EPR spectroscopy: A tool to characterize gamma-irradiated foods

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Summary
The γ-radiation induced changes in ground black pepper (*Piper nigrum* L.), allspice berries (*Pimenta officinalis* L.), ginger root (*Zingiber officinale Rosc.*), dried clove buds (*Caryophyllus aromaticus* L.) and dry oregano leaves (*Origanum vulgare* L.) exposed to doses from 2.5 to 30 kGy using ^{60}Co source were studied by EPR spectroscopy. Influence of the dose on the character of generated paramagnetic structures, as well as their thermal stability and life-time were investigated. EPR spectrum of all reference (non-irradiated) samples consists of a broad singlet line with unresolved hyperfine splitting, attributable to Mn(II) ions, upon which an additional narrow EPR signal (g = 2.0022; $\Delta B_{pp} \sim 1$ mT) is superimposed, assigned to stable semiquinone radicals produced by the oxidation of polyphenolics in plants. The analysis of individual EPR spectra of γ-radiation treated spice showed the formation of new paramagnetic structures of different origin (mostly cellulose and carbohydrate), which exhibited diverse thermal stability and life-time. Ethanolic extracts of reference spice samples showed considerable radical scavenging ability using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical as an oxidant. The influence of γ-radiation dose on the ability of spice extracts to terminate DPPH was also investigated. The results indicated that the antioxidant capacity was only slightly affected by the radiation treatment.

Keywords
EPR; γ-irradiation; spice; free radicals; thermal stability; life-time; DPPH scavenging ability

Spices frequently undergo a microbiological contamination resulting in the increased content of various microorganisms and their spores. To eliminate serious health risks, as well as to prolong the shelf-life of these products, the γ-irradiation treatment is accepted as a standard and safe sterilization procedure.

Although the application of γ-irradiation as a sterilization technique has recently found an enormous growth, there are only a limited number of papers dealing with the influence of higher doses of ionizing radiation on the changes in the structure, physical, chemical or biochemical properties of foods preserved in that way. From this point of view, exact studies of γ-irradiation food processing and its effects on safety, nutritional and organoleptic properties, are very important and strongly required [1-9].

In the past, many γ-irradiation detection methods (based on physical, chemical or biochemical techniques) have been developed in food inspection, which are most frequently used nowadays. Each method provides specific information about studied materials [9-12]. Some of them have been recently accepted by the European Committee for Standardization (CEN) as European standards, e.g. EN 1784:1996, EN 1785:1996, EN 1788:2001 [13-15].

In relation to the formation of paramagnetic species upon γ-irradiation food processing, EPR spectroscopy represents a unique detection technique for their characterization and investigation. In EN 1787:2000, CEN proposed the EPR method for the detection of foods containing irradiated cellulose. Later, four additional methods for the detection of irradiated foods have been standardized (i.e. EN 13708:2001, EN 13751:2002, EN 13783:2001 and EN 13784:2001). Application of EPR spectroscopy is limited by the life-time of radiolytically produced free radicals [3, 9, 10, 16-20].

The γ-radiation treatment of plant products containing cellulose leads to the generation of a three-line EPR signal, characterized by the

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g-value of 2.0060 ± 0.0005 and hyperfine splittings at about 3.00 ± 0.05 mT attributed in literature to 'cellulosic' radical [3, 9, 10, 21-25]. For the purpose of post-radiation identification of cellulose-containing foods, the presence of this signal was accepted as a marker of the γ-radiation treatment. However, it was observed that the EPR intensity of the 'cellulosic' triplet signal gradually decreased with storage time and the rate of disappearance depended on temperature, humidity, presence of oxygen and on further factors [9, 10, 21, 24-26].

Black pepper *(Piper nigrum* L.*)* is a widely used spice, with interesting pharmacological and other applications. The main alkaloid mostly responsible for its characteristic pungency is piperine, which constitutes from 2 to 7.4% of *Piper nigrum* cultivars. Piperine has anti-inflammatory properties and was tested in vitro as a protection agent against the oxidative damage by inhibiting or quenching free radicals, reactive oxygen species and hydroxyl radicals. Contrary to these positive effects, under specific conditions it can exhibit potentially mutagenic and carcinogenic behaviour, e.g. when piperine reacts with nitrites, which are also present in food [9, 27, 28].

Oregano (*Origanum vulgare* L.) is an aromatic spice native to the Mediterranean and Eurasia, containing various antioxidants, e.g. derivatives of phenolic acids, flavonoids, volatile oils, from which carvacrol and thymol influence most significantly its taste and aroma [29-33]. According to the phytochemical database [34], the number of different antioxidants in this spice reaches up to 34. Similarly as black pepper, in addition to its gastronomic applications, it is frequently used in the traditional medicine as an antiseptic and tonic agent [32, 33].

Allspice (*Pimenta officinalis* L.) originates from West India and Central American countries. Its berries contain from 2 to 5% of essential oils (depending much on the time of harvest). As the main components, eugenol, eugenol-methylether, and terpenes (myrcene, 1,8-cineol and α-phellandrene) have been reported [35-37]. It is widely used in pickling and in condiment mixtures, especially to improve the taste of meats. Due to the content of eugenol and its derivatives providing mild analgesic effect, allspice can be used as a component of plasters for treating neuralgia or rheumatism [36, 38-40].

Ginger *(Zingiber officinale Roscoe)* is the rhizome known in commerce under various names, originating from Africa. There exist more than 70 species of this spice genus [30]. The characteristic flavour is most probably depending on the presence of volatile oils. The mixture of gingerols of the formula $C_{16}H_{26}O_3$, is responsible for its pungency, showing also antioxidant properties [30]. Ginger extract showed such behaviour as well; it contains monoterpenes and sesquiterpenes, able to scavenge superoxide anion radicals and hydroxyl radicals [42-44]. Gingerol derived from ginger at high concentrations inhibits ascorbate-ferrous complex, which may induce lipid peroxidation [45]. It has also been suggested that ginger interferes with inflammatory processes. Moreover, ginger acts as a hypolipidemic agent in rabbits fed nutrients containing cholesterol [46, 47]. Feeding rats with ginger elevated significantly the activity of hepatic cholesterol 7a-hydroxylase which is a rate-limiting enzyme in the biosynthesis of bile acids and stimulates the conversion of cholesterol to bile acids leading to the excretion of cholesterol from the body [48].

Clove *(Caryophyllus aromaticus* L.*)* are dried flower buds, originated from a clove-tree, native to Moluccas Island and Southern Philippines. At present, the spice is cultivated both in the West and East Indias, in tropical Africa, and in Brazil. Approximately seven-eighths of the world's clove supply is grown in Zanzibar. The flower buds are collected manually when they are bright red, and subsequently are exposed to smoke and artificial heat before being exposed to the sun [30, 49]. The most important clove components are volatile oils, containing mostly tannin (from 10 to 13%) and resins, mostly caryophyllin, eugenin and eugenol. Clove is frequently used in traditional and 'official' therapy. It is safe and effective against vomiting during pregnancy; increases circulation of the blood and promotes digestion and nutrition. Aroma extracts isolated from clove buds exhibit antimicrobial and antioxidant activity [49-51].

This study is focused on the application of EPR spectroscopy in the characterization of changes in the above-mentioned spice samples upon γ-radiation treatment. The changes in five different spice matrices, induced by γ-radiation treatment, are mutually compared. Additionally, the ability of spice ethanolic extracts to terminate DPPH free radicals was studied and evaluated.

MATERIAL AND METHODS

Solid samples characterization

Commercially available samples of ground black pepper (Vietnam; dry matter content 87.9% w/w), dried oregano leaf from Cambidi, Izmir (Turkey, dry matter content 90.1% w/w), ground allspice (Mexico; dry matter content 86.8% w/w), ground ginger roots (dry matter content 89.1%) and ground clove buds (dry matter content 82%)

from India were used. The spice samples were irradiated using ^{60}Co source at doses from 2.5 up to 30 kGy according to commercial practices at Artim, Prague, Czech Republic. All samples were stored in closed bags in darkness at 6 °C and relative humidity of 60%.

Ethanolic extracts of spices

The extracts of individual spices were prepared as follows: 1 g of dry spice sample was placed into 15 ml vessel; 10 ml of spectroscopic-grade ethanol was added, the flask caped and contents gently stirred for 5 min. Afterwards the flasks were placed into a box and stored there in darkness at 10 °C for 24 h. Subsequently, the solid phase was separated from the liquid by filtration and the coloured liquid extracts were used in the EPR measurements.

EPR measurements

Thin-wall quartz EPR tubes (internal diameter of 3 mm, length of 150 mm, and wall thickness about 0.1 mm) were used for the measurements of solid samples, enabling the formation of cylindrical samples with identical dimensions. The individual spice sample (100 mg) was placed in the tube (sample column heights: ground black pepper, 2.6 ± 0.2 cm; oregano, 5.2 ± 0.2 cm; allspice, 3.0 ± 0.2 cm; ginger, 2.5 ± 0.2 cm; clove, 1.8 ± 0.2 cm); and then inserted into a standard TE_{102} (ER 4102 ST) rectangular cavity of an EMX X-band EPR spectrometer or into the cavity of e-scan EPR spectrometer (Bruker BioSpin, Karlsruhe, Germany) and the EPR spectrum was recorded at 298 K. The temperature-dependent measurements were carried out using a Bruker temperature control unit ER 4111 VT assembled to EMX EPR spectrometer. The accurate filling procedure of EPR cells resulted in good reproducibility of measurements (relative EPR intensity standard deviation of ± 5% for five independent experiments). The response and settings of EPR spectrometers was checked by means of solid 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Strong pitch standards (Bruker) daily before the experiments.

The test of spice's extracts ability to terminate DPPH free radical was performed as follows: A defined volume of ethanolic extract was mixed with the same volume of DPPH solution in ethanol (initial concentration of DPPH in system, $c_{0(DPPH)} = 1\times10^{-4}$ mol.dm^{-3}), the mixture was immediately mixed with 1 ml of air and transferred into the EPR flat cell. The EPR measurements started exactly 3 min after the DPPH and spice extract mixing.

Simulation analysis of EPR spectra

The experimental EPR spectra processing and simulation was carried out using WIN EPR and SimFonia programs (Bruker). The integral intensities of EPR signals were obtained by double integration of the spectrum. The multi-component experimental EPR spectra simulation was evaluated as a linear combination of individual EPR spectra simulations using a least-squares minimization procedure (Micromath® Scientist Program, Micromath, Saint Louis, Missouri, USA). The statistical parameters of the calculation procedure served for the determination of the simulation quality, i.e. correlation of the experimental and simulated spectra. The relative concentration of individual paramagnetic species was evaluated from the contributions of individual simulations to experimental spectrum after double integration.

RESULTS AND DISCUSSION

Identification of radicals formed upon γ-irradiation in solid spice samples

All the EPR measurements started setting the magnetic field width to 500 mT in order to detect the presence of transition metals in the samples. The spectra obtained (Fig. 1) are characterized by a broad singlet line with unresolved hyperfine splitting attributed to Mn(II) ions frequently present in samples of plant origin [9, 10, 32, 33, 55], upon which one or more sharp EPR signals are superimposed (* in Fig. 1). The experimental spectra of non-irradiated (reference) samples were characterized by one stable narrow EPR signal (g = 2.0022, $\Delta B_{pp} \sim 1$ mT) typical for the semiquinone radicals produced by the oxidation of plant polyphenolics [9, 10, 32, 33, 52].

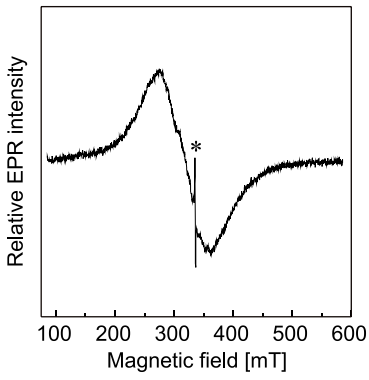

Fig. 1. X-band EPR spectrum of ground black pepper irradiated at dose of 7.5 kGy, measured one week after the radiation process using 0.633 mW microwave power at 298 K.

Tab. 1. Spin Hamiltonian parameters of γ-radiation induced EPR signals observed in spice samples γ-irradiated at doses from 2.5 to 30 kGy.

EPR signal (Origin)	*g*-Value	Hyperfine splittings [mT]	ΔB_{pp} [mT]	Spice
Carbohydrate	$g_\perp = 2.0060$ $g_{\|\|} = 2.0032$	$A_\perp = 0.85$ $A_{\|\|} = 0.70$ (2H)	0.67	Black pepper, oregano, allspice, ginger, clove
Carbohydrate	$g_\perp = 2.0060$ $g_{\|\|} = 2.0050$	$A_\perp = 0.50$ $A_{\|\|} = 0.40$ (1H)	0.40	Black pepper, clove
Cellulose	$g_\perp = 2.0029$ $g_{\|\|} = 2.0014$	$A_\perp = 3.00$ $A_{\|\|} = 1.80$ (2H)	1.20	Black pepper, oregano, allspice, ginger, clove

The application of γ-radiation leads to changes in chemical structure of spice matrices, producing new types of paramagnetic species analogous for all investigated spice species. Simulation of EPR spectra obtained from black pepper samples revealed the formation of three paramagnetic species, i.e. the triplet and doublet assigned to 'carbohydrate' radical structures and the typical three-line 'cellulosic' signal [9, 10]. The paramagnetic signals identified in individual irradiated spice samples, along with their spin Hamiltonian parameters ($g_\perp, g_{||}; A_\perp, A_{||}; \Delta B_{pp}$) used in simulations, are summarized in Table 1. The detailed simulation analysis of EPR spectra of the reference ginger sample and the sample irradiated at the dose of 30 kGy, is depicted in Fig. 2.

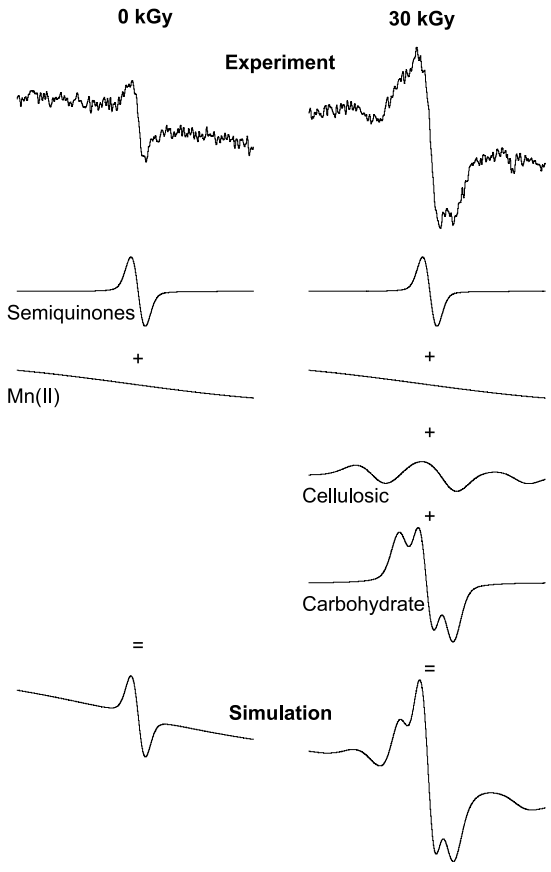

Fig. 2. A schema demonstrating simulation analysis of experimental EPR spectra of reference (non-irradiated) ginger sample and of sample γ-irradiated at dose of 30 kGy.
Spectra recorded 2 weeks after γ-radiation treatment using 0.633 mW microwave power at 298 K; simulation parameters are given in Table 1).

By evaluating the EPR spectra of all studied spice matrices we have confirmed that the integral EPR intensity of γ-radiation induced radical species is related to the γ-radiation dose and can be well fitted using the model of saturation curve (Fig. 3a). Figure 3b demonstrates the changes in relative concentration of individual radical species in relation to the absorbed dose of γ-irradiation, obtained from the simulation of ground ginger samples EPR spectra. It can be supposed that all the radical structures are generated in processes of cellulose polymer chain cleavage during the exposure to γ-radiation initially producing the 'cellulosic' radical species. The subsequent process impacts the glucose units producing different types of 'carbohydrate' radicals. The proposed structures of potential 'carbohydrate' paramagnetic species formed in γ-irradiated spice samples are in good correlation with the data suggested by KORKMAZ and POLAT [53] for γ-radiation treated red pepper.

Thermal treatment of γ-irradiated spices

The EPR spectra of semiquinones identified in the reference samples of black pepper, oregano leaves, ginger and clove showed only negligible changes upon increasing temperature from 298 K to 353 K. On the other hand, the stability of γ-radiation induced radicals in these spices is strongly temperature-dependent, as their EPR signals undergo significant and irreversible changes. Generally, the

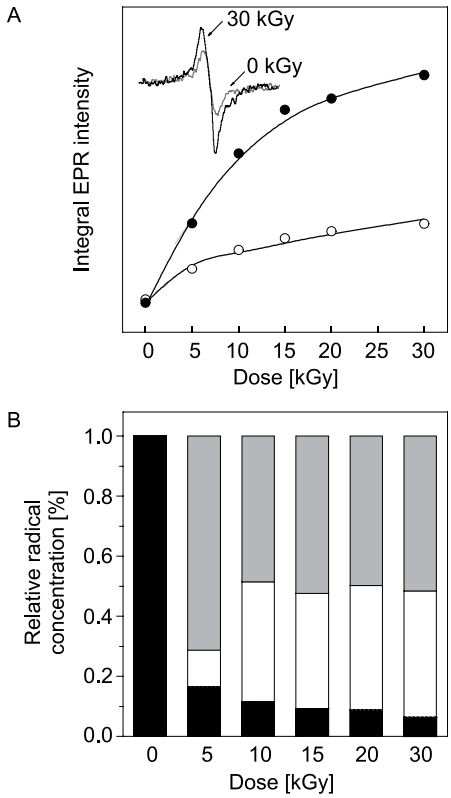

Fig. 3.

A. The dependence of integral EPR intensity of clove (○) and ginger (●) spice samples on γ-radiation dose.

EPR spectra were measured using 0.633 mW microwave power two weeks after γ-radiation treatment at 298 K. Inset represents experimental EPR spectra of non-irradiated and irradiated clove sample (dose of 30 kGy; magnetic field sweep width, SW = 8 mT).

B. Illustration of relative decrease of semiquinone radicals (■) and simultaneous dose-dependent formation of 'cellulosic' (■) and 'carbohydrate' (□) radical structures upon the γ-radiation treatment of ginger samples obtained from the simulation of experimental EPR spectra.

study of thermal stability of γ-induced EPR signals represents the valuable tool for the assessment of previous γ-radiation treatment of cellulose-containing samples, regardless to the contradictions to the results published [21, 22, 25, 26].

Different behaviour was found for allspice samples, where semiquinone radicals showed a moderate increase of their relative concentration, as depicted in Figure 4a-c. The experimental EPR spectra measured at 298 K (Fig. 4a) or 353 K (Fig. 4b) revealed the increase of semiquinone signal with temperature growth, while the γ-induced signals corresponding to 'cellulosic' and 'carbohydrate' structures extinct. This trend is unambiguously demonstrated in Fig. 4c which presents the temperature changes of integral EPR intensity for reference and samples γ-irradiated at various doses. The reference sample reflected only negligible variation in the range of 298-323 K, while further increase of temperature caused the integral intensity increase up to 40%. Contrariwise, the decline of integral EPR intensity of γ-irradiated samples was measured, and a detailed simulation analysis of temperature-dependent changes of allspice sample irradiated at dose of 30 kGy confirmed the significant decrease of 'carbohydrate' radical concentration with simultaneous increase of semiquinone and 'cellulosic' radicals upon thermal treatment from 298 K to 353 K (Fig. 4d). Continuous temperature increase up to 373 K lead probably to thermal decomposition of each sample and to the formation of new, thermally induced radicals, resulting in slightly increased EPR spectra intensity. Such behaviour is in accord with the previously published data of FRANCO et al. [54]. In order to characterize the thermal stability of identified radicals, the dependence of their individual relative concentrations obtained from the simulation analysis of EPR spectra on temperature was fitted to the Arrhenius model, and the values of activation energy were used as the marker of thermal stability as was mentioned in our previous papers [9, 10].

Evaluation of life-time of γ-radiation induced radicals

The γ-radiation induced paramagnetic structures show only limited life-times [1, 3, 9, 21, 25]. This phenomenon can, along with the thermal instability of γ-radiation induced radicals, significantly influence the application of EPR spectroscopy as a reliable dosimetric method [21, 22, 25, 26].

In view of these facts, the changes in EPR spectra of studied spices upon the storage time (up to 20 weeks after the irradiation process) were also monitored. We found that the storage interval after radiation treatment of black pepper and oregano samples influenced only the EPR spectra of γ-irradiated samples and that of the reference remained unchanged. Simulation of the experimental spectra of ground black pepper showed that the decrease of EPR spectra intensity on storage time for the γ-radiation-induced signals can be described by a formal first-order kinetic model, and the calculated individual half-lives confirmed the lowest stability of 'cellulosic' triplet signal, in accord with previously published data [1, 3, 21, 25]. Fully compatible results were obtained by the analysis of EPR spectra of both ground ginger and cloves. In contrast, allspice samples showed the

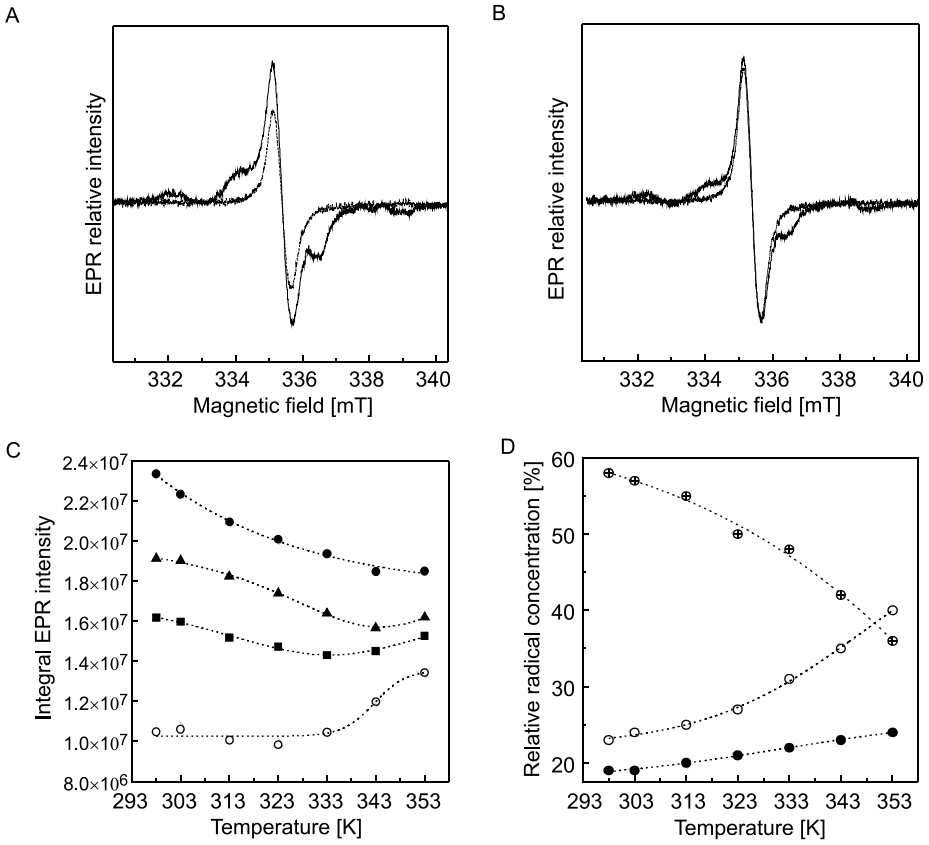

Fig. 4.

A. The experimental EPR spectra of reference allspice samples (dotted line) and of sample γ-irradiated at dose of 30 kGy (full line), measured at 298 K.

B. The experimental EPR spectra of reference allspice samples (dotted line) and of sample γ-irradiated at dose of 30 kGy (full line), measured at and 353 K.

C. Temperature dependence of integral EPR intensity of reference allspice sample (○) and of samples γ-irradiated at doses of 5 kGy (■), 10 kGy (▲) and 30 kGy (●) measured 3 weeks after the radiation treatment at 298 K.

D. Temperature dependence of relative concentrations of semiquinone (○), 'cellulosic' (●) and 'carbohydrate' (⊗) paramagnetic species, obtained from the simulation analysis of experimental EPR spectra of allspice sample γ-irradiated at dose 30 kGy and measured 3 weeks after the radiation treatment.

decline of γ-radiation induced radicals, whereas the relative EPR signal intensity of semiquinones increased upon the prolonged storage time.

For illustration, the dependence of radical concentrations on storage time after the irradiation, obtained from the EPR spectra of ground ginger sample irradiated at dose of 30 kGy, is depicted in Fig. 5.

Radical scavenging ability of alcoholic extracts of spices

The influence of γ-radiation dose on the radical-scavenging and antioxidant ability of spice samples was monitored in the ethanolic extracts using DPPH free radical as an oxidant (Fig. 6).

Previously, the application of DPPH as a radical-scavenger was described [9, 52]. In addition, it can also act as an electron acceptor from antioxidants. Several electron transfer reactions of DPPH with phenols, amines and other compounds were described in the literature [55, 56].

The time course of EPR spectra was recorded for 15 min. Under the given experimental conditions, the EPR spectrum of DPPH free radical represents a five-line signal as depicted in the inset of Fig. 7. Ethanol was used instead of spice extract as a blank against which changes in time-course of EPR spectra intensity were compared. The decay of DPPH signal resulting mostly from termination reactions with the antioxidants present in spice ex-

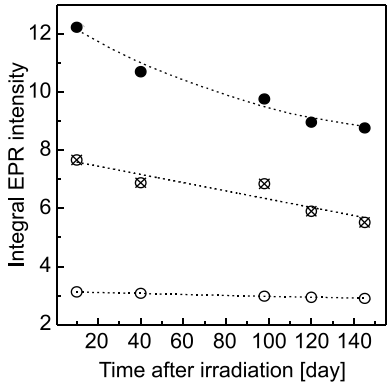

Fig. 5. Time dependence of integral EPR spectra intensity of semiquinone (○), 'cellulosic' (●) and 'carbohydrate' (⊗) paramagnetic species, obtained from the simulation analysis of experimental EPR spectra of ground ginger sample γ-irradiated at dose of 30 kGy and measured 20 weeks after the radiation treatment at 298 K.

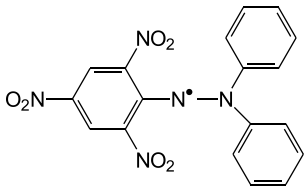

Fig. 6. Structure of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable free radical.

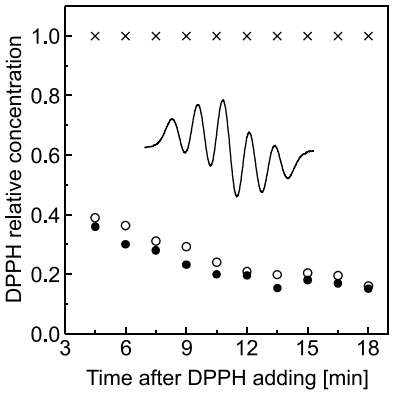

Fig. 7. The time dependence of relative DPPH concentration after its mixing with clove ethanolic extracts prepared from samples irradiated at doses: × blank experiment (ethanol); O reference 0 kGy; ● 30 kGy. Inset represents EPR spectrum of DPPH in ethanol (magnetic field sweep width, SW=6 mT).

tracts was monitored in time (Fig. 7) and was fitted to the exponential function to obtain the corresponding values of DPPH half-time.

Previously, we had found that the radical-scavenging activity of ethanolic extracts prepared from non-irradiated black pepper sample was indistinguishable from that of γ-radiation treated samples at doses of 5 up to 10 kGy. However, extracts prepared from black pepper samples processed at higher doses of γ-radiation (20 and 30 kGy) revealed the lowered ability to terminate DPPH [9]. Extracts prepared from other investigated spices (oregano, allspice, ginger, clove) did not demonstrate any correlation between the absorbed dose of γ-irradiation and their radical-scavenging abilities (Fig. 7).

CONCLUSIONS

EPR spectroscopy confirmed that γ-radiation treatment of cellulose-containing spice samples resulted in the dose-dependent generation of paramagnetic species of different structures and properties. Their behaviour is significantly affected by temperature, relative humidity and storage conditions. All these factors have to be taken into account when surveying the changes induced by the absorption of γ-radiation. The differences between spices indicate that although the spice matrices are very similar, they represent complex systems, and the individual impact of γ-irradiation is strongly influenced by the presence of characteristic specific spice constituents.

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