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EFFECT OF GAMMA RADIATION ON AFLATOXIN LOAD, AMINO ACID AND FATTY ACID COMPOSITION OF *ORYZA SATIVA* L.

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Abstract

Crops which are stored for more than a few days become a potential target for mould growth and mycotoxin formation by the species *Aspergillus*. The present study investigated an effective extraction, clean up and analysis methodology for aflatoxins, amino acids and fatty acid methyl esters (FAME) in rice (*Oryza sativa* L) samples collected from Faisalabad Division, Pakistan The extraction solvent (acetonitrile & water) gave ≥ 85% recovery in spiked cereal samples using a MycoSep-226 column. The sensitivity (LOD) of HPLC (FLD) was higher as compared to HPLC UV/Vis after derivatization of sample extract with trifluoroacetic acid (TFA). After treatment with γ-irradiation (0, 2, 4, 6 kGy), more than 95% reduction in AFB1 was observed in the samples. Effectiveness of γ-irradiation in rice samples contaminated with high concentrations of aflatoxin (AFB_1) and total aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* was significantly ($p<0.05$) dependant on radiation dose. Initial fungal population of rice samples was evaluated at 2.4×10^6 CFU g^{-1} while maximum reduction percentage was observed at radiation dose 6 kGy (6.4 CFU g^{-1}) when compared to the control sample. Amino acid profiles remained predominantly unchanged except for an increase ($p<0.05$) in leucine at 4 kGy. Rice fat, after irradiation, showed minor changes in the composition of fatty acid methyl esters. Effect of dose on palmitic (16:0), stearic (18:0), linoleic (18:2), linolenic (18:3) and arachidic (20:0) acids was statistically non significant whereas, olenic acid showed a significant change in concentration. The results indicate that γ-irradiation is a good technique for removing contaminants such as aflatoxins from cereal commodities. The total biomass (CFU/g) showed linear behaviour as dose level of gamma irradiation was increased.

Introduction

Rice (*Oryza sativa* L.) is an important cereal food stuff and forms an important part of the human diet. It is a major food source in several Asian and African countries (Park *et al*., 2005; Rabbani *et al*., 2010). These plants are native to tropical and sub tropical southern Asia and Africa (Crawford & Shen, 1998; Khan *et al*., 2013). Rice is a staple food and is the second most consumed cereal grain. It provides more than one fifth of the calories of human consumption (Smith, 1994; Pervaiz *et al*., 2010) In Pakistan it is major export item accounting for 6.1% of total export earning over the last five years. Rice was cultivated on an area of 2.51 million hectares and its production was 5.56 million tons.

Ionization, or radiation energy, has been effectively used for inhibition of sprouting, destruction of food borne insects and parasites, delay of physiological ripening, extension of shelf life and improvement of food quality (Radomyski, *et al*., 1994; Thayer, 1996; Shah *et al*., 2011). Irradiation offers broader avenues for sanitizing different ingredients as compared to thermal treatments at a competitive cost (Farkas, 1998). It can be effective in reducing micro-organisms, viruses and fungus, and is a good method for inactivating pathogens in food materials. Several studies have been conducted regarding the natural occurrence of aflatoxins in rice (Stewart, 2001).

Gamma ray irradiation is now recognized worldwide as an effective method for maintaining quality of food and food products. Directive 1999/2/EC has established a list of foods and food ingredients which can be treated with ionizing radiation. The maximum overall average absorbed dose of 10 kGy is permissible and suitable for dried aromatic herbs, spices and vegetable seasonings. However, the FDA limit for culinary herbs, seeds, spices, vegetable seasonings and blends of these aromatic vegetable substances have been limited to 30 kGy. Irradiation is a very effective technique for the reduction of fungal load and mycotoxin reduction. The present study is designed to investigate the effect of gamma radiation on aflatoxin load, amino acid and fatty acid profile of rice.

Materials and Methods

Cereal sampling: Polished rice samples (n = 40) were obtained from urban, semi urban and rural areas of Faisalabad (Pakistan). The samples were ground, mixed and stored in plastic bags at-4°C prior to the analysis. All experimental procedures were carried out in the Pesticide Chemistry Laboratory, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan for mycotoxin analysis.

Procedure for aflatoxins analysis: Aflatoxins were extracted from the ground rice samples (25 g) with 100 ml acetonitrile/water (84;16,v/v) mixture containing 20% sodium chloride, by blending vigorously for 45 min in a high speed blender. After filtration and acidifying with acetic acid the mixture was then passed through a Mycosep 226 column with flow rate of 2ml/min. A portion of 2 ml of solution was evaporated to dryness with a stream of nitrogen at 60°C using a centrifuge glass tube. The residue was dissolved in 0.3ml of mobile phase solution. The analysis of the cleaned extracts were carried out using a Shimadzu HPLC system equipped with UV/Visible (SPD-

10 A) and florescence detectors (RF-530) with and without derivatization using Trifluroacetic acid (TFA). The system was validated/ standardized with known standards individually, and in mixture form. All analyses were performed in triplicate using Discovery C18 column (250 x 4.6 mm, 5µm), Supelco, USA in isocratic mode.

Total biomass in cereals: After irradiation, with Co 60 gamma radiation source (2, 4 and 6 k Gy), 10g samples were ground, thoroughly mixed with 90 mL of sterile distilled water and agitated in sterile tubes for 30 min. Spore counting was performed by plate-count technique in DRBC agar (OXOID) medium using each suspension in a serial dilution from 10^{-1} to 10^{-6}. After incubation, at 25 ± 1°C for 7 days, the counting was performed according to Pitt *et al.,* (1983) and the results expressed in colony forming units per gram of maize (cfu/g).

Determination of amino acids: Amino acid analysis was performed by the method of Anjum *et al.,* (2005). 13 mg of the rice sample was incubated with 3 ml of 6N HCl, under nitrogen purging, in a sealed tube at 110°C for 24 hours. After that, samples were dried by evaporation and 3 ml of sodium citrate buffer (0.2N, pH 4.25) was added and the mixture centrifuged at 2500 rpm for 15 minutes. The supernatant was collected and analysed with an amino acid analyser.

Determination of fatty acid methyl esters (FAME): Fatty acid composition of extracted oils, before and after irradiation, of cereal samples, was carried out following the procedure of Wang *et al.,* (2000) with some modifications. Extracted oils (200 mg) were taken in 50 mL screw capped Pyrex glass tubes (50 x 1 cm) to which 2 mL methanolic sulphuric acid was added and the contents heated at 80°C for 2h. After cooling at room temperature, 2 ml of distilled water was added to the mixture and fatty acid methyl esters were collected in petroleum ether. The solvent was evaporated to dryness and the residue was analyzed isothermally with gas chromatography (model 3920 Perkin Elmer, USA) under the following conditions: glass column 3 ft, diethylene glycol succinate as stationary phase, injection temperature 200°C, column temp 190°C isothermal, flame ionization detector temp 250°C and injection volume 0.3 µL. The identification of individual fatty acid methyl ester was determined by comparing retention times with those of authentic standards (PolyScience Analytical Standards Kits; Quant-Kits; 99.5%, USA).

Statistical analysis: Each sample was analysed in triplicate. The data was reported as mean (n = 3 x 3) ± SD (n = 3 x 3). An analysis of variance (ANOVA) was performed using Minitab 2000 version 13.2 statistical software (Minitab Inc. Pennsylvania, USA). Significant differences ($p<0.05$) of mean were calculated using Duncan's multiple range tests.

Results and Discussion

The effectiveness of γ-irradiation was studied in rice samples contaminated with a high concentration of aflatoxin (AFB_1) and total aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*. The samples were analyzed after radiation application and the data presented in Table 1.

The elimination of aflatoxin content in rice samples was significantly ($p<0.05$) dependant on the radiation dose. Reduction in aflatoxins was directly associated with the level of radiation dose.

The reduction percentage of aflatoxin load was significantly ($p<0.05$) high and maximum in all the samples treated at 6 kGy.

Moulds of the genera *Aspergillus* and *Penicillium* occur in different agricultural matrices and produce toxins which create health problems for humans and animals, resulting in economic losses. The effect of γ-irradiation on initial fungal population of rice samples was evaluated and the data presented in Table 2. It is evident that initial fungal load was 2.4 x 10^6 CFU g^{-1} and after irradiation at different doses (2-6 kGy), a significant ($p<0.05$) decrease in fungal population was observed. Rice samples irradiated at 6 kGy had a count of 6.4 CFU g^{-1} as compared to the control sample. Our results are in agreement with those of Aziz & Moussa (2002) who reported a significant decrease in fungal counts in fruits treated at a dosage level of 1.5 and 3.5 kGy when compared to the untreated samples.

Table 1. Effect of gamma irradiation on the reduction of aflatoxins in rice samples

Area	Initial level (AFB_1) µg kg^{-1}	Irradiation dose (kGy)		
		2	4	6
Urban	20 (25)[a]	12 (16)[b]	7 (9)[c]	0.5 (2)[d]
Semi-urban	25 (30)[a]	16 (22)[b]	8 (10)[c]	0.8 (3)[d]
Rural	35 (40)[a]	28 (30)[b]	11 (15)[c]	1.5 (3.5)[d]

Mean within a row superscripted by different letters are significantly different at $p<0.05$

Table 2. Effect of γ-irradiation on fungal load (CFU g^{-1}) of rice samples.

Treatments	Total biomass (CFU g^{-1})	Nature of packing
Control sample	2.4 x 10^6 ± 12^a	Polythene envelope
2 kGy	4.1 x 10^3 ± 5^b	Polythene envelope
4 kGy	7.2 x 10^2 ± 2^c	Polythene envelope
6 kGy	6.4 x 10^1 ± 3^c	Polythene envelope

Values are mean of triplicate samples ± standard deviation.
All samples were treated at ambient temperature (22 ± 0.5°C).
Means superscripted by different letters are significantly different at $p<0.05$

Irradiated samples of rice were analyzed for amino acid profiles and it was observed that, predominantly, there was no change in the amino acid composition of the irradiated samples when compared to the control sample (non-irradiated). The radiation exposure resulted in a significant ($p<0.05$) increase in leucine at 4 kGy. Other essential amino acids were non-significantly affected with radiation (Table 3). Similar results have been reported in the literature (WHO, 1994).

The results of rice fat after irradiation showed minor changes in the composition of fatty acid methyl esters. The control sample of rice (0 kGy) showed maximum concentration of C18:1, C18:2 and C16:0 respectively, and least concentration of C18:3 and C20:0 (Table 4). A small change in fatty acid methyl esters profile was detected in total saturated and polyunsaturated, while a significant ($p<0.05$) change in percentage concentration of monounsaturated fatty acids was observed (Table 5). This may be attributed to the breakdown of the double bonds at a high dose of irradiation (6 kGy) or with hidden artifacts. ANOVA indicated that the effect of dose on palmitic (16:0), stearic (18:0), linoleic (18:2), linolenic (18:3) and arachidic (20:0) acids was statistically non significant whereas, olenic acid showed significant change in concentration. The results are parallel to earlier data reported by Nergiz & Donmez, (2004) and Yoon-Ha Kim *et al*., (2012).

Table 3. Amino acid (mg/g) composition of non-irradiated and irradiated rice

Amino acids	0 kGy	2 kGy	4 kGy	6 kGy
Isoleucine	$26.27^{a} \pm 1.25$	$23.08^{a} \pm 0.85$	$25.56^{a} \pm 4.20$	$26.25^{a} \pm 1.25$
Leucine	$62.87^{a} \pm 3.05$	$66.18^{ab} \pm 3.26$	$69.72^{b} \pm 1.20$	$69.85^{b} \pm 2.56$
Lysine	$41.80^{a} \pm 2.45$	$39.68^{a} \pm 0.22$	$41.35^{a} \pm 0.56$	$41.65^{a} \pm 0.38$
Methionine	$11.87^{a} \pm 0.35$	$9.79^{a} \pm 2.44$	$11.67^{a} \pm 2.08$	$11.78^{a} \pm 2.15$
Phenylalanine	$40.00^{a} \pm 1.78$	$39.15^{a} \pm 0.82$	$37.95^{a} \pm 3.15$	$39.65^{a} \pm 3.68$
Threonine	$28.80^{a} \pm 0.86$	$28.96^{a} \pm 1.35$	$28.85^{a} \pm 1.80$	$28.65^{a} \pm 0.56$
Tryptophan	$13.67^{a} \pm 1.35$	$11.67^{a} \pm 2.85$	$52.05^{a} \pm 3.46$	$12.85^{a} \pm 2.08$
Valine	$40.00^{a} \pm 3.48$	$41.38^{a} \pm 1.05$	$40.15^{a} \pm 0.85$	$40.35^{a} \pm 0.25$
Glycine	$32.67^{a} \pm 2.08$	$29.88^{a} \pm 3.45$	$39.42^{a} \pm 1.65$	$33.05^{a} \pm 1.86$
Proline	$39.46^{a} \pm 0.29$	$45.90^{b} \pm 2.28$	$40.85^{a} \pm 0.82$	$40.35^{a} \pm 1.85$
Serine	$40.13^{a} \pm 3.58$	$42.56^{a} \pm 3.38$	$40.56^{a} \pm 1.68$	$40.76^{a} \pm 2.26$

Values are mean of triplicate analysis ± standard deviation.

Means within a row superscripted by different letters are significantly different at $p<0.05$

Table 4. Fatty acid methyl ester profile of rice after irradiation

Irradiation dose (kGy)	C16:0 Palmitic acid	C18:0 Steric acid	C18:1 Oleic acid	C18:2 Linoleic acid	C18:3 Linolenic acid	C20:0 Arachidonic acid
0	$1.5^{a} \pm 0.2$	$16.5^{a} \pm 0.5$	$42.4^{a} \pm 1.50$	$38.8^{a} \pm 0.80$	$0.5^{a} \pm 0.25$	$0.2^{a} \pm 0.1$
2	$1.5^{a} \pm 0.02$	$16.5^{a} \pm 0.2$	$38.1^{b} \pm 1.50$	$38.5^{a} \pm 1.02$	$0.5^{a} \pm 0.01$	$0.4^{a} \pm 0.04$
4	$1.4^{a} \pm 0.01$	$16.4^{a} \pm 0.4$	$32.5^{c} \pm 2.45$	$38.3^{a} \pm 2.40$	$0.2^{a} \pm 0.02$	$0.4^{a} \pm 0.05$
6	$1.5^{a} \pm 0.03$	$16.4^{a} \pm 0.1$	$28.1^{d} \pm 2.60$	$38.2^{a} \pm 1.75$	BDL	$0.5^{a} \pm 0.02$

Values are taken as mean ± standard deviation (SD) of a triplicate analysis

BDL = below detection limit

Means within a column superscripted by different letters are significantly different at $p<0.05$

Table 5. Overall effect of irradiation on total amount of fatty acids.

Irradiation dose (kGy)	Total saturated	Total Mzono unsaturated	Total polyunsaturated
0	18.20^{a}	42.40^{a}	39.30^{a}
2	18.25^{a}	38.05^{b}	38.80^{a}
4	18.24^{a}	32.45^{c}	38.45^{a}
6	18.48^{a}	28.06^{d}	38.15^{a}

Means in a column superscripted by different letters are significantly different at $p<0.05$

Conclusion

Cereal samples collected from central areas of Punjab were found to be highly contaminated with aflatoxins. Contaminated samples of rice were analysed with HPLC UV/Visible and HPLC-FLD in isocratic mode using RP-Column. The highest reduction was at dose 6 kGy. The nutritional quality of irradiated samples was also studied to determine fatty acid and amino acid composition in control and treated samples with gamma irradiation. It was found that there was no change before and after irradiation. A small change was observed in mono saturated fatty acid methyl ester but was not significant. Rural inhabitants of central areas of Punjab are consuming food contaminated with high aflatoxins therefore precautions should be administered to prevent loss of life.

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