Gamma irradiation effects on ochratoxin A: Degradation, cytotoxicity and application in food

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ABSTRACT

Ochratoxin A (OTA) is one of the main mycotoxins that can be found in food. The use of gamma radiation is a technique for preserving food that may exert some effects on mycotoxins. OTA was irradiated in its dry form, in aqueous and in methanolic solutions, and in wheat flour, grape juice and wine. Additionally, the toxicity of OTA irradiated in water was tested. In aqueous solutions, more than 90% of the OTA was degraded by γ-radiation doses ≥2.5 kGy, and a 2-fold reduction in OTA cytotoxicity was observed. In food matrices, the elimination of OTA by γ-radiation was found more difficult, as radiation doses of 30 kGy eliminate at most 24% of the OTA. Higher moisture content of food matrices did not substantially increase OTA elimination. It is concluded that OTA is very sensitive to irradiation in water solutions but resistant in its dry form and in food matrices.

1. Introduction

Mycotoxins are toxic secondary metabolites of filamentous fungi that are commonly found in foodstuffs. Ochratoxin A (OTA) is the most prevalent and toxic mycotoxin from the ochratoxin family. This mycotoxin is nephrotoxic and is classified in Group 2B by the IARC because it is considered a possible carcinogen in humans (IARC, 2002).

OTA is produced by a large number of *Aspergillus* and *Penicillium* species causing the widespread occurrence of OTA in many food and feedstuffs in several regions of the world (Oliveira, Zannini, & Arendt, 2014). In cool and temperate regions, OTA is primarily produced by *Penicillium* spp. and, in tropical and semitropical regions, it is primarily produced by *Aspergillus* spp. (Ringot, Chango, Schneider, & Larondelle, 2006). Cereals such as wheat, maize, barley and rice are the most important sources of OTA (Miraglia & Brera, 2002), but OTA can also be found in beans, nuts, coffee beans, dried fruits, spices (Ali, Hashim, & Shuib, 2015), beer, and wine (Di Stefano et al., 2015). Additionally, because of its long elimination half-life, OTA can also be present in some of the organs and tissues of livestock (Duarte, Lino, & Pena, 2011). For example, OTA was found in swine blood (Pozzo, Cavallarin, Nucera, Antoniazzi, & Schiavone, 2010), ham (Chiavaro et al., 2002) and traditional Croatian sausages (Markov et al., 2013). Occasionally, OTA is also found in milk (Skaug, 1999) and in dairy products (Dall'Asta et al., 2008). In humans, OTA was found in blood (Karima et al., 2010), urine (Gilbert, Brereton, & MacDonald, 2001) and milk (Galvano et al., 2008).

After oral administration, OTA is quickly absorbed from the gastrointestinal tract and enters the bloodstream, where it binds to serum proteins. OTA is subsequently metabolized in the liver and kidneys into many different products, and it is eliminated predominantly through the faecal and urinary routes (Ringot et al., 2006). Nonetheless, OTA can be reabsorbed into the kidneys and intestines, increasing its systemic redistribution towards the different tissues (Ringot et al., 2006). This characteristic of OTA greatly increases individual exposure to its toxic effects. In general, OTA is considered cytotoxic, nephrotoxic, immunotoxic, myelotoxic, teratogenic, carcinogenic, genotoxic and mutagenic (Pfohl-Leszkowicz & Manderville, 2007).

Because OTA constitutes an important risk for human and animal health, there is a need for strategies to reduce or eliminate OTA from food and feed. Most important approaches currently in use are preventive and include common good agricultural and storage practices employed at the pre-harvest and post-harvest stages (Abrunhosa, Paterson, & Venâncio, 2010; Amezqueta, Gonzalez-Penas, Murillo-Arbizu, & De Cerain, 2009; Hao et al., 2012). Nevertheless, when OTA is still produced several decontamination methods can also be implemented to inactivate or remove this mycotoxin from contaminated materials (Temba et al., 2016). In spite of available strategies, the study, improvement and implementation of innovative solutions, is still

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a need.

Food irradiation is a physical method of preserving foodstuffs, and its contribution to the control of mycotoxins has been addressed by several researchers (Calado, Venancio, & Abrunhosa, 2014; Di Stefano, Pitonzo, Cicero, & D'Oca, 2014; Hooshmand & Klopfenstein, 1995; Kumar, Kunwar, Gautam, & Sharma, 2012). Gamma rays are the preferred source of radiation for food because of their penetrating capability and high effectiveness in inactivating a wide diversity of microorganisms. In the field of mycotoxicology, gamma radiation is known to exert a direct action on mycotoxins by degrading them under certain conditions, and it has an indirect action through the inhibition or delay of fungal development (Calado et al., 2014). However, the mycotoxin-degrading capability of gamma radiation depends on factors such as matrix composition, water content, radiation dose, types of mycotoxin and its concentration, and is a contradictory subject. Concerning OTA, some studies have shown good elimination of this mycotoxin (approx. 50%) from several food and feed commodities (Ben Mustapha, Bousselmi, Jerbi, Ben Bettaïeb, & Fattouch, 2014; Jalili, Jinap, & Noranizan, 2010, 2012; Kumar et al., 2012). However, there are also studies in which the elimination does not exceed 25% (Di Stefano, Pitonzo, & Avellone, 2014; Di Stefano, Pitonzo, Cicero, et al., 2014; Domijan et al., 2015). Also, as far as we know, no studies have been done in liquid food systems. Moreover, the degradation of mycotoxins by gamma radiation may form end products that can still be toxic. Thus, studies involving the irradiation of mycotoxins should be complemented with toxicological assays to evaluate the safety of the radiolytic end products, individually or in total. As far as we know, these evaluations have not been reported for OTA.

In vitro cytotoxicity assays using cells have the advantage of minimizing animal use, allowing for the testing of a wider range of chemicals and concentrations (Eisenbrand et al., 2002). However, the use of cells in toxicity testing requires rapid and sensitive cell viability assays. Viability tests in microwell plates that employ fluorescent dyes have several desirable features. At present, many indicator dyes are commercially available; therefore, there is a large range of cellular parameters that can be monitored (Dayeh, Chow, Schirmer, Lynn, & Bols, 2004). Examples of indicator dyes are Alamar Blue (AB) for changes in energy metabolism, carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) for evaluating membrane integrity, and neutral red (NR) for evaluating lysosomal function (Dayeh, Lynn, & Bols, 2005). OTA causes liver toxicity, and human hepatoma HepG2 cells were reported to retain many of the properties of primary liver cells; this human cell line has been previously used to test OTA toxicity (Li et al., 2014).

The purpose of the present study was (i) to investigate the radiolytic effect of gamma radiation doses on OTA standards in its dried form or in solution, (ii) to evaluate the cytotoxicity of the irradiated mycotoxin in water, (iii) to test the radiolytic effects of gamma radiation on OTA-containing food matrices with different moisture contents such as wheat flour, grape juice and wine.

2. Materials and methods

2.1. Chemicals and reagents

Methanol and acetonitrile were obtained from Merck (Lisbon, Pt). An OTA standard (O1877-5MG) was purchased from Sigma-Aldrich (Sintra, Pt). Ultraglutamine 1 (200 mmol l^{-1}) (l-Gln), foetal bovine serum (FBS), penicillin and streptomycin (P/S) (10,000 U ml^{-1}, 10 mg ml^{-1}), non-essential amino acids (NEAA) 100X, Trypsin-EDTA (200 mg L^{-1} EDTA, 17,000 U trypsin l^{-1}), and cell culture EMEM (Eagle's Minimum Essential Medium) were sourced by Lonza (Barcelona, ES). Phenol red-free Minimum Essential Medium (MEM) was purchased from PAN-Biotech (Aidenbach, DE). Alamar Blue, 5-carboxyfluorescein diacetate and acetoxy methyl ester (CFDA-AM) were purchased from Life Technologies (Madrid, ES). Neutral red (3-amino-7-

dimethylamino-2-methyl phenanzine hydrochloride) solution (0.33%), sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO) and glacial acetic acid were acquired from Sigma-Aldrich (Madrid, ES). Ethanol was purchased from Panreac (Barcelona, ES). High-purity water (> 18 MΩ/cm) was obtained from a Milli-Q Element A10 Century (Millipore Iberia, ES). Wheat flour reference materials that were naturally contaminated with OTA at concentrations of 23.2 ± 3.3 µg kg^{-1} (O-W-823) and 93.7 ± 9.6 µg kg^{-1} (O-W-821) were from Trilogy and were purchased from Ambifood (Porto, Pt). Red wine (alcohol content of 13.5%) and red grape juice were purchased from a local Portuguese store. OTA immunoaffinity columns (OchraTest™ WB) were supplied by Vicam (Nixa, USA).

2.2. Preparation of samples containing OTA standards

A stock solution of OTA at a 1 mg ml^{-1} concentration was prepared by dissolving a commercial standard of OTA in 5 ml of methanol and stored at -20 °C until use. To prepare the OTA samples at 2.5 µmol l^{-1}, the appropriate amounts of stock were pipetted into clean amber 2-ml vials. The samples were then evaporated at 50 °C under a gentle nitrogen stream. To study the effects of irradiation, three different types of mycotoxin samples were prepared to obtain different moisture contents. A set of samples was left dry, another set was resuspended in 1 ml of deionized water (H_2O_{dd}) and the last set was resuspended in 1 ml of water/methanol (50:50, v/v). After being prepared, the samples were kept in amber vials and stored at −20 °C until they could be irradiated. For the cytotoxicity studies, OTA samples at a 200 µmol l^{-1} concentration in H_2O_{dd} were prepared as described above. An increase in the mycotoxin concentration was necessary to detect its cytotoxicity.

2.3. Preparation of food samples containing OTA

The moisture content of wheat flour reference materials was measured using a Radwag Mac 50/1/NH moisture analyser. The flour with 23.2 µg OTA kg^{-1} (Matrix A) contained 10.6% moisture, and the one with 93.7 µg OTA kg^{-1} (Matrix B) contained 11.2% moisture. Falcon tubes containing 2 g of each reference material were prepared in triplicate for non-irradiated control (0 kGy) and irradiated samples (gamma radiation doses of ≈ 2, 10 and 30 kGy) and each moisture content (≈ 11%, 15%, 20% and 35%) to be tested. The moisture of the reference materials was adjusted by adding H_2O_{dd} to the tubes, and the final moisture content of the samples was confirmed by using the moisture analyser mentioned before. Wine and grape juice samples were supplemented with OTA standard at a concentration 20 and 100 µg l^{-1}. For each one, falcon tubes containing 10 ml of sample were prepared in triplicate to be irradiated at the same doses of wheat. After being prepared, the samples were stored at −20 °C until they could be irradiated.

2.4. Irradiation process

The irradiations were performed at room temperature in a Co-60 experimental equipment (Precisa 22, Graviner Manufacturing Company Ltd., UK) with four sources and a total activity of 165 TBq (4.45 kCi), located at Campus Tecnológico e Nuclear (Bobadela, Portugal). The average dose rate was 1.6 kGy h^{-1}, as previously determined by Frick reference dosimeter (E1026-95, 1995). Two routine dosimeters (Amber Perspex dosimeters, Batch X, from Harwell Company, U.K.) were used in each irradiation experiment to estimate the doses absorbed by the samples. The absorbance and thickness of Amber Perspex dosimeters were measured in a UV–VIS Spectrophotometer (UV 1800, Shimadzu, USA) at 603 nm and in a micrometer (Mitutoyo America Corporation, USA), respectively, to estimate the dose according to a previous calibration curve. Samples of OTA (dried, aqueous and methanolic) were irradiated at the absorbed gamma radiation doses of 0.4, 0.9, 1.7, 2.5, 5.4 and 8.6 kGy. To evaluate the cytotoxicity after the irradiation,

aqueous solutions of 200 µmol l^{-1} OTA were exposed to gamma radiation doses of 2.4 and 10.3 kGy. To the samples of wheat flour reference materials, grape juice and wine, gamma radiation doses of 2.4, 10.1 and 30.5 kGy were applied. Non-irradiated controls (0 kGy) were also prepared for each condition and sample type. All experiments were irradiated in triplicate. After irradiation, food samples were stored at −20 °C until analysis.

2.5. Determination of OTA in samples

After irradiation and before the analysis by high-performance liquid chromatography with fluorescence detection (HPLC-FD), the dried OTA samples were resuspended in 1.5 ml of HPLC mobile phase, and aqueous and methanolic OTA samples were diluted with 0.5 ml of the mobile phase. The HPLC mobile phase was a mixture of water/acetonitrile/acetic acid (99:99:2, v/v/v) which was filtered and degassed with a 0.2 µm membrane filter (GHP, Gelman).

The determination of OTA in the wheat flour reference materials was performed according to a method recommended by the immunoaffinity column supplier (Vicam) with few modifications. In brief, 8 ml of acetonitrile/water (60:40, v/v) were added to each falcon tube, the samples were homogenized with an Ultraturrax (T-25, Ika) at 15,000 rpm for 30 s, and the extracts were centrifuged at 7200 RCF for 10 min. Then, 5 ml of the supernatant was diluted with 20 ml of phosphate-buffered saline buffer (PBS), and the solution was filtered using a PES syringe filter (0.45 µm, GVS). Ten ml of filtrate was passed through an OchraTest column at a flow rate of 1–2 drops/s, which was then washed with 10 ml of PBS and 10 ml of H$_2$O$_{dd}$. The mycotoxin elution was performed by passing 2 ml of HPLC grade methanol that was collected in a vial. Finally, the residue was evaporated at 50 °C under a gentle stream of nitrogen and redissolved in 1 ml of mobile phase for HPLC analysis.

The determination of OTA in the grape juice and wine was also performed according to the method recommended by Vicam. Briefly, for matrices containing 20 µg l^{-1}, a sample of 5 ml was diluted with 15 ml of a solution containing PEG (1%) and NaHCO$_3$ (5%); for matrices containing 100 µg l^{-1}, a sample of 1 ml was diluted with 19 ml of a solution of PEG. The dilutions were mixed and filtered through a Whatman 1.5 µm glass microfibre filter. Ten ml were passed through an OchraTest affinity column at a rate of about 1–2 drop/s. The column was washed with 5 ml of washing solution (2.5% NaCl and 0.5% NaHCO$_3$) and 5 ml of distilled water. Finally, the mycotoxin elution was done as described before and sample prepared for HPLC-FD analysis.

The HPLC-FD analysis was performed according to a modified version of the method described in Serra, Mendonca, Abrunhosa, Pietri, and Venâncio (2004). The HPLC system was comprised of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector (λ_{exc} = 333 nm and λ_{em} = 460 nm), a Varian 850-MIB data system interface and a Galaxie chromatography data system. The chromatographic separation was performed using a 21 min isocratic run on a C18 reversed-phase YMC-Pack ODS-AQ analytical column (250 × 4.6 mm I.D., 5 µm) that was fitted with a pre-column of the same stationary phase. The flow rate was set to 0.8 ml min^{-1} and the column temperature was set to 30 °C. The injection volume was 50 µl. The OTA was identified by its retention time (18.5 min) and quantified by comparing the peak areas with its respective calibration curve, which was prepared by dissolving the required amount of working standard solution in the mobile phase. The gain of the fluorescence detector was set to 100 or 1 depending on the OTA's working concentration range (0.012–2.5 µmol l^{-1} or 1.2–250 µmol l^{-1}), and for each case a calibration curve was prepared. The limits of detection (LOD) and quantification (LOQ) were calculated as 3 and 10 times the signal-to-noise ratio, respectively. For the low working concentration range, the LOD and LOQ were 0.004 and 0.012 µmol l^{-1}, respectively. For the high working concentration range, the LOD and LOQ were 0.4 and 1.2 µmol l^{-1}, respectively.

2.6. Fluorescence spectrum of irradiated OTA standards

To evaluate if the aromatic rings of the OTA molecule were degraded by irradiation and to check if any degradation product with an aromatic structure was formed, the fluorescence spectra of irradiated OTA standards were acquired using a Cytation 3 imaging reader (Biotek, USA) as follows. One hundred µL of each irradiated sample was transferred into a black 96-well microplate (Greiner); its emission spectra (λ_{em}) were acquired between 360 and 700 nm (excitation at 333 nm), and its excitation spectrum (λ_{exc}) were acquired between 280 and 400 nm (emission at 460 nm).

2.7. Cytotoxicity studies

The Hep G2 cell line was obtained from American Type Culture Collection (ATTC) (Manassas, VA, USA) and cultured in 75 cm^2 Cell Star Cell Culture flasks (Greiner Bio-One GmbH, Frickenhausen, DE) in EMEM supplemented with 1% NEAA, 10% P/S, 1%[1] L-Gln and 10% FBS (which is referred to as EMEM + in the following text). The flasks were incubated at 37 °C in a humidified 50 g kg^{-1} CO$_2$ atmosphere and split twice a week using PBS/EDTA and trypsin.

A Hep G2 cell suspension (5 × 10^5 cells ml^{-1}) with EMEM + was seeded into transparent, flat-bottom 96-well plates (Greiner Bio-One GmbH, Frickenhausen, DE) by adding 100 µl of cell suspension to each well. The plates were incubated for 24 h and exposed to irradiated and non-irradiated OTA samples for 48 h. The OTA samples were prepared and irradiated as described previously, dried in a speed vac concentrator (Savant SPD 111 V) and dissolved in 1 ml of EMEM + supplemented with 5% of DMSO (to improve the solubility of the residues). Then, a 1:4 dilution was performed with EMEM+, and the resulting solution was applied to a cell culture plate in which 6 successive serial dilutions (with a dilution factor of 2) were performed. As a positive control, a subset of wells was treated with increasing concentrations of SDS (15.6–500 µmol l^{-1}, dilution factor 2/3). Cells that were treated with EMEM + served as a negative control, whereas cells treated with 0.5% (v/v) DMSO/EMEM + were the vehicle control.

The AB, CFDA-AM and NRU assays were performed on the same set of cells. The assays were conducted according to the protocol described by Lammel, Boisseaux, Fernández-Cruz, and Navas (2013). Prior to adding the reactants, the exposure medium was removed and the cells were rinsed twice with 200 µl of PBS. Then, 100 µl of phenol red-free MEM containing 1.25% (v/v) AB and 4 µmol l^{-1} CFDA-AM was added to each well. The 96-well plates were incubated for 30 min in the dark as described above. The fluorescence intensity was subsequently measured at λ_{exc} = 532 nm and λ_{em} = 590 nm for the AB assay, and at λ_{exc} = 485 nm and λ_{em} = 535 nm for CFDA-AM assay using a microplate reader (Tecan Genios, Tecan Group Ltd., Männedorf, CH). After that, the medium was removed and the cells were washed once with PBS. One hundred µL of NR solution (0.03 mg ml^{-1} in phenol red-free MEM) was added per well, and the plates were incubated for 1 h in the dark as described above. After the incubation period, the NR solution was removed, the cells were rinsed twice with 200 µL of PBS, and the NR that was retained in the cells was extracted with 150 µL of an acidified solution composed of ethanol/Milli-Q water/acetic acid (50:49:1, v/v/v). The NR fluorescence was measured at λ_{exc} = 532 nm and λ_{em} = 680 nm. The fluorescence values were corrected for the cell-free control and normalized against the vehicle control. The fluorescence spectra of OTA samples (irradiated or not) did not show any significant fluorescence at the excitation/emission wavelengths that were used in the AB (532/590 nm), CFDA-AM (485/535 nm) and NRU (532/580 nm) assays.

2.8. Statistical analysis

A statistical analysis was performed using SPSS Statistics for Windows (Version 22.0, Armonk, NY: IBM Corp.). To evaluate the
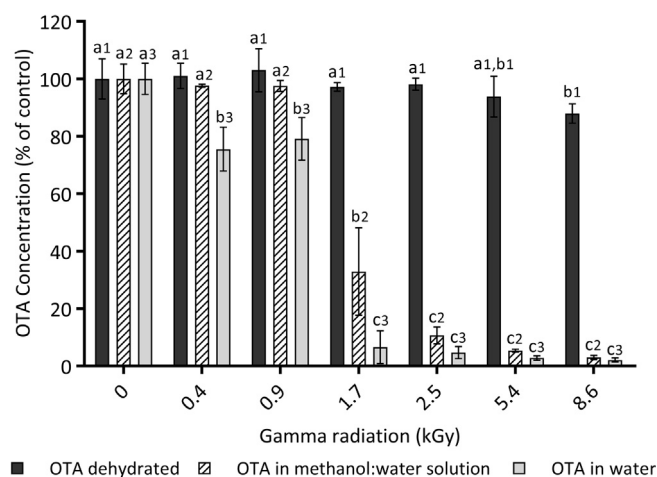
Fig. 1. Effects of incremental gamma radiation doses on the OTA concentration (2.5 μmol L^{-1}) under different conditions. Statistically significant differences for each sample of OTA (dried, aqueous and methanolic) are indicated with different letters, by analysis of variance (two-way ANOVA, Duncan's posthoc test, $P < 0.05$).

significant differences between the OTA concentrations in non-irradiated and irradiated samples, the means were compared by using a two-way ANOVA analysis of variance followed by Duncan's posthoc test for standard samples and Dunnett's test for food matrices. The results of the cytotoxicity assays represent the means and standard errors (SEM) of at least three independent experiments, in which each treatment was applied in triplicate. Significant differences among treatments were determined by one-way repeated measures analysis of variance (rmANOVA, p < 0.05). A posthoc Dunnett's test was used to compare the different treatments with respect to the control group. All the data were tested beforehand for normality (Shapiro-Wilk's test, p < 0.05) and equal variance (p < 0.05).

3. Results and discussion

3.1. Effect of irradiation on OTA standards

The radiolytic effects of gamma radiation doses on samples containing 2.5 μmol L^{-1} of OTA are presented in Fig. 1. There a dose-dependent reduction of the mycotoxin, which was more pronounced in samples of OTA dissolved in water, can be observed. In this case, the lowest applied radiation dose (0.4 kGy) was sufficient for achieving a 25% reduction in the OTA concentration, and the highest dose tested (8.6 kGy) to achieve a 98% reduction. However, the irradiation of dried OTA was less effective because no significant reductions were observed for doses between 0.4 and 5.4 kGy. In its dried form, the maximum radiation dose tested (8.6 kGy) only eliminated 12% of the OTA. In the assays that were performed in water/methanol solutions, significant reductions in OTA were achieved only with radiation doses higher than 1.7 kGy, but the OTA reduction at 8.6 kGy was almost the same (97%) as it was in water (98%).

These results show that the presence of water during the irradiation process increases OTA elimination and is in accordance with the results of Kumar et al. (2012). The higher effect of gamma radiation in the presence of water can be explained by water radiolysis. During this process, the ionization of water causes the splitting of water molecules into positively charged water radicals (H$_2$O$^+$) and negative free solvated electrons (e$^-$), which lead to the formation of the reactive species e−aq, H$^\cdot$, HO$^\cdot$, HO$_2$$^\cdot$, OH$^-$, H$_3O^+$, H$_2$, and H$_2O_2$ after several recombination and cross-combination reactions (Le Caër, 2011). These chemical species are reactive against double bonds, especially the ones found in aromatic or heterocyclic rings, and they initiate the hydrolysis of these compounds (Jalili et al., 2012).

Although the water content has a very important role in the

irradiation efficacy, the irradiation process can also be affected by other factors. The radiation dose, the type of mycotoxin, the mycotoxin concentration, and the existence of other compounds or matrix components are factors that also influence the success of irradiation (Calado et al., 2014). For example, from the experiments that were conducted with 2.5 μmol L^{-1} and 200 μmol L^{-1} of OTA in water, it can be deduced that higher concentrations of mycotoxin require higher doses of irradiation to achieve satisfactory levels of elimination. In 2.5 μmol L^{-1} samples, OTA eliminations of 93% and 98% were achieved at doses of 1.7 kGy and 8.6 kGy, respectively; while for 200 μmol L^{-1} samples, radiation doses of 2.4 kGy and 10.3 kGy only led to reductions of 81% and 80%, respectively (data not shown).

Another important difference that was observed between those two types of samples was the existence of detectable radiolytic products in irradiated 200 μmol L^{-1} samples (Fig. 2) that were not detected in the irradiated 2.5 μmol L^{-1} samples nor in the non-irradiated ones. These radiolytic products were found to be more abundant in samples that were irradiated with low doses (Fig. 2B) than in samples irradiated with higher ones (Fig. 2C), indicating that the resulting products are themselves degraded by higher doses of radiation. These unidentified degradation products are present in small quantities but can eventually still be toxic and constitute an additional food safety risk if low doses of radiation are used for the treatment of food products.

To seek out additional radiolytic compounds that could not be detected by HPLC-FD, fluorescence spectra of the samples were obtained. The spectra of the 2.5 μmol L^{-1} samples are presented in Fig. S1 and those of the 200 μmol L^{-1} samples are found in Fig. S2. These figures show that the excitation and emission spectra before and after the irradiation process are quite similar. Both spectra exhibit one single maximum (333 nm for excitation and 460 nm for emission) and no peak shift. A strong reduction in the fluorescence intensity that is dependent on the radiation dose is also evident in irradiated samples that contain water. These results strengthened the hypothesis that the aromatic rings of OTA are degraded by irradiation and show that no relevant compounds that still contain fluorescent aromatic rings were obtained after the process.

3.2. Cytotoxicity of irradiated OTA standards

There are several studies that claim that gamma radiation is a promising method of reducing the mycotoxin contents of food and feed. Nonetheless, the decrease in mycotoxin toxicity after irradiation has been poorly studied. Therefore, cytotoxicity studies were conducted with irradiated and non-irradiated aqueous samples to evaluate if the degradation of OTA by irradiation corresponded to the elimination of its toxicity.

The cytotoxic effect of OTA standards on Hep G2 cells after 48 h of incubation was measured through CFDA-AM, AB and NRU and is shown in Fig. 3. The cytotoxicity assays were performed with serial half-dilutions of irradiated OTA samples dissolved in cell culture medium.

The CFDA-AM assay is an indirect measure of cell integrity. It is based on the conversion of CFDA-AM to its fluorescent product 5-carboxyfluorescein (5-CF) by cytosolic esterase, which is only retained in cells with an intact plasma membrane (Dayeh et al., 2005). With this assay, a non-significant decrease in cell viability was observed after OTA exposure (Fig. 3A – black bars). Similarly, no significant differences in cell viability were observed with solutions of irradiated OTA in which a degradation of approx. 80% was measured. These results suggest that the plasma membrane of HepG2 cells was unaffected by OTA or irradiated OTA, and it suggested that this assay discriminates OTA toxicity poorly.

Alamar Blue is a commercial preparation of the dye resazurin, which is reduced to a fluorescent form by viable cells. Its diminished reduction indicates an impairment in cellular metabolism (Dayeh et al., 2005). Exposing HepG2 cells to non-irradiated OTA for 48 h resulted in a significant decrease in the fluorescence intensity, indicating a
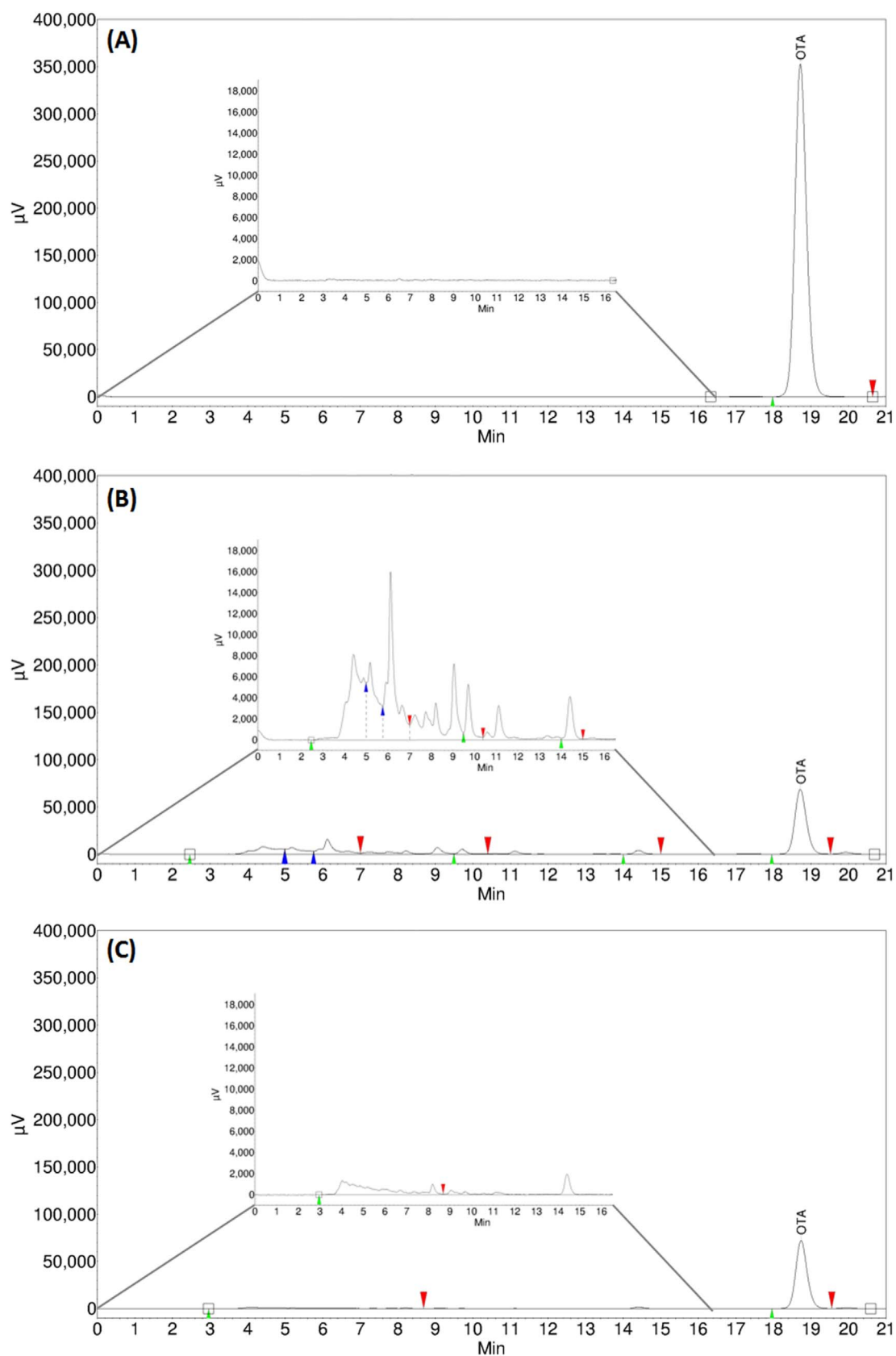
Fig. 2. HPLC-FD chromatograms corresponding to OTA (200 μmol L^{-1}) in water (A) without gamma radiation, (B) after 2.4 kGy and (C) after 10.3 kGy gamma radiation treatments.

(A) OTA in water- CFDA-AM

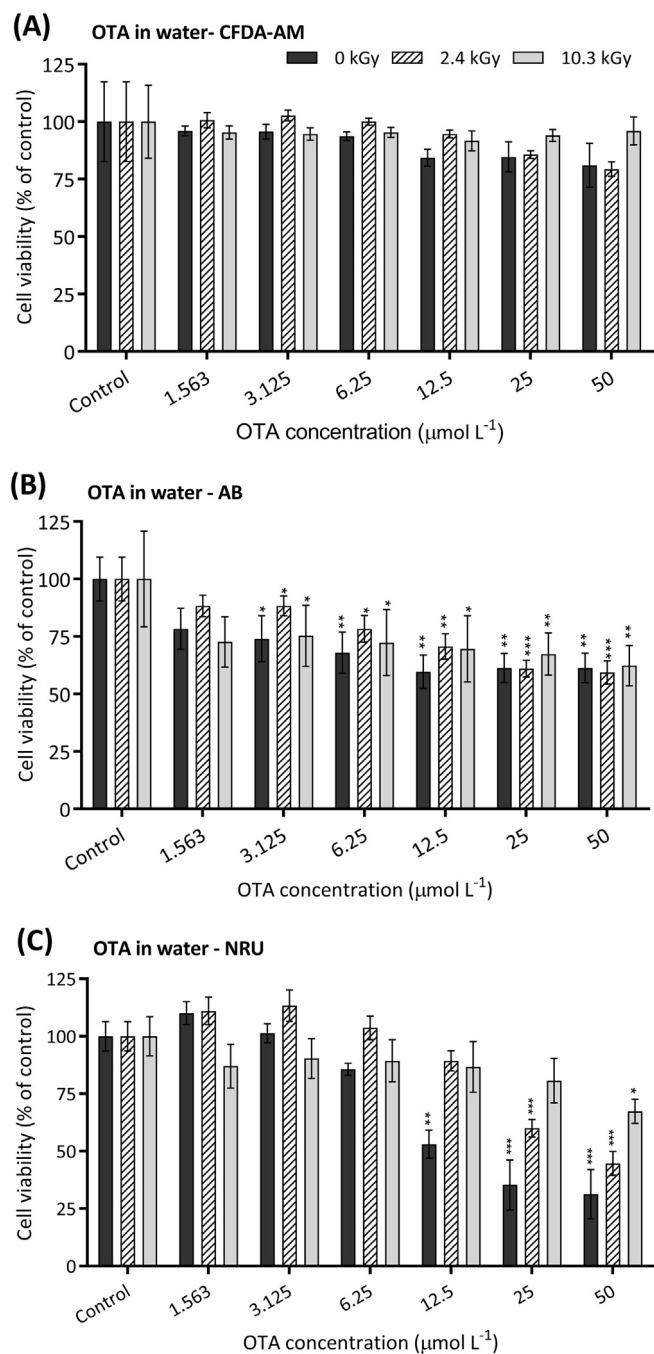**(B)** OTA in water - AB

(C) OTA in water - NRU

Fig. 3. Effects of OTA samples on Hep G2 cell viability as assessed by means of (A) CFDA-AM, (B) Alamar Blue (AB) and (C) Neutral Red uptake (NRU) assays. The bars represent the means and standard errors of the mean (SEM) of at least three independent repetitions. Statistically significant differences with respect to the vehicle control are indicated as follows: $^* P < 0.05$; $^{**} P < 0.01$; $^{***} P < 0.001$ (one-way rmANOVA, Dunnett's Post-hoc test).

reduction in cell metabolic activity (Fig. 3B – black bars). However, the same trend in cytotoxicity was observed with irradiated samples. This finding may be related to the residual content of OTA that was present in the samples. This test also showed poor efficiency for discriminating OTA toxicity between irradiation doses.

Neutral red (NR) is used to evaluate lysosomal function because this assay is based on the accumulation of NR in functional lysosomes. The OTA cytotoxicity in HepG2 cells according to the NRU assay is presented in Fig. 3C (black bars). Exposing HepG2 cells to non-irradiated OTA for 48 h resulted in a significant decrease in the fluorescence

intensity and therefore reduced cell viability, indicating clearly that OTA interferes with pinocytosis and cellular lysosomal activity. On the contrary, the irradiated OTA solutions showed a cytotoxicity that was significantly lower than that of the controls and from non-irradiated OTA. Differences were also evident between radiation doses. The cell viability was lower in samples that were irradiated with 2.4 kGy than in samples irradiated with 10.3 kGy, showing that the decrease of OTA toxicity was dependent on the radiation dose. However, an identical cytotoxicity would be expected because the amount of OTA eliminated in both of these samples was quite similar (80 and 81%, respectively). The unexpected higher toxicity of 2.4 kGy samples could result from the presence of OTA radiolytic products (unidentified peaks that were detected in Fig. 2B), which may themselves be toxic. Additionally, the full elimination of OTA toxicity was not observed after irradiation, even at a gamma radiation dose of 10.3 kGy, but a complete degradation of OTA was also not achieved after this irradiation treatment.

The use of different cytotoxicity assays should provide complementary information regarding the mechanism of toxicity (Simarro-Doorten, Bull, van der Doelen, & Fink-Gremmels, 2004). In comparing the three cytotoxicity assays used here, it was evident that the NRU is the most sensitive test for evaluating OTA cytotoxicity in HepG2 cells. The least sensitive test was the CFDA-AM. These results are in accordance with the ones obtained by Simarro-Doorten et al. (2004), which verified that NRU is a more sensitive test than AB for measuring OTA cytotoxicity.

To the best of our knowledge, there is only one study that addresses the cytotoxicity of irradiated OTA. Kumar et al. (2012) verified a similar toxicity trend in intestinal epithelial cells with MTT assays. These authors verified that cells that were treated with irradiated OTA at 10 kGy had a 7-fold increase in cell viability when compared with cells treated with non-irradiated OTA. In our results, approx. a 2-fold increase in cell viability was verified with the NRU assay for the same dose. These differences could be related to the initial concentration of OTA that was used. Kumar et al. (2012) irradiated samples containing 6 $\mu mol\,L^{-1}$ to 25 $\mu mol\,L^{-1}$ of OTA and, in this study, samples containing 200 $\mu mol\,L^{-1}$ of OTA were irradiated. So, it may be expected that more residual OTA and OTA radiolytic products were present in our samples.

3.3. Irradiation of food samples containing OTA

As confirmed in the first part of this study, water plays an important role in the efficiency of OTA radiolysis and on the subsequent reduction of its toxic effects. Therefore, it was hypothesized that water radiolysis may also promote the elimination of OTA and of its toxicity in foods if enough moisture is provided. To test the effect of radiation on food matrices, assays with wheat flour, grape juice and wine containing OTA were performed. Two reference wheat flour materials with known certified initial concentrations of OTA (23.2 ± 3.3 $\mu g\,kg^{-1}$ and 93.7 ± 9.6 $\mu g\,kg^{-1}$) were tested, as were four different moisture levels (approx. 11%, 13%, 18% and 32%) for each reference material. Grape juice and wine with two different initial concentrations of OTA (approx. 20 $\mu g\,kg^{-1}$ and 100 $\mu g\,kg^{-1}$) were also tested because liquid food matrices may occasionally contain OTA.

The effects of gamma radiation on OTA-contaminated flour at different moisture levels are presented in Fig. 4. For samples containing around 23 $\mu g\,kg^{-1}$ of OTA (Fig. 4A), no statistically significant reductions of the mycotoxin were observed for all the experimental conditions tested. However, significant reductions were observed for samples containing around 93 $\mu g\,kg^{-1}$ of OTA (Fig. 4B). In this case, the efficiency of irradiation increased with the increase of wheat flour moisture and radiation dose, a maximum elimination of 24% with 30.5 kGy of radiation and 32% of moisture being achieved. Jalili et al. (2010) also observed more elimination of OTA and aflatoxins in food samples containing higher concentrations of mycotoxin than in samples containing lower amounts. This trend most likely results from the
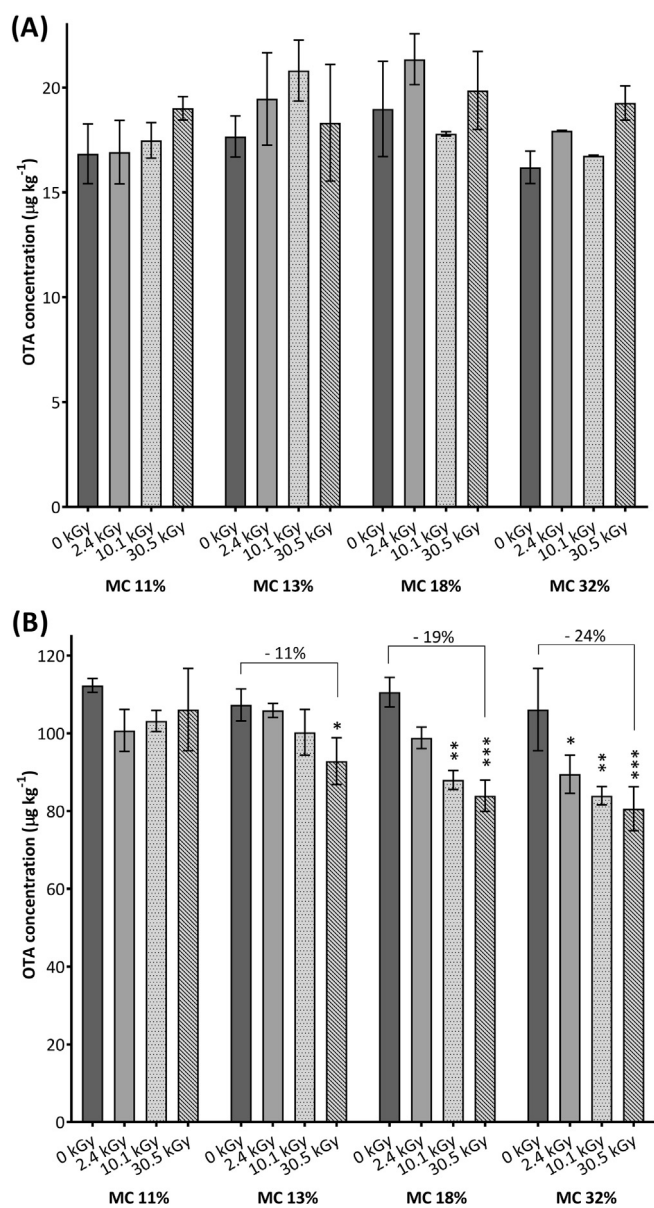
Fig. 4. Effects of gamma radiation on OTA in naturally contaminated wheat flour with different moisture contents and initial OTA concentrations of (A) $23.2 \pm 3.3\ \mu g\ kg^{-1}$ and (B) $93.7 \pm 9.6\ \mu g\ kg^{-1}$. Statistically significant differences with respect to non-irradiated control are indicated as follows: $^{*}\ P < 0.05$; $^{**}\ P < 0.01$; $^{***}\ P < 0.001$ (two-way ANOVA, Dunnett's posthoc test).

Fig. 5. Effects of gamma radiation on OTA in grape juice and wine containing initial OTA concentrations of (A) $20\ \mu g\ L^{-1}$ and (B) $100\ \mu g\ L^{-1}$. Statistically significant differences with respect to non-irradiated control are indicated as follows: $^{*}\ P < 0.05$; $^{**}\ P < 0.01$; $^{***}\ P < 0.001$ (two-way ANOVA, Dunnett's posthoc test).

protective effect of food principal components. For the same amount of matrix, less mycotoxin will certainly be more protected from radiation effects than more mycotoxin. Even so, the elimination of OTA observed in wheat flour was drastically inferior to the one observed in pure water, suggesting that a large amount of water would be necessary to totally eliminate the mycotoxin from flour.

To test this hypothesis we decided to irradiate two liquid food samples that may occasionally contain OTA (grape juice and wine). Thus, it was possible to test whether the presence of higher water contents in the food matrix (between 80 and 90%) would be sufficient to substantially improve the elimination of OTA by radiation. The results obtained for grape juice and wine are presented in Fig. 5. Surprisingly, it was found that the elimination of OTA in these matrices was also low, between 11 and 23%, depending on the matrix and radiation dose tested. These findings clearly show that irradiation has a limited effect on OTA in real food samples, even if they are liquid matrices with water contents higher than 80%. The presence of
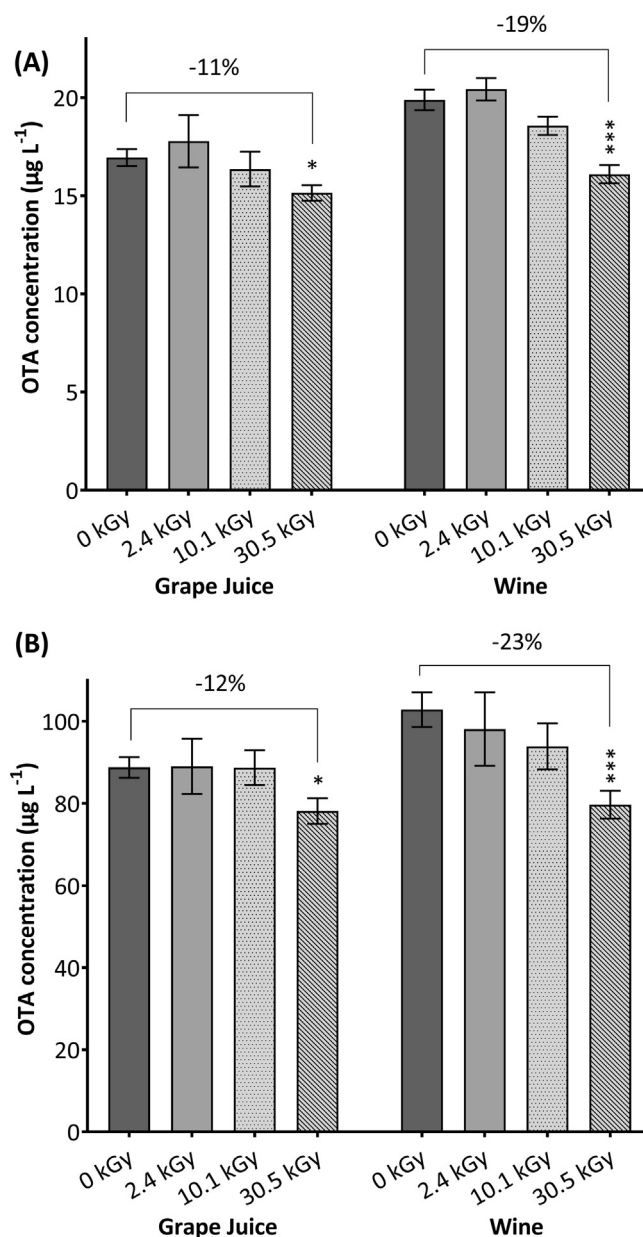
scavengers in food matrices that may react with free radicals from water radiolysis are probably protecting the mycotoxin, reducing the radiation damage. These compounds, which are present in greater amounts than OTA, may also be absorbing the radiation energy, reducing the amount of primary reactive radicals generated from water radiolysis. For example, in grape juice and wine, a reduction of their red colour with increasing doses of radiation (Fig. S3) was clearly observed, suggesting that radiation had a destructive effect on anthocyanins and other flavonoids that are usually the main source of red colour in grape-derived products. The degradation of other flavonoids (kaempferol and quercetin) by gamma radiolysis has been demonstrated (Marfak et al., 2002; Marfak et al., 2003). The protective effect of food matrix components may also explain why the elimination of OTA was lower in grape juice (up to 12%) than in wine (up to 23%). The higher content of dissolved solids (°Brix) and total solids in grape juice compared to wine may have better protected OTA from the effects of radiation.

In summary, from our data it can be concluded that gamma radiation can effectively eliminate OTA in water solutions using a dose as low as 1.7 kGy. However, its effect on real food samples is quite moderate even in food matrices that contain a high moisture content, not exceeding 24%. Additionally, a dose of radiation 3 times higher than the maximum dose recommended for most of the food products (10 kGy) was necessary to achieve those levels of reduction. The results obtained contradict data from Mehrez and co-authors (2016), which reported OTA reductions of 35.5% and 47.2% with an irradiation dose of 8 kGy in wheat grains containing 14% and 16% of moisture, respectively. In this case, the authors irradiated full grains of wheat that were spiked with OTA. So, it is likely that the uneven distribution of OTA in wheat grains may have affected the subsequent quantifications of the mycotoxin. In the current study, a certified wheat flour reference material naturally contaminated with OTA was used. Wheat flour is an extremely uniform matrix and the homogeneous distribution of OTA is certified by the producing laboratory. Even so, we observed some variability in OTA determinations as can be observed in Fig. 4. Therefore, in this type of study, the matrix homogeneity and the uniform distribution of the mycotoxin in the matrix are considered to be of extreme importance. The correct determination of absorbed doses is also important.

Nonetheless, our data is in agreement with Di Stefano, Pitonzo, and Avellone (2014), Di Stefano, Pitonzo, Cicero, et al. (2014), who conducted irradiation studies with OTA in poultry feed and obtained reductions of only 23.9% with a dose of 15 kGy; and with Domijan et al. (2015), who verified an OTA reduction of only 22.5% with a 10 kGy dose on different dry-cured meat products. Furthermore, our results are in accordance with the observation of Hooshmand and Klopfenstein (1995) for aflatoxin B1 in ground feed grains (wheat, maize and soybeans), since they did not observe significant reduction in this mycotoxin with radiation doses up to 20 kGy. In these studies, the samples were blended to obtain homogeneous matrices before the irradiation process.

4. Conclusions

In conclusion, gamma irradiation was shown to eliminate significant amounts of OTA when this mycotoxin is dissolved in water but its elimination resulted only in a 2-fold decrease of sample toxicity. It was also shown that dried OTA is extremely resistant to radiation doses of up to 8.6 kGy. In food matrices, OTA was also found more radioresistant than in pure water. In spite of increasing the moisture content of wheat flour, the maximum elimination achieved was 24% with a radiation dose of 30 kGy. In liquid matrices such as grape juice and wine, no further improvements in OTA elimination were achieved. Therefore, it can be concluded that gamma radiation is not a feasible technology for the detoxification of OTA in foods.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.07.136.

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