



## Seeing the unseen: degradation measured by EPR spectroscopy

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Material degradation results from complex interactions between physical, chemical, and biological degradation processes that often involve free radicals. Some of the processes that compromise product stability are heat (thermal degradation and thermal oxidative degradation when in the presence of oxygen), light (photodegradation), oxygen (oxidative degradation), and weathering (generally UV degradation).

Product stability during its entire shelf life is an important matter and stability studies do not only cover the physicochemical properties but also explain the safety and efficacy of the products during their entire shelf life. Forced degradation (stress testing) studies are studies in which stress or accelerating conditions are applied to the product. They help to facilitate product development, manufacturing, production, and packaging where knowledge of free radical behavior can be used to improve shelf life and product quality.

Electron paramagnetic resonance (EPR) spectroscopy is the only analytical technique capable of detecting species with free radicals in a direct and non-invasive manner. At the same time the technique is completely 'blind' to samples without free radicals (or unpaired electrons) so it is very specific. It is very versatile and can be applied to gaseous, liquid, or solid samples over a large range of temperatures. EPR can be used to detect, quantify, and monitor the intrinsic generation of short-lived species and is therefore extremely useful for detecting free radical degradation in polymers, foods and beverages, pharmaceuticals, and environment, etc. The magnitude of the EPR signal directly correlates with the level of degradation in the product of interest so the analysis is very straight forward. Applications widened as spin traps were developed to 'capture' the very short-lived free radicals, effectively fixing them into a more stable form that could be measured by EPR. A common method for spin-trapping involves the addition of radical to a nitron spin trap resulting in the formation of a spin adduct, a nitroxide-based persistent radical, that can be detected using EPR. The spin adduct usually yields a distinctive EPR spectrum that identifies the free radical that is trapped. The result was a move for EPR to become widely used not only in industry but also in vitro and

in vivo studies in the medical and pharmaceutical fields.

The aim of this paper is to present a general overview of the diversity of EPR applications when it comes to degradation reactions.

### I. Pharmaceuticals

Therapeutic drugs require a well characterized shelf-life to ensure correct dosage and patient safety. Developing successful formulations depends on a thorough understanding of their chemical and physical stability and properties. Heat, light, oxygen, moisture, sterilization processes, impurities, and excipient interactions are some of the factors that can compromise product stability. Moreover, all these

factors may cause degradation of the Active Pharmaceutical Ingredients (APIs), excipients, or formulations resulting in loss of product potency or toxic by-product generation. Degradation processes quite often involve free radicals and transition metals that are responsible for most of the damage that occurs in drug products.

EPR spectroscopy is successfully used to monitor free radical formation during degradation of pharmaceutical products, identify the radical intermediates, and help determine the reaction mechanism. It can determine the root cause of degradation, measure the extent of degradation, and predict long-term stability characteristics of the APIs, excipients, and formulations. Because of EPR's inherent high sensitivity the measurements are very quick and only use a small quantity of the API or drug formulation, so the technique could be used during the early drug development phase.

Excipients are substances other than the pharmacologically active drugs or prodrugs which are included in the manufacturing process or are contained in a finished pharmaceutical product dosage form. These excipients improve the properties of the drug, either by enhancing the therapeutic effect of the APIs or by facilitating the manufacturing process and are typically the major components in a pharmaceutical product. Not only could direct degradation of the APIs diminish the action of the product, but degradation of excipients can also affect the efficacy of the drug either by altering its physicochemical properties or by

reacting with the APIs. It is therefore crucial to assess the stability of such components after they undergo industrial processes which could affect their stability. Polysorbates are one of the most commonly used excipients in formulations of biopharmaceuticals and it is known that polysorbates are prone to degradation by autoxidation which is a free radicals chain process. EPR data shows that degradation occurs and is detected under a variety of storage conditions leading to formation of free radicals. The primary concern is that these radicals can readily oxidize and degrade proteins, leading to potentially severe and undesired effects in patients (Figure 1).

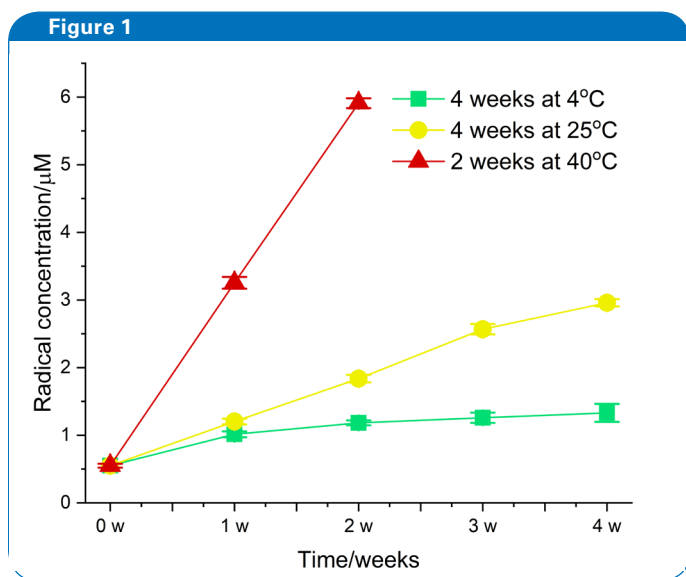


Figure 1. Radical concentration in polysorbate 20 stored under different storage conditions

Identification and quantification of detected free radicals in polysorbates is a key step in understanding the mechanism of their autoxidation. With our EPR quantification package (SpinCount and SpinFit, **Bruker patent!**) implemented in Bruker Xenon software, the task of identifying and quantifying the polysorbate radicals is both straightforward and precise (Figure 2).

Another recent work was focused on the effects of  $\gamma$ - and X-irradiation on L-histidine, an excipient typically used in parenteral formulations as a buffering agent and a stabilizer for subcutaneous, intramuscular and peritoneal injections [2]. The authors concluded that irradiation induces deamination of histidine and formation of C-centered radical (Figure 3). Furthermore, spin trapping showed also regeneration of the radical long after the initial dissolution of the irradiated material. Fenton-type chemistry involving strong oxidants generated during the irradiation process and catalyzed by trace metals from a standard sterile syringe needle, was implicated in this process.

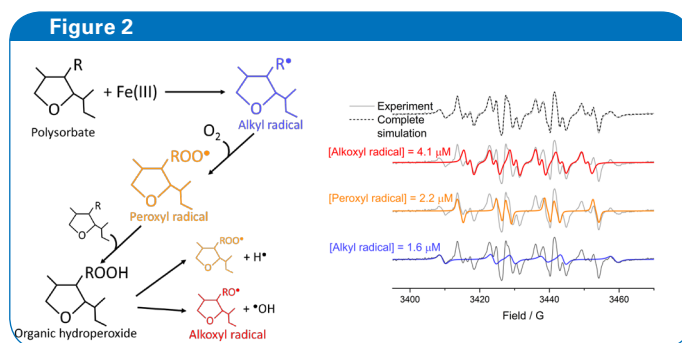


Figure 2. Left panel: An EPR spectrum of spin trapped radicals detected in polysorbates and the complete simulation are presented on the top in grey. From the simulated spectrum (the dotted trace) three different radicals were identified using the SpinFit module – alkoxy (simulated in red), peroxy (simulated in orange), and alkyl (simulated in blue) radicals. The concentration of each radical species was determined using the SpinCount module. Right panel: The proposed mechanism of autoxidation in polysorbates strongly correlates with the EPR data. The reaction scheme was adopted from Ref. [1].

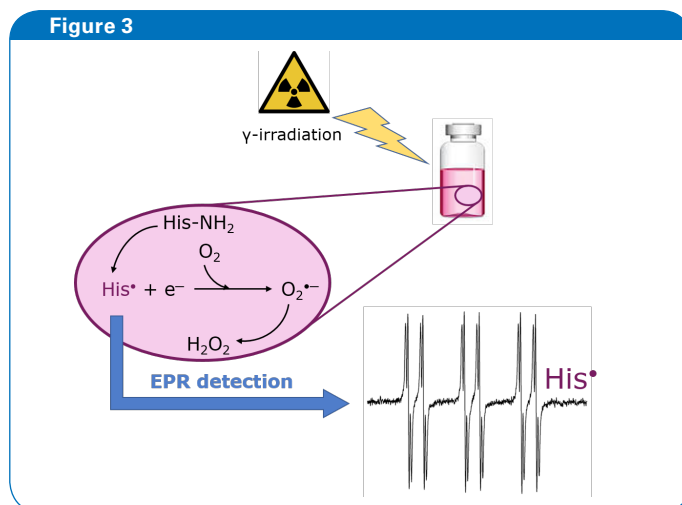


Figure 3. Deamination of L-histidine upon  $\gamma$ -irradiation (adopted from Ref. [2])

According to the authors, knowing the behavior of the reactive degradation products in solution is essential when dealing with excipients intended for parenteral formulations. Avoiding the regeneration of the radicals in solution is necessary in order to eliminate the potential for radical-induced degradation of other drug components, such as APIs in particular, in a complete pharmaceutical formulation. Unintentional injection of free radical containing solutions into patients could also have direct toxicological implications. While a thorough analysis of each irradiation sterilized product remains necessary, assessing the effects of  $\gamma$ -irradiation on single drug ingredients is an essential first step towards the analysis of multi-component systems.

## II. Antioxidant efficacy

The need of antioxidants in materials and products has been recognized for many decades. Antioxidants inhibit the oxidation of other molecules by reducing free radical intermediates and preventing the proliferation of reactive oxygen species. Therefore, antioxidants are undeniably a requirement in every product. However, there is an open question: what are the long-term effects of antioxidants? Are they able to provide products with the necessary lasting protection that really matters to prevent degradation and reduced shelf-life? EPR is a tool to evaluate antioxidant activity. It allows the determination of antioxidant efficacy taking different parameters into account: their activity and kinetics, and the impact of a typical stress testing on their performance. For example, UV-filters are the key ingredients of cosmetic sunscreen formulations that absorb specific wavelengths of ultraviolet radiation [3]. Sunscreens should ideally protect from sunburn/erythema induced by UVB and from the genotoxic/oxidant effects of both UVA and UVB which contribute to skin photodamage. For a sunscreen's optimum performance and efficacy, the first and foremost requirement is that the UV-filters should remain effective during the entire period of exposure. The EPR signal of a stable radical (TEMPOL) was followed for 25 min of UV irradiation in the presence of different UV-filters showing their efficiency to reduce the radical (Figure 4). Clearly UV-filter D was the most effective one at quenching free radicals in the sunscreen formulation.

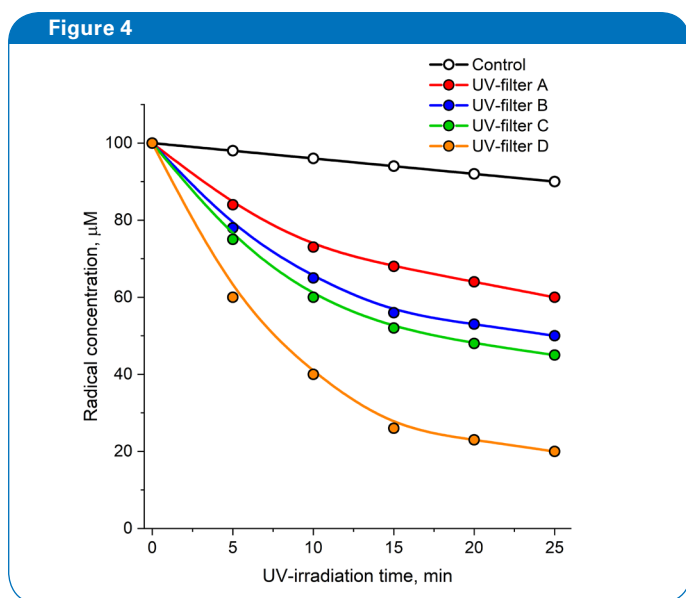


Figure 4. UV-filter efficacy determined by EPR. The ability of the UV-filters to quench free radicals increases in the following order: A < B < C < D.

## III. Polymer degradation

The ubiquity of free radicals, including biradicals, triplet states, and point defects in polymer systems, has made EPR spectroscopy an indispensable tool in polymer science among other mainstream analytical techniques. The scope of EPR

spectroscopy has broadened particularly due to the growing trend to produce polymers with enhanced stability, to follow the charge transfer process in conducting polymers, to decipher the complex nanoscale dynamics of polymers, to fathom out the reaction mechanisms and kinetics of complex polymer reactions in a more facile way, and to carry out comprehensive structural and conformational analyses. EPR spectroscopy is being applied in polymer science in numerous ways, including, but not limited to, exploring the structure, conformation, and dynamics of polymer chain segments, degradation or defect studies, charge transfer properties, kinetics and reaction mechanisms especially of free radical polymerization reactions, and EPR imaging of the polymer matrix.

For example, ultra-high molecular weight polyethylene (UHMWPE) is a semi-crystalline polymer that has been used for over four decades as a bearing surface in total joint replacements. It has been shown that UHMWPE is susceptible to oxidative free radical degradation following gamma radiation sterilization with subsequent loss of mechanical properties. While contemporary UHMWPE sterilization methods have been developed to reduce or eliminate the free radical degradation, post irradiation thermal treatment of the polymer is required to ensure the oxidative stability of joint implants in the long term. EPR studies [4] from Harvard Medical School showed that: (i) annealing below the polymer melting point preserves the mechanical properties but the residual free radicals trapped in the crystalline regions are not completely eliminated, leading to oxidation in the long-term and (ii) annealing above the melting point (150°C) eliminates the free radicals but leads to a decrease in mechanical properties through loss of crystallinity during the melting process (Figure 5).

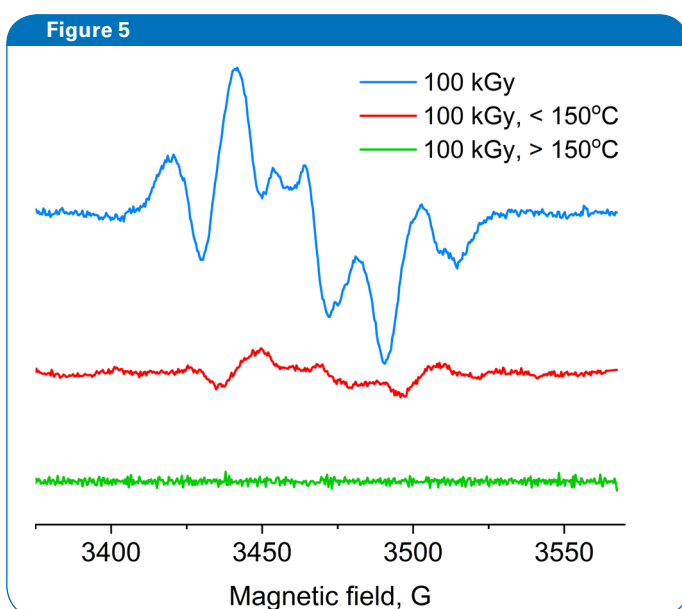


Figure 5. EPR spectra of 100 kGy irradiated, unmelted UHMWPE (blue trace), pressure treated (red trace), and melted at 150°C at ambient pressure (green trace) (adopted from Ref. [4]).

#### IV. Materials: defects and impurities

EPR spectroscopy is a suitable method to investigate paramagnetic defects and impurities in materials. In the past three decades photovoltaic (PV) modules have experienced an average price reduction of 20% for every doubling of the accumulated sales. The availability of sufficiently pure silicon or polymers (for organic PVs) has been an important limiting factor for rapid growth. Therefore, there is a strong need for low-cost technologies for production of silicon and polymer synthesis for PV applications. However, such low-cost production will most likely compromise the purity of the resultant silicon or polymer. It is crucial to have accurate specifications for concentrations of defects and impurities without risking the production yield and cost targets, and achieving shorter energy pay-back times.

Recent EPR studies on polycrystalline Si films revealed two types of defects – grain boundary and intra-grain defects distinguished by their g-values. The analysis of the asymmetric EPR spectra revealed the existence of intra-grain defects located at  $g = 2.0032$  (yellow trace in Figure 6) in addition to grain boundary defects at  $g = 2.0055$  (red trace in Figure 6). In addition, quantitative EPR analysis was applied to present the total spin concentration per  $\text{cm}^3$  as a function of the average grain size of poly-Si films in their crystallized state and after applying post-crystallization treatments (rapid thermal annealing and hydrogen passivation) [5].

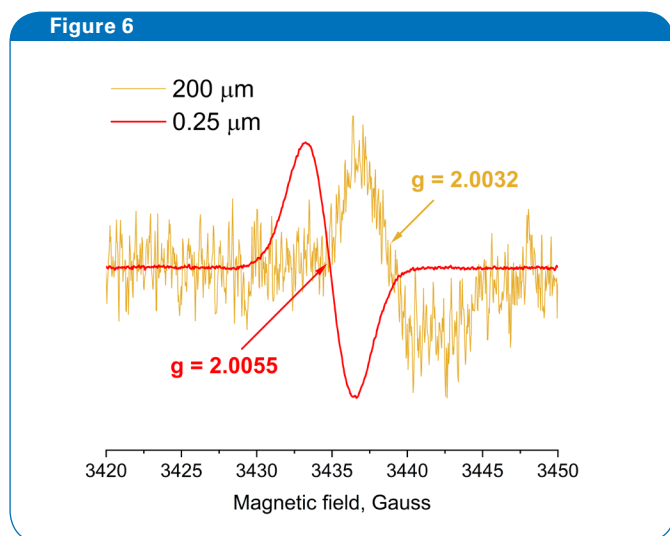


Figure 6. EPR spectra of polycrystalline Si film with 0.25  $\mu\text{m}$  and 200  $\mu\text{m}$  large grains (adopted from Ref. [5]).

#### V. Food degradation

Free radical reactions are very common in food and beverages and they play a major role in food degradation. However, there are also studies showing that free radicals generated during food processing may play key functional roles in texturization, flavor formation, and other reactions contributing to food properties and characteristic qualities. There-

fore, identifying and understanding reactions that are free radical-mediated in foods and beverages is crucial. Learning to control these reactions associated with food qualities and shelf-life stability give us sufficient reason to be interested in EPR detecting and studying radicals in foods and beverages.

An example of food degradation is rancidity of edible oils. It arises during storage or transport and is due to the free-radical mediated oxidation of unsaturated fatty acids that affects quality and determines, in many cases, the shelf-life of the oil. Fatty acid degradation occurs in three well-documented phases: (i) initiation, (ii) propagation, and (iii) termination. The oxidized compounds that result at the end of the process give the oil an undesirable odor and taste. With the help of EPR stress testing, oxidation profiles and accurate calculation of free radical concentrations provide a measure for oxidative resistance at each stage of the production process. This enables manufacturers to make rapid and informed process control decisions to optimize product shelf-life.

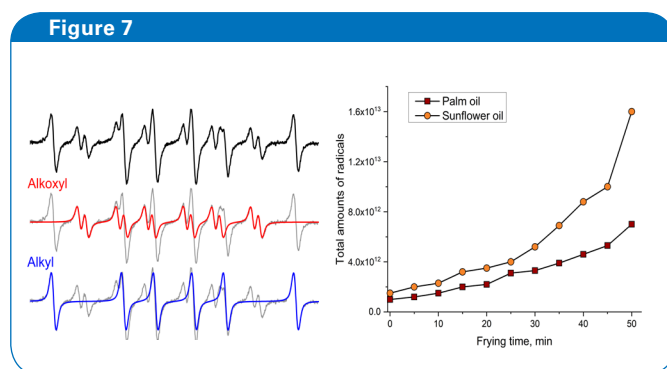


Figure 7. Left panel - EPR spectrum of frying oil heated at  $180^\circ\text{C}$ . Two radicals species were identified (alkoxy and alkyl radicals). Right panel – quantitative EPR data collected for palm and sunflower frying oils (adopted from Ref. [6]).

A recent EPR study was published on edible oil oxidation during the frying process and the impacts of oil with different fatty acid composition on free radicals are evaluated [6]. Two types of radicals (alkoxy and alkyl) are identified and quantified by using EPR (Figure 7). The results show an increase of formed free radicals in frying oils over time which is correlated to other physicochemical results and the authors conclude that EPR is a very sensitive method to detect oxidative changes in frying oil systems. It further shows that the amounts of free radicals detected by EPR in sunflower oil are higher than those in palm oil which correlates with the higher amounts of volatile compounds related to lipid thermal oxidation than those in palm oil.

Another work using EPR spectroscopy shows the impact of UV-irradiation and thermal treatment on starch samples [7]. The process of radical formation, studied by quantitative EPR, occurring to a larger extent in barley starch, confirms its lower stability compared to oat starch structure (Figure 8). The amount of EPR detected radicals correlates well with alterations of starch structures and their functional properties. EPR also reveals that starch degradation leads to three different radical species identified and assigned to C-centered polysaccharide radicals, depending on the treatment. It is concluded that depolymerization of starch chains is more significant in the case of thermal treatment, whereas UV irradiation is a very effective agent of oxidation. In practice, the identification and quantification of thermally- or UV-induced free radicals by EPR is considered as a predictive tool in starch structure alterations.

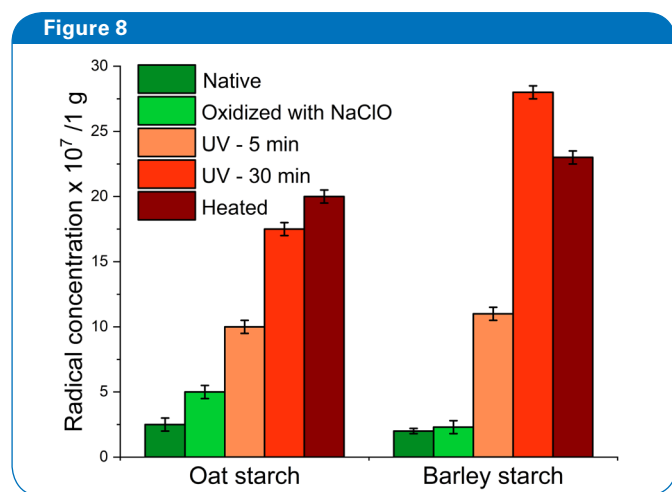


Figure 8. Radical concentration in barley and oat starches during chemical-, thermal-, and UV-degradation (adopted from Ref. [7]).

Food irradiation (the application of ionizing radiation to food) is a technology that improves the safety of foods by reducing or eliminating the health risk associated with food-borne pathogens such as Salmonella and to prolong shelf life (sprout inhibition, delay of ripening). In fact, ionizing radiation inhibits the division of microorganisms and creates so-called radiolytic products as well as free radicals. In a dry environment these radicals are relatively stable. The European Union has accepted the EPR as a standard method for the detection of irradiated food containing bone (EN 1786, 1996), cellulose (EN 1787, 2000) or granulated sugar (EN 13708, 2001). However, it has been estimated that the potential of EPR in this field is much broader. This EPR application is a useful and successful tool to evaluate the irradiation treatment of six different types of cheese [8]. It was shown that irradiation treatment (1 – 4 kGy) lead to formation of  $\alpha$ -carbonyl radicals of free amino acids, peptides or proteins and the EPR signal correlated with the absorbed dose, storage time, and the type of cheese (Figure 9).

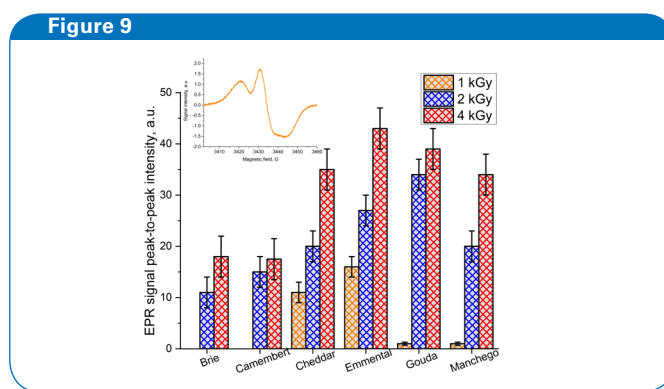


Figure 9. Accumulation of free radicals in cheese irradiated with E-beam at 1, 2, and 4 kGy (adopted from Ref. [8]).

## VI. Beverage degradation

It is well established that free radicals are reaction intermediates in the degradation processes of beer. EPR is one of the ASBC methods of analysis of oxidative resistance in beer and is based on a spin trapping experiment in which free radicals form covalently bound adducts with the spin trapping reagent (9). The accumulation of these adducts is detected by EPR during the forced oxidation period (150 min @60°C) and directly reflects the resistance (or lack of resistance) of the beer to oxidation. What happens is, under these conditions naturally occurring antioxidants (mostly polyphenols) in beer delay radical-induced oxidation causing a lag time. Lag time is the period it takes until all the antioxidants in the beer are consumed and radicals can be detected by EPR. This metric obtained from the assay is used to quantitatively assess a beer's oxidative resistance (Figure 10).

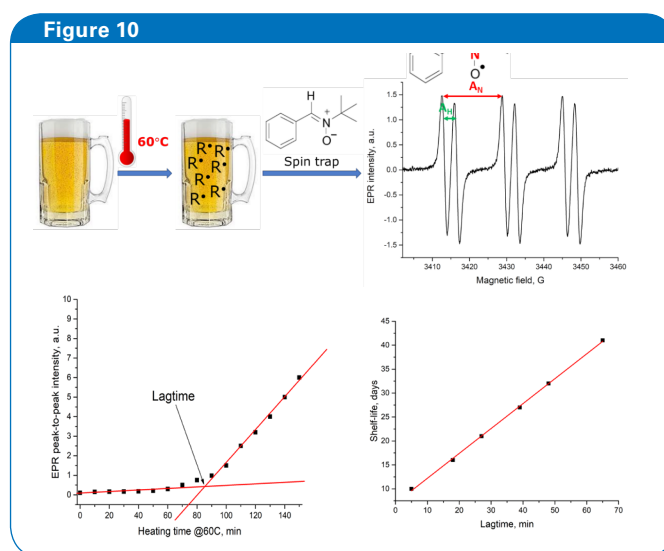


Figure 10. Upper panel: Example of an EPR spectrum of the PBN spin adduct obtained in beer. It was recorded after 150 min of aerobic forced aging at 60 °C. Lower panel: Example of a lag time calculation and its correlation with shelf-life.

## Conclusion

In this white paper, we have reviewed some of the important EPR applications used for the studies of pharmaceuticals, polymers, food and beverages, and other materials, by observing endogenous radicals and by initiation, detection, and identification of radicals via stress testing. The use of EPR for analysis of degradation levels in industry is

growing rapidly. New methodologies in initiation and detection of free radicals result in better understanding of mechanisms involved in degradation processes. The high sensitivity and versatility of EPR makes this technique a valuable tool in different industries and further applications are expected to emerge in the future.

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