 mM (Black bars); DMPO 0.6 μM and Fe(II)-Quin complex 10 μM (pale grey bars); DMPO 0.6 μM and Fe(II)-Quin complex 10 μM and phosphate buffer 20 mM (dark grey bars). Bars marked by unlike letters differ significantly by Fisher's least significant difference (LSD) ($P \leq 0.05$).

Figure(s)

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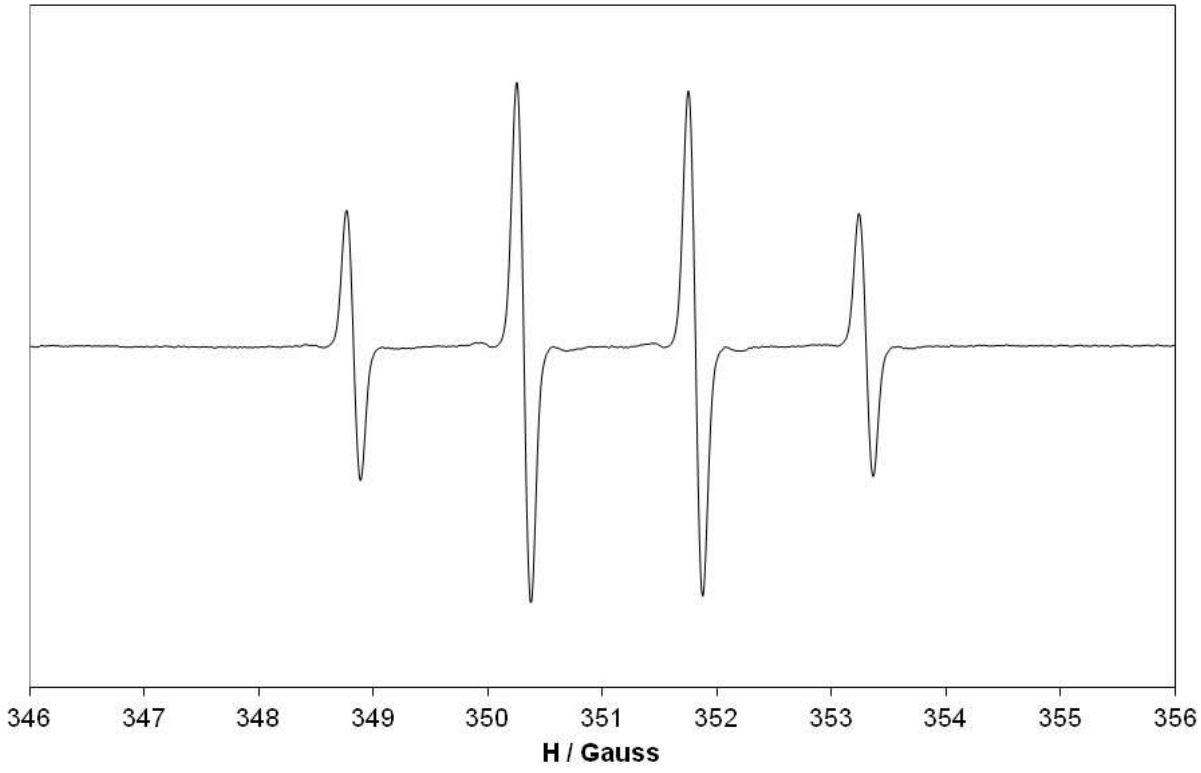

Figure 1

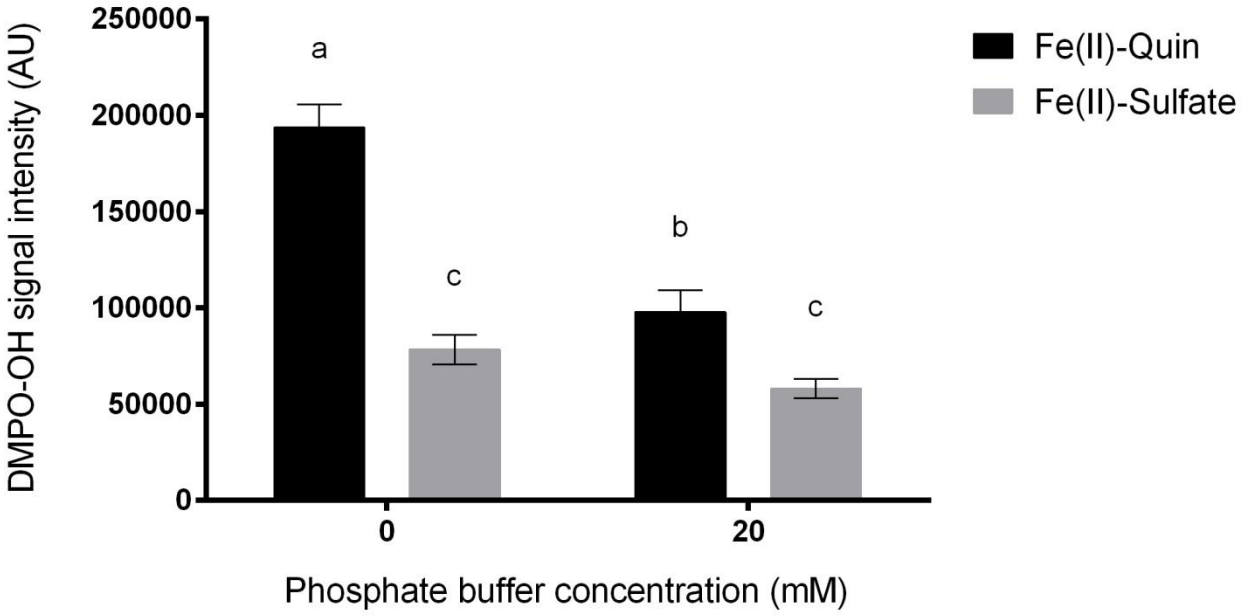
DMPO-OH signal intensity (AU)
250000
200000
150000
100000
50000
0
a
b
c
c
0
20
Phosphate buffer concentration (mM)
Fe(II)-Quin
Fe(II)-Sulfate

Figure 2

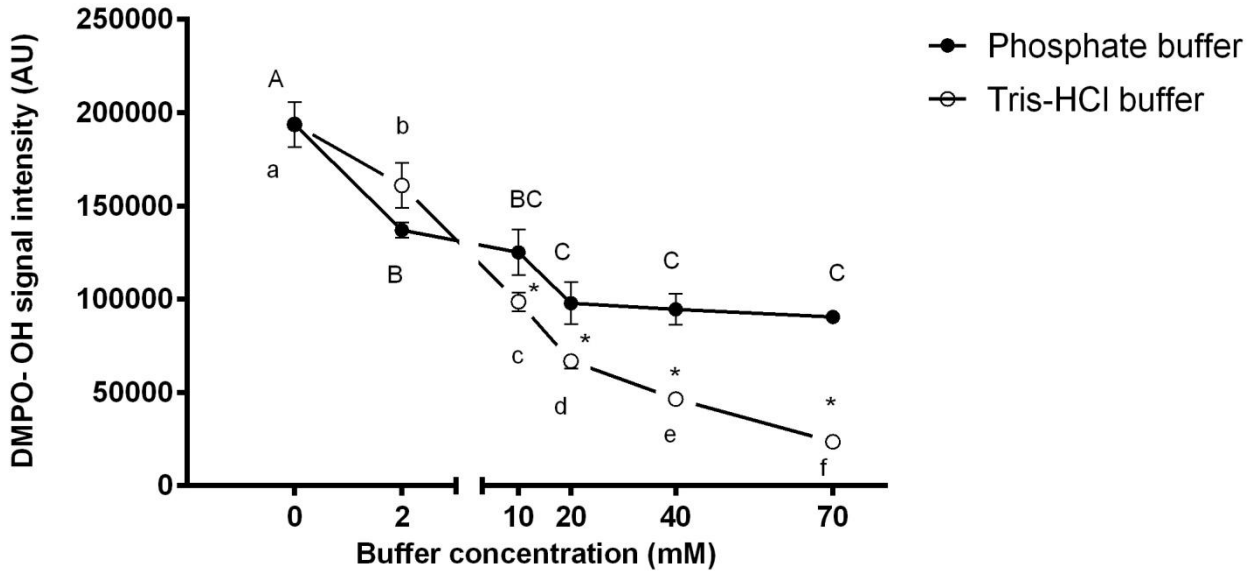
DMPO- OH signal intensity (AU)
250000
200000
150000
100000
50000
0
A
a
b
B
BC
C
C
C
c
d
e
f
*
*
*
*
0
2
10
20
40
70
Buffer concentration (mM)
Phosphate buffer
Tris-HCl buffer

Figure 3

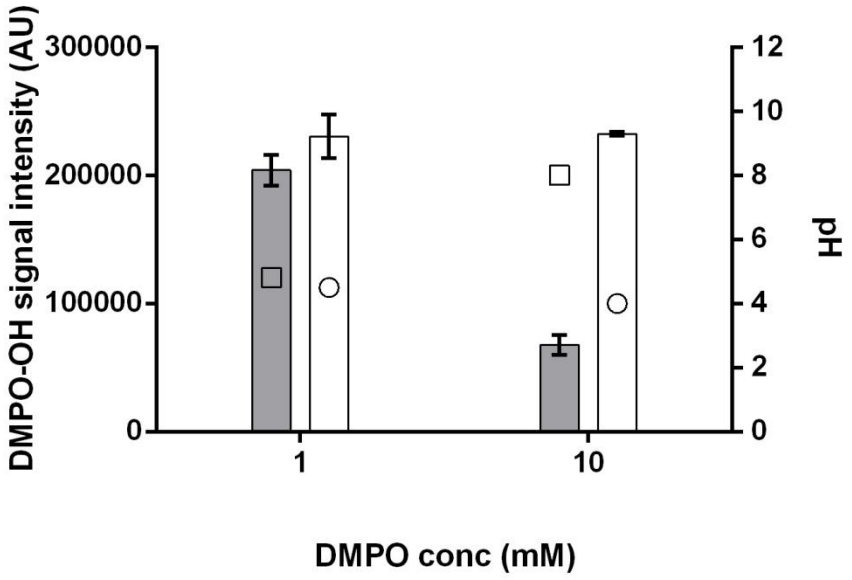
DMPO-OH signal intensity (AU)
300000
200000
100000
0
12
10
8
6
4
2
0
pH
1
10
DMPO conc (mM)

Figure 4

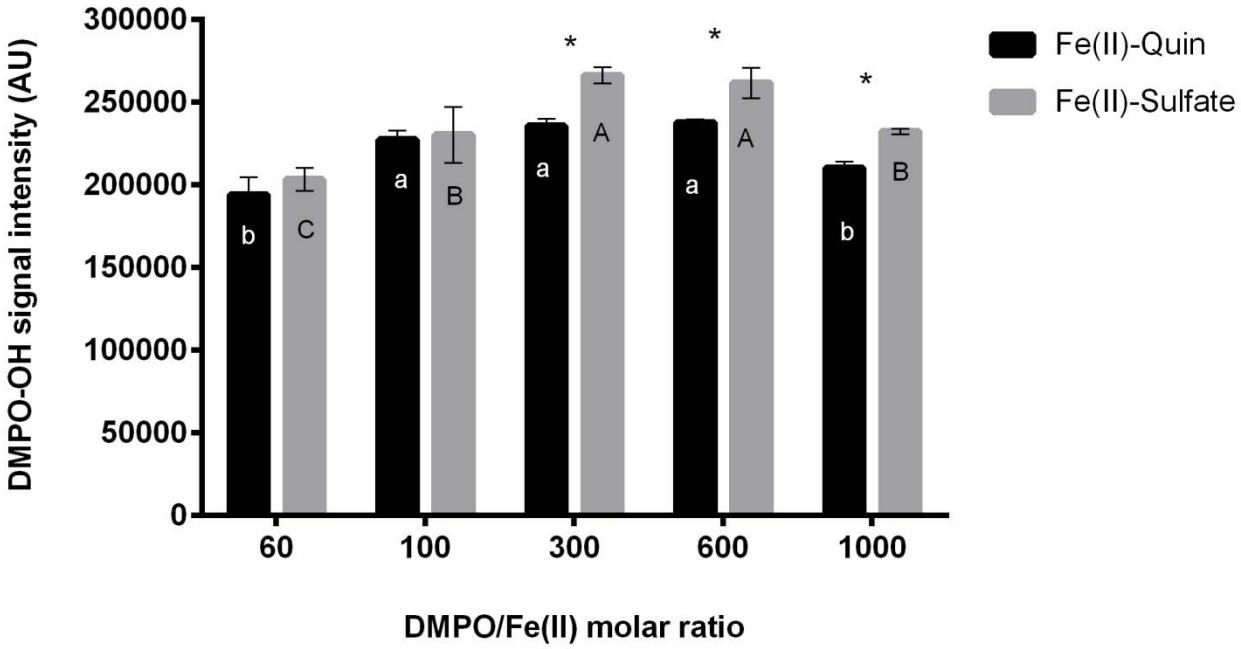
DMPO-OH signal intensity (AU)
300000
250000
200000
150000
100000
50000
0
60
100
300
600
1000
DMPO/Fe(II) molar ratio
Fe(II)-Quin
Fe(II)-Sulfate
b
C
a
B
a
A
a
A
b
B
*
*
*

Figure 5

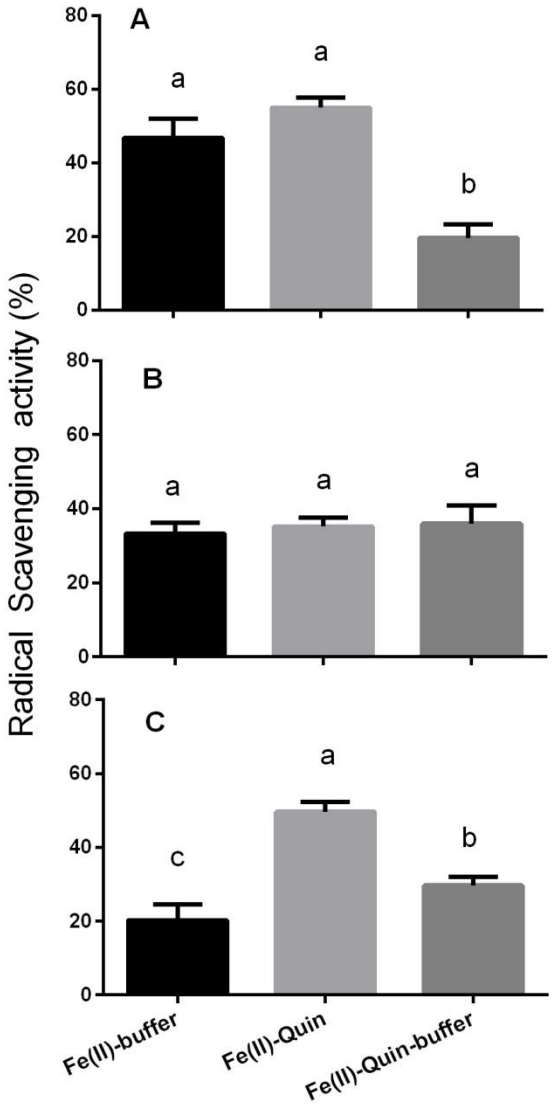
A
B
C
a
a
b
a
a
a
c
a
b
80
60
40
20
0
Radical Scavenging activity (%)
Fe(II)-buffer
Fe(II)-Quin
Fe(II)-Quin-buffer

Figure 6