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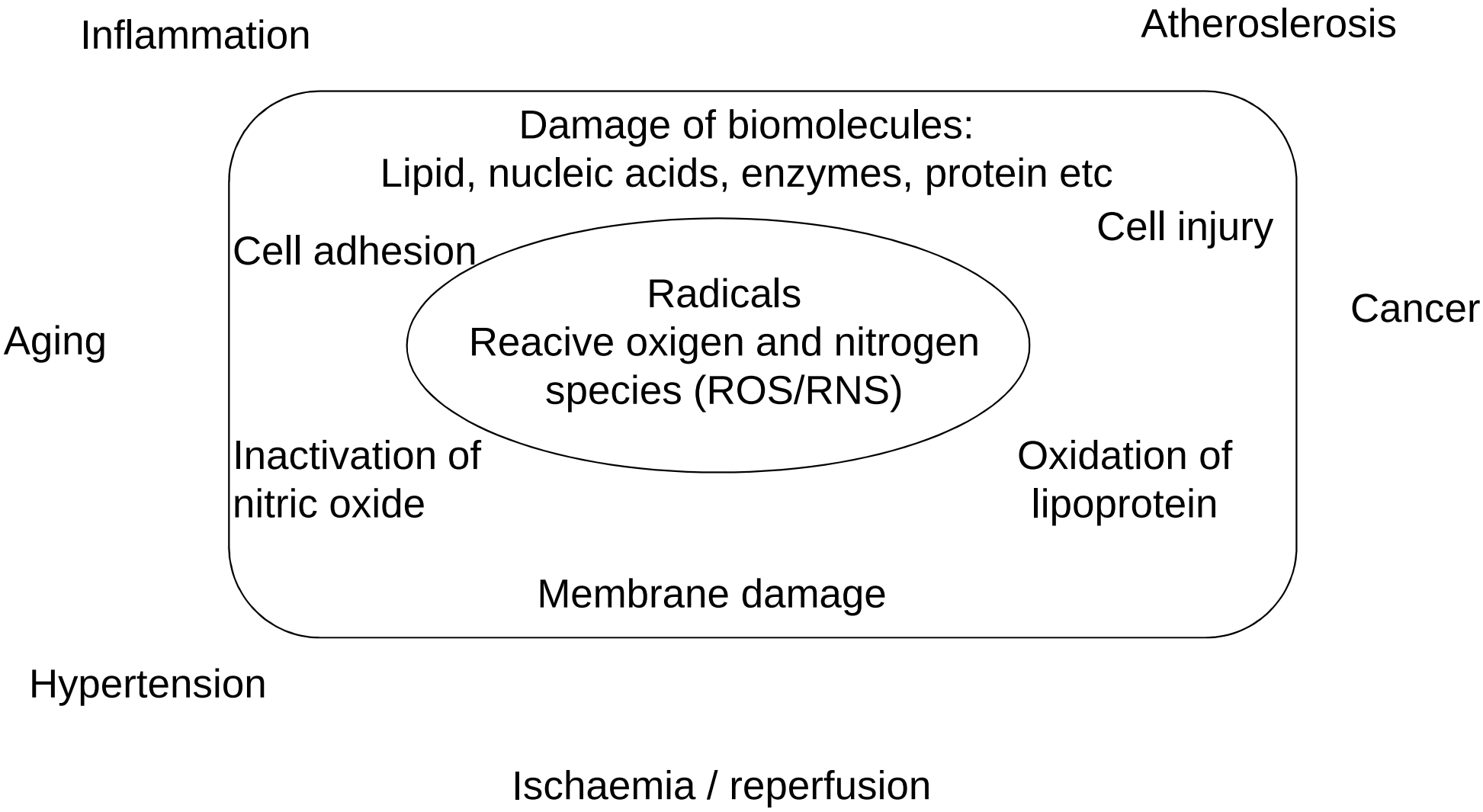

Graphical abstract

Review article

Electron spin resonance as a tool to monitor the influence of novel processing technologies on food properties

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Running head: Influence of novel technologies on food properties and electron spin resonance

Abstract

Background: Nowadays, electron spin resonance (ESR) is widely used as a powerful, non-destructive and very sensitive technique for the detection of free radicals in food systems. It can be applied for the direct identification of highly reactive oxygen species, organic and inorganic paramagnetic species and screening of food for potential toxicity. Its applications cover investigating food oxidative stability and properties of irradiated foods including fruits and vegetables, meats and fishes, spices, cereal grains, and oil seeds.

Scope and approach: This review aims at providing specialists in food science and industry with the fundamentals of ESR spectroscopy, typical radicals present in foods and their sources, ESR modalities, and detailed account for the use of the technology for evaluation of the physicochemical and nutritional properties of foods. Examples illustrating ESR applications for the evaluation of the effects of innovative and emerging technologies (ionizing radiation, high pressures, pulsed electric fields, cold plasma and ultrasonication) are discussed.

Key findings and conclusions: ESR can be used for the identification/quantification of free radicals in foods, for spin-label oximetry, estimation of free radical scavenging, food stability, and chelating activity, with particular interest for food processed using innovative technologies, with the main advantages of its high sensitivity, specificity, and low amounts of sample needed and nowadays many types of ESR instruments are commercially available. However, due to the different nature of foods, the development of novel ESR techniques and methods of analysis specially designed to study foods is of great interest in the future.

Keywords: Electron spin resonance; ESR; free radicals; novel processing technologies; high pressure processing; pulsed electric fields

1. Introduction

Free radicals are molecular species that contain an unpaired electron in the atomic orbital area paramagnetic group of molecular species. Due to their independent existence, free radicals are mostly unstable, highly reactive, and can either donate or accept an electron from other molecules. The free radical reactions are very typical for biological systems (Yoshikawa, Naito, & Kondo, 1997). For example, the transformation of O_2 into H_2O includes the formation of superoxide O_2•- and hydroxyl HO• short-lived radicals with a lifetime in the nanosecond to millisecond range (Yoshikawa, Naito, & Kondo, 1997).

Table 1 summarises the half-life and rate constants of biological reactive species (both free radical and oxidant species) (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013). Free radicals are unstable and are highly reactive oxygen species (ROS) promoting changes in DNA and cell damage, lipid and protein oxidation as well as cancer development and other oxidative stress-related diseases. The free radicals are known to attack important constituents of foods such as nucleic acids, proteins, carbohydrates, lipids, pigments and vitamins. The presence of these radicals accelerates oxidation processes, leading to decomposition of food constituents, the formation of oxidized products, development of off-flavor/odor, deterioration of pigments and useful nutrients that lead to reduction in the shelf-life and eating quality of foods (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013). Hydroxyl free radical has the shortest half-life among the various free radicals and oxidants (**Table 1**), but from a biologically point of view the hydroxyl radical is regarded as the most damaging free radical species due to it high reaction constant rates (**Table S1**) and indiscriminate reaction with neighbouring biomolecules (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013). It is worth noting that the reaction rate constants of the hydroxyl radical with proteins (collagen and albumin) are generally higher than individual amino acids (Table S1), which highlight its damaging role in biological systems. Furthermore, the ability of the hydroxyl radical to

oxidise antioxidants, fatty acids, protein, and aminoacids indiscriminately, which lead to extensive damage to neighbouring biomolecules. The half-life of superoxide and alkoxyl radicals are higher than the hydroxyl radical (Table 1) and they are important contributors to oxidative processes. Molecular oxygen has the highest half-life and reaction rate constant among the various oxidants listed in Table 1.

Food processing, which usually involves a series of mechanical, physical and chemical transformations of raw ingredients, may enhance the formation of free radicals in the food products and cause drastic changes in their quality. Therefore, over the recent years, there has been a growing awareness about free radical formation during food processing due to consumers' increasing demand for healthy food products, free from artificial chemicals and preservation of their natural and bioactive nutrients. This has, to some extent, favoured technological developments in non-thermal food processing, i.e. food processes carried out at ambient or near ambient temperatures, unlike thermal processing or cooking that require high temperature and cause major quality changes in foods (Rawson et al., 2011).

To date, very little work has been undertaken to identify the nature and unravelthe chemistry of the free radicals produced in foods subjected to novel non-thermal food processes(Ahn, Akram, Kim, & Kwon, 2013; Bolumar et al., 2014; Zhang, Yang, Zhao, Liang, & Zhang, 2011). A vast body of literature has highlighted the chemistry aspects of free radicals, their roles in human health and disease, as well as the possibility of annihilating radicals with adverse effects (Favier, Cadet, Kalyanaraman, Fontecave, & Pierre, 1995; Hiramatsu, Yoshikawa, & Inoue, 1997; Morello, Shahidi, & Ho, 2002; Rani & Yadav, 2015; Uppu, Murthy, Pryor, & Parinandi, 2010).

Earlier reviews have discussed the potential of ESR for estimating free radical scavenging capacity, food oxidative stability, determination of Cu^{2+} chelating capacity (Yu & Cheng, 2008), and properties of irradiated foods including meat, fruits, vegetables, spices,

cereal grains, and oilseeds (Shukla, 2016). However, literature pertinent to the application of ESR for evaluating radical formation during alternative processing approaches, such as ionizing radiation, high pressure, pulsed electric fields, ultrasound, and microwave, is scarce. ESR measurement can be a useful strategy to understand the chemical reactions at cellular level and to establish a relationship between free radical formation and healthy functional products. For example, a correlation of radical formation measured by ESR with inflammation, atherosclerosis, cancer, damage of biomolecules (e.g. lipids, nucleic acids, enzymes, protein, etc.) could be established by using *in vivo* models.

This review provides an overview of the current status of the use of ESR spectroscopy in topical nutraceutical and food research activities. The main focus is paid to recent advantages of ESR technique for free radical analysis in foods processed using innovative processing technologies, including ionizing radiation, high pressure, pulsed electric fields, ultrasound, cold plasma treatment, and microwaves.

2. Typical radicals present in foods and their sources

There are extensive reviews (Andersen & Skibsted, 2008; Kristensen, Kröger- Ohlsen, & Skibsted, 2002; Kumar, 2011; Shukla, 2016) and books (Favier et al., 1995; Gutteridge & Halliwell, 2015; Hiramatsu et al., 1997; Laher, 2014; Minisci, 1997; Pryor, 1984; Rani & Yadav, 2015; Roberfroid & Calderon, 1995; Uppu et al., 2010) discussing the mechanisms involved in free radical formation, their types and sources in biological and food systems.

2.1. Types of radicals

A wide variety of free radicals and other reactive oxygen and nitrogen species (ROS/RNS) can be found in food systems. Oxygen can produce different toxic species and activate reactions involved in the degradation of biomolecules such as lipids, nucleic acids,

and proteins. The chemistry of ROS has been reviewed in detail (Pierre, 1995). Radical ROS includes hydroxyl (HO•), superoxide (O_2•-), peroxy (RO_2•), and alkoxy (RO•) radicals. Non-radical hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and singlet oxygen ($^1O_2$) can also evolve in radical or radical-mediated reactions (Morello et al., 2002). RNS such as nitric oxide radicals, have been implicated in various physiological processes and they are very reactive towards molecular oxygen, superoxide radical, organic radicals, and transition metals (Garrel & Fontecave, 1995).

2.2. Formation of radicals

There are different internal and external sources of radicals in food systems (Kumar, 2011). The internal sources include mitochondrial activity as a major source of enzymes that generate free radicals as by-products of their activity (**Table 2**) (Bekhit et al., 2013). Several dehydrogenases, such as dihydroorotate dehydrogenase, glycerol-3-phosphate dehydrogenase, succinate dehydrogenase, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase as well as reductases (NADH:ubiquinone reductase, succinate-cytochrome c reductase and cytochrome b5 reductase) that are located in mitochondria, remain active postharvest and are able to produce several radicals and oxidants (**Table 2**). The interactions between these enzymes and their substrates become easier during postharvest storage as the integrity of mitochondria is lost over time. The generation of free radicals in biological materials through this pathway is important and can cause significant quality defects in fresh produce, e.g. fresh meat (for more information please see Bekhit et al., 2013). Furthermore, reactions involving Fe^{2+}, Cu^{2+}, and other transition metals; ischaemia/reperfusion; and inflammation (among others in plants and/or animal systems). The external sources include: non-enzymatic reactions of the oxygen with organic compounds, reactions initiated by ionizing radiations,

action of cigarette smoke, and exposure to environmental pollutants, radiations, ultraviolet light, and ozone, treatment with certain drugs, pesticides, and industrial solvents.

3. ESR modalities

3.1. Principle mechanism of ESR

The ESR was first discovered in 1944 in Kazan University by Zavoisky, (1944). This technique is based on the absorption of the microwave radiation by a paramagnetic sample (materials with unpaired electrons) placed in an external magnetic field. ESR is a useful technique for the detection of free radicals and other paramagnetic species such as transition metals. Position and shape of ESR lines are strongly dependent on the nature of the radicals. The electron-Zeeman interaction between unpaired electron(s) and an applied magnetic field is expressed via *g*-values. The g-value is analogous to the chemical shift in Nuclear Magnetic Resonance (NMR). The g-value extracted from ESR spectrum is an important characteristic that depends on the nature of the radical under consideration (for example, for a free electron, $g = 2.0023$). However, the ESR spectrum is often complicated by the hyperfine structure formed in the presence of neighbouring magnetic nuclei, such as ^{1}H, ^{13}C, ^{14}N, ^{19}F, etc. Thus, calibration of the ESR spectroscopy instrument is a necessary step.

For calibration of an ESR instrument, a suitable reference material has to be employed, e.g., a powder containing Mn^{2+} ions in lime (CaO) (Negut & Cutrubinis, 2017). The Mn^{2+} ion has effective spin $S = 5/2$, nuclear spin $I = 5/2$ and its ESR spectrum consists of a hyperfine sextet (**Figure S1**) (De Biasi & Grillo, 2014). The lines are spaced by ≈9mT and the third and fourth lines with *g*-value of 2.0292 and 1.9760, respectively, are commonly used for the calibration (De Biasi & Grillo, 2014).

The main advantages of ESR include its high sensitivity and specificity. This technique also requires relatively small amounts of sample. For example, using conventional

X-band (with the frequency of about 9.1–9.7 GHz) concentration of radicals≈2-3 µM can be detected for a 25 µL sample (Abbas, Babić, & Peyrot, 2016). Another advantage of the ESR method is the simplicity of sample preparation (Schaich, 2002).

3.2. ESR measurement

For detection and identification of free radical metabolites, ESR can be applied asa direct or indirect method. Biological semiquinone radical with a *g*-value of around 2.004 and a line width of approximately 5G on fungal spores of *Penicillium digitatum* can be kinetically analyzed in situ during atomic oxygen generated plasma electric discharge at real time and the decay of the ESR signal is possibly linked to the inactivation of the fungal spore (Ishikawa et al., 2012). Characteristic ESR signalsarisen from Fe^{3+}state and peroxy radical (RO_2•) on haemoglobin or myoglobin (Libardi, Skibsted & Cardoso, 2014;Jongberget al., 2014) were detected during atomic hydrogen, nitrogen, and oxygen exposure on raw horse meat during non-thermal processing (HPP, PEF, etc.). Therefore, these signals can be used as an indicator of a balance between inactivation of microorganisms and deterioration of food nutritional status (Kitada et al., 2017, Kitada et al. 2018).The *direct* application of ESR is possible for the relatively long-lived radical species while the *indirect* application of ESR uses spin trapping and spin labelling techniques. The spin trapping technique is based on the formation of long-lived and ESR-detectable spin adducts as a result of the reaction of a short-lived reactive free radical R• with a diamagnetic molecule (Mason, 1997).

$$R\bullet + \text{spin trap} \rightarrow \text{spin adduct}\bullet$$

The spin adduct (usually a nitroxide) should be a relatively long-lived radicalproduct. The signal intensity of the spin adducts, as observed in an ESR spectra, is directly proportional to the concentration of the formed free radicals R•.

The chemical structures of the popular spin traps DMPO (5, 5-dimethyl-1-pyrroline N-oxide) and PBN (*N*-tert-butyl-α-phenylnitrone) are presented in **Figure S2**. The structure of other spin traps developed for biological studies can be found in the available literature (Hawkins & Davies, 2014).

Information about the hyperfine splitting of the spin adducts for popular spin trapsis well known (Buettner, 1987). The splitting patterns in ESR spectra of spin adducts can provide useful information about the structure and identity of the trapped radicals. The spin trapping technique was initially developed to study biological compounds containing highly reactive and short-lived superoxide (O_2•−) and hydroxyl (HO•) radicals and radical formation on proteins, lipids, and polysaccharides (Abbas et al., 2016; Davies, 2016; Hawkins & Davies, 2014). The trapping efficiency and stability of the resulting adducts depend on the type of the radicals and the applied spin adducts.

The spin labelling technique is based on using special spin labels (stable free radicals). This technique can be used for the determination of the concentration of dissolved oxygen in foods. The chemical structures of the most popular water-soluble N-containing nitroxide radicals, PDT (4-oxo-2, 2, 6, 6-tetramethylpiperidine-d16-1-oxyl) and CTPO (3-carbamoyl-2, 2, 5, 5-tetramethyl-3-pyrroline-1-yloxyl) are presented in **Figure S3**.

The non-volatile nitroso spin trap, 3, 5-dibromo-4-nitrosobenzenesulfonate (DBNBS) is useful for detecting pyrolysis radicals which are formed in high-temperature interfacial regions produced by ultrasonic cavitation (Kondo, Krishna, & Riesz, 1989). As reported in sonolysis of dimethyl sulfoxide (DMSO)-water mixtures, the spin adducts of DBNBS-SO3 and –CH3 can be detectable (Kondo, Kirschenbaum, Kim, & Riesz, 1993).

4. Evaluation of the physicochemical and nutritional properties of food

There are different ESR techniques for the evaluation of the concentration ofdissolved oxygen, and determining free radical scavenging, stability, and chelating activity of food ingredients. Among the main advantages of ESR reported in the available literature, one of the most important benefits is the ease of detection and identification of free radicals generated by chemical or biological systems by observing the spectrum of a spin adduct. Moreover, it also allows the quantification of free radicals by comparing the peak area to those obtained from stable radicals and to carry out kinetic analyses as well as to determine the formation and elimination velocities of a free radical (Kohno, 2010). In addition, the characteristics (*g*-value, alignment, line width, ΔW, among others) of the free radicals can be also determined using ESR (Kohno, 2010).

On the other hand, some main drawbacks are found with the technique, for example ESR does not allow the detection of a free radical when it reacts immediately with a molecule different from the spin-trapping agent. Moreover, spin adducts can be neutralized when a reducing agent is present and a new spin adduct can be generated if a spin adduct is decomposed, thus the difficulty of the identification of the free radicals. It is also difficult to determine the electron distribution and the molecular structure of the free radical when the hyperfine coupling constant, 2-(4-carboxyphenyl)-4, 4, 5, 5- tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) is the only ESR parameter determined for a spin adduct (Kohno, 2010). Since carboxy-PTIO reacts selectively with NO• radical and this reaction yields 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-l-oxyl (carboxy-PTI) and NO• radical, the carboxy-PTIO reaction system can be used for detection of NO• radical (Kurake et al., 2017; Uchiyama et al., 2015). It is worth mentioning that in some applications, the ESR techniques are only qualitative, and not quantitative (Zhou, Yin, & Yu, 2005).

4.1. Spin label oximetry

ESR spin label oximetry technique has wide applications for detection of dissolved oxygen in foods (Subczynski & Swartz, 2005). The technique is based on the collision between paramagnetic oxygen O_2 and a spin label (stable free radicals). The extent of spin exchange influences the line width for the spin label and it depends on the concentration of dissolved oxygen. It allows real-time monitoring of generation or consumption of O_2 in food systems. Water-soluble N-containing nitroxide radicals, namely PDT (Yin et al., 2009) and CTPO (Hyde & Subczynski, 1984) are common spin labels used for the ESR oximetry. Spectra for these spin labels are widely available and hence calibration procedures for these techniques are well established. The ESR oximetry has been applied to study oxygen uptakes and lipid oxidation in emulsions, in fatty acid model systems and liposome systems and to evaluate oxygen permeation through an oil-encapsulated glassy food matrix (for a review see Zhou, Yin, & Lo, (2011)). Data on oxygen solubility and diffusivity in food and different oxygen quantification methods including ESR oximetry have been reviewed (Pénicaud, Peyron, Gontard, &

Guillard, 2012).

4.2. Free radical scavenging

The formation of free radicals and their scavenging by antioxidants in foods canbe evaluated using different the assay procedures (Karadag, Ozcelik, & Saner, 2009; Shivakumar & Yogendra Kumar, 2017). Nowadays, the use of ESR techniques for these purposes is considered to be reliable and sensitive in radical quenching (Cömert & Vural, 2017). For example, the antioxidant capacity of a large number of varieties of fruits (strawberry, mulberry, lemon, banana, etc.) to scavenge 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical was evaluated using spectrophotometric and ESR measurements (Zanget al., 2017). The results obtained from the two methods were found to be highly correlated. It was

demonstrated that in some cases (for the sample with a colour similar to that of DPPH or non-transparent sample) ESR spectroscopy might be more suitable for determining the antioxidant capacity of fruits. In fact, the use of ESR technique to evaluate both radical scavenging activity and antioxidant properties in foods have shown high correlation for various food products, which include antioxidant drink (Hiramatsu et al. 2013), medicinal tea (Pejin & Kien-Thai, 2013), betanin of red beet (Esatbeyoglu et al., 2014), polyphenols of wine compounds (De Beer, Joubert, Gelderblom, & Manley, 2017; de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2016), coffee (Kameya, 2017), herbal materials (Wojtowicz, Krupska, & Zawirska-Wojtasiak, 2017), peptides of soybean meats (Sami, 2017), and other liquid foods and beverages (Smirnov, 2017). Therefore, ESR technique has become an integral part for food analysis that provide valuable information regarding the antioxidant properties of a food material.

The ability of ESR spectroscopy to differentiate between the antioxidant activity of soluble and insoluble/bound phenolic fractions extracted from winemaking by- products pre-treated with cell-wall degrading enzymes was demonstrated (Camargo et al., 2016). The antioxidant activity with respect to DPPH and hydroxyl radical scavenging activity showed a good correlation with specific phenolic compounds found in each extract fraction exposed to two different enzyme-assisted extraction treatments. **Figure 1** presents examples of ESR signals used for the evaluation of the ability of the phenolics extracted from the control (devoid of enzyme) and the starting material pre-treated with Pronase to scavenge hydroxyl radicals (the higher the ESR signal, the lower the scavenging activity) (Camargo et al., 2016). The ratio observed between the fraction containing soluble and insoluble-bound phenolics increased upon enzyme treatment of the starting material. The similar trends were observed for pre-treatment of the starting materials with Viscozyme.

4.3. Food stability

The quality and nutritional properties of foods during processing and storage can be directly related to free radical-mediated oxidation of lipids. The process of oxidative deterioration of lipids by direct attack of carbon-carbon double bonds, especially in PUFAs (polyunsaturated fatty acids), with free radicals in a process that is known as lipid peroxidation (Ayala, Muñoz, & Argüelles, 2014). The chemical mechanisms and methods of analytical determination of the extent of lipid peroxidation are widely discussed in the literature (Catala, 2012; Repetto, Semprine, & Boveris, 2012). The products of an oxidative breakdown in foods have high toxicity and for their determination, different assays have been developed. However, these assays may be rather complex and require multistep sample preparations. For example, aldehyde and ketone derivatives and the measurement of the carbonyl groups is regarded as an important marker for protein or lipid oxidation caused by reactive species. Several spectrophotometric, immunochemical and chromatography methods (Rimbach et al., 1999; Estévez, Ollilainen, & Heinonen, 2009) have been reported with varying levels of sensitivity and ability to identify individual carbonylated by-products of the oxidation process. Other methods more relevant to lipid oxidation rely on determination of volatile compounds generated as end products of the oxidation reaction, such as 4-hydroxy-2-nonenal (4-HNE)], are frequently investigated and quantified using HPLC, GC and ELISA.

4.4. Chelating activity

Ions are commonly found in foods and have significant nutritional value (e.g.,Fe^{2+} and Cu^{2+}), display a high catalytic activity and they can accelerate the oxidative reactions and result in the generation of free radicals. Natural chelates have an affinity for metal ions and they can bind to these metals. Recently, the role of chelates have attracted significant attention in nutrition (Kratzer & Pran, 2018). Some examples of the application of ESR

technique for the determination of chelating activity of food components have also been presented, since the formation of chelating complexes alters the ESR spectra.

The chelating properties of five phenolic acids (*p*-coumaric, ferulic, syringic, and vanillic acids) that are commonly present in wheat grain and fractions, were evaluated against Fe^{2+} and Cu^{2+} using spectrophotometric and ESR measurements (Zhou, Yin, & Yu, 2006). It was demonstrated that these phenolic acids differed in their capacity to form chelating complexes. The correlations between the radical-scavenging capacity, chelating capacity against transition metals and structure of phenolic acids were discussed. ESR measurement has been used for the evaluation of Cu^{2+} chelating activities and radical-scavenging properties of botanical extracts from black peppercorn, nutmeg, rosehip, cinnamon, and oregano leaf (Su et al., 2007) and wheat bran extracts (Zhou et al., 2005). For wheat bran extracts significant radical scavenging and chelating capacities were detected due to significant levels of phenolic acids, tocopherols, and carotenoids (Zhou et al., 2005).

5. Evaluation of the effects of food processing operations

It is important to note that the concentration of radicals in native food systems canbe rather low and their level increases with processing. For example, the number of radicals per gram amounted to about 1014-1015 for unroasted coffee beans, 1016 for roasted coffee, and 1017 for spent coffee grounds (the waste product from brewing coffee) (Rosenthal, 1998). These results demonstrated that free radicals can effectively be produced by different food processing operations. The formation of the radicals in the processed food material should be carefully monitored to ensure nutrients retention in the food after processing.

5.1. Ionizing radiation

Irradiation with moderate ionizing energy (≤10 kGy) is frequently used to produce biocide effects and to prevent the bacterial growth in foods (ISO14470, 2011; Stefanova, Vasilev, & Spassov, 2010). Irradiation can be performed with ^{60}Co gamma rays, and X-ray or accelerated electrons. A comprehensive book that covers different aspects of food irradiation, processing, and sterilization, as well as legislation and market aspects was recently published (Ferreira, Antonio, & Cabo Verde, 2018). Nowadays, the ESR is the principal method of detection of free radicals in irradiated foods to ensure safety and treatment efficacy.

A typical examples of the ESR spectra of un-irradiated (0 kGy) and irradiated (10 kGy) food materials (complex seasoning) containing Mn^{2+} are presented in **Figure 2** (Ahn, Akram, Kim, & Kwon, 2013). Note that the manganese ions are important for biochemical processes of green plants as cofactors of proteins and enzymes. The typical sextet Mn^{2+} signals were observed. Upon irradiation, complex ESR spectra were observed and the signals due to Mn^{2+} showed overlapping with the radiation-induced ESR signals.

Different examples for the application of ESR to study irradiated fruits, vegetables, tea leaves, seeds, spices and herbs, food containing bones, crystalline sugar, sauces, and beverages have been already reported (Shukla, 2016). Therefore, for more details on the analysis and technical information, we refer the reader to this recently published book (Shukla, 2016).

5.2. High pressure processing

High pressure (HP) processing involves the application of hydrostatic pressures > 100 MPa at ambient temperature to inactivate microorganisms and inhibit oxidative enzymes, while retaining the inherent quality attributes of the food material (Oey et al. 2008). Food products (in the form of liquids or semi-solids) are pre-packed and loaded into a chamber vessel and the vessel is then closed and filled with a pressure-transmitting medium such as

water or food-grade solutions (e.g. castor oil, silicone oil, sodium benzoate, ethanol, and glycol). The food products are held inside the vessel under pressure for a predefined duration, followed by system depressurisation before opening the vessel and unloading the food products (Tao et al. 2014).

High pressure (HP) processing has been shown to initiate lipid oxidation in freshmeats (Bolumar, Andersen, & Orlien, 2014), and a greater amount of volatiles linked to fatty acid oxidation has been detected in HP-treated fruit juices and vegetable purees (Kebede et al., 2013; Vervoort et al., 2013). Therefore, a reliable assessment of process- induced changes in HP processed food is of a major importance in the context of legislative aspects of this innovative non-thermal processing technology. In this respect, the potential involvement of any specific radical intermediates during HP that might be involved in lipid oxidation can be thoroughly examined with the aid of ESR spectroscopy.

Figure 3 presents examples of the EPS spectra (first derivatives) of the DMPO (a) and PBN (b) spin-adducts formed in beef loin and chicken breast processed by HP treatment (Bolumar et al., 2014). The spin traps DMPO and PBN were added to minced beef and chicken meats and then hp treatment was applied. For DMPO spin trap, the spectra had a shape of an isotropic spectrum with a high degree of line broadening due to slow rotational mobility (**Figure 3a**). For PBN spin trap an ESR spectra with the typical shape of a nitroxyl radical powder spectrum were observed (Figure **3b**). The powder spectrum evidenced that the formed spin adducts are immobilized in random orientations in a solid matrix. The level of spin adducts was higher in the beef loin compared to the chicken breast, which might be related to the higher iron content in beef compared to chicken, reflecting a higher level of radicals formed in the beef loin during pressurization. The formation of new free radical species in chicken meat during HP processing (400-800 MPa, 5-40 °C for 10 min) has been reported in various studies (Bolumar, Andersen, & Orlien, 2011; Bolumar et al., 2014;

Bolumar, Skibsted, & Orlien, 2012; Mariutti, Orlien, Bragagnolo, & Skibsted, 2008). Based on ESR spin trap spectroscopy investigation, it can be deduced that radicals formation in HP meat, as initiators of lipid oxidation under HPP exposure is a time-dependent process (following a first-order reaction) (Bolumar et al., 2011) and interestingly, it has been clearly revealed that both protein- and iron-derived radicals were formed and accumulated at the sarcoplasmic and myofibrillar muscle fractions during HP processing (Bolumar et al., 2014). Furthermore, increasing the processing temperature and time at atmospheric pressure and during HP processing of chicken meat has been shown to promote greater formation of radicals (Bolumar et al., 2012). Therefore, ESR could be employed as a reliable technique to assist optimization of HP processing for various foods, targeting to minimize the occurrence of lipid oxidation.

5.3. Pulsed electric fields

Recent studies on the use of pulsed electric fields (PEF) processing in foodresearch demonstrate the food industry is interested in this technology that can assist different food operations such as extraction, drying, freezing, osmotic treatment, improve safety, and cause texture modifications. PEF treatment at a high electric field strength in order of 20-100 kV/cm with very short duration pulses (between µs and ms), can be used for inactivation of bacteria and sterilization of liquid foods. PEF processing at high electric field can induce polarisation of water molecules with dissociation of them into the ions (Boussetta, Soichi, Lanoiselle, & Vorobiev, 2014). This would possibly lead to the subsequent formation of free radicals during PEF treatment, but there is a lack of studies evaluating this phenomenon in available literature. For detection of free radicals induced by a pulse discharge, ESR technique can be successfully applied (Tahara & Okubo, 2014). It is worth noting that at present time, pulse discharge technologies are recognized as a cost-effective and

environmentally friendly for the destruction of microorganisms in contaminated potable water and wastewater (Yang & Cho, 2012).

ESR technique with DMPO spin trap was used to detect the generation of free radicals in phosphate buffer and in an oleic acid emulsion after PEF processing (Zhang, Yang, Zhao, Liang, & Zhang, 2011). The concentration of hydrogen peroxide in phosphate buffer after PEF treatment were 0.177×10^{-6} at 30 kV/cm, and 1.858×10^{-6} at 35 kV/cm. This work evidenced that PEF had a potential role as initiator of free-radical reaction. The effects of PEF on oxidation of oleic acid were also studied (Zhao et al., 2011). Hydrogen radicals were detected by ESR technique using the DMPO spin trap. Note that the DMSO can trap carbon-centered and oxygen-centered radicals generated in chemical and biochemical systems. **Figure 4** shows examples of ESR spectra of DMPO adducts of oleic acid without PEF treatment and after PEF treatment 30 kV/cm for 400 μs. The ESR signal was practically absent for the control sample (**Figure 4a**) but was very intense for the sample under PEF treatment (**Figure 4b**). For PEF treated sample the spectra contain a triplet and each triplet line is further split into another triplet with intensities of 1:2:1. The study confirmed the oxidation of oleic acid under PEF treatment and generation of hydrogen radicals. Following PEF, a gradual quality deterioration of an oleic acid emulsion occurred, as indicated by the increase in the peroxide and carbonyl values of the PEF- treated oleic acid (Zhang et al., 2011; Zhao et al., 2011).

The oxidative effects of nanosecond PEF treatment (1–13 kV/cm, 300 ns) in cells and cell-free media were demonstrated (Pakhomova et al., 2012). It was shown that nanosecond PEF triggers oxidation both extracellularly (electrochemically) and intracellularly mediated by biochemical reactions.

In an advancement of the PEF technology, electrical-insulation breakdown was shown to take place by application of high electric fields and generation of electrical discharges.

Under ambient atmosphere, electrons in the high electricity discharge effectively collide with background-molecules such as nitrogen, oxygen, and water, leading to dissociation of these molecules. A rich variety of gaseous and aqueous reactive oxygen and nitrogen species (RONS) are produced by the electrical discharge (Takeda, Ishikawa, Tanaka, Sekine, & Hori, 2017). Evidence for the generation of hydrogen peroxide H_2O_2 and short-living active species (HO, H, O, $^1O_2$, HO_2, O_2–) resulting from the dissociation of water activated by an underwater electrical discharge has been reported (Hong, Huh, Ma, & Kim, 2018). Basically, when oxygen atoms are generated by the electrical discharge of atmospheric air remotely from liquids such as water, saline, biological liquids, water molecules dissolved in organic constituents such as lipids, peptides, and proteins, reaction of these biological compounds with oxygen atoms occurs at the gas-liquid interface (Hong et al., 2018; Kobayashi et al., 2017). In contrast, when the discharge is in direct contact with the liquids, more effective dissociations of the dissolved organics occur by irradiations simultaneously of high-energetic photons, large- amount of RONS, electrically charged species, as well as high-electric fields (Kurake et al., 2017; Uchiyama et al., 2018).

The generation of free radicals and ROS/RNS after a PEF application can be viewed in a positive or negative way, depending on the intended application of this non-thermal technology. For instance, PEF can effectively inactivate microorganisms in food systems possibly due to the PEF-induced cell electroporation effect that has led to the extensive formation of highly reactive free radicals from chemical species in the microbial cell (Sitzmann, 1995), which is regarded as positive outcome of the process. On the other hand, PEF has been reported to modify the chemical conformation of the antioxidant compounds and their antioxidant properties due to free radicals formation. For instance, the formation of free radicals (HO•) after PEF (5-35 kV/cm, unipolar square 40 µs pulses, continuous operating mode at a flow rate of 60 mL/min, 0.8-7.2 ms treatment time) has been associated

with the conversion of vitamin C isomer from enol- to keto-form (Zhang et al., 2015), thus modifying the vitamin C structure without significantly decrease its total content. Furthermore, changes in the structural conformation induced by PEF have enhanced the antioxidant properties of vitamin C. However, PEF-induced reactive species (H_2O_2 or hydroxyl radicals) have different effects on polyphenols such as anthocyanin. For example, cyanidin-3-glucoside purified from red raspberry has been reported to lose its stability after PEF treatment (1.2-3.0 kV/cm, 300 exponentially decaying 300 µs pulses for 1 Hz), as indicated by the increased formation of chalcone due to the opening of the pyrylium ring (Zhang et al., 2008). Moreover, it has been observed that weak chemical bonds present in the structure of amino acids, proteins, and polysaccharides, such as hydrogen, disulphide, and hydrophobic bonds, are susceptible to break down after PEF exposure (Han et al., 2012; Liu, Zeng, Deng, Yu, & Yamasaki, 2011; Perez & Pilosof, 2004). This observation can also be partially explained by H_2O_2 or free radical formation due to PEF treatment.

The antioxidant activity of a peptide with sequence Gln-Asp-His-Cys-His (QDHCH) of pine nut (*Pinus koraiensis*) was improved by PEF treatment (at E=5-20 kV/cm) (Liang, Zhang, & Lin, 2017). It was demonstrated that hydroxyl radicals scavenging activity of QDHCH was increased after PEF processing as detected using ESR technique. PEF has no effect on the basic structure of QDHCH, but it influenced the secondary structure of QDHCH.

5.4. Ultrasound

Ultrasound is a nonthermal processing technology that involved continuous agitation of food material at ultrasonic frequencies (>20 kHz) using an ultrasonic bath or probe. One of the earliest works by Vercet, Lopez, & Burgos, (1998) in examining enzyme inactivation effect of manothermo-sonication (MTS), a combined treatment of heat and ultrasound (20 kHz frequency) under moderate pressure, was able to deduce that one of the MTS enzyme

inactivation mechanisms involved the interaction between the free radicals produced by water sonolysis with amino acid residues. The work showed further that free radical production rate increases linearly with increasing ultrasound amplitude (from 20 and 145 μm) and decreased when increased temperature and pressure combination (70 °C/200 kPa vs. 130 °C/500 kPa) was applied.

The work of Makino, Mossoba, & Riesz, (1983) was among the first in the literature to demonstrate the feasibility of using ESR spin trapping spectroscopy technique to study the radicals' formation in an aqueous medium (sonicated water saturated with argon) following ultrasound sonication. It was clear that hydroxyl (HO•) and hydrogen atom radicals (H•) were the two most abundant ultrasound-induced free radicals formed in the aqueous medium investigated (Kondo et al., 1989).

The recent work of Zhang et al. (2015) performed with ESR spin trapping spectroscopy with DMPO was able to reveal increasing formation of 1-hydroxyethyl radicals during sonication of red wine, while only HO• radicals were detected in DMPO (control) solution during sonication. Comparing the types of spin adducts detected in both ultrasound-processed DMPO (control) solution and red wine, it is possible to postulate that 1-hydroxylethyl radicals were formed due to ethanol oxidation via the ultrasound-generated HO• in water. Thus, this work provided the first direct evidence to uncover the formation of 1-hydroxyethyl free radical in red wine exposed to ultrasound.

Influence of ultrasound-assisted thermal processing (thermo-sonication) on the physicochemical and sensorial properties of beer was investigated (Deng et al., 2018). ESR was employed to monitor changes in the generation of free radicals and it was demonstrated that thermo-sonication clearly improves the oxidative stability of beer determined by ESR spectroscopy.

5.5. Cold plasma treatment

Cold plasma treatment is a novel technology that uses partially ionized gases that contain a mixture of neutral and charged species with temperature close to room temperature. The technology has attracted a lot of attention due to its efficacy in reducing/eliminating microorganisms and viruses (Takamatsu et al., 2015). The technology basic mode of action is mainly related to the generation of reactive species and their effects on bacteria. Depending on the intensity of treatment and the gas used, a wide range of reactive species (e.g., UV photons, charged particles, free radicals, and oxidants)are generated that contribute to the antimicrobial activity and its successful use on fresh and dry food products (Barba, Koubaa, do Prado-Silva, Orlien, & Sant'Ana, 2017; Gavahian, Chu, Mousavi Khaneghah, Barba, & Misra, 2018; Hertwig, Meneses, & Mathys, 2018). The use of nitrogen as the source gas of reactive species appear to be the most effective to inactivate microorganisms due to the high hydroxyl radical generated using nitrogen (Takamatsu et al., 2015). ESR has been used to measure several short lived radical species such hydroxyl radical (< 100 ms), peroxynitrous (~1 ms and superoxide and hydroperoxyl radicals (< 10 s) in liquid solutions (Attri et al., 2015; Ikawa, Tani, Nakashima, & Kitano, 2016). ESR has been used to measure free radicals generated in plasma treated liquids (Jablonowski et al., 2015), but no use of the technology has been reported in real foods. There is a large potential to utilize ESR to determine the depth of cold plasma penetration by investigating free radical formation at sub-surface layers to ensure proper decontamination process. Another potential use of the technology is to determine the concentration and nature of free radicals generated by cold plasma treatment in relation to undesirable changes in treated foods. This is an important aspect, particularly in milk and dairy products (Coutinho et al., 2018).

6. Conclusion

The goal of this review was to classify and describe applications of various ESR spectroscopic techniques for free radical analysis in foods processed using emerging technologies. The typical radicals present in foods, their types and sources (both internal and external) were discussed. The ESR techniques have become very popular for the identification of free radicals in different types of foods, including fruits and vegetables, meats and fishes, spices, cereal grains, and oilseeds. These techniques can be applied for spin-label oximetry, estimation of free radical scavenging, food stability, and chelating activity. Moreover, they can be employed to detect and quantify free radical species in food processed using innovative operations assisted by ionizing radiation, high pressures, pulsed electric fields, ultrasonication, and microwaves. The main advantages of ESR for applications in food systems include its high sensitivity and specificity. Nowadays, many types of ESR instruments are commercially available, this technique requires relatively small amounts of sample and analyses can be easily and rapidly done in scientific and industrial laboratories. However, the ESR data for foods are typically affected by the nature of the material, type of applied treatment and especially the water content in foods, complicating the detection and quantification of radicals. Therefore, the development of novel ESR techniques and methods of analysis specially designed to study foods is greatly desirable in future.

Conflict of interest

There are no conflicts to declare.

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Figure captions

Fig 1. Examples of ESR signals of soluble and insoluble-bound phenols affected by Pronase enzymatic pre-treatment on winemaking by-products. The higher the ESR signal, the lower the scavenging activity as demonstrated by the content of DMPO-OH adducts (with permission from (De Camargo et al., 2016)).

Fig. 2. Typical examples of the first derivative ESR spectra of un-irradiated (0 kGy) and irradiated (10 kGy) food materials (complex seasoning) containing Mn2+. Irradiation was performed with ^{60}CO gamma-ray source (with permission from (Ahn, Akram, Kim, & Kwon, 2013)).

Fig. 3. Examples of ESR spectra of the DMPO (a) and PBN (b) spin-adducts formed in beef loin and chicken breast during HP-treatment (with permission from (Bolumar, Andersen, & Orlien, 2014)).

Fig. 4. Examples of ESR spectra of DMPO adducts of oleic acid without PEF treatment (a, control) and after PEF treatment 30 kV/cm for 400 μs (b). For PEF treated the spectrum contains two triplet patterns. The first triplet peaks are caused to one nitrogen atom of the DMPO adduct with a hyperfine coupling constant of a_N=1.65 mT and the second triplet peaks are caused by the two identical β-protons ($a_{H\beta}$ =2.23 mT) of the DMPO adduct (with permission from (Zhao et al., 2011)).

Table 1. The half-life and rate constants of biological reactive species.

Species		Symbol	Half-life (s) at 37°C	Rate constant* ($M^{-1}s^{-1}$)
Radicals				
	Semiquinone radical	$Q^{\cdot}$	$>10^{2}$	-
	Peroxyl radical	$ROO^{\cdot}$	$>1 \times 10^{-2}$	-
	Superoxide radical	$O_2^{\cdot}$	$>1 \times 10^{-6}$	< 0.3
	Alkoxyl radical	$RO^{\cdot}$	$>1 \times 10^{-6}$	-
	Hydroxyl radical	$HO^{\cdot}$	$>1 \times 10^{-9}$	
	Perhydroxyl	$HOO^{\cdot}$	1-30	-
	Nitric oxide radical	$NO^{\cdot}$	1–30	9.1×10^{9}
	Carbonate radical anion	$CO_3^{\cdot}$		1.2×10^{8}
	Azide	$N_3^{\cdot}$	10^{-5}—10^{-6}	$<10^{7}$
Oxidants				
	Molecular oxygen	O_2	$>10^{2}$	1.9×10^{10}
	Lipid peroxide	ROOH	$>10^{2}$	-
	Singlet oxygen	$^1O_2$	$>1 \times 10^{-6}$	2×10^{7}
	Hydrogen peroxide	H_2O_2	10	1×10^{-2}
	Ozone	O_3	9×10^{3}	5×10^{6}
	Peroxynitrite	$ONOO^-$	$10\text{-}20 \times 10^{-3}$	-
	Hypochlorous acid	HOCl		3.8×10^{7}

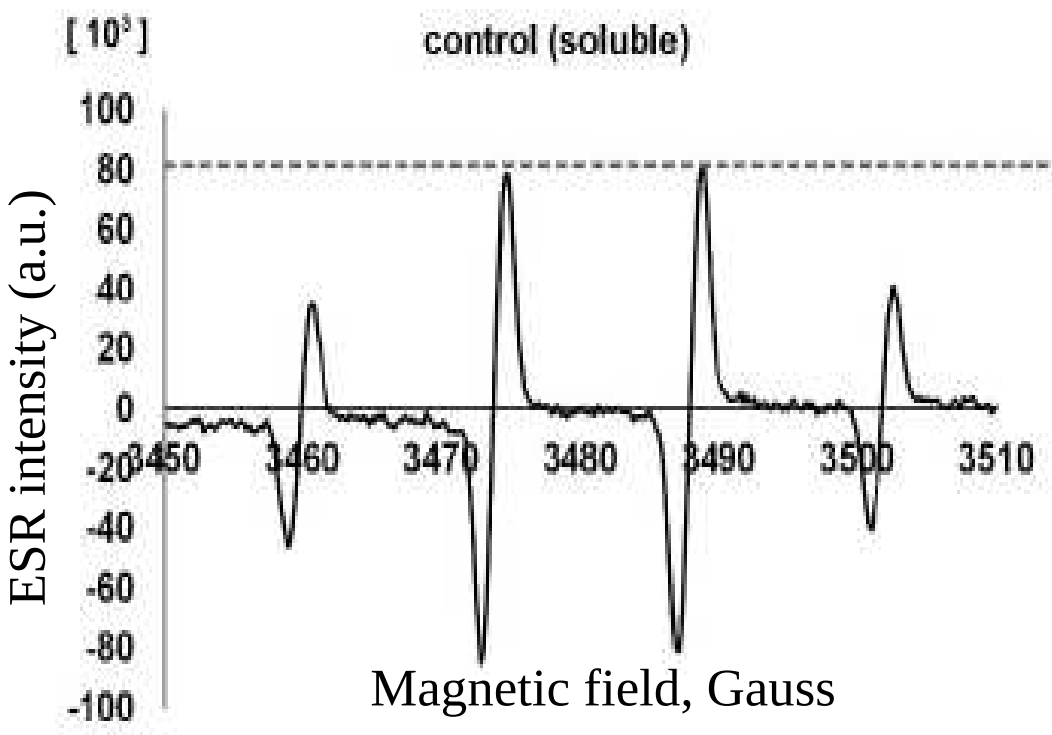
*= rate constant with methionine. Source: Bekhit et al. (2013)

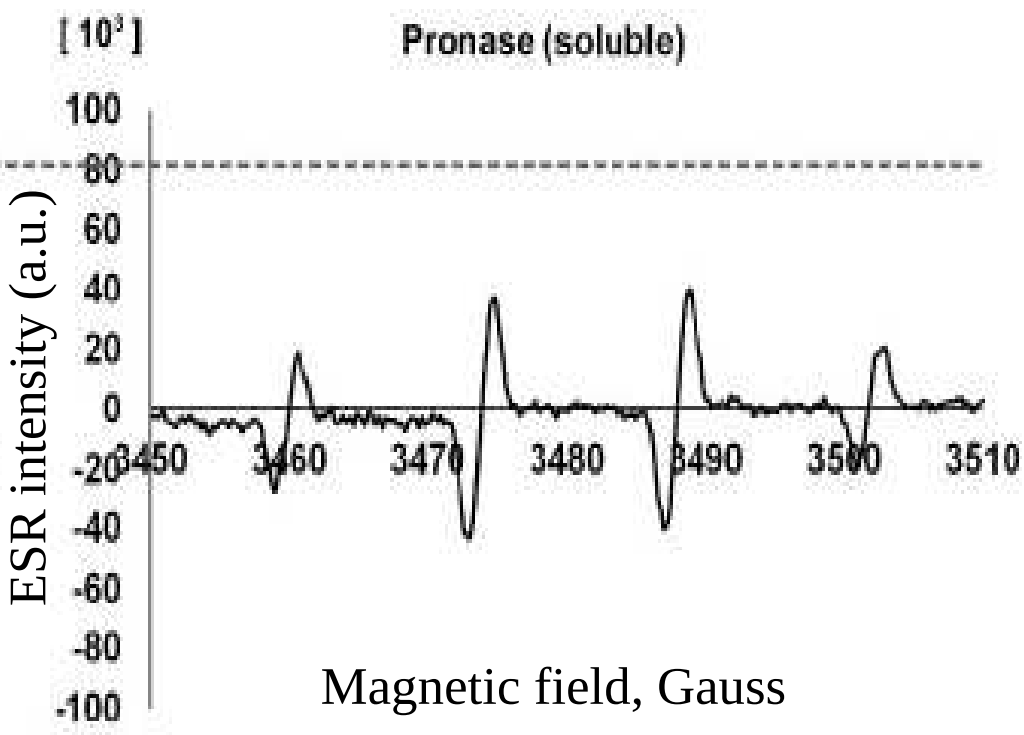
Enzyme	Radical generated	Enzyme Function	Location
NADH oxidase	$O_2^{\cdot}$	Unknown function	Muscle
NAD(P)H oxidase (EC 1.6.3.1)	H_2O_2		Sarcoplasmic Reticulum
Dihydroorotate dehydrogenase (EC 1.3.3.1 or EC 1.3.99.11)	H_2O_2, $O_2^{\cdot}$	Catalyzes conversion of dihydroorotate to orotate, a step in the	Mitochondria

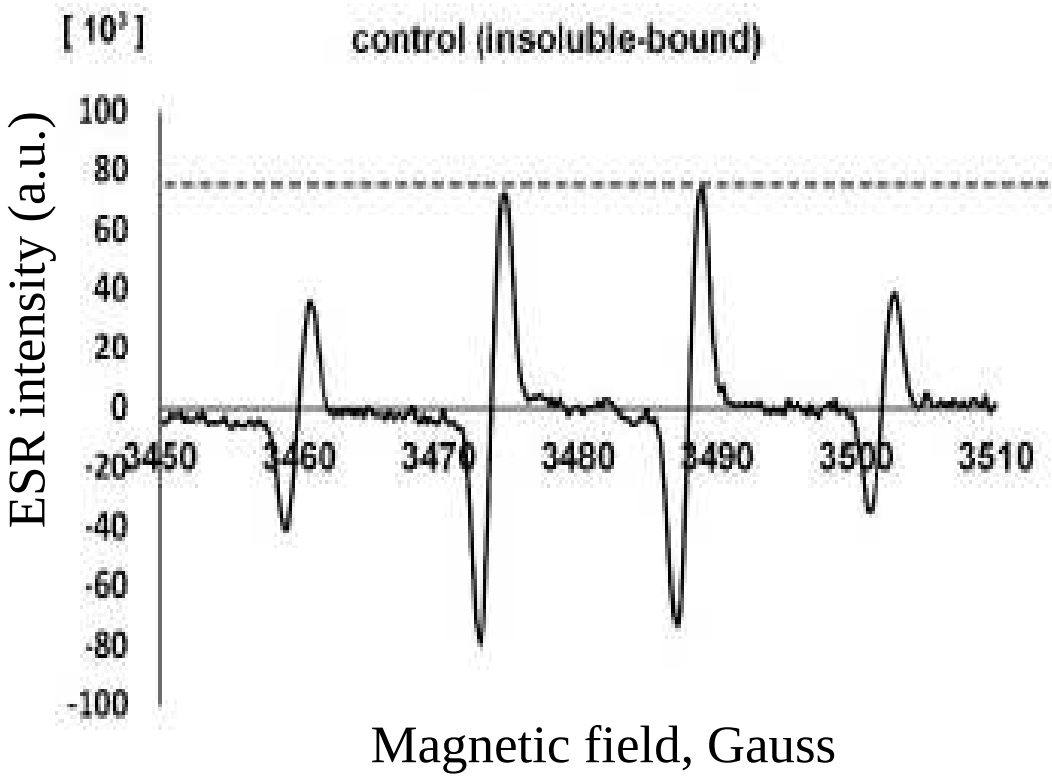
Table 2. Enzymatic systems involved in free radical generation.

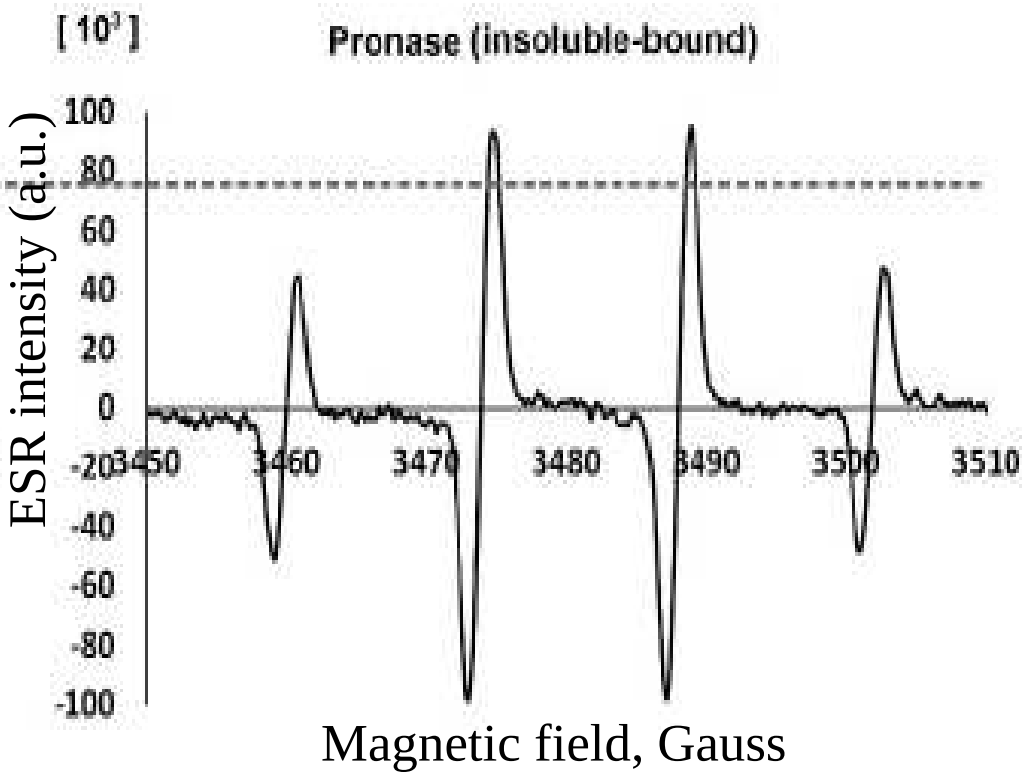
		synthesis of pyrimidine nucleotides	
Glycerol-3-phosphate dehydrogenase (EC 1.1.99.5)	H_2O_2	FAD-containing enzyme catalyses oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate, utilizing mitochondrial coenzyme Q as an electron acceptor	
Succinate dehydrogenase (EC 1.3.5.1)	ROS	Oxidizes succinate to fumarate using coenzyme Q as an electron acceptor	Mitochondria Complex II
Aconitase (EC 4.2.1.3)	$HO^{\cdot}$	Catalyzes conversion of citrate to isocitrate as part of the tricarboxylic acid cycle	Mitochondria
α-Ketoglutarate dehydrogenase complex [multiple copies of three enzymes: α-ketoglutarate dehydrogenase (EC 1.2.4.2), dihydrolipoamide succinyltransferase (EC 2.3.1.12), and lipoamide dehydrogenase (EC 1.6.4.3].	H_2O_2, $O_2^{\cdot}$	Catalyzes oxidation of α-ketoglutarate to succinyl-CoA using NAD+ as an electron acceptor	Mitochondria
Pyruvate dehydrogenase (EC 1.2.4.1)	H_2O_2, $O_2^{\cdot}$	Multiple functions. See Brenda website	Mitochondria
Cytochrome *b*5 reductase (EC 1.6.2.2)	$O_2^{\cdot}$ at a rate of ~300 nmol/min /mg protein.	It oxidizes cytoplasmic NAD(P)H and reduces cytochrome *b*5 in the outer membrane	Mitochondria
Monoamine oxidases (EC 1.4.3.4)	H_2O_2	Catalyzes oxidation of biogenic amines and the oxidative deamination of primary aromatic amines along with long-chain diamines and tertiary cyclic amines	Outer mitochondrial membrane
Succinate-cytochrome *c* reductase system (may be EC 1.6.2.1)	$O_2^{\cdot}$		Mitochondria
NADH:ubiquinone reductase (EC 1.6.5.3)	$O_2^{\cdot}$	Oxidizes NADH, produced predominantly by the tricarboxylic acid cycle in the mitochondrial matrix, and reduces ubiquinone in the inner mitochondrial membrane.	Mitochondria
Nitric oxide synthase (EC 1.14.13.39)	NO	Multiple see Brenda website	Mitochondria

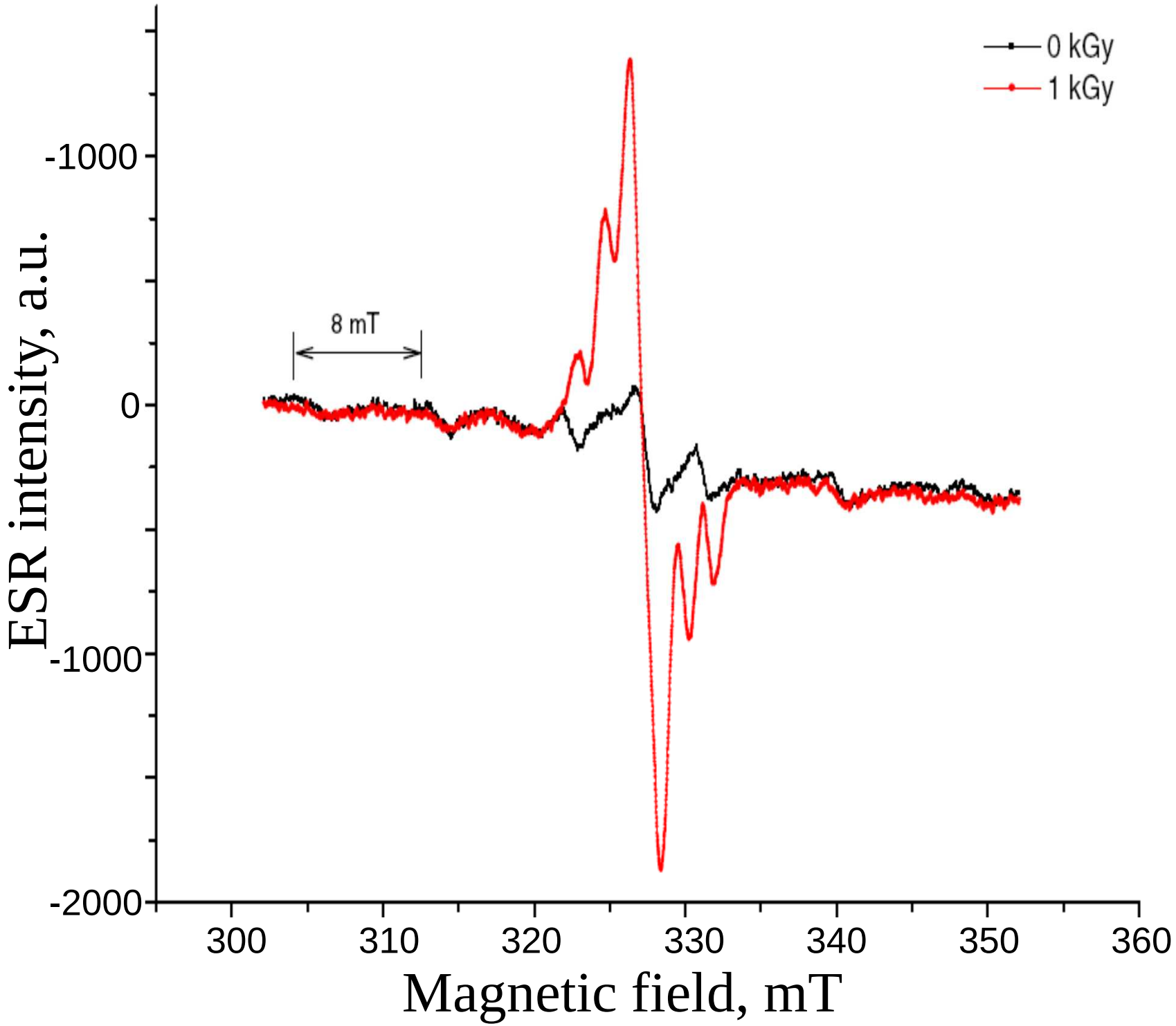
Source: Bekhit et al. (2013)


[10³]
control (soluble)
ESR intensity (a.u.)
100
80
60
40
20
0
-20
-40
-60
-80
-100
3450
3460
3470
3480
3490
3500
3510
Magnetic field, Gauss

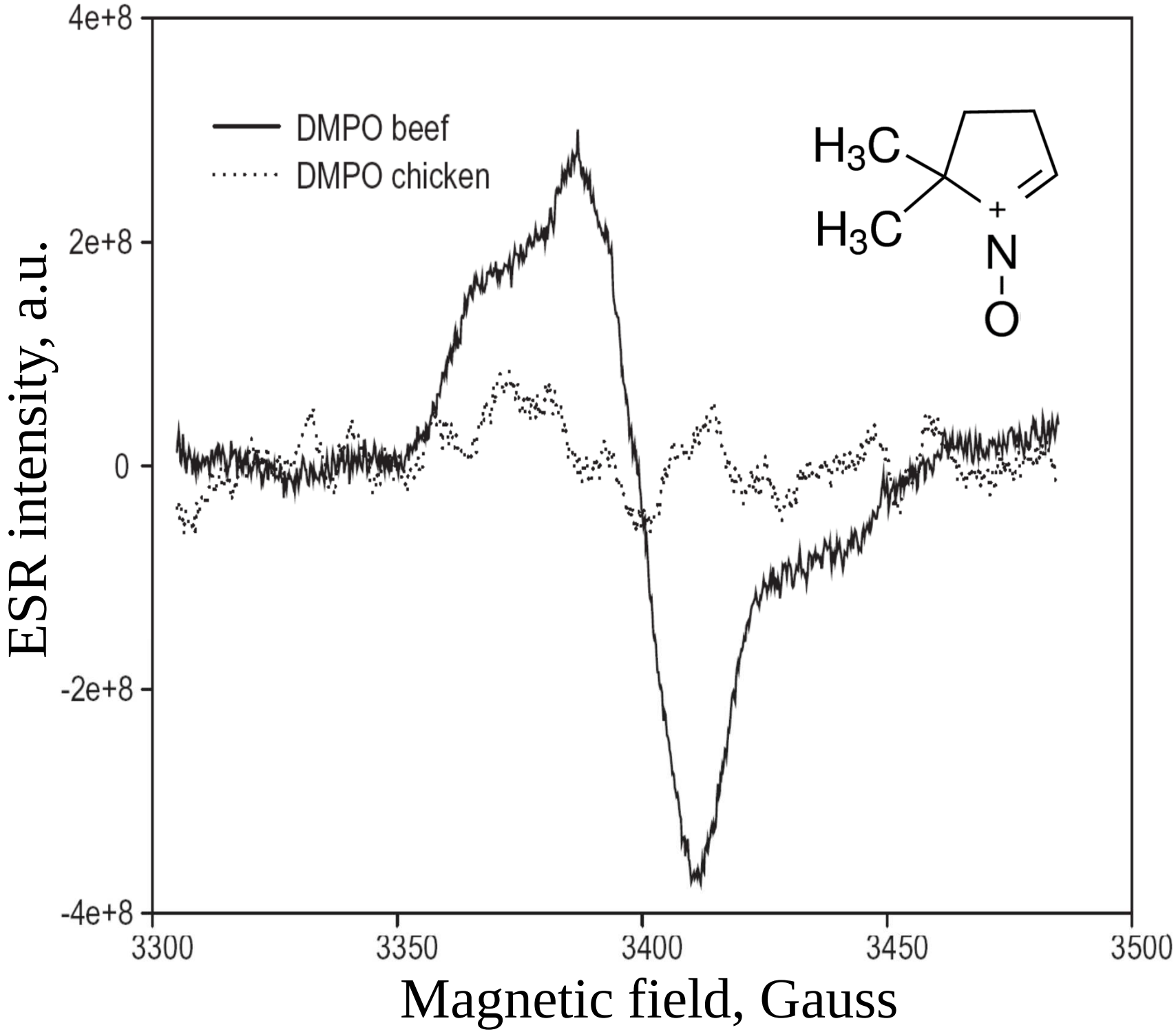

[10³]
Pronase (soluble)
ESR intensity (a.u.)
100
80
60
40
20
0
-20
-40
-60
-80
-100
3450
3460
3470
3480
3490
3500
3510
Magnetic field, Gauss


[10³]
control (insoluble-bound)
ESR intensity (a.u.)
100
80
60
40
20
0
-20
-40
-60
-80
-100
3450
3460
3470
3480
3490
3500
3510
Magnetic field, Gauss

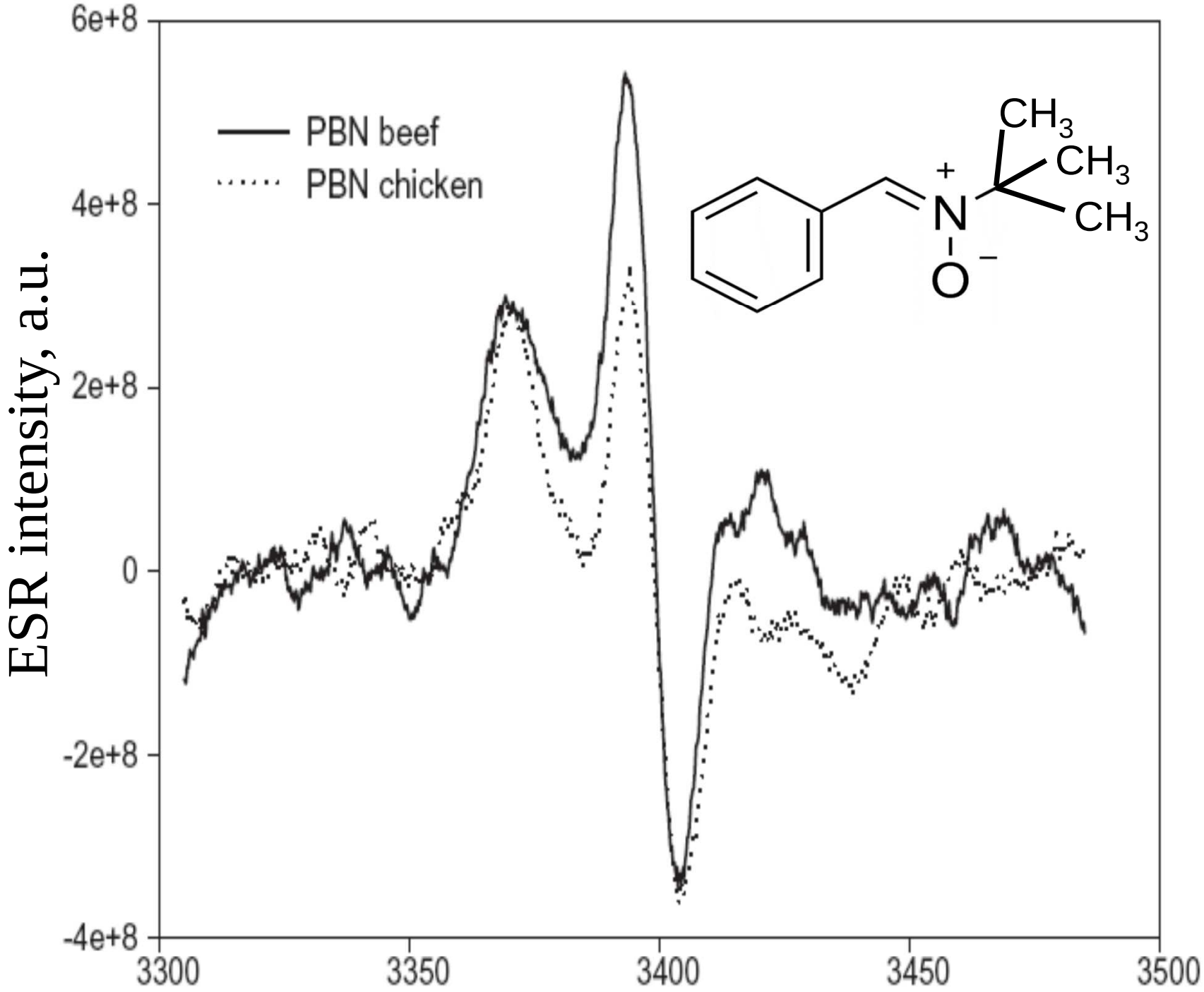

[10³]
Pronase (insoluble-bound)
ESR intensity (a.u.)
100
80
60
40
20
0
-20
-40
-60
-80
-100
3450
3460
3470
3480
3490
3500
3510
Magnetic field, Gauss


0 kGy
1 kGy
8 mT
-1000
0
-1000
-2000
ESR intensity, a.u.
300
310
320
330
340
350
360
Magnetic field, mT

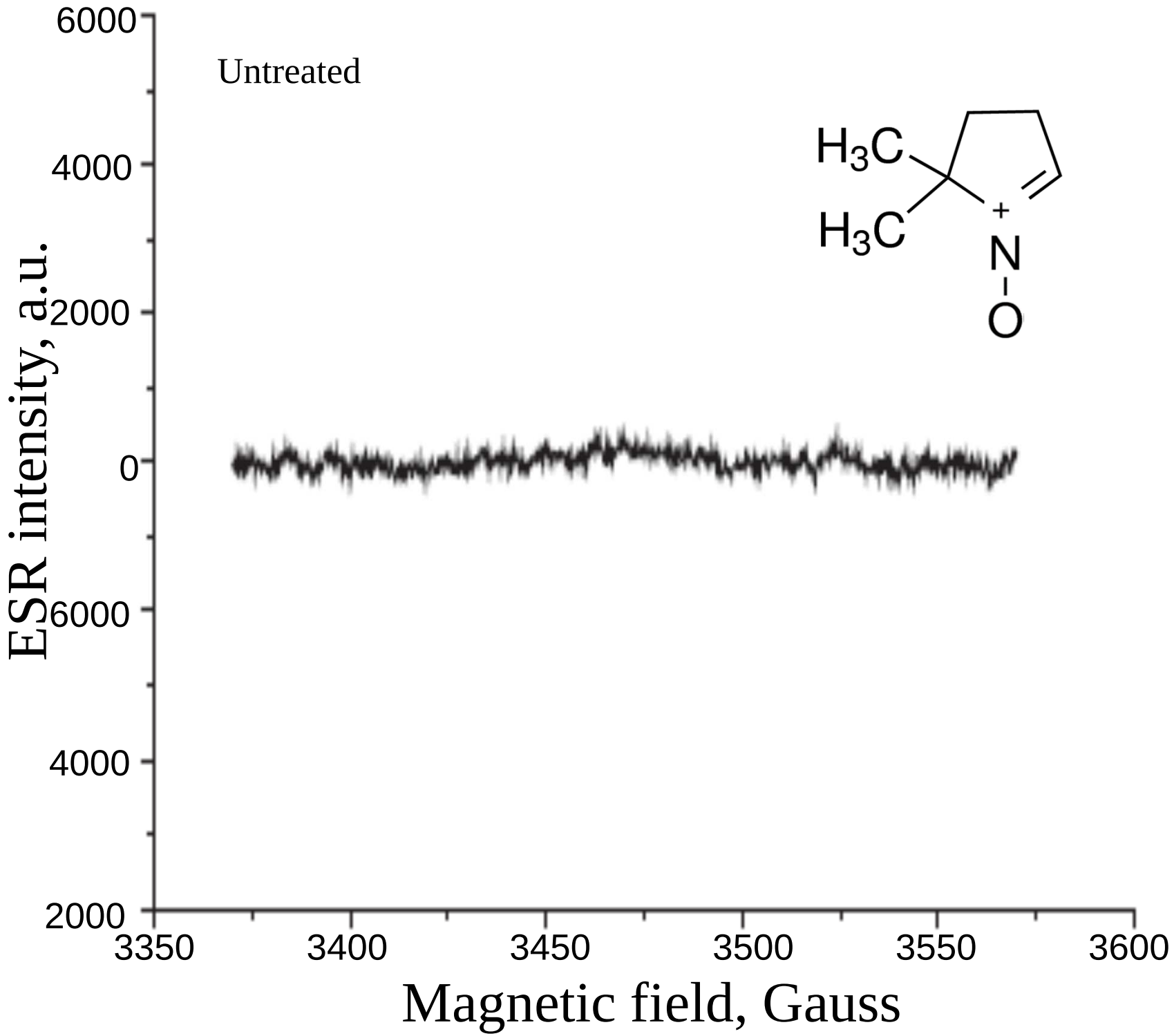
a)

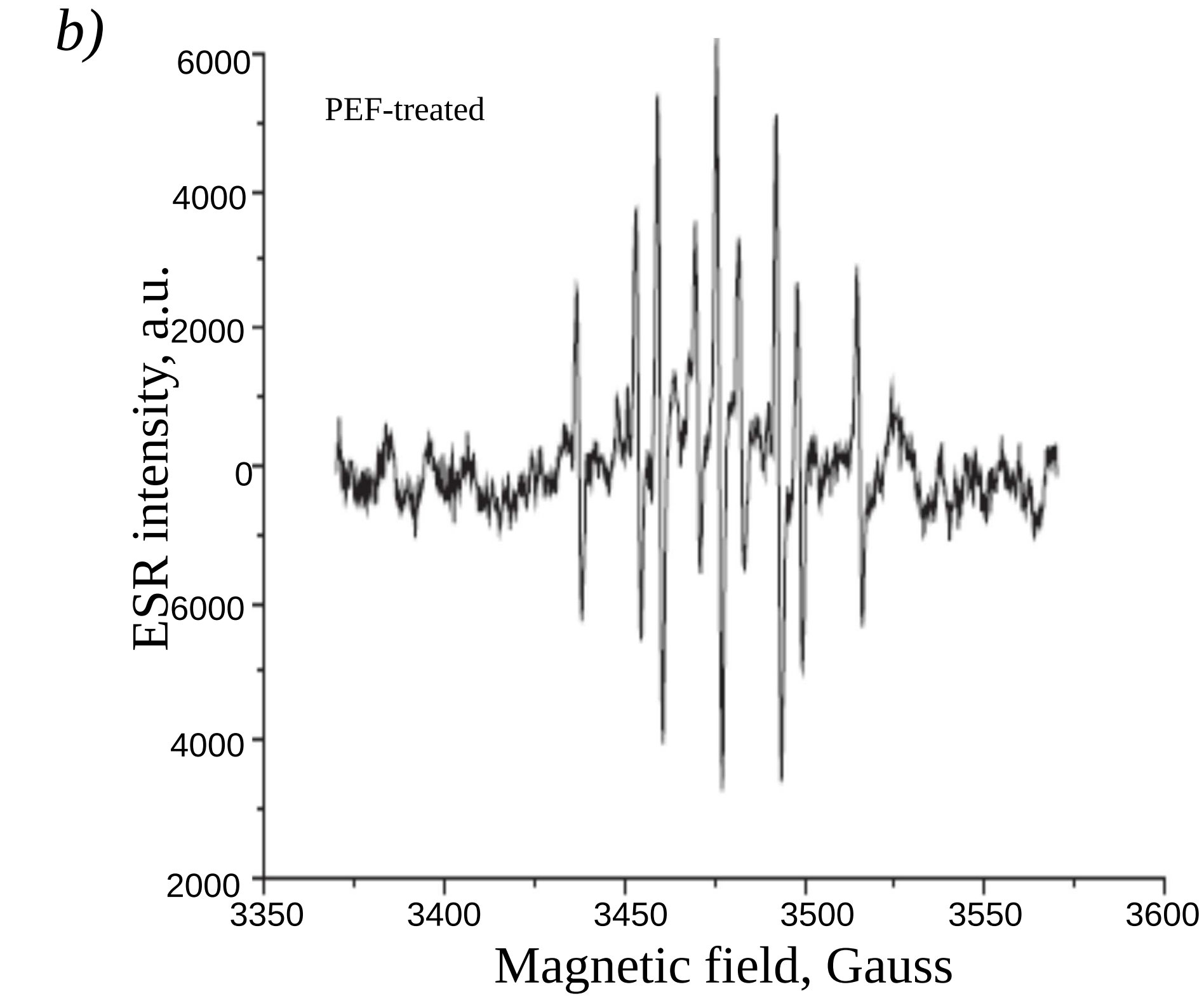

4e+8
2e+8
0
-2e+8
-4e+8
ESR intensity, a.u.
DMPO beef
DMPO chicken
H3C
H3C
N
O
3300
3350
3400
3450
3500
Magnetic field, Gauss

b)


6e+8
4e+8
2e+8
0
-2e+8
-4e+8
ESR intensity, a.u.
PBN beef
PBN chicken
CH3
CH3
CH3
N
O
3300
3350
3400
3450
3500
Magnetic field, Gauss

a)


Untreated
6000
4000
2000
0
6000
4000
2000
ESR intensity, a.u.
3350
3400
3450
3500
3550
3600
Magnetic field, Gauss
H3C
H3C
+
N
O


b)
PEF-treated
6000
4000
2000
0
6000
4000
2000
ESR intensity, a.u.
3350
3400
3450
3500
3550
3600
Magnetic field, Gauss

Highlights

- Electron spin resonance (ESR) as a tool to identify/quantify free radicals in foods
- ESR as a novel analytical possibility to evaluate potential food toxicity
- Physicochemical and nutritional properties of food can be accessed by ESR
- ESR can be used to evaluate the effect of novel food processing technologies
- ESR is a robust and non-invasive technology for food analysis