Using gamma irradiation to attenuate the effects caused by drying or freezing in *Macrolepiota procera* organic acids and phenolic compounds

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Abstract

Macrolepiota procera (Scop.) Singer, commonly parasol mushroom, is an appreciated wild edible species. Due to its very perishable nature, *M. procera* must be processed to extend its shelf life. The chemical changes caused by common processing types should be avoided to maintain the wholesomeness of organoleptic features. Irradiation might be used as a preservation methodology due to its safety, cost effectiveness and ability to ensure hygienic and sensory quality. Furthermore, when combined with other preservation technologies, irradiation exhibits an attenuating effect over the chemical changes caused by some of those treatments *per se*. Herein, the effects of irradiation of *M. procera* processed samples (frozen, dried and fresh) were evaluated considering changes in organic acid and phenolic compound profiles. Detected contents of phenolic were much lower than those of organic acids. Differences caused by processing type, specifically the lower levels of total organic acids and phenolic acids in dried and frozen samples, were larger than those observed for stronger irradiation doses, which did not cause remarkable changes, except for a slightly lower content of phenolic acids in non-irradiated samples. This larger effect was statistically confirmed in the performed linear discriminant analysis. Besides its slighter influence, irradiation showed potential usefulness to be used as complementary preservation technology since it attenuated the lowering effects of dehydration and freeze treatment over specific organic acid contents.

Keywords: Irradiation; Processed mushrooms; *Macrolepiota procera*; Organic acids; Phenolic compounds; LDA

Introduction

Macrolepiota procera (Scop.) Singer is one of the most popular mushrooms, being considered an excellent edible species, highly appreciated for its nutritional and culinary values (Polese, 2005). In view of the very perishable nature, fresh mushrooms have to be processed to extend their shelf life. Among the various methods employed for preservation, canning is the most frequently adopted method in commercial scale (Walde et al. 2006), but drying is also a common method for preserving mushrooms (Giri and Prasad, 2007) and freezing is becoming increasingly popular (Jaworska and Bernás, 2009; Jaworska and Bernás, 2010). Drying is perhaps the oldest technique known by mankind for preservation of food commodities for long duration. It is a comparatively cheaper method (Rama and Jacob, 2000; Walde et al. 2006), applied to decrease the moisture content of food to a level that can prevent the growth of mould and fungi and thus minimize microbial degradation. Food freezing is among the most efficient and adequate preservation methods, in which most of the liquid water changes into ice, which greatly reduces microbial and enzymatic activities (Haiying et al. 2007). Several studies indicate irradiation as a possible methodology to increase the shelf life of fresh mushrooms (Koorapati et al., 2004; Akram and Kwon, 2010). It can be a safe and cost effective method to enhance shelf-life and ensure hygienic and sensory quality (Fernandes et al. 2012, 2013a).

Previously, our research group evaluated the effects of different processing technologies (freezing, drying and gamma irradiation) on chemical and antioxidant parameters of the wild mushroom *M. procera*, and irradiation was the processing technology with the highest ability to maintain the chemical characteristics of the fresh samples (Fernandes et al. 2013b). Moreover, *M. procera* gamma irradiation attenuated the effects caused by drying or freezing (*e.g.*, combining the freeze treatment with a 0.5 kGy dose preserved tocopherols). Rather than a preservation methodology, gamma irradiation emerged as a

useful adjuvant for other preservation techniques such as freezing or drying (Fernandes et al. 2013c). Nevertheless, the mentioned reports did not assess the effects on organic acids or phenolic compounds, which are important molecules in mushrooms (Valentão et al. 2005; Ribeiro et al. 2006; Barros et al. 2009; Vaz et al. 2011; Barros et al. 2013).

Phenolic compounds might provide health benefits by reducing risk of chronic diseases due to their free radicals scavenging activity, singlet oxygen quenching or chelating effects. The antioxidant properties of phenolic compounds have also been related to the increased stability of food products, or to the antioxidant defense mechanisms of biological systems (Wright et al. 2001; Vaz et al. 2011).

Organic acids play a determinant role in maintaining fruit and vegetable quality and organoleptic characteristics and have also been used in their quality control (Cámara et al. 1994; Barros et al. 2013). Oxalic acid is very common in natural matrices, occurring also in animals; despite their biological functions, attention should be paid to the fact that calcium oxalate is the most common component of kidney stones. Quinic acid is a crystalline acid more common in plants, being often used as a versatile chiral-starting material for the synthesis of new pharmaceuticals; malic acid contributes to a pleasantly sour taste, and is often used as a food additive. Citric acid is known to be very important in the prevention of mushroom browning and to extend its shelf life, due to its antibacterial and antioxidant properties (Brennan et al., 2000). Fumaric acid is important because of its antioxidant, antimicrobial and acidifying properties (Ribeiro et al. 2008). The nature and concentration of these compounds are also important factors in mushrooms flavor (Valentão et al. 2005; Ribeiro et al. 2006).

Accordingly, the effects of irradiation of *M. procera* processed samples (frozen, dried and fresh mushrooms) were accessed regarding organic acids and phenolic compound profile and contents.

Materials and methods

Samples and samples irradiation

Macrolepiota procera fruiting bodies were obtained in Trás-os-Montes, in the Northeast of Portugal, in November 2011.

The samples were divided in three groups with nine mushrooms per group with different stages of maturation, and further submitted to different processing technologies: freezing (at -20º C in a freezer) and drying (at 30 ºC in an oven); the third group was kept fresh (stored at 4 ºC in a refrigerator). Each group was further subdivided in three subgroups: control (non-irradiated, 0 kGy); sample 1 (0.5 kGy) and sample 2 (1.0 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. 2013c). The estimated doses after irradiation were 0.6±0.1 kGy and 1.1±0.1 kGy for samples 1 and 2, respectively, at a dose rate of 2.3 $kGyh^{-1}$. For simplicity, in the text, tables and figures, we considered the values 0, 0.5 and 1 kGy, for non-irradiated and irradiated samples, respectively.

After irradiation, all the samples were freeze-dried (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh), mixed to obtain homogenate samples and promptly analyzed.

Standards and reagents

For irradiation: To estimate the dose and dose rate of irradiation a chemical solution sensitive to ionizing radiation was used, the Fricke dosimeter, prepared in the lab following

the appropriate standard (American Society for Testing and Materials, 1992). 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% was of HPLC grade from Lab-Scan (Lisbon, Portugal); other solvents were of analytical grade purity and were also supplied by Lab-Scan. Standards of phenolic compounds (protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids), cinnamic acid and organic acids (oxalic acid, quinic acid, malic acid, citric acid and fumaric acid) were from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Organic acids identification and quantification

Samples (~1.5 g) were extracted by stirring with 25 mL of meta-phosphoric acid (25 °C at 150 rpm) for 25 min and subsequently filtered through Whatman No. 4 paper. Before analysis by ultra-fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), the sample was filtered through 0.2 µm nylon filters. Organic acids were determined following a procedure previously optimized and described by the authors (Barros et al. 2013).

Analysis was performed by ultrafast liquid chromatography (UFLC) coupled to a photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Cooperation). Detection was carried out in a PDA, using 215 nm and 245 as preferred wavelengths. The organic acids were quantified by comparison of the area of their peaks recorded at 215 nm with those of calibration curves obtained from commercial standards of

each compound. The results were expressed in mg per g of dry weight (dw) (except for fumaric acid, expressed in µg/g dw).

Phenolic compounds identification and quantification

Each sample (~1.5 g) was extracted with methanol:water (80:20, v/v; 30 mL) at -20 °C for 6 h. After sonication for 15 min and filtered through Whatman nº 4 paper. The residue was then extracted with two additional 30 mL portions of the methanol:water mixture. Combined extracts were evaporated at 40 ºC under reduced pressure to remove methanol. The aqueous phase was submitted to a liquid-liquid extraction with diethyl ether (3 × 30 ml) and ethyl acetate (3 × 30 mL). The organic phases were evaporated at 40 °C to dryness, re-dissolved in water:methanol (80:20, v/v; 1 mL), followed by filtering through a 0.22 µm disposable LC filter disk for HPLC analysis.

Phenolic compounds were determined in the UFLC system mentioned above, as previously described by the authors (Barros et al. 2009). DAD detection was carried out using 280 nm and 370 nm as preferred wavelengths. The phenolic compounds were characterized according to their UV spectra and retention times, and comparison with authentic standards. For quantitative analysis, calibration curves were prepared from different standard compounds. The results were expressed in µg per g dw.

Statistical analysis

Two samples from each subgroup (details in section *2.1. Samples and samples irradiation*) were extracted with *m*-phosphoric acid (for organic acids) or with acetone:water (80:20) (for phenolic compounds and cinnamic acid extraction). Each purified extract was injected twice in the HPLC system.

The results were analyzed by means of an analysis of variance (ANOVA) with Type III sums of squares performed using the GLM (General Linear Model) procedure of the SPSS software, version 18.0. The dependent variables were analyzed using 2-way ANOVA, with "processing type" (PT) and "gamma irradiation dose" (ID) as factors. Since a significant interaction (PT×ID) was detected for all cases, the two factors were evaluated simultaneously by the estimated marginal means plots (EMM) for all levels of each single factor.

Further, a linear discriminant analysis (LDA) was used to compare the effect of the PT and ID on organic acids, phenolic compounds and cinnamic acid. 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 processes, where the inclusion of a new variable is preceded by making sure that 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 the significance of canonical discriminant functions, 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. For each ID and or PT, three samples were analysed, with all the assays being also carried out in triplicate. The results are expressed as mean value±standard deviation (SD).

Results and discussion

The values for each individual parameter are presented as the mean value of each PT, considering different applied ID, and also the mean value of each ID, considering the results for all PT. This approach allows understanding the real influence of each factor,

independently of the applied ID, as well as the most suitable ID to be applied, independently of the chosen PT. With no exception, PT×ID interaction was a significant ($p < 0.001$) source of variation for all the quantified compounds. Accordingly, and despite presenting the least squares means for both effects, no multiple comparisons could be performed. Nevertheless, from the analysis of the EMM plots (data shown only in specific cases) some overall conclusions can be outlined.

Organic acids

The UFLC-PDA analysis showed that all samples presented a profile composed of five organic acids: oxalic, quinic, malic, citric and fumaric acid, with malic acid as the main compound (**Table 1**). The obtained profiles were qualitatively similar to those reported previously (Barros et al. 2013), despite some quantitative differences, which might be related with the different collecting location. The interaction among PT×ID was a significant ($p < 0.001$) source of variation for all the quantified organic acids. Accordingly, the classification obtained by multiple comparisons tests could not be performed. Nevertheless, from the analysis of the EMM plots some particular tendencies could be identified. For instance, quinic acid presented the lowest values in frozen samples and in samples irradiated with 1 kGy (**Figure 1A**); malic acid presented highest values in fresh samples (**Figure 1B**); fumaric acid (**Figure 1C**), like quinic acid, showed minimal values in frozen samples. In terms of total organic acids, no particular tendency could be observed; the interaction among factors is evident, as it can be seen by the intersection of lines in **Figure 1D**. According to the identified tendencies, the variance caused by PT overcomes the effect of ID, but both factors induced only slight changes in organic acids. In fact, organic acids are known to have a lower susceptibility to change during processing than other components such as pigments and flavor compounds (Cámara et al. 1994). In

order to obtain a clearer understanding of the effect of ID and PT on organic acids profiles, different LDA were applied. The discriminant ability of the differences obtained in those profiles can be inferred from the obtained classification performance, assessed by the percentage of correctly classified groups. The higher influence of PT was confirmed in the performed LDA assays, once 100.0% of the samples were correctly classified, both for the original groups and for the cross-validation procedure. The classification ability was quite lower for ID, resulting in 75.0% of accuracy for the original groups and 66.7% for the cross-validation procedure. In both cases, two significant (p<0.001 for the Wilks' λ test) discriminant functions, including 100.0% of the variance of the experimental data in all cases, were defined. Regarding PT (**Figure 2A**), function 1 (90.9%) and function 2 (9.1%) were mostly correlated with fumaric acid (dried>fresh>frozen) and malic acid (fresh>frozen>dried), respectively. Fumaric, malic and quinic acids were selected as discriminant variables. In the case of ID (**Figure 2B**), function 1 (80.2%) and function 2 (19.8%) were more highly correlated with citric acid (showing tendency to be higher in samples irradiated with 1 kGy) and oxalic acid (showing tendency to be higher in samples irradiated with 0.5 kGy), respectively. Besides these two, quinic and fumaric acids were also selected as discriminant variables.

Phenolic acids

The results obtained show that *M. procera* contain very small amounts of phenolic acids (**Table 2**), which are in agreement with values commonly found in mushrooms (Valentão et al. 2005).

The interaction among PT×ID was a significant (p < 0.001) source of variation for all the quantified phenolic acids. Accordingly, the results could not be classified by multiple comparisons tests. However, analyzing the estimated margins mean plots, allowed

identifying some general tendencies. For instance, protocatechuic acid had highest values on fresh samples and in non-irradiated samples (**Figure 3A**); *p*-hydroxybenzoic and *p*-coumaric acids presented highest values in fresh samples (**Figures 3B and C**); the results obtained for total phenolic acids are in line with the observed for each individual molecule. As it can be concluded from **Figure 3D**, dried samples tended to present lower amounts of phenolic acids; furthermore, irradiation (1.0 kGy dose in particular) exerted a notorious protective effect on total phenolic acids content. The lowest value for cinnamic acid (**Figure 3E**), was also obtained in dried samples. Similarly to observations for organic acids, differences caused by PT were larger than those corresponding to ID. To clarify this conclusion, two additional LDA were applied. The higher influence of PT was confirmed, since 100.0% of the samples were correctly classified, both for the original groups as well as for the cross-validation procedure, regarding this factor. The classification ability was quite lower for ID; in fact, no qualifying variables were selected in this case. The discriminant model obtained for PT was defined by two significant (p<0.001 for the Wilks' λ test) discriminant functions, including 100.0% of the variance of the experimental data (**Figure 4**). Function 1 (88.9%) and function 2 (11.1%) were mostly correlated with *p*-coumaric acid (fresh>dried>frozen) and total phenolic acids (maximum value in fresh samples), respectively. Cinnamic acid was rejected as discriminant variable.

Consumer research is a key activity to evaluate the acceptance or liking of a determined product. This represents, in fact, important information regarding product decisions, such as the development and marketing of new products, the reformulation of existing products, the acceptance of suppliers and processes or the establishment of quality control specifications (Krishnamurthy et al., 2007). However, this type of descriptive tests requires a well-trained panel and tends to be expensive (Choi, 2013). Accordingly, we are conducting preliminary assays in several mushroom species, which are intended to be

aggregated and submitted to sensory panels simultaneously. Nevertheless, we have assayed the effect on nutritional composition (Fernandes et al., 2012, 2013a, b, c), concluding that there were no significant differences among the assayed parameters.

Conclusions

Herein, phenolic compounds and organic acids profiles were characterized in samples submitted to different processing types and irradiation doses. Comparing with organic acids, phenolics are present in notably lower contents. According with the observed changes, irradiation might be a useful complementary preservation technology since it induced less significant effects when compared with common techniques like dehydration or freeze treatment. Furthermore, some pronounced effects of these processing types were attenuated by irradiation: the lower amounts of oxalic acid in fresh samples, malic acid in dried samples and citric acid in fresh and dried samples, was significantly mitigated by irradiation treatment. In an overall perspective, it is possible to conclude that irradiation alone, especially 1.0 kGy dose, is the best option to preserve total organic acids and total phenolic acids.

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Figure 1. Interactions between processing type (PT) and/or gamma irradiation dose (ID) effects on the organic acids of *M. procera* samples. Quinic acid (A), malic acid (B), fumaric acid (C), total organic acids (D).

Figure 2. Discriminant scores scatter plot of the canonical functions defined for organic acids results according with PT (A) and ID (B).

Figure 3. Interactions between processing type (PT) and/or gamma irradiation dose (ID) effects on the phenolic compounds of *M. procera* samples. Protocatechuic acid (A), *p*-hydroxybenzoic acid (B), *p*-coumaric acid (C), total phenolic acids (D), cinnamic acid (E).

Figure 4. Discriminant scores scatter plot of the canonical functions defined for phenolic compounds results according with PT.

Table 1. Organic acids composition of *Macrolepiota procera* samples submitted to different processing types (PT) or gamma irradiation doses (ID). The results are presented as mean±SD.

		Oxalic acid (mg/g dw)	Quinic acid (mg/g dw)	Mallic acid (mg/g dw)	Citric acid (mg/g dw)	Fumaric acid (µg/g dw)	Total organic acids (mg/g dw)
PT	Fresh	4.4±0.4	10±2	28±4	4±4	2±1	49±7
	Dried	7±1	11±7	13±6	2±1	5.1±0.5	38±2
	Frozen	8±3	3±1	21±1	6±1	1.6±0.1	41±6
	p-value (n=12)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
GID	0 kGy	6±1	12±7	20±12	3±2	3±2	44±7
	0.5 kGy	8±4	7±2	19±4	4±3	3±2	41±5
	1 kGy	5±1	5±2	23±4	6±3	3±1	42±8
	p-value (n=12)	<0.001	<0.100	<0.001	<0.001	<0.001	<0.001
PT×GID	*p*-value (n=36)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

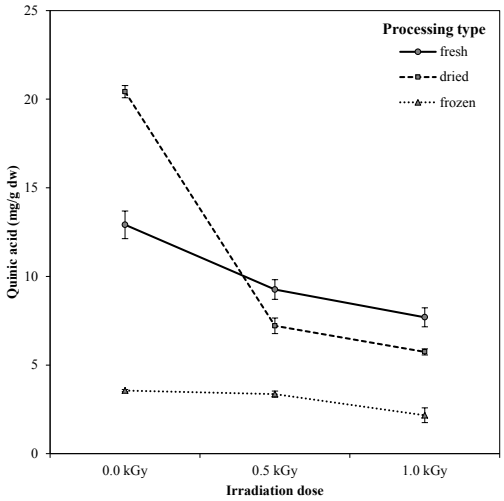
dw - dry weight.

Table 2. Phenolic and cinnamic acids composition of *Macrolepiota procera* samples submitted to different processing types (PT) or gamma irradiation doses (ID). The results are presented as mean±SD.

		Protocatechuic acid (µg/g dw)	*p*-Hydroxybenzoic acid (µg/g dw)	*p*-Coumaric acid (µg/g dw)	Total phenolic acids (µg/g dw)	Cinnamic acid (µg/g dw)
PT	Fresh	8±3	1.1±0.4	1.9±0.2	11±3	3±1
	Dried	nd	0.5±0.1	1.5±0.1	2.0±0.1	1.5±0.2
	Frozen	2.0±0.5	0.2±0.1	nd	1.8±0.5	3.5±0.3
	p-value (n=12)	<0.001	<0.001	<0.001	<0.001	<0.001
GID	0 kGy	5±5	0.7±0.5	1±1	7±7	2±1
	0.5 kGy	3±3	0.4±0.2	1±1	4±4	2±1
	1 kGy	2±2	0.6±0.5	1±1	4±3	3±1
	p-value (n=12)	<0.001	<0.100	<0.001	<0.001	<0.001
PT×GID	*p*-value (n=36)	<0.001	<0.001	<0.001	<0.001	<0.001

dw - dry weight; nd - not detected.

Figure 1.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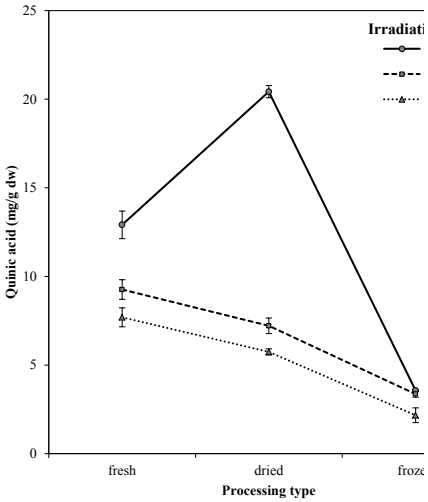
0.5 kGy
1.0 kGy
Irradiation dose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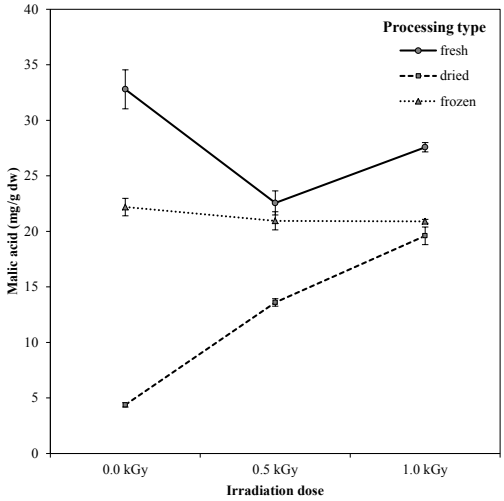
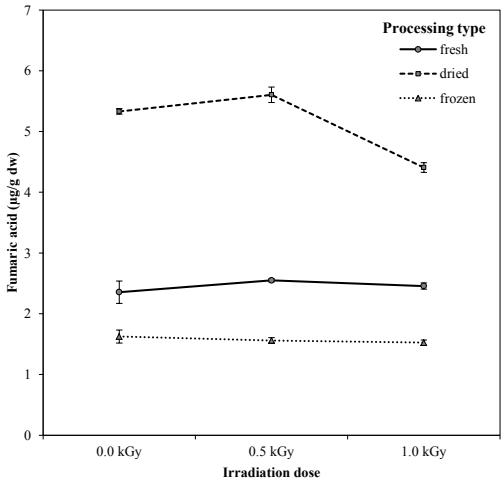

Processing type
fresh
dried
frozen
Malic acid (mg/g dw)
0.0 kGy
0.5 kGy
1.0 kGy
Irradiation dose

B


Processing type
fresh
dried
frozen
Fumaric acid (μg/g dw)
0.0 kGy
0.5 kGy
1.0 kGy
Irradiation dose

C

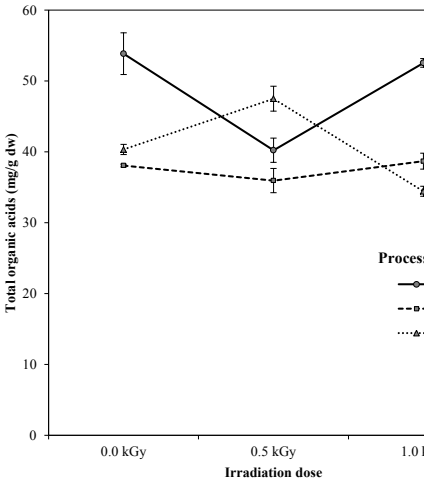
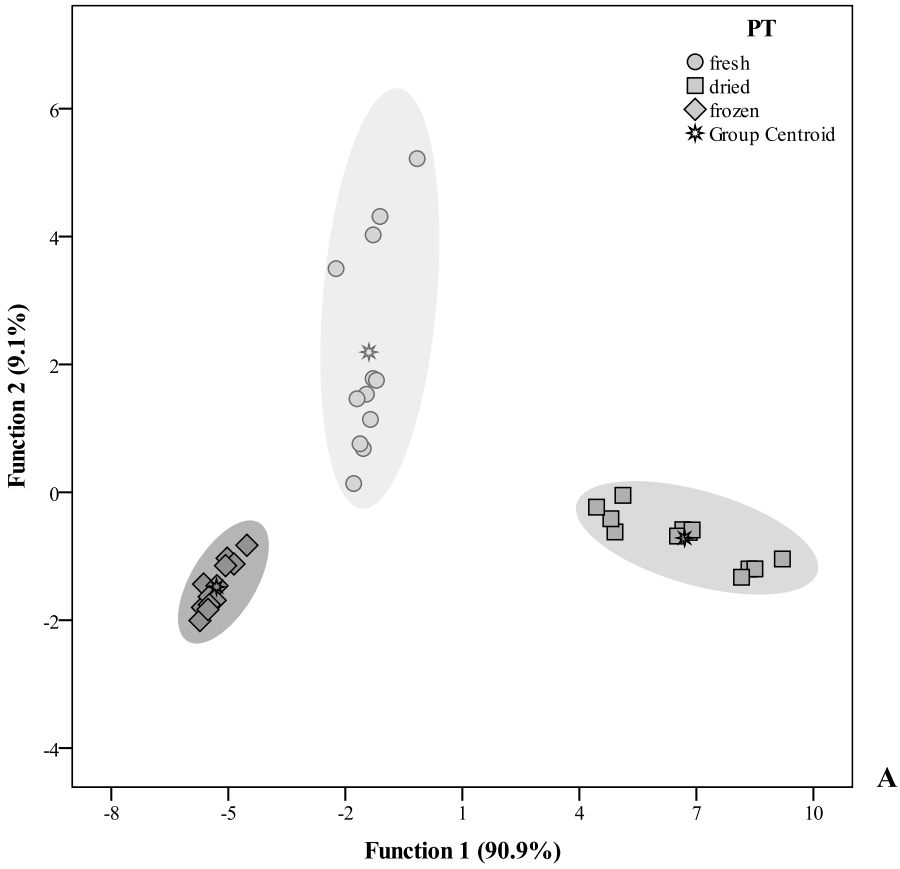
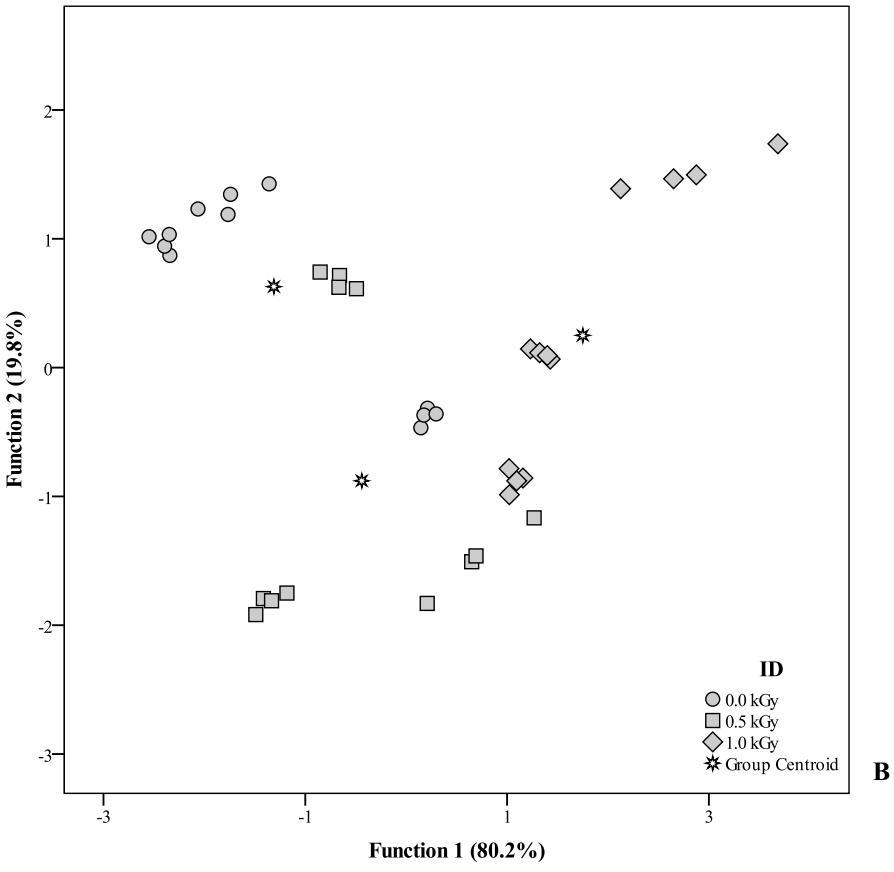

Proces
Total organic acids (mg/g dw)
0.0 kGy
0.5 kGy
Irradiation dose

Figure 2.


PT
fresh
dried
frozen
Group Centroid
Function 2 (9.1%)
Function 1 (90.9%)
6
4
2
0
-2
-4
-8
-5
-2
1
4
7
10

A


ID
0.0 kGy
0.5 kGy
1.0 kGy
Group Centroid
Function 2 (19.8%)
Function 1 (80.2%)
2
1
0
-1
-2
-3
-3
-1
1
3

B

Figure 3.

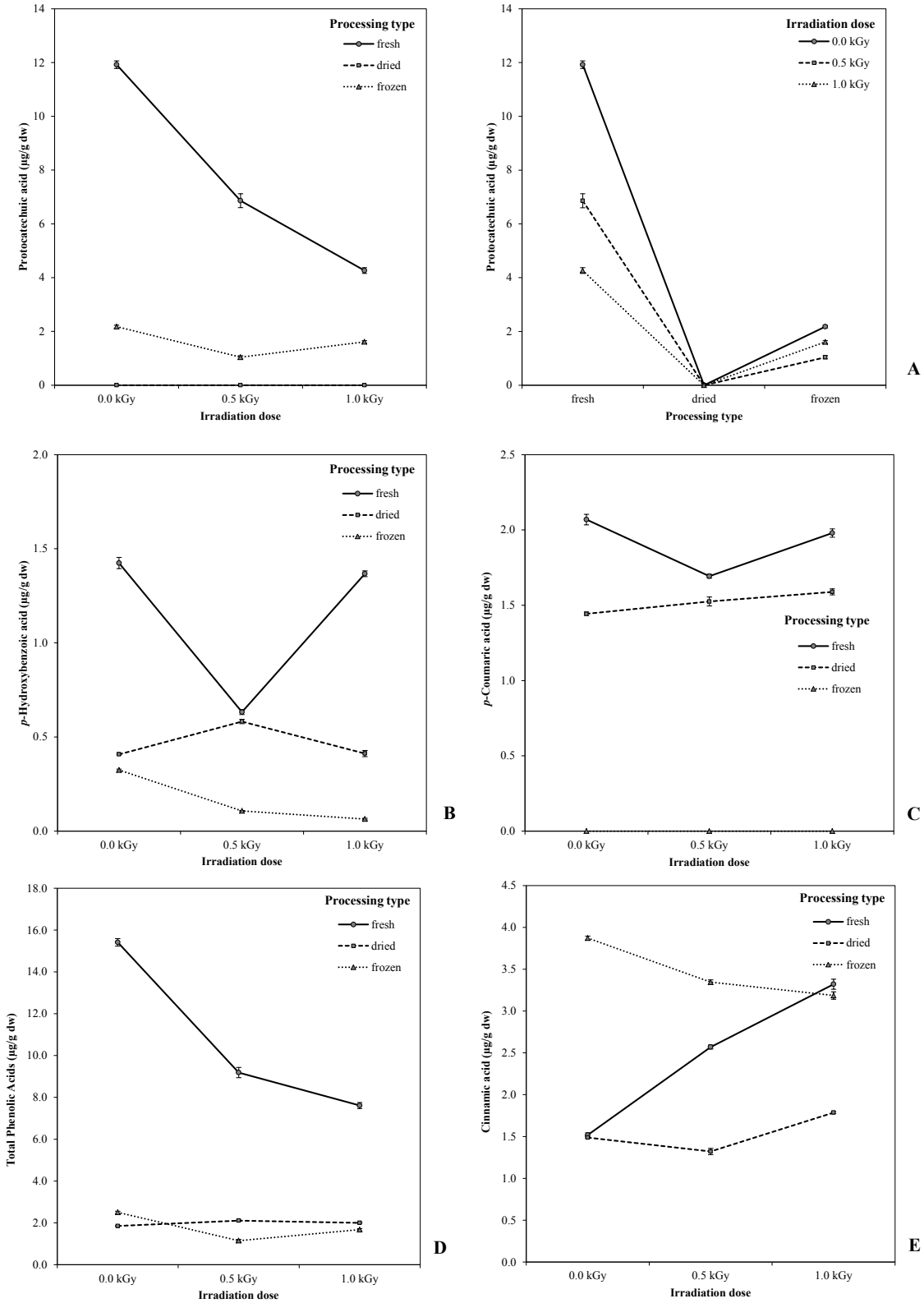
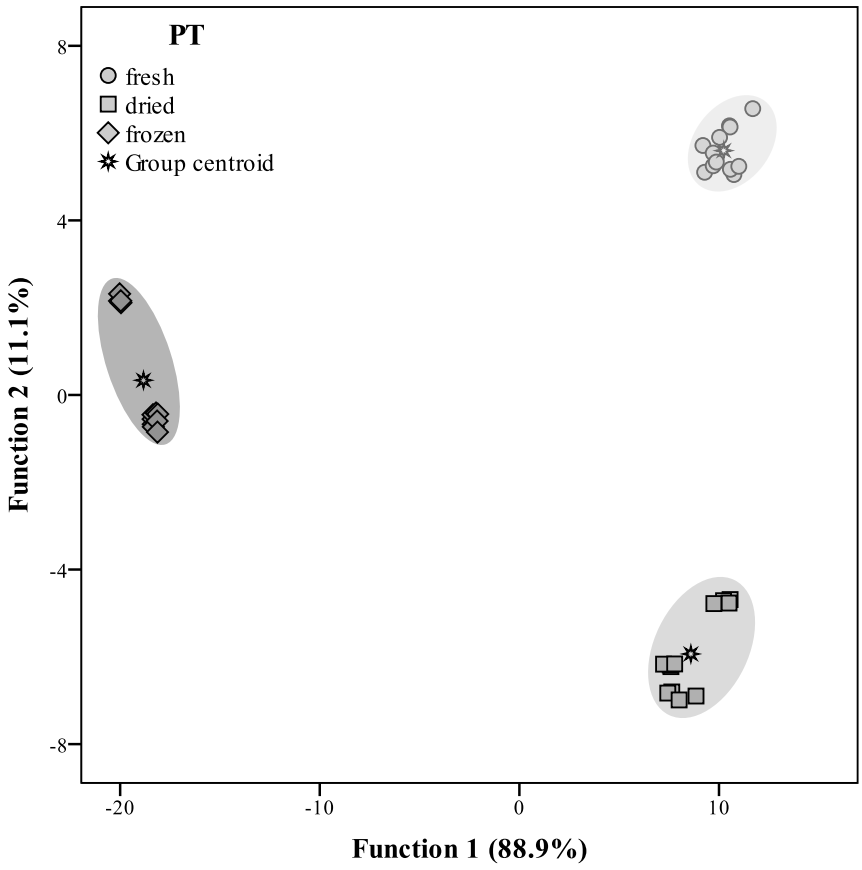

Protocatechuic acid (μg/g dw)
Irradiation dose
0.0 kGy
0.5 kGy
1.0 kGy
Processing type
fresh
dried
frozen
A
p-Hydroxybenzoic acid (μg/g dw)
B
p-Coumaric acid (μg/g dw)
C
Total Phenolic Acids (μg/g dw)
D
Cinnamic acid (μg/g dw)
E

Figure 4.


PT
fresh
dried
frozen
Group centroid
8
4
0
-4
-8
-20
-10
0
10
Function 2 (11.1%)
Function 1 (88.9%)