Irradiation as a novel approach to improve quality of *Tropaeolum majus* L. flowers: benefits in phenolic profiles and antioxidant activity

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Abstract

Edible flowers are increasingly used in food preparations, requiring new approaches to improve their conservation and safety. Food irradiation, particularly electron beam and gamma irradiation, is legally recognized to extend shelf life, improve hygienic quality and disinfest foods. Garden nasturtium (*Tropaeolum majus* L.) flowers are widely used in food preparations, being also known for their antioxidant properties and high content of phenolics. The purpose of this study was to evaluate the dose-response effects of gamma and electron beam irradiation (unirradiated and doses of 0.5, 0.8 and 1 kGy) on its antioxidant activity and phenolic compounds. Kaempferol-*O*-hexoside-*O*-hexoside was the most abundant compound, while pelargonidin-3-*O*-sophoroside was the major anthocyanin. The flowers showed high antioxidant activity, in particular as reducing agents. The interaction among the effects of irradiation dose and irradiation technology was a significant source of variation for all parameters. In general, irradiated samples gave higher antioxidant activity, maybe due to their higher amounts of phenolic compounds. Anthocyanins were the sole compounds negatively affected by irradiation. These differences were reflected in the linear discriminant analysis, which allowed the perfect separation of the applied doses, as also both irradiation technologies. Accordingly, irradiation represents a feasible technology to preserve the quality of edible flowers.

Keywords: Tropaeolum majus; edible flowers; phenolic compounds; antioxidant activity; Linear Discriminant Analysis

1. Introduction

Edible flowers have been used in the culinary arts to add flavour and garnish for hundreds of years. In many locations worldwide, the use of flowers as foods is an old tradition. Currently, people use various shapes, colours and flavours of flowers in order to enhance the sensory and nutritional qualities of foods, and many of them have biologically active substances (Mlcek & Rop, 2011). Edible flowers are increasingly being used in meals as an ingredient in salads or garnish, entrees, drinks and desserts. Recipes with flowers have been applied to different food matrices namely, tea, baking, sauces, jelly, syrup, flavoured liquors, vinegars, honey, and oils (Creasy, 1999; Felippe, 2004).

The increasing application of edible flowers in various branches of food technology requires new approaches to improve conservation and safety of these products. The extension of post-harvest storage, preserving the quality of the plants, will benefit the industrial development as well as the health of consumers (Rop, Mlcek, Jurikova, Neugebauerova & Vabkova, 2012). Edible flowers are highly perishable products and must be free from diseases and insect pests, which represents a challenge because they must grow without the use of any chemical pesticide (Newnam & O'Conner, 2009). Food irradiation is a method that can be used for the extension of shelf life of perishable commodities, improvement of hygienic quality, disinfestation of insects and food safety (Farkas, 2006).

Low doses of ionizing radiations do not cause any significant alteration on the sensory properties of food. Safety and efficiency of food irradiation have been recognized by several authorities such as World Health Organization- WHO, International Atomic Energy Agency- IAEA and Food Agriculture Organization- FAO (Farkas, 2006; Farkas & Mohácsi-Farkas, 2011).

The electron beams generated by accelerators have superior dose rates than gamma rays, provide higher efficiency and higher throughput, requiring short treatment time and being achievable by applying low cost processes without production of nuclear waste (Wei et al., 2014). Nevertheless, electron beams penetrate the products only in a limited extension, while gamma rays can penetrate in a greater depth (Gomes et al., 2008).

Garden nasturtium (*Tropaeolum majus* L.) has richly colloured red, orange, and yellow flowers, with predominance of the orange phenotype. These flowers have strong spicy flavour watercress and are great in salads, sauces, grilled dishes and stuffed preparations (Creasy, 1999; Garzón & Wrolstad, 2009). The antioxidant activity and anthocyanins composition in petals of orange garden nasturtium flowers from Colombia have been previously studied (Garzón & Wrolstad, 2009). The aqueous extracts revealed antioxidant properties and relatively high contents of total phenolics and ascorbic acid; furthermore, three anthocyanins derived from cyanidin, delphinidin and pelargonidin were found , although only the majority one was tentatively identified as pelargonidin-3-*O*-sophoroside (Garzón & Wrolstad, 2009). Total phenolic and flavonoids content, and antioxidant properties of hydroalcoholic extracts of garden nasturtium flowers and leaves were also reported (Santo, Martins, Tomy & Ferro, 2007; Rop et al., 2012). Bazylko et al. (2013) and Bazylko, Parzonkoa, Jez, Osinska and Kiss (2014), attributed the scavenging activity of aqueous and hydroethanolic extracts from garden nasturtium herb obtained in Poland to the presence of cinnamoylquinic acids, primarily chlorogenic acid, and vitamin C. Furthermore, garden nasturtium flowers have been considered excellent dietary sources of lutein, which reduces the risk of macular degeneration (Niizu & Rodriguez-Amaya, 2005), and vegetative parts of garden nasturtium are also characterized by containing high concentrations of the aromatic glucosinolate glucotropaeolin to which antimicrobial and anticarcinogenic properties have been

attributed (Kleinwachter, Schnug & Selmar, 2008; Schreiner, Krumbein, Mewis, Ulrichs & Huyskens-Keil, 2009; Bloem, Haneklaus & Schnug, 2013).

Nevertheless, nothing is known about the effects of irradiation on the activity and composition of garden nasturtium. Thus, the purpose of this study was to evaluate the dose-response effects of gamma and electron beam irradiation (unirradiated and doses of 0.5, 0.8 and 1 kGy) on the antioxidant activity and phenolic compounds of garden nasturtium flowers obtained in Brazil.

2. Materials and methods

2.1. Samples

Samples of fresh flowers of *Tropaeolum majus* L. (commercialized inside polyethylene bags) were purchased from a local market in São Paulo, Brazil, in September 2013. Flower petals presenting different phenotypes (yellow, orange and red) were used.

2.2. Samples irradiation

Gamma irradiation. The samples were irradiated at Nuclear and Energy Research Institute - IPEN/CNEN (São Paulo, Brazil), using a ^{60}Co source Gammacell 200 (Nordion Ltd., Ottawa, ON, Canada), at room temperature, with a dose rate of 1.258 kGy/h, at doses of 0 (control), 0.5, 0.8 and 1 kGy. Harwell Amber 3042 dosimeters were used to measure the radiation dose. After irradiation, samples were lyophilized (Solab SL404, São Paulo, Brazil) and kept in the best conditions for subsequent use.

Electron beam irradiation. Samples were irradiated at Nuclear and Energy Research Institute - IPEN/CNEN (São Paulo, Brazil), using an electron beam accelerator

(Dynamitron, Radiation Dynamics Inc., Edgewood, NY, USA), at room temperature. The applied doses were 0.5 kGy (dose rate: 1.11 kGy/s, energy: 1.400 MeV, beam current: 0.3 mA, tray speed: 6.72 m/min), 0.8 kGy (dose rate: 1.78 kGy/s, energy: 1.400 MeV, beam current: 0.48 mA, tray speed: 6.72 m/min) and 1.0 kGy (dose rate: 2.23 kGy/s, energy: 1.400 MeV, beam current: 0.6 mA, tray speed: 6.72 m/min). After irradiation, samples were lyophilized and kept in the best conditions for subsequent use.

2.3. Chemical characterization of the extracts

Analysis of non-anthocyanin phenolic compounds. The powdered flower samples (~0.5 g) were extracted by stirring with 20 mL of methanol:water 80:20 (*v/v*), at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman No. 4 paper. The residue was then re-extracted with additional portions (20 mL) of methanol:water 80:20 (*v/v*). The combined extracts were evaporated at 35 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) to remove methanol. The aqueous phase was lyophilized and 10 mg were re-dissolved in 2 mL of 20% aqueous methanol and filtered through a 0.22-μm disposable LC filter disk for high performance liquid chromatography (HPLC-DAD-MS) analysis. The extracts were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies, Santa Clara, CA, USA) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred

wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet (Chahdoura et al., 2014).

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500 V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to record full scan spectra to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1500. Analysis in EPI mode was further performed in order to obtain the fragmentation pattern of the parent ion(s) detected in the previous experiment using the following parameters: DP -50 V, EP -6 V, CE -25 V, and collision energy spread (CES) 0 V.

The phenolic compounds present in the flower samples were characterized according to their UV and mass spectra and retention times compared with commercial standards when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known concentrations (1-100 μg/mL) of different standards compounds: *p*-coumaric acid (y = 884.6x + 184.5; R^2 = 0.999); 5-*O*-caffeoylquinic acid acid (y = 313.0x - 58.20; R^2 = 0.999); myricetin (y = 741.4x - 221.6; R^2 = 0.999); quercetin-3-*O*-rutinoside (y = 282.0x - 0.3459; R^2 = 1.000); kaempferol-3-*O*-rutinoside (y = 239.2x - 10.59; R^2 = 1.000).

Quantification was performed based on DAD results from the areas of the peaks recorded at 280 nm or 370 nm and results were expressed in mg per g of extract.

Analysis of anthocyanins. Each sample (~0.5 g) was extracted with 20 mL of methanol containing 0.5% TFA, and filtered through a Whatman nº 4 paper. The residue was then re-extracted with additional 20 mL portions of 0.5% TFA in methanol. The combined extracts were evaporated at 35 ºC to remove the methanol, and re-dissolved in water. For purification, the extract solution was deposited onto a C-18 SepPak® Vac 3 cc cartridge (Phenomenex, Torrance, CA, USA), previously activated with methanol followed by water; sugars and more polar substances were removed by passing through 10 mL of water and anthocyanin pigments were further eluted with 5 mL of methanol:water (80:20, *v/v*) containing 0.1% TFA. The extract was concentrated under vacuum, lyophilized, re-dissolved in 1 mL of 20% aqueous methanol and filtered through a 0.22-μm disposable LC filter disk for HPLC analysis.

The extracts were analysed in the HPLC system indicated above using the conditions described by García-Marino, Hernández-Hierro, Rivas-Gonzalo and Escribano-Bailón (2010). Separation was achieved on an AQUA® (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 μm, 150 mm × 4.6 mm i.d) thermostatted at 35 ºC. The solvents used were: (A) 0.1% TFA in water, and (B) 100% acetonitrile. The gradient employed was: isocratic 10% B for 3 min, from 10 to 15% B for 12 min, isocratic 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min and from 30 to 35% for 5 min, at a flow rate of 0.5 mL/min. Double detection was carried out by DAD, using 520 nm as the preferred wavelength, and MS using the same equipment described above. Zero grade air served as the nebulizer gas (40 psi) and turbo gas (600 ºC) for solvent drying (50 psi). Nitrogen served as the curtain (100 psi) and collision gas (high).

Both quadrupols were set at unit resolution. The ion spray voltage was set at 5000 V in the positive ion mode. EMS and ESI methods were used for acquisition of full scan spectra and fragmentation patterns of the precursor ions, respectively. Setting parameters used for EMS mode were: declustering potential (DP) 41 V, entrance potential (EP) 7.5 V, collision energy (CE) 10 V, and parameters for EPI mode were: DP 41 V, EP 7.5 V, CE 10 V, and collision energy spread (CES) 0 V.

The anthocyanins present in the samples were characterised according to their UV and mass spectra and retention times, and comparison with available standards. For quantitative analysis, a calibration curve was obtained by injection of known concentrations (50-0.25 μg/mL) of delphinidin-3-*O*-glucoside (y = 557274x + 126.24; R^2 = 0.9997), cyanidin-3-*O*-glucoside (y = 630276x - 153.83; R^2 = 0.9995) and pelargonidin-3-*O*-glucoside (y = 268748x - 71.423; R^2 = 1.0000). Quantification was performed based on DAD results from the areas of the peaks recorded at 520 nm and results were expressed in μg per g of extract.

2.4. Antioxidant activity

The hydromethanolic extract described above was used for the antioxidant activity assays. A stock solution of 20 mg/mL was used and successive dilutions were made and submitted to *in vitro* antioxidant assays. The sample concentrations (mg/mL) providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH and β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (ferricyanide assay), respectively (Barros et al., 2013). Trolox was used as a positive control.

Reducing power. 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 in an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA).

DPPH radical-scavenging activity. The reaction mixture consisted of different concentrations (30 μL) of the extract solutions and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol/L) in different wells of a 96 well microplate. The mixture was left to stand in the dark for 30 min, and the absorbance was measured at 515 nm (microplate reader mentioned above). The radical scavenging activity (RSA) was calculated as the percentage of DPPH discoloration: $\%RSA=[(A_{DPPH} - A_S)/A_{DPPH}]\times100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

Inhibition of β-carotene bleaching or β-carotene/linoleate assay. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and 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 test tubes containing fraction/extract solutions with different concentrations (0.2 mL). The tubes were shaken and incubated at 50°C in a water bath. As soon as the emulsion was added to each tube,

the zero time absorbance was measured at 470 nm (Analytik 200-2004 spectrophotometer, Jena, Germany). β-Carotene bleaching inhibition was calculated using the following equation: (absorbance after 2 h of assay/initial absorbance)×100.

2.5. Statistical analysis

For each sample, three independent extractions were performed, and each of them was injected in duplicate for the chromatographic analysis or assayed in triplicate for the spectrophotometric readings. The results were expressed in mg per g (μg per g, for anthocyanins) of extract (dw), as mean values±standard deviation (SD).

The fulfilment 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 a determined phenolic compound had significant differences among the assayed doses or irradiation technologies). 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. Results regarding the comparison of electron beam and gamma irradiation were classified using a simple *t*-test, since there were fewer than three groups.

In order to obtain a combined analysis, a linear discriminant analysis (LDA) was used to compare the effect of IT and ID on antioxidant activity and phenolic compounds. 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, in which the

inclusion of a new variable come after ensuring that all variables selected previously remain significant (Maroco, 2003; López, García & Garrido, 2008). With this approach, it is possible to identify the significant variables obtained for each factor. 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.

3. Results and discussion

The characterization of the phenolic compounds was performed by HPLC-DAD/ESI-MS analysis, and data of the retention time, λ_{max}, deprotonated molecule, main fragment ions in MS^2, tentative identification and concentration of phenolic acid derivatives and flavonoids are presented in **Table 1**. The HPLC phenolic profiles recorded at 280 nm (A) and 370 nm (B) of the control (unirradiated) samples are given in **Figure 1**.

UV and mass spectra obtained by HPLC-DAD-ESI/MS analysis showed that this *Tropaeolaceae* species is characterized by the presence of phenolic acids (hydroxycinnamoyl derivatives) and flavonoids. The analysis of the MS^2 fragments revealed *O*-glycosides of flavonols (myricetin, quercetin and kaempferol) and anthocyanins (delphinidin, cyanidin and pelargonidin). Glycosides substituents consisted hexosyl and dihexosyl units, as deduced from the losses of 162 and 324 Da. The presence of hydroxycinnamoyl derivatives and flavonoid glycosides is coherent with the results obtained in Polish varieties of this species (Bazylko et al., 2013).

Compound **1** was identified as 3-*O*-caffeoylquinic acid based on its mass spectral data, taking into account to the hierarchical keys described by Clifford, Johnston, Knight, & Kuhnert (2003). Following the same criteria, compound **3** was identified as 5-*O*-caffeoylquinic acid by comparison with a standard and according to its MS^2

fragmentation pattern as reported by Clifford et al. (2003) and Clifford, Knight, & Kuhnert (2005). Compound **2** was identified as 3-*p*-coumaroylquinic acid, yielding the base peak at *m*/*z* 163 ([*p*-coumaric acid-H]$^-$) with lower intensity for the ion at *m*/*z* 191 ([quinic acid-H]$^-$) (Clifford, Zheng & Kuhnert, 2006). Compounds **5** and **6** showed similar MS^2 fragmentation pattern presenting the base peak at *m*/*z* 191 and a weak fragment at *m*/*z* 163, coherent with 5-*p*-coumaroylquinic acid (Clifford et al., 2006). These compounds were tentatively assigned as the *cis* and *trans* isomers, respectively, based on the observation that hydroxycinnamoyl *cis* derivatives are expected to elute before the corresponding *trans* ones, as observed after UV irradiation (366 nm, 24 h) of hydroxycinnamic acids in our laboratory (Barros, Dueñas, Carvalho, Ferreira & Santos-Buelga, 2012). The presence of 3- and 5-caffeoylquinic acid and 3- and 5-*p*-coumaroylquinic acid in vegetative parts of *T. majus* was also described by Bazylko et al. (2013).

Compound **4** presented a pseudomolecular ion [M-H]$^-$ at *m*/*z* 641, releasing MS^2 fragments at *m*/*z* 479 and 317 from the sequential loss of two hexosyl moieties (-162 mu). Based on these characteristics and its flavonol-like UV spectrum, the compound was tentatively identified as myricetin-*O*-hexoside-*O*-hexoside. Similar reasoning allowed assigning compounds **7** and **8** as quercetin-*O*-hexoside-*O*-hexoside and kaempferol-*O*-hexoside-*O*-hexoside, respectively. The precise identity and position of the sugar substituents could not be established in any of these flavonols. In previous phytochemical studies, the occurrence of quercetin and kaempferol glycosides in *T. majus* was also reported (De Medeiros et al., 2000; Mietkiewska et al., 2004; Zanetti, Manfron & Hoelzel, 2004; Bazylko et al., 2013), however, as far as we know, the presence of a myricetin derivative is described here for the first time. Compounds **9-11** were identified as anthocyanin derivatives (**Figure 2A**). Compound **9** was identified as

delphinidin-*O*-dihexoside based on its mass spectra, which showed an MS^2 signal at *m/z* 303 (delphinidin; $[M-324]^+$, loss of a dihexosyl moiety). The same loss was also verified for compounds **10** and **11**, which produced fragment ions at *m/z* 287 (cyanidin; $[M-324]^+$) and *m/z* 271 (pelargonidin; $[M-324]^+$). The ratios $E_{440}/E_{\lambda_{max}}$(29% for delphinidin, 32% for cyanidin and 44% for pelargonidin) were also coherent with previously reported values (Garzón & Wrolstad, 2009). Similarly, the UV-Vis spectrum of compound **11** also complies with the expected shape for pelargonidin, which is characterized by a prominent shoulder in the 410-450 nm range (**Figure 2B**).

The presence of three anthocyanins derived from cyanidin, delphinidin and pelargonidin in garden nasturtium flowers was already reported by Garzón and Wrolstad (2009), from which only the majority one was tentatively identified as pelargonidin-3-*O*-sophoroside. In our case, the nature and position of the sugar substituents in the detected anthocyanins could not be established, although in accordance with the previous authors, compound **11** might be speculated to correspond to pelargonidin-3-*O*-sophoroside.

The effect of the irradiation dose (ID) (0.0, 0.5, 0.8 and 1.0 kGy) and irradiation technology (IT) (electron beam and cobalt-60), as well as the interaction of both factors (ID×IT), were assessed by evaluating changes in phenolic compounds profiles and antioxidant activity. With this approach, it was intended to find the most suitable ID, independently of the used source, as well as determining which of the irradiation technologies is the most adequate (independently of the applied dose) considering the phenolic compounds contents and the antioxidant activity of garden nasturtium flower extracts.

Table 2 shows the quantified amounts of phenolic compounds reported as mean value of each ID for both IT, as well as mean value of each IT including the entire ID.

Kaempferol-*O*-hexoside-*O*-hexoside was the most abundant compound in all samples. Actually, this compound represents more than 50% of all phenolic compounds quantified in each sample. On the other hand, *trans*-5-*p*-coumaroylquinic acid was the minor compound in all cases, excepting, of course, the quantified anthocyanins. Pelargonidin-3-*O*-sophoroside was the most abundant anthocyanin, representing approximately 65% of the quantified anthocyanins.

The interaction among factors (*i.e.*, ID and IT) was statistically significant ($p<0.001$), not allowing classifying each of them individually. Even though, the influence of each factor acting individually was also significant, especially concerning ID, as it can be deduced from the *p*-values. In fact, some particular tendencies could be identified from the analysis of the estimated margins mean plots (data not shown) obtained in the GLM procedures. For instance, 3-*O*-caffeoylquinic, 5-*O*-caffeoylquinic, *cis*-5-*p*-coumaroylquinic and *trans*-5-*p*-coumaroylquinic acids tended to be higher in samples irradiated with 1 kGy, while the anthocyanin derivatives were higher in non-irradiated samples (independently of the source). Despite the low applied doses, which induced some effect on the levels of some phenolic compounds, the anthocyanins content weas significantly reduced, in percentages that might be considered expectable for these irradiation levels (Alighourchi, Barzegar & Abbasi, 2008). On the other hand, anthocyanins profile is affected by the food matrix, structural features, and the processing conditions (Torskangerpoll & Andersen, 2005). However, the detected chromatographic differences were not reflected by noticeable changes in the flowers' colour, as deduced from the direct observation of the irradiated samples. Regarding IT, kaempferol-*O*-hexoside-*O*-hexoside leaned toward higher values in samples irradiated with electron beam (independently of applied dose). Actually, gamma irradiation seems

to affect negatively the levels of this compound whereas the opposite happens with electron beam.

Concerning their antioxidant activity (**Table 3**), *T. majus* flower extracts were particularly active reducing agents, showing also good radical scavenging activity, followed by their ability to inhibit β-carotene bleaching. The interaction among factors was again significant in all cases, while the individual effect of ID was not significant in the reducing power (p = 0.112), in line with the observed for IT effect over DPPH radical-scavenging activity (p = 0.973) and inhibition of β-carotene bleaching (0.244). The main differences for each factor were the lower EC_{50} values regarding inhibition of β-carotene bleaching observed in samples irradiated with 1 kGy and the higher reducing power showed by samples submitted to gamma irradiation.

In order to better understand the effects of ID and IT on the antioxidant activity and phenolic compounds amounts, two linear discriminant analysis were applied. The significant independent variables (results for antioxidant activity assays and phenolic compounds contents) were selected using the stepwise method of the LDA, according to the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.05$) were kept in the analysis.

In the case of ID effect, three significant functions were defined (**Figure 3**), which encompassed 100.0% of the observed variance (first, 61.23%; second, 38.12%; third, 0.65%). As it can be seen in Fig. 3, the tested groups (0.0 kGy, 0.5 kGy, 0.8 kGy and 1.0 kGy) were completely separated. The variables 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, *cis*-5-*p*-coumaroylquinic acid, *trans*-5-*p*-coumaroylquinic acid, DPPH scavenging activity and reducing power were not selected as discriminant by the model. Function 1 was mostly correlated to pelargonidin-3-*O*-sophoroside and β-carotene bleaching inhibition. As it can be observed by the markers distribution,

function 1 separated mainly samples irradiated with 0.5 and 1 kGy, as confirmed by the means of canonical variance (MCV: 0.0 kGy, 11.151; 0.5 kGy, -19.502; 0.8 kGy, -7.726; 1.0 kGy, 21.653). Function 2, was again more strongly correlated with pelargonidin-3-*O*-sophoroside, but also with delphinidin-*O*-dihexoside, contributing to individualize unirradiated samples (MCV: 0.0 kGy, 30.967; 0.5 kGy, -1.340; 0.8 kGy, -3.567; 1.0 kGy, -10.486). Finally, function 3 was more correlated with cyanidin-*O*-dihexoside and quercetin-*O*-hexoside-*O*-hexoside, proving its contribution to separate samples irradiated with 0.8 kGy (MCV: 0.0 kGy, 0.039; 0.5 kGy, 1.810; 0.8 kGy, -2.543; 1.0 kGy, 0.713), despite the lower percentage of variance explained by this function. In terms of classification performance, all samples were correctly classified, either for original grouped cases, as well as for cross-validated grouped cases.

Regarding IT, the discriminant model selected 2 significant functions (**Figure 2B**), which included 100.0% of the observed variance (function 1: 90.21%, function 2: 9.79%). In this case, the tested groups (unirradiated, e-beam and Co-60) were also completely individualized, despite the lower number of selected variables (3-*O*-caffeoylquinic acid, 3-*p*-coumaoylquinic acid, *trans*-5-*p*-coumaoylquinic acid, myricetin-*O*-hexoside-*O*-hexoside, quercetin-*O*-hexoside-*O*-hexoside, kaempfeol-*O*-hexoside-*O*-hexoside and DPPH scavenging activity were not considered as discriminant variables by the obtained model). Function 1 separated mainly unirradiated samples (MCV: unirradiated, 29.016; electron beam, -5.420; cobalt-60, -4.252), being more strongly correlated to the three detected anthocyanin derivatives (compounds 9-11), reflecting the similarity of the results obtained for each IT (**Table 2**), while function 2 allowed the separation of electron beam and cobalt-60 irradiated samples (MCV: unirradiated, -0.437; electron beam, -4.143; cobalt-60, 4.289), showing the highest correlation with reducing power, which was previously indicated as being

higher in gamma irradiated samples. The classification performance was also completely accurate for original grouped cases and for cross-validated grouped cases. According to the obtained results, the antioxidant activity was not relevantly weakened by irradiation; in fact, some irradiated samples gave higher antioxidant activity than the corresponding control. This increase might be explained by the higher amounts of phenolic compounds in irradiated samples, specially observed in phenolic acids, but also in kaempferol-*O*-hexoside-hexoside. These higher amounts might probably be explained by a certain protective effect of irradiation, which may affect the atmosphere (especially, decreasing the O_2 percentage to produce atomic oxygen) inside the polyethylene bags containing the flowers, when compared to unirradiated samples. Actually, among the assayed parameters, the anthocyanins were the unique compounds negatively affected by irradiation, together with the higher sensibility of kaempferol-*O*-hexoside-*O*-hexoside to gamma irradiation in comparison to electron beam. The observed differences were reflected in the linear discriminant analysis, which allowed the complete separation of the applied doses, and also the applied technologies. The effects of each factor were significantly different, since the correlations among discriminant functions and selected variables were different within each analysis. Hence, the obtained profiles might have the additional advantage of discriminate among irradiated and unirradiated garden nasturtium samples.

Overall, the applied irradiation treatments seemed to represent a feasible technology to preserve the quality of edible flower petals considering the requirements imposed by their increasing uses. Irradiation might be useful to expand the post-harvest storage, preserving the quality of the edible flowers, promoting their industrial development as well as the health of consumers.

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References

Alighourchi, H., Barzegar, M., & Abbasi, S. (2008). Effect of gamma irradiation on the stability of anthocyanins and shelf-life of various pomegranate juices. *Food Chemistry*, *110*, 1036-1040.

Barros, L., Dueñas, M., Carvalho, A.M., Ferreira, I.C.F.R., & Santos-Buelga, C. (2012). Characterization of phenolic compounds in flowers of wild medicinal plants from Northeastern Portugal. *Food and Chemical Toxicology*, *50*, 1576-1582.

Barros, L., Pereira, E., Calhelha, R.C., Dueñas, M., Carvalho, A.M., Santos-Buelga, C., & Ferreira, I.C.F.R. (2013). Bioactivity and chemical characterization in hydrophilic and lipophilic compounds of *Chenopodium ambrosioides* L. *Journal of Functional Foods*, *5*, 1732-1740.

Bazylko, A., Granica, S., Filipek, A., Piwowarski, J., Stefańska, J., Osińska, E., & Kiss, A.K. (2013). Comparison of antioxidant, anti-inflammatory, antimicrobial activity and chemical composition of aqueous and hydroethanolic extracts of the herb of *Tropaeolum majus* L. *Industrial Crops and Products*, *50*, 88-94.

Bazylko, A., Parzonkoa, A., Jez, W., Osinska, E., & Kiss, A.K. (2014). Inhibition of ROS production, photoprotection, and total phenolic, flavonoids and ascorbic acid content of fresh herb juice and extracts from the leaves and flowers of Tropaeolum majus. *Industrial Crops and Products*, *55*, 19-24.

Bloem, E., Haneklaus, S., & Schnug, E. (2013). *Tropaeolum majus* L. - Life Cycle and Optimum Harvest Time for Highest Glucotropaeolin Contents. *Phyton-Annales Rei Botanicae*, *53*, 305-319.

Chahdoura, H., Barreira, J.C.M., Barros, L., Santos-Buelga, C., Ferreira, I.C.F.R., & Achour, L. (2014). Phytochemical characterization and antioxidant activity of *Opuntia microdasys* (Lehm.) Pfeiff flowers in different stages of maturity. *Journal of Functional Foods*, *9*, 27-37.

Clifford, M.N., Johnston, K.L., Knight, S., & Kuhnert, N.A. (2003). A hierarachical scheme for LC-MS^n identification of chlorogenic acids. *Journal of Agricultural and Food Chemistry*, *51*, 2900-2911.

Clifford, M.N., Knight, S., & Kuhnert, N.A. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS^n. *Journal of Agricultural and Food Chemistry*, *53*, 3821-3832.

Clifford, M. N., Zheng, W., & Kuhnert, N. (2006). Profiling the chlorogenic acids of aster by HPLC–MSn. *Phytochemical Analysis*, *17*, 384-393.

Creasy, R. (1999). The Edible Flowers Garden. Periplus Editions, Boston.

De Medeiros, J.M., Macedo, M., Contancia, J.P., Nguyen, C., Cunningham, G., & Miles, D.H. (2000). Antithrombin activity of medicinal plants of the Azores. *Journal of Ethnopharmacology*, *72*, 157-165.

Farkas, J. (2006) Irradiation for better foods. *Trends in Food Science and Technology*, *17*, 148-152.

Farkas, J., & Mohácsi-Farkas, C. (2011). History and future of food irradiation. *Trends in Food Science and Technology*, *22*, 121-126.

Felippe, G. (2004). Entre o jardim e a horta, Editora Senac, São Paulo.

García-Marino, M., Hernández-Hierro, J.M., Rivas-Gonzalo, J.C., & Escribano-Bailón, M.T. (2010). Colour and pigment composition of red wines obtained from comaceration of *Tempranillo* and *Graciano* varieties. *Analytical Chimica Acta*, *660*, 134-142.

Garzón, G.A., & Wrolstad R.E. (2009). Major anthocyanins and antioxidant activity of Nasturtium flowers (*Tropaeolum majus*). *Food Chemistry*, *114*, 44-49.

Gomes, C., Silva, P., Chimbombi, E., Kim, J., Castell-Perez, E., & Moreira, R.G. (2008). Electron-beam irradiation of fresh broccoli heads (*Brassica oleracea* L. *italica*). *LWT - Food Science and Technology*, *41*, 1828-1833.

Kleinwachter, M., Schnug, E., & Selmar, D. (2008). The Glucosinolate-Myrosinase System in Nasturtium (*Tropaeolum majus* L.): Variability of Biochemical Parameters and Screening for Clones Feasible for Pharmaceutical Utilization. *Journal of Agricultural and Food Chemistry*, *56*, 11165-11170.

López, A., García, P., & Garrido, A. (2008). Multivariate characterization of table olives according to their mineral nutrient composition. *Food Chemistry*, *106*, 369-378.

Maroco, J. Análise Estatística, com utilização do SPSS. (2003) Edições Sílabo, Lisboa, Portugal.

Mietkiewska, E., Giblin, E.M., Wang, S., Barton, D.L., Dirpaul, J., Brost, J.M., Katavic, V., & Taylor, D.C. (2004). Seed-specific heterologous expression of a nasturtium FAE gene in Arabidopsis results in a dramatic increase in the proportion of erucic acid. *Plant Physiology*, *136*, 2665-2675.

Mlcek, J., & Rop, O. (2011). Fresh edible flowers of ornamental plants - A new source of nutraceutical foods -a review. *Trends in Food Science and Technology*, *22*, 561-569.

Newnam, S.E., & O'Conner, A.S. (2009). Edible Flowers. CSU Extension, 7237, 1-5.

Niizu, P.Y., & Rodriguez-Amaya, D.B. (2005). Flowers and leaves of *Tropaeolum majus* L. as rich sources of lutein. *Journal of Food Science*, *70*, 605-609.

Rop, O., Mlcek, J., Jurikova, T., Neugebauerova, J., & Vabkova, J. (2012). Edible flowers - a new promising source of mineral elements in human nutrition. *Molecules*, *17*, 6672-6683.

Santo, A., Martins, I., Tomy, S., & Ferro, V. (2007). Anticoagulant in vitro effect of hidrotethanolic extract of edible leaves and flowers of Tropaeolum majus L. (Tropaeolaceae) on human plasma. *Latin American Journal of Pharmacy*, *26*, 732-736.

Schreiner, M., Krumbein, A., Mewis, I., Ulrichs, C., & Huyskens-Keil, S. (2009). Short-term and moderate UV-B radiation effects on secondary plant metabolism in different organs of nasturtium (*Tropaeolum majus* L.). *Innovative Food Science and Emerging Technologies*, *10*, 93-96.

Torskangerpoll, K., & Andersen, Q.M. (2005). Colour stability of anthocyanins in aqueous solutions at various pH values. *Food Chemistry*, *89*, 427-440.

Wei, M., Zhou, L., Song, H., Yi, J., Wu, B., Li, Y., Zhang, Che, F., Wang, Z., & Gao, M., Li, S. (2014). Electron beam irradiation of sun-dried apricots for quality maintenance. *Radiation Physics and Chemistry*, *97*, 126-133.

Zanetti, G.D., Manfron, M.P., & Hoelzel, S.C.S. (2004). Análise morfo-anatômica de *Tropaeolum majus* L. (*Tropaeolaceae*). *IHERINGIA Série Botânica*, *59*, 173-178.

Table 1. Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, relative abundances of fragment ions and tentative identification of the phenolic compounds in *Tropaeolum majus* extracts.

Compound	R_t (min)	λmax (nm)	Molecular ion (*m/z*)*	MS^2 (*m/z*)	Tentative identification
1	5.4	326	353	191(100), 179(69), 173(16), 135(44)	3-*O*-Caffeoylquinic acid
2	7.1	310	337	191(33), 163(100), 119(48)	3-*p*-Coumaroylquinic acid
3	8.4	326	353	191(100), 179(3), 173(2), 135(3)	5-*O*-Caffeoylquinic acid
4	13.1	356	641	479(3), 317(100)	Myricetin-*O*-hexoside-*O*-hexoside
5	13.4	312	337	191(100), 173(10), 163(11), 119(5)	*cis*-5-*p*-Coumaroylquinic acid
6	14.3	306	337	191(100), 173(9), 163(13), 119(2)	*trans*-5-*p*-Coumaroylquinic acid
7	16.0	354	625	463(4), 301(100)	Quercetin-*O*-hexoside-*O*-hexoside
8	18.5	348	609	447(8), 285(100)	Kaempferol-*O*-hexoside-*O*-hexoside
9	12.9	524	627	303(100)	Delphinidin-*O*-dihexoside
10	15.3	518	611	287(100)	Cyanidin-*O*-dihexoside
11	17.6	502	595	271(100)	Pelargonidin-3-*O*-sophoroside

Table 2. Quantification (mg/g of dried plant) of the phenolic compounds in *Tropaeolum majus* extracts according to the irradiation dose (ID) and irradiation technology (IT).

Compound	Tentative identification	Quantification (mg/g of extract)								
		Irradiation dose (ID)				*p*-value	Irradiation technology (IT)		*p*-value	ID×IT
		0 kGy	0.5 kGy	0.8 kGy	1.0 kGy	(n = 12)	e-beam	^{60}Cobalt	(n = 24)	*p*-value (n = 48)
1	3-*O*-Caffeoylquinic acid	1.56±0.01	1.9±0.1	2.2±0.4	2.7±0.2	<0.001	2.0±0.5	2.2±0.5	0.127	<0.001
2	3-*p*-Coumaroylquinic acid	0.93±0.02	1.1±0.4	1.3±0.4	1.5±0.1	<0.001	1.0±0.3	1.4±0.3	<0.001	<0.001
3	5-*O*-Caffeoylquinic acid	1.95±0.04	2.1±0.3	2.1±0.4	3.0±0.2	<0.001	2.3±0.5	2.3±0.4	0.913	<0.001
4	Myricetin-*O*-hexoside-*O*-hexoside	0.62±0.01	0.9±0.5	0.54±0.02	0.7±0.1	0.035	0.8±0.4	0.5±0.1	0.001	<0.001
5	*cis*-5-*p*-Coumaroylquinic acid	0.38±0.01	0.4±0.1	0.40±0.03	0.6±0.1	<0.001	0.5±0.1	0.4±0.1	0.012	<0.001
6	*trans*-5-*p*-Coumaroylquinic acid	0.29±0.01	0.30±0.05	0.35±0.05	0.45±0.04	<0.001	0.3±0.1	0.36±0.05	0.199	<0.001
7	Quercetin-*O*-hexoside-*O*-hexoside	0.78±0.01	1.2±0.5	0.7±0.1	0.9±0.1	0.016	1.1±0.5	0.7±0.2	0.003	<0.001
8	Kaempferol-*O*-hexoside-*O*-hexoside	9.8±0.1	12±8	9±3	13±1	0.063	14±4	8±3	<0.001	<0.001
		Quantification (µg/g of extract)								
9	Delphinidin-*O*-dihexoside	3.2±0.1	1.6±0.3	1.7±0.5	1.9±0.2	<0.001	2±1	2±1	0.352	<0.001
10	Cyanidin-*O*-dihexoside	0.21±0.01	0.14±0.01	0.12±0.02	0.16±0.02	<0.001	0.16±0.04	0.16±0.04	0.729	<0.001
11	Pelargonidin-3-*O*-sophoroside	5.8±0.1	2.6±0.4	3.1±0.4	4.7±0.2	<0.001	4±1	4±1	<0.001	<0.001

Table 3. Antioxidant activity (EC_{50} values, mg/mL) of *Tropaeolum majus* extracts according to the irradiation dose and irradiation technology.

Assay	EC_{50} values (mg/mL of extract)								
	Irradiation dose (ID)				*p*-value	Irradiation technology (IT)		*p*-value	ID×IT
	0 kGy	0.5 kGy	0.8 kGy	1.0 kGy	(n = 12)	e-beam	^{60}Cobalt	(n = 24)	*p*-value (n = 48)
Reducing power	0.32±0.01	0.29±0.04	0.31±0.05	0.32±0.02	0.112	0.34±0.02	0.28±0.02	<0.001	<0.001
DPPH radical-scavenging activity	0.64±0.05	0.69±0.05	0.68±0.04	0.66±0.04	0.047	0.67±0.05	0.67±0.05	0.973	<0.001
Inhibition of β-carotene bleaching	1.0±0.1	1.5±0.5	1.3±0.4	0.6±0.3	<0.001	1.0±0.5	1.2±0.3	0.244	<0.001

Figure 1. HPLC chromatograms of the phenolic compounds from unirradiated samples of *Tropaeolum majus* flowers recorded at 280 nm (A) and 370 nm (B). **1**: 3-*O*-Caffeoylquinic acid; **2**: 3-*p*-Coumaroylquinic acid; **3**: 5-*O*-Caffeoylquinic acid; **4**: Myricetin-*O*-hexoside-*O*-hexoside; **5**: *cis*-5-*p*-Coumaroylquinic acid; **6**: *trans*-5-*p*-Coumaroylquinic acid; **7**: Quercetin-*O*-hexoside-*O*-hexoside; **8**: Kaempferol-*O*-hexoside-*O*-hexoside.

Figure 2. (A) HPLC chromatograms of the anthocyanins from unirradiated samples of *Tropaeolum majus* flowers recorded at 520 nm. **9**: Delphinidin-*O*-dihexoside; **10**: Cyanidin-*O*-dihexoside; **11**: Pelargonidin-3-*O*-sophoroside. (B) UV spectrum of the major anthocyanin (compound **11**).

Figure 3. Mean scores of different irradiation doses (**A**) or irradiation technology (**B**) projected for the discriminant functions defined from phenolic compounds profiles and antioxidant activity assays results.