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Analytical Methods

Detection of caraway and bay leaves irradiation based on their extracts' antioxidant properties evaluation

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ABSTRACT

Antioxidant properties of extracts prepared from native (non-irradiated) ground caraway (*Carum carvi*, L.) and bay leaves (*Laurus nobilis*, L.) samples, as well as from those γ-irradiated by Co^{60} source at doses from 5 to 30 kGy were studied by EPR and UV–VIS spectroscopy, and expressed as Trolox Equivalent. Ferric reducing power, thiobarbituric acid reactive substances and total phenolic compounds content of each extract were characterised, as well. In addition, character of radicals formed upon the γ-irradiation in solid phase was studied by means of EPR spectroscopy. For the first time, multivariate statistical methods were used for γ-irradiation detection. The experimental data obtained from UV–VIS and EPR, were successfully used in canonical, step-wise and *k*th-neighbour discriminant analyses for the differentiation and classification of γ-irradiated samples from those of reference. More than 92% predictability of γ-irradiation was achieved by cross-validation tests for both caraway and bay leaves samples, exposed even at low radiation doses.

1. Introduction

Irradiation of food and food products including spices is effectively used for insects and microbial contamination elimination, serving thus for many years as a food preservation technique. According to the respective directive of European Community in which a list of foods and food ingredients that can be treated with ionizing radiation was established. In the same document, the maximum overall average absorbed dose applicable for dried aromatic herbs, spices and vegetable seasonings sterilisation was set to 10 kGy, in good agreement with Codex Alimentarius General Standard for irradiation (Codex Stan, 2003; Directive 1999/3/EC). Limitation of US Food and Drug Association (FDA), on the other hand, extent the maximum limit to 30 kGy (Code of Federal Regulation, 2004).

Caraway (*Carum carvi*, L.) is one of the commonly used spices for food preparations. Similarly to other spices, its primary active constituent represents volatile oil (4–6% on average), which itself consists from carvone and limonene. The oil fraction from both caraway seeds and herbs contains considerable amounts of oxygenated monoterpenes. Caraway aldehyde was found to be the main component of seed oil (53.6%) as well as of herb oil (40.5%) (El-Sawi & Mohamed, 2002).

Bay leaves (*Laurus nobilis*, L.) are evergreen shrub or dried leaves native to Asia. Besides a gastronomy application, this spice is used as an insecticide, or in traditional medicine, suppressing high blood sugar, migraine, headaches, bacterial and fungal infections, gastric ulcers, but found also other application (Ali-Shtayeh, Yaniv, & Mahajna, 2000; Baytop, 1984; Elmastas, Ozturk, Gokce, Erenler, & Aboul-Enein, 2004; Gülçin, 2006; Kang et al., 2002; Papachristos & Stamopoulos, 2002; Yoshikawa et al., 2002). It consists mostly of essential oil including 1, 8 cineol, eugenol, acetyl eugenol, methyl eugenol, α- and ß-pinene, phellandrene, linalool, geraniol and terpineol. Aroma of the leaves is influenced mostly by the content of terpenes, cinnamic acid and its methyl ester (Demo, Petrakis, Kefalas, & Boskou, 1998; Elmastas et al., 2004; Kang et al., 2002).

A noticeable increase of information on potential human health benefits, revealing also antioxidant properties of both, caraway and bay leaves can be found in the literature (Bonanni, Campanella, Gatta, Gregori, & Tomassetti, 2007; Conforti, Statti, Uzunov, & Menichini, 2006; Fatemeh, Kadivar, & Keramat, 2006; Hinneburg, Dorman, & Hiltunen, 2006a; Hinneburg, Dorman, & Hiltunen, 2006b; Politeo, Jukič, & Miloš, 2006; Satyanarayana, Sushruta, Sarma, Srinivas, & Subba Raju, 2004; Thippeswamy & Naidu, 2005; Škerget et al., 2005). On the other hand, only few contributions possess the information about the effect of γ-irradiation or heat processing on their antioxidant properties. In this context, few recently published papers pointed on the effect of irradiation at doses up to 50 kGy on antioxidant activity of caraway and laurel leaf.

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A review published by Chmielewski dealt besides the other aspects, with an impact of γ-irradiation at dose of 10 kGy on the stability of main biologically active substances of spices (Chmielewski, 2005).

Chemical changes induced by γ-irradiation were investigated in 11 pure aroma compounds typically found in spices by Sjövall et al. involving GC and GC–MS (Sjövall, Honkanen, Kallio, Latva Kala, & Sjoberg, 1990). Respective spice samples were irradiated at doses of 0, 10, and 50 kGy. As a result of γ-irradiation, significant decrease (4–13%) in linalool and α-terpineol content, typical components of coriander and bay leaves was detected.

Black cumin samples irradiated at doses from 2.5 to 10 kGy were studied by Arici, Colak, and Gecgel (2007). The increasing radiation dose causes an increase of both free fatty acid and peroxide values, whereas oil contents, iodine numbers, refraction index and Rancimat values decreased. In the composition of fatty acids, *trans* fatty acid levels increased while the percentages of unsaturated fatty acids decreased.

Antioxidant properties of caraway, cumin, anise and fennel essential oils extracted from untreated, γ-irradiated and microwaved seeds were evaluated by Farag and Khawas (1998). As they found, neither γ-irradiation at 10 kGy nor microwave treatments affects the antioxidant status of the essential oils under study in any way.

Respecting the fact that the application of γ-radiation on spices leads to the production of paramagnetic species an electron paramagnetic resonance (EPR) spectroscopy is considered to be the unique detection technique for their characterization and investigations (EN 13708: 2001; EN 13751: 2002; EN 13783: 2001; EN 13784: 2001; EN 1787: 2000; Horváthová, Suhaj, Polovka, Brezová, and Simko, 2007; Polovka, Brezová, & Šimko, 2007; Polovka et al., 2006; Raffi et al., 2000; Suhaj, Rácová, Polovka, & Brezová, 2006; Sádecká & Polovka, 2008).

Abdel-Fattah used EPR spectroscopy to distinguish the irradiated from non-irradiated caraway seeds. Additive re-irradiation moreover produced a reproducible dose–response function, which can be used to assess the initial dose by back-extrapolation. The stability of the radiation-induced EPR signal of irradiated caraway was studied over a storage period of 6 months. As he confirmed, the exponential fit to the data cannot be used without a correction of decay of free radicals (Abdel-Fattah, 2002).

Multivariate analysis represents valuable tool which enable the categorization of different food samples via the consideration of many variables that can be measured, often in a single analytical level. The analysis of chromatographic, electrophoretic or elemental data was previously effectively used for the discrimination of many kinds of foods, e.g., of cheeses and cheese-derived products or wines, based on the different geographical origin, varieties or quality (Koreňovská & Suhaj, 2005; Koreňovská & Suhaj, 2007; Rodríguez, Alaejos, & Romero, 1999; Suhaj & Koreňovská, 2007). In spite of this, its application on the assessment of γ-irradiated systems is only poorly described in the literature. Canonical discriminant analysis was previously used for the discrimination of γ-irradiated carbohydrates in honeys and fructose or starch gels (Kizil & Irudayaraj, 2006; Kizil & Irudayaraj, 2007).

Our previous research was focused on the study of antioxidant activity changes due to the exposure of solid spices to γ-radiation. (Horváthová et al., 2007; Horváthová, Suhaj, & Šimko, 2007; Polovka et al., 2006, 2007; Suhaj et al., 2006). The influence of absorbed dose on the character of radical species formed in several solid spice samples exposed to γ-radiation, their life-time as well as thermal stability and other characteristics were studied, as well (Polovka et al., 2006; Polovka et al., 2007; Suhaj et al., 2006).

In this contribution, antioxidant properties of extracts prepared from non-irradiated ground caraway and bay leaves as well as from those γ-irradiated by Co^{60} source at doses from 5 to 30 kGy were studied by EPR and UV–VIS spectroscopy. Moreover, character of radical species formed upon the γ-irradiation in solid spice samples was studied. All the experimental data were processed by multivariate statistic calculations, employing methods of principal components (PCA), canonical (CDA), step-wise and *k*th-neighbour discriminant analysis and classification.

2. Materials and methods

2.1. Samples characterisation

2.1.1. Solid samples characterisation

Commercial powdered dry caraway *(Carum carvi)* originated from Austria and bay leaves *(Laurus nobilis)* from Turkey were used in the experiments. Both raw grounded spices harvested in year 2006 were provided by Kotanyi, GmbH, Vienna, Austria. Spice samples were irradiated according to commercial practices at Artim, Ltd. (Prague, Czech Republic) by means of ^{60}Co source at average doses of 5, 10, 20 and 30 kGy, at a dose rate 2 kGy h^{-1}. The range of doses applied was chosen in respect to the Directive EC 1999/3 as well as FDA limitations for spices irradiation (Code of Federal Regulation, 2004; Directive 1999/3/EC). The properties of γ-radiation processed samples were compared to that of respective reference (non-irradiated, 0 kGy) samples. All the spice samples were stored in polyethylene bags at laboratory conditions (darkness; temperature, 25 °C, relative humidity, 40%) between the experiments.

2.1.2. Extract preparation

Extracts used in UV–VIS experiments were prepared as follows: 2 g of individual spice sample was mixed with 50 ml 80% (v/v) aqueous methanolic solution. The mixture was shaken 1 h at 25 °C using a laboratory shaker (Innova 2000, New Brunswick Scientific, Edison, New Jersey, USA) at 3.3 Hz and subsequently the solid phase was removed by filtration.

Extracts for EPR experiments were prepared by mixing of exactly 0.4 g spices with 10 ml ethanol of spectroscopic grade (Merck, Germany), to keep the concentration comparable to those used in UV–VIS experiments. The suspension was then gently stirred and stored in darkness for 24 h at room temperature. Subsequently, the solid spice particles were removed by filtration.

2.2. UV–VIS experiments

UV–VIS spectrophotometer Specord M40 (Carl Zeiss, Jena, Germany) with accessory was used for absorbance measurements. All the experiments were realised in a quartz square cell (path length, 1 cm) under the following conditions: spectral bandwidth, 10 cm^{-1}; integration time, 1 s; gain, 1. All measurements were performed at temperatures from 22 °C to 25 °C. Absorbance of pure methanol was measured in blank experiment.

2.2.1. ·DPPH radical-scavenging assay

·DPPH assay was modified according to Bandonienė, Murkovic, Pfanhauser, Venskutonis, and Gruzdiéne (2002) and Lebeau et al. (2000). Exactly 0.5 ml of methanolic caraway extract (0.05 ml, bay leaves extract) was placed into 25 ml methanolic solution of ·DPPH and absorbance at 515 nm was measured after 5 min at 25 °C. Radical-scavenging activity of extract was expressed as the percentage and calculated as:

$$\% = \frac{A_{0(\cdot\mathrm{DPPH})} - A_{t(\mathrm{Sample})}}{A_{0(\cdot\mathrm{DPPH})}} \times 100, \quad (1)$$

where: $A_{0(\cdot\mathrm{DPPH})}$, $A_{t(\mathrm{Sample})}$ is the absorbance of pure ·DPPH solution, or the absorbance of solution containing sample extract in chosen time *t*.

The calculation of ·DPPH radical-scavenging ability from Eq. (1) did not reflects neither the different volumes of methanolic extracts of respective spices, nor the dilution factors. These ones were taken into account by the expression of ·DPPH radical-scavenging ability of extracts as their Trolox equivalent antioxidant capacities (TEAC) using the following equation:

$$\mathrm{TEAC} = \frac{\left(A_{0(\cdot\mathrm{DPPH})} - A_{t(\mathrm{Sample})}\right) * V_{(\cdot\mathrm{DPPH})}}{\varepsilon_{515} * V_{(\mathrm{Sample})}} * \nu * Z, \quad (2)$$

where: $A_{0(\cdot\mathrm{DPPH})}$, $A_{t(\mathrm{Sample})}$ is the absorbance of pure ·DPPH solution, or the absorbance of sample extract in chosen time t; $V_{0(\cdot\mathrm{DPPH})}$ is the volume of ·DPPH added to the system; $V_{(\mathrm{Sample})}$ is the volume of sample added to the system; ε_{515} is the molar extinction coefficient ($\varepsilon_{515(\cdot\mathrm{DPPH}} = 4.1\ \mathrm{mmol^{-1}\ dm^3\ cm^{-1}}$); ν is the stoichiometric coefficient of the reaction between ·DPPH and TROLOX ($\nu = 1/2$); Z is the dilution factor.

This approach enables also the comparison of antioxidant activity tests performed by UV–VIS and EPR spectroscopy.

2.2.2. Thiobarbituric acid number

Oxidative reaction products determined as thiobarbituric acid number (TBARS) were analysed according to method suggested by Zin (2002). To 1 ml of respective spice extract, 20% aq. of trichloroacetic acid (2 ml) and of 0.67% aq. thiobarbituric acid solution (2 ml) were added. This mixture was then placed in a boiling water bath for 10 min. After cooling to ambient temperature, the mixture was centrifuged at 3000 rpm (500*g*) for 20 min. Thiobarbituric acid number was determined as an absorbance of supernatant at 532 nm.

2.2.3. Ferric reducing power (FRP)

Determination of ferric reducing power of spice extracts, in fact their ability to reduce the Fe^{3+} to Fe^{2+}, was performed according to Chyau, Tsai, Ko, and Mau (2002). Spice methanolic extract (100 μl) was mixed with 1.9 ml of distilled water; 2 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2 ml of 1% potassium ferricyanide, and the mixture was incubated at 50 °C for 20 min. After that, 2 ml of 10% trichloroacetic acid was added and the mixture was centrifuged at 3000 rpm (500*g*) for 10 min. Upper layer (1 ml) was mixed with 1 ml of distilled water and 0.2 ml of 0.1% ferric chloride, and the absorbance at 700 nm was measured after 1 min.

2.2.4. Total phenolic compounds (TPC) content

Content of total phenolic compounds was determined using the Folin–Ciocalteau modified method (Chaovanalikit & Wrolstad, 2004). 100 μl of respective spice extract was diluted to 15.9 ml of distilled water and 1 ml of Folin–Ciocalteau reagent (Merck, Hohenbrunn, Germany) was added. After 3 min, 3 ml of 20% of sodium carbonate was added and the content was mixed. As the result of reaction, intensive violet colour was developed. The absorbance at 755 nm was measured after 60 min. The same procedure was repeated using a standard solution of gallic acid. The results were expressed as gallic acid equivalent (GAE) in mg/100 ml of extract.

2.3. EPR experiments

Experiments were performed using a portable X-band EPR spectrometer e-scan (Bruker BioSpin, GmbH, Karlsruhe, Germany) with accessory.

2.3.1. Radical-scavenging activity of spices' extracts

A defined volume of respective ethanolic extract was mixed either with the solution of ·DPPH in ethanol (initial concentration of ·DPPH in system, $c_{0(\cdot\mathrm{DPPH})} = 0.1\ \mathrm{mmol/dm^3}$), or the solution of $ABTS^{\cdot+}$ in water, prepared as previously suggested by Re et al. (1999). The concentration of $ABTS^{\cdot+}$ stock solution was 85–100 μmol/dm^3 determined via the measurement of UV absorbance at 735 nm using the value of molar extinction coefficient, $\varepsilon_{735\ \mathrm{nm}} = 14.8\ \mathrm{mmol^{-1}\ dm^3\ cm^{-1}}$ (Arts, Haenen, Voss, & Bast, 2004).

The mixture was purged with 1 ml of air and immediately transferred into the EPR flat cell. The EPR measurements started exactly 3 min after the ·DPPH or $ABTS^{\cdot+}$ addition and a set of 10 EPR spectra was recorded during the next 15 min. Each experimental spectrum represents an average of 30 individual scans. The experiments were performed in triplicate mode. The relative standard deviation among the individual measurements was less than 5%.

2.3.2. EPR measurements with solid samples

Spice sample (100 ± 0.5 mg) was placed in the thin-wall EPR quartz tube (internal diameter, 3 mm) and cylindrically shaped column was formed (sample column heights: 2.1 ± 0.2 cm (caraway) and 2.0 ± 0.2 cm (laurel leaves), respectively. Then the tube was inserted into the standard rectangular cavity of EPR spectrometer and EPR spectrum was recorded at 298 K as an average of 20 individual scans. The response and settings of EPR spectrometers was checked by means of solid ·DPPH standard (Bruker) daily before the experiments. The g-values were determined with uncertainty of 0.0005 by simultaneous measurement of a Bruker certified reference standard ($g = 1.98$) and respective spice sample in the NG holder of EPR spectrometer in a similar conditions like when double-resonance cavity is used.

2.3.3. Processing of EPR spectra

The experimental EPR spectra processing and simulation was carried out using *WIN EPR* and *SimFonia* programs (Bruker), respectively. The integral intensities of EPR signals were obtained by double integration of the spectrum. The multi-component experimental EPR spectra were evaluated as a linear combination of individual EPR spectra simulations using a least-squares minimization procedure with the *Scientist* (MicroMath) program.

The statistical parameters of the calculation procedure (coefficient of determination and correlation) serve as a determination of the simulation quality, i.e. correlation of the experimental and simulated spectra. The relative concentration of the individual paramagnetic species was evaluated from the contributions of the individual simulations to experimental spectrum after double integration.

2.4. Multivariate statistics

To distinguish the irradiated spice sample from the respective reference (non-irradiated) one, multivariate statistic calculations, employing methods of canonical, stepwise and *k*th-neighbour discriminant analysis and classification were performed by means of Unistat® (Unistat, London, United Kingdom) statistical software, taking into consideration all the experimental data obtained both from UV–VIS and EPR experiments. The convergence criteria of discriminant analysis were chosen for a standardized proximity matrix with maximum number of iteration, 50. The following stepwise selection criteria were used: tolerance – 0.001, F statistic: F to enter – 3.8416, F to remove – 2.7056.

The recognizability of discriminant model was determined as the percentage of the correctly classified samples in the training data set. In addition, the predictability was tested, as the percentage of the samples correctly classified in the leave-one-out cross-validation approach as suggested by Berrueta, Alonso-Salces, and Héberger (2007).

3. Results and discussion

3.1. UV–VIS experiments

As follows from previously published data, the dry matter content and extraction yield are parameters that must be taken into account in order to correctly evaluate the antioxidant activity of spices and their extracts. These values may alter as a result of either radiation impact or post-irradiation storage of spices (Ayed, Yu, & Lacroix, 1999; Huang & Mau, 2006; Kim, Yook, & Byun, 2000).

The dry matter content of solid spices and their extraction yields were determined before each measurement. Data obtained clearly indicated that dry matter content values of both bay leaves and caraway samples determined immediately after the irradiation and after 6 months of post-irradiation storage differ only negligibly and there was no obvious dependence between the dry matter content and absorbed radiation dose. Its average values ranged from 92.1% to 92.9% w (caraway) and from 92.5% to 93.3% (bay leaves).

On the other hand, the yield of extraction was slightly decreased upon the dose of γ-radiation absorbed by the respective solid sample. This effect is more significant in caraway samples, in which the difference in extraction yields reached up 6%, whereas in the case of bay leaves, only 3% difference of extraction yields was noticed, comparing the extracts prepared from reference sample and from that γ-irradiated at 30 kGy. As a result of half year post-irradiation storage, the meaningful, about 10% increase of extraction yields of both spices was noticed. It can be supposed that the changes of spices extrability partially contribute to changes of antioxidant activity caused by irradiation and storage.

The effects of γ-irradiation and storage on the antioxidant activity of methanolic extracts prepared from γ-irradiated grounded caraway and bay leaves were characterised by ·DPPH free radical. In addition, the extracts were tested in TBARS and FRP assays and their TPC content was determined, as well.

The results of ·DPPH radical-scavenging ability of caraway and bay leaves extracts are depicted on Fig. 1a. As follows from data presented, the absorption of γ-radiation dose in a range from 5 up to 30 kGy did not cause any significant changes in the ability of caraway methanolic extracts to quench the ·DPPH free radical, since practically no differences between the extracts prepared from reference and irradiated samples were observed, without regards to whether they were prepared immediately after the irradiation, or after 6 months of post-irradiation storage.

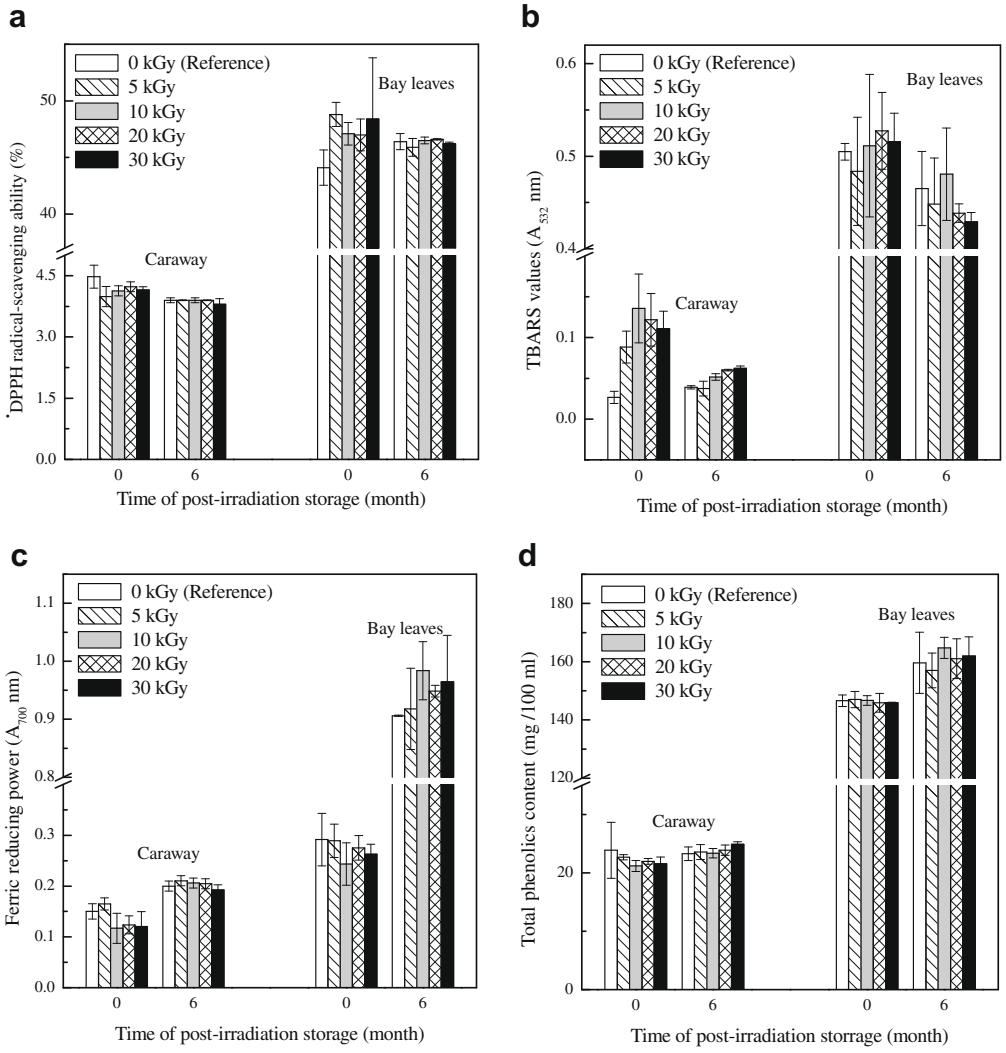

Fig. 1. Effect of γ-irradiation and post-irradiation storage on (a) ·DPPH radical scavenging activity; (b) thiobarbituric acid reactive substances values (TBARS); (c) ferric reducing power (d) the total content of phenolic compounds (TPC); of extracts prepared from caraway and bay leaves samples.

Under the identical conditions, the extracts prepared from bay leaves samples possessed approximately 10 times higher radical-scavenging activity than that from caraway. Nevertheless, ·DPPH radical-scavenging ability of these extracts is only in minor way influenced by the γ-radiation, as the difference between extract of reference and of sample treated at 30 kGy reached 5%. After a half-year post-irradiation storage, the differences in radical-scavenging ability between the extracts prepared from γ-irradiated and reference bay leaves samples disappeared.

In recently published papers, the increase of ·DPPH radical-scavenging activity of extracts from irradiated herbs and spices is relatively rare. It was observed, e.g. in the rosemary leaves powder extract prepared from sample exposed to 30 kGy (Pérez, Calderón, & Croci, 2007). On the contrary, our previous experiments with extracts prepared from ground black pepper samples treated at doses from 5 to 30 kGy revealed that the ·DPPH radical-scavenging activity decreased with the increasing radiation dose (Polovka et al., 2006; Polovka et al., 2007; Suhaj et al., 2006).

$TEAC_{\cdot DPPH}$ values calculated from UV–VIS experiments (Eq. (2), Table 1), confirmed again that the ·DPPH radical-scavenging ability of both caraway and bay leaves extract is only negligibly influenced by the absorption of γ-radiation. Caraway extracts prepared immediately after the γ-irradiation, possessed the average $TEAC_{\cdot DPPH}$ values of 0.7 ± 0.05, whereas those of bay leaves ranged from 8.3 ± 0.8 (extracts from sample treated at doses from 5 up to 30 kGy) to 9.2 (reference). Although the difference in ·DPPH radical-scavenging ability between the extract prepared from control bay leaves sample and from sample γ-irradiated at 30 kGy is more significant than when expressed in% values, there is not a clear evidence of any relationship between the $TEAC_{\cdot DPPH}$ value and the absorbed dose of γ-radiation.

The effect of γ-irradiation and post-irradiation storage on the content of oxidative products of methanolic extracts of spices expressed as TBARS is shown in Fig. 1b. Experiments performed immediately after the irradiation revealed that extracts prepared from γ-irradiated caraway samples possessed significantly higher TBARS values than that prepared from the reference. The highest, approximately fivefold increased TBARS value was found for extract prepared from sample treated at 10 kGy. These findings are in good agreement with our previously published data, as the similar trend was observed also in methanolic extracts of black pepper and oregano γ-irradiated at doses from 5 up to 30 kGy (Horváthová et al., 2007; Suhaj et al., 2006). As a result of post-irradiation storage, a meaningful decrease of initial TBARS was found, accompanied by the disappearance of dose-dependent differences described above.

In contradiction, the TBARS values determined in extracts prepared from bay leaves samples are approximately 5 times higher, but only negligible effect of γ-irradiation on oxidative products formation was found.

We moreover found, that the application of γ-radiation did not affect the FRP of methanolic extracts neither of caraway nor bay leaves extracts, as finally depicted on Fig. 1c. Applying this assay, the same finding was previously found in extracts of several other herbs and spices treated by ionizing radiation, e.g.: in oregano, powdered ginseng or in dry rosemary leaves (Byun, Son, Yook, Lo, & Kim, 2002; Horváthová et al., 2007; Pérez et al., 2007). On the contrary, γ-irradiation at doses from 5 up to 30 kGy led to the decrease of black pepper extracts reducing power (Suhaj et al., 2006).

Post-irradiation storage of both reference and γ-irradiated caraway samples led to significant, 25% increase of their extracts' FRP. The storage of bay leaves samples tripled the ferric ions reducing potential of respective extracts. It can be concluded that the increased values of FRP resulting from post-irradiation storage of spices are in close relationship with the increased extraction yield (Table 1).

Phenolic compounds represent a very substantial part of antioxidants in spices. In many studies, the antioxidant properties as well as health beneficial properties of food samples are assigned just to them (Aruoma, 2003; Cantos, Espín, & Tomás-Barberán, 2002; Czyzowska & Pogorzelski, 2002; Garcia-Alonso, Rimbach, & Sasai, 2005; Ju & Howard, 2003; Kris-Etherton et al., 2004; Mira, Silva, Rocha, & Manso, 1999; Park & Surh, 2004; Rice-Evans, Miller, & Paganga, 1996; Sanchez-Moreno, 2002; Shetty & McCue, 2003; Stanner, Hughes, Kelly, & Buttriss, 2004; Tiwari, 2004; Vitaglione & Fogliano, 2004). Therefore, the effect of γ-irradiation and storage on the content of total phenolic compounds in methanolic extracts of caraway and bay leaves was evaluated, as depicted on Fig. 1d. The content of phenolics related to 1 g of dry matter of caraway extracts prepared from reference reached maximum of 5 mg, while that of bay leaves, 35 mg. The last value is comparable to the values presented in several previously published papers (Fatemeh et al., 2006; Hinneburg et al., 2006a; Hinneburg et al., 2006b; Thippeswamy & Naidu, 2005). As clearly illustrated on Fig. 1d, the increasing radiation dose practically did not affect the total phenolic content neither in extracts of caraway nor bay leaves samples. These results are in good agreement with previously published papers. Pérez et al. performed the study of the influence of γ-radiation treatment on antioxidant activity of rosemary extracts (Pérez et al., 2007). As they found, γ-irradiation of rosemary leaves powder at dose of 30 kGy remained the phenolic content of its extract unchanged.

On the other hand, some authors pointed out, that γ-irradiation led to the increase of soluble phenols content in extracts of some other spices (Horváthová et al., 2007; Variyar, Bandyopadhyay, & Thomas, 1998; Variyar, Raghavendra, Lokesh, & Akhilender, 2004), that could being explained by better extractability of irradiated spices (Ayed et al., 1999) and/or by destructive oxidation process induced by the absorption of ionizing radiation, resulting in breaking of the chemical bounds of polyphenols and thereby releasing soluble phenols of low molecular weight (Adamo et al., 2004). We suppose, that these phenomena are responsible also for the increase of FRP values, as discussed above.

A half year post-irradiation storage did not affect significantly the content of soluble phenols in caraway, but resulted in about 10% increase of polyphenolic compounds in extracts prepared from the reference bay leaves sample or from samples exposed to γ-radiation.

Table 1
Values of Trolox equivalent antioxidant capacity (TEAC) of caraway and bay leaves extracts evaluated from UV–VIS and EPR experiments performed immediately after the γ-irradiation and after 6 months of post-irradiation storage.

Radiation dose (kGy)	$TEAC_{DPPH}$ (mmol Trolox/g of dry matter)				$TEAC_{ABTS^{\cdot+}}$ (mmol Trolox/g of dry matter)
	UV–VIS experiments		EPR experiments		EPR experiments
	0 Months	6 Months	0 Months	6 Months	0 Months
Caraway					
0	0.8	0.7	4.7	4.2	9.2
5	0.7	0.7	4.8	5.1	8.2
10	0.7	0.7	4.4	4.0	8.6
20	0.7	0.7	4.3	4.2	8.4
30	0.7	0.6	4.4	4.5	7.8
Bay leaves					
0	9.2	8.5	19.8	19.7	19.6
5	7.7	8.4	21.3	21.2	19.3
10	8.0	8.5	20.1	20.05	17.6
20	7.8	8.5	17.9	18.5	13.3
30	7.8	8.4	16.4	16.4	13.3

3.2. EPR experiments

3.2.1. Antioxidant properties of caraway and bay leaves' extracts

·DPPH assay was used also to monitor the influence of γ-irradiation of caraway and bay leaves samples on the antioxidant properties of their ethanolic extracts by means of EPR spectroscopy. This assay was recently successfully applied to test the antioxidant properties of various food and beverages (Delincée & Soika, 2002; Polovka, 2006; Polovka, Brezová, & Staško, 2003; Staško, Brezová, Biskupič, & Rapta, 2007; Staško, Polovka, Brezová, Biskupič, & Malík, 2006), but also to test the radical-scavenging activity of extracts prepared from black pepper, ginger or clove treated at γ-radiation doses up to 30 kGy (Horváthová et al., 2007; Polovka et al., 2006; Polovka et al., 2007; Suhaj et al., 2006).

The dependence of relative ·DPPH concentration on time after its addition to experimental system, containing either pure ethanol (control experiment) or extract prepared from reference caraway sample and from samples exposed to γ-radiation, is depicted on Fig. 2a. As can be expected, immediately after ·DPPH addition to experimental system containing either caraway or bay leaves extract, the termination of this stable radical mostly by the extracts component occurred, resulting in the corresponding time-dependent decay of ·DPPH EPR signal.

Two approaches were used in order to quantify the radical-scavenging action of ethanolic extracts. First, the decay of ·DPPH was fitted to first-order kinetic model (Eq. (3)) which best fit to the experimental data, and the formal first order rate constants were calculated (Fig. 2b) (Polovka et al., 2006; Polovka et al., 2007; Suhaj et al., 2006).

$$\ln\left(I_{\mathrm{EPR}}\right) = \ln\left(I_{\mathrm{EPR}}^{t=0}\right) + k' * t, \tag{3}$$

where: I_{EPR} is the double integrated EPR spectrum; k' is the formal first order rate constant (min^{-1}); t is the time after ·DPPH addition into experimental system (min).

Calculated values of k' reflected the ability of spices extract to terminate ·DPPH. As follows from data presented on Fig. 2b, ·DPPH radical-scavenging ability of caraway spice extracts was practically not affected by the absorption of γ-radiation. In comparison to extract prepared from reference sample, only slight decrease of ·DPPH termination rate was noticed in extracts from samples irradiated at 5 and 10 kGy. Post-irradiation storage did not influence meaningfully the ability of caraway extracts to terminate ·DPPH, as well. The similar behaviour revealed also the extracts prepared from bay leaves (data not presented).

In the second approach, $\mathrm{TEAC}_{\cdot\mathrm{DPPH}}$ values were calculated using the modified Eq. (2):

$$\mathrm{TEAC}_{\cdot\mathrm{DPPH}}^{\mathrm{EPR}} = \frac{c_{0(\mathrm{DPPH})} * \left(I_{0(\mathrm{DPPH})} - I_{t(\mathrm{Sample})}\right) * V_{(\mathrm{DPPH})}}{V_{(\mathrm{Sample})}} * v * Z, \tag{4}$$

where: $c_{0(\bullet\mathrm{DPPH})}$ is the concentration of ·DPPH stock solution; $I_{0(\bullet\mathrm{DPPH})}$, $I_{t(\mathrm{Sample})}$ is the double integrated EPR spectrum, recorded in reference sample; and in sample extract in chosen time t, respectively; $V_{(\bullet DPPH)}$ is the volume of ·DPPH/$\mathrm{ABTS}^{\cdot+}$ added to the system; $V_{(\mathrm{Sample})}$ is the volume of sample added to the system; v is the stoichiometric coefficient of the reaction between ·DPPH and TROLOX ($v = 1/2$); Z is the dilution factor.

Double integral of EPR spectrum (I_t) recorded exactly at 10.5 min after the ·DPPH addition into experimental system was used in calculation. As follows from $\mathrm{TEAC}_{\cdot\mathrm{DPPH}}$ values presented in Table 1, extracts of caraway samples reach TEAC values around 4.5 ± 0.5 mmol/g, independently on whether the extract was prepared from γ-irradiated sample or not. In good agreement with UV–VIS experiments, extracts prepared from bay leaves samples possessed generally higher TEAC values, about 19 mmol/g. While the extracts prepared immediately after the radiation process did not reveal any meaningful change of ·DPPH radical-scavenging ability, as a result of post-irradiation storage, the TEAC values slightly decreased, especially in extracts prepared from γ-irradiated samples.

While comparing the $\mathrm{TEAC}_{\cdot\mathrm{DPPH}}$ values evaluated from UV–VIS and EPR experiments, it can be concluded that values obtained from EPR experiments were significantly higher than those from UV–VIS, especially in the case of caraway extracts. These differences probably followed from different sensitivity and principles of both spectroscopic techniques, and/or from the differences in extracts composition. Dominant role has the content and structure of individual polyphenols in individual extracts.

Miller et al. suggested the application of $\mathrm{ABTS}^{\bullet+}$ cation-radical, generated e.g., via chemical reaction with potassium persulphate or ascorbic acid, on the evaluation of antioxidant properties of foods or water-soluble phenols (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993). It was previously successfully employed e.g., for liquor or wine samples antioxidant properties characterisation (Castillo et al., 2000; De Beer, Joubert, Gelderblom, & Manley,

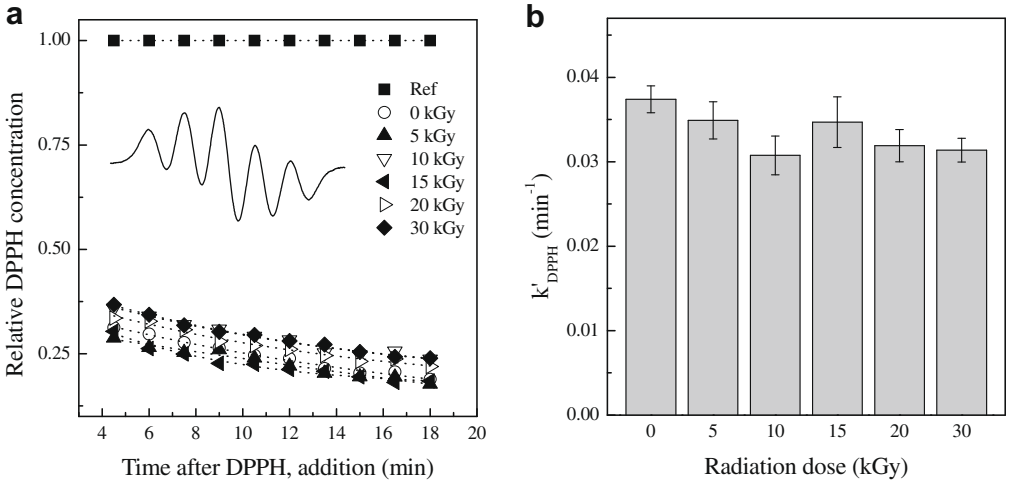

Fig. 2. (a) The dependence of relative ·DPPH free radical concentration on time after its addition to caraway ethanolic extracts prepared from reference caraway sample and from samples γ-irradiated at doses from 5 up to 30 kGy. Extracts were prepared immediately after the radiation process. Inset represents EPR spectrum of ·DPPH in ethanol. (b) The dependence of formal first-order rate constants of •DPPH decay on absorbed dose of γ-radiation evaluated for caraway ethanolic extracts.

2003; Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000; Soleas, Tomlinson, Diamandis, & Goldberg, 1997; Staško et al., 2006; Staško et al., 2007; Van den Berg, Haenen, Van den Berg, & Bast, 1999). Similarly like in the case of ·DPPH assay, extracts prepared from both caraway and bay leaves revealed significant capability to terminate this cation-radical. Values of formal first-order rate constants revealed only negligible influence of γ-irradiation on radical-scavenging ability of caraway extracts. On the other hand, results obtained for bay leaves indicated the considerable decrease of *k*' values of extracts prepared from bay leaves samples γ-irradiated at doses above 10 kGy. Identical trend followed also from $TEAC_{ABTS^{\cdot+}}$ values, evaluated in the same manner as described above for ·DPPH. As a result of γ-irradiation, a dose-dependent decrease of $TEAC_{ABTS^{\cdot+}}$ from 19.9 mmol/g (reference sample) to 13.3 mmol/g of dry matter (sample irradiated at 30 kGy) was observed (Table 1).

Results obtained from UV–VIS experiments are in good correlation with those from EPR experiments. Regarding the differences of $TEAC_{\cdot DPPH}$ and $TEAC_{ABTS^{\cdot+}}$ values obvious mainly in extracts prepared from caraway, it should be noted here, that they probably followed from the differences in redox potential of $ABTS^{\cdot+}$ and ·DPPH ($E^{\varnothing}_{(\cdot DPPH/DPPH^{-}\cdot)} = 0.43$ V; $E^{\varnothing}_{(ABTS^{\cdot+}/ABTS^{\cdot})} = 0.68$ V, measured against standard hydrogen electrode) which influenced the reactivity of these compounds, or from the differences in the composition of polyphenols (Scott, Chen, Bakac, & Espenson, 1993). The same conclusion was made by Staško et al. when investigated the antioxidant properties of Tokay wines (Staško et al., 2006).

3.3.1. The influence of γ-irradiation on solid caraway and bay leave samples

In order to obtain as many characteristics on studied samples that would be subsequently used in the multivariate statistics as even possible, solid samples were characterised by EPR spectroscopy, as well.

The detailed simulation analysis revealed that EPR spectra of both reference samples represents broad singlet line with unresolved hyperfine splitting, attributable mostly to Mn^{2+} ions, upon which the additional sharp EPR line is superimposed, previously assigned to stable semiquinone radicals produced by the oxidation of polyphenolic compounds present in plants (Jezierski et al., 2002; Merdy, Guillon, Dumonceau, & Aplincourt, 2002; Morsy & Khaled, 2001; Morsy & Khaled, 2002; Pedersen, 2002; Polovka et al., 2003; Polovka et al., 2006; Polovka et al., 2007; Suhaj et al., 2006; Sádecká & Polovka, 2008; Ukai & Shimoyama, 2003). In addition, the presence of low-intensive EPR singlet line was noticed in caraway reference sample, attributable to radicals generated during the grinding process. Previously, the production of free radicals by the grinding process of sugars was described e.g. by Yordanov and Georgieva (2004). The effect of mechanical treatment on radical production in lactose and carboxymethylcellulose was described by Raffi et al. (2002). The presence of radicals originating from grinding process was noticed also in γ-irradiated black pepper and clove samples (Polovka et al., 2006; Polovka et al., 2007; Suhaj & Horváthová, 2007).

The exposure of samples to γ-irradiation caused the changes of chemical and physical properties leading to the dose-dependent production of additional paramagnetic structures. As follows from detail simulation analysis of obtained spectra (data not shown), different, mostly cellulosic and carbohydrate radical structures were identified, in accord with several recently published papers (Bayram & Delincée, 2004; Delinceé & Soika, 2002; Formanek et al., 1999; Horváthová et al., 2007; Polovka et al., 2006; Polovka et al., 2007; Raffi et al., 2000; Sádecká & Polovka, 2008; Suhaj & Horváthová, 2007; Yordanov & Gancheva, 2000; Yordanov et al., 1998). These radicals originate either from cleavage processes of cellulose matter (laurel leaves) and/or of other polysaccharides forming the skeleton of plant structures and their cells, as the cellulosic radicals were not detected in γ-irradiated caraway samples.

The dependence of double-integrated EPR spectra on absorbed dose presented at Fig. 3 can be effectively used by competent food-control authorities for dosimetric purposes, enabling the estimation of previously absorbed dose (Horváthová et al., 2007; Polovka et al., 2006; Polovka et al., 2007; Suhaj & Horváthová, 2007).

As a result of irreversible decay of radical structures induced by γ-radiation, the EPR spectra intensity of irradiated samples decreased gradually, whereas the signal intensity of both reference samples remains practically unaffected upon a half-year of post-irradiation storage (Fig. 3). Simulation analysis of experimental spectra recorded in regular time intervals during the storage exhibit the lowest stability of cellulosic radicals in laurel leaves (half life ~10 weeks) followed by carbohydrate radicals, which stability ranged from 20 up to 60 weeks, in good agreement with our previously published papers (Horváthová et al., 2007; Polovka et al., 2006; Polovka et al., 2007; Suhaj & Horváthová, 2007). These experiments stressed out the importance of half-lives of radical structures estimation in order to improve the reliability of absorbed dose estimation.

3.4. Multivariate statistic

EPR spectroscopy as well as other experimental methods was recognised as valuable dosimetric techniques. Respecting the problems connected with the analysis of spice samples, other, easy-to use procedures of γ-irradiation detection are strongly required. A multivariate statistics represents a method of choice, as the results of various analytical techniques can be effectively used for the discrimination of samples.

Experimental data were statistically evaluated by the multivariate PCA and discriminant analysis, using the model suitable for the visualisation, classification and prediction of possible γ-irradiation of samples under study.

Results of principal component analysis of reference (non-irradiated) caraway and bay leaves' samples and of samples γ-irradiated at doses from 5 up to 30 kGy, are depicted on Fig. 4. As follows from data presented, an unambiguous positive differentiation of caraway samples from those of bay leaves was achieved,

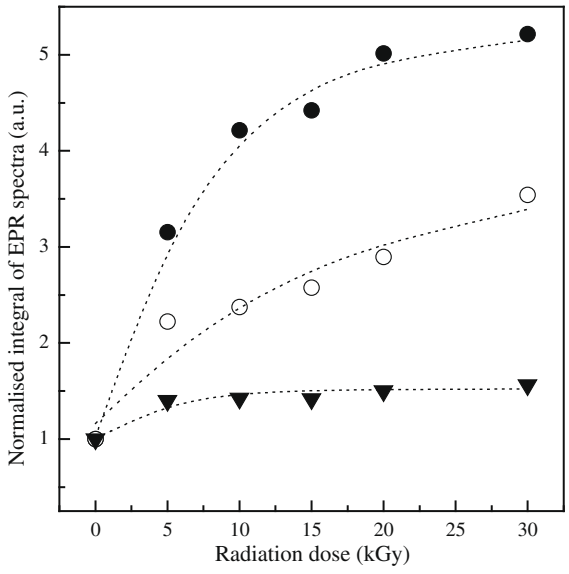

Fig. 3. The dependence of integral EPR intensity of caraway samples on radiation dose evaluated for EPR spectra measured at 298 K using 0.633 mW microwave power two days (●), 1 months (○) and 6 months (▼) after irradiation.

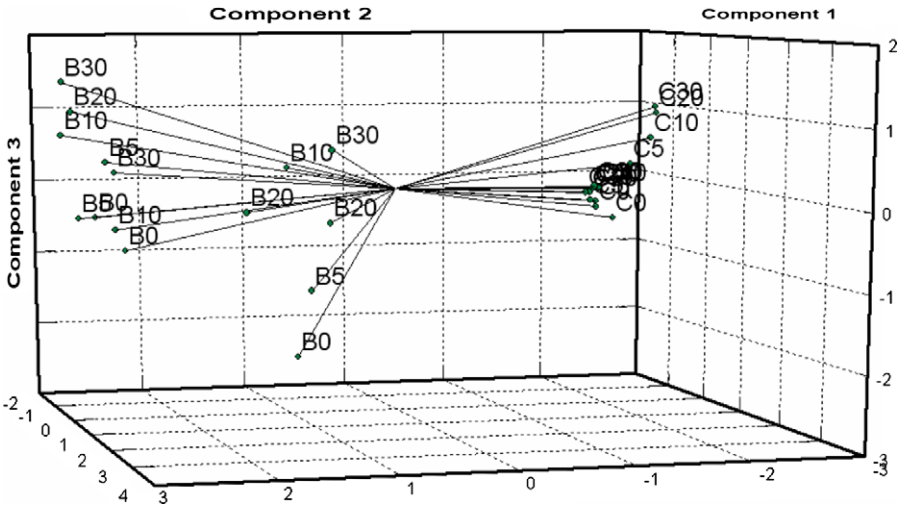

Fig. 4. Principal component analysis of reference (non-irradiated) caraway (C) and bay leaves (B) samples and of samples γ-irradiated at doses from 5 up to 30 kGy. All experimental data were used as variables for principal components construction.

using all experimental data as variables for principal components construction. These results followed from the very different values of antioxidant characteristics obtained both by UV–VIS and EPR for caraway and bay leaves. As followed from variance table (data not presented), first three principal components explained cumulatively 97% of the whole system variability. Eigenvectors indicated that in the first principal component, the total variability is dominantly influenced by TPC content, whereas in the second and third

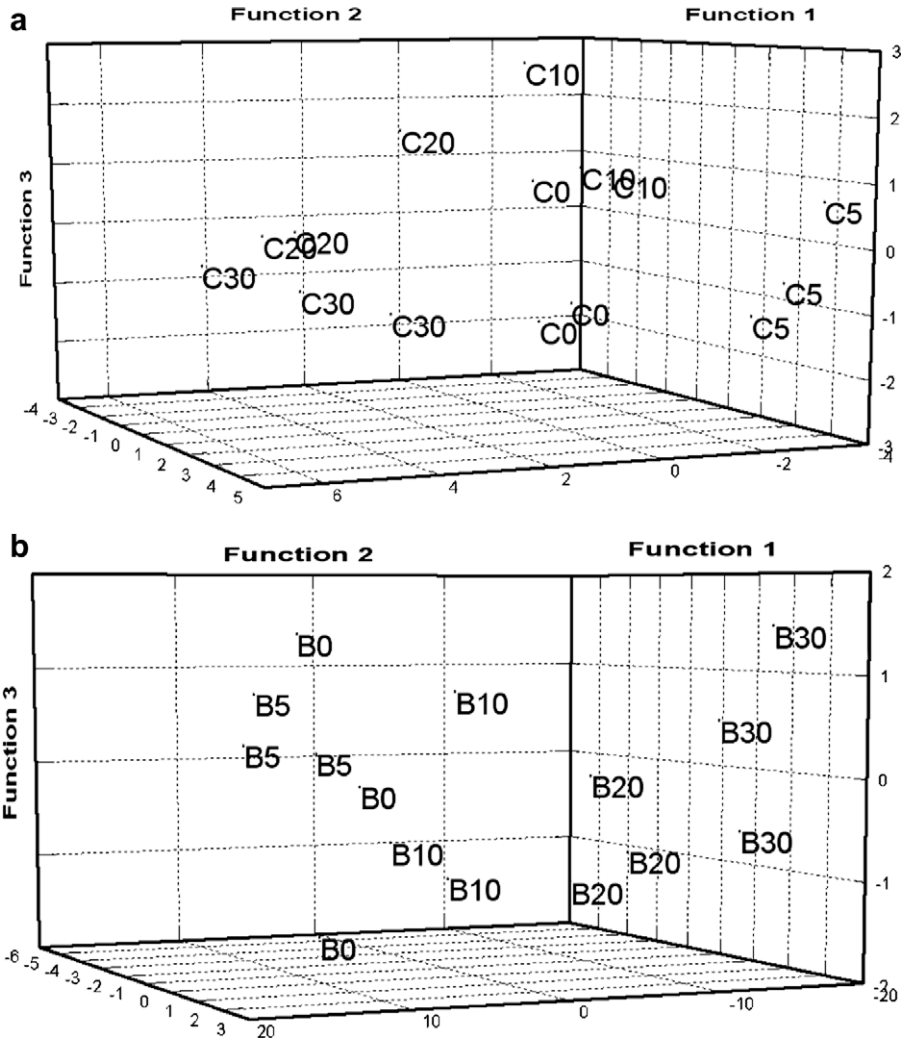

Fig. 5. Canonical discriminant analysis of reference (non-irradiated) caraway (a)) and bay leaves (b)) samples and of samples γ-irradiated at doses from 5 up to 30 kGy.

one, relative EPR intensities of solid samples had the most significant weight. Using the PCA approach, spice samples were clearly differentiated according to their species, without respect on whether they were exposed to γ-irradiation, or not.

To distinguish the native (non-irradiated) samples from those exposed to γ-radiation, canonical discriminant analysis was performed, using all the experimental data as variables for discrimination functions construction. As followed from the results obtained, the recognizability of 93% and the predictability of 92% were achieved for caraway samples classification according to the applied doses of γ-radiation, respectively. Three most important discriminant functions explaining cumulatively about 99% of the whole system variability were used for the visualisation of statistical results (Fig. 5a). According to the values of CDA standardised coefficients, the most important role in the first discriminant function revealed the values of FRP and TBARS, whereas in the second and third one, FRP and relative intensities of solid-state EPR spectra. Meaningful correlations with the third function were found also for $TEAC_{\cdot DPPH}$ values, obtained both from EPR and UV–VIS experiments. Stepwise discriminant analysis selected only the EPR $TEAC_{\cdot DPPH}$ values as the most important marker for discrimination procedure. *K*th-neighbour discriminant analysis proved, that for $k = 1$, 100%, recognizability and 93% predictability of the γ-irradiated and non-irradiated caraway samples classification were achieved, respectively.

Similar results were found in the case of bay leaves' samples, for which the recognizability reached up 100% in both, CDA and *k*1st-neighbour classification (Fig. 5b). The predictability of 96% and 93% was obtained, respectively. In addition, statistical results confirmed, that the discrimination procedure was influenced by the same variables as in the case caraway samples classification.

4. Conclusion

Extracts of both, caraway and bay leaves samples revealed significant antioxidant and radical-scavenging properties, only slightly influenced either by the absorption of γ-irradiation, or subsequent half-year pos-irradiation storage. In spite of these, the differences were sufficient for the successful differentiation and classification of γ-irradiated samples from the native (non-irradiated) ones, even at low γ-irradiation dose of 5 kGy. $TEAC_{\cdot DPPH}$ values evaluated from EPR experiments were found as the most important marker for discrimination procedure. This approach could be effectively used by relevant food control authorities as a tool to distinguish correctly the non-irradiated spices from those exposed to the γ-radiation.

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