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Fungal Mycotoxins Reduction by Gamma Irradiation in Black and White Pepper

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Abstract

Gamma ray was applied to reduce mycotoxins, i.e. Ochratoxin A (OTA) and aflatoxins B1, B2, G1 and G2 (AFB1, AFB2, AFG1 and AFG2) in black and white pepper. The aim of this study was to evaluate the efficacy of gamma radiation for decontaminating ochratoxin) and aflatoxins residues in artificially contaminated black and white pepper samples. The moisture content of the pepper samples was set at 10% or 16%, and the applied gamma dose ranged from 5 to 20 kGy. Mycotoxin levels were determined by High-Performance Liquid Chromatography (HPLC) after Immune Affinity Column (IAC) chromatography. Both the gamma irradiation dose and moisture content showed significant effects ($p<0.05$) on mycotoxin reduction. The maximum toxin reductions, found at16%moisture content and 20 kGy, were 57.2%, 53.4%, 40.4%, 48.6% and 44.2% for OTA, AFB1, AFB2, AFG1and AFG2, respectively.

Keywords: Black and white pepper; Mycotoxins; Gamma ray

Introduction

Mycotoxins are naturally-occurring toxins produced by certain fungi that can grow on foods such as cereals, nuts, dried fruits, spices and legumes under certain environmental conditions. The most commonly observed mycotoxins include the aflatoxin (B_1, B_2, G_1 & G_2) and ochratoxin A. Aflatoxins have been shown to cause cancer of the liver in laboratory animals and to directly damage DNA. They are also considered to cause liver cancer in humans, particularly in a number of developing countries, where high levels of aflatoxins are found in some staple foods [20].

For spices there are two groups of mycotoxins of concern, Aflatoxins (AFs) and Ochratoxin A (OTA). Aflatoxins are the most toxic group of mycotoxins that are produced by some Aspergillus species (Aspergillus flavus, Aspergillus parasiticus and the rare Aspergillus nomius). Several types of AFs are produced in nature belonging to a group called the difuranocoumarins, but only four, Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1) and Aflatoxin G_2 (AFG_2) are naturally found in foodstuffs. AFB_1 is the most potent genotoxic and carcinogenic AFs and amongst the most commonly found in agricultural products [18]. AFB_1 and naturally occurring mixtures of AFs have been classified by the International Agency for Research on Cancer as Group I (carcinogenic to humans) [19], with a role in aetiology of liver cancer, notably among subjects who are carries of hepatitis B virus surface antigens [14].

Ochratoxin A is a kidney toxin, produced mainly by Penicillium verrucosum in temperate climates and Aspergillus ochraceus and the rare Aspergillus carbonarius inwarm and tropical countries that can contaminate agricultural products prior to harvest or more commonly during storage. This compound has been shown to have nephrotoxic effects on all mammalian species and has been associated with fatal human kidney disease, referred to as Balkan Endemic Nephropathy and with an increased incidence of tumours of the upper urinary effect (EFSA, 2006). The IARC has classified OTA as a probable human carcinogen (Group 2B) based on sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans [14].

Due to the high toxicity of mycotoxins, many methods have been used to reduce or eliminate them from different foods. These methods can be classified as: (1) biological method by applying nontoxigenic strains of *Aspergillus flavus* or other nontoxigenic moulds [7]; (2) chemical method such as treatment with ammonia, sodium bisulfite, calcium hydroxide, formaldehyde and other common chemicals [5,21,23,24]; (3) physical method such as heating, extraction, adsorbing by adsorption agent [27] and radiation. The reduction using chemical method usually produces undesirable toxic residues and causes changes in nutritional, sensory and functional properties of food [22]. By far, physical method has been the most effective method for the reduction of mycotoxins from contaminated commodities. However, this technique is limited due to high cost of equipment and it needs additional management for the disposal of the toxic extracts.

Despite much public debate on the safety of irradiated foods, WHO, in September 1997, concluded that foods treated with doses greater than 10 kGy can be considered safe and nutritionally adequate when produced under established Good Manufacturing Practice [4].

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Spice irradiation is used to achieve some beneficial effects include disinfestations, improvement of shelf life and safety of spices [32]. There are a number of contrasting reports. Some researchers found that gamma ray treatment was effective in reducing mycotoxin concentration in different foods. [12,28,30,34]; however, some found it was not effective [10,11,13,26].

The present study was investigated the effect of gamma irradiation dosages and moisture content on the reduction of OTA, AFB_1, AFB_2, AFG_1 and AFG_2 in black and white pepper.

Materials and Methods

Sample Preparation and Irradiation

To investigate the effect of gamma doses and moisture content on mycotoxin reduction, samples of black and white pepper were spiked with AFB_1, AFG_1 and OTA at 60ng/g and with AFB_2 and AFG_2 at 18ng/g based on dry weight of pepper. The moisture content of pepper (8% –10%) was adjusted to 10% or 16% by the direct addition of a weighed amount of sterilized water. The moisture content in each sample was confirmed by the standard method of the AOAC (1990). Following thorough mixing, each sample was divided into 25g sub-samples, each of which was placed in a transparent plastic bag. The samples were irradiated at 5, 10, 15 or 20 kGy at 28±2C in a gamma chamber, which has ^{60}Co as the radiation source (Atomic Energy Authority, Cairo, Egypt). The experiments were conducted twice and triplicate samples were irradiated with the same dose each time.

Reagents and Materials

Methanol and acetonitrile used for sample preparation and mobile phase were HPLC-grade. Sodium chloride, potassium chloride, sodium hydroxide, Monobasic potassium phosphate and sodium phosphate dibasic were obtained from Sigmae Aldrich, while acetic acid, nitric acid and potassium bromide were from Merck (Darmstadt, Germany). In all analytical steps, ultrapure water produced by Direct-Q3 water purification system was used (Millipore). The immune affinity columns (Afla Test® and Ochra Test™) from Vicam (Water-worn, MA, USA). GF/A glass microfiber filter (125 mm) were from Whatman International (Kent, UK).

Standards

The mixed standards of AFB_1, AFB_2, AFG_1 and AFG_2 were provided from Supelco® (Bellefonte, PA, USA). The mixture in each ampoule consists of 1µg AFB_1, 0.3µg AFB_2,1 µg AFG_1 and 0.3 µg AFG_2 in 1 ml of methanol. A series of working standards (1-20 ng ml^{-1} for AFB_1 and AFG_1, and 0.3-6ng ml^{-1} for AFB_2 and AFG_2) were prepared freshly in LC mobile phase consisting of Water-acetonitrile-methanol (6/2/3, v/v/v).

The OTA standard was purchased from Sigma-Aldrich as a crystalline powder form. A series of working standards from 1 to 30 ng OTA ml^{-1} was prepared in LC mobile phase consisting of acetonitrile-water-acetic acid (47/51/2, v/v/v). The working standards of AFs and OTA were renewed every 2 weeks. They were used to calibrate the LC detector response and recovery studies.

AFs Analysis

Sample Extraction and IAC Clean-up

The samples were extracted and cleaned-up according to the AOAC Official method [31], with slight modifications. Fifty grams of spice samples were extracted with 300 ml methanol-water (8/2, v/v) and 5g NaCl using a Waring Blender (Waring Products Co., Connecticut, USA) at high speed for 1 min. The sample extract was filtered through Whatman no. 4 filter paper. Then, a 10 ml aliquot of filtrate was diluted with 60 ml Phosphate-Buffered Saline (PBS) and filtered once more through a glass microfiber filter and passed through an AflaTest® IAC attached on to a vacuum manifold (Agilent Technologies, Santa Clara, CA, USA). The column was washed two times with 10 ml of ultrapure water and dried with air. Subsequently, the AFs bound to the specific antibody were eluted by passing twice 0.5 ml of methanol through the IAC at a flow rate of 1-2 drops/s and collected in HPLC vials. The elute was evaporated until dryness at 45°C under N_2 stream and the residue was reconstituted in 1 ml of water-acetonitrile-methanol (6/2/3, v/v/v). The samples were stored at 4°C until analysis by HPLC-FD after post-column derivatisation.

OTA Analysis

Sample Extraction and IAC Clean-up

The samples were extracted according to Ahn *et al*. (2010), with some modifications. In detail, a portion of 25 g of spice samples was extracted with 200 ml of acetonitril-ewater solution (6/4, v/v), using high-speed blending for 1 min and then the extract was filtered through filter paper. A 5 ml aliquot of filtrate was mixed with 50 ml PBS solution and again filtered through a glass microfiber filter. The clean-up of samples was carried out with OchraTest™ IAC attached onto a vacuum manifold. After the final filtrate had passed through the IAC, the column was washed twice with 10 ml of ultrapure water and air was forced through the column. OTA was eluted from the column with 1 ml (2×0.5 ml) of methanol. The elute was evaporated until dryness at 45°C under N_2 stream and the residue was reconstituted in 1 ml of acetonitrile-water-acetic acid (47/51/2, v/v/v) for HPLC-FD analysis.

HPLC-FD Analysis

The HPLC apparatus was performed using a Shimadzu (Tokyo, Japan) liquid chromatographic system coupled to a fluorescence detector (Shimadzu RF-20AXL) equipped with an LC-20AD pump, a SIL-20AHT auto sampler, a DGU- 20A3 online degasser and a CBM-20Alite system controller. Shimadzu LC solution software was used for data acquisition and processing. Chromatographic separations were achieved using a reversed-phase InertsilODS-3column (5 µm, 250 × 4.6 mm i.d.) supplied by GL Sciences Inc, Tokyo, Japan. The column temperature was maintained at 35°C.

The mobile phase consisted of the mixed solution of water-acetonitrile-methanol (6/2/3, v/v/v) containing 0.12 g l^{-1} potassium bromide and 350 µl l^{-1} nitric acid (4 M) and was isocratically delivered at 1 ml min^{-1}. The injection volume was 100 µl. A post-column derivatisation with electrochemically generated bromine in Cobra cell (Coring System Diagnostics GmbH, Gernsheim, Germany) using a reaction tube of 340× 0.25 mm i.d. PTFE was used to enhance the fluorescence intensity of AFB_1 and AFG_1. Detection of AFs was carried out by fluorescence with excitation and emission wavelengths of 360 and 440 nm, respectively. The OTA content was quantified using HPLC with fluorescence detection system described for AFs. However, the column temperature was set at 45°C. An isocratic mobile phase of acetonitrile- water-acetic acid (47/51/2, v/v/v) was used with

a flow rate of 1 ml min^{-1}. Detection of OTA was carried out using excitation and emission wavelength of 333 and 460 nm, respectively.

Statistical Analysis

Analysis of Variances (ANOVA) was used to investigate the significant effects of moisture content and gamma dose on the reduction of mycotoxins in black and white pepper. Significant differences ($p<0.05$) of means were calculated using Duncan's multiple range tests. Data analysis and optimization procedures were performed using the Minitab v.14 statistical package.

Results and Discussion

The data presented in Tables (1) and (2) show the effect of gamma ray doses (0, 5, 10, 15, 20 kGy) at two different levels of moisture content (10,16%) on the percent reduction of AFB_1, AFB_2, AFG_1, AFG_2 and OTA in black and white pepper. Statistical analysis showed that there was no significant difference between the reductions of the evaluated mycotoxins in black and white pepper after irradiation. However, the results obtained from two-way ANOVA showed that the effects of the independent variables, gamma dose and moisture content, were significant ($p<0.05$). Irradiation of high moisture peppers did not cause any visual changes in product quality and the appearance of both samples (10% and 16% moisture content) was the same.

Table 1: Effect of gamma irradiation on aflatoxins and ochratoxin A (ng/g) in black and white pepper at 10% moisture.

Mycotoxin	Reduction % (Mean ± SD[a])				
	control	5 kGy	10 kGy	15 kGy	20 kGy
Black pepper					
OTA[b]	2.5±2.6	4.8±4.3	2.4±2.3	36.4±3.7	44.8±3.7
AFB[c]	2.5±2.5	3.8±1.5	2.6±1.3	28.1±2.5	35.3±4.1
AFB[d]	8.7±1.3	6.0±0.9	5.4±2.0	18.2±2.9	24.5±1.6
AFG_1[e]	6.8±2.8	5.7±2.0	5.1±2.8	22.2±2.4	40.6±2.9
AFG_2[f]	6.2±2.6	6.3±2.8	4.7±1.3	19.0±2.5	26.9±5.0
White pepper					
OTA	8.1±0.7	4.2±2.8	1.4±2.3	35.4±3.3	46.6±1.8
AFB_1	5.6±4.1	6.5±4.1	7.2±4.8	28.4±2.1	39.6±1.7
AFB_2	4.4±3.8	6.9±1.3	7.8±2.6	16.8±3.5	28.8±2.8
AFG_1	9.2±2.4	8.6±3.7	6.2±1.9	22.1±4.8	39.0±4.0
AFG_2	6.4±2.0	6.2±1.7	7.3±3.9	18.6±3.8	27.0±2.4

[a]Standard deviation. [b]ochratoxin A. [c]aflatoxin B1. [d]aflatoxin B2. [e]aflatoxin G1. [f]aflatoxin G2.

Table 2: Effect of gamma irradiation on aflatoxins and ochratoxin A (ng/g) in black and white pepper at 16% moisture.

Mycotoxin	Reduction % (Mean ± SD[a])				
	control	5 kGy	10 kGy	15 kGy	20 kGy
Black pepper					
OTA[b]	2.5±2.6	5.4±3.9	6.2±1.9	35.4±1.3	56.1±3.2
AFB[c]	2.4±2.5	4.8±1.8	2.8±1.6	36.3±4.5	47.2±1.8
AFB[d]	7.6±1.3	10.8±1.3	6.8±1.3	25.2±3.3	40.4±2.0
AFG_1[e]	6.6±2.8	10.2±3.1	6.2±2.2	38.9±1.5	47.4±2.8
AFG_2[f]	6.4±2.5	8.5±3.7	4.2±1.5	28.4±2.4	42.4±2.7
White pepper					
OTA	2.4±2.8	3.0±1.4	2.9±2.3	37.4±2.4	57.2±2.1
AFB_1	2.4±2.5	5.7±1.0	3.2±2.8	34.5±2.5	53.4±2.8
AFB_2	7.6±1.3	6.7±2.7	5.8±1.2	27.2±3.3	36.2±2.8
AFG_1	6.8±2.8	5.1±3.0	4.1±2.8	35.4±1.3	48.6±3.7
AFG_2	6.4±2.6	5.7±3.5	4.4±2.0	32.7±2.6	44.2±2.8

[a]Standard deviation. [b]ochratoxin A. [c]aflatoxin B_1. [d]aflatoxin B_2. [e]aflatoxin G_1. [f]aflatoxin G_2.

Effect of Moisture Content Regardless of Gamma Irradiation Dose and Type of Mycotoxin

The higher moisture content (16%) has more efficiently reduced the mycotoxin level relative to the lower moisture content (10%). The maximum reduction, regardless of gamma irradiation dose and type of mycotoxin was found to be 46.6±1.8% and 57.2±2.1% for moisture contents of 10% and 16%, respectively. The control samples showed 2.5% – 9.2% reduction which is related to recovery. In a related study, the recovery of studied mycotoxins was ranged from 72%– 101% [16]. Water appears to have an important effect on the destruction of Afs and OTA by gamma energy. Up on irradiation of foods, the primary reaction is the ionisation of water, which causes the water molecule to split into positively charged water radical and a negative free solvated electron. The water radical then decomposesin to a hydroxyl radical and a hydrogen ion. The reaction progresses until the endproducts of hydrated electrons, hydroxyl radicals, and hydrogenion and hydrogen atoms are formed [25]. The addition of free radicals to double bonds, especially to those in aromatic or heterocyclic rings, is an energetically positive reaction that is expected to occur in AFB_1 and G_1. The solvated electron may also add to the aromatic and heterocyclic rings, or to the carbonyl group of the lactone ring in the structures of Afs and OTA. These mechanisms would reduce the mutagenicity and toxicity of mycotoxins. In a related study, Temcharoen & Thilly (1982) reported that after treatment with 5–10 mrad gamma radiation, the contaminated peanut meal lost its toxic and mutagenic properties.

Effect of Gamma Doses on the Stability of Aflatoxins and OTA

The effect of gamma irradiation dose on mycotoxin reduction was significant ($p<0.05$). A proportional decrease in the concentrations of Afs and OTA ($p=0.000$) was noted by increasing gamma ray doses from 10 to 20 kGy.

In general, the yield of particular radiolysis products will linearly increase with dose; however, there can be deviations from such linearity, depending on the range of applied doses [35]. Farag *et al*. (1995) reported that the toxicity index values for infected yellow corn and peanuts were decreased by increasing the irradiation dose. In our results, the greatest reduction was obtained by irradiation at 20kGy, which ranged from 40.4±2.0 to 56.1±3.2 for AFB_2 and OTA, respectively, in black pepper and 36.2±2.8 to 57.2±2.1 in white pepper for AFB_2 and OTA, respectively. Recently, Aziz & Youssef (2002) showed that a dose of 15 kGy was sufficient for complete destruction of AFB1 in peanut, yellow corn, wheat and cotton seed meal. In a related study, the maximum reductions were obtained at 60 kGy for OTA, AFB_1 and AFG_1 at 52%, 43% and 40%, respectively [16].

In contrast with our results, Hooshmand and Klopenstein (1995) reported that irradiation doses up to 20 kGy did not significantly affect the AFB_1 content in wheat, corn or soybeans. A related study showed that peanut meal contained in a thin polyethylene bag exposed to gamma rays at a dosage of 25 kGy showed no apparent difference in toxin level from non-irradiated control meal when examined by a fluorescence test [10].

Of the different types of aflatoxins, AFB_1 and AFG_1 seem to be more sensitive to gamma radiation as compared to AFB_2 and AFG_2. This finding may be related to the 8,9 double bound presents in AFB_1 and G_1, which undergoes a reaction induced by the gamma ray. Mutluer & Erkoc (1987) reported that AFB_1 was the

most radio-sensitive of the four aflatoxins. The radio-sensi- tivity of the other aflatoxins was in increasing order: G_2, B_2, G_1. In a related study, the B_1 and G_1 toxins were completely destroyed at irradiation doses of 10 and 20 kGy, respectively (Farag *et al*., 1995). In a previous study on the reduction of mycotoxins in black pepper, found that AFB_1 and G_1 were more sensitive to the effects of the gamma ray [17]. It seems that more research is needed to investigate the effects of gamma rays on the different mycotoxins in different foods.

Conclusion

The effects of gamma irradiation ranging from 5 to 20 kGy and moisture content at 10% or 16% on the reduction of the mycotoxins OTA, AFB_1, AFB_2, AFG_1 and AFG_2 in black and white pepper were investigated. Both the gamma irradiation dose and moisture content showed positive significant effects ($p<0.05$) on mycotoxin reduction. The maximum reductions were found at 16% moisture content and a 30 kGy gamma ray dose (57.2 ± 2.1). The results showed that the gamma ray, even at 20 kGy and16% moisture content, was not sufficient to completely destroy the OTA and AFs. More research is needed to determine the shelf-life of pepper after irradiation and the quality of irradiated samples.

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