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Lipid modification processes induced by thiyl radicals

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HIGHLIGHTS

- The preparation procedure of LiH model systems affects the thiyl radical reactivity.
- Above 400 Gy lipid peroxidation was drastically reduced.
- Geometrical isomerization of LiH reached maximum at 2 kGy in equilibrium with air.
- In food irradiation doses up to 10 kGy may result in permanent lipid modifications.

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ABSTRACT

Polyunsaturated fatty acid (PUFA) oxidation by thiyl radicals ($RS^{\bullet}$) is believed to be responsible for some of the biological radiation damage. At the same time, $RS^{\bullet}$ can cause isomerization of PUFA double bonds with the formation of *trans* isomers. The aim of this study was to better understand the competition between lipid peroxidation and geometrical isomerization processes in biomimetic model system of linoleic acid in the presence of 2-mercaptoethanol using irradiation as a method for free radicals generation. In air-equilibrated conditions the propagation of lipid peroxidation was dominant up to the dose of 400 Gy, after which at higher doses up to 10 kGy the termination occurred with the predominance of geometrical isomerization. This study revealed that undesirable and permanent lipid modifications are possible at higher irradiation doses which should be considered in the planning of irradiation treatment of foods and feeds with high content of lipids and sulfur compounds.

1. Introduction

Because of their specific structural features unsaturated fatty acid molecules are uniquely suitable major constituents of biological membranes. However, those very same features make them at the same time uniquely vulnerable to free radical attack (Halliwell and Gutteridge, 2007). In the presence of sulfur compounds and S-centered radicals damage mechanisms may be modified in two opposite ways: lipid peroxidation is inhibited by thiols, while lipid isomerization is catalysed by S-centered radicals. Recently it has been shown that both processes proceed simultaneously and that lipid hydroperoxides and mono-*trans* fatty acids are formed to a comparable extent under oxidative conditions (Mihaljević et al., 2011, Ferreri and Chatgilialoglu, 2012).

Medical aspects of cellular damage related to peroxidation of unsaturated lipids have motivated the studies of lipid peroxidation in model membrane systems such as micelles and vesicles (Barclay and Vinqvist, 1994; Barclay, 1993; Miyashita, 2014; Niki, 2012; Sargis and Subbaiah, 2003). Although structurally much simpler than bilayers of phospholipids, fatty acids in micelles undergo fundamentally the same processes associated with oxidative modifications of lipids. Therefore, fatty acid in micelles remain suitable models for biomimetic chemistry studies (Breslow, 1998).

In this work competing processes of lipid peroxidation and geometrical isomerization were studied by gamma radiolysis of lipid model systems consisting of fatty acid in micelles.There are many reports showing that free radicals formed by gamma radiation produce oxidative modifications and/or isomerization altering molecular properties of lipids which results in disturbance and loss of functional properties of biomembranes (Khalil and Milochevitch, 2005; Kale and Sitasawad, 1990; Shadyro et al., 2002). However, besides our recently published paper (Mihaljević et al., 2011), no studies of the simultaneous occurrence of fatty acid peroxidation and geometrical isomerization in model systems are available. Special attention in this work is given to thiyl radicals formed under conditions where they are the main reactive species. Our previous investigations were carried out at very low

Abbreviations: LiH, Linoleic acid; PUFA, Polyunsaturated fatty acids; LiOOH, Lipid hydroperoxide; PB, Phosphate buffer; $RS^{\bullet}$, Thiyl radicals

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doses which would, nevertheless, be sufficient to induce chemical changes preceding biological consequences *in vivo* (Mihaljević et al., 2011). In the present work we extended the dose range to establish how the two lipid modification processes are mutually related at high doses.

2. Materials and methods

2.1. Chemicals

Linoleic acid (LiH), >99% pure, was purchased from Aldrich Chemicals. Nonionic surfactant polyoxyethylenesorbitan monolaurate (Tween®-20; Sigma–Aldrich, low-peroxide, low carbonyls), 2-mercaptoethanol (Sigma–Aldrich) and sodium dihydrogen phosphate (PB) (Sigma, ≥98%) were used as received. Ferrous sulfate ($FeSO_4 \times 7H_2O$) and potassium thiocyanate by Merck, and all other chemicals used were of analytical reagent grade purity. Water was triply distilled, and solutions of $FeSO_4 \times 7H_2O$ and sodium dihydrogen phosphate were prepared daily in redistilled water.

2.2. Methods

Model system containing mixed surfactant micelles and buffer was prepared by slow solubilization of LiH in non-ionic surfactant micelles previously formed by mixing Tween®-20 and PB, pH 6.5. The composition of the investigated systems was typically 5.0×10^{-4} M LiH, 2.8×10^{-4} M Tween®-20 and 5.0×10^{-3} M PB (pH∼5) (control). Two different systems with typical composition of mixed micelles were prepared: System A in which 2-ME was added just before irradiation and System B in which 2-ME was incorporated with LiH during the micelle formation. Model lipid systems were irradiated in equilibrium with air or after saturation with N_2O at room temperature using panoramic ^{60}Co source at the Ruđer Bošković Institute (Zagreb, Croatia). The applied dose rate was 260 Gy/min. The dose rate was established with the ethanol-chlorobenzene dosimetry system (Ražem et al., 1985) and calculated daily taking into account the radioactive decay of ^{60}Co. After irradiation lipid components were extracted with a solvent mixture of Ψ(CH_2Cl_2:MeOH)=2:1. An aliquot of the sample was taken out for the quantitative determination of LiOOH by spectrophotometric ferric thiocyanate method (Mihaljević et al., 1996). All measurements were performed by UV/vis spectrophotometer Varian Cary 4000. Conjugated diene oxidation products which absorb around 232 nm could not be observed since the absorbance of 2-ME interfered at this wavelength.

The rest of the lipid extract was treated with an ethereal solution of diazomethane in order to transform linoleic acid to the corresponding methyl esters (Glastrup, 1998). Varian 450 gas chromatograph equipped with a flame ionization detector and a Rtx-2330 (90% biscyanopropyl/10% phenylcyanopropylpolysiloxane) capillary column (105 m × 0.25 mm) was used with the following oven program: temperature started from 180 °C, held for 35 min, followed by increase of 10 °C min^{-1} up to 250 °C and held for 5 min. Methyl esters were identified by comparison with the retention times of authentic samples, which are commercially available and their distribution was determined.

3. Results and discussion

Thiyl radicals were generated from 2-ME in lipid micelles by free radicals initially formed by the radiolysis of water at pH 5.0. Radiolysis of neutral water leads to e_{aq}^-, $HO^\bullet$ and $H^\bullet$ with the known radiation chemical yields (G)/μmol J^{-1} as shown in Eq (1) (Buxton, 2008). The $HO^\bullet$ radicals (and part of $H^\bullet$ atoms) participate in the reactions with thiol and Tween®-20/LiH giving thiyl and alkyl radicals $R^\bullet$, respectively [(Eqs (2) and (3)]. It can be supposed that the abstraction of hydrogen atom by $HO^\bullet/H^\bullet$ giving $R^\bullet$ could occur preferentially in micellar medium, because of the proximity of unsaturated acyl chains (Patterson and Hasegawa, 1978; Al-Sheikhly et al., 2004). The $H^\bullet$ atom should be quenched by oxygen too [Eq. (4)]. On the other hand, e_{aq}^- are partitioned between oxygen and thiol [Eqs (4) and (5)]. Assuming that the concentration of O_2 in solution in equilibrium with air will be not less than 2.66×10^{-4} M (*i.e.*, equal to or less than in air-saturated aqueous solution), having 2.8×10^{-3} M 2-ME and taking into consideration respective rate constants with e_{aq}^- and $H^\bullet$, (Buxton et al., 1988; Ross et al., 1998), the main products will be peroxyl radicals [Eq. (6)] and superoxide radical anion [Eq. (4)]. (Porter, 1986; Schöneich et al., 1992). While contributions of the reactions of considerably less reactive superoxide radical anion could not be considered (Gebicki and Bielski, 1981), in acid aqueous media at pH∼5 perhydroxyl radical, as well as peroxyl and thiyl radicals, are expected to react with LiH generating the bisallylic radical $Li^\bullet$ [Eq. (7)] (Porter, 1986; Schöneich et al., 1992).

In the first propagation step molecular oxygen adds to $Li^\bullet$, whereas in the second propagation step $LiOO^\bullet$ abstracts hydrogen atom from the bisallylic position at rate k_p to generate $Li^\bullet$ which is the rate-determining step in the propagation sequence [Eqs (8) and (9)] (Porter, 1986). The termination steps involve recombination of radicals [Eqs (10)–(12)].

3.1. Initiation steps

$$H_2O \rightsquigarrow e_{aq}^-(0.27),\ HO^\bullet(0.28),\ H^\bullet(0.062) \quad (1)$$

$$HO^\bullet/H^\bullet + HOCH_2CH_2SH \rightarrow H_2O/H_2 + HOCH_2CH_2S^\bullet \quad (2)$$

$$HO^\bullet/H^\bullet + \text{TWEEN-20/LiH} \rightarrow H_2O/H_2 + R^\bullet \quad (3)$$

$$H^\bullet/e_{aq}^- + O_2 \rightarrow H^+ / O_2^{\bullet -} \quad (4)$$

$$e_{aq}^- + HOCH_2CH_2SH \rightarrow HOCH_2CH_2^\bullet + HS^- \quad (5)$$

$$R^\bullet + O_2 \rightarrow ROO^\bullet \quad (6)$$

$$HOO^\bullet/ROO^\bullet/HOCH_2CH_2S^\bullet + LiH \rightarrow ROOH/HOCH_2CH_2SH + Li^\bullet \quad (7)$$

3.2. Propagation steps

$$Li^\bullet + O_2 \rightarrow LiOO^\bullet \quad (8)$$

$$LiOO^\bullet + LiH \overset{k_p}{\Longrightarrow} LiOOH + Li^\bullet \quad (9)$$

3.3. Termination steps

$$Li^\bullet + Li^\bullet \rightarrow \text{non-radical products} \quad (10)$$

$$Li^\bullet + LiOO^\bullet \rightarrow \text{non-radical products} \quad (11)$$

$$LiOO^\cdot + LiOO^\cdot \overset{2k_t}{\Longrightarrow} \text{non} - \text{radical products} \quad (12)$$

Under aerobic conditions lipid peroxidation process proceeded rapidly and reached maximum at 400 Gy in LiH micelles (control)

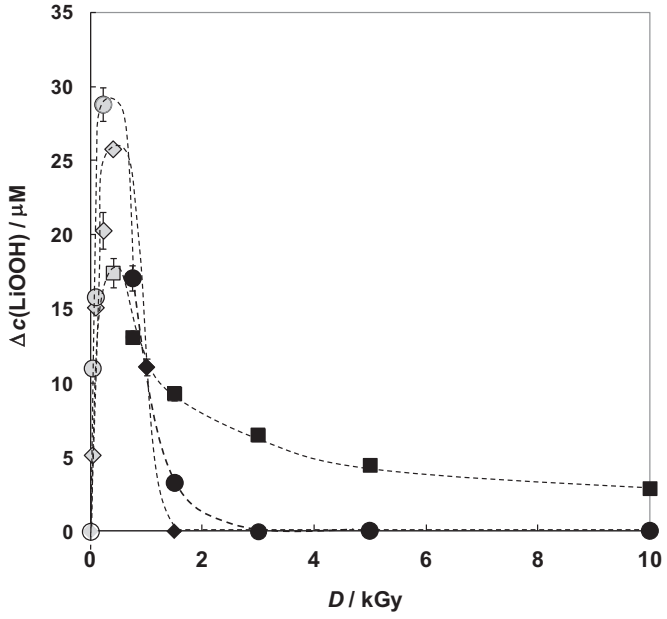

Fig. 1. The concentration of LiOOH formed as a function of the irradiation doses in aerobic conditions: (♦) LiH micelle (control); (•) system A (micelle+2-ME); (■) system B (incorporated 2-ME); c(LiH)=5×10^{-4} M; c(Tween®-20)=2.8×10^{-4} M; c(PB)=5.0×10^{-3} M; c(2-ME)=2.8×10^{-3} M, pH 5. Gray symbols represent data from reference Mihaljevic et al., 2011.

and in both systems A and B (Fig. 1). After further dose increase up to 10 kGy lipid peroxidation decreased.

The process of geometrical isomerization was saturated in both lipid model systems at about 2 kGy dose. The disappearance of 9*c*,12*c*-18:2 isomer of LiH was accompanied by the formation of 9*t*,12*t*-18:2 isomers after an induction dose of about 400 Gy in both systems. While 9*c*,12*c*-18:2 isomer decreased exponentially, the intermediate isomers 9*t*,12*c*- and 9*c*,12*t*-18:2 passed through maxima at about 1 kGy under aerobic conditions (Fig. 2). The existence of these maxima in both systems A and B indicates the reaction occurring by a consecutive mechanism. The relative amounts of 9*t*,12*t*-, 9*c*,12*t*- and 9*t*,12*c*-18:2 isomers were not significantly different at higher irradiation doses in both model systems. The formation of 9*t*,12*t*-18:2 isomers in both aerobic systems A and B were characterized by similar induction doses of about 400 Gy.

As expected, under N_2O-saturated conditions LiH peroxidation was below detection limit (data not shown). Based on the rate constants of 1.2×10^{10} and 9.1×10^{9} M^{-1} s^{-1} for the reaction of e_{aq}^- with 2-ME (2.8 mM) and N_2O (20 mM) respectively, (Buxton et al., 1988; Ross et al., 1998), 85% of hydrated electrons are trapped by N_2O to increase the formation of HO• radicals [Eq. (13)].

$$e_{sol}^- + N_2O + H_2O \rightarrow N_2 + HO^- + OH^\bullet \quad (13)$$

The HO• radicals and H• atom should be partitioned between thiol and Tween®-20/LH to give thiyl and alkyl radicals [Eqs (2) and (3)]. In the absence of oxygen alkyl radicals are essentially trapped by thiol to give extra thiyl radicals, which are known to isomerize double bonds of PUFA. Under these experimental conditions peroxidation of LiH could not proceed and consequently, only isomerization in both lipid systems A and B took place (Fig. 3). Although the influence of the system preparation procedure on lipid isomerization was not significant under aerobic conditions (Fig. 2), in Fig. 3 it can be seen that under N_2O-saturated conditions this influence on the isomerization level was more pronounced. Fig. 3 While isomerization in system A exponentially decreased with irradiation dose (Fig. 3(a)), this process decreased

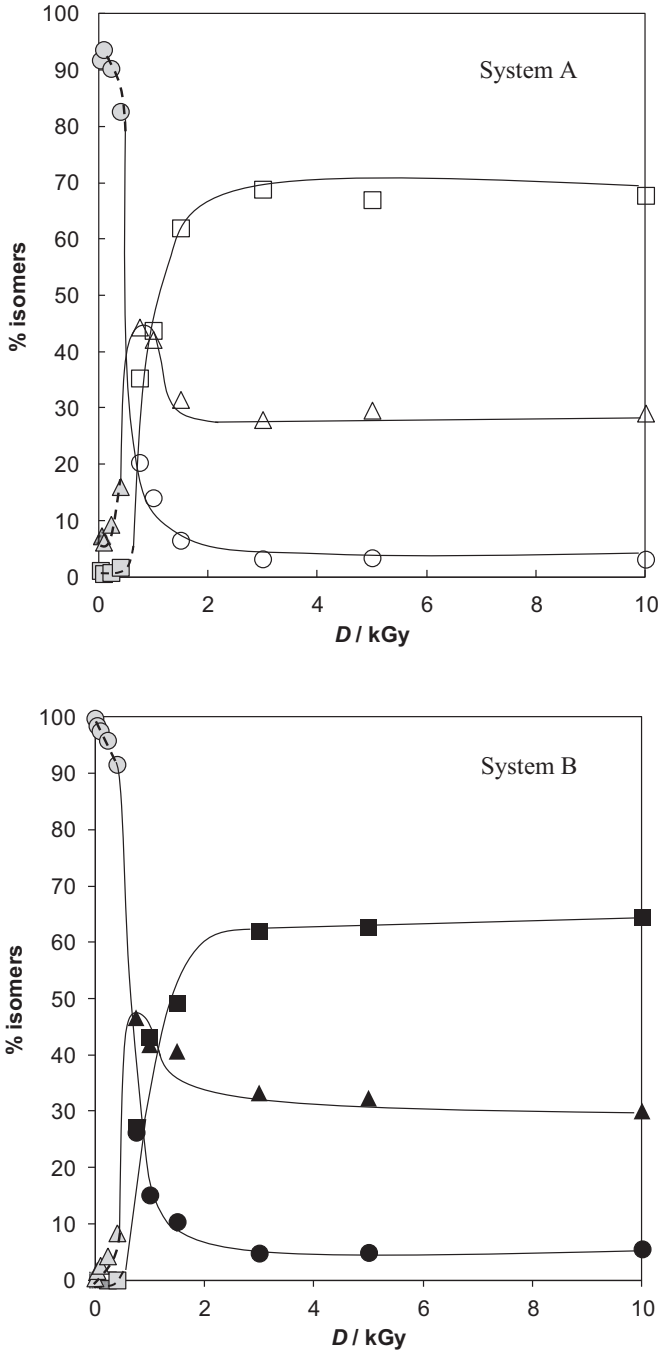

Fig. 2. Dose dependence of the LiH isomers modified by irradiation of the system A (empty symbols) and system B (filled symbols) under aerobic conditions; 9*c*,12*c*-18:2 (○,•), 9*t*,12*c*-18:2+9*c*,12*t*-18:2+9*t*,12*t*-18:2 (□,■); c(LiH)=5×10^{-4} M c(Tween®-20)=2.8×10^{-4} M; c(PB)=5.0×10^{-3} M; c(2-ME)=2.8×10^{-3} M, pH 5. Dashed lines (gray symbols) represent data from reference Mihaljevic et al., 2011.

almost linearly up to 0.8 kGy in system B (Fig. 3(b)). At the same time, the induction dose for the formation of 9*t*,12*t*-18:2 isomers at about 300 kGy was observed only in deaerated system B. The obtained results show that the procedure for the preparation of model systems has an important effect on the reactivity of formed thiyl radicals. The micelles, inside which 2-ME was incorporated (B), were more resistant to isomerization than the micelles with 2-ME added just before irradiation (A).

Isomerization has been correlated to the formation of diffusible thiyl radicals which are able to move from the aqueous phase and reach the lipid phase without being trapped in the interior of micelles.

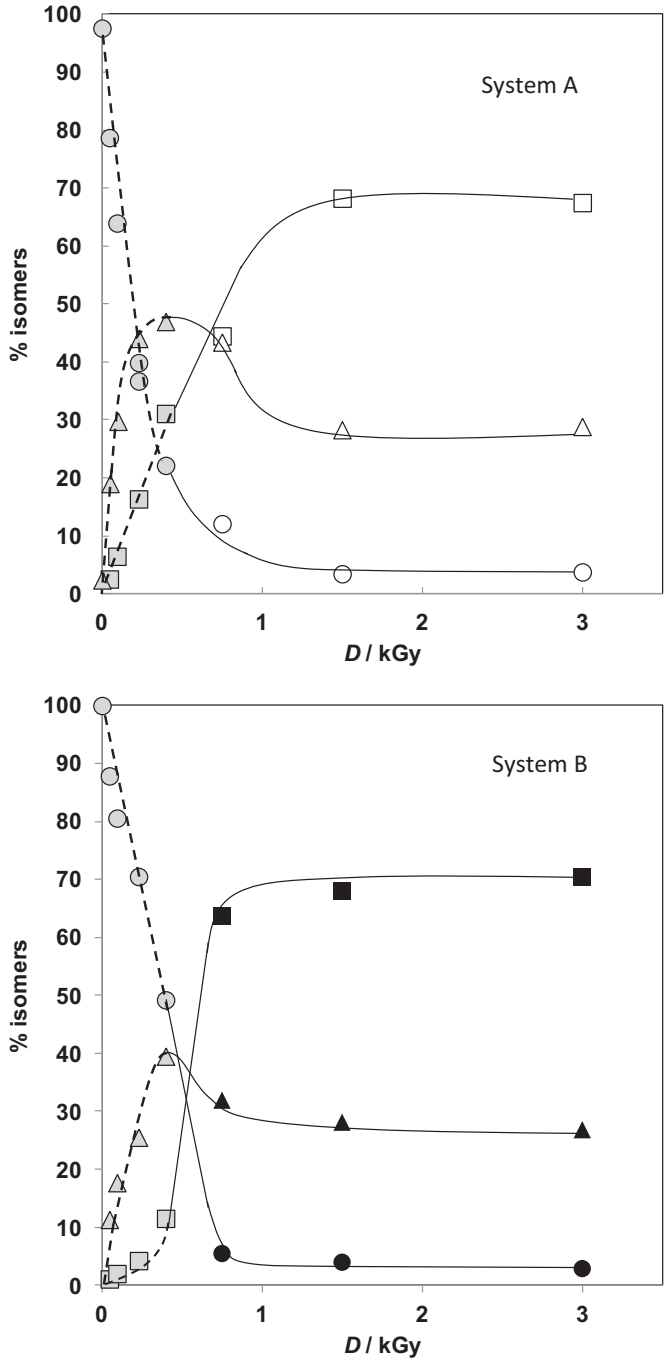

Fig. 3. Dose dependence of the LiH isomers formation in System A and System B under anaerobic conditions; 9*c*,12*c*-18:2 (○, •), 9*t*,12*t*-18:2 (□,■), 9*c*,12*t*-18:2+9*t*,12*c*-18:2 (Δ,▲); c(LiH)=5×10^{-4} M; c(Tween®-20)=2.8×10^{-4} M; c(PB)=5.0×10^{-3} M; c(2-ME)=2.8×10^{-3} M, pH 5. Dashed lines (gray symbols) represent data from reference Mihaljevic et al., 2011.

Our results suggest that the supramolecular structure of self-organized systems LiH/Tween®-20/2-ME in water is very important. In other words, in the micelles of the System B, thiol was more effective free radical scavenger because it was closer to fatty acid during the propagation of lipid peroxidation, whereas thiol distribution was not effective to the same extent in the System A.

4. Conclusions

In the past few decades lipid peroxidation and geometrical isomerization processes in biomimetic aqueous systems have received significant attention. Both processes are highly sensitive to molecular environment where the interfacial interactions between lipid molecules, water, surface active compounds and other types of molecules such as sulfur compounds in their immediate vicinity may play an important role on the outcome of the two competitive processes (Miyashita, 2014., Ferreri et al., 2005.). In this study radical initiation in water compartment of mixed micelles was obtained by gamma irradiation up to 10 kGy where thiyl radicals were in substantial excess relative to other formed radicals. They efficently initiated structural modifications of PUFA depending on molecular structure of self-organized systems with LiH in water. Our results indicated that in equilibrium with air peroxidation increased with dose up to about 400 Gy. Above this dose peroxidation was drastically reduced, probably because of the interruption of propagation of free radical chain mechanism caused by the loss of physical integrity of micelles. The System B, in which 2-ME was incorporated into micelle, was characterized by an induction dose to isomerization in both aerobic and anaerobic conditions demonstrating more protective activity of thiol which was incorporated deeper into the micelles.

The integrative approach of lipid peroxidation and isomerization should be considered for the examination of free radical reactivity of thiol-containing biological or model lipid systems. Understanding of radical processes that are involved in permanent structural modifications of biomembranes in cells is essential because both processes consequently have damaging effects on cell membranes functions in living organisms. Well known peroxidation process of PUFA has been shown to be responsible for the biological damage resulting from lipid oxidation under oxidative stress. On the other hand, the physiological role and relevance of lipid *cis-trans* isomerization in humans are still to be clarified, since *trans*-fatty acids were considered only to derive from the diet, particularly through food of animal origins and partially hydrogenated fats and oils (Sebedio and Christie, 1998; Ferreri et al., 2001; Ferreri and Chatgilialoglu, 2012). In this work the investigation of the model systems consisting of LiH demonstrated that *trans*-isomers could be also formed under radical stress in cells due to the thiyl radical-catalyzed *cis-trans* isomerization even under condition of equilibration with air.

However, *cis-trans* isomerization induced by irradiation in our model systems did not increase with an increase of irradiation dose beyond about 2 kGy in equilibrium with air and 1 kGy in the absence of air. This effect of gamma irradiation on the structural modification of LiH might be relevant to food irradiation. In food irradiation treatment higher doses (up to 10 kGy) might be required, depending on the contamination level of pathogenic organisms in food. Besides fats some irradiatied foods may contain higher content of water and sulfur compounds. Any of these components of food could participate in reactions with free radicals generated by ionizing radiation, resulting in the undesirable and permanent lipid modifications. The nature and amount of radiation chemical changes is the principal criterion for judging the wholesomeness of irradiated food (WHO, 1997).

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