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Analysis of organic acids in electron beam irradiated chestnuts (*Castanea sativa* Mill.): Effects of radiation dose and storage time

Márcio Carocho [a], Lillian Barros [a], Amilcar L. Antonio [a,b,c], João C.M. Barreira [a,d], Albino Bento [a], Iwona Kaluska [e], Isabel C.F.R. Ferreira [a,*]

[a] CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança, Apartado 1172, 5301-855 Bragança, Portugal
[b] IST/ITN, Instituto Tecnológico e Nuclear, Estrada Nacional 10, 2686-953 Sacavém, Portugal
[c] Departamento de Física Fundamental, Universidade de Salamanca, Plaza de la Merced, 37008 Salamanca, Spain
[d] REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia da Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[e] Centre for Radiation Research and Technology, Institute of Nuclear Chem. and Technology, Dorodna str. 16, 03-195 Warsaw, Poland

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ABSTRACT

Since 2010, methyl bromide, a widely used fumigant was banned from the European Union under the Montreal Protocol guidelines, due to its deleterious effects on health and risk to the environment. Since then, many alternatives for chestnut conservation have been studied (hot water dip treatment being the most common), among them, electron beam irradiation has been proposed as being a safe, clean and cheap alternative. Herein, the effects of this radiation at different doses up to 6 kGy and over storage up to 60 days in the amounts and profile of nutritionally important organic acids were evaluated. Chestnuts contained important organic acids with quinic and citric acids as main compounds. Storage time, which is traditionally well accepted by consumers, caused a slight decrease on quinic (13–9 mg/g), ascorbic (1.2–0.8 mg/g), malic (5–4 mg/g), fumaric (0.4–0.3 mg/g) and total organic (33–26 mg/g) acids content. Otherwise, irradiation dose did not cause appreciable changes, either individually or in total (28–27 mg/g) organic acid contents. Electron beam irradiation might constitute a valuable alternative for chestnut conservation.

1. Introduction

Organic acids are low weight molecules and are considered to be any organic carboxylic acids with a general structure R-COOH. Depending on their dissociation properties and the number of carboxylic groups, they can carry varying negative charges, thereby allowing the complexation of metal cations in solution (Dibner and Buttin, 2002; Jones, 1998). Citric, succinic, fumaric and malic acids play an important role in the Krebs cycle, being essential for human metabolism, but they have much more applications; citric acid is a crystal thickener in bones (Hu et al., 2010), succinic acid is known to help on diabetes treatment (Pari and Saravanan, 2007), fumaric acid is effective against psoriasis and inflammation and can be used as a neuro and chemoprotector (Baati et al., 2011), and malic acid is reported to have a bactericidal effect (Raybaudi-Massilia et al., 2009).

Other organic acids, like oxalic and quinic acids are important metabolites in plants, displaying important roles on their metabolism, nonetheless oxalic acid is part of pharmaceutical preparations and used for desloughing of wounds and ulcers (Lian et al., 1999), while quinic acid is a stronger antioxidant than butylated hydroxyl toluene (BHT) (Hung et al., 2006). Shikimic acid takes part in the shikimate pathway, being essential for vegetable metabolism therefore producing L-phenylalanine and L-tryptophane witch are essential amino acids for humans (Krämer et al., 2003). Finally, ascorbic acid, one isoform of vitamin C, is a naturally occurring organic acid which is essential against scurvy, a powerful antioxidant and quite effective against hypertension (Duffy et al., 1999).

Chestnuts are a widely consumed nut around the world, being China the biggest producer. Portugal represents 4% of its worldwide production with a gross weight of 22105 tons of chestnut in 2010, and an income of 15 M€ (INE, 2011). To maintain the quality and extend shelf-life of these nuts it is essential to apply adequate conservation methods. Until 2010, fumigation with methyl bromide (CH_3Br) was the most common technique, until the European Union banned its use for allegedly being toxic to operators and for the negative effects on the environment (Official Journal of the EU, 2008). Following this decision, many other conservation techniques have been introduced, for instance, hot water dip, heat treatment and other fumigants, but they still represent quite a number of limitations and setbacks (Fields and White, 2002). Irradiation is recognized by international organizations as a valid conservation alternative and allowing pest free products (Bhat et al.,

* Corresponding author. Tel.: +351 273 303219; fax: +351 273 325405.
E-mail address: iferreira@ipb.pt (I.C.F.R. Ferreira).

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2008; Nagar et al., 2012; World Health Organization, 1991). The legally recognized ionized irradiation sources include gamma rays (high-energy photons) emitted by the radioactive elements cobalt-60 (^{60}Co) or cesium-137 (^{137}Cs) with high penetrating power, high-energy electron beams with limited penetration depth generated from electron accelerators operated at or below an energy level of 10 MeV and X-rays generated from bombardment of a metal target by electrons at or below an energy level of 5 MeV (Stefanova et al., 2010).

Our research group has proved that gamma radiation does not significantly alter the antioxidant, chemical and nutritional parameters of chestnuts for the tested doses, typical used for fruit conservation (Antonio et al., 2011; Barreira et al., 2012; Fernandes et al., 2011a; Fernandes et al., 2011b). Likewise, electron beam radiation proved to have slight effects on chestnuts antioxidant potential (Carocho et al., 2012a) and nutritional parameters (Carocho et al., 2012b). Other authors have studied the influence of irradiation in diverse food products (Girennavar et al., 2008), analyzing the effects on different bioactive compounds including organic acids (Kim et al., 2004; Lisińska and Aniołowski, 1991; Reyes and Cisneros-Zevallos, 2007). Nevertheless, to our knowledge, there are no studies on the effects of electron beam irradiation on chestnuts organic acids, and given their importance in human nutrition, it would be beneficial that these compounds would not undergo any variations. In this study we report the effects of electron beam irradiation (doses of 0- control, 0.5, 1, 3 and 6 kGy) and storage time (0- control, 30 and 60 days) on a series of organic acids, namely oxalic, quinic, malic, ascorbic, citric, fumaric, succinic and shikimic acid, using Ultra-Fast Liquid Chromatography and Photodiode Array detection (UFLC-PDA). The applied doses, which are in accordance with the legally permitted maximum doses (Stefanova et al., 2010), have the additional advantage of putatively ensure the biological safety. In fact, electron beam radiation proved to have an important effect in destroying the weevil larvae (Todoriki et al., 2006) reducing also yeast and mold.

2. Materials and methods

2.1. Standards and reagents

The standards of organic acids (L(+)-ascorbic acid; citric acid; malic acid; oxalic acid; shikimic acid; succinic acid; fumaric acid; quinic acid) were purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples and samples irradiation

Chestnuts (*Castanea sativa* Mill.) samples from Longal cultivar belonging to Castanha da Terra Fria PDO were collected in orchards located in Vinhais (41°50′N, 7°00′W), Trás-os-Montes, in northeastern Portugal, in the second fortnight of October during the crop year of 2011. Well-formed chestnuts without any physical injury in the outer skin were selected among those collected randomly in the orchard.

They were divided in five groups: control (non-irradiated, 0 kGy), and the other four to be irradiated at 0.5, 1, 3 and 6 kGy with fifteen units per group.

The irradiation was performed at the INCT – Institute of Nuclear Chemistry and Technology – in Warsaw, Poland. To estimate the dose during the irradiation process three types of dosimeters were used, a graphite calorimeter, and two routine Gammachrome YR and Amber Perspex dosimeters, from Harwell company (UK). The irradiation took place in a e-beam irradiator of 10 MeV of energy with a pulse duration of 5.5 μs, a pulse frequency of 440 Hz, the average beam current was 1.1 mA, the scan width of 68 cm, the conveyer speed in the range 20–100 cm/min and a scan frequency of 5 Hz. The absorbed dose was 0.53, 0.83, 2.91 kGy and 6.10 kGy, with an uncertainty of 20% for the two first doses, 15% for the third dose and 10% for the last dose. To read the Amber and Gammachrome YR dosimeters, spectrophotometric methods were used. For the Graphite calorimeter dosimeter the electrical resistance was read and converted in dose according to a previous calibrated curve. For simplicity, from now on we refer only the exact value for the dose: 0, 0.5, 1, 3 and 6 kGy.

After irradiation the samples were analyzed immediately or stored at 4 °C (refrigerator) for 30 and 60 days. Before analyses, they were pilled, reduced to powder and lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas, USA).

2.3. Organic acids extraction and analysis

Samples (~2 g) were extracted by stirring with 25 mL of meta-phosphoric acid (25 °C at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis by UFLC-PDA, the samples were filtered through 0.2 μm nylon filters (Barros et al., 2013). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Coperation). Separation was achieved on a SphereClone (Phenomenex) reverse phase C_{18} column (5 μm, 250 mm × 4.6 mm i.d) thermostatted at 35 °C. The elution was performed with sulfuric acid 3.6 mM using a flow rate of 0.8 mL/min. Detection was carried out in a PDA, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per g of dry weight.

2.4. Statistical analysis

Organic acids extraction was performed in triplicate and each sample was injected two times in UFLC-PAD. The results are expressed as mean values ± standard deviation (SD).

Analysis of variance (ANOVA). ANOVA with Type III sums of squares was 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 the main factors "irradiation dose" (ID) and "storage time" (ST). The interaction effect among the factors (ID × ST) was always significant; thereby, the two factors were evaluated simultaneously by the estimated marginal means plots for all levels of each single factor.

Principal components analysis (PCA). PCA was applied as pattern recognition unsupervised classification method. PCA transforms the original, measured variables into new uncorrelated variables called principal components. The first principal component covers as much of the variation in the data as possible. The second principal component is orthogonal to the first and covers as much of the remaining variation as possible, and so on (Patras et al., 2011). The number of dimensions to keep for data analysis was evaluated by the respective eigenvalues (which should be greater than one), by the Cronbach's alpha parameter (that must be positive) and also by the total percentage of variance (that should be as higher as possible) explained by the number of components selected. The number of dimensions considered for PCA was chosen in order to allow meaningful interpretations, and by ensuring their reliability.

3. Results and discussion

The effect of electron beam irradiation (ID) (0.0, 0.5, 1.0, 3.0 and 6.0 kGy) and storage time (ST) (0, 30 and 60 days), as well as the interaction of both factors, were assessed by evaluating changes in organic acids composition. Studying the combined effect of both factors (ID and ST), it is possible to understand the influence of each single factor without having biased results.

Table 1 shows the organic acids composition data reported as mean value of each irradiation dose (ID) along the different storage times, as well as mean value of each storage time (ST) for the different irradiation doses.

At the beginning of the experiment, the main organic acids found in all treatment groups were quinic (13 ± 3 mg/g) and citric (12 ± 3 mg/g) acids. The obtained profiles were qualitatively similar to those presented in works dealing with the metabolite composition of chestnut upon cooking (Gonçalves et al., 2010; Ribeiro et al., 2007), despite the absence of *cis*-aconitic in the present samples. Nevertheless, the detected amounts are higher than those presented in the pointed publications, most likely due to the applied extraction method. ST × ID interaction was a significant ($P < 0.001$) source of variation for all the assayed compounds. Accordingly, no multiple comparisons tests could be performed. Nevertheless, from the analysis of the estimated margins mean plots some particular tendencies could be identified. For instance, quinic (Fig. 1a) and ascorbic (Fig. 1b) acids tended to be lower after 60 days of storage, while malic (Fig. 1c) and fumaric (Fig. 1d) acids leaned toward higher values in samples non-submitted to storage. The variance caused by the assayed irradiation doses is slighter, and did not allow indication of any particular tendency. Moreover, it is known that heat induced reactions between nitrogen-free carboxylic acids and sugars are the most affecting parameters in