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## EPR Methods Applied on Food Analysis

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Additional information is available at the end of the chapter

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### Abstract

An overview of the different methodologies developed so far for the investigation of paramagnetic species in foods is presented. Electron paramagnetic resonance spectroscopy (EPR), also known as electron spin resonance spectroscopy (ESR), is the primary technique toward the development of methods for the exploration of EPR-sensitive species, such as free radicals, reactive oxygen species (ROS), nitrogen reactive species (NRS), and C-centered radicals and metal ions. These methods aim for: (a) quantification of radical species, (b) exploration of redox chemical reaction mechanisms in foods, (c) assessment of the antioxidant capacity of food, and (d) food quality, stability, and food shelf life. For these purposes, different radical initiations and detections have been used in foods depending on both the chemistry of the target system and the kind of information required, listed in: the induction of radicals by (a) microwave, UV, or  $\gamma$ -radiation; (b) heating; (c) addition of metals; and (d) use of oxidants.

**Keywords:** EPR, free radicals, food, antioxidants, spin traps, time-dependent EPR

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### 1. Introduction

In the last few years, the applications of the magnetic resonance techniques, particularly nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR), in food chemistry have enormously increased [1–5].

EPR spectroscopy is a sensitive and versatile technique for analyzing molecules that contain unpaired electrons, such as paramagnetic metal ions and organic radicals. The formation of organic radicals in foods is an indication of food degradation occurring mainly due to oxidation reactions. Metal ions present in foods are able to catalyze oxidation of the food components by activating  $O_2$  to produce reactive oxygen species (ROS). In addition to the analysis of

the paramagnetic species in foods, EPR can be used for the evaluation of the food stability and shelf-life. In order to perform such studies, acceleration of the radical production and degradation in food is needed. Several methods have been applied for the production of radicals in foods, including irradiation with microwave, UV, or  $\gamma$ -radiation, heating, and addition of oxidants. Stable organic radicals, such as tyrosyl and semiquinone radicals, can be detected directly by EPR. However, for the detection of transient radicals, spin traps are employed in order to be measured by EPR spectroscopy. The life of the short-lived radicals can also be extended by rapid freezing of the samples after their generation. In addition, time-resolved EPR can be used for the detection of short-lived radicals. Valuable information is acquired for the mechanisms involved in these reactions by measuring the EPR signal *vs* time.

The main objective of this chapter is the discussion of methods for food analysis by cw X-band EPR, including the observation of endogenous unpaired electronic spin species and the initiation and detection of free radicals in foods.

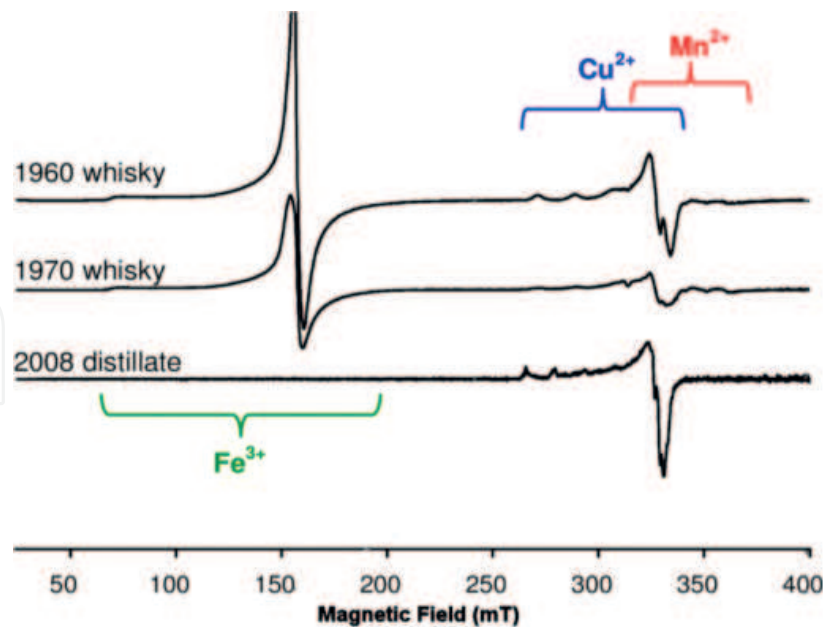
## 2. Endogenous unpaired electronic spin species in foods

### 2.1. Metal ions in food

Foods contain metal ions originated either from the raw starting materials or from contamination with metals from metallic containers or from contamination with metals during food processing [6–9]. EPR spectroscopy is particularly sensitive in detection of  $\text{Fe}^{\text{III}}$ ,  $\text{Mn}^{\text{II}}$ , and  $\text{Cu}^{\text{II}}$  metal ions, which can be found in food materials, because of their relative long relaxation times.  $\text{Fe}^{\text{III}}$  gives at X-band EPR a singlet at  $\sim 160$  mT,  $\text{Mn}^{\text{II}}$  a six-line hyperfine pattern due to the coupling of the unpaired electrons with  $^{55}\text{Mn}$  nucleus (spin  $I = 5/2$ ) at 300–350 mT, while  $\text{Cu}^{\text{II}}$  gives quartet hyperfine splitting after coupling with  $^{63}\text{Cu}$  nucleus (spin  $I = 3/2$ ) for the isotropic spectra at room temperature at 250–320 mT. The axial anisotropic EPR spectra of  $\text{Cu}^{\text{II}}$  nucleus consist of four peaks for the magnetic field aligned along the  $z$  axis and one peak for the magnetic field aligned along  $xy$  plane. One example was provided by Drew et al. who employed cw X-band EPR to explore the origin of the metal ions in Scotch whiskies [7].

The EPR spectrum of a frozen whiskey, depicted in **Figure 1**, shows the presence of all three metal ions.

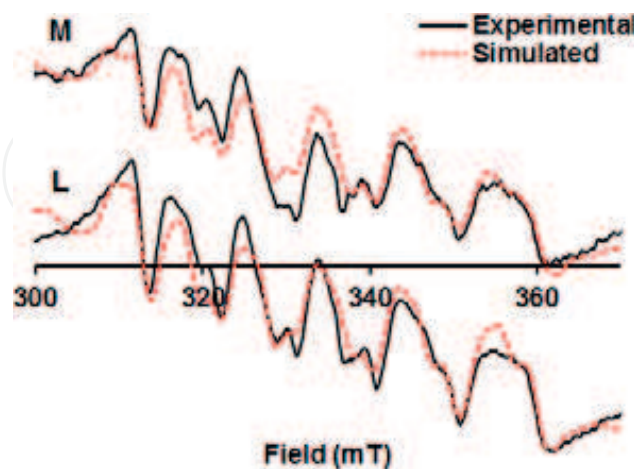
The EPR spectra of  $\text{Mn}^{\text{II}}$  is of particular interest because  $\text{Mn}^{\text{II}}$  is present at almost all the foods of plant origin [10]. The signal of the frozen solutions of the symmetric  $[\text{Mn}^{\text{II}}(\text{H}_2\text{O})_6]^{2+}$  consists of six narrow lines with additional small peaks between the six main components due to forbidden transitions. However, the EPR signal of  $\text{Mn}^{\text{II}}$  is significantly different from  $[\text{Mn}^{\text{II}}(\text{H}_2\text{O})_6]^{2+}$  when  $\text{Mn}^{\text{II}}$  is coordinated to small ligands or large biomolecules mainly because of changes in zero field splitting (ZFS) parameters [11, 12]. These EPR data can be obtained from the simulations of the experimental spectra and they can be used for investigating the coordination environment around  $\text{Mn}^{\text{II}}$  in foods. However, foods are complicated biosystems and metal ions might interact with several molecules creating around them various environments [13] of different symmetry.



**Figure 1.** Cw X-band EPR spectra of a 2008 distillate and as-bottled aged whiskeys from 1960 to 1970. After the permission of Prof. SC Drew.

Thus, the  $\text{Mn}^{\text{II}}$  EPR signal is complicated and fitting of the signal by considering one  $\text{Mn}^{\text{II}}$  species is not possible in most of the cases. In order to analyze the multicomponent EPR signals, researchers combine EPR and separation techniques and analyze the EPR signals of simpler-paramagnetic fractions [14].

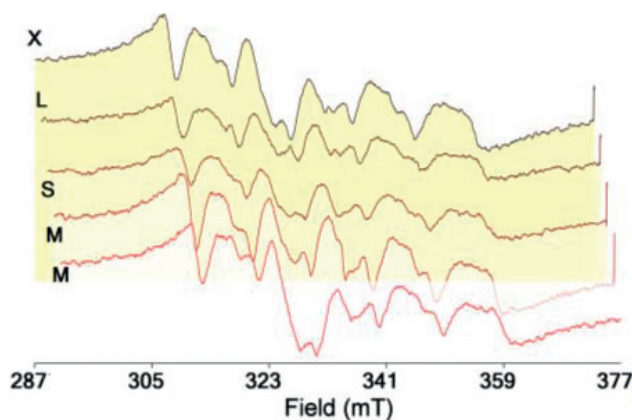
Trials to fit the  $\text{Mn}^{\text{II}}$  EPR signal of two Cypriot wines using Easyspin 5.2.8 [15] (**Figure 2**) did not result in a perfect match with the experimental spectra revealing multiple  $\text{Mn}^{\text{II}}$  species in the wines.



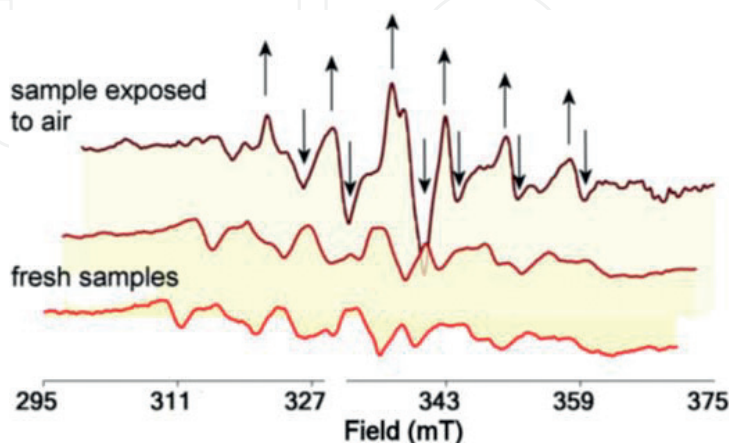
**Figure 2.** Experimental (black continues lines) and simulated (red dashed lines) cw X-band EPR spectra of two Cypriot wines from the grapes varieties Lefkada (L) and Maratheftiko (M) at 110 K. For the simulations were used the following parameters: (L)  $g = 1.999$ ,  $A = 258$  MHz,  $D = 530$  MHz, and  $E = 192$  MHz; (M)  $g = 1.999$ ,  $A = 257$  MHz,  $D = 564$  MHz, and  $E = 210$  MHz.

These EPR spectra features of the metal ions, which are originated from the various environments occurring for metal ions in foods, might be used for the food classification such as geographical or botanical discrimination. An example of the use of  $\text{Mn}^{\text{II}}$  X-band EPR spectroscopy for the discrimination of Cypriot wines from various grape varieties is shown in **Figure 3** (unpublished results). In addition to the characteristic shape of the spectrum, the quantity of  $\text{Mn}^{\text{II}}$  in each wine can be measured from the double-integrated spectra in the presence of standard [14, 16] information that can be additionally used as a variable for the wine discrimination.

The  $\text{Mn}^{\text{II}}$  cw X-band EPR spectra are also useful for analyzing the degradation of the food [10, 17]. An example of the alternation of the  $\text{Mn}^{\text{II}}$  signal in the wines up to exposure to air is shown in **Figure 4**. After the exposure, a new signal is appeared at  $g = 2.000$  and  $A \sim 185$  MHz. Such signals have been assigned to multinuclear manganese clusters of higher oxidation states than  $\text{Mn}^{\text{II}}$  as previously reported for studies in solutions of model  $\text{Mn}^{\text{II}}$  compounds after their exposure to  $\text{O}_2$  [18, 19]; therefore, similar clusters might be formed also in wines.



**Figure 3.** Cw X-band EPR spectra of various Cypriot wines from the grape varieties Xynisteri (X), Lefkada (L), Shiraz (S), and Maratheftiko (M) at 110 K.



**Figure 4.** Cw X-band EPR spectra of two fresh samples and one sample exposed to atmospheric oxygen for 1 day of the Cypriot wine from the grape variety Maratheftiko (M) at 110 K.

The presence of free ions, such as  $\text{Fe}^{\text{III}}$  and  $\text{Cu}^{\text{II}}$ , might accelerate degradation of foods, through Fenton reactions, leading to undesirable taste, color, or food spoilage [20–26]. Sometimes the removal of excessive free ions from foods is required in order to preserve their quality [8]. Metal chelators have found to inhibit the oxidation and increase the stability of model wines [27]. On the other hand, addition of metal ions in foods emerges reactive radical species that can be detected by EPR and used further for food characterization.

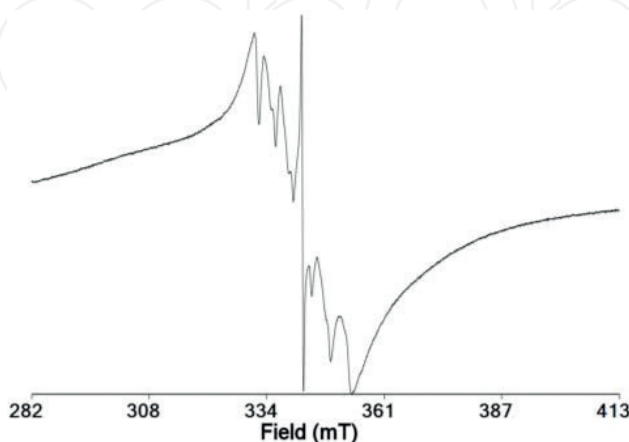
## 2.2. Organic radicals

In addition to metallic radicals, foods might contain persistent organic radicals formed by the exposure of food in atmospheric oxygen or the food preparation processes. Metal ions might play an important catalytic role in the formation of organic radicals. For example, although X-band EPR spectrum of fresh tea leaves gives at  $g = 2.000$  only the sextet of  $\text{Mn}^{\text{II}}$ , the ground tea from tea bags gives a sharp peak due to the stable semiquinone radical, in addition to the  $\text{Mn}^{\text{II}}$  peak (Figure 5).

An extensive EPR study of dry tea leaves from various origins has shown that except the semiquinone radicals, stable carbohydrate radical can also be detected [28]. The same study showed that the type of radical is depended on the content of flavan-3-ols in tea. The teas owned the highest content of flavan-3-ols (unfermented teas) form carbohydrate radicals, whereas fermented teas have high quantities of semiquinone radicals.

Troup et al. have investigated the organic radicals formed in roasted coffee beans and the brewed coffee solutions by EPR spectroscopy [14]. They have assigned the radicals to high-molecular-weight phenolic compounds present in the coffee brew and melanoidin compounds generated in the course of the Maillard reaction from reducing sugars and amino acids.

Phenolics are also the compounds which form radicals in red wines [29]. In addition, stable radicals were detected directly in the extracts of carrot root, celery stalk, cress shoots, cucumber, parsley, and cabbage leaf appeared upon maceration. The EPR signal is a double peak in the EPR spectrum, attributed to the monodehydroascorbyl radical formed in the aqueous



**Figure 5.** Cw X-band EPR spectra of ground tea from tea bags at room temperature.



solution. A wide single peak overlays the above signals in some samples and is attributed to the stressed biotic or abiotic conditions [30].

In general, fresh foods, protected from the oxidation, do not form organic radicals. However, such radicals might be induced and used for the characterization of food shelf-stability.

### 3. Induction and monitoring of radicals in foods

#### 3.1. Methods for induction of radicals

Several methods have been used for the induction of free radicals in foods, including irradiation with UV, microwaves, or  $\gamma$ -radiation, heating, addition of ozone, metal ions, or other oxidants. The EPR signal of stable radicals formed in food could be monitored directly, whereas unstable radicals can be measured indirectly with the addition of spin traps.

The use of EPR spectroscopy to monitor radicals in  $\gamma$ -radiated foods is a common practice which is very well documented in the literature [31–39]. The most of the studies were focused on consumer safety due to the use of this method in some countries for food product sterilization.

Microwave irradiation also causes formation of radicals in foods which can be monitored by EPR spectroscopy [40]. X-band EPR studies of the effect of microwave radiation on rice flour and rice starch [41–43] have shown the formation of tyrosyl and semiquinone radicals, after food irradiation, localized in the starch and the protein fraction of rice flour. These radicals exist in the native rice flour; however, their intensity increases exponentially by increasing microwave power and radiation time. The authors have proposed that transition metal redox process might be associated with the formation of the radicals [42, 43]. On the other hand, the rate of radical generation in flour starch is not related to the microwave power and irradiation time but increases rapidly at about 100°C [41].

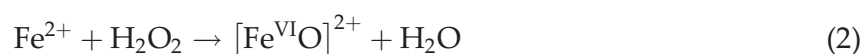
UV-irradiation is a very popular technique for the generation of radicals measured by EPR [44–46]. Foods are directly irradiated with UV-light [47–49] or after the addition of a photosensitive radical initiator in foods [50, 51]. The radicals, produced from UV-irradiation, usually are trapped by spin traps before being measured by EPR. However, there are examples of direct measurement of stable radicals formed in food. For example, UV-irradiation of grains resulted in the formation of reactive oxygen species and stable semiquinone and phenoxyl radicals [49]. In addition to the formation of organic radicals, the  $\text{Mn}^{\text{II}}$  and the  $\text{Fe}^{\text{III}}$  EPR signals alternate, pointing to a disturbance of the biomolecules' structures.

The thermal stability of foods, in particular, edible oils, is a property associated with the storage life of food stuff explored through various spectroscopic methods and rancimat analysis [52–57]. The thermal process of foods generates radicals that can be detected by EPR spectroscopy. An example of heating-induced radical formation is the coffee beans roasting with formed radicals to be monitored in real time [14, 58, 59]. Goodman et al. have shown that the organic radicals

produced from the heating of coffee beans are dependent on the variety of the bean, but the experimental data were not enough to support an explanation. In addition, they noticed that the quantity of radicals is higher at the presence of O<sub>2</sub>, and the oxidation rate of beans is considerably higher during the cooling process [58]. The radicals produced from the heating of edible oils are trapped with radical traps such as *N-tert-butyl-α-phenylnitron* (PBN). Monitoring the signal of the PBN spin adducts by EPR consists a promising method for the determination of the lipid oxidation lag phase but not suitable for the lag phase of hydroperoxides and thus oil shelf-life [60]. The formation of free radicals in edible oils is catalyzed by unsaturated lipids, and in this autoxidation mechanism, there is a direct involvement of β-carotene and chlorophyll [61]. The EPR spectra of the heated oils showed also the formation of α-tocopheryl radical, suggesting that the α-tocopheryl radical might be used as an alternative marker for studying the oxidation state of edible oils [61, 62]. The EPR spectra of edible oils heated at 180 °C in contact with metals suggested that iron and aluminum do not significantly affect the oils. On the other hand, heating the oil with copper resulted in the dissolution of large quantities of Cu<sup>II</sup> in the oil promoting the decomposition of primary oxidation products, while increasing the buildup of secondary oxidation products [63].

Ozone is a nonthermal technology with promising application in food processing. It is primarily used as a disinfectant and antimicrobial agent for food safety applications and for food preservation [64–66]. However, processing of foods with ozone results in the formation of radicals that can be detected with EPR [67, 68]. The ozonation of grains was found to be safe for the consumers; however, the application of ozone directly on food products containing crushed grains, for instance, meal, might pose a threat to consumers.

The initiation of radicals with addition of metal ions or with the addition of metal ions with H<sub>2</sub>O<sub>2</sub> (Fenton-like reagents) is also a usual strategy for the characterization of foods. The formation of radicals with the Fenton reagents is based in the reactions (1) and (2).



However, in addition to Fenton reagent, other reagents [69], reacting like the Fenton reagent, such as Co<sup>II</sup>/H<sub>2</sub>O<sub>2</sub>, Cu<sup>I</sup>/H<sub>2</sub>O<sub>2</sub> [70], and K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> [71], might be used. Usually, the radicals formed from the reaction with the Fenton reagents are trapped by spin traps and monitored by various spectroscopies including EPR. This methodology has been applied on several types of foods including plant extracts [72], strawberry fruit [73], sugar and other molecules found in foods [74], edible oils [48, 75], tea [76], wines [27], etc. Investigation of the reactivity of Fe<sup>II</sup> complexes with quinolinic acid as Fenton reagent has shown that Fe(II)-Quin produces more hydroxyl radicals and is more stable than Fe(II) alone [72]. In addition, metal ions being in the form of salts are insoluble in lipids; thus, in order to be used as radical initiators in lipids, they require their solubility to be increased by the addition of emulsifiers [77–79]. Recently, Drouza et al. have synthesized lipophilic metal complexes soluble in oils that initiate radicals in the presence of oxygen [3], whereas α-tocopherol is used as a marker for the investigation of the olive oils' stability.



### 3.2. Addition of radicals

A common use of EPR spectroscopy is the addition of reactive organic radicals, usually DPPH<sup>•</sup>, galvanoxyl radical, ABTS<sup>•+</sup>, TEMPO, TEMPOL, or Fremy's salt for the determination of the antioxidant activity of foods [80–84]. The EPR signal is reduced after the addition of radicals in oil because of the reduction of the radicals from the antioxidant food components, and the antioxidant activity can be calculated from Eq. (3) or more complicate mathematical equations [85–89].

$$\text{Inhibition activity}\% = (A_0 - A)/A_0 \times 100 \quad (3)$$

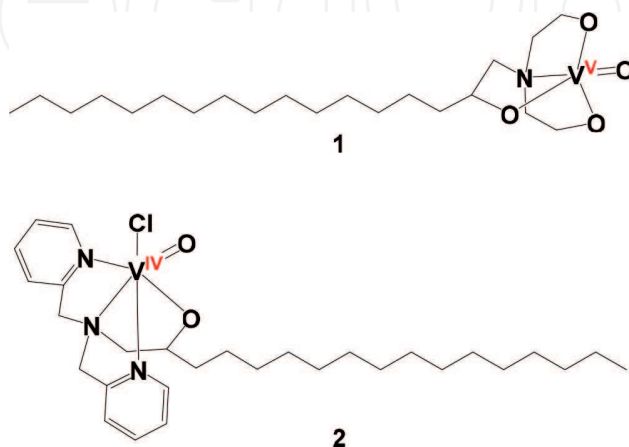
where  $A_0$  and  $A$  are the double integrals of the signal of the control and the sample after the addition of the antioxidant, respectively.

Stable radicals can also be added as probes. The EPR signal of the radical is dependent on the environment around the radical, thus structural information can be acquired. The radical probes could be organic [90–94] or inorganic [13]. The X-band EPR spectra of aqueous solutions containing extracts of green or black tea and Cu<sup>II</sup> showed the formation of six complexes, probably of Cu<sup>II</sup> with amino acids. The interactions of Cu<sup>II</sup> with teas are pH dependent. At high pH, the Cu<sup>II</sup> ions form complexes with polyphenols [13].

### 3.3. Lipophilic metal initiators

Although metal ions have been used as insoluble salts to induce free radicals in edible oil samples, a novel approach has been presented by the utilization of lipophilic metal complexes as radical initiators for the oxidation of lipids in olive oils, targeting the activation of  $\alpha$ -tocopheryl radical naturally contained in edible oils [3].

The new metal initiators consist the V<sup>V</sup> and V<sup>IV</sup> complexes, **1** and **2** (Figure 6), containing a lipophilic tail enabling them to perfectly dissolve in the oil matrix. This has been presented as an advantage of the new method because it allows the retaining of the chemical environment neighboring the polar phenols as it is in the bulk pure oil. Thus, phenols are allowed to participate in the free radical interplay between the redox species unaffected by any phase

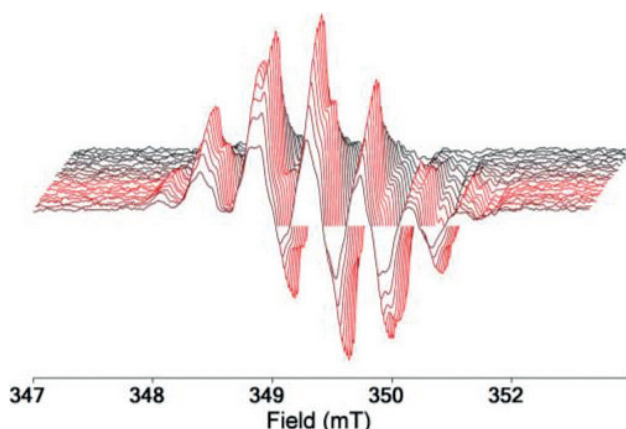


**Figure 6.** Vanadium (IV/V) complexes **1** and **2**.

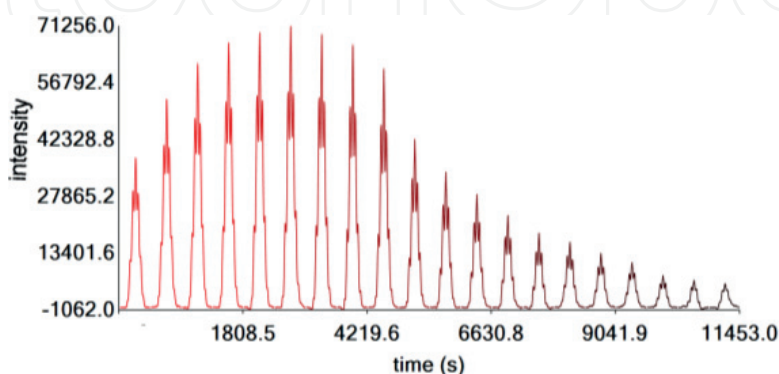
change discontinuation as it occurred in the case of the emulsions. In this method, the evolution of the phenol scavenging activity is recorded versus time revealing information for all the time framework of the food exposure to radicals (**Figures 7 and 8**).

The particular metal ion, vanadium, was selected because it participates in redox reactions, producing radicals and stabilizing semiquinone radicals [95–97], and activate molecular dioxygen [98, 99]. Cw X-band variable temperature (VT)-EPR spectroscopy reveals strong interactions between complex **2** and phenols suggesting that such interactions in the presence of O<sub>2</sub> might promote the initiation of the radicals.

The effect of the polar phenols naturally contained in the edible oils on the dioxygen activation and the free radical production was explored by a key experiment based on the monitoring of the intensity of the EPR  $\alpha$ -tocopheryl signal in the presence and/or the absence of the polar phenols. The subtraction of the polar phenols resulted in (i) the reduction of maximum intensity of the EPR signal of  $\alpha$ -tocopheryl radical and (ii) the decrease of the time needed for the occurrence of maximum intensity,  $t_m$ , for the same edible oil. This new method has been applied for evaluating the age of olive oil or the storage period associated with the amounts of the polar phenols, which are decomposed by the increase of the storage time, using the



**Figure 7.** X-band EPR spectrum of virgin olive oil (0.500 g) *vs* time after addition of **1** (100  $\mu$ L, 7.00 mM) at RT. The time period between two adjacent spectra is 6.5 min.



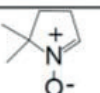
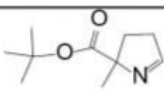
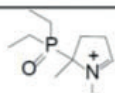
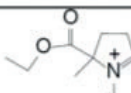
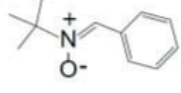
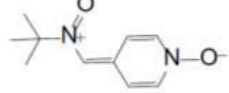
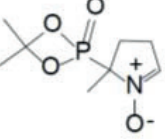

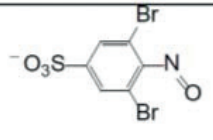
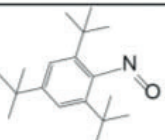
**Figure 8.** First integral X-band EPR spectrum of virgin olive oil (0.500 g) *vs* time after addition of **1** (100  $\mu$ L, 7.00 mM) at RT. The time period between two adjacent spectra is 6.5 min.

abovementioned two spectral characteristics as evaluating parameters. The mechanism of the radical initiation by **1** and **2** complexes was further investigated by spin trap experiments.

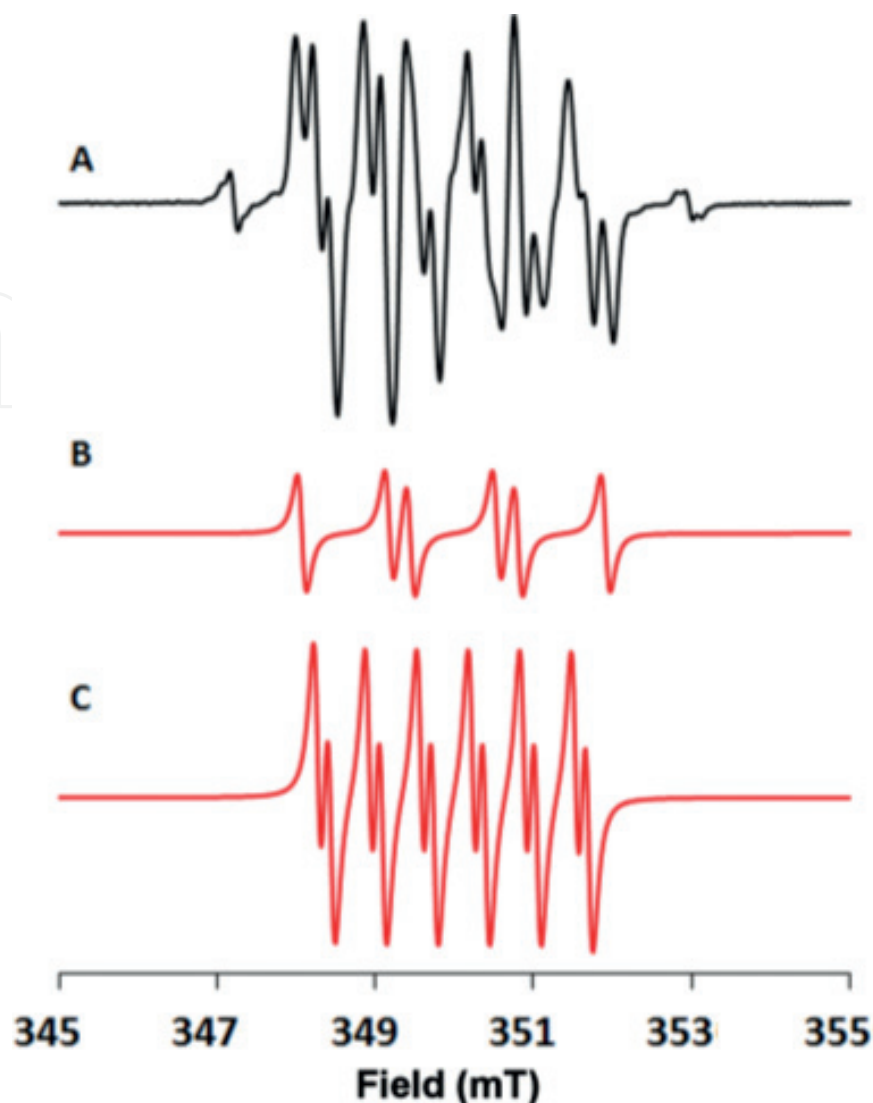
### 3.4. Radical traps

The life time of organic free radicals is usually very short because they undergo bimolecular self-reaction. Spin trap technique has been developed since 1968 for the detection and identification of the transient free radicals. Spin traps are diamagnetic molecules exerting a particular high affinity for reactive radicals, to which reactive radicals rapidly add to form persistent spin adducts, detectable in the EPR spectroscopy. Typically, there are two types of molecules serving as spin traps, the C-nitroso compounds and the nitrones; some of them are shown in **Table 1**.

The first one, the C-nitroso compounds are organic nitroxides which upon reaction form the spin adduct through addition of organic part of the radical directly on the nitrogen atom [100, 101]. This proximity to the unpaired electron occupying the  $p^*$  orbital of N atom of the functional group generates additional hyperfine coupling because of the presence of the neighboring magnetic nuclei of the added free radical. These hyperfine coupling parameters can provide structural information for the identification of added radical. The spin adducts of C-nitroso compounds in general have longer life times but bound less types of radicals, usually the C-centered ones, than nitrones [102]. The second type of spin traps, nitrones are organic molecules reacting with free radicals very fast, close to the diffusion-controlled limit, forming spin adducts by the bound of the added radical to the unsaturated C atom next to the N atom

Nitrone spin traps			
			
DMPO	BMPO	DEMPO	EMPO
			
PBN	POBN	CYPMPO	
Nitroso spin traps			
			
MNP (2-methyl-2-nitrosopropane)	DBNBS	TBN	

**Table 1.** Spin traps commonly used for detection and identification of free radicals.



**Figure 9.** (A) X-band EPR spectra of a solution of 200  $\mu\text{L}$  **1** (7.0 mM, in  $\text{CH}_2\text{Cl}_2$ ) 0.5 g pomace olive oil and 100  $\mu\text{L}$  DMPO (30.0 mM DMPO in  $\text{CH}_3\text{OH}$ ) at 5 min, (B, C) simulated spectra of the two components of the experimental spectra ( $A_N = 1.37$  and  $A_H = 1.06$  mT (DMPO-OOR) and with  $A_N = 1.31$ ,  $A_{H\beta} = 0.65$ , and  $A_{H\gamma} = 0.17$  mT (DMPO-OR)).

of the functional group [101–103]. It appears that this type of traps is widely used because they can form spin adducts with a wide range of radical species, such as peroxy ( $\text{HOO}^\bullet$ ), alkoperoxy ( $\text{ROO}^\bullet$ ), alkoxy ( $\text{RO}^\bullet$ ), hydroxy ( $\text{HO}^\bullet$ ), acyloxy radicals, as well as with other heteroatom-centered radical, including halogen atoms. The prime drawback for this type of traps is the poor information provided by their EPR spectra: the unpaired electron gives hyperfine coupling in the very best cases only from nitrogen nuclei of the function group and the  $\beta$ -proton, but not from the added radical. Thus, identification of the free radical goes through comparison of the under examination EPR spectra with undoubtedly characterized spectra obtained from the spin adducts of the prototype radicals.

An example of the use of DMPO for the detection of the alkoperoxyl and the alkoxy lipid radicals is shown in **Figure 9**. The spectrum was acquired 5 min after the addition of DMPO, and the vanadium complex **1** in olive oil. Deconvolution of the spectra fits to the alkoperoxyl lipid radical adduct of DMPO (DMPO-OOR) ( $A_N = 1.37$  and  $A_H = 1.06$  mT) in 33%, and the

alkoxyl lipid radical adduct of DMPO (DMPO-OR) of ( $A_N = 1.31$ ,  $A_{H\beta} = 0.65$ , and  $A_{H\gamma} = 0.17$  mT) in 77%, and a minor unknown carbon adduct of DMPO (DMPO-CRR'R'').

## 4. Conclusions

In this chapter, we have reviewed the main cw X-band EPR methodologies used for the study of foods, by observing endogenous unpaired electronic spin species and by the initiation and detection of radicals in foods. The use of EPR for analysis of foods is growing up rapidly. New methodologies in initiation and detection of radicals have resulted in the better understanding of the mechanisms involved in food oxidation processes. The high sensitivity and versatility of EPR makes this technique a valuable tool in food science, and further applications are expected to emerge in the future.

The cw EPR methods used for the characterization of foods are based on the recording of endogenous metal ion or organic radical preexisting in food or the initiation of radicals that can be detected directly or by the addition of radical traps. This chapter is an overview of these methods focusing to the research of the last 15 years.

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## Notes/Thanks/Other declarations

The cw X-band EPR spectra in this review were acquired on an ELEXSYS E500 Bruker spectrometer at resonance frequency  $\sim 9.5$  GHz and modulation frequency 100 MHz. Figures were produced by the software MultiSpecEPR (the software has been developed by Prof. AD Keramidas).

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