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Electron spin resonance – A spectroscopic method for determining the antioxidative activity

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Introduction

General aspects

Electron paramagnetic resonance (EPR) or nowadays more often named electron spin resonance (ESR) is used for the investigation of paramagnetic substances [1]. A paramagnetic state of a chemical compound is given, when it has an (unpaired) free electron. Especially for radicals, transition metals, and irregular crystal structures this fact is most abundant. In transition metals and crystal structures unpaired electrons are relatively stable and therefore easy to determine. Most of the free radicals, however, are very reactive and do not accumulate to high-enough levels to be measured by ESR directly.

From nutritional and physiological point of view free radicals and the scavenging of them by antioxidants are widely discussed [2]: antioxidants are needed to prevent the formation and oppose the actions of reactive oxygen and nitrogen species (ROS resp. NOS), which are generated *in vivo* and cause damage to lipids, proteins, and even the DNA. These reactions are thought to be reasons for various degenerative diseases and ageing. To support the endogenous antioxidant defense systems (superoxide dismutases, H_2O_2-removing enzymes, metal-binding proteins), diet-derived antioxidants seem to be important for maintaining health. Beside the well-known key role of vitamins E and C, many other dietary compounds have been suggested to be important antioxidants. Especially plant phenolic compounds have grown attention in recent years [3].

A number of analytical methods are available for the detection and quantitation of ROS/NOS [4]. These methods represent a wide variety of techniques, including electrochemical measurements [5], chemiluminescence [6], colorimetric analysis (*e.g.*, Trolox equivalent antioxidant capacity (TEAC) assay, [7]) and ESR. Nowadays ESR is widely used and seems to be the prerequisite tool for detecting and identifying free radicals generated by *ex vivo* or *in vivo* chemical reactions. The progress in ESR spectroscopy in the last ten years was enormous. The very large devices used in the past could be reduced substantially to benchtop devices which are much more easy to handle. For *in vivo* measurements 3-D spectrometers are available. Localization of the formation or degradation of radicals in organs of whole laboratory animals like rats and mice is possible [8]. The techniques frequently used in ESR spectroscopy are the stabilization of free radicals with so-called spin traps or spin labels as well as the use of naturally stabilized radicals for the determination of the antioxidative activity of natural compounds like vitamins and secondary plant metabolites, here, especially phenolic compounds.

Theoretical aspects of ESR

The basis of the ESR spectroscopy is the absorption of microwave energy by unpaired electrons, when they are in a magnetic field. As a result of their ability to rotate – this is the so-called "spin" – unpaired electrons are magnetic. In the magnetic field applied there are two possibilities of

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Abbreviations: DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; **DMPO**, 5,5-dimethyl-1-pyrroline *N*-oxide; **DPPH**, 2,2-diphenyl-2-picrylhydrazyl; **ESR**, electron spin resonance spectroscopy; **NOS**, nitrogen oxygen species; **PBN**, *N-t*-butyl-α-phenylnitrone; **POBN**, α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone; **ROS**, reactive oxygen species; **TEAC**, Trolox equivalent antioxidant capacity; **TEMPONE-H**, 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidin

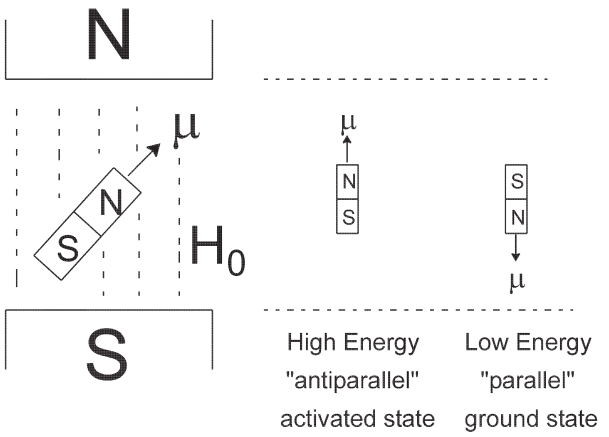

Figure 1. Spin orientation in an applied magnetizing field. μ = Bohr magneton; H_0 = magnetizing field.

orientation of the spin. In the so-called "parallel" state (or ground state) the orientation in the magnetic field only corresponds to the outer magnetic field attraction. In the "antiparallel" state (or activated state) the repulsion in the magnetic field dominates. This phenomenon is called Zeeman splitting (Fig. 1).

The magnetic interaction energy ΔE between the both energetic states is linear, depending on the magnetizing field:

$$\Delta E = h \times \nu = g \times \mu_B \times H_0 \quad (1)$$

(h = Planck's constant; ν = frequency of the (microwave) radiation; g = Landé factor; μ_B = Bohr magneton; H_0 = magnetizing field).

As a result of Eq. (1), there are two possibilities of performing the measurements. Either the frequence of the microwave radiation is changed while the magnetizing field remains constant or the magnetizing field gets changed with a constant microwave radiation. From technical points of view the second possibility is much easier to realize. Due to the modulation of the magnetizing force an ESR spectra illustrates the difference of the microwave amplitude between the maximum and the minimum of the magnetizing field (ΔI), divided through the amplitude of the magnetizing field (ΔH). So it represents the first derivative of the absorbance spectra of the microwave radiation (Fig. 2). A sample only absorbs microwave energy when resonance is reached. This is given when the energy difference ΔE between parallel and antiparallel state of unpaired electrons is equal to the energy of the constant irradiated microwave frequence (Fig. 3). Depending on this frequence one can differentiate diverse frequence bands, whereas the X band spectrometers with 9–10 GHz are used preferably.

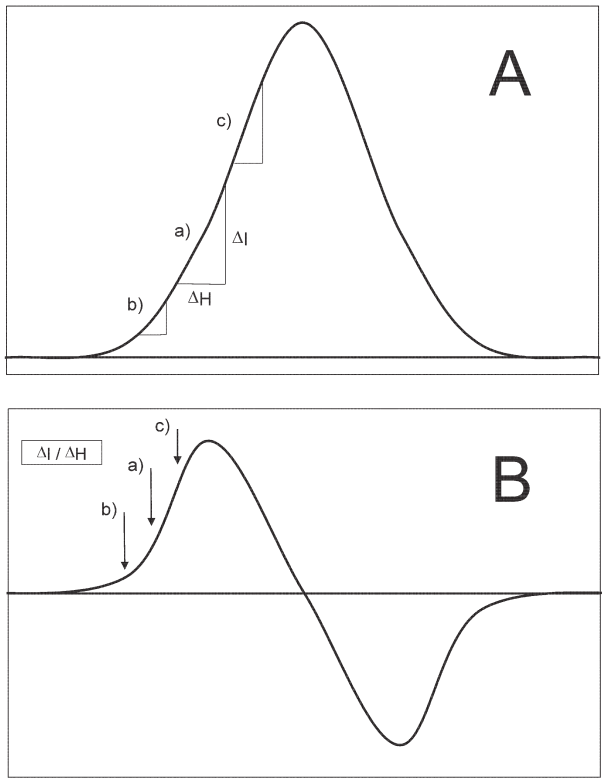

Figure 2. (A) Absorbance spectra (I, intensity) of the microwave energy depending on the magnetizing field (ΔH). (B) The differential scan of (A) (1. derivative) represents the ESR-signal.

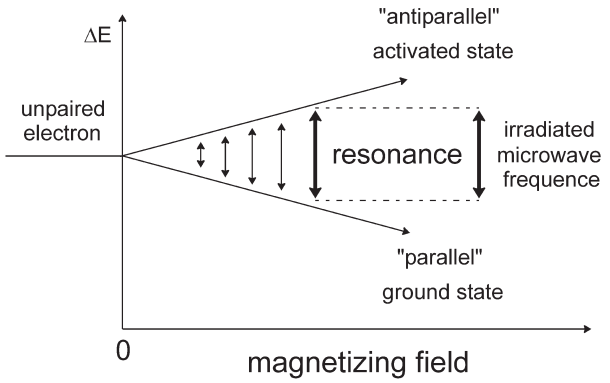

Figure 3. Resonance of an electron depending on the microwave energy (ΔE) and the magnetizing field.

Experimental methods

Samples

Beside pure analyte solutions even solid samples or suspensions are suitable for the ESR measurements. The choice of an appropriate sample device which consists of a synthetic,

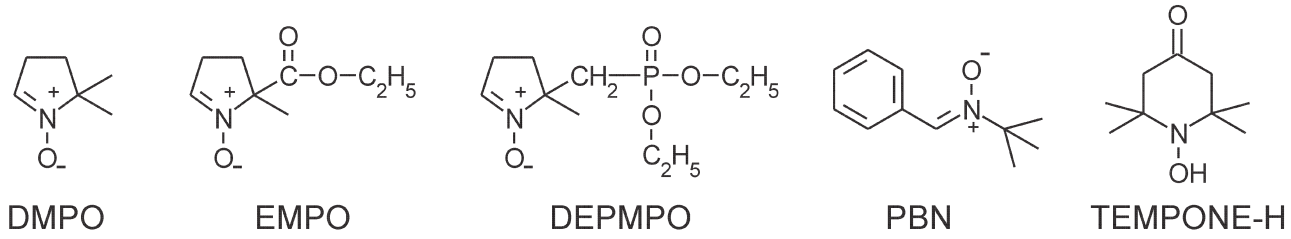

Figure 4. Structures of the most frequently used spin traps. DMPO: 5,5-dimethyl-1-pyrroline *N*-oxide; EMPO: 2-ethoxycarbonyl-2-methyl-1-pyrroline *N*-oxide; DEPMPO: 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; PBN: *N*-*t*-butyl-α-phenylnitrone; TEMPONE-H: 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidin.

pure fused silica, is depending on several factors: sample amount, concentration of the paramagnetic species and water content of the sample. The latter is a limiting factor for solid samples; when it is too high, all the microwave energy is absorbed and resonance of the sample can not be reached. For a qualitative analysis the whole sample device (a capillary) is filled in order to get the maximum intensity of the ESR signal. For the quantitative analysis of solid samples they have to be homogeneous. That can be achieved by simple grinding or milling. For liquid samples the sample devices can be capillaries or flat cells. The samples are pure solutions, suspensions or thin pastes. Here the limiting factor is the dielectricity constant of the solvent used. Depending on the dielectric properties the microwave energy is absorbed by the solvent and resonance of the sample can not be reached. Tissue or cell culture samples are preferably measured in flat cells in order to gain higher resolution. For such samples it is important to keep in mind sedimentation effects during the measurements. *In vivo* measurements using 3-D spectrometers are noninvasive and were performed in large tomographs. This technique is very similar to the magnetic resonance tomography used in medicine which determines the resonance of ^{1}H instead of unpaired electrons.

Spin trapping

As already mentioned, free radicals are highly reactive, possessing a short half-life in the range of nanoseconds to seconds (*e.g.*, hydroxylradicals 1 ns, nitric oxide up to 10 s). Due to methodological reasons this time period is too short to handle the samples adequately. As a result the so called spin trapping technique was developed [9]. A spin trap is an organic molecule which is able to stabilize a radical by forming a spin trap radical reaction product. These adducts have longer half-lives and are therefore easier to measure. Figure 4 gives an overview of the spin traps usually used. They can be differentiated in specific and nonspecific spin traps (Fig. 5).

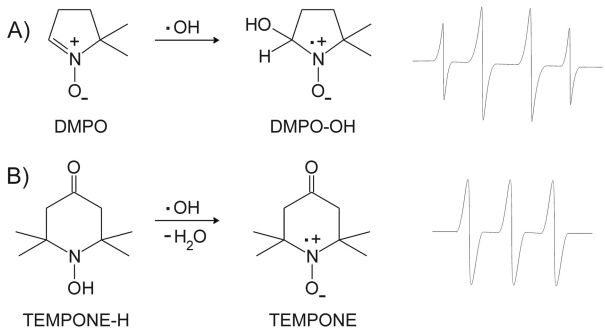

Figure 5. Examples for (A) specific (DMPO) and (B) nonspecific (TEMPONE-H) spin trapping.

Specific spin traps react with radicals at a double bond, resulting in a spin trap radical adduct, whose ESR signal is characteristic for identifying the original radical. Due to interactions between the electron spins and the nuclear spins of atoms of the surrounding area there are different ESR signal profiles. While atoms with an even number of protons and neutrons do not show any splitting of the signal, atoms with an odd number of protons or neutrons (like hydrogen or phosphorus) lead to a doublet signal formation. Odd numbers of protons and neutrons (as given for nitrogen) show triplet signal formation. As the most spin trap radical adducts are nitroxyl radicals, triplet ESR signals are very frequent, resulting from the stabilization at the nitrogen atom. Depending on the kind of atoms at the β-site every signal of the triplet may split into further doublets and triplets. The atoms at the γ-site of the molecules only influencing the signal slightly. Figure 6 illustrates the splitting profile of a hydroxyl 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) adduct: the triplet signal, resulting from the nitroxyl radical, splits into three further doublet signals with an intensity pattern of 1:2:2:1. Specific spin traps, like DMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO), 2-ethoxycarbonyl-2-methyl-1-pyrroline *N*-oxide (EMPO) (Fig. 4), are usually used in concentrations of 0.1 mol/L. In biological systems like cells or tissues

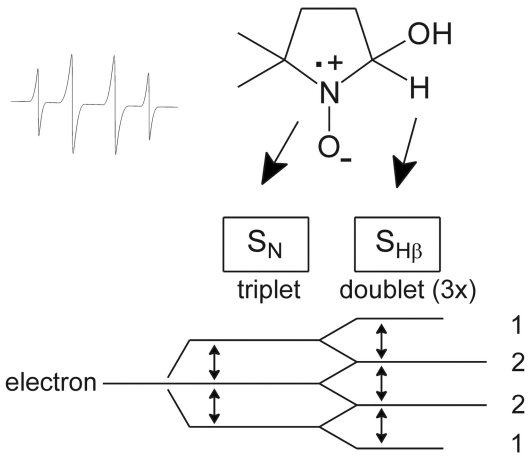

Figure 6. Splitting profile of hydroxyl radical DMPO adducts. Triplet splitting of the signal caused by the nitrogen; further doublet splitting due to the interactions with the hydrogen atom at the β-site. As a result there is a four-line signal with an intensity pattern of 1:2:2:1.

the concentration should be reduced, because of toxic effects [10, 11].

Unspecific spin traps are often hydroxylamines, which possess the advantages of a higher reaction velocity and a better stability of the resulting adducts [12]. These spin traps are more effective and can be used in much lower concentrations (0.5 – 10 mM/L). During the reaction the hydrogen atom of the hydroxylamine gets abstracted. The signal does not split into further signals, because of the lack of potential coupling partners at the β-site (Fig. 5, 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidin (TEMPONE-H)).

Beside these typical spin trapping reactions, it is also possible to trap radicals with metal complexes by coordinative binding. For example, ferrous-bis-diethyldithiocarbamate complexes or hemoglobin can be used to bind nitric oxide: The radical binds to the Fe-(II) center, thus stabilizes, without changing the oxidation state of the Fe and the properties of the radical [13].

For the choice of an appropriate spin trap there are some points which have to be considered, *e.g.*, solubility of the spin trap, stability of the spin trap radical adduct formed, pH of the system, parallel presence of transition metals or oxidative resp. reductive agents. Spin trap solutions always should be prepared freshly and highly concentrated in order to allocate enough trapping capacity. Especially the influence of reductive agents seems to play a major role in biological samples. Ascorbate, urate, and glutathione may reduce both the free rdaical and the spin trap radical adduct to ESR-silent species [2]. Rizzi *et al.* [14] presented a new spin trap, TAM OX063, a triarylmethyl radical which traps superoxide anion without being influenced by bioreduction.

In food quality assurance the spin trapping technique is used to identify oxidation products of radical nature or to verify the beginning of a radical formation after the endogenous antioxidants have been consumed. For example, in beer the development of the oxidation during storage was investigated using *N-t*-butyl-α-phenylnitrone (PBN) as a spin trap [15]. The antioxidants present in beer, *e.g.*, ingredients such as polyphenols, melanoidins, and sulfur dioxide, eliminate all radicals till the antioxidative capacity is depleted. After this period, the so-called lag time, the radicals form only spin trap radical adducts (Fig. 7). So, the lag-time represents the extent of the antioxidative activity and as a consequence it correlates with the stability of stored food.

For many biological samples, such as blood, urine, cell homogenates, *etc.*, the chemical systems used for applications in food analysis are also practical. Using α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (POBN) as a spin trap it was shown for chloroform extracts of rat lungs that asbestos leads to oxidative damage [16]. But there are also possibilities to measure antioxidative activity *in vitro* where whole intact cells of cell culture are applied. For example, in HL-60 leucemic cells DMPO was used to monitor prooxidant resp. antioxidant activity and cytotoxicity of selected synthetic resveratrol analogues [17]. In freshly isolated and cultured rat pancreatic islets spontanous formation of oxygen radical species was detected using DMPO for spin trapping [18]. In endothelial cells formation of free radicals plays a major role in vessel physiology and pathology. For the determination of the important superoxide anion DMPO was also used [19].

One of the most important phenomena in oxidative damage is the oxidation of unsaturated fatty acids. Lipid peroxidation of, *e.g.*, cell membranes and lipoproteins can be monitored by using DMPO and POBN [20]. DMPO is able to permeate through liposomal membranes and traps the radicals in liposomes resulting from the reaction with hydrogen peroxide [21]. The influence of oxidative stress also affects macromolecules, such as proteins and DNA. Though spin trapping was developed for investigating small radicals, in recent years it was also recognized to be suitable for detecting macromolecule radical formation. Oxidation can occur at both the protein backbone and on the amino acid side-chains, with the ratio of attack dependent on a number of factors. Especially oxidation of methionine plays an important role *in vivo*, during biological conditions of oxidative stress, as well as for protein stability *in vitro*. For example, Alzheimer's disease is related to ROS-induced oxidation of methionine in β-amyloid peptide [22].

With the classical spin traps DMPO, DEPMPO, and PBN it is difficult to resolve hyperfine structures. Instead of the

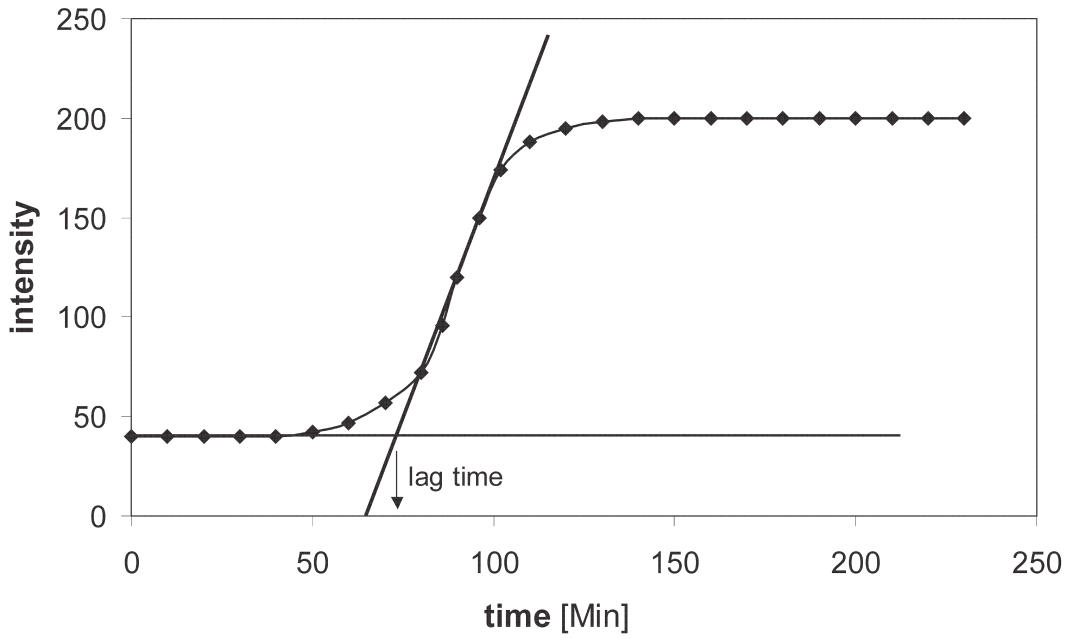

Figure 7. Determination of the lag-time: time when the endogenous antioxidative capacity is depleted, followed by a continuous formation of spin trap radical adducts.

nitrone spin traps nitroso spin traps were applied. The advantage is a direct attachment of the radical to the nitroso nitrogen atom and is therefore in close proximity to the unpaired electron. As a result, hyperfine couplings are much easier to identify [23].

Proteolytic digestion leads to the release of peptides from the initial protein species, some of which still having the spin trap attached. The advantage of this treatment is a better mobility of the fragments, leading to sharper ESR spectra. These peptides may be used to detect the sites of protein radical formation [24]. The spin trapping of protein radicals is further improved by the coupling to immunoassays. Polyclonal antibodies have now been developed which bind to protein adducts of the nitrone spin trap DMPO. As a result, the detection of protein radicals is more sensitive [25, 26]. Further aspects of the analysis of protein radicals using ESR measurements are reviewed by Davies *et al.* [23].

In *ex vivo* approaches whole intact tissues or even organs can be used for the localization of radicals. For example, DMPO and POBN have been used to trap free radicals in human skin biopsies and hair after treatment with *t*-butylhydroperoxide, UV-light, and laser radiation [27–29]. In liver biopsies free radicals, generated during various diseases, can also be detected by using bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)-decandioate as a spin trap [30]. Isolated rat hearts were perfused and the coronary effluent was "trapped" with ferrous-bis-diethyldithiocarbamate to detect nitric oxide formation [31]. Table 1 gives an overview of several investigations using different spin traps for the determination of the antioxidative acivity in food and biological samples.

The trapping of radical species *in vivo* is also possible. Most of the techniques used are noninvasive because of oral administration of the respective traps and scanning of the whole organism in large tomographs. Oxidative stress in the brain of rats was studied with 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (MC-PROXYL), which is a blood-brain barrier-permeable spin trap [8]. 3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL) was used to study hepatic oxidative stress [36]. In parallel it is also possible to perform anatomic analysis by following the distribution of the spin traps in different organs [37, 38]. Further aspects of *in vivo* ESR measurements are reviewed by Fuji and Berliner [39]. Applicability to humans is still very limited because of the unknown toxicity at the levels that would be required for radical trapping *in vivo*.

Spin labeling

Spin labels (or spin probes) are stable radicals which have the ability to give informations about the microenvironment of the target radical. Beside the determination of the radical, the measurement of polarity, viscosity, pH value, and even oxygen concentration is possible with selective spin labels.

Table 1. Examples for ESR measurements in food and biological samples using spin traps

Sample	Spin trap[a)]	Radical formation through ...	Ref.
Cheese	DMPO	Light	[32]
Beer	PBN	Heat	[15]
Onion extract	DMPO	a) Xanthine/xanthine oxidase b) $FeSO_4$ / H_2O_2	[33]
Sempervivum tectorum extract	DEPMPO	UV radiation	[34]
Inflammatory oxidative stress	PP-H	Lipopolysaccharide stimulation	[12]
Oxidative DNA damage	Vitamin C	Cigarette smoke	[35]
Lung damage	POBN	Asbestos	[16]
Vascular nitric oxide	Fe-II-$(DETC)_2$	Nitrogen monoxide synthase	[13]
Human skin biopsies	POBN	UV radiation	[27]
Human skin biopsies	DMPO	UV radiation	[28]
Liver biopsies	TEMPDD	Various liver diseases and injuries	[30]
Rat heart nitric oxide	Fe-II-$(DETC)_2$	Cold cardioplegia	[31]

a) PP-H = 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidine; DETC = diethyldithiocarbamate; POBN = α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; TEMPDD = bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)-decandioate

The polarity of the solvent influences the absolute position of the spin label signal (*g*-value) as well as the interactions between the electron spins and the nuclear spins. As a result, the signal splitting profile changes. With decreasing polarity the *g*-value increases. An increase in polarity leads to stronger interactions. A high viscosity of the spin label environment leads to a reduced rotation velocity, resulting in preferences of the spin orientation. In the spectra this is illustrated by a broadening of the outer signals – the so-called anisotropy. Labeling fatty acids of membranes makes the viscosity (membrane fluidity) time-dependently determinable [40, 41]. The binding orientation of enzymes on phospholipids vesicles can be investigated by using a water-soluble spin relaxation agent, chromiumoxalate [42, 43]. There are some imidazolin and imidazolidine nitroxyl radicals which are very effective pH sensors. pH values from 0 to 14 are determinable with an accuracy of 0.05 units [44]. By means of the ESR signal width the oxygen partial pressure in tissues can be measured [45].

Stabilized radicals

As mentioned above, radicals have a very short half-life. But there are also some radicals which are stabilized due to their structure or other conformational reasons. Early ESR investigations on the Maillard reaction showed the existence of stable radicals, whose formation depended on the concentration of the reactants. These radicals reached a maximum in thermo-induced browning reactions. The hyperfine structure of the ESR signal was supposed to be similar to pyrazine cation radicals [46]. Ten years later it was shown that this intermediate of the Maillard reaction is a pyrazonium radical, determined directly during the roasting of coffee. It can be used as an indicator of a proceeding Maillard reaction [47].

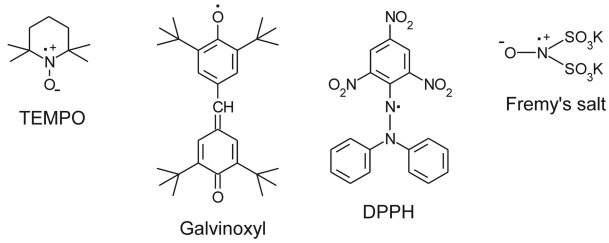

Figure 8. Structures of the most frequently used synthetic stabilized radicals. TEMPO: 2,2,6,6-tetramethylpiperidin-1-xyloxyl; galvinoxyl: 2,6-di-*tert*-butyl-α-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-tolyoxyl; DPPH: 2,2-diphenyl-2-picrylhydrazyl; Fremy's salt: potassiumnitrosodisulfonate.

In plant food the oxidation of phenolic compounds leads to quinones, whose intermediate products, the semiquinones, are of radical nature. Some of them are stabilized under the conditions of a low water containing matrix, where a disproportionation to hydroquinones and quinones is reduced. These semiquinones can also be consulted for the freshness of plant food.

Beside the detection of the stabilized foodborne radicals, such radicals are often used to determine the antioxidative properties of all kinds of food. Figure 8 illustrates the most common synthetic stabilized radicals. Their time-dependent degradation by the antioxidants of the samples can be measured. The resulting function further depends on the concentration of both, radical and antioxidant.

There are two different antioxidative parameters which are determinable from the degradation kinetics (Fig. 9). (i) The antioxidative potential represents the velocity of the radical degradation. It is determined by measuring the concentration

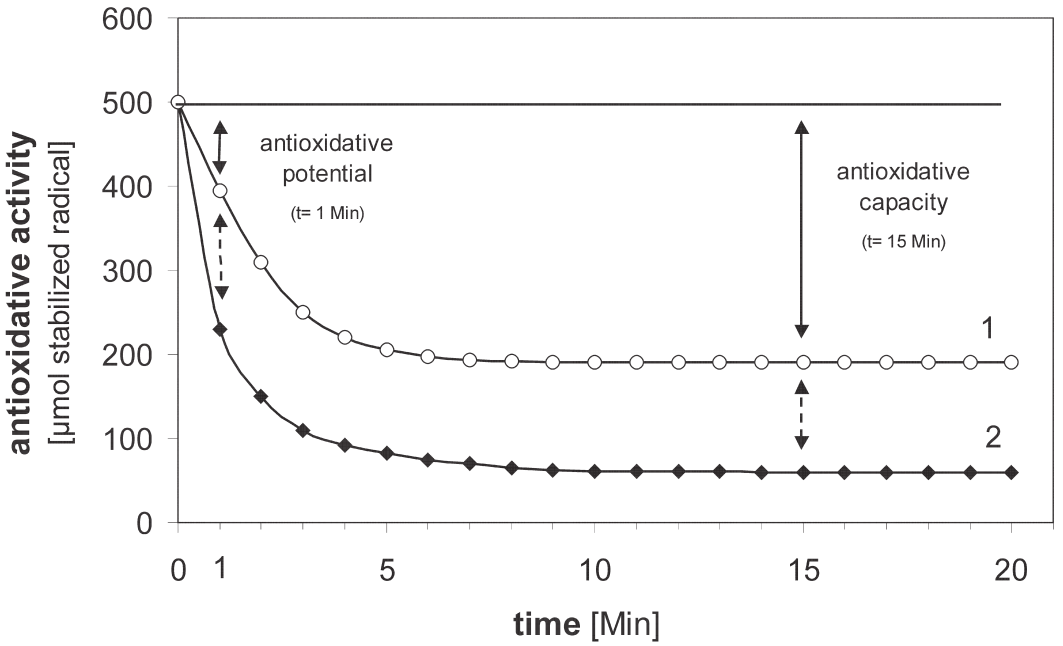

Figure 9. Kinetics of a synthetic stabilized radical degraded by two different antioxidants (1 and 2). Antioxidative potential (µmol radical degraded per minute) and antioxidative capacity (µmol radical per µmol antioxidant).

Table 2. Examples for ESR measurements in food samples using stabilized radicals.

Sample	Stabilized radical	Solvent	Ref.
Tea extract	Galvinoxyl	Ethanol	[48]
Sea buckthorn	Fremy's salt	Phosphate buffer, pH 7.4	[49]
Red wine	Fremy's salt	Ethanol	[50]
Wheat extract	DPPH	Benzene	[51]
Cheese	TEMPO	Without solvent (solid ESR)	[32]

TEMPO = 2,2,6,6-tetramethylpiperidin-1-xyloxyl

of the radical degraded by a constant amount of antioxidant during the first minute of the reaction. As a result the antioxidative potential describes a kinetic phenomenon and is given in µmol degraded radical per minute. (ii) The antioxidative capacity is the amount of radical which is degraded after a certain time by a given concentration of an antioxidant (Fig. 9, for example 15 min). The result has the unit µmol degraded radical per µmol antioxidant. In most cases the concentration can not be defined (*e.g.*, mixtures of antioxidants in food), here one can only talk of antioxidative activity. Table 2 shows some examples of the use of stabilized radicals for determining antioxidative properties of food.

Primarily foodstuffs rich in plant phenolic compounds, such as tea, wine, *etc.*, are a main target for investigating antioxidative properties, because they have a naturally high antioxidative capacity. Especially flavonoids show a high number of diverse structures (more than 4000 substances), due to the variations at the C-ring of the flavonoid skeleton and several positions which are often glycosylated [52]. All these substances may possess different antioxidative activities. The influence of such structural alterations on the antioxidative capacity can be illustrated for aqueous systems by using Fremy's salt as a stabilized radical for the ESR spectroscopy. As a result, a structure-dependent assignment of the antioxidative activity can be given for all kind of samples like plant extracts, fruit juices, *etc.* For example, sea buckthorn juice possesses many flavonoids, but not all of them contribute to its high antioxidative activity. Only the flavonols like quercetin, rutin, and isorhamnetin, which can be oxidized to *ortho*- or *para*-quinoid structures have a high antioxidative capacity. The isorhamnetin-3-glycosides, although present in high concentrations in sea buckthorn juice, are not able to form these quinoid struc-

tures, resulting in a very low antioxidative capacity. Therefore, they do not contribute to the antioxidative activity of sea buckthorn [53]. A chromatographic fractionation of the sea buckthorn pomace showed a high amount of proanthocyanidins, which also have high antioxidative activites [53, 54]. Beside the monomeric fractions (flavan-3-ols), the dimeric, the trimeric, as well as the oligomeric proanthocyanidin fractions showed higher antioxidative capacities compared to vitamin C or Trolox, a vitamin E analogue. With an increasing degree of polymerization the antioxidative capacity decreased, following the order: monomeric proanthocyanidins ≈ dimeric proanthocyanidins > trimeric proanthocyanidins > polymeric proanthocyanidins.

It was shown for selected flavonols that the results differed depending on the stabilized radical used [48]. Beside steric hindrance resulting from the varying structures of the radicals, differences in redox potential as well as in reaction conditions like solvent, pH, or solubility are reasons for this behavior [48]. Choosing the appropriate radical might therefore be difficult. However, these chemical systems may not reflect the antioxidant capacity in heterogeneous cellular environments, but it is possible to compare the antioxidative properties of several foodstuffs when using standardized conditions (solvent, stabilized radical, *etc.*).

The *in vitro* importance of flavonoids was tested by applying selected isoflavonoids in a endothelial cell model system using 2,2-diphenyl-2-picrylhydrazyl (DPPH) and galvinoxyl stabilized as synthetic radicals. As a result it was found that the isoflavonoids tested do not substantially contribute to the antioxidative activity by might be important because of increasing cellular glutathion levels [55]. In a heterogenous phase of red blood cells and phosphatidylcholine liposomes the already mentioned flavan-3-ols and procyanidins were able to recycle tocopheroxyl radical after oxidation with DPPH [56].

Evaluation of the ESR measurements

For a qualitative assessement the profile of the ESR signals is important. As mentioned above, the use of spin traps leads to characteristic profiles. If there are any overlaying effects of different spin trap radical adducts, a spectra substraction or simulation will be necessary. For a profile fully unknown, there are several databases in which the signals can be searched.

As an ESR spectra is the first derivative of the absorbance spectra of the microwave radiation, a quantitative evaluation can be performed by two different approaches: (i) by a classical integration of the amplitude or (ii) by determining the intensity of the signal. For the integration, the area under the curve correlates with the number of radicals. If all the radical species are of the same nature, the line width remains constant. In this case the intensity can also be used for quantification. As described above, deformation of the outer signals due to interactions or system conditions (pH, viscosity) is likely to take place. For signals with more lines only the middle line or the left line is used for evaluation.

Concluding remarks

Although measurement of paramagnetic substances is not a new method, only for a short time ESR spectroscopy has become more and more a state-of-the-art method for determining antioxidative properties of foodstuffs and biological samples. In comparison to the more often used colorimetric assays, such as the TEAC assay, it is much more specific due to the formation of characteristic signals. By using ESR spectroscopy, the differences between the numerous substances contributing to the total antioxidative activity can be characterized, resulting in a more precise assessment of the antioxidative activity of the respective samples. These measurements have to be performed under standardized conditions, because the use of varying approaches (different stabilized radicals, solvents, *etc.*) may lead to different results which are hardly comparable.

The *in vitro* and *ex vivo* determination of reactive species is possible in cells from cell culture, whole tissues, or even organs. Especially by using the spin trapping technique formation of free radicals or occurring secondary radicals (*e. g.*, lipid or protein radicals) can be monitored. One has to keep in mind that the spin trap radical products giving the ESR signal can be rapidly removed in cells, *ex* or *in vivo* by enzymatic metabolism and direct reduction. The enormous development of noninvasive methods allows to follow the distribution of the spin traps in whole bodies of labortory animals. Techniques for monitoring reactive species in humans are limited because of the lack of appropriate spin traps. Pharmacokinetic data and toxicity at the levels required for spin trapping *in vivo* are still largely unknown and the use of high doses is important in order for the trap to compete with endogenous molecules for a reactive free radical.

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