Current Topics in Biophysics 2002, 26(1), 93-99

EPR INVESTIGATION OF IRRADIATED SEEDS USING NITROXIDE SPIN PROBES

M. MARAL SÜNNETÇİOĞLU[1], DILEK DADAYLI PAKTAŞ[2], İSMAIL ERCAN[3]

[1]Department of Physics Engineering, Hacettepe University, 06532, Beytepe, Ankara, Türkiye;
[2]Department of Physics, Faculty of Art and Science, Zonguldak Karaelmas University, 67100, Zonguldak, Türkiye;
[3]TAEA, Ankara Nuclear Research and Training Center, 06100, Ankara, Türkiye.

Membrane permeability changes in wheat and rice embryos as a result of irradiation and re-irradiation were investigated in a temperature range of 223-303 K, using spin probe technique. Aqueous solutions of 4-oxo-TEMPO (TANON) together with line broadening material potassium ferricyanide and 16-doxyl stearic acid (16-DS) were used in the studies. For TANON, the high field line is well resolved into water and lipid parts. The intensity ratio of these peaks I_{water} /I_{lipid} was followed as a function of time for 150 minutes. The values were calculated from recorded spectra. Dependent on the absorbed dose of an exponential decay was observed for both types of seeds. The decay of rice was slower than wheat. Since rice was irradiated in the husk, a decrease in the absorbed dose is an expected result. Irradiated seeds were re-irradiated at the same absorbed doses and effects of this second irradiation investigated. In the studied temperature range, differences were observed between irradiated and control samples for both TANON and 16-DS.

INTRODUCTION

In food science and technology irradiation has wide application. During storage, protection of cereals against microorganisms is provided this way. Overall average dose on foods is currently 10 kGy (Bögl, Regulla & Suess, 1988). Radiation processing of food is legal in many countries but it is still prohibited in some others. In order to prevent any excessive or unnecessary treatment, it is important to determine the degree to which the product has been irradiated. There are several analytical detection methods such as Electron Spin Resonance (EPR), thermoluminescence (TL), chemical and biological methods (McMurray, 1996; Ikeya, 1993). Among them EPR is a well known method with its sensitivity and accuracy. Its detection is based on the presence of paramagnetic species in the material (Onderlinden & Strackee, 1974; Desrodiers, 1996). In foods, radicals produced as a result of irradiation and the decay of these radicals followed by EPR. However, in cereals the decay of the number of radicals to their background levels take approximately a month (Hunter, Hutton & Troup, 1988; Munoz, Adem, Burillo, Gleason & Murrieta, 1994; Murrieta, Munoz, Adem, Burillo, Vazquez & Cabrera, 1996). Germination tests can provide information until 12 months (McMurray, 1996). When the number of free radicals in the system is insufficient for an EPR study, EPR spin probe technique enables the investigation of the system by introducing a molecule carrying an unpaired electron. Nitroxide radicals are widely used as spin probes or spin labels in many branches of science such as chemistry, biology and medicine (Kocherginsky & Swartz, 1995; Berliner, 1976; Berliner & Reuben 1989). Spin probe technique in detection of irradiated seeds is a new application in this area. (Dadaylı, Sünnetçioğlu, Köksel & Çelik, 1997; Sünnetçioğlu, Dadaylı, Çelik & Köksel, 1999) This technique enables the discrimination of irradiated seeds even after long storage times. After the first applications, other studies were also performed on irradiated seeds using various spin probes (Wang & You, 2000; Sünnetçioğlu, Dadaylı, Çelik & Köksel, 1998). Recent studies on seeds concentrated on molecular mobility changes.The effect of these changes on ageing of seeds and stability of foods were searched using echo detected EPR (ED EPR)(Dzuba, Glovina & Tsvetkov, 1993; Buitink, Dzuba, Hoekstra & Tsetkov, 2000; Buitink, Hemminga & Hoekstra, 1999; Buitink, Leprince, Hemminga, Hoekstra, 2000; Hemminga & van der Dries, 1998; Hoekstra, Wolkers, Buitink, Golovina, Crowe & Crowe, 1997), and saturation transfer EPR (ST EPR). Irradiation of foods can be thought as an artificial ageing mechanism but at the same time it is necessary for sterilisation purposes. Therefore investigation of the effects of irradiation in terms of molecular mobility is important to lengthen the shelf life of seeds and other foods.

The aim of the current study is to investigate the applicability of the previously introduced method Sünnetçioğlu, Dadaylı, Sugar & Bingöl, 1997) for

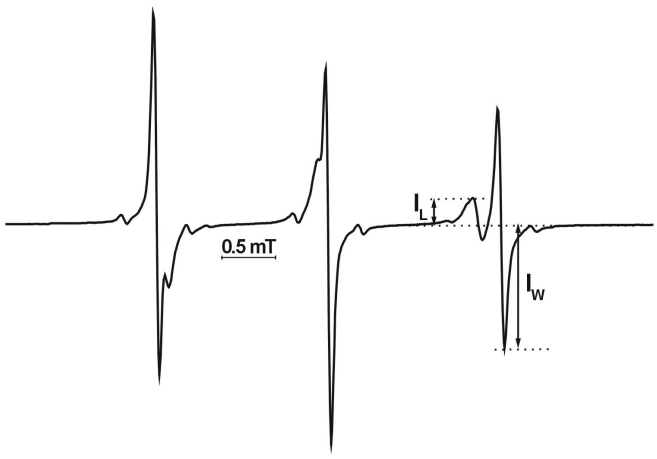

Fig. 1. Measured intensities of high field resonance line.

various seeds using different spin probes and also to search the temperature response of control and irradiated seeds.

MATERIALS AND METHODS

Wheat (*Triticum aestivum L.* -Pehlivan) and rice (*Oryza sativa*-Osmancık) seeds harvested in 1997 were used in the present studies. Germination tests were carried out and 98% germination for wheat and 92% for rice was obtained. Irradiation of the samples was performed at Sarayköy Nuclear Research Institute of TAEA using γ-irradiation from calibrated ^{60}Co source. Samples received 1.0, 2.5, 5.0, 10.0, 20.0 kGy doses. First irradiation of the samples were performed at the beginning of January, 2001, and later at the end of April a part of irradiated samples were re-irradiated at the same doses. For spin probe studies, after irradiation, samples were stored in a steel cabinet at room temperature for 30 days. Experiments were performed between February and July. Dry embryos of wheat and rice seeds were used in the studies. Spin probes 4-oxo-TEMPO (TANON) and 16-Doxyl-stearic acid (16-DS) were provided from Sigma-Aldrich. Aqueous solutions of TANON prepared at 1mM concentration and used with and without addition of 100

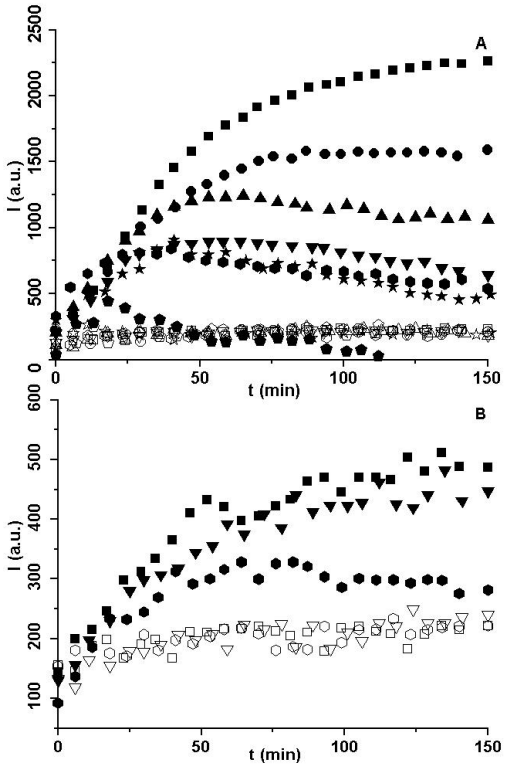

Fig. 2. Rehydration curves for A) wheat and B) rice embryos. In the figure L and W indicates lipid and aqueous parts respectively (solid). Control (squares), 1 kGy (circles), 2.5 kGy (up triangles), 5 kGy (down triangles), 10 kGy (hexagons), 20 kGy (pentagons), re-irradiated 5 kGy (stars).

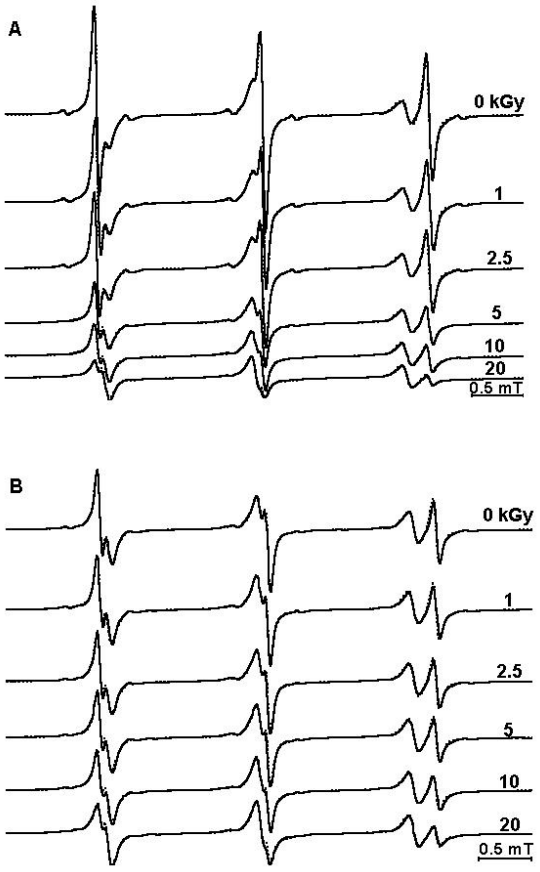

Fig. 3. The spectra recorded with the samples prepared according to method II. Solid line: experimental, dotted line: calculated. A) wheat B) rice.

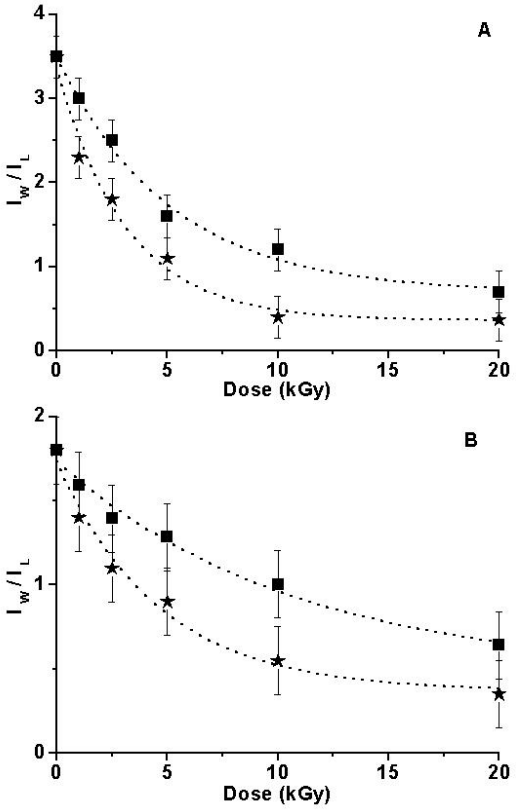

Fig. 4. The decay of the I_W/I_L ratio against dose for first (squares) and second (stars) irradiation. (A) wheat (B) rice.

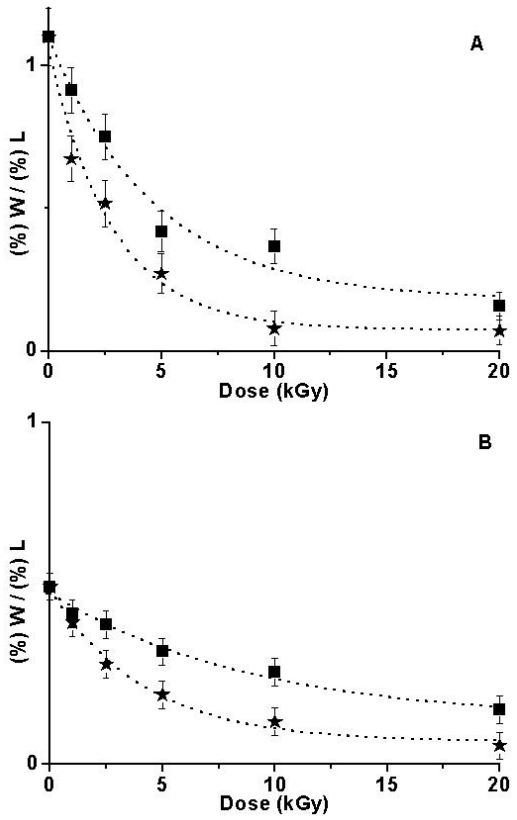

Fig. 5. The decay of the percentage ratio of TANON from aqueous intracellular and lipid regions for first (squares) and second (stars) irradiation. (A) wheat (B) rice.

mM line broadening material $K_3Fe(CN)_6$ (Potassium Ferricyanide). Line broadening material was used to discriminate the signal from intracellular regions. At first, rehydration behavior of the wheat and rice embryos was followed by recording the spectra approximately every 6 minutes for 150 minutes at all irradiation doses. For re-irradiated samples 2.5 and 5 kGy doses were studied. These studies performed using single embryos and repeated for two times.

Secondly, 5 dry embryos were soaked for 150 minutes in the spin label solution in a glass tube and then taken onto glass plates using a paint brush. Samples were air dried for 15 minutes and their spectra were recorded. Both irradiated and re-irradiated samples were studied in the whole dose range. This type of the experiments was repeated at least three times.

For 16-DS, line brodening material was not used and after soaking in the prepared solution, embryos were washed five times with distilled water.

Table. The fitted values for the regions of embryo.

SAMPLE	LINE WIDTHS (mT)			A_N	A_C	Gaussian	I_N / I_C
m_I	+1	0	-1	(mT)	(mT)	(%)	
TANON / Region I	0.037	0.037	0.048	1.600	0.606	25	25
TANON / Embryo lipids	0.067	0.067	0.083	1.455	0.540	10	37

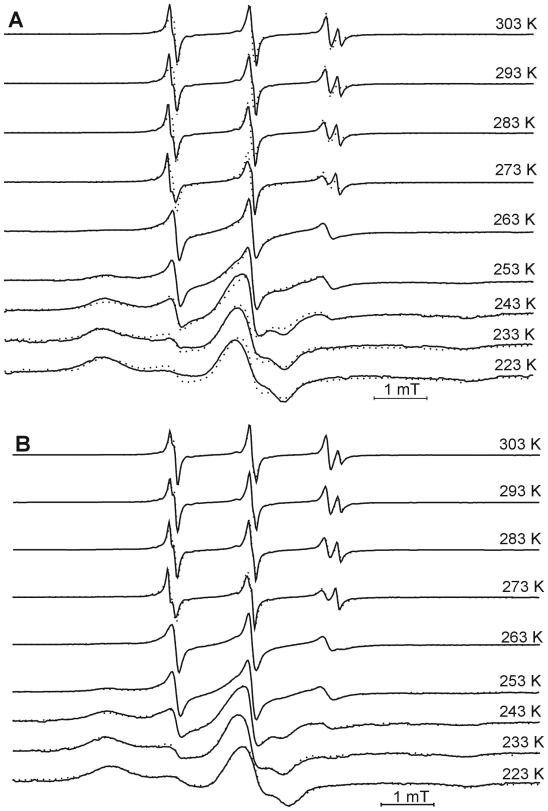

Fig. 6 Temperature dependent spectra of control (solid line) and 10 kGy(dotted line) samples in aqueous TANON solution. A) wheat B) rice.

Temperature dependent differences in wheat and rice seeds were investigated in the range of 223-303 K using the second method explained above. In the studies control and 10 kGy samples were used.

The spectra were recorded by use of a Bruker EMX spectrometer using the following spectral conditions; modulation amplitude: 0.05 mT, microwave power: 2 mW. For studies with 16-DS modulation amplitude: 0.1 mT, microwave power: 10 mW. All experiments were performed at temperatures controlled to ± 1 K.

RESULTS AND DISCUSSION

Intensity-time (rehydration) curves for TANON were drawn for both wheat and rice embryos at all doses studied. In the spectra high field line ($m_I = -1$) is well resolved into the lipid (L) and water (W) components. However, to prevent the errors from overlapping effects at the bases of the peaks, instead of peak to peak intensities I_W, I_L, values shown in Figure 1 were used in the measurements (Golovina, Tikhonov & Hoekstra, 1997). The signal from lipid parts was almost constant for

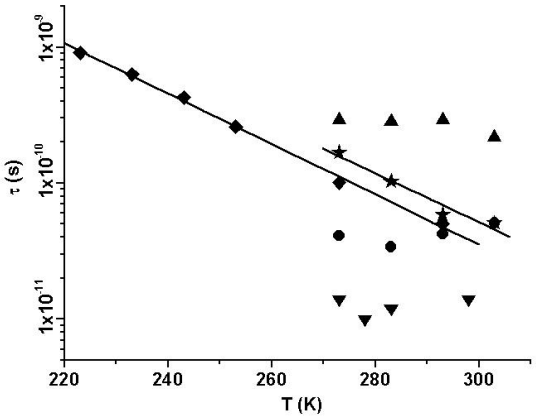

Fig. 7 The change of correlation times of TANON against temperature: in wheat lipids (stars), region II (up triangles), region I (circles), in models: sunflower oil (squares) , water (down triangles).

all samples except small deviations. Therefore average value of lipid signal intensity was calculated and all signal intensities from aqueous parts were arranged in a way to leave the signal intensity from lipid parts constant. For both wheat and rice embryos dose dependent order was observed (Fig. 2). In the intensity-time curves of rice, signal intensity was low and dose dependent differences were not much pronounce in comparison to wheat. The reason is thought to be the screening effect of the outer cover of rice. The rehydration behaviour of Pehlivan was similar to previously studied kinds Kunduru and Gerek. (Sünnetçioğlu, Dadaylı, Sungur & Bingöl, 1997). In Fig. 2A the rehydration of the re-irradiated 5 kGy sample was also shown. The curve-shape of this sample closely resembles the 10 kGy sample indicating the additive effect of dose.

In the studies according to the second method, the dose dependent order can clearly be followed in the spectra (Fig 3A, B). As I_W/I_L ratio was drawn against dose an exponential decay could be directly observed (Fig 4A, B). In a recent study the exponential decay of the signal was shown by simulation of spectra using 4-hydroxy-TEMPO (TANOL). In Fig. 4A and B the decay of re-irradiated samples were also pointed. The decay rate in re-irradiated samples is almost twice the single irradiated ones. During the measurements of control samples, a slight decay in the I_W/I_L ratio was observed and therefore mean values were used in the evaluations.

As a second test of exponential behaviour, the simulation of spectra was performed for all samples and in the whole dose range. In the simulations, the signals of TANON from (i) aqueous parts of the healthy cells (region I); (ii) aqueous parts of the damaged cells (including the line

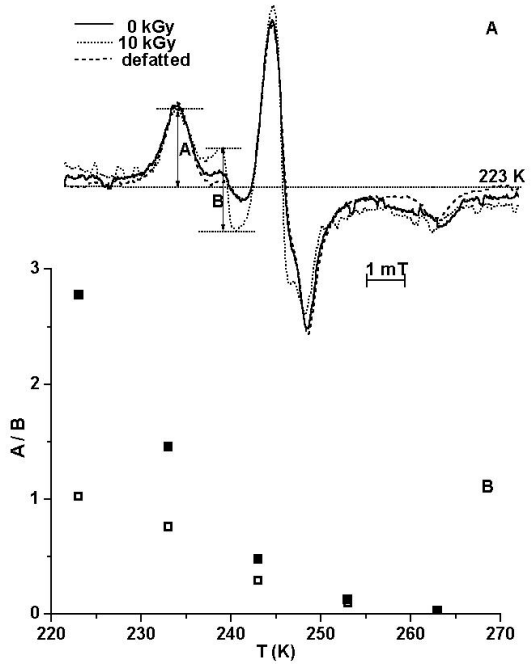

Fig.8 (A) 223 K spectra of control, 10 kGy and defatted wheat embryos. (B) A/B values against temperature: control (solid squares), 10 kGy (open squares).

broadening material)(region II); (iii) lipid parts were summed (Sünnetçioğlu & Dadaylı, 2000). For TANON, the ^{13}C satellites are clearly observable in the spectra. Therefore, in the present simulations these peaks also taken into account. Results are listed in the Table and Fig. 3A, B. The values given in the Table were obtained by simulation of control and 20 kGy samples. For ^{13}C satellites the same line widths and Gaussian percentages were used and intensity ratio of nitrogen to carbon was also given. The values obtained were used for both wheat and rice embryos. Keeping the fitted values constant, percentages of TANON in aqueous (region I) and lipid parts at all doses were calculated. The change of the percentage ratio of these parts was drawn against dose (Fig. 5A, B). From both spectral intensities and water percentages, similar results were obtained for decay constants. The error bars in Figures 4 and 5 were calculated from the distribution of the experimental data. The mean standard deviation for simulations was 0.02.

Low temperature spectra of the control and 10 kGy embryos of wheat and rice were also investigated to receive information about mobility changes. Two nitroxides TANON and 16-DS were used in the studies. Three line spectrum was observed for TANON between 303-263K. However at 263 K the first signs of slow motion became visible in the spectra. Interestingly, the differences between control and 10 kGy samples were minimal only at 263 K. Below and above this temperature differences were observed apparently for wheat embryos. For rice, the differences were again small (Fig. 6A, B). Model studies were also performed for TANON in sunflower oil and water. The spectra of the aqueous sample below 273 K were a single line character similar to powder.

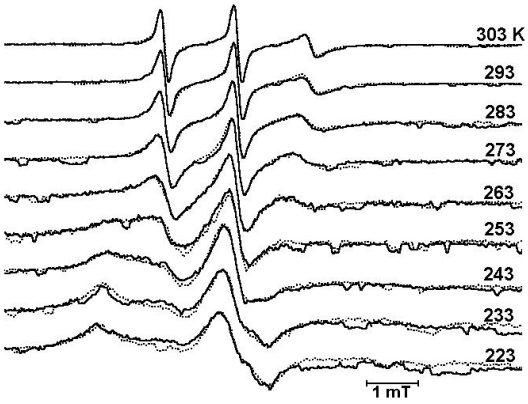

Fig. 9 Temperature dependent spectra of control (solid line) and 10 kGy (dotted line) samples in aqueous solution of 16-DS.

The evaluation of spectra was performed in two ways. In the 268-303 K range rotational correlation times were calculated according to (Knowles, Marsh & Rattle, 1998)

$$\tau_R = 6.5 \cdot 10^{-6} \Delta B_0 [(\Delta B_{-1} / \Delta B_0)^{1/2} - 1]$$

The line widths were calculated from simulation of spectra for the three regions. The results were shown for wheat seeds together with model study data for TANON in sunflower oil and water at 1mM concentration. A consideration of Fig. 7 reveals the similarity of the change of correlation times against temperature for sunflower oil and wheat lipids. The correlation times were slightly higher for wheat lipids. As seen from the figure, the correlation times of TANON in aqueous parts are quite different from each other. In the temperature range of calculations their correlation times are approximately constant. These values were calculated for control samples only. For 10 kGy sample the line widths of region I and lipids are the same and there is a change in the line width of the signal from region II. For lower temperatures the change of *A*/*B* ratio for irradiated and control samples were indicated in Fig. 8A. The difference in the *A*/*B* ratio of control and 10 kGy wheat samples is pronounced between 223 and 250 K (Fig. 8B). The effect of irradiation at low temperatures seems to liquefy the lipid environment. Another experiment was performed by extraction of

storage lipids of embryo. Extraction was performed as stated in a previous study (Dzuba *et al.*, 1993). For defatted embryos a difference could not be observed between control and 10 kGy samples (Fig. 8A).

For 16-DS, temperature dependent studies indicated some differences, as well. Direct spectral evaluation is possible by the use of nitroxide spin probes having smaller line widths. These differences were increased as the temperature decreased (Fig. 9).

CONCLUSION

The studies verified in a practical way detection of irradiated seeds even after long term storage. The use of nitroxides having smaller line widths provides direct detection of irradiation from intensity ratios. Since irradiation is widely used for sterilisation and preservation purposes on foods, the observed differences at low temperatures is important. Therefore, further studies are necessary on irradiated seeds and foods at low temperatures. Studies have been continue about the effects of irradiation at lower irradiation doses and at low temperatures.

Acknowledgements

We thank TUBITAK for the financial support of the project TBAG-1867(199T107).

REFERENCES

Berliner L. J. (1976). *Spin Labeling: Theory and Applications*. Academic Press: New York.

Berliner L. J. & Reuben J. (1989). *Biological Magnetic Resonance*, Plenum Press, New York.

Bögl K. W., Regulla D. & Suess M. (1988). *Health Impact, Identification and Dosimetry of Irradiated Foods.* MMV Medicine Verlag: München.

Buitink J., Dzuba S. A., Hoekstra F. A. & Tsvetkov Yu. D. (2000). Pulsed EPR Spin-Probe Study of Intracellular Glasses in Seed and Pollen. *J. Magn. Reson.*, **142**, 364-368.

Buitink, J., Hemminga M.A. & Hoekstra F.A. (1999). Characterization of molecular mobility in seed tissues: An Electron Paramagnetic Resonance Spin Probe study. *Biophysical Journal*, **76**, 3315-3322.

Buitink J., Leprince O., Hemminga M.A. & Hoekstra F.A. (2000). Molecular mobility in the cytoplasm: An approach to describe and predict lifespan of dry germplasm. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 2385-2390.

Dadaylı D., Sünnetçioğlu M. M., Köksel H. & Çelik S. (1997). Detection of irradiated wheat using the electron paramagnetic resonance spin probe technique. *Cereal Chem.*, **74**., 375-**[last page]**.

Desrodiers M. F. (1996). Current Status of the EPR Method to Detect Irradiated Food. *Appl. Radiat. Isot*., **47**, 1621-**[last page]**.

Dzuba S. A., Golovina Ye. A. & Tsvetkov Yu. D. (1993). Echo induced EPR spectra of spin probes as a method for identification of glassy states in biological objects. *J. Magn. Reson*., **B 101**, 134-138.

Golovina E. A., Tikhonov A. N. & Hoekstra F. A. (1997). An Electron Paramagnetic Resonance Spin-Probe Study of Membrane-Permeability Changes with Seed Aging. *Plant Physiol*., **114,** 383-389.

Hemminga M.A. & van den Dries, I.J. (1998). *Spin label applications to food science in biological magnetic resonance.* Vol. 14, *Spin Labeling* The Next Millenium, edited by Berliner L. J., Chapter 8

Hoekstra F. A., Wolkers W. F., Buitink J., Golovina E. A., Crowe J. H. & Crowe L. M. (1997). Membrane Stabilization in the Dry State. *Comp. Biochem. Physiol*., **117A**, 335-341.

Hunter C. R., Hutton D. R. & Troup G. J. (1988). Monitoring Free Radicals in γ-irradiated foods. *Search*, **19**, 198-199.

Ikeya, M. (1993). *New Applications of Electron Spin resonance: Dating, Dosimetry and Microscopy*. World Scientific Publishing Co. Pte. Ltd.: Singapore.

Knowles P. F., Marsh D. & Rattle, H. W. E. (1998). *Magnetic Resonance of Biomolecules.* John Wiley & Sons: Bath.

Kocherginsky N., H. M. Swartz. (1995). *Nitroxide Spin Labels, Reactions in Biology and Chemistry*. C. R. C Press.

McMurray C. H. (1996). Detection Methods for Irradiated Foods-Current Status. *Special Publication* no. 171, The Royal Society of Chemistry: Cambridge.

Munoz, E. P., Adem, E., Burillo,G., Gleason, R.V., Murrieta, H.S. (1994). ESR studies of irradiated ground corn as a dosimeter, *Radiat. Phys. Chem*., **43**(4), 311-313.

Murrieta H. S., Munoz E. P., Adem E., Burillo G., Vazquez M. & Cabrera, E. B. (1996). Effect of Irradiation Dose, Storage Time and Temperature on the ESR Signal in Irradiated Oat, Corn and Wheat. *Appl. Radiat. Isot*. **47**, 1657.

Onderlinden D. & Strackee L. (1974). ESR as a tool for the identification of irradiated material. [In:] *International Colloquium on the Identification of Foodstuffs* (p. 127-140). Commision of the European Communities: Luxembourg.

Sünnetçioğlu M. M. & Dadaylı D. (2000). The use of simulation in the EPR spin probe technique for detection of irradiated seeds. *Talanta*, **53**, 69-74.

Sünnetçioğlu M. M., Dadaylı D., Çelik S. & Köksel H. (1998). Application of the Electron Paramagnetic Resonance Spin Probe Technique for Detection of Irradiated Wheat. *Cereal Chem.*, **75**, 875-878.

Sünnetçioğlu M. M., Dadaylı D., Çelik S. & Köksel H. (1999). Use of EPR spin probe technique for detection of irradiated wheat. *Applied. Radiat. Isot*., **50**, 557-560.

Sünnetçioğlu M. M., Dadaylı D., Sungur R. & Bingöl G. (1997). An EPR study of wheat seeds by the use of nitroxide spin probes. *J. Plant. Physiol*., **151**, 196-200.

Wang Z. & You R. (2000). Changes in wheat germination following γ-ray irradiation: an in vivo electronic paramagnetic resonance spin probe study. *Environ. Exp. Botany*, **43**, 219-225.