Influence of gamma irradiation in the antioxidant potential of chestnuts (*Castanea sativa* Mill.) fruits and skins

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Abstract

As seasonal products chestnuts have to be postharvest treated to increase their shelf-life. The most common preservation method for chestnuts is the chemical fumigation with methyl bromide, a toxic agent that is under strictly use under Montreal Protocol due to its adverse effects on human health and environment. Food irradiation is a possible feasible alternative to substitute the traditional quarantine chemical fumigation treatment. This preliminary study evaluated the influence of gamma irradiation in the antioxidant potential of chestnut fruits and skins, through several chemical and biochemical parameters. The bioactive compounds (phenolics and flavonoids) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, reducing power and inhibition of β-carotene bleaching capacity were determined. The obtained results seem to indicate that the storage favoured chestnuts antioxidant potential. Furthermore, the application of gamma irradiation also seems to be advantageous for antioxidant activity, independently of the dose used (0.27 ± 0.04 kGy or 0.54 ± 0.04 kGy).

Keywords: Irradiated chestnuts; Gamma irradiation; Antioxidant activity; Phenolics/Flavonoids.

1. Introduction

Portugal is one of the most important chestnut producers, with nearly 25% of European production. Trás-os-Montes region represent 75.8% of Portuguese chestnut crops and 84.9% of chestnut orchards area (23338 ha). The best development conditions are found at altitudes higher than 500 m and winter low temperatures, as in the "Terra Fria Transmontana" region (Northeast of Portugal) in which 12500 ha are used for chestnut cultivation (Portuguese Agricultural Statistics, 2009).

There are two main problems related to chestnuts preservation: weight losses due to dehydration and development of insects and microorganisms. Methyl Bromide (MeBr) fumigation has been used traditionally for chestnuts preservation. However, according to Montreal Protocol it will be banned due to its harmful environment and health effects. Another conservation process is heat treatment, but it is time consuming and has a low efficiency. Therefore, an alternative conservation process is urgently needed. Food irradiation has been successfully used for fruit disinfestations (CAC/RCP, 2003; UNEP, 2006; Pinto et al., 2007). This technique has recently been considered as an alternative to fumigation, as it reduces considerably the amount of product lost during post-harvest period due to rotting, resulting from the development of fungi and moulds. Furthermore, this technology is environmentally friendly, in contrast to the traditional use of fumigants (e.g.: methyl bromide), not leaving any type of chemical residues on fruits or environment. Nevertheless, irradiation is a method that must be studied in detail, since the results vary significantly within different fruit species, exposure time (doses) and geometry (dose uniformity) (Belchior et al., 2007; Kim et al., 2007).

Chestnuts are important sources of polyphenolic antioxidants that have high free radical scavenging properties being associated to protective effects against coronary heart disease (Engler & Engler, 2006), cancer (Nichenametla et al., 2006), neurodegenerative diseases (Lau

et al., 2005) and osteoporosis (Weaver & Cheong, 2005). Particularly chestnut fruits (Ribeiro et al., 2007) and chestnut leaves (Calliste et al., 2005) contain those compounds.

Our research group has reported the antioxidant potential of different extracts of *Castanea sativa* Mill. (flowers, leaves, skins and fruits) (Barreira et al., 2008). Nevertheless, little research has been done in the influence of irradiation on antioxidant properties of chestnuts, and particularly on Portuguese varieties nothing has been reported.

Herein, we describe the influence of irradiation process (at two different doses and along storage time) in antioxidant properties (radical scavenging activity, reducing power and inhibition of β-carotene bleaching) and antioxidants contents (phenolics and flavonoids) of fruits and skins stored at 4 ºC for 2 months.

2. Materials and methods

2.1. Standards and reagents

To prepare the acid aqueous Fricke dosimeter solution the following reagents were used: ferrous ammonium sulphate (II) hexahydrate, sodium chloride and sulphuric acid, all of them purchased from Panreac S.A. (Barcelona, Spain) with purity pa (pro-analysis), and water treated in a Milli-Q water purification system (Millipore, model A10, 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), gallic acid and (+)-catechin were purchase from Sigma (St. Louis, MO, USA). Methanol and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples and samples irradiation

Chestnuts cv. *Longal* samples were obtained from Trás-os-Montes, in the Northeast of Portugal. They were divided in three samples (control, sample 1, sample 2) with fifteen units per sample.

Previous to chestnuts irradiation, a dosimetric study was performed using a chemical solution sensitive to ionizing radiation, called Fricke dosimeter.

2.2.1. Preparation of Dosimeters

To estimate the doses it was used a chemical solution sensitive to ionizing radiation, Fricke dosimeter, which is a reference standard dosimeter within the range 40 to 400 Gy. The Fricke dosimeter is widely used in the calibration of radiation processing and provides a reliable means of absorbed doses measurement in water, based on an oxidation process of ferrous ions to ferric ions in acidic aqueous solution by ionizing radiation.

The glass lab material used to prepare the solution and the flasks for Fricke were washed with RBS solution, then well rinsed with distilled water and used when were well dried.

Following the standard (ASTM E1026, 1992), 0.392 g of ferrous ammonium sulfate and 0.058 g of sodium chloride was dissolved in 12.5 mL of 0.4 mol L^{-1} sulfuric acid diluted to 1 L in a volumetric flask with 0.4 mol L^{-1} sulfuric acid at 25 ^{0}C. The resultant dosimetric solution has the following composition: 1×10^{-3} mol L^{-1} ferrous ammonium sulfate; 1×10^{-3} mol L^{-1} sodium chloride and 0.4 mol L^{-1} sulfuric acid.

The solution was then air saturated, bubbled with pure oxygen during 10 min, and the flask covered with aluminum foil and kept in the dark till the irradiation process. Before filling the ampoules to irradiate they were rinsed three times with the unirradiated solution.

Five dosimeters of Pyrex® glass tubes were filled with 15 mL of Fricke solution. This dosimeter volume was chosen in accordance with the thickness of chestnut fruit samples.

Irradiations were performed on the 4th level of the Cobalt-60 Gammacell (Precisa 22, Graviner Manufacturing Company Ltd). The ^{60}Co irradiation facility, shown in **Figure 1A**, consists of a rectangular cavity with the following dimension: 65 cm × 50 cm × 20 cm (h × d × w) and surrounded with a lead protection barrier. Four ^{60}Co sources, with a total activity of 305 TBq (8.233 kCi) in November 2009, are positioned in stainless-steel tubes located in the lateral walls of the chamber, in positions directly facing each other, about 30 cm above the chamber floor. The movement of the sources in the 50 cm long tubes is controlled by an automatic mechanism.

Fricke dosimeters were placed at the corners and centre of a rectangle in a area approximately equal to the sample bag, as shown in **Figure 1B**.

After irradiation, the absorbance (Ai) of the irradiated solution was determined by a spectrophotometer (Shimadzu mini UV 1240) set at 305 nm wavelength.

The equation used to estimate the absorbed dose, D, was (ASTM E1026, 1992; IAEA, 2002):

$D_{Fricke} = (278\ \Delta A) / ([1+0.007(T - 25)][1+0.0015(T' - 25)])$,

where ΔA is the difference in absorbance measured at 305 nm, between irradiated and non-irradiated solution; T is the solution temperature (°C) during the spectrophotometric measurements and T’ is the irradiation temperature (°C).

The temperature of the irradiated dosimeter solution during spectroscopic measurement was measured with a thermocouple and a digital multimeter (Fluke, model 179, with a resolution of 0.1 °C). The temperature T’ is the ambient temperature inside the chamber and was measured with a thermocouple during the irradiation.

2.2.2. Chestnuts irradiation

After irradiation geometry dose rate estimation, the samples 1 and 2, each one with fifteen chestnuts, were placed into polyethylene plastic bags and irradiated for 1 h and 2 h, respectively.

The irradiations were performed in a ^{60}Co experimental equipment described above.

2.3. Antioxidant activity assays.

The samples were stored at 4 ºC for 0 days, 30 days and 60 days. A sub-sample from each of the treatments was obtained at each time point and analyzed (control, sample 1, sample 2 - **Figure 2A**).

Fruits were separated from the skins (**Figure 2B**) and the samples were dried in an oven at ~ 30 ºC. A fine dried powder (20 mesh) (1.5 g) was extracted twice with methanol (30 mL) for 1 h. After filtration and evaporation of the methanol, the extracts were re-dissolved in methanol at a concentration of 20 mg mL^{-1} and analysed for phenolics and flavonoids contents, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, reducing power and inhibition of β-carotene bleaching, following previously described procedures (Barros et al., 2010).

2.3.1. Determination of antioxidants content

For phenolics, an aliquot of 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^{-1}, 4 mL). The tubes were vortexed for 15 s and allowed to stand for 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm (AnalytikJena 200-2004 spectrophotometer). Gallic acid was used to calculate the standard curve (9.4×10^{-3} -1.5×10^{-1} mg mL^{-1}), and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

For flavonoids, an aliquot (0.5 mL) of the extract solution was mixed with distilled water (2 mL) and subsequently with $NaNO_2$ solution (5%, 0.15 mL). After 6 min, $AlCl_3$ solution (10%, 0.15 mL) was added and allowed to stand further 6 min, thereafter, NaOH solution (4%, 2 mL) was added to the mixture. Immediately, distilled water was added to bring the final volume to 5 mL. Then the mixture was properly mixed and allowed to stand for 15 min. The intensity of pink colour was measured at 510 nm. (+)-Catechin was used to calculate the standard curve (4.5×10^{-3} - 2.9×10^{-1} mg mL^{-1}) and the results were expressed as mg of (+)-catechin equivalents (CE) per g of extract.

2.3.2. *DPPH radical-scavenging activity*

This methodology was performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture in each one of the 96-wells consisted of one of the different concentrations of the extracts (30 µL) and aqueous methanolic solution (80:20 v/v, 270 µL) containing DPPH radicals (6×10^{-5} mol L^{-1}). 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 discolouration 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. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Trolox was used as standard.

2.3.3. Reducing power

The different concentrations of the extracts (0.5 mL) were mixed with sodium phosphate buffer (200 mmol L^{-1}, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). 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 deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in the Microplate Reader described above. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against extract concentration. Trolox was used as standard.

2.3.4. Inhibition of β-carotene bleaching

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40ºC 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 50ºC 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: (β-carotene content after 2h of assay/initial β-carotene content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against extract concentration. Trolox was used as standard.

2.4. Statistical analysis

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software, version 18.0 (SPSS, Inc.). The dependent variables were analyzed using 2-way ANOVA, with the main factors "irradiation

dose” (ID) and “storage time” (ST). When a (ID×ST) was detected, the two factors were evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistical significant interaction was verified, means were compared using Tukey’s honestly significant difference (HSD) multiple comparison test.

Furthermore, a linear discriminant analysis (LDA) was used to assess the classification of different storage times and irradiation doses in different groups according with antioxidant activity assays results and phenols and flavonoids contents. A stepwise technique, using the Wilks’ λ method with the usual probabilities of F (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination procedures, where before selecting a new variable to be included, it is verified whether all variables previously selected remain significant (Maroco, 2003; López et al., 2008). With this approach, it is possible to identify the significant variables obtained for each sample. To verify which canonical discriminant functions were significant, the Wilks’ λ test was applied. A leaving-one-out cross-validation procedure was carried out to assess the model performance.

All statistical tests were performed at a 5% significance level. All the assays were carried out in triplicate. The results are expressed as mean values with standard deviation (SD).

3. Results and discussion

3.1. Irradiation studies

An estimation of dose was performed using Fricke chemical dosimeter solution as described above. The estimated values for the different positions are presented in **Table 1**.

In food irradiation the dose distribution inside the chamber and the dose uniformity ratio must be well characterized to control the irradiation process.

The results highlighted that the material could be rotated to obtain a better uniform dose, as is a standard practice in commercial units. However, the dose uniformity ratio obtained is in conformity with the good practices for food irradiation (Directive 1999/2/EC, 1999).

In this experimental setup the dose uniformity ratio, the ratio of maximum to minimum absorbed dose in the production lot, obtained is similar to one ($D_{max}/D_{min} = 1.3$).

Samples were exposed to 1 and 2 h of irradiation, therefore, using the average dose rate this would equivalent to 0.27 and 0.54 kGy, respectively.

3.2. Antioxidant potential

Figures 3 and **4** give the phenolic and flavonoid contents in irradiated chestnut fruits and skins, after different storage times (0, 30 and 60 days). **Table 2** (fruit) and **3** (skins) show the antioxidant activity EC_{50} values data reported as mean value of each irradiation dose over three different storage times, as well as mean value of all irradiation doses within each storage time. The antioxidant activity was measured by different biochemical assays: scavenging activity on DPPH radicals (measuring the decrease in DPPH radical absorption after exposure to radical scavengers), reducing power (measuring the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form) and inhibition of β-carotene bleaching (by neutralizing the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated β-carotene models).

Irradiated chestnut skins showed higher phenolic and flavonoid contents (**Figures 3 and 4**), as well as higher antioxidant activity (lower EC_{50} values; **Tables 2 and 3**) than chestnut fruits, which is in agreement to our previous results in non-irradiated samples (Barreira et al., 2008).

The results in table 2 show that storage time×irradiation dose interaction was a significant ($P < 0.001$) source of variation for all the performed antioxidant activity assays. Therefore,

although the least squares means are presented for the main effects, no multiple comparisons were performed.

Moreover, both main factors (storage time and irradiation dose) show a significant effect (P < 0.001). Nevertheless, from the analysis of the plots of the estimated margins means, for each antioxidant assay, some general conclusions can be pointed out. For instance, EC_{50} values were higher for all the assayed antioxidant activity methods (except for inhibition β-carotene bleaching in chestnut skins) when samples were analyzed promptly (0 days of storage). In other way, the samples irradiated with 0.54 kGy demonstrated the lowest EC_{50} values for reducing power in chestnut fruits and scavenging activity on DPPH radicals, reducing power and inhibition of β-carotene bleaching in chestnut skins. The results indicate that the use of gamma irradiation seemed to increase antioxidant potential of fruits and skins. Nevertheless, the acquired results showed that the dose used (0.27±0.04 kGy or 0.54±0.04 kGy) did not show significant influence in those parameters. Along storage (up to 60 days) the studied parameters followed the same tendency in control and irradiated sample fruits. The phenolic and flavonoid contents of both skins and fruits increased with storage, which seems to exert a more significant effect than the irradiation dose.

The results were evaluated through linear discriminant analysis (LDA) to evaluate which were the most discriminative variables regarding antioxidant potential and bioactive compounds contents. All independent variables selected by the stepwise procedure were statistically significant according to the Wilks‟ λ test (P < 0.05).

The LDA was performed using the results of the antioxidant activity assays and those obtained from phenolic and flavonoid contents. Considering chestnut fruits and antioxidant activity assays, the stepwise LDA resulted in a discriminant model with two significant (P < 0.001 for the Wilks' λ test) discriminant functions. These two functions explained 100.0% of

the variance of the experimental data (the first explained 99.2% and the second 0.8%) (**Figure 5A**).

The first function separates primarily 0 days from 30 and 60 days (means of the canonical variance (MCV): 0 days = 6.344, 30 days = -3.662, 60 days = -2.682), and revealed to be more powerfully correlated with reducing power. The second function had low discriminant ability, and did not reach a clear separation among 30 and 60 days (MCV: 0 days = 0.052, 30 days = 0.474, 60 days = -0.526), showing to be more correlated with DPPH scavenging activity. The model demonstrated a good classification performance, allowing to correctly classifying 86.4% of the samples for the original groups, as well as for the cross-validation procedure.

Similar results were obtained with different sets of parameters (data not shown), proving the high influence of storage time on antioxidant activity and bioactive compounds availability. Satisfactory classification performances were always achieved: 85.2% for the original groups, as well as for the cross-validation procedure, with chestnut fruits and phenolic and flavonoid contents; 77.8% for the original groups and 75.3% for the cross-validation procedure, with chestnut skins and antioxidant activity assays; 100% for the original groups, as well as for the cross-validation procedure, with chestnut skins and phenolic and flavonoid contents.

Otherwise, the irradiation dose presented much lower discriminant power. Considering chestnut fruit and antioxidant activity assays, the stepwise LDA resulted in a discriminant model with two significant ($P < 0.001$ for the Wilks' λ test) discriminant functions. These two functions explained 100.0% of the variance of the experimental data (the first explained 83.9% and the second 016.1%) (**Figure 5B**).

The similarity among results was reflected in the MCV proximity, either for function 1(MCV: 0 kGy = -0.913, 0.27 kGy = 1.005, 0.54 kGy = -0.092) and function 2 (MCV: 0 kGy = -0.278, 0.27 kGy = -0.208, 0.54 kGy = 0.485). The model demonstrated a weak classification

performance, allowing to correctly classifying 55.6% of the samples for the original groups, as well as for the cross-validation procedure. This lack of effectiveness was also observed in the other LDA tests regarding irradiation doses: no variables were qualified for the analysis with chestnut fruit and phenolic and flavonoid contents; 55.6% for the original groups and for the cross-validation procedure, with chestnut skins and antioxidant activity assays; 46.9% for the original groups, as well as for the cross-validation procedure, with chestnut skins and phenolic and flavonoid contents.

These results highlight the higher influence of storage time over antioxidant activity and bioactive compound availability when compared with the irradiation dose used.

Overall, the storage time was more significant to chestnuts antioxidant potential than the irradiation dose. The activity of some antioxidant defences (non-enzymatic or enzymatic) present in chestnuts apparently increased along the storage time, in response to the oxidative stress inherent to the storage process. The application of gamma irradiation proved to be advantageous for the assayed antioxidant methods, probably due to an increase in the availability of antioxidant compounds such as polyphenols previously linked to the cell wall.

Further studies will be done in order to elucidate the interactions herein reported and also the influence of irradiation in chemical composition and nutritional value of chestnuts fruits. The study will be extended to include more positions in the chamber and to consider other irradiation doses.

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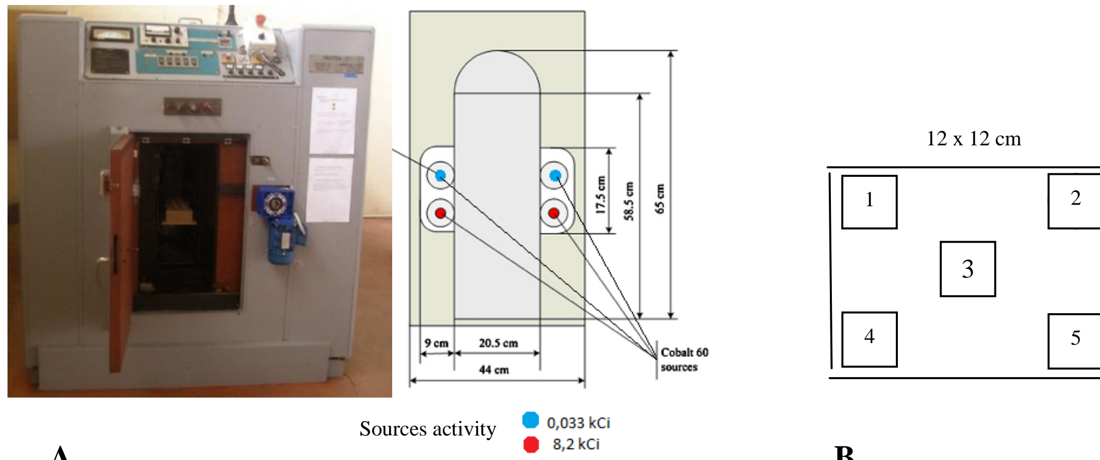
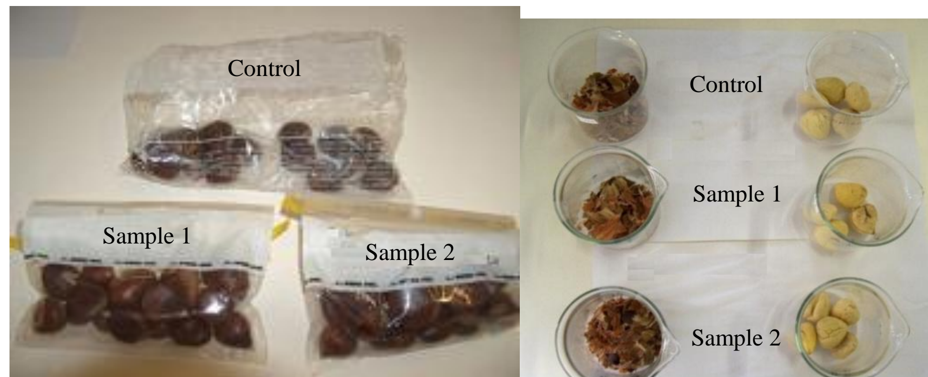
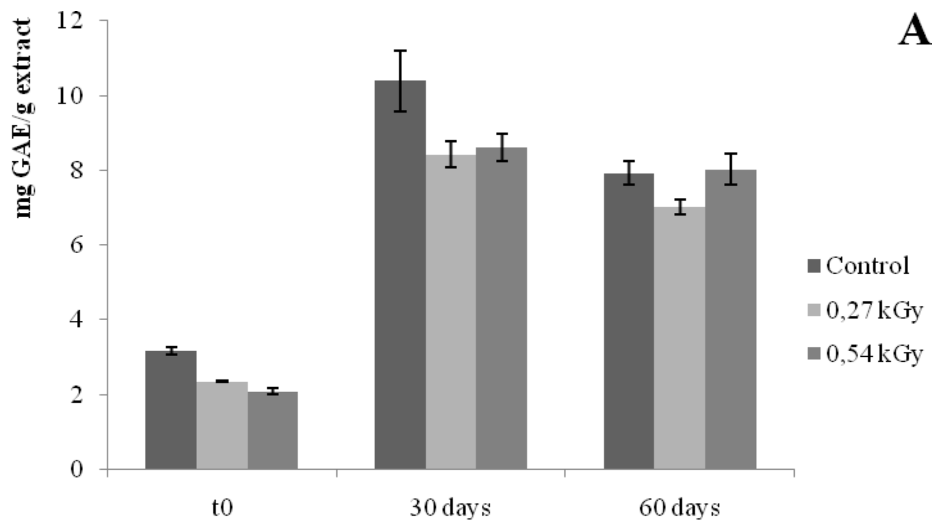


Figure 1. A. Irradiation chamber: activity of sources and dimensions (Belchior et. al., 2007); **B.** Irradiated area and dosimeter positions.



A. **B.**

Figure 2. A. Chestnuts: Control (without irradiation), Sample 1 (0.27 kGy), Sample 2 (0.54 kGy). **B.** Chestnuts after peeling (fruits and skins): Control, Sample 1, Sample 2.



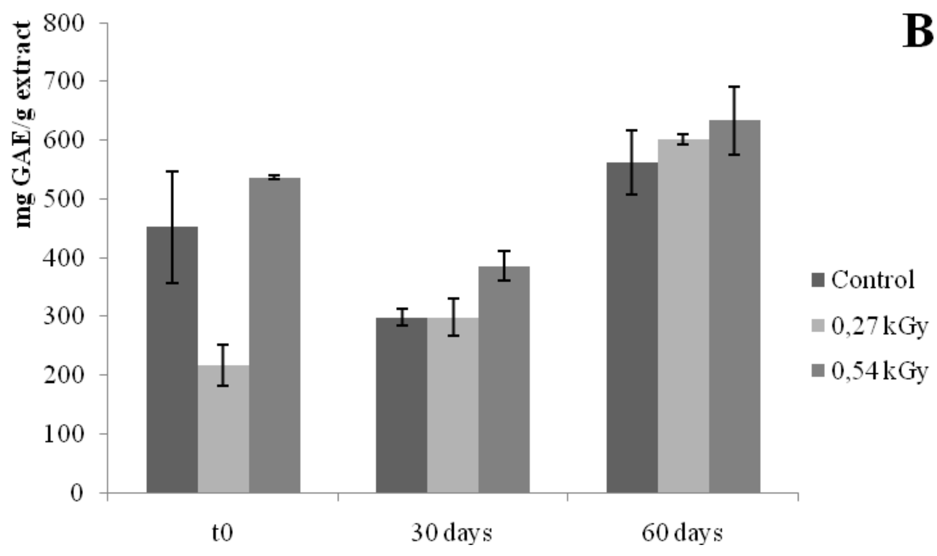
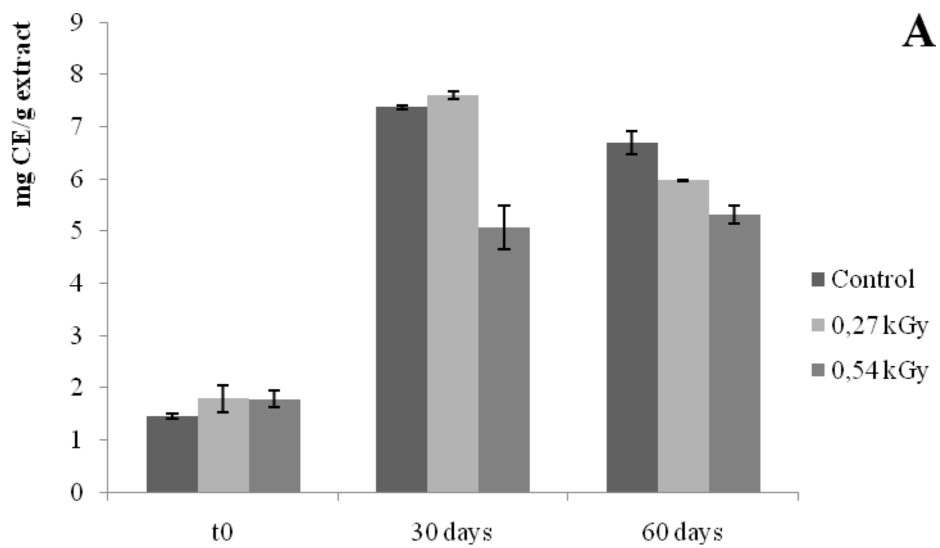


Figure 3. Phenolics content in chestnut fruits (A) and skins (B).



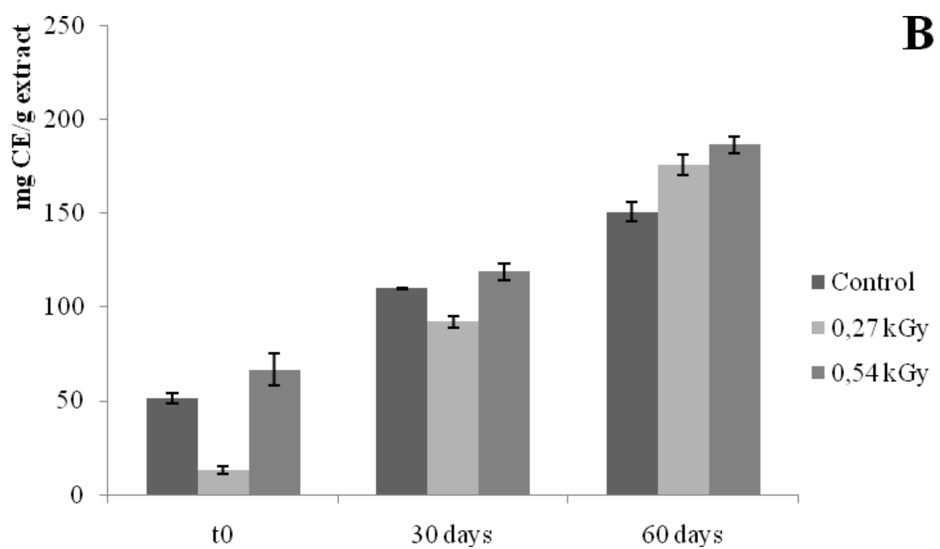
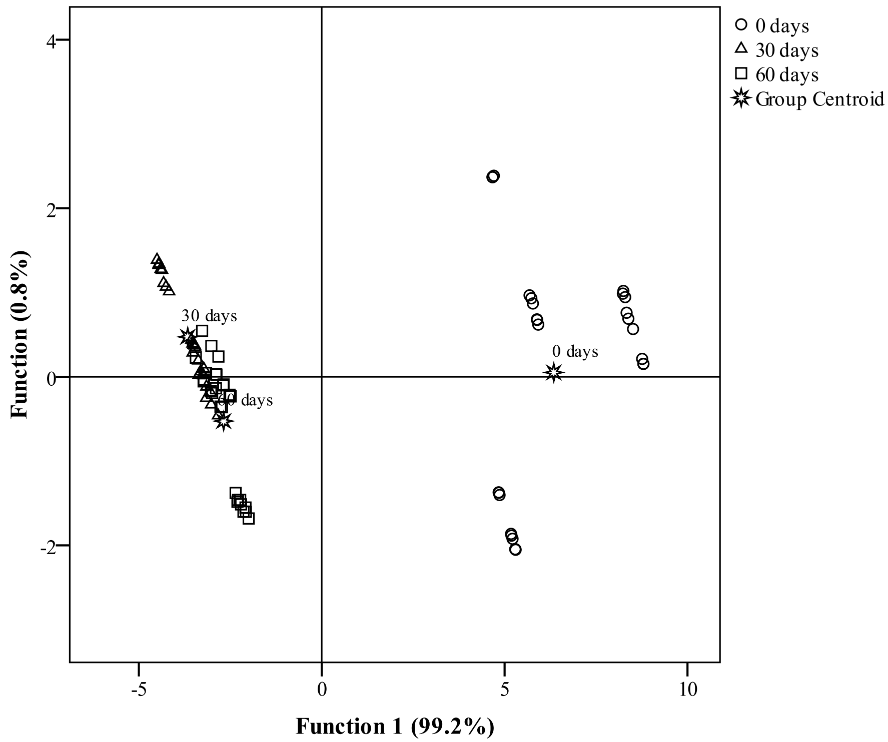


Figure 4. Flavonoids content in chestnut fruits (A) and skins (B).



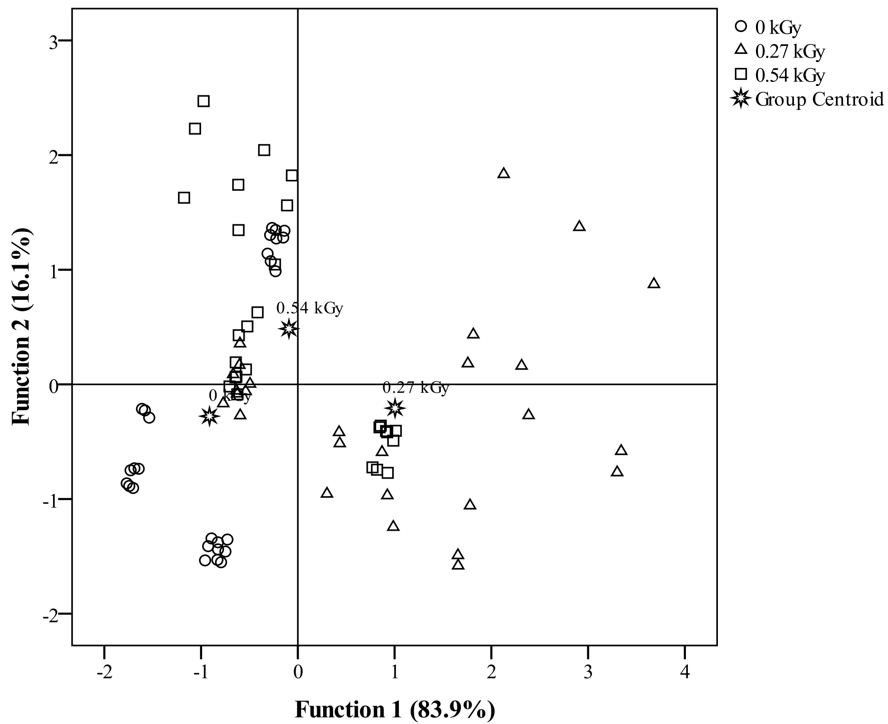


Figure 5. Canonical analysis of A) storage times influence on antioxidant activity of chestnut fruits; B) irradiation doses influence on antioxidant activity of chestnut fruits.

Table 1. Dose rate distribution in irradiated samples area.

Dosemeter position	1	2	3	4	5
Dose rate (kGy h^{-1})	0.30	0.29	0.29	0.23	0.23
$\mathbf{D_{mean}}$ (kGy h^{-1})	0.27 ± 0.04				

Table 2. Chestnut fruits antioxidant activity (EC_{50} values, mg mL^{-1}) according with irradiation dose (ID) and storage time (ST).

		DPPH scavenging activity	Reducing Power	β-Carotene bleaching inhibition
ST	0 days	45.38±9.36	8.59±1.10	2.18±0.94
	30 days	16.67±1.18	3.44±0.08	1.74±0.43
	60 days	14.96±2.43	3.60±0.12	1.20±0.03
	P-value (n=27)	<0.001	<0.001	<0.001
ID	0 kGy	20.95±9.08	4.71±1.85	1.23±0.04
	0.27 kGy	26.61±18.12	5.20±2.42	2.21±0.96
	0.54 kGy	29.45±18.12	5.72±3.03	1.68±0.37
	P-value (n=27)	<0.001	<0.001	<0.001
ST × ID	*P*-value	<0.001	<0.001	<0.001

Table 3. Chestnut skins antioxidant activity (EC_{50} values, μg mL^{-1}) according with irradiation dose (ID) and storage time (ST).

		DPPH scavenging activity	Reducing Power	β-Carotene bleaching inhibition
ST	0 days	66.98±22.57	38.07±11.40	59.00±14.46
	30 days	43.60±5.04	25.65±2.76	161.90±86.99
	60 days	32.20±2.54	22.58±0.79	72.94±20.68
	P-value (n=27)	<0.001	<0.001	<0.001
ID	0 kGy	44.87±10.29	27.69±4.63	137.88±75.29
	0.27 kGy	60.07±27.67	34.68±13.74	105.75±69.78
	0.54 kGy	37.84±7.41	23.93±2.40	50.21±7.33
	P-value (n=27)	<0.001	<0.001	<0.001
ST × ID	*P*-value	<0.001	<0.001	<0.001