THE GAMMA IRRADIATION EFFECT ON THE ANTIOXIDANT AND LIPID OXIDATION ACTIVITY OF ALMOND (*PRUNUS DULCIS L.*) OIL

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ABSTRACT

Almond nuts (Prunus dulcis, Amygdalus subgenus inside the Prunus genus, Rosaceae family) are cultivated globally and 30 % of their worldwide production is derived from the Mediterranean region. The almond nut's main components, like lipids (oleic, linoleic, palmitic, palmitoleic, and stearic acid), proteins, soluble sugars, minerals, fibrous, and phytochemicals, are responsible for ROS/RNS detection. In recent years, food irradiation has been used to protect against microorganisms, oxidative processes, and radiation-induced toxicity, and this methodology is internationally recognized for effective long-term storage. The ROS/RNS detection by Electron paramagnetic resonance spin - trapping provides useful information on the susceptibility to oxidative stability of gamma (γ) - irradiated food/oils for comparative purposes.

In the current study by using two different methods in vitro were evaluate and compared radical- scavenging abilities and lipid oxidative stability of almond oil, at 0 kGy, 10 kGy, 25 kGy radiation, re-oxidized at 50°C. It was found that a 10 kGy irradiation dose increased membrane protection activity in the lipid phase. 10 kGy irradiated almond oil showed high reproducibility, on the 1 min - 60 min (57.3 μmol kg^{-1} oil), while 25 kGy irradiated extracts showed a slight decrease. Based on the EPR signals, 10 kGy irradiated almond oil show almost commensurate singlet line intensity, but with a slight change in the g value (g = 2.0054; o -semiquinone radical originates from the polyphenol substances), as opposed to 25 kGy doses decreased signal intensity and change the g = 2.0050, compared to non-irradiated oil.

Keywords: almond oil, PBN-adducts, radiation.

INTRODUCTION

Almond nut (*Prunus dulcis*, belongs to the *Amygdalus* subgenus inside the *Prunus genus*, *Rosaceae* family) is cultivated globally and 30 % of its worldwide production derived from the Mediterranean region [1, 2]. In their investigations, Roncero et al. comment, that the main components in almond nut are: lipids (oleic, linoleic, palmitic, palmitoleic and stearic acid), proteins, soluble sugars, minerals, fibrous and phytochemicals [2]. In ripe almonds, fatty acids are in the triglycerides (> 98 %) form, which leads to a low acidity index, i.e. the sequence and quantity of almond oil triglycerides determine the ultimate functional properties [2]. Alasalvar and Pelvan, and Pasini et al., found that quantitatively small unsaponifiable fractions (sterols, aliphatic alcohols and vitamins), complex lipids (phospholipids and glycolipids) and fat-soluble bioactive substances

(tocopherols, tocotrienols, phospholipids, phytosterols, tertinopels, phytostanols, phytostanols with phytostanols with of great importance for the biological, antioxidant and nutritional role of almond oil [3, 4]. The almonds or almond oil use in the diets, reduces the saturated fatty and trans fatty acids absorption and increases the linoleic and oleic acid consumption, while leading to weight loss and reduced blood LDL cholesterol [5]. Nut and almond oil consumption reduces the blood lipid levels, decrease cardiovascular disease, supports intestinal transit, lowers blood pressure, prevents anemia and cancer, protects against free radicals, etc. [6, 7]. Mahfouz, draws attention to the fact that almonds are susceptible to infection by molds, insects, larvae, fungi and their storage is carried out through chemical, biological and combined controls [1].

In the last decade, food irradiation has been used to protect against microorganisms, oxidative processes and radiation-induced toxicity, and this methodology is internationally recognized for effective long - term storage. Almonds contain high unsaturated fatty acids levels, prone to lipid peroxidation and oxidative disorders in radiation. After radiation (5, 10, 25 kGy), the levels of free radicals (ROS/RNS), esters, aldehydes and ketones sharply increase as a result of the radiolysis process and unsaturated fatty acids suffer damage. After radiation, the ROS/RNS levels, esters, aldehydes and ketones increase sharply as a result of the radiolysis process and these compounds responsible for unsaturated fatty acids structural changes [8]. The Directive 1999/3/EUN of the EP established a Community list of food and food ingredients that maybe treated with ionizing radiation and maximum overall average absorbed dose could be 10 kGy for food containing cellulose (nuts, berries, dry herbs and spices, vegetable seasonings), meat and fish bones and food containing crystalline sugars (dried figs, mangoes, papayas and raisins) [9].

Electron paramagnetic resonance (EPR) identified different inorganic, organic, transition metal species, chemical composition changes and spectrum shapes difference, as a time, temperature or radiation type function [10]. EPR is the promising *in vitro* method for γ-irradiated free radicals detection by stable spin-adduction to determine the lipids oxidative stability in nuts/nuts oil under slightly accelerated storage conditions/exogenous factors that promote oxidation processes (humidity, temperature at 60°C, oxygen content in the storage atmosphere, ultraviolet radiation (UV - B), etc.) [11, 12].

The main purpose of this study using *in vitro* EPR spectroscopy were to evaluate and compare radio-modulatory abilities and lipid oxidation properties in almond oil during storage at 50°C oxidation, before and after 10 and 25 kGy exposure.

EXPERIMENTAL

Almond samples preparation and oil extraction

In the study, almond nuts (*Prunus dulcis*, without shell) samples were purchased from local markets in Southern province in Bulgaria. All samples (250 g/ package) were transferred into self-sealable low-density polyethylene (LDPE) pouches for long storage irradiation procedure. The samples were kept in a refrigerator (± 4°C) until irradiation exposure to 10 kGy and 25 kGy.

The 30 g per almond samples (0 kGy; 10 kGy and 25 kGy) were ground and extracted with hexane for 8 h in the Soxhlet apparatus [13]. In brief, the lipid sample is weighed quantitatively and dissolved in 2 mL chloroform - methanol (1:1) mixture. After addition of 0.04 mL 50 % potassium iodide solution (50 %, KI) the mixture is incubated in dark for 2 min. Subsequently, 5 mL distilled water and starch solution (3 drops) was added and the separated iodine is titrated with 0.002 N $Na_2S_2O_3$. The solvent was distilled under a vacuum and the residue was weighed, calculated per fat content and 10 % stock oil/hexane solution were used for analyses.

Chemicals

Cholesterol (1:1 molar ratio), Soyalecithin, R-Phenyl-N-tert-butylnitrone (PBN) (purity > 97 %) were purchased from Sigma Chemicals, USA. Deionized and distillated water was used for all experiments. Other chemicals used were analytical or HPLC grade.

γ-irradiation procedure

Two parallel almond nuts samples (100 g each) were γ-irradiated at 10 kGy and at 25 kGy using ^{60}Co source with 8200 Ci activity (National Centre Radiobiology and Radiation Protection, Sofia, Bulgaria). The γ-ray facility has a mobile irradiation chamber with 4.0 L volume and dimensions: 13.5 cm diameter and 22 cm height. During the irradiation, the chamber rotates on its vertical axe. For the study of the absorbed dose

distribution Alanine dosimeters (Kodak BioMax) were used, measured by ESR spectrometer E^{-scan} Bruker and calibrated in units of absorbed dose in water. In each point, three dosimeters were placed. Non - irradiated almonds, 10 kGy and 25 kGy irradiated nuts were used in the experiment.

Membrane protection activity against γ-irradiation - induced damage in lipid phase

Cholesterol and Soyalecithin (1:1 molar ratio) were suspended in chloroform. A thin film was developed by complete chloroform evaporation in a rotary evaporator (Buchi, New castle, USA) at 40°C. The film was subjected to hydration in (0.1 M, pH 7.4) PBS and were incubated in (40°C) water bath for 4 h. The stock solution was diluted with PBS to the final concentration in terms of phospholipid content [14]. Different treated almond oil samples (0 kGy, 10 kGy and 25 kGy irradiated), liposome only (untreated), radiation only (10 kGy; 25 kGy), liposome + alomond oil; liposome + 10 kGy alomond oil and liposome + 25 kGy almond oil were evaluated for the malondialdehyde (MDA) levels, the final membrane degeneration product. After incubation for 1h at 37°C, 10 % TCA and 0.5 % thiobarbituric acid, 1:1 ratio and 0.025 M NaOH were added. The mixture was heated in water bath (50ºC) for 1h and absorbance was measured at 535 nm [15].

Direct EPR spectroscopy evaluation of oxidative stability of almond oils under irradiation storage conditions

Six months after 10 kGy and 25 kGy irradiation storage, the three oils as well as the non-irradiated almond oil were examined by direct EPR (X-band - EMX ^{micro}, Bruker, Germany) spectroscopy. Spectral processing and g - value calculation was performed with Bruker WIN -EPR and Sim - Fonia software. The following EPR settings were used: center field 3513.50 G, microwave power 20.03 mW, modulation amplitude 10.00 G; gain 2 x 10^2; time constant 327.68 ms; sweep time 61.44 s.

Oxidation conditions after γ - irradiation - induced damage in lipid phase

PBN - adducts formations were investigated by directly PBN dissolving in the three oils samples (0 kGy; 10 kGy; 25 kGy), at 215 mg g^{-1} oil concentration, by stirring at room temperature, and 0.5 mM PBN alone were used as control. To test the γ - irradiation induced damages in lipid phase and lipid oxidation stability; the samples were oxidized in air at 50°C in the dark, by using P_2O_5, and 0 % humidity [11]. The each almond oils were divided into 3 independent tubes (aliquots 0.5 mL^{-1}) and was left open. The tubes were placed into a desiccator containing P_2O_5, and the desiccator was placed in an oven. The air in the desiccator was replaced during each sampling. Three tubes of each oils were taken periodically, the contents were mixed, and examined triplicate on different time intervals by EPR.

Statistical Analysis

EPR spectral processing was performed using Bruker Win - EPR and Sim - fonia Software. Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., one-way ANOVA, Student t - test to determine significant difference among data groups. The results were expressed as means ± standard error (SE). A value of $p < 0.05$ was considered statistically.

RESULTS AND DISCUSSION

γ-irradiation is currently used for disinfestation, pasteurization, and sterilization of food products and is an effective way of inactivating foodborne pathogens [8]. Gecgel et al., accentuate that γ-irradiation high doses may result in food texture changes and oxidative membrane - lipids degradation [16]. 10 kGy is the maximal dose allowed for commercial food processing [16]. γ-irradiation produces free ROS/RNS responsible for color, taste changes, or nutritional value of irradiated foods. In addition, Čolić et al., comment that monounsaturated (MUFA, 60 %) and polyunsaturated fatty (PUFA, 30 %) acids and large vitamin E and fiber amounts in the almonds and almond oil structure are prone to lipid peroxidation and changes in oxidative stability [17]. Phospholipids, a cellular membrane major component, form lipid bilayers in the membranes and act synergistically with tocopherols to delay the lipid oxidation processes [17]. Membrane macromolecules oxidation leads to persistent oxidative disorders and intracellular processes obstruction [18].

γ-irradiation increase membrane protection activity in lipid phase

In fact, almond oil polyphenols and flavonoids,

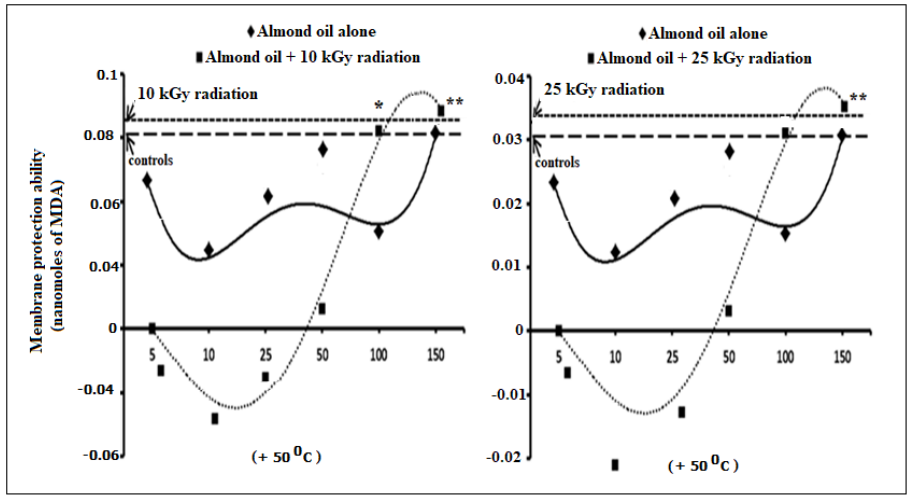

Fig. 1. Membrane protection ability analysis (%) of almond oil alone, almond oil + 10 kGy radiation and almond oil + 25 kGy radiation utilizing an artificial membrane system (liposome). A significant decrease in the malondialdehyde (MDA) formation was recorded at 150 μg/mL^{-1} of 10 kGy and 25 kGy irradiated almond oil samples, ** $p < 0.05$ and ** $p < 0.004$, respectivelly. The lipid peroxidation activity is expressed as nanomoles of MDA formation. Each experiment was performed in triplicate and was repeated three times.

concentrated at the lipid interface, have lipid oxidation inhibition by reactive lipid - peroxyl radical suppression and reduce ROS/RNS initiation of the additional lipid peroxidation [19]. The membrane system (artificial liposomes) was utilized to estimate the almond oil possibility to protect the membrane lipids of liposomes against 10 kGy and 25 kGy oxidation. The most effective dose for peroxidation inhibition in the tested liposome system was 100 μg mL^{-1}. Non-irradiated almond oil exhibited significantly higher membrane protection in lipid phase at 150 μg/mL^{-1} for both, 10 kGy and 25 kGy irradiated samples, 0.083 ± 0.17%; **$p < 0.05$ and 0.039 ± 0.06%; ** $p < 0.004$, respectively (Fig. 1). The maximal lipid oxidation protection was registered in the test region at 100 - 150 μl/mL^{-1} for both, 10 kGy and 25 kGy samples.

Interestingly, 10 kGy oil samples indicated two-fold, and 25 kGy almond oil samples indicated one-fold higher anti-lipid oxidation, compared to non-irradiated almond oil ($p < 0.05$). Exogenous stressors (50°C and 10 kGy, 25 kGy radiation) promote endogenous enzymatic oxidation, such as lipoxygenase (LOX). LOX activation usually occurs after plant tissue destruction and is accompanied by sequential processes of fatty acid release and lipid oxidation initiation [20]. We assume that the highest membrane protection in the lipid phase after 10 kGy irradiation is due to the stable almond oil antioxidant activity and LOX un-activation [20]. 10 kGy radiation only does not activate LOX, but also protects against residual lipid auto -oxidation processes, i.e. prevents the hydroperoxides and volatile compounds accumulation that lead to organoleptic changes in food. On the other hand, Salcedo et al., comment that the nuts/nut oils polyphenolic compounds - phenolic acids, flavonoids, tannins - proanthocyanidins and hydrolyzable are with potent ROS/RNS ability and able to break the lipoperoxidation multiplication chain [20]. Regarding these findings, it could be concluded that in 10 kGy irradiated almond oil antioxidant compounds are localized within the intra/cellular membrane and act as ROS/RNS oxidative preventers, decrease in malondialdehyde inhibition in the lipophilic environment, after accelerated storage conditions [21, 22].

During storage, lipid oxidation is responsible for the nuts oil quality loss, causing toxic by-products such as reactive carbonyl compounds (RCC), which generate lipid oxidation advanced end products, potentially harmful to human health [7, 23]. The lipid oxidation degree depends on the unsaturation degree, the antioxidants and metals (copper and iron) presence, and contact of nut oils with exogenous stressors, like oxygen, temperature, and radiation [23]. Exposure to these factors leads to the free radicals induction, as central and highly reactive intermediates. Direct lipid

radicals detection by EPR is not possible due to their very short half -life. Lipid radicals (·L) can be detected indirectly by EPR, by stable spin - adducts formation [24]. The radical's detection by EPR inhibition was used to assess early/late oxidative events in oils and various foods, and to assess the rate and lipid oxidation pathway. In this regard, resistance to PBN spin - adducts has shown utility in detecting antioxidant efficacy; in determining the lipid oxidation initiation, and as a method often used for oxidative changes assessment in foods, nuts, and almond, walnut, peanut oils [11, 25, 26]. EPR spin - trapping has also been applied as a test to determine oxidative stability of food and oil lipids under accelerated conditions at 50°C - 60°C temperatures for 16 days or after 4 weeks [11, 25, 26], and after radiation exposure. As expected: 1) less radical accumulation was attributed to less oil oxidation and greater oxidative stability; and 2) the induction period decreased and the number of radicals at a fixed time decreased with storage time [11, 25, 26].

In a recent report, we sought to elucidate the extent to which the oxidative stability and lipid radical stability in almond oil, including non-irradiated and exposed to 10 kGy, 25 kGy radiation, is maintained even under accelerated storage conditions (at 50°C for 24 h).

10 kGy irradiation activate lipid oxidation - stress tolerance under accelerated storage conditions

EPR signals were detected in all tested almond oil samples before radiation (0 kGy), at six months after irradiation (10 kGy, and 25 kGy radiation), and under accelerated storage conditions, at 50°C for 24 h. The Fig. 2A presents the EPR spectra of the untreated almond oil.

As expected, in the untreated almond oil samples of raw almond materials a singlet almost symmetric signal with g = 2.0042 was well visible, with intensity depending on the specific almond oils matrix. It is accepted, that this EPR signal originates from stable o-semiquinone radical structures produced by polyphenols oxidation, attributable to non-irradiated food/oils from plant origin. Similar results were registered in investigations of peanuts and hazelnuts shell before radiation [10, 27]. EPR spectra with the same central line increased intensity were registered in both irradiated oil samples (Fig. 2A). 10 kGy irradiated almond oil show almost commensurate singlet line intensity, but with a slight change in the g value (g = 2.0054), as opposed 25 kGy doses decreased signal intensity and change the g = 2.0050. The satellite "cellulose - like" lines (9 G peak to peak), specific after irradiation, were visible in the 10 kGy almond oil, but in 25 kGy the left line appeared not

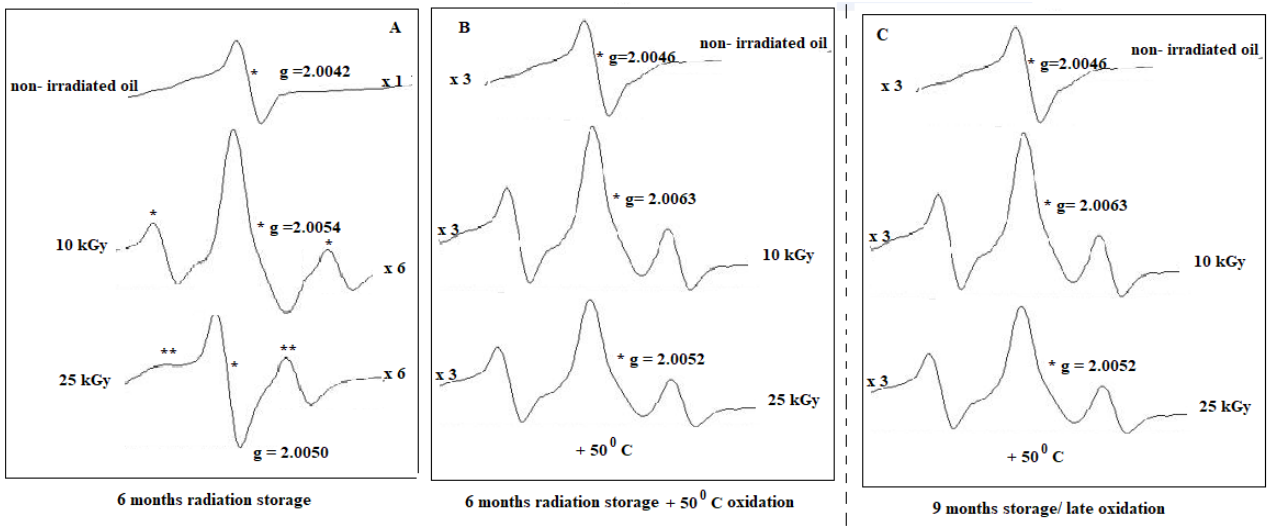

Fig. 2. Direct EPR spectroscopy evaluation of almond oils oxidative stability under irradiation storage conditions, recorded: (A) on 6 months after irradiation; (B) on 6 months after irradiation and re-oxidized at 50°C for 24 h; (C) on 9 months after irradiation and re-oxidation. Each experiment was performed in triplicate and was repeated three times.

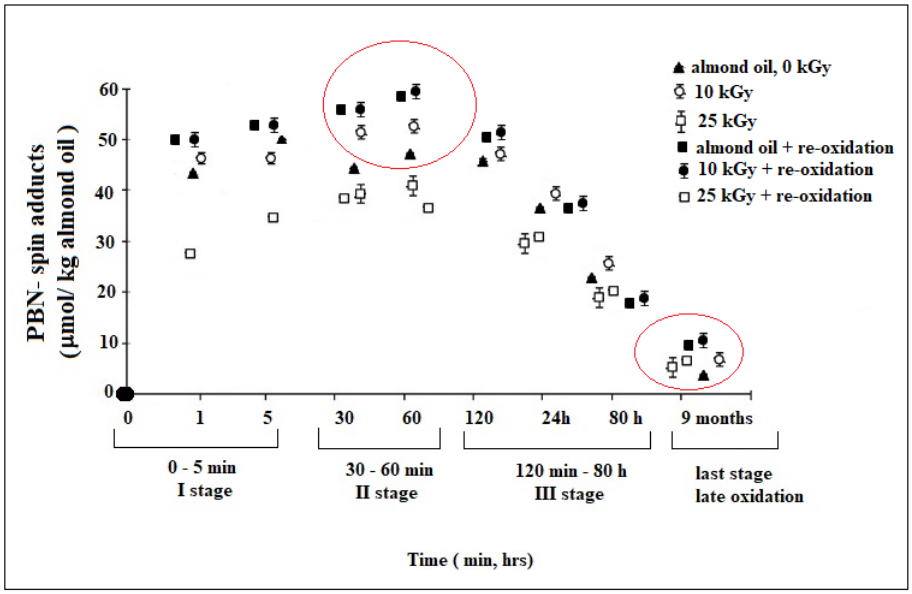

Fig. 3. PBN - adducts formation during oxidation stages of almond oil, almond oil alone (non-irradiated, 0 kGy), almond oil + 10 kGy, almond oil + 25 kGy, almond oil + re-oxidation at 50°C, almond oil + 10 kGy / re-oxidation at 50°C, almond oil + 25 kGy / re-oxidation at 50°C, respectively. Each experiment was performed in triplicate and was repeated three times.

completely resolved [10, 27]. The different, 10 kGy and 25 kGy dose irradiation did not cause a drastic difference in the induced free radicals amount, which is evidenced by the slight difference in the spectral intensity. Better results were obtained in samples of irradiated almond oil, reoxidized at 50°C for 24 h. 10 kGy irradiated almond oil gives the best results, even after 6 months of storage (Fig. 2B). Gamma - specific satellite lines were clearly expressed in both, 10 kGy and 25 kGy irradiated oils, and the left satellite line seemed resolved perfectly. It should be emphasized that when comparing the spectral lines, the intensities after the re-oxidation at 6 months storage at 10 kGy and 25 kGy and at 9 months storage (Fig. 2C) did not differ significantly, both in the central line and in the satellite lines. In recently reported results by Chiappinelli et al., Tomaiuolo et al., and Bortolin et al., for hazelnuts, walnuts, and irradiated almonds, the intensity of the satellite lines, and the central peak decreases over time, but they are still registered, even and two years after irradiation [28, 29, 30].

The lipid peroxidation of almond oils re-oxidized at 50°C for 24 hrs was monitored illustrated by the PBN-adducts concentrations during the oxidation time (Fig. 3). Compared to the initial PBN (3.71 mmol/ kg oil) concentration, the maximal concentration of detected radicals is in the range of 52 µmol/kg oil, in most almond oils in the range of 30 min - 60 min. On the other hand, the results of 57.3 µmol/kg oil in the 10 kGy irradiated almond oil show high reproducibility, on the 30 min - 60 min, on the II stage. It is interesting to note that PBN - adducts were maximal detection at minute 1st and 60th thin non - irradiated, 10 kGy, 25 kGy, and in almond oil only re-oxidized at 50°C, while 25 kG showed significant signal minimization ($p < 0.004$). All samples showed the first period - rapid PBN - adducts formation at 1 min, followed by a period in which the formation was equalized and again rapid PBN adducts formation at 60 min. Although the radical concentrations were significantly lower at 25 kGy radiation, the profiles of the lipid peroxidation curves in all samples were very similar throughout the oxidation period (Fig. 3). 3 months (*9th months*) storage after reoxidation at 50°C, the PBN adducts decrease sharply; therefore the primary and residual lipid oxidation in the oils probably subsides. This is further evidenced by the rancidity lack. It is important to note that in the non-irradiated but oxidized almond oil, the transformation of already formed lipid peroxides into aldehydes and ketones is also not observed. The high antioxidants, α-tocopherol, and metal ions content in almond oil may explain the higher oxidative stability and rancidity lack [8].

The investigated oils profiles in Fig 3 appear to be

useful only during the radical formation initial stages, to 60 min. In agreement with other investigations a significant ESR signal detection or a sudden radical formation increase indicated different oxidative stability of the non - irradiated, 10 kGy, 25 kGy irradiated oils [11, 25, 26, 30]. Velasco et al., [11] and Velasco et al., [25] found that the PBN-adducts effect on the lipid oxidation depends on the oil type, as well as on the different PBN ability to react with peroxyl radicals in the different oils, which is determined by the antioxidants and α-tocopherol concentrations. Therefore the equal α-tocopherol concentrations found in the non-irradiated and 10 kGy almond oil results in the equal PBN-fficacy and the equal peroxyl radical inhibition frequency. The PBN effect increased in the lower amounts presence of α-tocopherol. This assumption is supported by the fact that the bitter almond α-tocopherol content was lower at 60°C storage, but the other antioxidants presence hinders the propagation of lipid peroxidation step, slowing down the radical-adducts formation [12, 23].

In addition, our results showed that no significant differences in almond oil lipid content between un-irradiated, 10 kGy - irradiated and re-oxidised almond oils. Our results are in agreement with previous study of Gecgel et al., the authors did not detect significant changes in the water, lipids, ash, and carbohydrate contents and on the nuts oxidation after 5, 7, 10 kGy [16].

CONCLUSIONS

In the present study antioxidant and oxidative stability of irradiated almond oils at 10 kGy and 25 kGy were compared to almond oil alone and to 50°C re-oxidation storage. There was a statistically significant difference ($p < 0.004$) in 10 kGy increases activity, probably by stable antioxidant and lipid contents. In conclusion, the equal antioxidant concentrations in the non-irradiated and 10 kGy almond oil results in the equal PBN - efficacy, high oxidative stability, and the equal peroxyl radical inhibition frequency, to wit the almond oil saves its important lipid characteristics.

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