EPR Lab

Faculty of Physical Chemistry
University of Belgrade

BioScope Labs

www.bioscope.ffh.bg.ac.rs

EPR Lab

- **EPR Laboratory** is an integral part of the **Center for Physical Chemistry of Biological Systems and BioScope Labs Consortium**, located at the **Faculty of Physical Chemistry, University of Belgrade**. The Center represents the scientific and research team of experts in various aspects of up-to-date problems in the field of physical chemistry of biological systems.
- The Center aims to educate young researchers through master, doctoral and post-doctoral programs in the field of biophysical chemistry at the Faculty of Physical Chemistry, University of Belgrade.

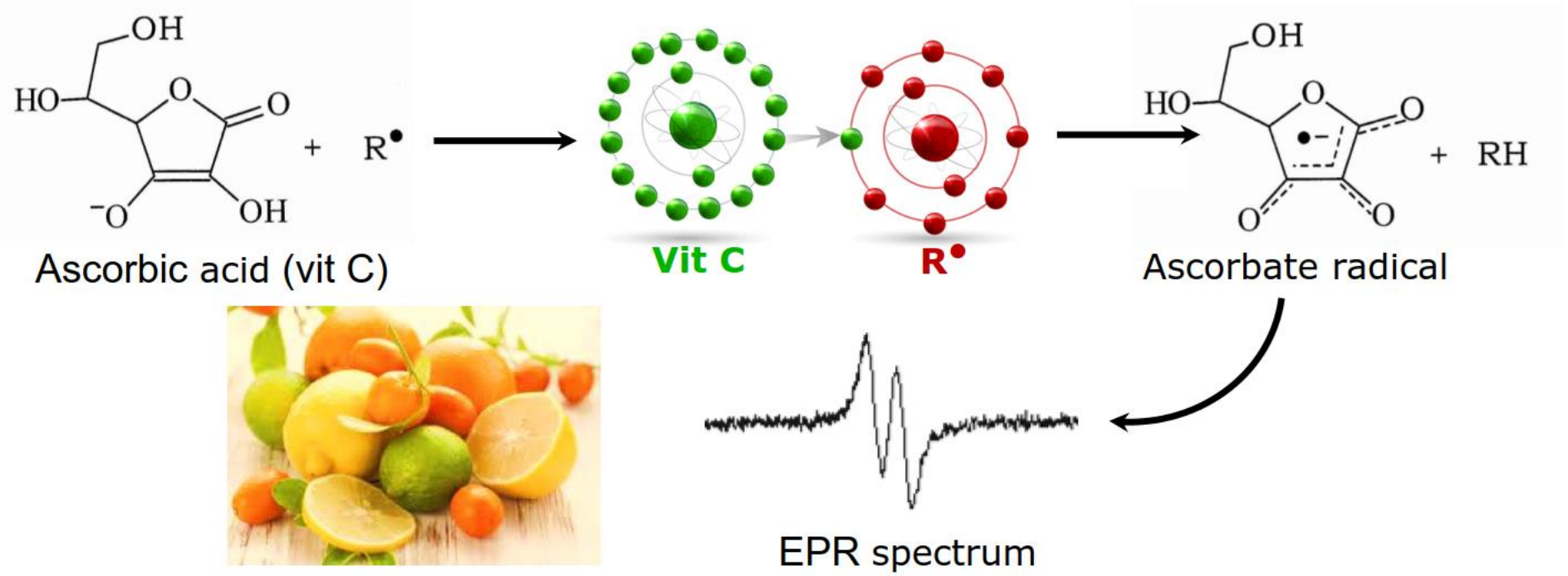
Equipment:

Bruker Biospin Elexsys II E540EPR spectrometer

- Two microwave bridges for X- (9.5GHz) and L-band (1.2GHz).
- Two resonators for X-band and three resonators for L-band.
- Accessories for measuring at liquid N_2 (100-350K) and liquid He (4-100K).
- Full EPR spectroscopy and EPR imaging capabilities.

What is EPR?

- EPR is a magnetic resonance technique that detects unpaired electrons in paramagnetic substances.
- Unpaired electrons occur in free radicals and many transition metals.
- EPR is the only technique that unambiguously detects free radicals.

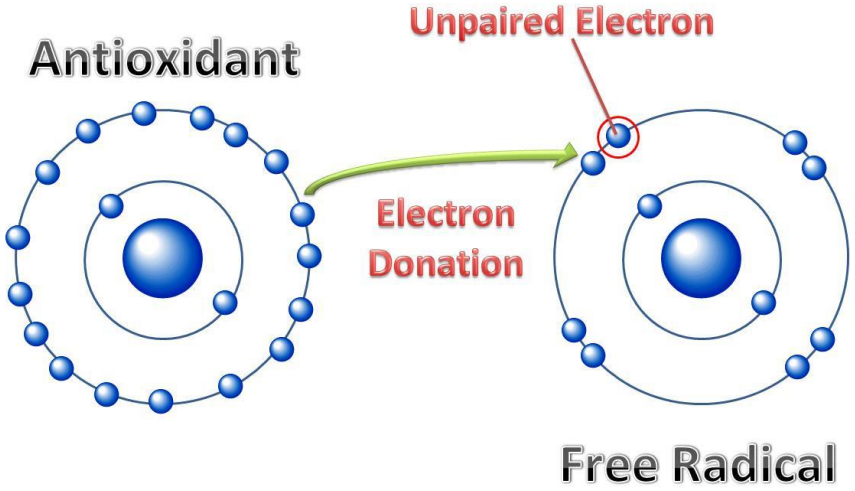


EPR Lab

Free radicals and antioxidants

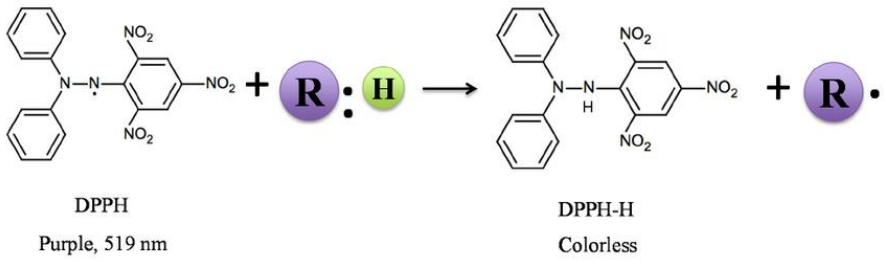
- Free radicals (e.g. hydroxyl radical, superoxide, etc.) are notorious reactive species, which are generated in all biological systems.
- Radicals can provoke damage to DNA, proteins, and cell-membranes, and are related to many pathophysiological conditions.
- To fight increased production of free radicals and to deal with radical-related diseases, efficient antioxidants ought to be developed.

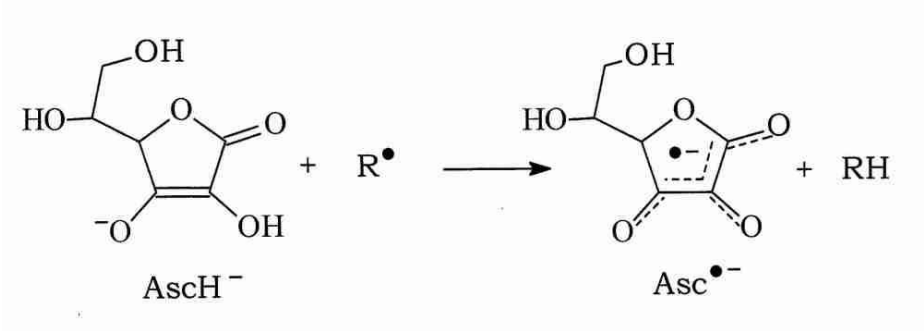
- Antioxidants are molecules capable of slowing or preventing the oxidation of other molecules, by removing or dismutating free radicals.
- Antioxidants can be found in plants, traditional medicaments, or can be chemically synthesized compounds.

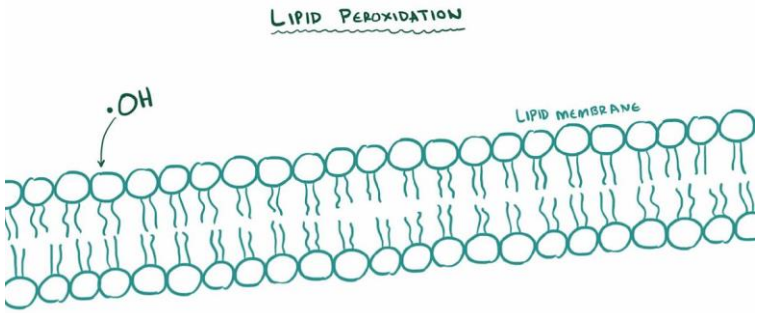


EPR methods

Determining antioxidative capacity







- EPR spectroscopy could be used to detect, quantify production and removal of various free radical species by antioxidants.
- Current investigations are mainly based on using different types of biological-irrelevant organic radicals (like DPPH) to evaluate antioxidative capacity of different antioxidants.
- Our investigations are focused to:

1. Determine antioxidative capacity of antioxidants to remove **biological-relevant ROS like $^{\bullet}OH$, $^{-}O_2^{\bullet}$, $NO^{\bullet}$, $Asc^{\bullet}$ $ONOO^{-}$** etc. (generated by well-known radical-generating systems).
2. Determine antioxidative capacity of antioxidants to remove commercially available long-lived radicals.
3. Determine the ability of antioxidants to prevent lipid peroxidation induced by ROS and RNS (using liposomes as artificially constructed membranes).

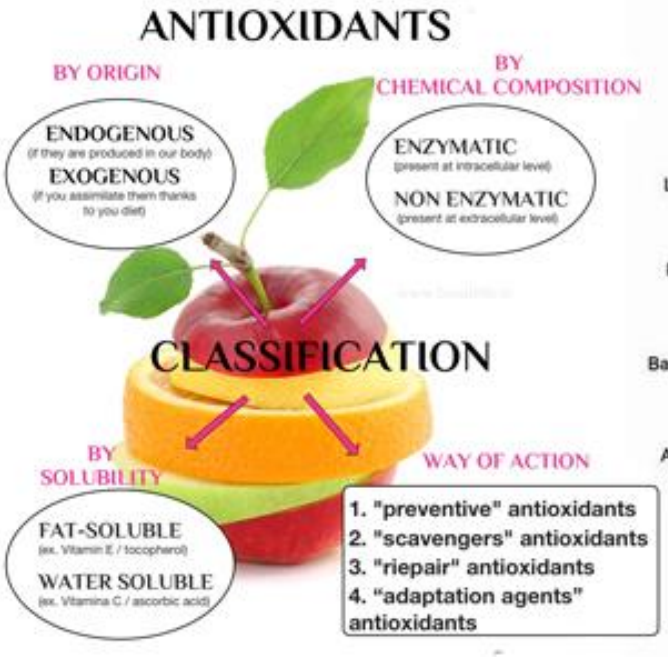
EPR methods

Determining antioxidative activity for water insoluble antioxidants

What about water-insoluble antioxidants?

- Many most effective antioxidants are water-insoluble and determining their antioxidative capacity towards ROS and RNS is a difficult task.

- Using EPR, we are able to determine antioxidative capacity of any compound, including ones which are insoluble in water.
- This method is exclusively developed by **EPR Lab** as a part of **BioScope Labs Consortium**.

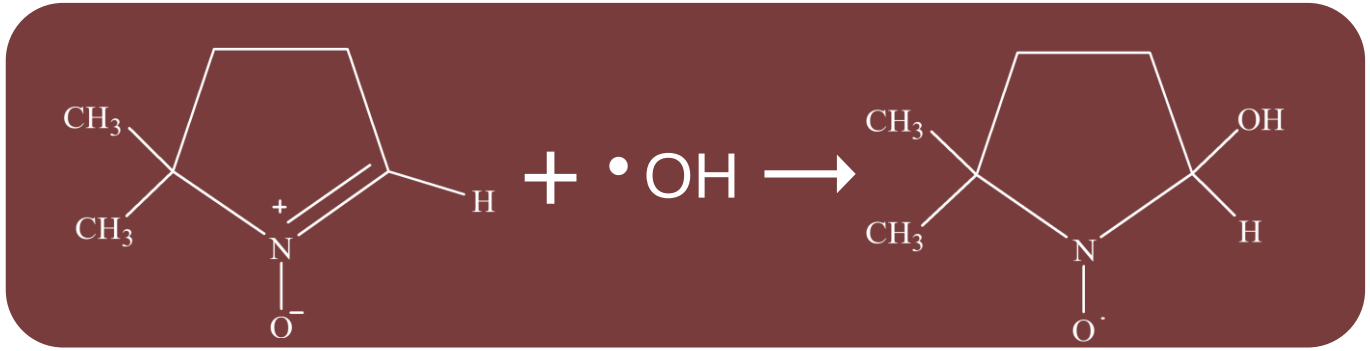


EPR methods

Determining antioxidative activity towards short-lived radicals

EPR spin-trapping

- Oxidative stress induced by the production of short-lived radical species contributes to numerous pathophysiological conditions such as development of cancer, neurodegenerative, and cardiovascular diseases.
- A variety of measurements of oxidative stress markers in biological systems have been developed; however, many of these methods are not specific and can produce artefacts.
- EPR is a unique tool that allows measurements of short-lived free radicals like $^{\cdot}OH$, $^{-}O_2^{\cdot}$, $NO^{\cdot}$ etc.
- The method developed for this purpose is known as EPR spin-trapping (see picture below).
- Using selected spin-traps we are able to detect production of short-lived radicals, as well as to determine antioxidative activity of selected antioxidants towards short-lived ROS or RNS in extra- or intra-cellular space.



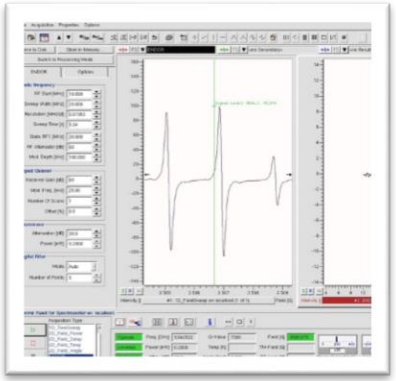
Spin trap | Short-lived radical | Long-lived spin adduct

EPR methods

Determining antioxidative activity *in vivo*

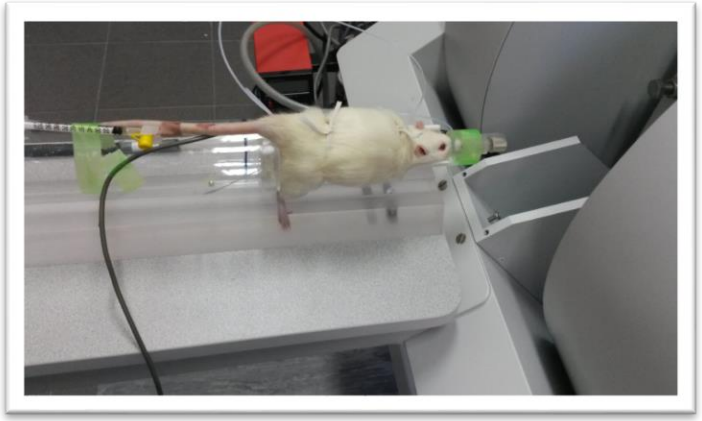
What about *in vivo* experiments?

- Experiments towards determination of antioxidative activity of different compounds (and the visualisation of their performance) could also be executed *in vivo* using EPR spectroscopy and imaging.

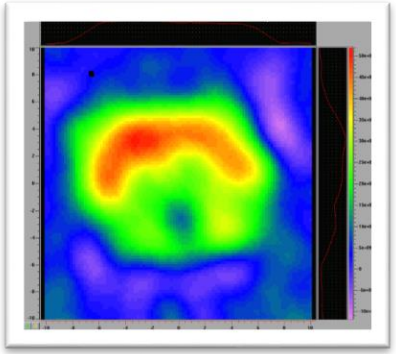


Free radicals *in vivo* EPR spectroscopy

- Using L-band EPR spectroscopy and imaging, we are capable to test whether selected antioxidants also work in *in vivo conditions.*
- All experiments including animals (mice and rats) are performed in accordance with the "Animal testing regulation laws".



In vivo EPR measurements



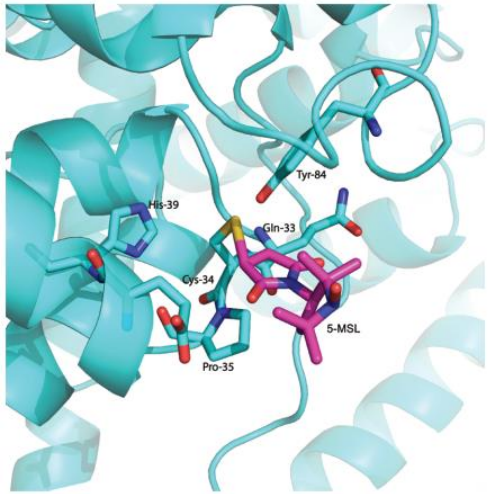
Free radicals *in vivo* EPR imaging

EPR methods

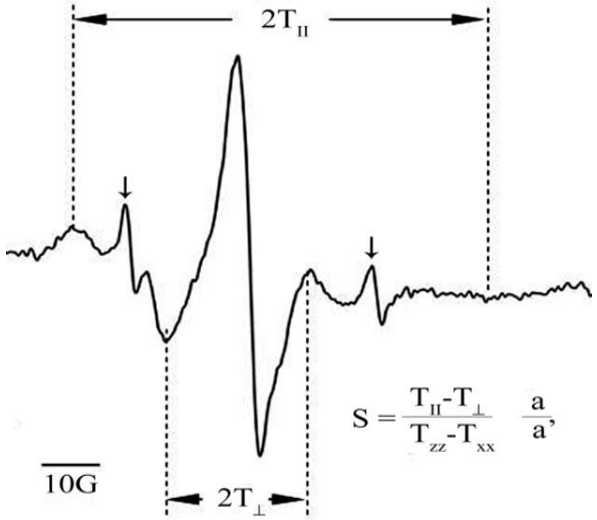
Spin-labelling

EPR spin-labelling of membranes and proteins

- The method in which specially designed EPR active molecules called spin-labels are used.
- These molecules are used to label the membrane (plant, animal or liposome) in order to evaluate the properties of the membrane (e.g. fluidity).
- This method could reveal the existence of the process of lipid peroxidation by different ROS.
- Spin labelling method could also be used for labelling proteins and evaluation of their conformational changes.



Spin-label 5-MSL covalently bound to Cys-34 of BSA



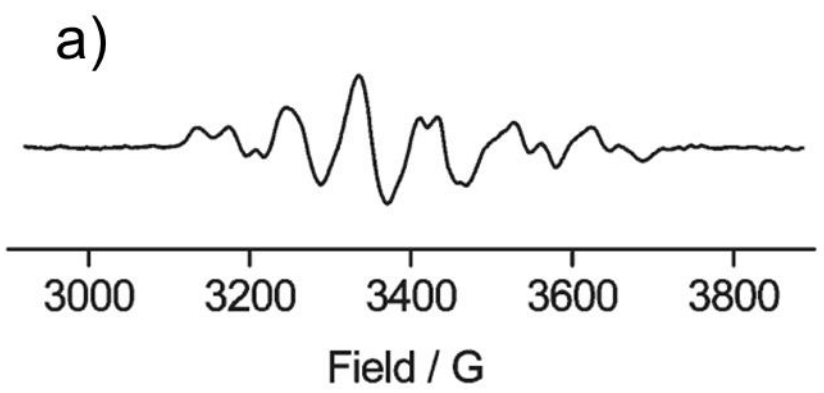
The EPR spectrum of 5-MSL bound to BSA

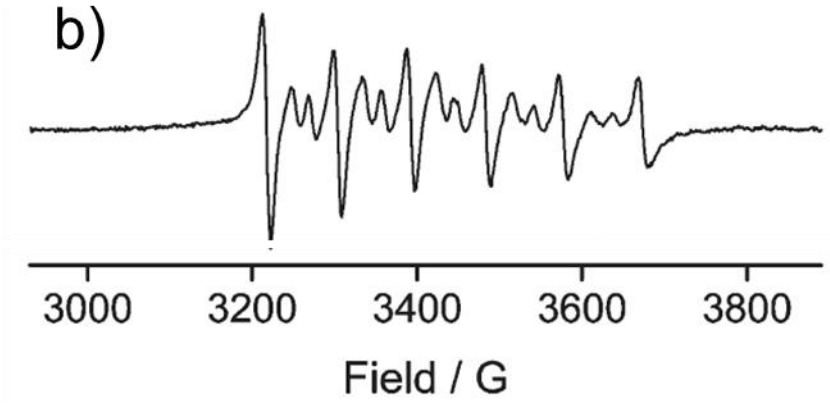
EPR methods

EPR or metalloproteins and protein radicals

Low temperature studies of metalloproteins and protein radicals

- These types of EPR measurements are performed at low temperatures, 77K (liq.N_2), and 4K (liq.He).
- We can identify metal-coordination features of metalloproteins that contain transition metal ions (e.g. V, Cr, Mn, Fe, Ni, Cu), metal oxidation states, and types of ligands.
- We can detect and quantify thiyl and tyrosyl radicals in different enzymes.
- Image on the left shows 20K EPR spectra of a) the Mn(III)Fe(III) dimetal center in the R2 subunit of *Chlamydia trachomatis*ribonucleotide reductase (RNR), and b) octahedrally bound Mn(II) in reconstituted mouse Y177F R2 RNR mutant protein.



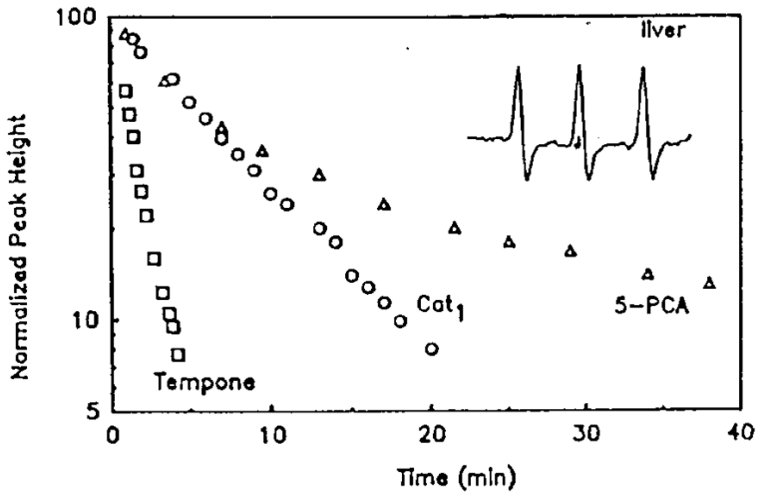


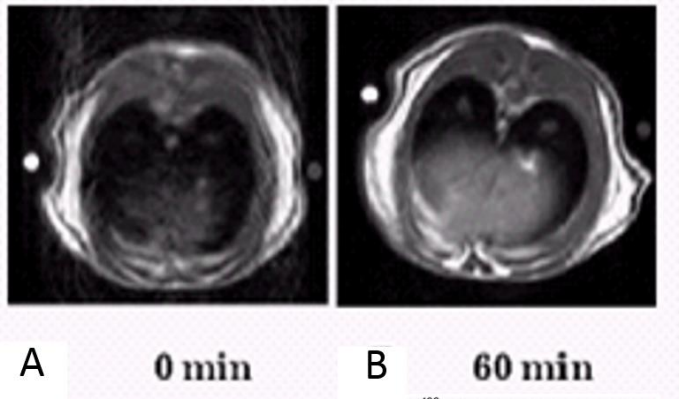
EPR methods

In vivo spectroscopy of small animals for detection of NO and ROS

Investigating the pharmacokinetics of nitroxides

- These experiments include investigating the pharmacokinetics of nitroxides measured using L-band EPR resonators in which small animals (e.g. mice or rats) are placed.
- EPR signal diminishes due to the clearance but also due to the reduction by endogenous scavengers.
- This way we can monitor BBB permeability (e.g. BBB damage in neurodegenerative diseases) or the total antioxidative capacity of target tissues or organs.
- The paramagnetic properties of nitroxides (or trapped radicals) could simultaneously serve as MRI contrast agents.



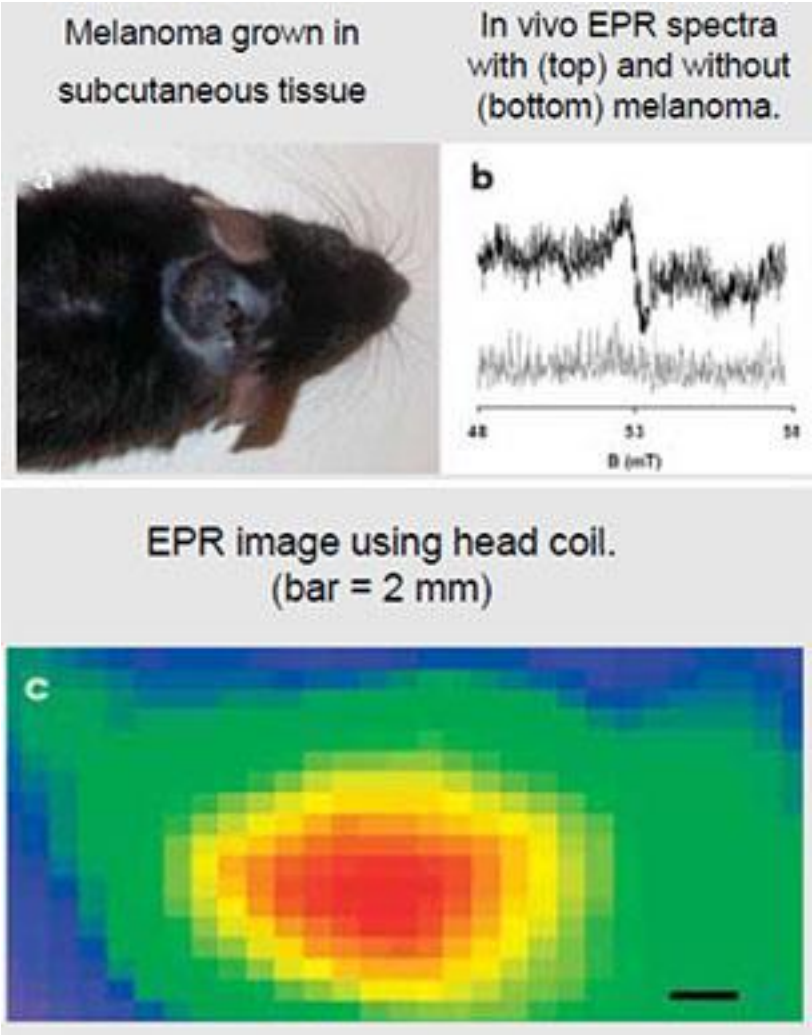


EPR methods

EPR spectroscopy and imaging – topical applications

Topical applications - skin

- Directly monitor the effect of drugs on skin (by detecting drug induced radical formation under pertinent therapeutic conditions).
- Explore the effect of UV light on skin (UV light presents potent oxidative stress in the skin).
- Monitor topical applications of liposomes as delivery system of hydrophilic substances through the skin (nitroxides as surrogate drugs).
- Early detection skin malignant melanoma at initial stage of development.
- Monitor controlled release from implantable devices – nitroxides as surrogate drugs.



Pharmaceutical applications of EPR

Why detecting free radicals and transition metals in drug products?

- There are at least 5 areas of interest where EPR spectroscopy is beneficial:

I. Detecting and evaluating degradation
- Photo
- Thermal
- Chemical

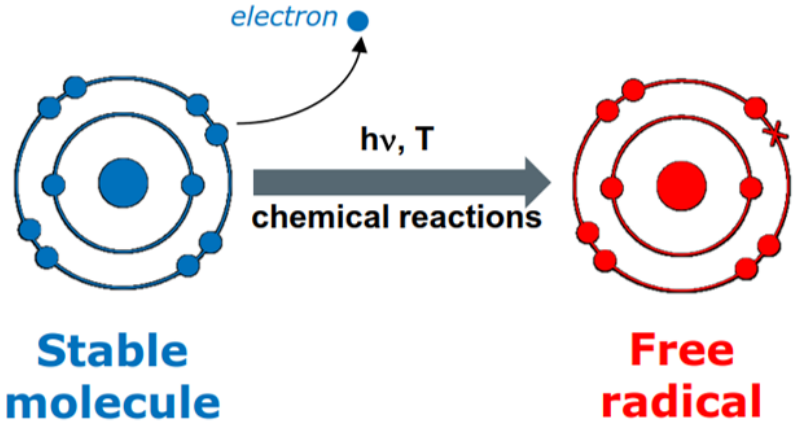
II. Optimizing stability & shelf-life
- Drug stability
- Antioxidant
- Effectiveness

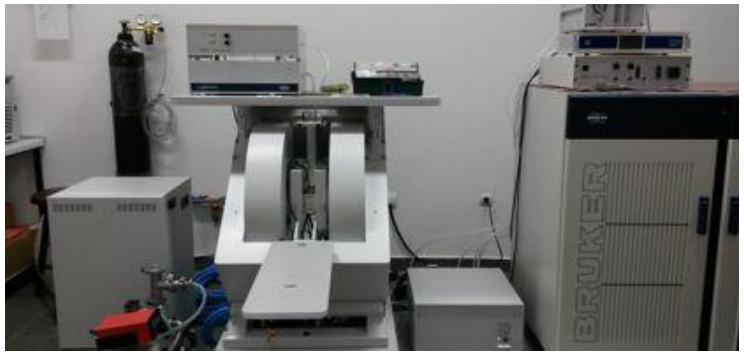
III. Reaction monitoring
- Yield optimization
- Green chemistry
- Chemical

IV. Sterilization processes
- Irradiation
- Thermal

V. Paramagnetic impurity profiling
- Trace elements
- Toxic by-products

Free radicals & Transition metals

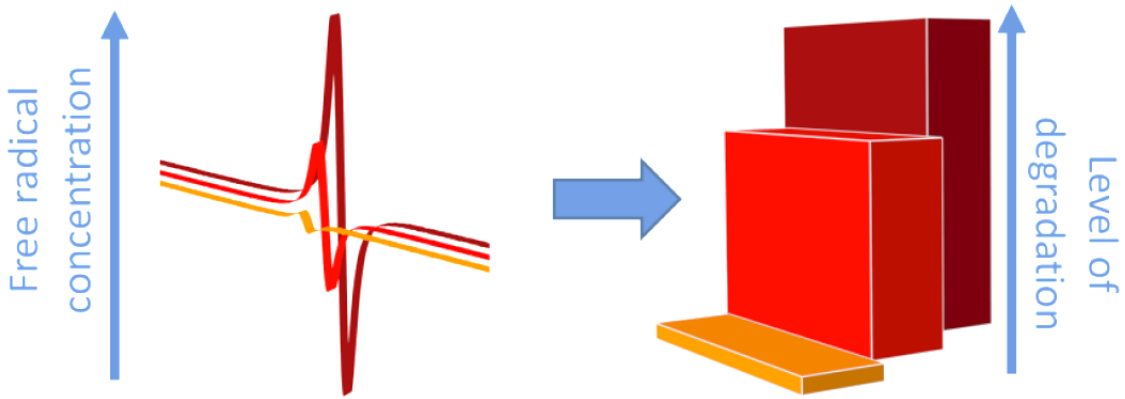




Pharmaceutical applications of EPR

I. Detecting and evaluating degradation

- Factors that compromise pharmaceutical product stability:
  - Heat
  - Light (UV, Vis)
  - Oxygen
  - Moisture
  - Sterilization processes
  - Impurities
  - Excipient interactions
- All these factors may cause degradation of APIs, excipients, or formulations
- Degradation processes quite often involves free radicals and transition metals
- Degradation correlates with the EPR signal:

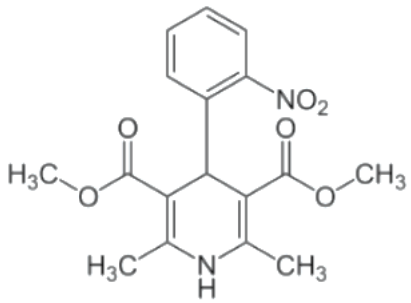


API (Active Pharmaceutical Ingredient)

Pharmaceutical applications of EPR

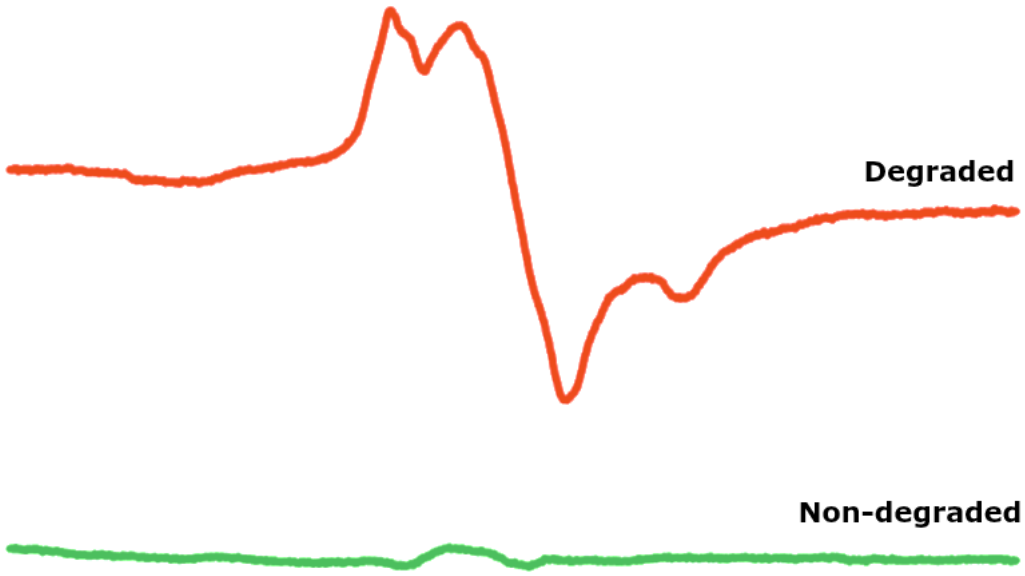
I. Detecting and evaluating degradation

- **How we can help you?**
  - By determining the root cause of degradation in drug products
  - By measuring the extent of degradation of APIs, excipients, and formulations
  - By predicting long-term stability characteristics of the APIs, excipients, and formulations



Nifedipine

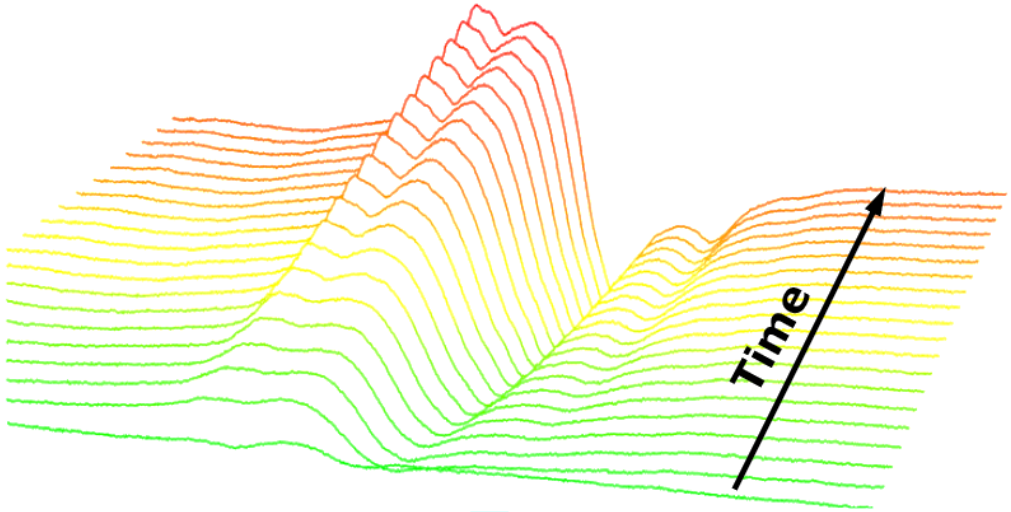
Photodegradation of Nifedipine after exposure to light shows the formation of N-based free radical. The amount of free radicals corresponds to the level of degradation

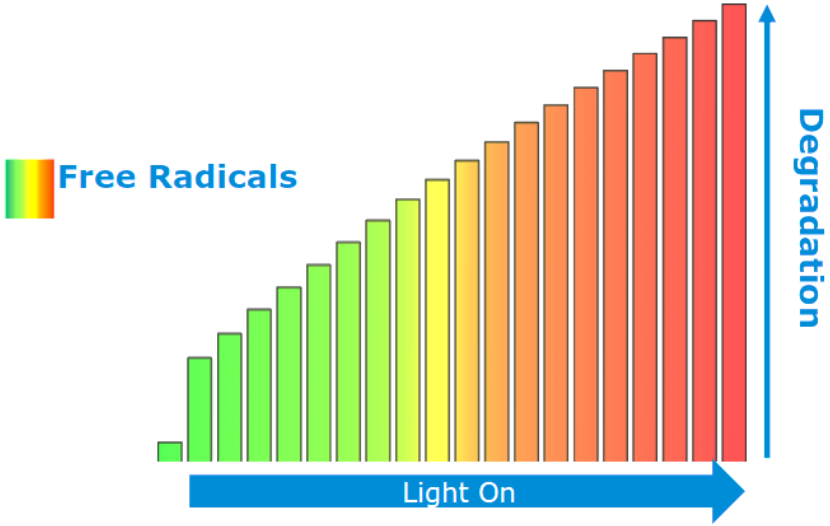


Pharmaceutical applications of EPR

I. Detecting and evaluating degradation

- The time evolution of the N-based radical can be followed by EPR:





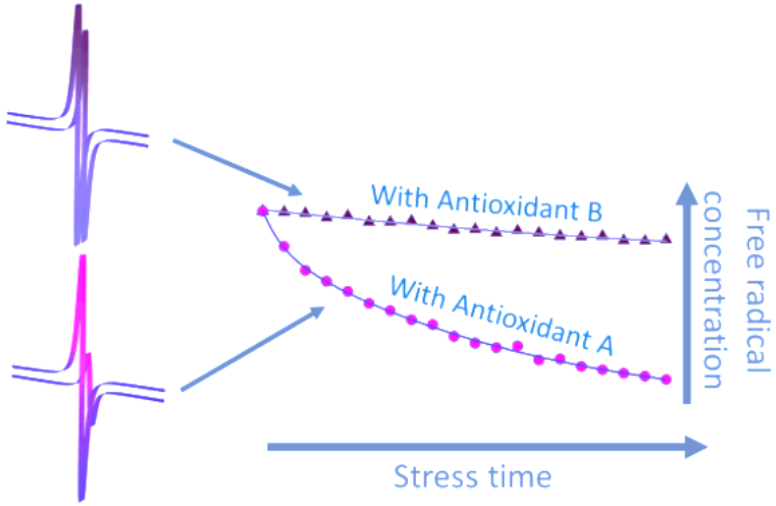
- The increasing amount of radicals shows the level of API degradation and can be used to predict stability of the product.

Williams H.E. and Claybourn M. (AstraZeneca), Predicting the photostability characteristics of active pharmaceutical ingredients using electron paramagnetic resonance spectroscopy, *Drug Dev. Ind. Pharm.* **(2012)** 38(2) 200

Pharmaceutical applications of EPR

II. Optimizing stability and shelf-life

- Stress testing is a form of forced oxidation/degradation and is used to predict drug stability
- In stress testing the drug product is exposed to heat, light or chemical agents
- Some of the goals achieved by stress testing are:
  - Understanding degradation pathways
  - Determining the intrinsic stability and shelf-life
  - Developing stable formulations
  - Evaluating antioxidant efficiency



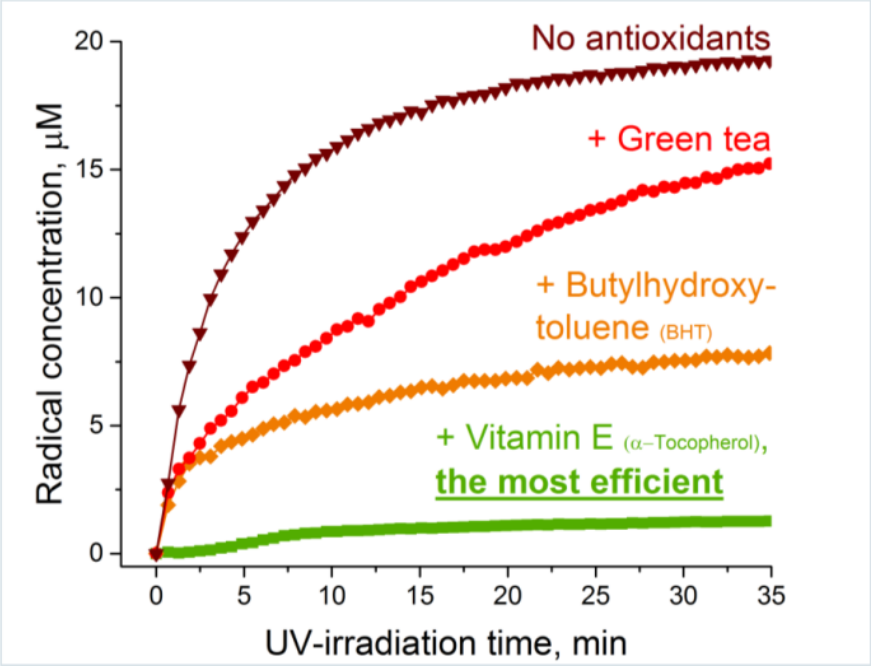
Antioxidant A is more effective than antioxidant B at quenching the free radicals in the drug formulation

Pharmaceutical applications of EPR

II. Optimizing stability and shelf-life

- **How we can help you?**

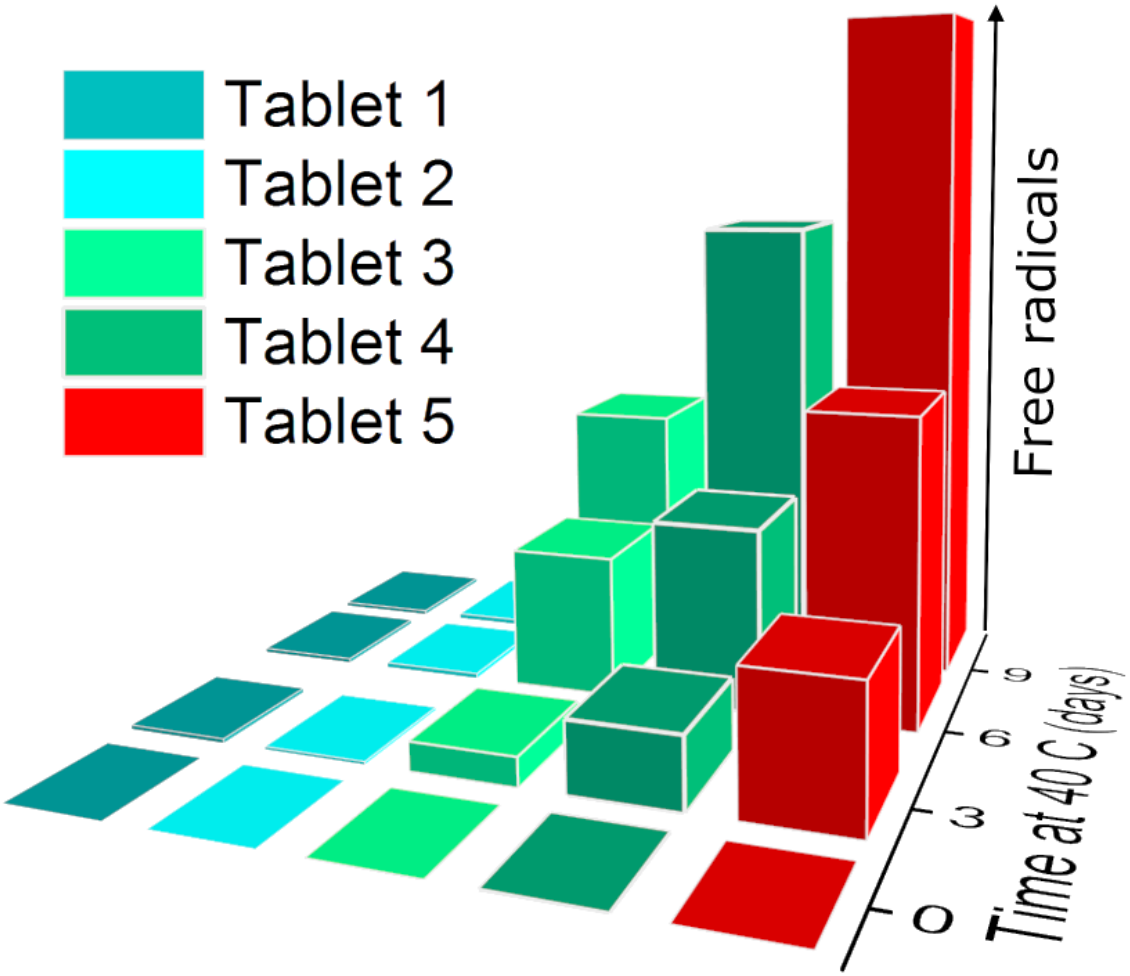
  - By monitoring processes that produce and involve free radicals which predicts long-term stability (photo-, thermo-, chemical-) of drug products
  - By using minimal sample quantities in early development phase of new APIs
  - By determining the antioxidant efficiency to quench free radicals with well established assays



Evaluation of antioxidants' effect on a skin care product during UV-irradiation shows vitamin E to be the most efficient antioxidant.

Pharmaceutical applications of EPR

II. Optimizing stability and shelf-life



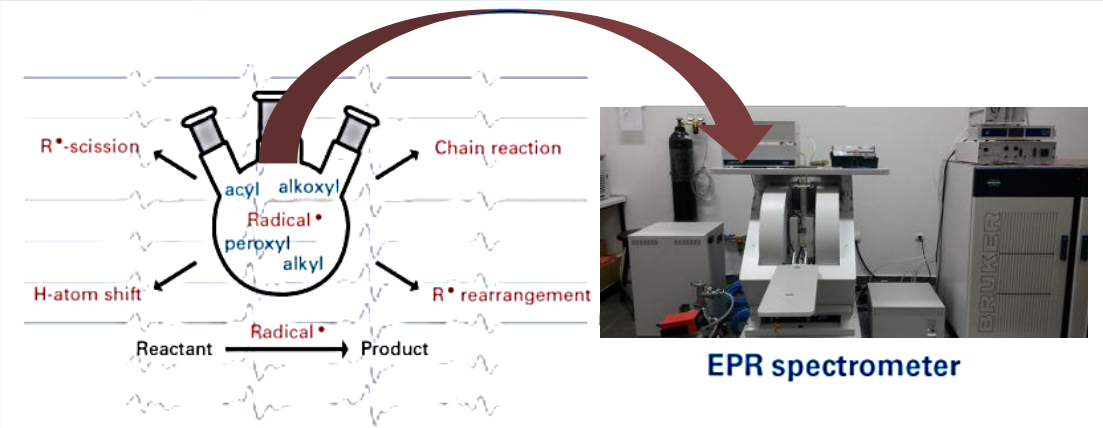
Case study: Shelf-life

- Five tablet formulations stress tested at 40 °C over a time course of 10 days
- Increasing free radical formation detected in tablets 3-5 indicates a reduced shelf-life
- Tablets 1 & 2 with low radical formation have better potential for drug development

Pharmaceutical applications of EPR

III. Reaction monitoring

- Reaction monitoring is critical for process understanding, optimization and scaling up
- Understanding reaction mechanism leads to cost saving and ensuring the quality of the final product
- Kinetic information and models allows one to predict conditions, enabling effective process optimization, risk assessment and control
- Chemistries involving radicals and transition metals are integral components of maximizing product yield and minimizing the environmental footprint



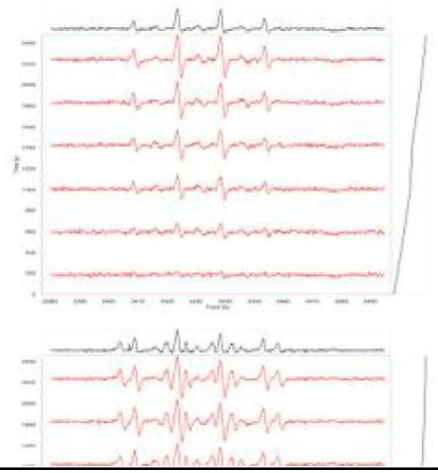
Pharmaceutical applications of EPR

III. Reaction monitoring

- **How we can help you?**

Hydroxyl Radical Generation by Ultrasonic Irradiation

As an example of closed loop reaction analysis, the radical generation initiated by ultrasonic irradiation was analyzed. When hydrogen peroxide (H_2O_2) is added to water and ultrasonic irradiation is performed, OH radicals are generated. These OH-radicals can be observed by using the spin trapping reagent DMPO (Fig. 4 upper). When methanol is added, both OH- and carbon centered radials are generated from the water and methanol (Fig. 4 lower). The radical identities were determined by SpinFit and their concentration by SpinCount. The kinetics are analyzed by the Xenon software.



- By identifying reaction intermediates (free radicals and transition metals) to obtain mechanistic information
- By answering key chemical questions: reaction yield and reaction kinetics
- By straightforwardly generating data to build kinetics models
- By quantifying free radicals and metals over the course of the reaction

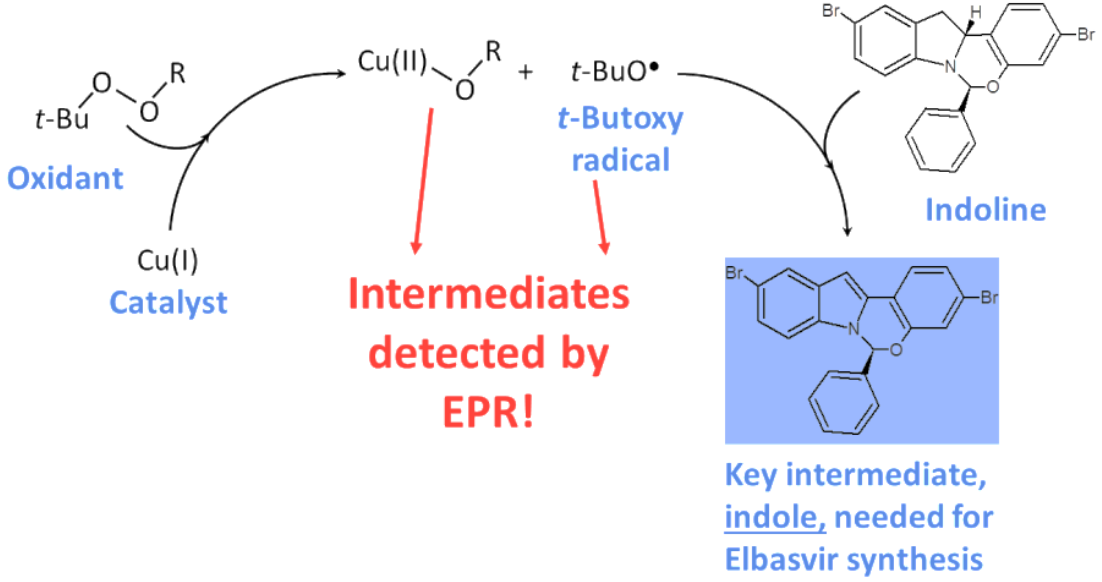
Mangion I. et. al. (Merck), Using electron paramagnetic resonance spectroscopy to facilitate problem solving in pharmaceutical research and development. *J. Org. Chem.* **(2016)** 81 6937

Pharmaceutical applications of EPR

III. Reaction monitoring

An example from Merck:

- An indole intermediate is a synthetic challenge in the production of a new Hepatitis C drug (Elbasvir)
- A proposed mechanism suggests that the catalyst Cu(I) is oxidized to form Cu(II) and tert-butoxy radical
- A novel green chemistry synthesis with high efficiency (92% indole yield) is accomplished



Simplified proposed mechanism of indoline oxidation

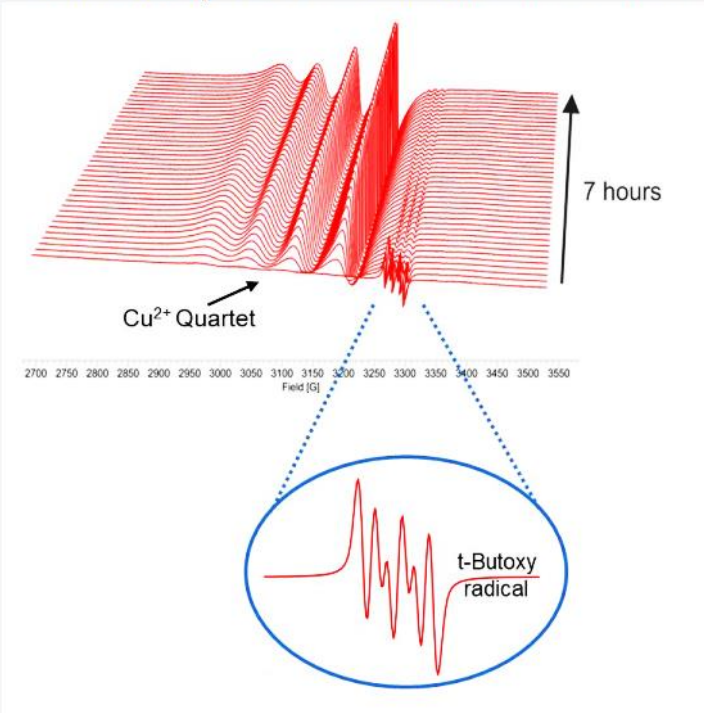
Peng F. et. al. (Merck), A mild Cu(I)-catalyzed oxidative aromatization of indolines to indoles, *J. Org. Chem.* **(2016)** 81 10009

Pharmaceutical applications of EPR

III. Reaction monitoring

An example from Merck:

- Monitoring the reaction confirms oxidation of the EPR silent Cu(I) to the EPR active Cu(II)
- Cu(II) signal reaches a plateau after ~ 3 hours indicating completion of the reaction
- t-Butoxy radical is detected as well by EPR using a spin trap
- Quantitative EPR analysis of both intermediates provides information about the synthesis efficiency



Peng F. et. al. (Merck), A mild Cu(I)-catalyzed oxidative aromatization of indolines to indoles, *J. Org. Chem.* **(2016)** 81 10009

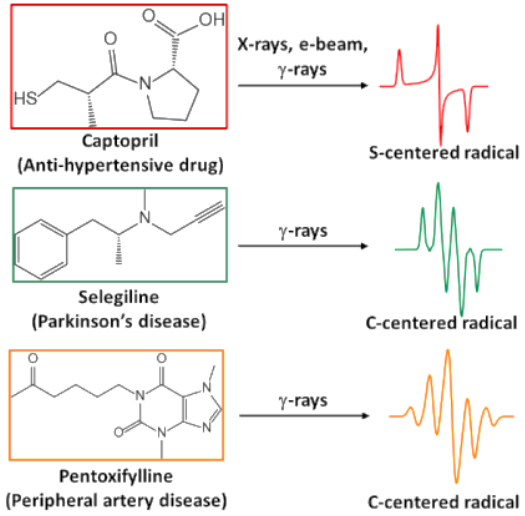
Pharmaceutical applications of EPR

IV. Sterilization processes

- Proper sterilization of APIs, excipients, final drug formulations, lab equipment, and medical devices is important for pharma manufacturers

- However, sterilization processes can generate radicals that:
  - Are responsible for degradation of the irradiated materials
  - Cause alteration of the physico-chemical properties of the sterilized product
  - Decrease drug potency by partial decomposition during sterilization
  - May be a toxicological hazard

Examples:



- Gamma-irradiation of drugs in the solid-state (Captopril, Selegiline, Pentoxifylline) induces S-or C-centered free radicals.

- Identifying the structure of radicals provides a better understanding of the mechanism of radiolysis.

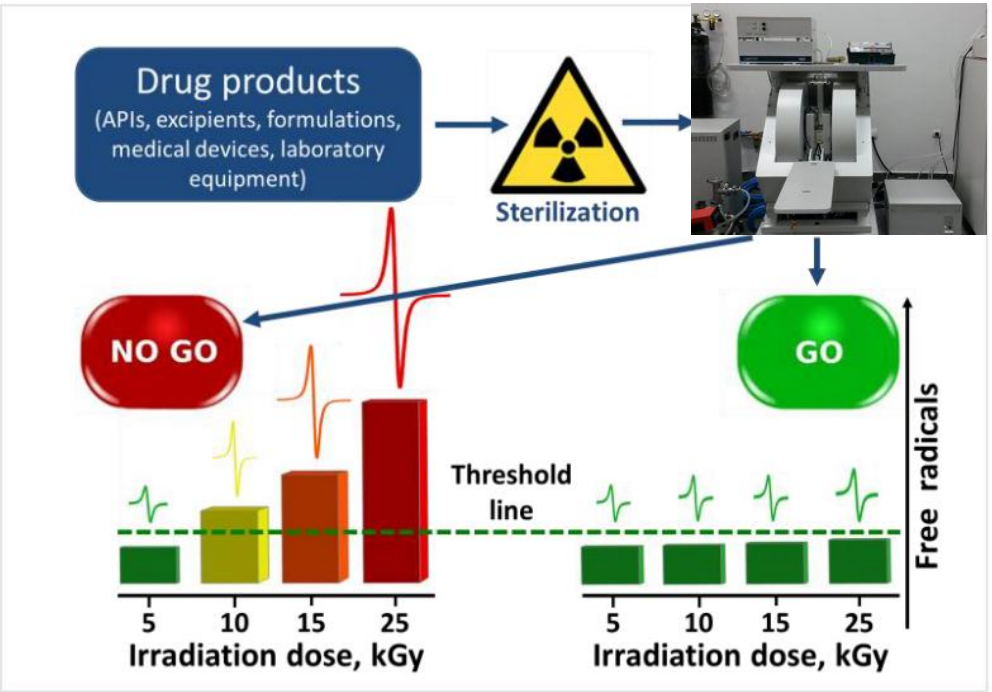
- Quantification of radical amount enables one to establish a threshold for the radiosterilization of these drugs.

Engalytcheff A. et al., *Radiat. Res.* **(2004)** 162 616
Köseoĝlu R. et al., *Appl. Radiat. Isot.* **(2003)** 58 63

Pharmaceutical applications of EPR

IV. Sterilization processes

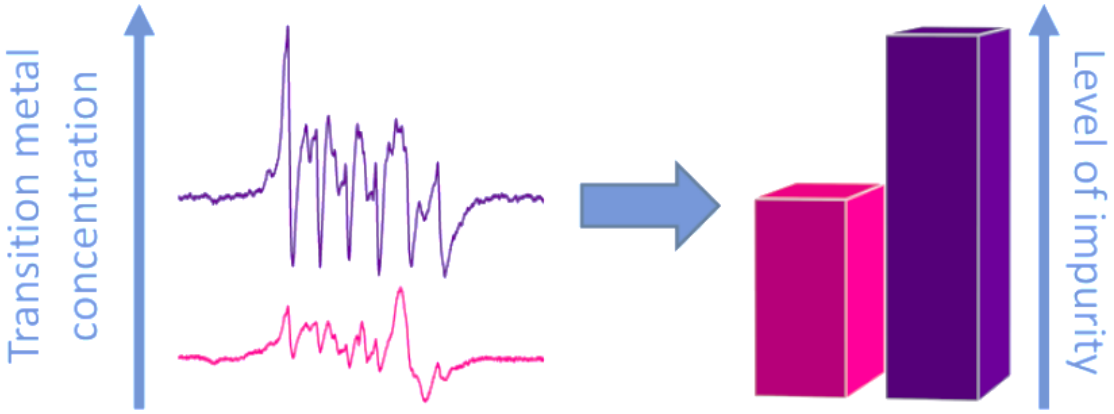
- **How we can help you?**
  - By determining stability of drug products after sterilization
  - By characterizing free radicals and identifying their source
  - By providing easy 'go/no go' decisions based on the quantification of free radicals for quality control and assurance



Pharmaceutical applications of EPR

V. Paramagnetic impurity profiling

- Drug impurities can arise from either the APIs or excipients, or both
- They can be also introduced to the the drug product during formulation processes, packaging, and storage
- Impurities have many unwanted effects such as:
  - Decreasing the therapeutic effect
  - Lowering the product shelf-life
  - Inducing toxicity

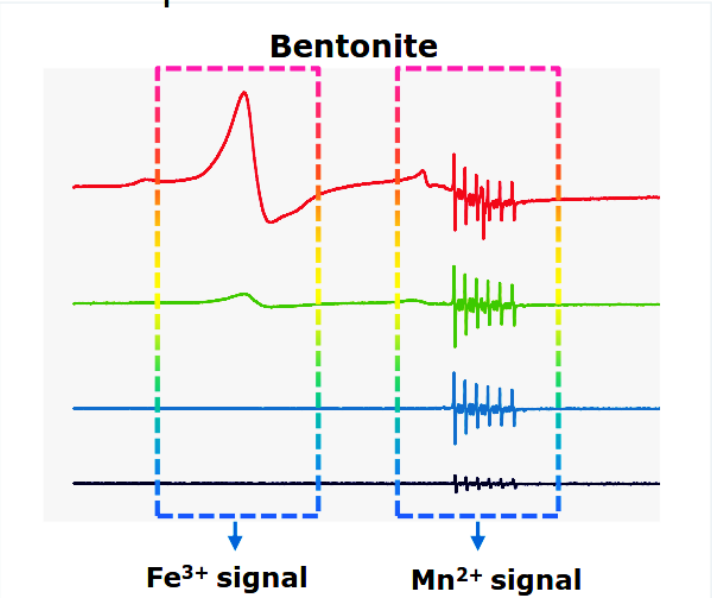


Metal concentration correlates with the EPR signal

Pharmaceutical applications of EPR

V. Paramagnetic impurity profiling

- **How we can help you?**
  - By detecting and identifying traces of transition metals
  - By monitoring drug degradation processes that produce and involve free radicals
  - By observing the production of free radicals catalyzed by transition metals or other impurities



Trace analysis: Impurity identification and control

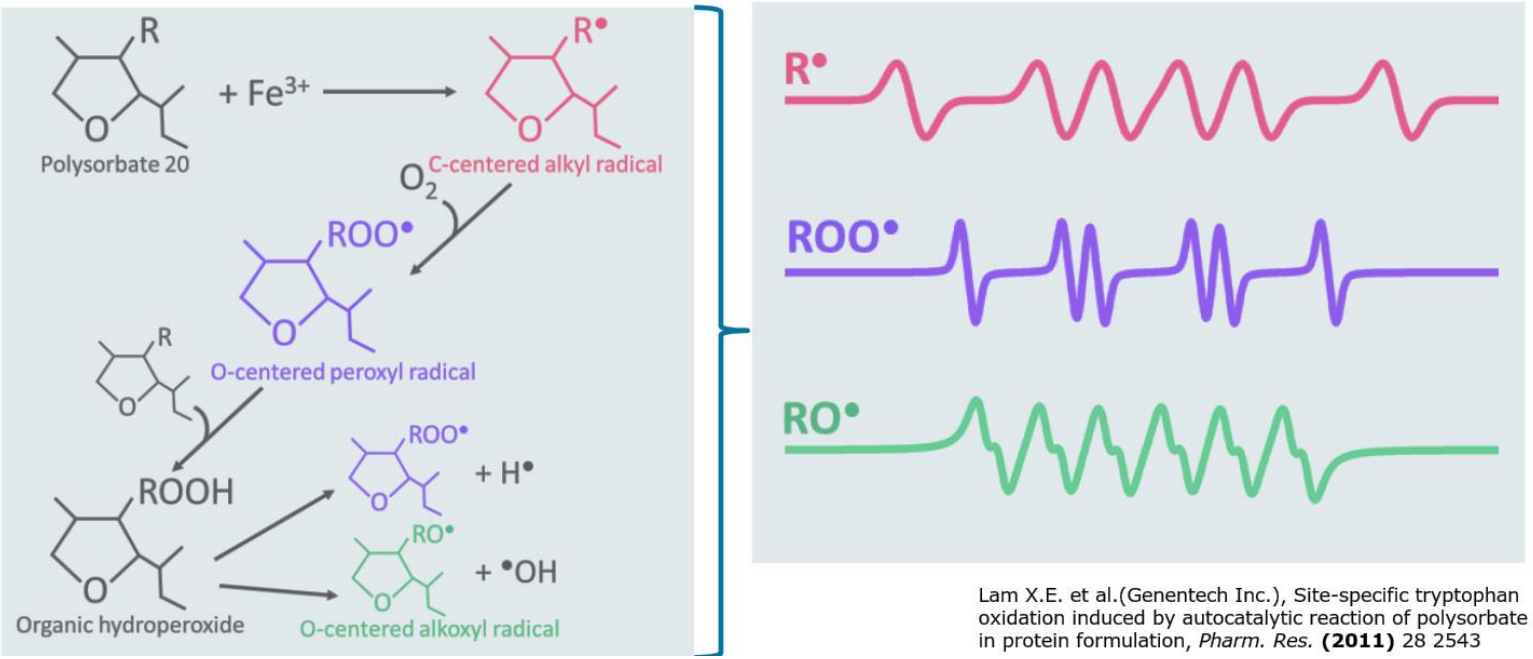
- Manganese (Mn^{2+}) and iron (Fe^{3+}) are present at trace levels in the excipient bentonite, commonly used as a filler in tablets.
- With the EMXnano impurity concentrations can be determined.
- Increasing amounts of metals accelerate the degradation of APIs and excipients.

Pharmaceutical applications of EPR

V. Paramagnetic impurity profiling

Case study: Tween 20 autoxidation

- Polysorbate 20 used in drug formulation as a stabilizer undergoes autoxidation.
- Autoxidation is catalysed by transition metals and results in side-chain cleavage and free radical formation.
- With EPR, we can detect, identify and quantify the free radical impurities.



Lam X.E. et al.(Genentech Inc.), Site-specific tryptophan oxidation induced by autocatalytic reaction of polysorbate 20 in protein formulation, *Pharm. Res.* **(2011)** 28 2543

EPR Lab

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