Study of chemical changes and antioxidant activity variation induced by gamma-irradiation on wild mushrooms: comparative study through principal component analysis

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Abstract

Mushrooms are especially sensitive to senescence, browning, water loss and microbial attack. Furthermore, wild species are characterized for their seasonality, demanding the development of suitable preservation technology. Gamma-irradiation was previously tested in wild *Lactarius deliciosus*, being verified that its application up to 1 kGy did not imply significant changes in chemical parameters. Herein, the effects of higher gamma-irradiation doses, typically used in natural food matrices like fruits or vegetables, were assessed in *Boletus edulis* Bull.: Fr. and *Hydnum repandum* L.: Fr. by checking for changes in nutritional parameters, free sugars, tocopherols, fatty acids, organic acids and antioxidant activity indicators. To have representative samples, the used carpophores were collected in different maturity stages, using the same number of specimens for each stage and also for each mushroom species. The specific effects of each tested irradiation were evaluated in an integrated manner through principal component analysis. The correspondent biplots indicate that differences caused by gamma-irradiation are enough to separate irradiated and non-irradiated samples of both mushrooms. Nevertheless, nutritional profiles were not affected in high extension, indicating that gamma-irradiation, up to the doses used in this work, might represent a useful mushroom conservation technology.

Keywords: Wild mushrooms; Gamma irradiation; Chemical composition; Antioxidant activity; Principal component analysis

1. Introduction

Mushrooms are highly perishable food matrices mainly due to the high water content (approx. 90%), being easily deteriorated due to senescence, browning, water loss and microbial attack (Jolivet et al., 1998). Furthermore, wild mushrooms such as *Boletus edulis* Bull.:Fr. and *Hydnum repandum* L.:Fr. are strictly seasonal, causing difficulties in their distribution and marketing as fresh products; the level of loss in similar matrices during marketing could be as high as 40% (Lacroix & Ouattara, 2000). Therefore, mushrooms need special care to keep quality and freshness. The northeast of Portugal, due to its climatic conditions and flora diversity, is one of the European regions with a high variety of wild mushrooms, some of them with great gastronomic significance (Martins, Baptista, Sousa, Meireles, & Pais, 2002). *B. edulis* and *H. repandum* are among the most commonly consumed wild mushrooms. Their popularity is mainly due to sensory qualities, in particular aroma, taste and texture. Moreover, it should be highlighted that wild species are considered add-value foods for commercialization in the markets of France and Spain (Martins et al., 2002).

Irradiation is recognized as a safe and effective method of preservation used worldwide to extend the shelf life of raw foods (*e.g.* fruits and vegetables, spices, grains, meat or seafood) (Andrews et al., 1998; Fernandes, Antonio, Oliveira, Martins, & Ferreira, 2012a). In fact, more than 26 countries are using the process on a commercial scale (Stevenson, 1994; Lacroix & Ouattara, 2000). Different cultivated mushrooms (mainly from *Agaricus*, *Lentinula* and *Pleurotus* genera) were previously studied using gamma-irradiation as a potential conservation technology (Fernandes et al., 2012a). Nevertheless, few studies were published regarding the application of gamma-irradiation in wild mushrooms. In a recent investigation studying the effects of gamma-irradiation on the chemical composition, antioxidant activity and physical parameters of fresh *Lactarius deliciosus* L. wild edible

mushroom, we conclude that up to 1 kGy this technology was effective in maintaining chemical composition and controlling the deterioration of fresh samples (Fernandes et al., 2012b; Fernandes et al., 2012c).

Herein, gamma-irradiation doses up to 2 kGy were applied to *B. edulis* and *H. repandum*. In fact, a dose of 2.0 kGy is usually the optimal dose that fruits and vegetables may tolerate to keep their quality intact (without suffering loss of firmness, change in flavor/taste, physiological breakage or accelerated ripening) (Lacroix & Ouattara, 2000). In addition, the elimination of mold and pathogenic bacteria from mushrooms can be achieved with 2.0 kGy dose, increasing their shelf life from 2 to 8 days when stored at 10ºC (Skou, Beett, & Lundsten, 1974; Lacroix & Ouattara, 2000). Accordingly, the effects on nutritional parameters, free sugars, tocopherols, fatty acids, organic acids and antioxidant activity were evaluated individually in each assayed mushroom. Furthermore, principal component analysis was applied to verify which specific parameters were more affected by each assayed gamma-irradiation dose.

2. Materials and methods

2.1. Standards and reagents

For irradiation: To estimate the dose and dose rate of irradiation it was used a chemical solution sensitive to ionizing radiation, Fricke dosimeter, prepared in the lab following the standards (ASTM, 1992) and Amber Perspex dosimeters (batch V, from Harwell Co., UK). To prepare the acid aqueous Fricke dosimeter solution the following reagents were used: ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, USA).

For chemical analyses: Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Lab-Scan (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, organic acids, tocopherol and sugar standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA).

For antioxidant potential analysis: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Standards trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and gallic acid were from Sigma (St. Louis, MO, USA). Methanol and all other chemicals were of analytical grade and obtained from common sources. Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples and samples irradiation

B. edulis and *H. repandum* fruiting bodies (carpophores) were collected in different maturity stages; however, the same number of specimens belonging to each maturity stage was selected for both mushrooms. All mushrooms were collected in Trás-os-Montes (Northeast of Portugal) in November 2012.

B. edulis and *H. repandum* fresh samples were divided in three groups (each species) with three mushrooms per group. Each group corresponds to: control (non-irradiated, 0 kGy); sample 1 (1 kGy) and sample 2 (2 kGy).

The estimated dose rate for the irradiation position was obtained with Fricke dosimeter, and the irradiation of the samples was performed in a Co-60 experimental chamber with four sources, total activity 267 TBq (6.35 kCi) in November 2011 (Precisa 22, Graviner Manufacturing Company Ltd, U.K.), following the procedure previously described by the authors (Fernandes et al., 2012c). The estimated doses, dose rates and dose uniformity

ratios (Dmax/Dmin) were: 1.14±0.23 kGy, 1.71 kGy/h, 1.72 and 1.99±0.32 kGy, 1.49 kGy/h, 1.44, for *B. edulis*; and 1.02±0.04 kGy, 1.53 kGy/h, 1.08 and 1.66±0.46 kGy, 1.24 kGy/h, 1.98, *H. repandum*. For simplicity, in the text, tables and graphs we considered the values 0, 1 and 2 kGy, for non-irradiated and irradiated samples of both mushroom species. All the samples were lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenized samples for subsequent analysis.

2.3. Chemical parameters

2.3.1. Nutritional value. Moisture, protein, fat, carbohydrates and ash were determined following the AOAC procedures (AOAC, 1995). Moisture content was evaluated by lyophilization (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), crude protein content (*N*×4.38) of the samples was estimated by the macro-Kjeldahl method according to Léon-Guzmán et al. (1997); the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ^{o}C using a Chamber furnace Lenton Thermal Designs Ltd, model ECF 12/22. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × ($g_{protein}$) + 3.75 × ($g_{carbohydrate}$) + 9 × (g_{fat}).

2.3.2. Free sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) after the extraction procedure described by Heleno et al. (2011), using melezitose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and a RI

detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

2.3.3. Fatty acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID), after the extraction and derivatization procedures described previously (Heleno et al., 2011). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a FID at 260 ºC and a Macherey-Nagel column 50% cyanopropylmeythyl 50% phenylmethylpolysiloxane (30 m × 0.32 mm ID × 0.25 µm d*f*). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

2.3.4. Tocopherols. Tocopherols were determined after an extraction procedure previously described, using tocol as IS (Heleno, Barros, Sousa, Martins, & Ferreira, 2010). The analysis was carried out in the HPLC system described above connected to a fluorescence

detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II normal-phase column (250 × 4.6 mm; YMC Waters) operating at 30 ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method, and tocopherols content was further expressed in mg per 100 g of dry weight (dw).

2.3.5. Organic acids. Organic acids were determined following a procedure previously optimized and described by the authors (Barros, Pereira, & Ferreira, 2013). Analysis was performed by ultra fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Cooperation). Detection was carried out in a PDA, using 215 and 245 nm as preferred wavelengths. The organic acids were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight (dw).

2.4. Antioxidant parameters

2.4.1. Extraction procedure. Lyophilized powdered mushrooms samples (1 g) were stirred with methanol (30 mL) at 25 ºC at 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of methanol. The combined methanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210; Flawil, Switzerland), re-dissolved in methanol at 20 mg/mL (stock solution) for *B. edulis* and 40 mg/mL for *H. repandum*; and stored at 4ºC for further use. Successive dilutions were made from the stock solution and submitted to *in vitro* assays already

described by the authors (Heleno et al., 2010) to evaluate the antioxidant activity of the samples. The sample concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations. Trolox was used as standard.

2.4.2. DPPH radical scavenging activity. This methodology was performed using an ELX800 Microplate Reader (Bio-Tek). The reaction mixture in each one of the 96-wells consisted of one of the different concentrations of the extracts (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: % RSA = $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution.

2.4.3. Reducing power. Two different procedures were used to evaluate the reducing power:

A) The first methodology was performed using the Microplate Reader described above. The different concentrations of the extracts (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). For each concentration, the mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48-wells, as also deionized water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm.

B) The second methodology followed the Folin-Ciocalteu assay. The extract solution (1 mL) was mixed with Folin-Ciocalteu reagent (5 mL, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were vortex mixed for 15 s and allowed to stand for 30 min at 40 ºC for color development. Absorbance was then measured at 765 nm. Gallic acid was used to obtain the standard curve (0.0094-0.15 mg/mL), and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

2.4.4. Inhibition of β-carotene bleaching. β-carotene (2 mg) was dissolved in chloroform (10 mL) and 2 mL of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40 ºC under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the extracts (0.2 mL). The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation: (absorbance after 2 h of assay/initial absorbance) × 100.

2.4.5. TBARS (thiobarbituric acid reactive substances) assay. Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized with a Polytron in ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000*g* for 10 min. An aliquot (100 µL) of the supernatant was incubated with the different concentrations of the samples solutions (200 µL) in the presence of $FeSO_4$ (10 mM; 100 µL) and ascorbic acid (0.1 mM; 100 µL) at 37

ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 µL), followed by thiobarbituric acid (TBA, 2%, w/v, 380 µL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively.

2.5. Statistical analysis

All analyses (extractions) were performed in triplicate; each replicate was quantified also three times. Data were expressed as means±standard deviations.

The fulfillment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro-Wilk's, and the Levene's tests, respectively. For each parameter, significant differences among mean values were checked by Welch's statistics (p<0.05 means that the mean value of the evaluated parameter of at least one irradiation differs from the others). In the cases where statistical significance differences were identified, the dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

Principal components analysis (PCA) was applied as pattern recognition unsupervised classification method. PCA transforms the original, measured variables into new uncorrelated variables called principal components. The first principal component covers as much of the variation in the data as possible. The second principal component is orthogonal to the first and covers as much of the remaining variation as possible, and so on (Patras et al., 2011). The number of dimensions to keep for data analysis was evaluated by

the respective eigenvalues (which should be greater than one), by the Cronbach's alpha parameter (that must be positive) and also by the total percentage of variance (that should be as higher as possible) explained by the number of components selected. The number of dimensions considered for PCA was chosen in order to allow meaningful interpretations, to ensure their reliability.

All statistical tests were performed at a 5% significance level using the SPSS software, version 18.0 (SPSS Inc).

3. Results and discussion

Results regarding the evaluated chemical parameters and antioxidant activity assays are presented in **Tables 1-6**.

Nutritionally (**Table 1**), *B. edulis* and *H. repandum* showed similar profiles, presenting water as predominant component (≈93% in *B. edulis*; ≈95% in *H. repandum*) and carbohydrates (≈70% in *B. edulis*; ≈75% in *H. repandum*) as major compounds in dry mass basis, in agreement with previous works (Ouzouni & Riganakos, 2007; Heleno et al. 2011). Fat contents were very low, highlighting the dietary interest of both species, especially in view of their low dry mass contents. Concerning the effects of gamma-irradiation, the most noticeable change was observed in protein content, which decreased with irradiation for both mushrooms. Actually, proteins are among the most reliable irradiation indicators, especially due to degradation reactions such as scission of the C-N bonds in the backbone of the polypeptide chain or splitting of the dissulfide bonds, and physical changes like unfolding and aggregation (Molins, 2001). The remaining nutritional parameters showed very slight changes in response to the applied gamma-irradiation doses; dry mass, in particular did not reveal significant (p=0.775 for *B. edulis*; p=0.156 for *H. repandum*) variation among irradiated and non-irradiated samples.

Free sugars profiles (**Table 2**) were quite different among the assayed species: trehalose was the main (≈ 14 g/100 g dw) sugar in *B. edulis*, a value similar to the previously reported by our research group (Heleno et al., 2011), while mannitol predominated (≈ 14 g/100 g dw) in *H. repandum*. The identified sugars decreased with irradiation in all cases. Sugars are known as being good indicators of a suitable conservation technology due to their sensibility to technical practices (Barreira, Pereira, Oliveira, & Ferreira, 2010). Irradiation, in particular, is known for causing several changes in sugars, such as melting point decreases, reduction in optical rotation and browning. Furthermore, sugars may suffer degradation, producing a mixture of gases consisting primarily of H_2 and CO_2, together with traces of CH_4, CO and H_2O. The relative proportions depends on the type of sugar irradiated and the absorbed dose (Molins, 2001), as verified in this case, where 1 kGy caused the minimization of sugar contents.

Tocopherol contents (**Table 3**) suffered the most marked effect in all quantified isoforms (α-, γ- and δ-tocopherol) presenting maximum values in samples irradiated with 1 kGy. Interestingly, the same irradiation dose minimized free sugars and maximized γ- and δ-tocopherol, a result that might be explained by the packaging atmosphere changes as a result of sugars degradation, since degradation of tocopherols is highly related with the availiability of free oxygen. α-Tocopherol, present in low amounts, was only detected in non-irradiated samples.

In what regards fatty acids (FA), 25 individual molecules were quantified in both mushroom species. **Table 4** presents the individual FA quantified above 0.2% in each mushroom species (C8:0, C10:0, C12:0, C14:1, C15:0, C17:0, C18:3, C20:3, C20:5, C22:1, C23:0 and C24:1 in both mushrooms, besides C6:0, C17:1 and C22.0 in *B. edulis* and C14:0 in *H. repandum* were also quantified, but in percentages lower than 0.2%). The most abundant FA in both mushrooms were palmitic acid (C16:0), oleic acid (C18:1) and

linoleic acid (C18:2), as it is common in these species (Kalač, 2009; Heleno et al., 2011). The high percentage of the latter should probably be associated with the highly appreciated organoleptic characteristics of these mushrooms, since linoleic acid is the precursor of oct-1-en-3-ol, known as "fungi alcohol", the main aromatic component in fungi (Maga, 1981). It became evident that irradiation cause a decrease in unsaturated fatty acids. Irradiation may change lipid profile by catalyzing their reaction with molecular oxygen (autoxidation) or by the action of high-energy radiation itself, more evidently in both cases in unsaturated molecules (Nawar, 1986). In fact, the general mechanism of lipids radiolysis is thought to involve primary ionization, followed by migration of the positive charge either toward the carboxyl carbonyl group or double bonds (Molins, 2001), thus enhancing unsaturated fatty acids degradation.

The profiles in organic acids (**Table 5**) were different among the two assayed mushrooms, particularly in what regards malic acid, which was absent in *B. edulis*. However, citric acid was the predominant organic acid, except in *H. repandum* samples irradiated with 2 kGy. Irradiated samples (excluding oxalic acid in *B. edulis*) presented higher organic acids values, as it was previously verified for tocopherols.

The antioxidant potential of *B. edulis* and *H. repandum* was used as a measure of their bioactivity. Five *in vitro* chemical and biochemical assays were used: scavenging effects on DPPH radicals (measures the decrease in DPPH radical absorption after exposure to radical scavengers), reducing power (conversion of a Fe^{3+}/ferricyanide complex to Fe^{2+}, further denominated as Prussian blue assay, and Folin-Ciocalteau method), inhibition of β-carotene bleaching (measures the capacity to neutralize the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated β-carotene models) and inhibition of lipid peroxidation in brain cells homogenates (measures the color intensity of MDA-TBA complex formed at the endpoint of the reaction). As mentioned in

previous reports, *B. edulis* also gave higher antioxidant activity than *H. repandum* (Heleno et al., 2010; Heleno et al., 2011). Performed assays (**Table 6**) indicate that irradiated samples tended to have lower scavenging activity and reducing power, but higher lipid peroxidation inhibition. The observed decrease might be related with free radicals resulting from lipid radiolysis, which was higher in irradiated samples. On the other hand, the increase in lipid peroxidation inhibition is probably associated to the higher amount of tocopherols (powerful lipophilic antioxidants) detected in irradiated samples.

To verify which parameters were more related with the detected differences, a principal components analysis (PCA) was applied to each mushroom species. The plot of component loadings (**Figure 1**) for *B. edulis*, indicates that the first two dimensions (first: Cronbach's α, 0.980; eigenvalue, 24.793; second: Cronbach's α, 0.962; eigenvalue, 17.447) account for most of the variance of all quantified variables (50.60% and 35.61%, respectively). Groups corresponding to each irradiation level (0 kGy, 1 kGy and 2 kGy) were clearly separated. Group corresponding to 0 kGy was more positively correlated to proteins, α-tocopherol, C6:0, C18:2, PUFA and TBARS (*i.e.*, it presented high contents in these parameters and was a weak inhibitor of TBARS formation); and more negatively correlated to fat, carbohydrates, C15:0, C17:1, C18:1, β-carotene and DPPH (*i.e.*, it presented low contents in these parameters and was a strong DPPH scavenger). 1 kGy was more positively correlated to ash, C23:0, C24:0, MUFA, fumaric acid and reducing power (Prussian blue assay, PBA); the highest negative correlations were associated to energy, fructose, C20:0, C20:5 and reducing power (Folin-Ciocalteau assay, F-CA); it should be noted that the correlations with reducing power are not conflicting since high values in PBA and low values in FC-A both mean lower reducing power. Objects corresponding to 2 kGy were

mostly characterized by high contents in C10:0, C12:0, C14:0, C16:0, C17:0 and C20:3 and low contents in C20:2, C22:1, C24:1 and oxalic acid.

In what concerns *H. repandum* (**Figure 2**) objects corresponding to each irradiation level were also clearly separated. The first two dimensions (first: Cronbach's α, 0.986; eigenvalue, 28.386; second: Cronbach's α, 0.907; eigenvalue, 8.855) include also most of the variance of all quantified variables (61.71% and 19.25%, respectively). In this case, group corresponding to 0 kGy was more positively correlated to fat, energy, mannitol, trehalose, α-tocopherol, C18:2, C18:3, C20:1 and PUFA, and more negatively correlated to C6:0, C8:0, C14:1, C16:0, C17:0, C18:0, C18:1, C20:0, C22:0, C22:1, C24:0 and oxalic acid. Objects corresponding to 1 kGy were more positively correlated to C24:1, MUFA, citric acid and reducing power (F-CA); no negative correlations were detected. Finally, 2 kGy group was mostly characterized by having high scores in carbohydrates, C10:0, C12:0, C14:0, C15:0, C16:1, fumaric acid, malic acid, β-carotene bleaching prevention, TBARS formation inhibition and reducing power (PBA) and low contents in dry matter, proteins, C20:2, C20:3 and C20:5.

Overall, it might be concluded that irradiation caused changes in minor individual compounds. The spatial distribution of PCA biplot markers in different clusters (corresponding to each irradiation dose) confirmed that irradiation exerted marked effects over the assayed parameters. Fatty acids in particular, seemed to be the most affected components, since their component loadings were often correlated with the defined objects. Nevertheless, despite the detected differences in individual compounds, the results of nutritional parameters (the most relevant in terms of mushroom acceptability by consumers) were less affected, indicating an interesting potential of gamma-irradiation to be used as an effective conservation technology. Furthermore, considering previous

research outcomes, irradiation minimizes the effects caused by storage time, being definitely indicated to be applied to mushrooms.

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Table 1. Proximate composition and corresponding energetic value of *B. edulis* and *H. repandum* samples submitted to different gamma irradiation doses. The results are presented as mean±SD[a].

		Dry matter (g/100 g fw)	Fat (g/100 g dw)	Proteins (g/100 g dw)	Carbohydrates (g/100 g dw)	Ash (g/100 g dw)	Energy (kcal/100 g dw)
Boletus edulis	0 kGy	7±1	4.3±0.3 b	23±2 a	65±2 b	7.9±0.1 b	390±2 ab
	1 kGy	7.3±0.4	4.6±0.1 a	15±1 b	72±1 a	8.6±0.2 a	389±1 b
	2 kGy	7±1	4.5±0.1 ab	16.0±0.4 b	71.5±0.5 a	7.9±0.2 b	391±1 a
Homoscedasticity[b]	*p*-value	0.004	<0.001	0.001	<0.001	0.003	0.022
One-way ANOVA[c]	*p*-value	0.775	0.002	<0.001	<0.001	<0.001	0.003
Hydnum repandum	0 kGy	6±2	4.6±0.1 a	14.1±0.2 a	72±1 b	9±1 c	385±4 a
	1 kGy	6±1	4.0±0.2 b	12.2±0.1 b	72.7±0.3 b	11.1±0.2 a	376±1 c
	2 kGy	4.5±0.1	4.0±0.1 b	8±1 c	77±1 a	10.4±0.1 b	378±1 b
Homoscedasticity[b]	*p*-value	0.001	0.894	0.003	0.051	<0.001	<0.001
One-way ANOVA[c]	*p*-value	0.156	<0.001	<0.001	<0.001	<0.001	<0.001

fw- fresh weight; dw- dry weight.

[a]Different letters in each column and for each mushroom indicate significant differences among mean values of each gamma irradiation level.

[b]Homoscedasticity among cultivars was tested by means of the Levene test: homoscedasticity, p-value>0.05; heteroscedasticity, p-value<0.05.

[c]$p<0.05$ meaning that the mean value of the evaluated parameter of at least one irradiation differs from the others (in this case multiple comparison tests were performed).

Table 2. Sugars composition of *B. edulis* and *H. repandum* samples submitted to different gamma irradiation doses. The results are presented as mean±SD[a].

		Fructose (g/100 g dw)	Glucose (g/100 g dw)	Mannitol (g/100 g dw)	Trehalose (g/100 g dw)	Total sugars (g/100 g dw)
Boletus edulis	0 kGy	0.41±0.01 b	1.57±0.03 a	1.13±0.03 a	17.7±0.1 a	20.9±0.1 a
	1 kGy	0.21±0.02 c	0.80±0.02 c	0.32±0.02 c	11.3±0.1 c	12.6±0.1 c
	2 kGy	0.45±0.02 a	1.29±0.03 b	0.67±0.01 b	14.1±0.1 b	16.5±0.1 b
Homoscedasticity[b]	*p*-value	0.080	0.424	0.016	0.067	0.036
One-way ANOVA[c]	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001
Hydnum repandum	0 kGy	nd	nd	13.0±0.2 a	4.4±0.1 a	17.4±0.2 a
	1 kGy	nd	nd	12.5±0.1 b	4.25±0.02 b	16.8±0.1 b
	2 kGy	nd	nd	12.6±0.1 b	4.3±0.1 b	16.9±0.1 b
Homoscedasticity[b]	*p*-value	-	-	0.007	<0.001	0.209
One-way ANOVA[c]	*p*-value	-	-	<0.001	<0.001	<0.001

dw- dry weight; nd- not detected.
[a]Different letters in each column and for each mushroom indicate significant differences among mean values of each gamma irradiation level.
[b]Homoscedasticity among irradiation doses was tested by means of the Levene test: homoscedasticity, *p*-value>0.05; heteroscedasticity, *p*-value<0.05.
[c]$p<0.05$ meaning that the mean value of the evaluated parameter of at least one irradiation differs from the others (in this case multiple comparison tests were performed).

Table 3. Tocopherols composition of *B. edulis* and *H. repandum* samples submitted to different gamma irradiation doses. The results are presented as mean±SD[a].

		α-Tocopherol (μg/100 g dw)	γ-Tocopherol (μg/100 g dw)	δ-Tocopherol (μg /100 g dw)	Total tocopherols (μg /100 g dw)
Boletus edulis	0 kGy	1.5±0.1	25±2 b	33±4 b	59±2 b
	1 kGy	nd	85±3 a	58±1 a	143±3 a
	2 kGy	nd	nd	24±1 c	24±1 c
Homoscedasticity[b]	*p*-value	<0.001	<0.001	<0.001	<0.001
One-way ANOVA[c]	*p*-value	<0.001	<0.001	<0.001	<0.001
Hydnum repandum	0 kGy	2.19±0.03	nd	8.9±0.2 c	11.1±0.2 c
	1 kGy	nd	nd	113±1 a	113±1 a
	2 kGy	nd	nd	80±2 b	80±1 b
Homoscedasticity[b]	*p*-value	<0.001	-	0.007	0.045
One-way ANOVA[c]	*p*-value	<0.001	-	<0.001	<0.001

dw- dry weight; nd- not detected.
[a]Different letters in each column and for each mushroom indicate significant differences among mean values of each gamma irradiation level.
[b]Homoscedasticity among irradiation doses was tested by means of the Levene test: homoscedasticity, *p*-value>0.05; heteroscedasticity, *p*-value<0.05.
[c]$p<0.05$ meaning that the mean value of the evaluated parameter of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed).

Table 4. Fatty acids composition (relative percentages) of *B. edulis* and *H. repandum* samples submitted to different gamma irradiation doses. The results are presented as mean±SD[a].

		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:0	C20:1	C20:2	C24:0	SFA	MUFA	PUFA
	0 kGy	0.100±0.002 c	7.6±0.1 c	0.63±0.01 b	2.89±0.02 b	34.1±0.2 c	51.7±0.2 a	0.38±0.02 a	0.88±0.02 b	0.38±0.02 a	0.21±0.01 b	11.6±0.1 b	35.9±0.2 c	52.5±0.2 a
Boletus edulis	1 kGy	0.118±0.001 b	7.8±0.1 b	0.71±0.01 a	2.59±0.03 c	37.4±0.1 a	48.1±0.2 b	0.30±0.01 b	1.07±0.02 a	0.37±0.02 a	0.34±0.01 a	11.7±0.1 b	39.5±0.1 a	48.8±0.2 c
	2 kGy	0.37±0.01 a	8.5±0.1 a	0.54±0.01 c	3.81±0.04 a	35.2±0.3 b	48.3±0.4 b	0.38±0.02 a	0.77±0.02 c	0.33±0.02 b	0.21±0.03 b	14.0±0.1 a	36.8±0.3 b	49.1±0.4 b
Homoscedasticity[b]	*p*-value	<0.001	0.004	0.010	0.243	0.023	0.015	0.490	0.392	0.751	0.04	0.237	0.018	0.027
One-way ANOVA[c]	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		C6:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:0	C20:1	C20:2	C22:0	C24:0	SFA	MUFA	PUFA
	0 kGy	0.11±0.01 c	13.1±0.1 c	0.24±0.01 c	2.24±0.03 c	37.2±0.3 b	39.3±0.3 a	0.21±0.02 b	5.7±0.1 a	0.33±0.01 a	0.20±0.01 b	0.18±0.01 b	16.6±0.1 c	43.2±0.2 b	40.2±0.3 a
Hydnum repandum	1 kGy	0.74±0.01 b	16.4±0.1 b	0.27±0.01 b	2.8±0.1 b	40.9±0.4 a	33.3±0.3 b	0.26±0.01 a	2.9±0.1 b	0.32±0.01 a	0.32±0.01 a	0.40±0.01 a	21.6±0.1 b	44.2±0.3 a	34.1±0.3 b
	2 kGy	1.0±0.1 a	17±1 a	0.30±0.02 a	2.9±0.1 a	40±1 a	33±1 b	0.26±0.02 a	2.5±0.1 c	0.28±0.03 b	0.31±0.02 a	0.39±0.04 a	23±1 a	43±1 b	34±1 b
Homoscedasticity[b]	*p*-value	<0.001	<0.001	0.127	0.029	0.007	0.092	0.291	0.082	0.015	0.028	<0.001	<0.001	0.001	0.087
One-way ANOVA[c]	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

dw- dry weight.

[a]Different letters in each column and for each mushroom indicate significant differences among mean values of each gamma irradiation level.

[b]Homoscedasticity among irradiation doses was tested by means of the Levene test: homoscedasticity, p-value>0.05; heteroscedasticity, p-value<0.05.

[c]p<0.05 meaning that the mean value of the evaluated parameter of at least one irradiation differs from the others (in this case multiple comparison tests were performed).

Table 5. Organic acids composition of *B. edulis* and *H. repandum* samples submitted to different gamma irradiation doses. The results are presented as mean±SD[a].

		Oxalic acid (g/100 g dw)	Malic acid (g/100 g dw)	Citric acid (g/100 g dw)	Fumaric acid (g/100 g dw)	Total organic acids (g/100 g dw)
Boletus edulis	0 kGy	0.485±0.004 a	nd	4.7±0.1 b	0.067±0.001 b	5.3±0.1 b
	1 kGy	0.36±0.01 b	nd	5.3±0.1 a	0.082±0.002 a	5.71±0.05 a
	2 kGy	0.18±0.1 c	nd	4.0±0.1 c	0.069±0.004 b	4.3±0.1 c
Homoscedasticity[b]	*p*-value	0.002	-	0.283	0.034	0.302
One-way ANOVA[c]	*p*-value	<0.001	-	<0.001	<0.001	<0.001
Hydnum repandum	0 kGy	0.0031±0.0002 c	3.47±0.03 b	3.82±0.03 b	0.61±0.01 b	7.90±0.04 b
	1 kGy	0.030±0.002 a	3.28±0.04 b	4.02±0.05 a	0.63±0.01 ab	7.96±0.05 b
	2 kGy	0.027±0.002 b	5.6±0.4 a	3.9±0.2 ab	0.65±0.04 a	10.2±0.4 a
Homoscedasticity[b]	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001
One-way ANOVA[c]	*p*-value	<0.001	<0.001	0.015	0.009	<0.001

dw- dry weight; nd- not detected.
[a]Different letters in each column and for each mushroom indicate significant differences among mean values of each gamma irradiation level.
[b]Homoscedasticity among irradiation doses was tested by means of the Levene test: homoscedasticity, *P*-value>0.05; heteroscedasticity, *P*-value<0.05.
[c]$p<0.05$ meaning that the mean value of the evaluated parameter of at least one irradiation differs from the others (in this case multiple comparison tests were performed).

Table 6. *In vitro* antioxidant properties obtained for the extracts *B. edulis* and *H. repandum* samples submitted to different gamma irradiation doses (mean±SD)[a]. Values are presented as EC_{50} values (mg/mL) for all assays except Folin-Ciocalteau, expressed as mg GAE/g extract.

			Reducing power		Lipid peroxidation inhibition	
		DPPH scavenging activity	Ferricyanide/Prussian blue assay	Folin-Ciocalteu assay	β-Carotene bleaching inhibition	TBARS formation inhibition
Boletus edulis	0 kGy	1.54±0.03 c	0.71±0.01 c	37±1 a	1.6±0.1 c	1.6±0.1 a
	1 kGy	2.22±0.03 a	0.96±0.02 a	30±1 b	2.5±0.3 a	0.53±0.03 b
	2 kGy	1.9±0.1 b	0.76±0.02 b	36±1 a	1.9±0.2 b	0.54±0.02 b
Homoscedasticity[b]	*p*-value	0.001	<0.001	0.018	<0.001	<0.001
One-way ANOVA[c]	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001
Hydnum repandum	0 kGy	31±1 c	2.47±0.05 c	6.8±0.2 b	3.8±0.1 b	0.9±0.1 b
	1 kGy	34±1 b	2.6±0.1 b	7.6±0.1 a	3.0±0.4 c	0.7±0.1 c
	2 kGy	39.6±0.5 a	2.8±0.1 a	6.9±0.2 b	8.3±0.4 a	1.1±0.1 a
Homoscedasticity[b]	*p*-value	0.022	0.051	0.069	0.197	0.002
One-way ANOVA[c]	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001

dw- dry weight.
[a]Different letters in each column and for each mushroom indicate significant differences among mean values of each gamma irradiation level.
[b]Homoscedasticity among irradiation doses was tested by means of the Levene test: homoscedasticity, *P*-value>0.05; heteroscedasticity, *P*-value<0.05.
[c]$p<0.05$ meaning that the mean value of the evaluated parameter of at least one irradiation differs from the others (in this case multiple comparison tests were performed).
EC_{50}- extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the Ferricyanide/Prussian blue assay. Concerning the Folin-Ciocalteu assay, higher values mean higher reducing power; for the other assays, the results are presented in EC_{50} values, what means that higher values correspond to lower reducing power or antioxidant potential.

Figure 1. Biplot of objects (irradiation doses) and component loadings (evaluated parameters) for *B. edulis*.

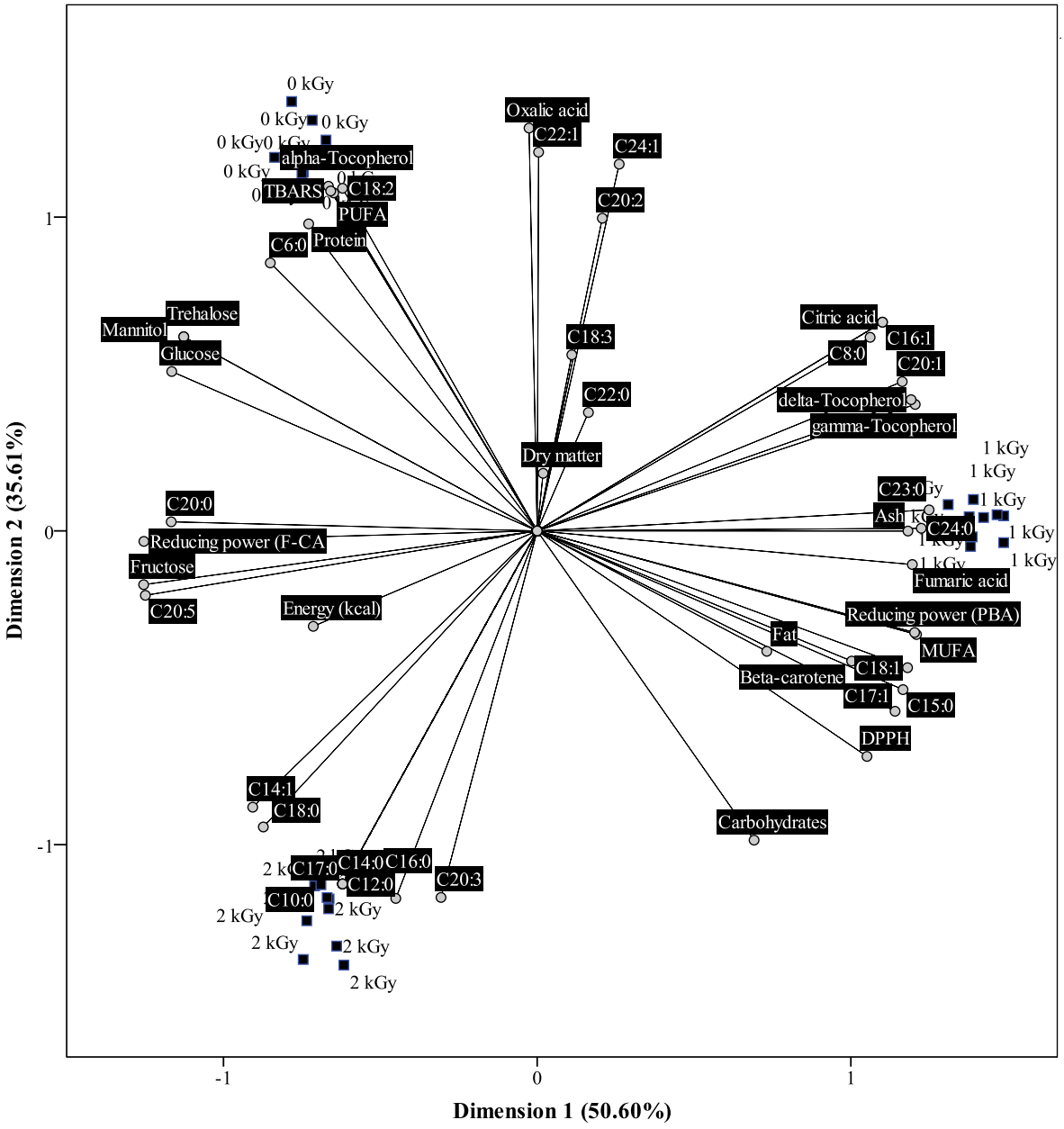

Figure 2. Biplot of objects (irradiation doses) and component loadings (evaluated parameters) for *H. repandum*.