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Gamma radiation inhibits the production of Ochratoxin A by *Aspergillus carbonarius*. Development of a method for OTA determination in raisins

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin of great interest to humans for its nephrotoxic effects. The development and validation of an OTA method determination in raisins is described by using High Pressure Liquid Chromatography (HPLC) with Fluorescence Detector (FD). The recovery of the method was 104% while the detection limit (DL) and quantification limit were 0.26 and 0.51 ng g^{-1}, respectively.

The effect of gamma irradiation at dose of 10 kGy on the production of OTA by *Aspergillus carbonarius* in raisins (*Vitis vinifera* L.) and on OTA in contaminated samples, was investigated. The OTA which was produced by *A. carbonarius* on the 12th day of incubation, after irradiation, showed that at dose level of 10 kGy, OTA production was not detectable (below the detection limit of the method) compared with the non-irradiated the same day. A dose of 10-kGy gamma radiation applied on 100 ng of OTA, in raisins reduced OTA contamination ~88%. According to the risk assessment analysis the Provisional Maximum Tolerable Daily Intake (PMTDI) of 5.8 ng OTA kg^{-1} bw, indicates that adult consumers are less exposed, 31.4–78.4 fold lower to OTA by the irradiated raisins.

1. Introduction

Aspergillus section *Nigri* (also known as black-spored aspergilli) are an important group of fungi because of their impact on food safety, medical mycology and biotechnology industry (Palencia, Klich, Glenn, & Bacon, 2009). The black aspergilli are among the most common fungi causing food spoilage and biodeterioration of other materials (Schuster, Dunn-Coleman, Frisvad, & Van Dijck, 2002). They have been isolated mainly from soil, but they also have been found in several other substrates such as vine grapes, vine fruits, peanuts (Magnoli, Violante, Combina, Palacio, & Dalcero, 2003; Magnoli et al., 2004; Magnoli et al., 2007).

Ochratoxin A (OTA) is a mycotoxin estimated to be a human carcinogen based on sufficient indications of studies on carcinogenicity in experimental animals (Report on Carcinogens, 2011). Therefore it has been classified by the International Agency for Research on Cancer (IARC, 1993) in group 2B. Ochratoxin A is also dangerous for human health due its nephrotoxic, immunotoxic, mutagenic and teratogenic effects (Walker, 2002). With regards to its chemical structure, the OTA molecule represents a phenylalanine-dihydroisocoumarine derivative, which is very stable in processing (Galvis-Sanchez, Barros, & Delgadillo, 2008). The European Union has established official regulations and guidelines for maximum levels of most mycotoxins and OTA in food stuffs such as cereals, coffee, dried vine fruits, wine and grape juices (European Commission, 2006). The maximum level of OTA in dried fruits for direct human consumption is 10 μg kg^{-1}. Furthermore, in a recent EFSA report, the significance of mycotoxins as a potential developing risk, due to climate change, was pointed out (EFSA, 2012).

Ochratoxin A is produced by fungal species mostly of the genus *Aspergillus* (*A. ochraceus, A. carbonarius* and *A. niger*) in warm climates such as southern Europe and by *Penicillium verrucosum* and *Penicillium nordicum* in more temperate climates (Pitt, Basilico, Abarca, & Lopez, 2000; Valero, FarrÈ, Sanchis, Ramos, & Marín, 2006). Black aspergilli are less susceptible than other species to the germicidal UV rays and the strong sunlight heating and thus become the dominant fungi in sun dried vine fruits (Covarelli, Beccari, Marini, & Tosi, 2012; Karbancioglu-Guler & Heperkan, 2008; Rotem, & Aust, 1991).

Abbreviations: OTA, Ochratoxin A; CFU, Colony forming units; CZA, Czapek Dox agar; EFSA, European Food Safety Authority; HPLC, High performance liquid chromatography; kGy, Kilogray; SD, Standard deviation; RSD, Relative standard deviation; TDI, Tolerable Daily Intake

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Several studies, conducted during the past period have shown that occurrence of OTA in many geographical regions is still a persistent problem in a great variety of foods such as coffee, cocoa beans, cereals, snacks, infant food, wines, grapes for wine production, red paprika, spices, dry fruits, beer, bread, flour, rice, pasta, beans, peas, olives and animal feed (reviewed by Nguyen & Ryu, 2014; Covarelli et al., 2012; Report on Carcinogens, 2011; de Magalhães, Sodré, Viscogliosi, & Grenier-Loustalot, 2011, Meletis, Meniades-Meimaroglou, & Markaki, 2007). The OTA occurrence in dried fruits such as dried vine products (sultanas, raisins, currants) has been reported in several studies, since sun dried fruits are more prone to contamination by toxinogenic fungi (Perrone, De Girolamo, Sarigiannis, Haidukowski, & Visconti, 2013; Covarelli et al., 2012).

It is well-known, that OTA contamination constitutes a serious health and economic problem not only in Southern European countries but also in other areas of the world with Mediterranean-type climates. Investigations have shown that dried vine fruit of Mediterranean origin often corresponds to the highest OTA levels (Caballero-Casero, Garcνa-Fonseca., & Rubio, 2012; Covarelli et al., 2012; Trucksess & Scott, 2008; Chulze, Magnoli, & Dalcero, 2006).

Considering the potential public health hazard due to exposure to toxigenic fungi, research is focused in preventing mold growth and mycotoxin production and gamma irradiation appears as an effective tool to decrease fungal counts and improve microbiological safety of foods (Farkas and Mohácsi-Farkas 2011; Refai et al., 1996).

The aim of this study was to evaluate the effect of gamma irradiation at a dose of 10 kGy in the production of ochratoxin A inoculated by *Aspergillus carbonarius* in raisins (*Vitis vinifera* L). In addition the in house development and validation of a method of OTA determination in raisins was applied. It is essential the interpretation of the experimental results to be sound and consequently the conclusions credible. Furthermore the risk assessment based on the OTA presence in raisins before and after irradiation was also estimate.

2. Materials and methods

2.1. Apparatus

An autoclave, Selecta Autester-E Dry (PBI Milano, Italy), an incubator WTB Binder (Tuttlinger, Germany), and a centrifuge Sorvall RC-5B (HS-4) (Norwalk, USA) were used during this study. HPLC was performed on a Hewlett-Packard 1050 Liquid Chromatography (pump and injection system) (Walborn, Germany) with a JASCO FP-920 fluorescence detector (Co. Ltd., Japan) and a HP integrator 3395. The HPLC column was C18 Nova-Pak (60 A, 4 μm, 4.6 × 250 mm) (Waters, Millipore; Milford, MA). The mobile phase for OTA [water+acetonitrile+acetic-acid (60+40+2)] determination, was filtered through Millipore HA VLP (0.45 mm) filters before use. Detection of OTA was carried out at l_{ex} 335 nm and l_{em} 465 nm. The flow rate was 1 ml min^{-1} and the retention time for OTA was 6.9 ± 0.06 min.

2.2. Reagents

Ochratoxin A standard was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The Millipore filters and the C_{18} Nova-Pak HPLC column were from Waters (Millipore; Milford, MA, USA). The Ochratest immunoaffinity columns were obtained from Vicam (Watertown, MA, USA). All reagents used were of analytical grade (Sigma-Aldrich) while HPLC solvents were of HPLC grade and were purchased from Fisher Chemical (Leicestershire, UK). Methanol and Acetic acid (pro analysis) were from Merck (Darmstadt, Germany).

2.3. Preparation of phosphate-buffered saline

Phosphate-buffered saline (PBS) was prepared by dissolving 0.2 g potassium chloride, 0.2 g potassium dihydrogen phosphate, 1.16 g anhydrous disodium hydrogen phosphate and 8.0 g of sodium chloride in 900 ml of distilled water. After adjusting the pH to 7.4 using 0.1 M HCl/0.1 M NaOH, if necessary, the solution was made up to 1000 ml (Daradimos, Markaki, & Koupparis, 2000).

2.4. Media

Czapek Dox agar (CZA) was prepared by dissolving 0.4 g of sodium nitrate (Merck), 0.1 g of potassium chloride (Merck), 0.1 g of magnesium sulfate (Merck), 0.002 g of ferric sulfate (Merck), 0.2 g of dipotassium phosphate (Merck), 6 g of sucrose (Merck), 3 g of agar (Merck), 0.002 g of zinc sulfate (Merck), and 0.001 g of copper sulfate (Merck) in 200 ml of distilled water, final pH 6.0–6.5 (Vergopoulou, Galanopoulou, & Markaki, 2001).

2.5. Preparation of spore inoculum

Aspergillus carbonarius (Bainer) Thom ATHUM 2854, (Culture Collection of Fungi, in the Mycetotheca of the University of Athens) was used throughout this study. The procedure of the inoculum preparation in our laboratory, is previously described in details by Kanapitsas, Batrinou, Aravantinos, and Markaki (2015). An inoculum of 100 conidia was used and raisins in petri dishes from all kinds were incubated for 12 days.

2.6. Experimental design

Samples of damaged raisins were inoculated with 100 conidia of *Aspergillus carbonarius*. In addition, control samples of non-inoculated raisins, were used throughout this study. All kinds of samples were irradiated at dose levels of 0 and 10 kGy. Ochratoxin A production by *A.carbonarius* in inoculated and non-inoculated raisins, irradiated and not irradiated was determined on the 0 and 12th day of observation (Fig. 1).

Moreover, treated samples of raisins contaminated with 100 ng of OTA were irradiated at dose level of 10 kGy. The OTA amount after irradiation was determined immediately the same day (day 0) against a non-irradiated control (Fig. 2).

In this study four samples (petri dishes containing 20 g of raisins) were examined for each case and for each day of observation A total number of forty (40) petri dishes was used for the

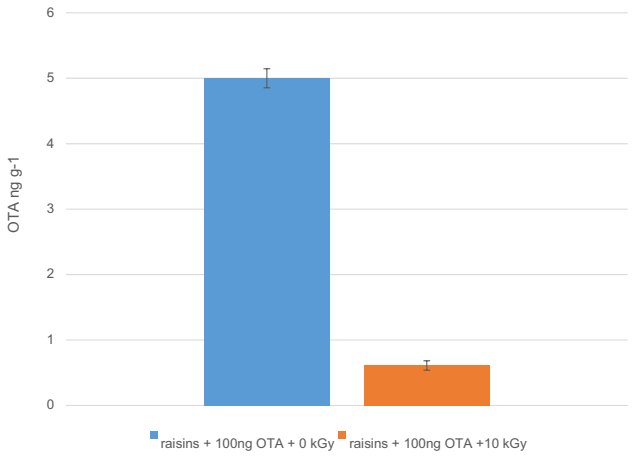

Fig. 1. Effect of γ- irradiation on OTA reduction in raisins originated from Corinth: (a) Spiked with 100 ng of OTA and not irradiated, (b) Spiked with 100 ng of OTA and irradiated with dose level of 10 kGy.