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Influence of pH, buffers and 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)

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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 ·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.

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 (·OH) is one of the major causes of oxidative damage (Sakai et al., 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 (Braga et al., 2016; Calliste, Trouillas, Allais, & Duroux, 2005; Staško, Polovka, Brezová, Biskupič, & Maĺık, 2006). 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 (Azman, Peiró, Fajarí, Julià, & Almajano, 2014; Brezová, Šlebodová, & Staško, 2009; Calliste et al., 2005; Fadda, Pace, Angioni, Barberis, & Cefola, 2016; Fadda & Sanna, 2015; Pérez-López, Pinzino, Quartacci, Ranieri, & Sgherri, 2014; Staško et al., 2006).

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

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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 (Braga et al., 2016; Debnath et al., 2011), 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 & 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).

2.2. Food extracts preparation

2.2.1. Java Green tea

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

2.2.2. Orange juice

Oranges (*Citrus sinensis* L. cv Hamlin) were harvested at the experimental orchard of the *Istituto di Scienze delle Produzioni Alimentari* located in central western Sardinia. The juice was obtained by squeezing 5 fruits per replication (five independent replicates) with a commercial juicer. Before analysis the juice was centrifuged (13,000 rpm for 15 min) and filtered (0.45 μm acetate cellulose filter).

2.2.3. Purple asparagus extracts

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

2.3. Spin trapping assay of the ·OH radical

2.3.1. Spin trapping assay

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

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

The DMPO-OH adduct was detected with a Bruker EMX spectrometer operating at the X-band (9.4 GHz) equipped with an HP 53150A microwave frequency counter using a Bruker AquaX capillary cell. With this cell it is possible to keep constant the value of Q (the quality factor of the resonator) during sample measurements thus allowing quantitative comparisons of the intensity of EPR signals to be made. Spin trapping experiments with DMPO can be used to calculate the concentration of the DMPO-OH adduct, demonstrating the reliability of the method (Eaton, Eaton, Barr, & Weber, 2010).

All the measurements were always compared to a blank sample measured under the same conditions.

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