“BABEŞ-BOLYAI” UNIVERSITY CLUJ-NAPOCA

Faculty of Physics

Laura Bolojan

Characterization of free radicals in biomedical and biopharmaceutical systems

PhD Thesis Summary

Scientific supervisor

Prof. univ. dr. Grigore Damian

Cluj-Napoca
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Acknowledgements

Development and scientific foundation of a doctoral thesis is possible only through an adequate guidance, made by professionalism and meticulosity. I owe special gratitude and thanks to Prof. Univ. Dr. Grigore Damian my scientific supervisor, for permanent support, understanding and encouragement to pursue in finishing this thesis. He provided me his knowledge and experience and a very valuable bibliographic material.

Also I would like to express my deepest gratitude to Prof. Dr. Heinz Jürgen Steinhoff, from University of Osnabrück (Germany), my scientific coordinator during mobility period, for his generosity, patience and understanding. I am very grateful for his contribution to my initiation into "mysteries" of high field ESR spectroscopy. I thank everyone who helped my during the internship period: Johann, Daniel, John and Christian. I thank to Cristina because she shared with me much of her experience, and also because she comforted my in loneliness moments.

I want to thank all those who, directly or indirectly, have helped me to refine the scientific approach and have supported me in completing it. I thank to Professor. Dr. David Leontin who made me understand and to like physics, and also because he supported me morally over time. Also thank to Mr. Vasile Miclăuş for enlightening advices provided in moments of wandering in the chemistry land. Special thanks to Mrs. Adriana Filip and Nicoleta Vedeanu, valuable contributors to experimental part.

I thank to Camelia because she was beside me during the long summer days and also thank to Luciana for generosity and empathy concern to me throughout the doctoral period.

I also thank for the financial support provided from programs co-financed by The Sectorial Operational Programme Human Resources Development, Contract POSDRU 6/1.5/S/3 – „Doctoral studies: through science towards society".

Last but not least, I am grateful to my beloved parents, because they supported my all of my life. They have encouraged my desire to evolve and they supported me with infinite and unconditional love. I dedicate this success to them. I thank those that give meaning to my life, my priceless daughter Maria (my Sun, Moon and Star) and my beloved husband Doru, who has the patience to bear my absence, to trust in me. I thank for their love and constant encouragement.

Content

Keywords: free radicals, antioxidants, spin traps, nitroxide radicals, electronic spin resonance spectroscopy (ESR), gamma irradiation.

Introduction

In the late 1950^{s} terms like free radicals and antioxidants, were almost unknown in biological and clinical sciences, the presence of radicals in biological materials being demonstrated only in 1954. For the first time in 1956 has established a link between the toxic effects of high levels of oxygen in aerobic organisms and ionizing radiation, suggesting the idea that oxygen toxicity is due to the formation of free radicals. Today, it is known that free radicals play an important role in the etiology of many diseases and many unexplained previous phenomena of disease. Thus, some of the conditions in which free radicals have a major influence are Alzheimer's disease, hypertension, myocardial ischemia, atherosclerosis and carcinogenesis. For these reasons, "measuring" the amount and type of radical species generated in various processes, is of major importance.

Technique that can be used to directly detect these species is electronic paramagnetic resonance spectroscopy (EPR or ESR). However, even this technique has its limitations, direct measurement is not always possible due to very short life time of radicals. One way to overcome this difficulty is to use spin trapping technique. In this technique, a diamagnetic compound, the spin trap is added to the system prior to radicals formation, forming an intermediate radical called spin adduct, which is less reactive than the initial trapped radical but still retains characteristics of trapped radical. Another method to study free radicals is by using stabilized nitroxide radicals called spin markers or "spin labels". The main feature of nitroxide radicals is given by the existence of a stable paramagnetic center consists of an unpaired electron localised at the bond between nitrogen and oxygen atoms. Spin markers are mainly used to study molecular dynamics in biological systems, to study the interface phenomena of colloid systems and also for analysis of antioxidant character of vitamins, minerals and other compounds and natural extracts.

On the other hand, is known that studies of the effects of high energy ionizing radiation on drugs, food or medical systems, are evolving due to multiple applications like medical sterilization and quality of food. However, by using ionizing radiation, system degradation may occur, by free radicals generation. ESR detection method for these systems succeed to reveal a number of private properties related to the electronic structure of paramagnetic defects formed in irradiated solid network.

In this paper ESR spectroscopy has been used as a method to study free radicals generated in different systems. Experimental results presented, by using ESR direct detection, were made on irradiated systems of biomedical interest such as antineoplastic agents (Purinethol), antidiabetic agents (Metformin hydrochloride) and photo-cured dimethacrylate-based dental resins. The aim was to establish if these systems contain or can form stable paramagnetic species, after sterilization with γ radiation, or after vitrification under visible radiation respectively. In case of dental resins studied, the experimental results reveal the presence of polymer radicals of methacryl derivatives stable over time, their concentration being dependent on the initiator system and the mixture of monomers used in the preparation of

composite resins. ESR measurements on fresh powders of Purinethol and Metformin hydrochloride irradiated with gamma rays, reveal the presence of several stable paramagnetic species, whose relative concentrations depend on the absorbed dose. After fitting and simulation of experimental spectra have been identified and characterized radicals generated by radiation in drugs.

By using spin trapping method, were identified free radicals generated in systems like: Fenton, xanthine-xanthine oxidase and potassium superoxide. Hydroxyl radicals generated in Fenton reaction were detected by employing high field (W band) and low field (X band) EPR spectroscopy, by usig PBN (N-tert-Butyl-α-phenylnitrone) as a spin trapping agent. Optimal spin trap concentration for our system was determined; also were looked methods to increase detection sensitivity by organic extraction and concentration of spin adducts. Due to the important role of superoxide radicals in cell damage, were searched for suitable methods for measuring this radical, using relatively new traps like DEPMPO-5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide and DIPPMPO-5-(Diisopropoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide, various superoxide generating systems like xanthine-xanthine oxidase system and potassium superoxide. In xanthine-xanthine oxidase system were detected and characterized superoxide radicals generated by this system using three kinds of spin traps: DMPO, DEPMPO, DIPPMPO. The aim was to determine optimal conditions for detection of superoxide radical in order to be analyzed by ESR spectroscopy at low and high frequency. Superoxide and hydroxyl radicals generated by potassium superoxide using spin trap DEPMPO were characterized. It was found that the stability of spin adducts is strongly dependent on the ratio between the amount of potassium superoxide in solution and the amount of spin trap used.

ESR studies have been also conducted on the antioxidant activity of some commercial juices and natural extracts (grape seed and Calluna Vulgaris) using nitroxide radical Tempol (2,2,6,6-tetramethyl-4-hydroxypiperidine-oxyl). Two commercial fruit juices (apple and grape juices) were investigated in order to check the correct labeling in the Romanian markets and to inter-compare the antioxidant activity of the studied juices and between the commercial juices and the natural juices. The obtained results have shown that both commercial juices are authentic fruit juices according to their labels. Further more, this study investigates the protective activity of red grape seeds (Burgund Mare variety-BM) and Calluna Vulgaris extracts (CV), on SKH-1 mice skin exposed to multiple doses of ultraviolet radiation (UV)-B. Was also evaluated if a topical application of the extract on mice skin before or after irradiation, can inhibit skin injury by modulating the antioxidant defence mecanisms. Our results suggest that both extracts might be potential chemo-preventive candidates in reducing the oxidative stress and apoptosis induced by multiple doses of UV-B in skin. It also notes that BM extract has an antioxidant character slightly stronger than CV extract, so it seems that BM extract is more effective in blocking chain reactions caused by ROS in the skin after multiple radiation exposure.

I. Characterization and generation of free radicals

I.1 Characterization of free radicals

Radicals (often, but unnecessary named as free radicals) are chemical species possessing an unpaired electron in the outer shell of the molecule so they are highly chemical reactive. Free radicals classification can be made by different criteria, a classification that includes the vast majority of radicals would be: [1]:

- Reactive oxigen species
- Nitrogen reactive species
- Aromatic compounds
- Quinonic and semiquinonic compounds
- Nucleic acids
- Thiyl radicals

I.2 Generation of free radicals

Generation of free radicals in biological systems is due to the action of internal and external factors. Internal sources of free radicals are metabolic processes in the body and especially processes associated metabolic disturbances accompanying various types of pathology (metabolic stress). External sources of free radicals (Fig.I.1) include ionizing radiation, UV radiation and microwaves, toxic metals (eg. Al and Cd in drinking water), smog, chemical food additives, tobacco smoke, air pollutants.

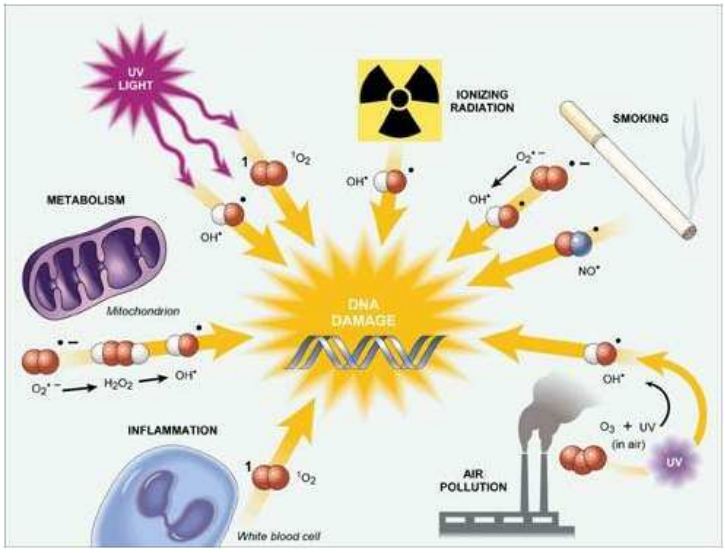


Fig. I.1. Free radicals sources (http://www.thefoodadvicecentre.co.uk/reference/free-radicals/

I.2.1 Homolysis

By homolytic cleavage of a covalent bonds, a molecule is fragmented in two fragments, each fragment retaining one electron. Homolytic cleavage is uncommon in biological systems because it requires a lot of energy, the source being either ultraviolet light, heat or ionizing radiation. Homolysis can be thermally, coupled or by electronic transfer.

I.2.2 Photolysis and radiolysis

Ionizing radiation and absorption of light promotes the production of large amounts of free radicals, depending on the absorbed radiation dose and exposure time. Free radicals are often formed by photolysis of chemical bonds due to absorption of a photon; as a result the molecule passes into a singlet or triplet excited state. Ionizing radiation generates radicals even after exposure, by indirect effect, due to water radiolysis in tissues. Radiolysis of water depends on the nature and energy of radiation and the presence of oxygen, because in the absence of oxygen, X and γ radiation does not decompose water.

I.2.3 Enzymatic reactions

Enzymes are natural substances produced by living cells playing the role of biocatalysts. Enzymatic reactions in the body give rise to free radical intermediates that react with each other or with other substances to form stable compounds.

I.2.4 Metabolism

Metabolism are all biochemical and energetic transformations that occur in the tissues of living organisms. Xenobiotic metabolism is the set of metabolic pathways that modify the chemical structure of xenobiotics (compounds foreign to an organism's normal biochemistry, such as drugs and poisons, pollutants, pesticides, etc.), leading to free radicals formation.

I.2.5 Free radicals in nature

Nitrogen and especially oxygen from the atmosphere easy form specific free radicals. Although ozone (O_3), is not a free radical, is a very powerful oxidizing agent. Also, NO and NO_2 existing in nature, are stable free radicals in relatively low concentrations (0.2 ppm NO_2 in smog). Flavin quinones is a group of semiquinone class, which can form free radical intermediates between oxidized and reduced forms of quinones.

I.2.6 ROS in human body

The most relevant radicals occured in biological regulatory processes are superoxide ($O_2^{\bullet}$) and nitric oxide ($NO^{\bullet}$). Superoxide anion formation process is either mediated by enzymes such as NAD(P)H oxidase and xanthine oxidase, or is nonenzimatic by redox reagents, such as semi-ubiquinone compound of the mitochondrial electron transport chain (Fig. I.2).

Superoxide dismutase converts enzymatic superoxide to hydrogen peroxide [2, 3]. In the presence of reducing transition metals (eg. iron or copper ions), hydrogen peroxide can be reduced to a very reactive radical species, hydroxyl radical [3].

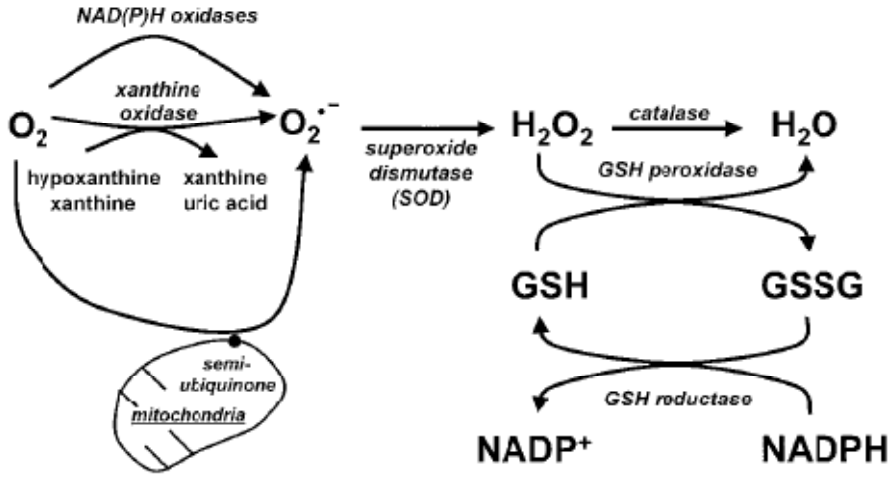


Fig. I.2 Pathways of reactive oxygen species (ROS) production and clearance. GSH-glutathione; GSSG-glutathione disulfide. [4]

II. Free radicals detection methods

There are many methods, direct or indirect, for detection and characterization of free radicals. The indirect methods include spectrophotometry and chemiluminescence.

II.1 Spectrofotometry

The principle of the method consists in irradiating the sample with different radiation wavelengths and recording the absorption spectrum (the radiation intensity as a function of wavelength). Molecular absorption spectrophotometry has applications both in qualitative and quantitative analysis.

II.2 Chemiluminiscence

The principle of this method consists in emission of light as a result of reactions between free radicals and a substance inserted into the system, the most used being luminol. The signal intensity is proportional to the amount of free radicalsl.

Direct methods detects free radicals formed during a chemical reaction, one of these methods being electronic spin resonance spectroscopy (ESR or EPR).

II.3 Electronic spin resonance spectroscopy (ESR)

ESR spectroscopy is based on transitions between Zeeman levels of a paramagnetic system (possessing an unpaired electron), located in a static magnetic field [5, 6]. There are a variety of ESR techniques, each with its own advantages. In the case of continuous wave spectroscopy (CW-EPR), the sample located in a static magnetic field is continuously irradiated with a microwave beam with a fixed frequency, while the magnetic field is swept trough the resonance condition. In CW-EPR can be used different frequencies of microwave radiation, called bands: S-band (3.5 GHz), X-band (9-10 GHz), etc.

ESR spectroscopy can reveal properties related to electronic structure of a paramagnetic defect formed in a solid irradiated network; also can be used to study the mechanism of radiolysis, to identify radical species induced by radiation in drugs or food [7-10]. Another utility of method is to study the antioxidant properties of substances or extracts, using nitroxide radicals[26-29].

II.3.1 CW-ESR measurements

ESR experiments presented in this paper were made to characterize free radicals in some biomedical and biopharmaceutical systems. X-band ESR spectra (9-10 GHz) were recorded with a BRUKER-BIOSPIN EMX^{micro} spectrometer equipped with a data acquisition computer. The samples analyzed were placed in quartz capillary tubes, Wilmad-labglass (10 cm length and an internal diameter of 1 mm) and the tubes were then placed in the center of the cavity resonator type TE_{102}.

Additionally, when magnetic field used in X-band was not high enough to resolve the anisotropy of g factor, measurements were performed in high field (~ 3 T). These measurements were made with a W band spectrometer (3.4 T, 95 GHz) built in the Biophysics laboratory at the University of Osnabrück (Germany). Samples were pipetted in quartz capillary with 0.6 mm inner diameter (VitroCom Inc, NJ, USA) for low temperature measurements and 0.2 mm for ambiental temperature measurements [30].

II.3.2 ESR detection methods of free radicals

Free radicals can be detected by ESR spectroscopy, either directly or indirectly depending on their mobility and stability and on the phase system where they were generated. Thus, in solids systems, free radicals can be detected directly because of their low recombination capacity [10]. In case of free radicals in the gas phase or liquid systems, detection is possible by indirect methods, due to their short life time (in order of microseconds). In this case, it is necessary to use a diamagnetic reagent of nitrones class called "spin trap" [12, 13] or "nitroxide radicals", called "spin labels") [11].

II.3.2.1 Applications of spin traps in ESR analysis of systems

Spin trapping method (ESR/ST) involves the addition of radical to a nitrone spin trap resulting in the formation of a spin adduct, a nitroxide-based persistent radical, that can be detected using electron paramagnetic resonance (EPR) spectroscopy.

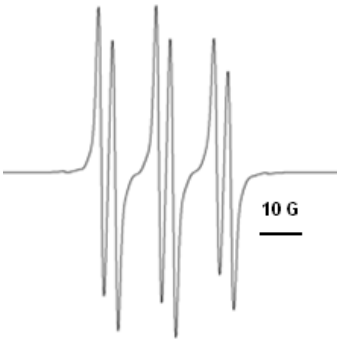


Fig. II.1 ESR spectrum of PBN-OH spin adduct

PBN (alpha-phenyl N-tertiary-butyl nitrone) is one of the first traps synthesized. Excepting the triphenylmethyl radical , PBN forms relatively long-lived spin adducts with various types of radicals but the differences between the hyperfine splitting constants for different radicals can be very small and therefore can not be differentiate. The ESR spectrum of the spin adduct with PBN is generally a triplet of doublets due to splitting by the nitrogen and the β proton (Fig. II.1).

Due to the lack of specificity of PBN, Janzen and Liu were synthesize in 1973 a new cyclic spin trap named **DMPO** (5,5-Dimethyl-1-pyrrolineN-oxide). The values of hyperfine splitting constants for DMPO adducts are higher and more sensitive to the radicals nature captured. ESR spectrum of DMPO-OH has four lines with intensities 1:2:2:1. (Fig. II.2 (a)), central lines being superimposed. For DMPO-OOH the lines are split due to the nitrogen and protons in β and γ positions (Fig. II.2 (b)).

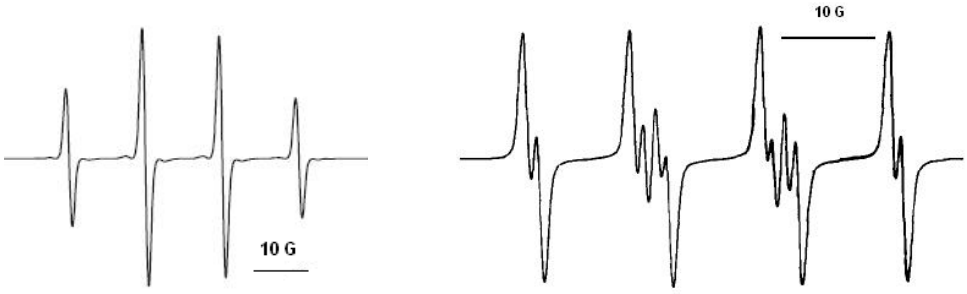


Fig. II.2 EPR spectra of DMPO-OH (a) and DMPO-OOH (b) adducts

One of the newly synthesized traps is **DEPMPO** (5-dietoxifosforil-5-metil-1-pirolin-N-oxid) [20-23], a phosphorylated derivative of the widely used DMPO. Higher stability of DEPMPO spin adducts yields a higher signal/noise ratio of DEPMPO adducts relative to DMPO adducts under identical experimental conditions. In addition, ^{31}P (I = 1/2) induces extra line splitting, leading to more complex but also more informative spectra when compared to DMPO [22-25] (Fig. II.3).

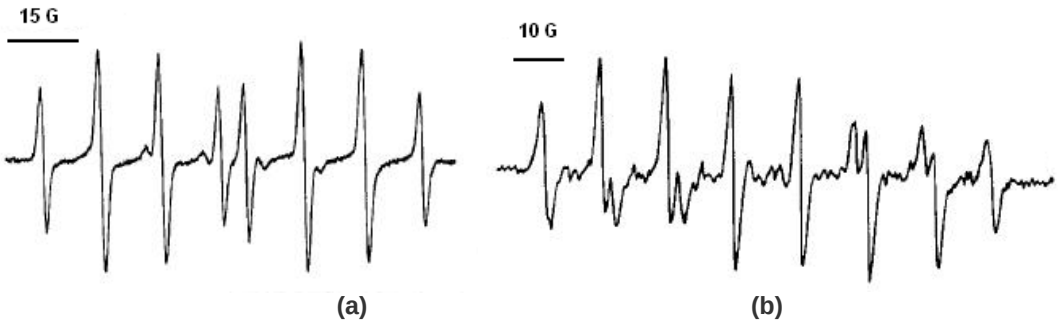


Fig. II.3 EPR spectra of DEPMPO-OH (a) and DEPMPO-OOH (b) adducts

II.3.2.2 Applications of nitroxide radicals in ESR analysis of systems

In the last decades, ESR spectroscopy of nitroxides has been intensively develope,d because nitroxides ESR spectra contain information about some features of molecular micro-environment of the neighborhood, such as polarity and mobility [11]. In case of nitroxide radicals the unpaired electron located predominantly on the nitrogen atom is responsible for hyperfine interaction with ^{14}N nucleus whose nuclear spin is I = 1 leading to a hyperfine splitting of the Zeeman lines in 2I +1 = 3 components. As a result, the ESR spectrum will have three lines (Fig. II.4), influenced by the mobility of the molecule.

Some of the many applications of these technique are: study of interface phenomena that occur in colloid systems, the mobility of a spin labelled molecule, analysis of the antioxidant nature of vitamins, minerals and other photochemical compounds [16, 17].

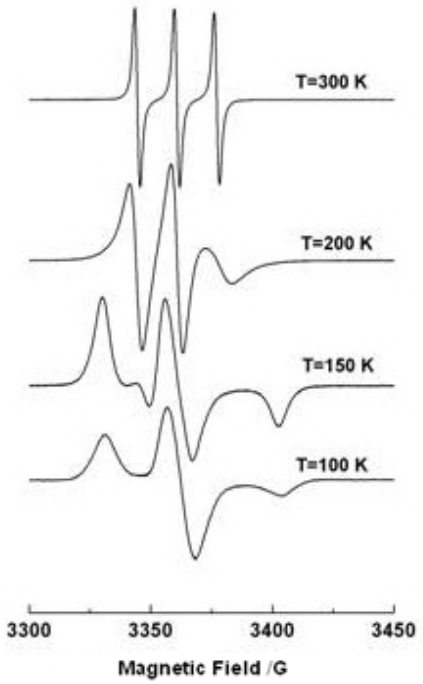


Fig. II.4 EPR spectra of Tempol recorded at different temperatures

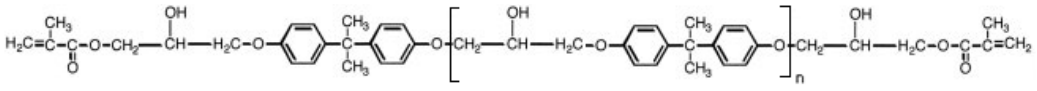
III. Direct detection by ESR spectroscopy of free radicals generated in some biopharmaceutical systems

Photopolymer composite resins are commonly used in dentistry as restoration materials for anterior and posterior teeth as dental fillings and prosthetic dentistry to prepare dental varnishes [31, 32]. During polymerization of dimethacrylate monomers employed in dental materials, the gel effect leads to entrapment of radicals and unreacted monomers in the crosslinked network, affecting physico-mechanical properties and the biocompatibility [33].

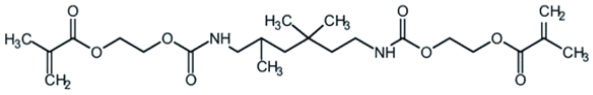
Gamma irradiation was proposed in the British Pharmacopoeia as a suitable sterilization method for sterilization of drugs and certain surgical materials and equipments with a 25 kGy dose. During irradiation of solid drugs, free radicals are formed and remain trapped in the matrix. Testing stress induced by γ radiation in drugs, can provide information about the degradation pathways and intrinsec stability of the molecule.

III.1 Dental resins

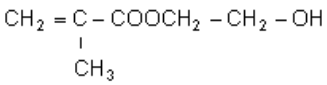
Six new experimental dental light-cured composites based on the Bis-GMA monomer - 2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy) phenyl]-propane, on the corresponding Bis-GMA dimer, 2-hydroxyethyl methacrylate (HEMA) and on 1,6-Bis-[2methacryloyloxyethoxycarbonyl-amino]-2,4,4-trmethylhexane (UDMA) have been employed, with three different photoinitiator systems. (Fig. III.1).



Bis-GMA$_{336}$ (n=0,1)



UDMA



HEMA

Fig. III.1 The chemical structures of methacrylate monomers used in the experiments

The composition of the experimental dental light-cured composites are presented in Table III.1.

T**abel III.1** Description of the samples examined in the present study

Monomer mixture + Hybrid filler / Initiation system	- Bis-GMA336 (12wt%) - HEMA (8wt%) + - Quartz (32wt%) - Sr/Zr glass (32wt%) - FHap (16wt%)	-Bis-GMA336 (7wt%) -UDMA (3wt%) -HEMA (10wt%) + - Quartz (32wt%) - Sr/Zr glass (32wt%) - FHap (16wt%)
-CQ - DMAEM	Sample 5	Sample 7
- CQ - - DMAEM - E-4-DMAB	Sample 6	Sample 8
- CQ - - E-4-DMAB	Sample 9	Sample 10

The cured composite samples were obtained by exposing the paste composite samples to a visible radiation in the wavelength range of 400-500 nm. The radiation was generated by an Optilux dental lamp, produced by Demetron Research Corporation USA.

Figure III.2 shows ESR spectra measured immediately after polymerization by irradiation. The nine lines of ESR spectra are typical, indicating the presence of polymer radicals of methacryl derivatives.

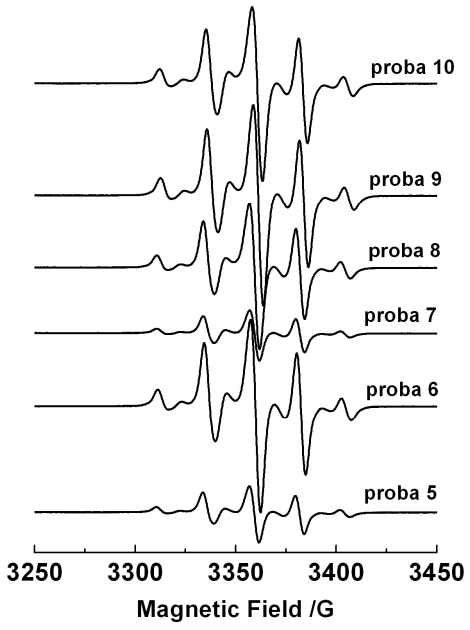


Fig. III.2 EPR spectra of dental resins after polymerization

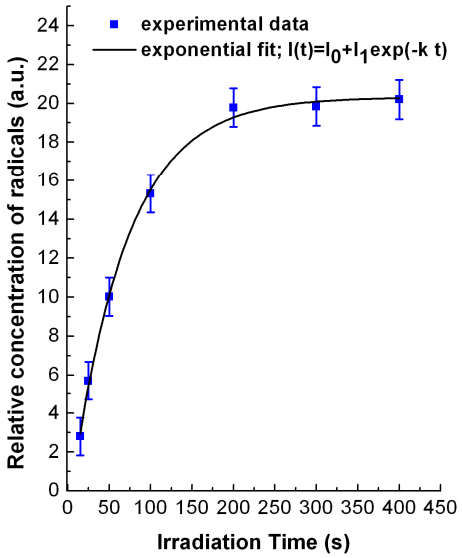


Fig. III.3 Dependence of relative signal intensity on the irradiation time

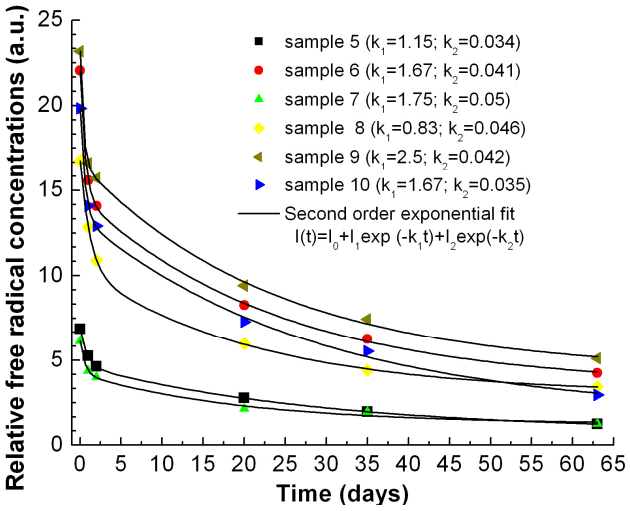


Fig. III.4 Time variation of relative signal intensity

Figure III.3 shows the kinetic of radical formation, depending on the irradiation time. One may note that the intensity of the ESR signals is dependent on the time of irradiation and that this dependence shows saturation behavior at ~ 3 minutes. Each sample was weighed and the concentration of free radicals has been reported per 1mg of sample. The integral intensities of EPR spectra were obtained by evaluating their double integrals using Origin 8 program [10].

From figure III.4 one can observe the general trend of decreasing the relative free radicals concentration in time, with different rates during samples aging, the concentration remaining constant after 60 days.

Summarizing the results we conclude that photopolymerization of dental resins at room temperature leads to the formation of long-life free radicals due to system vitrification. Within the limits of the present experiments, the radical concentration depends primarily on the initiation system and secondly on the composition of the monomers used in experimental composites [34].

III.2 Purinethol

Purinethol [1,7-dihydro-6H-purine-6-thione, $C_5H_4N_4S\times H_2O$] or 6-mercaptopurine, is an antineoplasic agent used in chemotherapy to treat acute leukemia and chronic granulocytic leukemia. Fresh Purinethol drug in the form of microcrystalline powder was exposed to γ-radiation from a ^{60}Co source (GAMMA CHAMBER 900) in ambient conditions. The ^{60}Co source gives a compact and uniform density of radiations and a moderate dose debit of 35 Gy/h evaluated by ferrous sulfate dosimetry. The absorbed dose of drugs was in the range of 0 to 25 KGy [35]. By ESR spectroscopy were detected and characterized radiation-induced radicals in the samples.

ESR X band (9-10GHz) spectra were recorded at room temperature with a field modulation of 100 KHz (Fig. III.5).

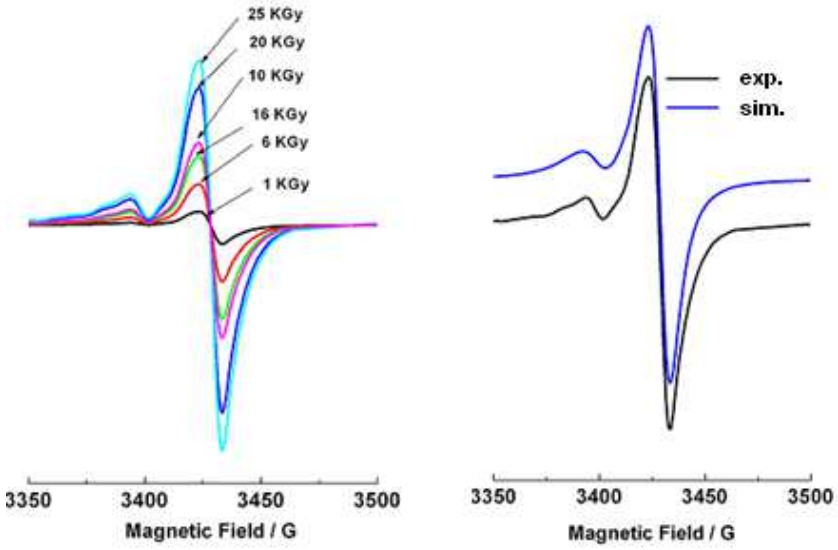


Fig. III.5 Experimental and simulated spectra of γ irradiated Purinethol

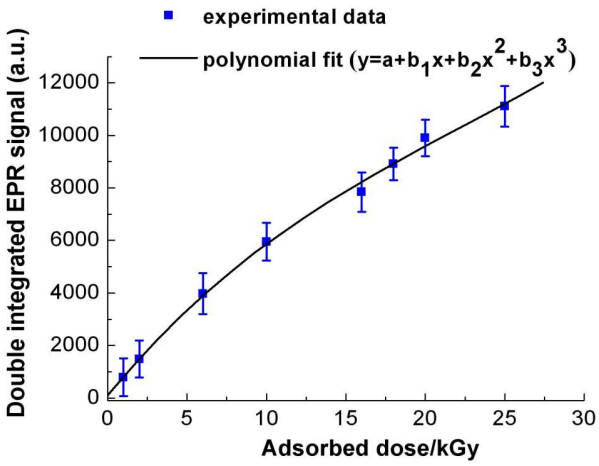


Fig. III.6 Dose–response curve of γ irradiated Purinethol

The anisotropic spectrum seems to belong mainly to sulphinyl radical RSO- with g_{zz}=2.0232 and $g_{xx}=g_{yy}$ =2.0048 formed by oxidation of the thiyl radical.This anisotropy in the EPR spectrum, are due probably, the localization of radical centers on both aromatic rings giving rise to a local axial arrangement. By computer simulation, using POWFIT program, we find that, besides thyl radical there is another two radical species with magnetic parameters g_1=1.998 and A_1≈3 G, and g_2=2.002 and A_2≈17 G (Fig. III.5). Both radical species is not a sulfur species but

rather an oxygen radicals centered on oxygen, interacting with a β and γ proton and it can be idenfied as free oxygen species.

The relative integral of the EPR absorption spectra intensities as a function of irradiation time (dose-response curve) was represented in (Fig. III.6). From the analysis of the dose-response curve, it can be concluded that γ-irradiation causes an increase in the amount of radicals with different parameters of generation and recombination.

III.3 Metformin hydrochloride

Metformin hydrochloride is an oral antihyperglycemic drug, belonging to the biguanide class, used in the management of non-insulindependent diabetes mellitus (type 2 diabetes mellitus) Exposure to gamma radiation sterilization process, and exposure to light, causes damages to its structure, and therefore it is important to know how radiations affect him. Microcrystalline powder of metformin was exposed to γ-radiation, the absorbed dose being in the range of 0-4 KGy .
EPR spectra registered for different irradiation time, is represented by a broad triplet with a ratio of signal intensity 1:2:1 (Fig. III.7).

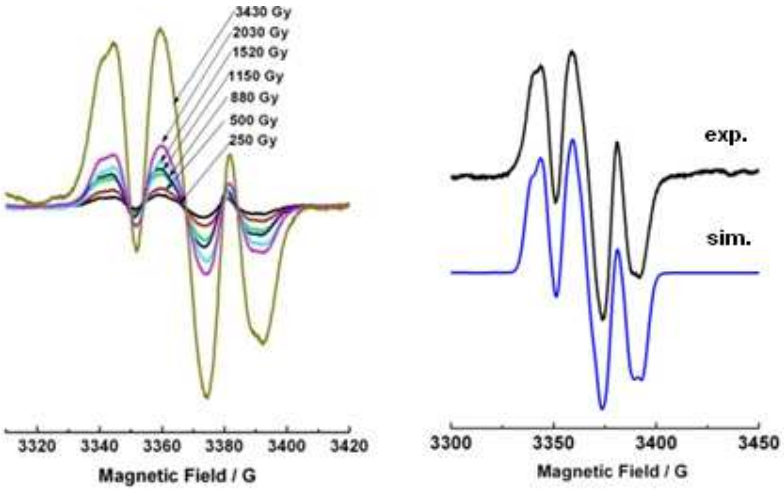


Fig. III.7 Experimental and simulated spectra of γ irradiated Metformin

By computer simulation, using POWFIT program, we can identify two nonequivalent magnetic species, with magnetic parameters:

- species 1: a_{CH2} = 25*,1* G, a_{NH} = 6,8 G and a_N= 10,4 G
- species 2: a_{CH2} = 18,5 G, a_{NH} = 16,7 G and a_N = 6,4 G.

The relative integral of the EPR absorption spectra intensities as a function of irradiation time (dose-response curve) was represented in (Fig. III.8).
From the analysis of the dose-response cure, it can be concluded that γ-irradiation causes an increase in the amount of radicals by a liniar function. This dependence shows that metformin can be used as an biodosimetric indicator [36].

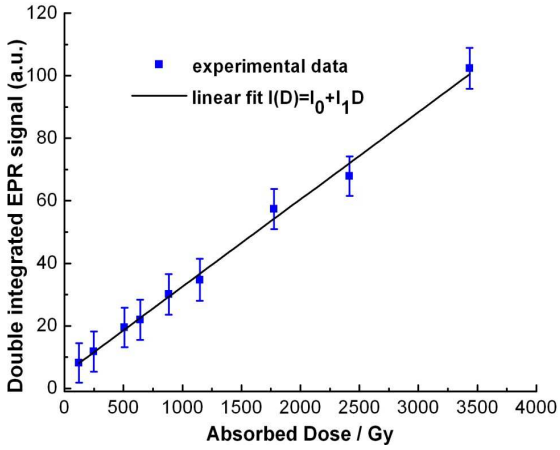


Fig. III.8 . Dose–response curve of γ irradiated Metformin

IV. Indirect detection of free radicals by „spin trapping" technique (ST/EPR)

Study of short-lived free radicals, as hydroxyl radical or superoxide in chemical and biological systems, is possible by ESR spectroscopy using spin trapping method. No other technique used in vivo proved to be as sensitive and specific, from analytical point of view, to detect free radicals [14, 15]. The systems chosen for the study of free radicals, by spin trapping technique, are Fenton reaction, xanthine-xanthine oxidase system and potassium superoxide. First system generates hydroxyl radicals, the second generates superoxide radicals and the last generates both types of radicals.

IV.1 Fenton reaction

The oxidation of organic substrates by iron(II) and hydrogen peroxide is called the "Fenton reaction". The nature of the oxidizing species obtained in Fenton reaction is still a subject of discussion, because the reaction it's common in both chemical and biological systems and in natural environment. The conclusion is that the reaction is the most likely mechanism for the generation of the highly reactive hydroxyl radical (•OH) in a biological system; also, this reaction is capable of generating higher oxidation states of the iron [37-40].

Were detected and characterized by ESR spectroscopy at low and high field (X-band and W band), radicals generated in Fenton reaction using PBN spin trap. Spin adduct formation reaction are depicted in fig. IV.1.

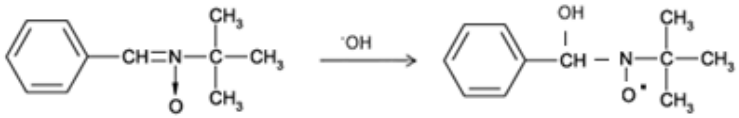


Fig. IV.1 Spin trapping of hydroxyl radical with PBN

After preparing the Fenton system, using various concentrations of PBN, ESR spectra were recorded in X band at room temperature (Fig. IV.2).

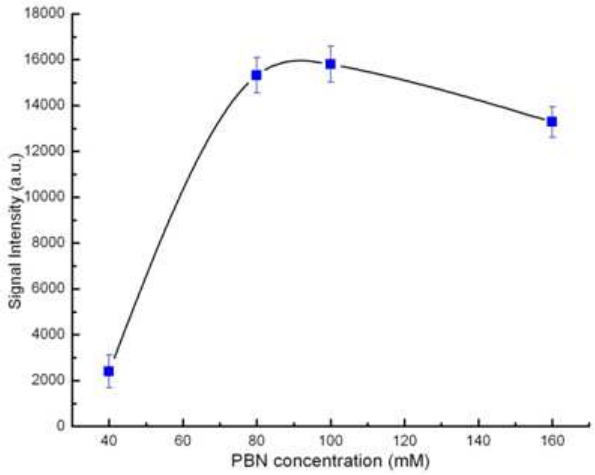


Fig. IV.2 EPR signal intensity as function of PBN concentration

To determine the optimal concentration of PBN, the dependence of signal intensity as function of the trap concentration was plotted. An optimal spin trap concentration is in range 80-100 mm. A method for increasing the concentration of paramagnetic centers in sample is organic extraction and concentration of spin adducts. The advantage of organic extraction (chloroform / methanol, 2:1 vol) is that the adduct is transferred into a medium with a lower dielectric constant (in this case chloroform).

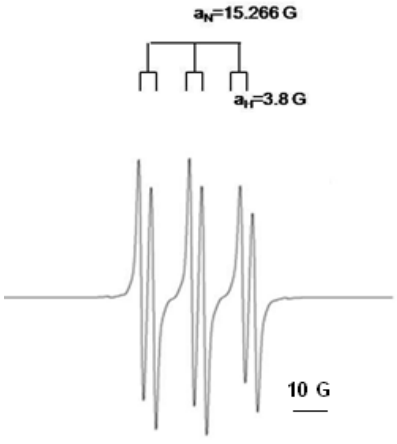


Fig. IV.3 EPR spectrum of PBN-OH˙ adduct at room temperature

ESR spectra at room temperature were recorded for concentrated sample; from spectrum analysis, were determined corresponding isotropic hyperfine splitting constants of nitrogen and hydrogen atoms: a_N = 15.266 G a_H = 3.8 G (Fig. IV.3). To resolve anisotropy, the ESR spectra at low temperature were recorded (Fig. IV.4).

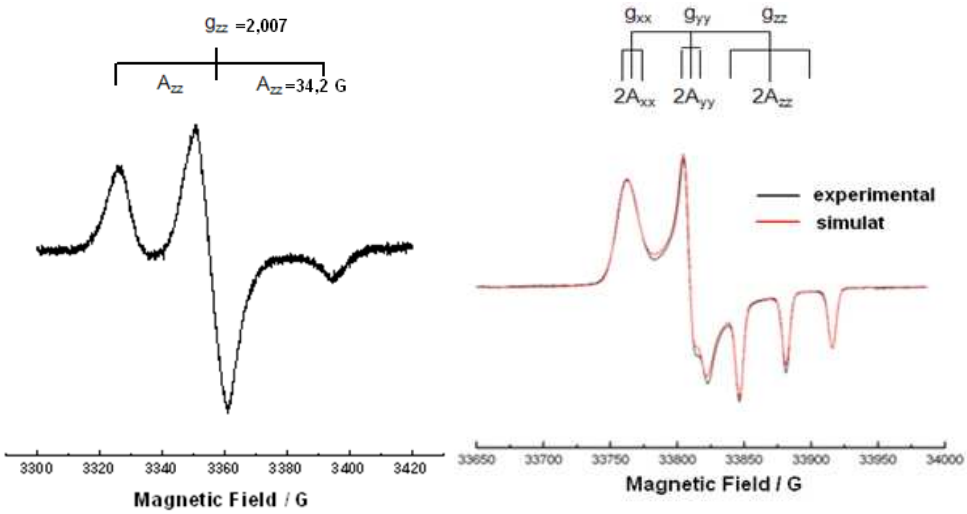


Fig. IV.4 X band and W band spectra of PBN-OH$^{\cdot}$ adducts, recorded at low temperature

Because the anisotropy is not completely resolved, the ESR spectra in high field (W band) were recorded (Fig. IV.4). Experimental spectrum was fitted and simulated with Easy Spin program. The values of magnetic parameters obtained were: g_{xx}=2.00916, g_{yy}=2.00614, g_{zz}=2.00221 and A_{xx}=4.32 G, A_{yy}=4.67 G and A_{zz}=34.68 G. In conclusion, a reliable and accurate method for characterization of free radicals generated in this system is the spin trapping method in high field.

IV.2 Potassium superoxide (KO_2)

Was studied potassium superoxide (KO_2) in alkaline solutions, which has been demonstrated to be a reliable source of superoxide, by EPR spectroscopy using DEPMPO spin trap, to find the best detection conditions of superoxide radicals. The superoxide radical was generated from solvation of KO_2 in an aprotic solvent (DMSO). This superoxide is a strong nucleophile and therefore it forms hydrogen bonds in water and undergoes rapid disproportion to HOO^- and HO^- in a concerted process. [44, 45]. The presence of superoxide in our samples was confirmed by the addition of the spin trapping agent DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) (Fig. IV.5), followed by ESR spectroscopy analysis (spin trapping technique). DEPMPO forms relatively stable spin adducts and is able to differentiate between different oxygen radical species. Higher stability of DEPMPO spin adducts yields a higher signal/noise ratio of DEPMPO adducts relative to DMPO adducts under identical experimental conditions.

In addition, ^{31}P (I = 1/2) induces extra line splitting, leading to more complex but also more informative spectra when compared to DMPO [23-25, 41].

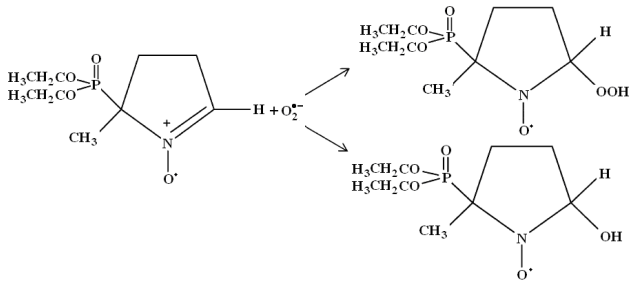


Fig. IV.5 Spin trapping of superoxide with DEPMPO

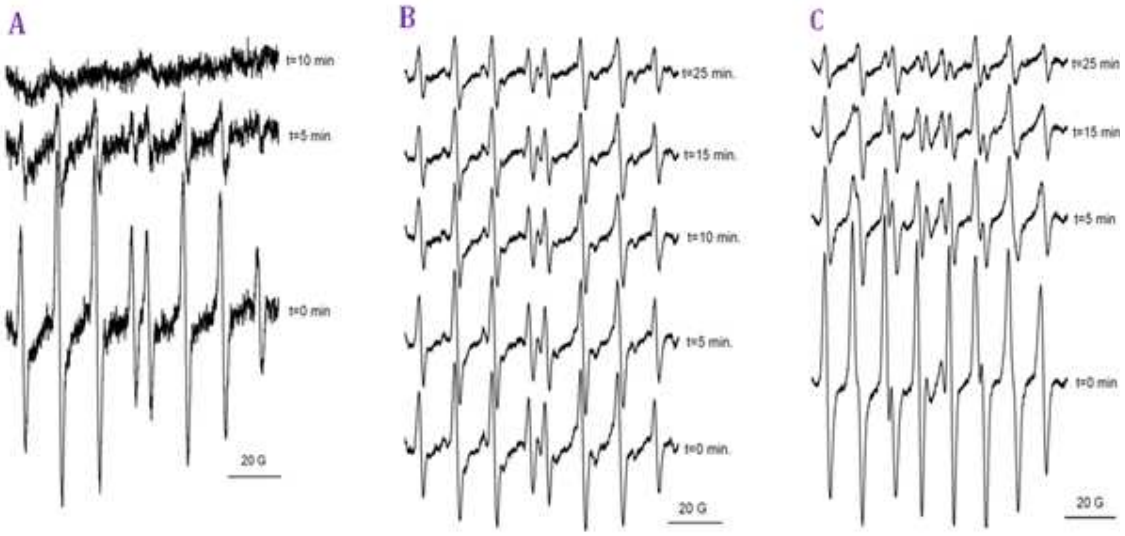


Fig. IV.6 Time evolution of the EPR spectra of samples A, B and C

Three solutions were prepared (A, B and C) with different KO_2/DEPMPO ratio (1 µl KO_2/10 mM DEPMPO- sample A, 10 µl KO_2/15 mM DEPMPO- sample B, 20 µl KO_2/20 mM DEPMPO-sample C). The EPR spectra were recorded about two minutes after the initiation of the reaction. (Fig. IV.6). We note that DEPMPO is able to differentiate between DEPMPO-OH şi DEPMPO-OOH aducts (Fig.IV.7).

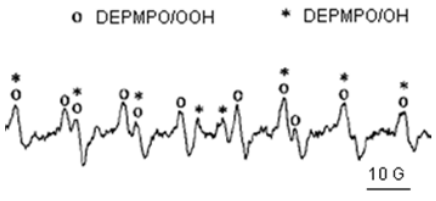


Fig.IV.7 Distinguish between adducts on spectrum

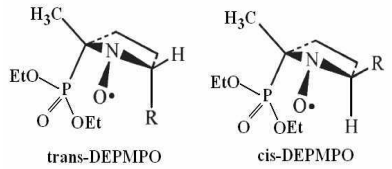


Fig.IV.8 Structure of DEPMPO-adducts conformers

Hyperfine splitting constants of adducts were determined by computer simulation of experimental spectra, by using WINSIM program (Fig. IV. 9) [47]. The results were registered in tabel IV.1. It seems to exist a fast exchange between two conformers (I and II, see Fig. IV.8) of the DEPMPO-superoxide spin adduct giving rise to the observed spectrum, rather than an exchange between the protonated and deprotonated forms of the DEPMPO-superoxide adducts [46].

Table IV.1 Hyperfine constants of DEPMPO-adducts

Hyperfine (G)	DEPMPO/OH				DEPMPO/OOH			
	Sample A		Sample B	Sample C	Sample A	Sample C		Sample B
	Conformer I	Conformer II				Conformer I	Conformer II	
a_N	14.12	14.04	14.53	14.4	-	13.01	13.07	13.07
a_H^β	13.26	13.28	14.07	13.26	-	10.63	11.15	9.22
a_P	50.4	50.62	51.38	47.42	-	49.46	50.68	49.6
a_H^γ(1H)	0.89	0.96	1.07	1.01	-	0.87	0.96	0.91
a_H^γ(6H)	0.43	0.44	0.39	0.41	-	0.34	0.41	0.48

Spectra evolution over time is depicted in figure IV.10. It seems that hydroxyl radicals predominate in the conditions of reaction A (low concentration of superoxide). Also, the signals of the DEPMPO/OH spin adduct disappear after 10 minutes, indicating a fast degradation of spin adducts. The EPR intensity of the signal corresponding to sample B decrease more slowly than for sample A, without changing the shape of the spectra, and can be measured even after 25 minutes. This seems to indicate a higher stability of the DEPMPO spin adducts in time.

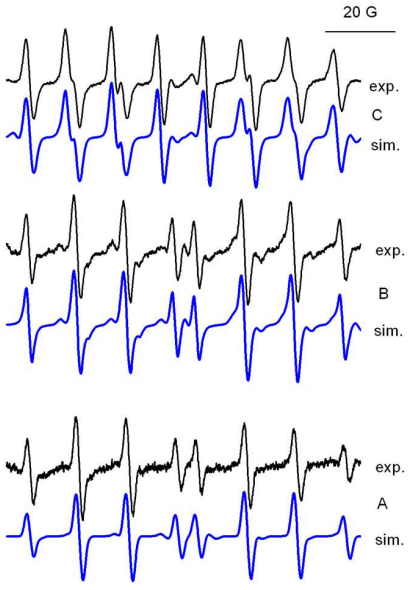


Fig. IV.9 Experimental and simulated EPR spectra of fresh solutions A, B and C

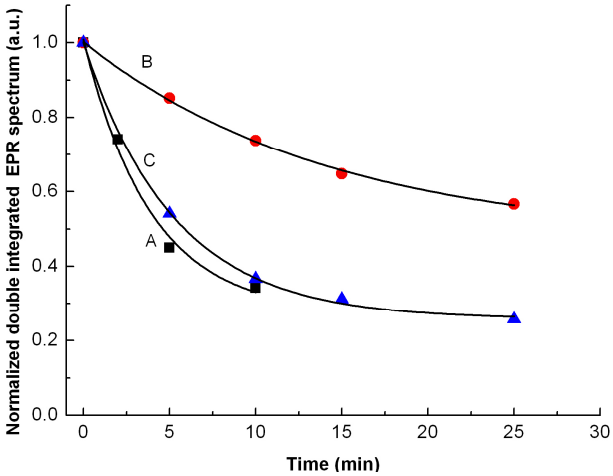


Fig. IV.10 Decay curves of the DEPMPO-adducts in time

In case of solution C, besides the fact that the overall intensity decreases faster than in reaction B, the shape of the spectrum changes over time. This may be interpreted to arise from a conversion of DEPMPO/OOH into DEPMPO/OH, by increasing the amount of oxygen in the sample.

IV.3. Xanthine-xanthine oxidase

Xanthine-xanthine oxidase system has a major biological importance, because it generates reactive oxygen species. Were studied radicals generated by this system using three kinds of spin traps: DMPO, DEPMPO and DIPPMPO. The aim was to determine optimal conditions for detection of superoxide radical in order to be analyzed by ESR spectroscopy at low and high field. Using the spin trap DMPO and DEPMPO, was found that the spin adducts half-life is very short (45 s and 15 minutes respectively) [41-43]. Since the high field measurements (W band) at low temperature requires a longer time to optimize the parameters, these measurements have not been achieved. By making the organic extraction using DIPPMPO (Fig. IV.11) (chloroform / methanol, 2:1 vol) was possible to measure in X and W band (Fig. IV.12).

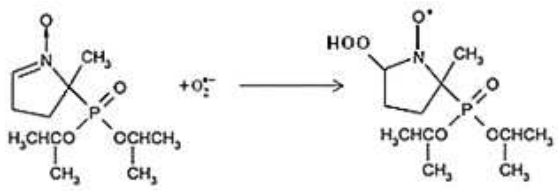


Fig. IV.11 Spin trapping of superoxide by using DIPPMPO

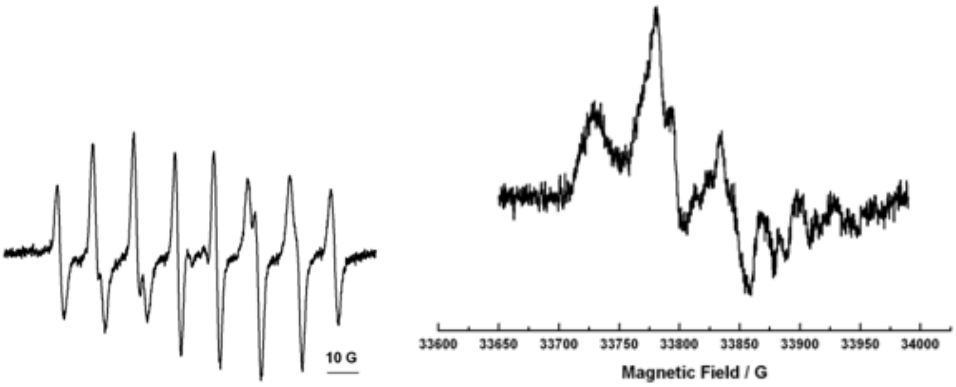


Fig. IV.12 EPR spectra of DIPPMPO-OOH at low and high field

V. Characterization of antioxidant compounds

V.1 Types of antioxidants

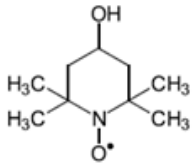
To prevent oxidation and damage caused by excessive concentrations of free radicals generated in biological systems, there are several groups of antioxidant agents such as: enzymatic antioxidants (enzymes), non-enzymatic antioxidants and exogenous compounds.

V.2 Oxidative stress

Free radicals in general and reactive oxygen species (ROS), nitrogen and chlorine in particular, are believed to have a major contribution to the development of several age-related diseases and the aging process itself [19, 48], by causing "oxidative stress" and "oxidative damage". For these reasons, considerable attention is given to the methods of "quantification" of damage and to identify compounds that can attenuate or even eradicate radicals effects. In this respect, of great interest is the study of antioxidant activity of various natural compounds and natural extracts as well as food and biological samples.

V.3 Determination of antioxidant activity

A technique widely applied in recent years to determine the antioxidant activity is ESR technique [26]. Thus, a method commonly applied to food quality assurance is spin trapping method, by using spin traps like PBN, DMPO, DEPMPO, POBN, etc. [49-53]. Another commonly used method for determining antioxidant activity is the use of stable nitroxide radicals (Fig. V.1).



TEMPOL

Fig. V.I Nitroxide radical Tempol

There are two distinct parameters wich are determined from the kinetics of degradation: antioxidative potential (µmol radical degraded per minute) and antioxidative capacity (µmol radical per µmol antioxidant) [54] (Fig. V.2).

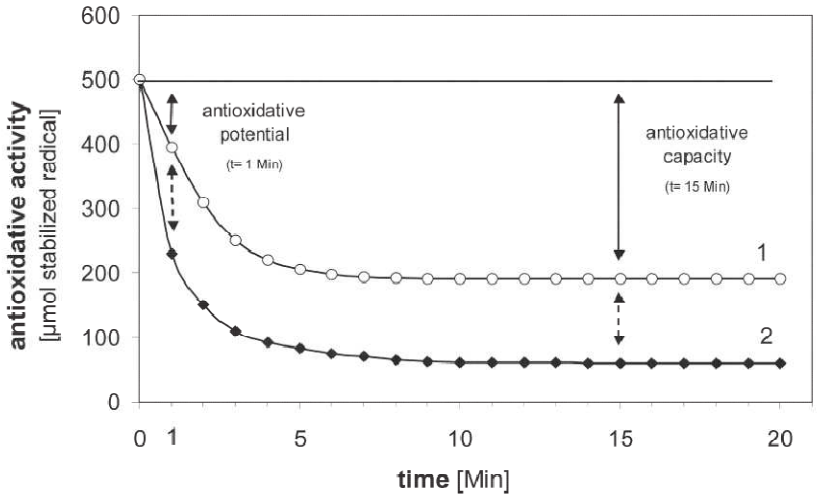


Fig. V.2 Kinetics of a synthetic stabilized radical degraded by two different antioxidants (1 and 2)

ESR spectroscopy is preferable to other techniques (such as Trolox-Equivalent Antioxidant Capacity-TEAC) because it is more specific, due to the formation of characteristic signals. Thus, one can distinguish between various substances which contribute to total antioxidant activity of a compound, resulting in a more precise evaluation of antioxidant activity of the compound.

V.4 Results

ESR studies have been conducted on the antioxidant activity of some commercial juices and natural extracts (grape seed and Calluna Vulgaris) using nitroxide radical Tempol (2,2,6,6-tetramethyl-4-hydroxypiperidine-oxyl) [55, 56-57]. Following oxidation, number of paramagnetic species decreases over time with different rates, depending on the amount of antioxidants present in the sample and their antioxidant potential. From EPR signal decay in time we can draw conclusions about antioxidant character of the compound.

V.4.1 Natural and commercial juices

Two commercial fruit juices (apple and grape juices) were investigated using EPR measurements in order to check the correct labeling in the Romanian markets and to inter-compare the antioxidant activity of the studied juices and between the juices and the natural juices. It was evidenced that the number of paramagnetic species decrease in time with different rates and this was correlated with the antioxidant activity of the studied juices (Fig. V.3). The rate of reaction between antioxidant compounds and Tempol was monitored based on the normalized double integrated residual EPR signal, which is correlated with the number of paramagnetic species in time (Fig. V.4).

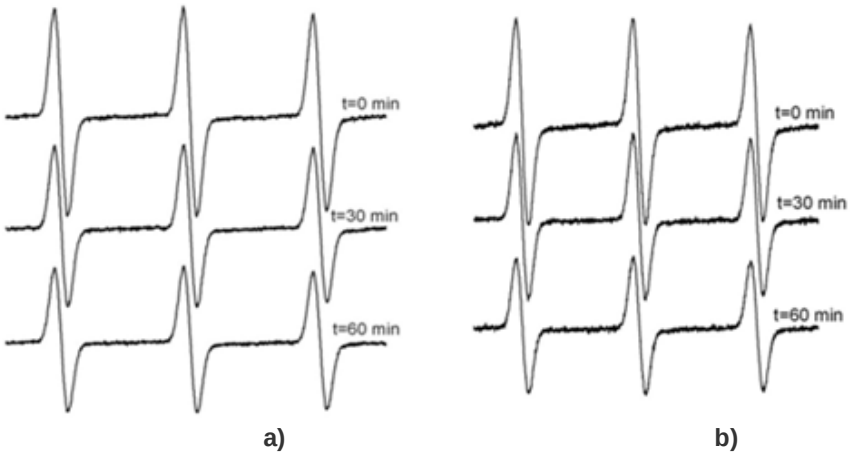


Fig. V.3 EPR spectra of Tempol at different time of incubation in fresh apple juice (a) and grape juice (b)

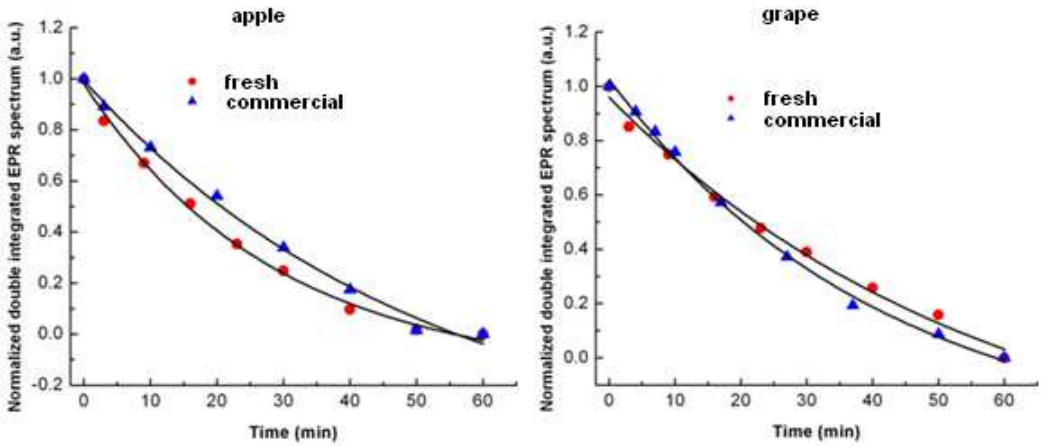


Fig. V.4 Normalized double integrated EPR spectra for fresh and commercial juices in function of time

Comparing the antioxidant characteristics of fresh apple juice with commercial apple juice we may say that fresh apple juice has the most significant antioxidant character (k_{fresh}=0.02, $k_{commercial}$=0.035). A similar situation was found in the case of grape juice;(k_{fresh}=0.017, $k_{commercial}$=0.024). In terms of antioxidant activity, it can be concluded that the studied juices have similar quality fresh juices [18].

V.4.2 Natural extracts

Because oxidative stress mediates the occurrence of adverse effects of radiation on the skin, regular intake or topical application of antioxidants are considered to be useful in reducing the harmful effects of exposure to radiation. Among many photochemoprotective agents, botanical origin antioxidants appear to be most promising.

Grape seeds

The study investigates the protective activity of red grape seeds (Burgund Mare variety) (BM) extracts in vivo on multiple doses of ultraviolet radiation (UV)-B-induced deleterious effects in SKH-1 mice skin [57]. Adaptive changes involving oxidative stress and antioxidant protection were analyzed. Was also evaluated if a topical application of the extract on mice skin before or after irradiation, can inhibit skin injury by modulating the antioxidant defence mecanisms.

The antioxidant activity of BM extract was evaluated by EPR technique, by monitoring Tempol concentrations after the addition of BM extract or gallic acid. The rate of reaction between antioxidant compounds and Tempol was monitored by using normalized double integrated residual EPR signal which is correlated with the number of paramagnetic species in time (Fig V.5) [17, 58].

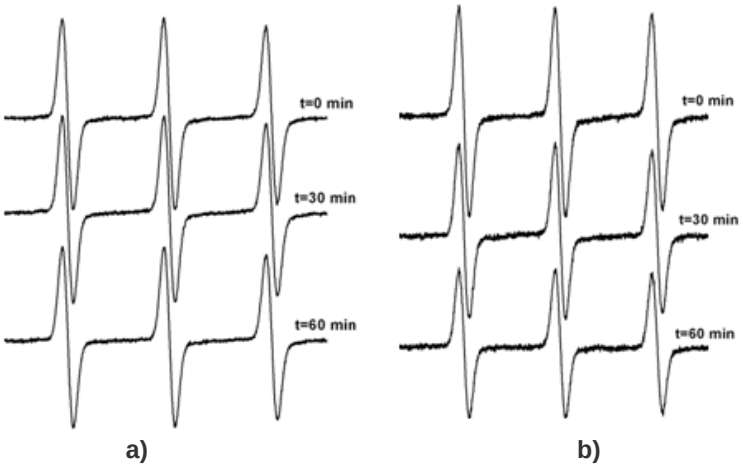


Fig. V.5 EPR spectra of Tempol at different time of incubation in (a) BM extract and (b) gallic acid.

Comparing the antioxidant characteristics of BM extract and gallic acid, we can observe that gallic acid extract has the most pronounced antioxidant character (Fig.V.6).

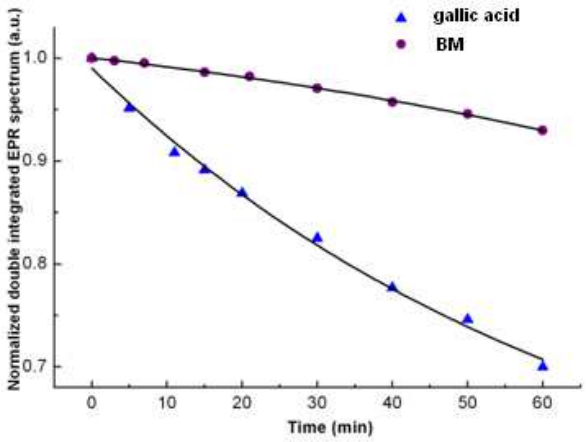


Fig. V.6 Normalized double integrated of EPR spectra for BM extract and gallic acid as function of time

Multiple doses of UV-B generates the formation of sunburn cells and increases glutathione peroxidase (GPx) and catalase (CAT) activities respectively glutathione (GSH) levels in skin. In group treated with 2.5 mg PF/cm^2 before UV-B irradiation, BM extract inhibits UV-B-induced sunburn cells, restores the superoxide dismutase (MnSOD) activity, increases insignificantly CAT and GPx activities. The BM 4.0 mg PF/cm^2 treatment decreases GSH level and reduces the percentage of CPDs positive cells in skin. Both doses of BM extract administered after UV-B irradiation increases the MnSOD and GPx activities and reduces the formation of sunburn cells in skin. Our results suggest that BM extract might be a potential chemo-preventive candidate in reducing the oxidative stress and apoptosis induced by multiple doses of UV-B in skin.

Calluna Vulgaris

It was investigated the activity of Calluna Vulgaris (CV) on skin exposed to multiple doses of UVB in SKH-1 mice, using the same methodology as in case of grape seeds extract. The antioxidant activity of CV extract was evaluated by EPR technique, by monitoring Tempol concentrations after the addition of CV extract or gallic acid (Fig.V.7). The rate of

reaction between antioxidant compounds and Tempol was monitored by using normalized double integrated residual EPR signal which is correlated with the number of paramagnetic species in time (Fig.V.8).

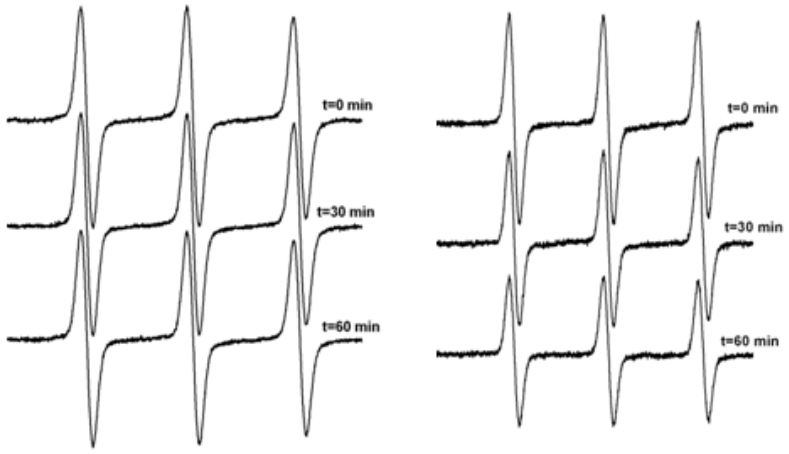


Fig. V.7 EPR spectra of Tempol at different time of incubation in (a) CV extract and (b) gallic acid.

Comparing the antioxidant activity of CV extract with gallic acid activity, we can observe that gallic acid has the most pronounced antioxidant character. It was also found that pretreatment with the extract causes a slight decrease in the number of burn cells, as it found in case of BM extract.

The results on both extracts, suggest that topical application of extracts (BM or CV), significantly suppresses damage induced by multiple exposure to UV-B radiation, by inhibiting DNA injury and apoptosis. It also notes that BM extract has an antioxidant character slightly stronger than CV extract (Fig. V.9), so it seems that BM extract is more effective in blocking chain reactions caused by ROS in the skin after multiple radiation exposure [59].

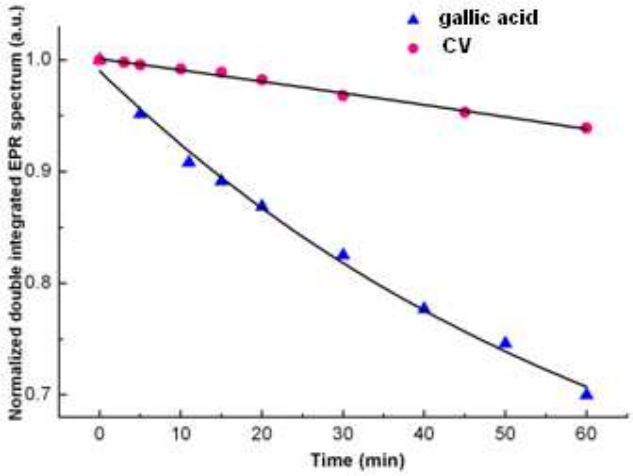


Fig. V.8 Normalized double integrated of EPR spectra for CV extract and gallic acid as function of time

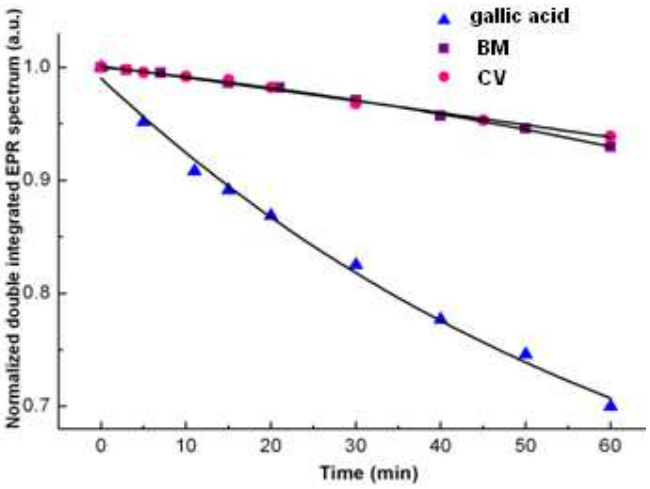


Fig. V.9 Normalized double integrated of EPR spectra for CV extract, BM extract and gallic acid, as function of time

Conclusions

- ESR spectroscopy is a sensitive technique for **direct** or **indirect** detection of free radicals in biological systems and pharmaceutical systems.
- Direct ESR measurements performed on powders of Purinethol and Metformin hydrochloride irradiated with gamma rays show the presence of several stable paramagnetic species, whose relative concentrations depend on the absorbed dose.
- The total ESR signal intensity is linearly dependent on the absorbed dose in case of Metformin, wich shows that this drug can be used as an biodosimetric indicator.
- Photopolymerization of dental resins at room temperature leads to the formation of long-life free radicals due to system vitrification. Within the limits of the present experiments, the radical concentration depends primarily on the initiation system and secondly on the composition of the monomers used in experimental composites.
- Hydroxyl radicals generated in Fenton reaction were detected and characterized by ESR spectroscopy at low and high field (X-band and W band), using PBN spin trap and extraction method with organic solvents.
- Radicals generated by xanthine-xanthine oxidase system were detected by ESR spectroscopy at low and high field, by using three kinds of spin traps: DMPO, DEPMPO and DIPPMPO.
- The superoxide radical was also generated from solvation of KO_2 in an aprotic solvent (DMSO). The presence of superoxide in our samples was confirmed by the addition of the

spin trapping agent DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) followed by ESR spectroscopy analysis (spin trapping technique).

- ESR studies have been also conducted on the antioxidant activity of some commercial juices and natural extracts (grape seeds and Calluna Vulgaris) using nitroxide radical Tempol. In terms of antioxidant activity, it can be concluded that the studied juices have similar quality fresh juices. In case of extracts, our results suggest that both extracts might be potential chemo-preventive candidates in reducing the oxidative stress and apoptosis induced by multiple doses of UV-B in mouse skin.

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