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Research Article

Analysis of aflatoxins; B1, B2, G1, and G2 in some rice samples of Bangladesh

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ABSTRACT

Identification and quantification of aflatoxins; B1, B2, G1, and G2 in twenty different rice samples were done by high-performance liquid chromatography coupled with a fluorescence detector. The rice samples were extracted with aqueous methanol and the extract was purified by immunoaffinity column by following the officially recognized ISO 16050 method. A post column derivatization device (Kobra cell) was used to make Bromo-derivative of the aflatoxins. The method for analysis of aflatoxins in the rice sample was validated in terms of selectivity, linearity, sensitivity, and recovery. Calibration curves were linear with a coefficient of variation $r^2 \geq 0.9998$, 09997, 0.9956, and 0.9969 for B1, B2, G1, and G2, respectively. The limit of detection (LOD) was 0.009, 0.006, 0.039, and 0.025 µg kg^{-1} and the limit of quantification (LOQ) was 0.025, 0.018, 0.116 and 0.075 µg kg^{-1} for B1, B2, G1, and G2, respectively. Recoveries (n=4) were carried out at two different spiking concentrations (1.39 and 2.77 µg kg^{-1} for B1, 0.49 and 0.98 µg kg^{-1} for B2, 1.56 and 3.12 µg kg^{-1} for G1 and 0.51 and 1.01 µg kg^{-1} for G2) and were ranged from 56.71±1.60-70.37±5.59%, 57.71±0.58-75.36±6.77%, 65.53±0.73-72.85±5.93% and 65.83±2.92-99.20±3.16% for B1, B2, G1, and G2, respectively. Aflatoxin B1, B2, G1, and G2 in rice samples were found to be present at 70, 60, 40, and 10%, respectively. The total aflatoxins (B1, B2, G1, and G2) in the rice samples were found to be in the range of trace- 3.54 µg kg^{-1}. The results revealed that 18 out of 20 samples contained the detectable number of aflatoxins. The total aflatoxins content was found to be below the tolerance level (4 µg kg^{-1}) according to the European Union (EU). So, the rice samples were safe for consumption, but the major concern is that a large population, especially in rural areas, consumes rice three times a day. If the rice sample contained aflatoxins even, below the present tolerance limit, there is a possibility of bad health implications from the consumption.

Introduction

Aspergillus flavus and *Aspergillus parasiticus* are fungi that produce aflatoxins (Reddy et al., 2009). According to the food and agriculture organization of the United Nations (FAO), mycotoxins, including aflatoxins, are responsible for the contamination of at least 25% of the world's cereal grains (Dowling, 1997). Human health can be affected by aflatoxins, and aflatoxicosis is a disease caused by aflatoxins. To assess the risk of aflatoxins in food, the provisional maximum tolerated daily intake of 1 ng kg^{-1} body weight/day can be used asthe guidance value (Yazdanpanah et al., 2013). Acute aflatoxicosis can cause sudden death and cancer and immune suppression can be happened by chronic aflatoxicosis (Magnussen and Parsi, 2013).

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Aflatoxins are produced by the fungal attack in a wide range of food commodities (Cristina et al., 2016). Bangladesh is a country that produces rice three times a year in the same field. The harvested paddies are dried by sunlight. If the rain comes during the harvesting period, paddy must be kept until rain gets over. Due to hot and humid weather, there is a high risk of fungal growth. Low-quality rice is much cheaper than high quality and is consumed mainly by low-income family members. There is a potential risk for the consumers of rice for aflatoxins. Various surveys conducted in different parts of the world indicated considerable levels of aflatoxins in rice (Gummert et al., 2009; Tanaka et al., 2007). It was found that there is a list of different countries (Sweden, Canada, India, Pakistan, Iran, United Arab Emirates, South Korea, Japan, Philippines, Columbia and Tunisia) where rice samples were analysed (Fredlund et al., 2009; Bansal et al., 2011; Reddy et al., 2009; Firdous et al., 2012; Feizy et al., 2010; Osman et al., 1999; Park et al., 2005; Tabata et al.,1993; Sales and Yoshizawa, 2005; Cespedez and Diaz, 1997; Ghali et al., 2008) for checking the presence of aflatoxins but no such data are available for Bangladesh. But heavy rains or high humidity could become susceptible to aflatoxin contamination in Bangladesh staple food (rice). Food safety demands rice samples should be free from chemical and biological contaminants. The present study was aimed to evaluate the growth of natural toxins in different kinds of rice (if any) from different parts of Bangladesh. High grade and inferior quality rice samples were chosen to identify aflatoxins, if any, present in the rice samples.

Materials and Methods

Grain samples

Twenty different rice samples (six were good and fourteen were of inferior quality) were purchased from different markets of Dhaka city, Kurigram, and Noakhali districts of Bangladesh from May to August 2016. The collected samples were labeled as R1 to R20; such as Swarna (R1), Nazirshail (R2 & R18), Paijam (R3), Minikit (R4, R9 & R17), BRRI28 (R5, R6, R10 & R11), BRRI29 (R7, R8, R12, R13 & R14), Heera (R15 & R16), Chinigura (R19 & R20).The good quality rice samples were R1, R4, R17, R18, R19 and R20. All the collected rice samples were ground into powder separately by a grinding machine, kept in twenty different zip-locked plastic bags, labeled, and stored in a freezer at -20^0 C until extraction was carried out.

Chemicals and Regents

The certified standard of aflatoxins, a mixture of B1 (99.00% purity), B2 (98.00% purity), G1 (99.70% purity), and G2 (99.00% purity) in benzene: acetonitrile (98:2) were purchased from Supelco, Sigma Aldrich, USA. Potassium chloride, potassium dihydrogen phosphate, anhydrous disodium hydrogen phosphate and nitric acid were purchased from Merck, Germany. Immunoaffinity column (Afla CLEAN™, LCTech) and potassium bromide were purchased from Scharlau, Spain. HPLC grade acetonitrile (ACN) and methanol were purchased from Merck, Germany. HPLC grade water (Milli-Q water), free from cations, anions, and hydrocarbons, was used to carry out the study.

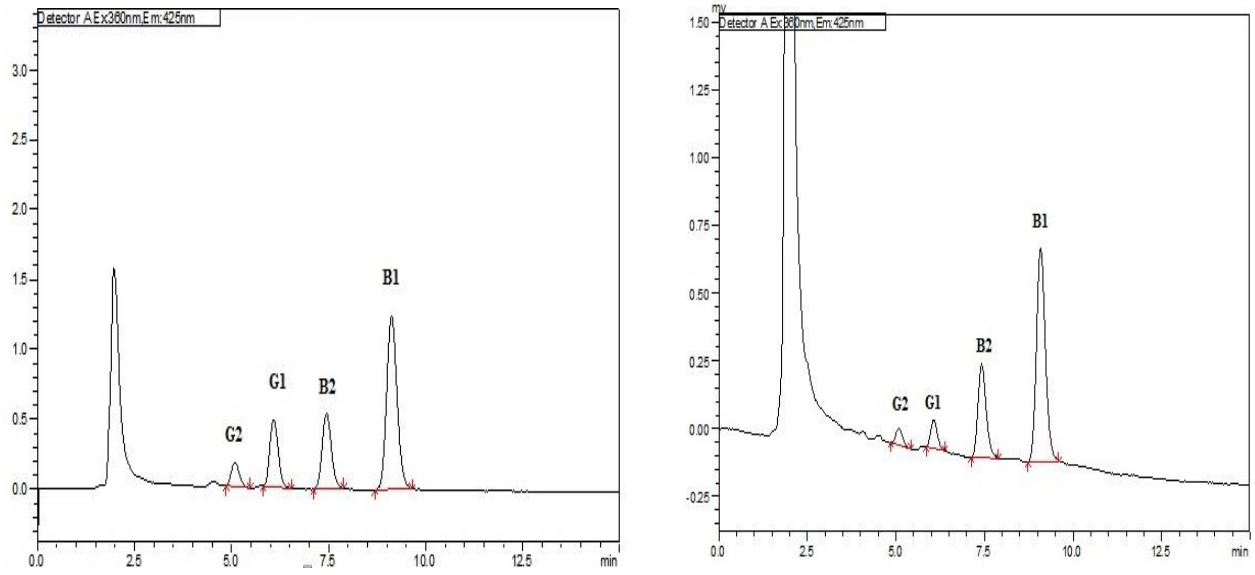
Extraction and Purification

Rice powder sample R1 (6.25 g; amount according to ISO 16050 method) was taken into a 50 mL Teflon tube, and a mixture of methanol-LC grade water in an 8:2 ratio (32 mL) was added to the sample. The mixture was vortexed for 5 min and filtered. From that extract, 3.5 mL was taken in a graduated screw cap test tube, mixed with 21.5 mL phosphate buffer saline (PBS), total volume was 25 mL. From that diluted extract in PBS, 20 mL was taken and loaded into the pre-conditioned immune-affinity SPE column. The PBS was removed by slow vacuum. The cleaned sample was eluted with methanol (1 mL x 3). All the collected rice samples (R2-R20) were extracted and cleaned up by following the same method.

Detection

Shimadzu Prominence Ultra-Fast Liquid Chromatograph (Prominence Degassing Unit DGU-$20A_{5R}$; Column Oven CTO-$10AS_{VP}$; Solvent Delivery Unit LC-20AD) having Fluorescence Detector (Shimadzu RF-20A) was used in the study. Separations were performed on a Shimadzu C18 (Shim-Pack VP-ODS; 150 mm x 4.6 mm i.d.; 5.0 µm; theoretical plate ≤ 2560) column for aflatoxins analysis. Post column-derivatization device (Kobra cell) was fitted between the LC column and the fluorescence detector to make the bromoderivative of the aflatoxins separated from the LC column. Elution was done by the isocratic system using aqueous phase as A (1 L contained 216.4 mg of KBr and 159.1 µL of concentrated HNO_3) and methanol as B. An Isocratic system was used to elute the samples from column (mobile phase A:B: 55:45). The column oven temperature was fixed at 40°C, the loop size was 20 µL, excitation and emission wavelengths of the detector (FLD) were set at 360 and 425 nm, respectively. The column flow rate was set at 1.0 mL min^{-1}.

The certified standard mixture of aflatoxins (B1, B2, G1, and G2) was dried and reconstituted in methanol. The concentration of the primary standard solution of aflatoxins B1, B2, G1, and G2 were 8.8, 3.1, 9.9, and 3.2 ng mL^{-1}, respectively. The primary standard solution of aflatoxins mixture was serially diluted to make ten different working standard solutions. The mixtures of working aflatoxins solutions were injected, retention times of B1, B2, G1, and G2 were found at 5.11, 6.12, 7.48 and 9.16 min, respectively (Fig.1). The limit of detection (LOD) and quantification (LOQ) were found to be 0.009 and 0.027, 0.006 and 0.018, 0.039 and 0.117 and 0.025 and 0.075 ng mL^{-1} for B1, B2, G1, and G2, respectively (Table 1). The calibration curve at different concentration levels was made using MS Excel software by plotting the area of the eluted standard vs concentration.



(a) (b)

Fig. 1. Chromatograms of certified standard aflatoxins (a) and rice sample (b)

Table 1. Correlation coefficients (r^2), LODs (Limit of detection) and LOQs (Limit of quantification) of aflatoxins

Aflatoxins	Linear range (ng mL^{-1})	r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
B1	0.009-4.40	0.9998	0.009	0.027
B2	0.006-1.55	0.9997	0.006	0.018
G1	0.005-0.25	0.9956	0.039	0.117
G2	0.005-0.25	0.9969	0.025	0.075

Analysis of Aflatoxins (B1, B2, G1, and G2)

The cleaned extract of twenty different rice samples was passed through the LC column and made into their respective Bromo derivatives in the Kobra cell, then was detected by a fluorescence detector. The Kobra cell was fitted between the HPLC column and the detector. The mobile phase contained the precursor of the derivatization agent, a potassium bromide and aflatoxins. The aflatoxins B1, B2, G1, and G2 were derivatized with electrochemically generated bromine in-situ to their brominated derivatives, which gave an enhanced fluorescence response and allowed the detection of the four individual toxins in very low concentration levels. The amount of four different aflatoxins was calculated using external calibration curves. Results are given in Table 2.

Recovery Experiment

The control rice powder sample (6.25 g) was taken into a 50 mL Teflon tube and 10 µL solution of aflatoxins mixture containing 8.8, 3.1, 9.9, and 3.2 ng mL^{-1} concentration of B1, B2, G1, and G2 was added. The spiked rice sample was shaken manually for 0.5 min and allowed to stay for 30 min at room temperature. Then the aflatoxins mixture was extracted and cleaned up by the same procedure as was done for experimental rice sample powder.

Four recovery experiments were done on the same day (intra-day recovery) at two different spiking levels of each standard. Similarly, four recovery experiments were done on another day (Inter-day) at two spiking levels by spiking the same amount of standard aflatoxins mixture. Percent recovery, standard deviation**,** and relative standard deviation were calculated, and the results are given in Table 3.

Results and Discussion

Aflatoxin Contamination in Rice Samples

A total of 20 samples were analysed, while 18 samples contained a detectable amount of aflatoxins (Table 2). Aflatoxin B1(in the range of 0.04 to 0.70 µg kg^{-1}), B2 (in the range of trace to 0.20 µg kg^{-1}), G1 (in the range of 0.22 to 1.82 µg kg^{-1}) and G2 (in the range of 0.12 to 1.56 µg kg^{-1}) were quantified in sample no. 17, 16, 6, and 4, respectively (Fig. 2).

Aflatoxins can contaminate a variety of agricultural and food commodities. In the present study**,** rice was chosen to analyse aflatoxins because it is our staple food. The analysis of aflatoxins in rice samples was done by following the established method (ISO 16050, 2003). The immune affinity column (3 mL; polypropylene) containing a gel material loaded with monoclonal antibodies against aflatoxins B1, B2, G1, and G2 were used in the present study for sample

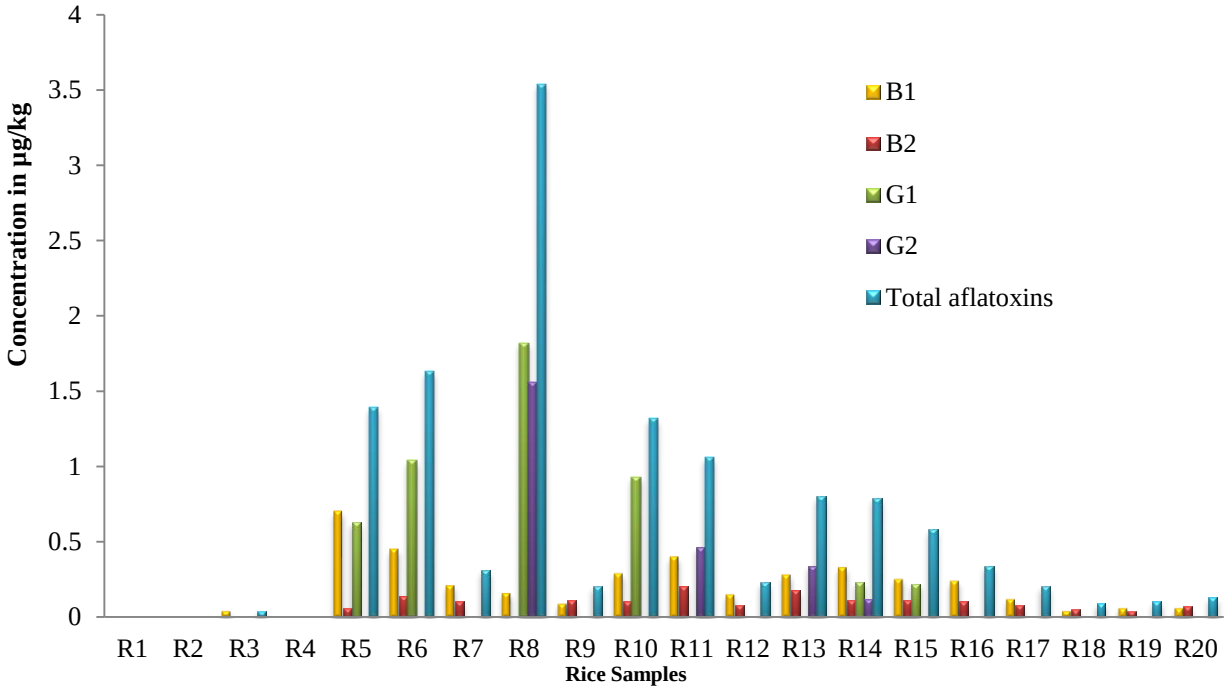


Fig. 2. Amount of Aflatoxins (µg kg^{-1}) in rice samples

clean-up purposes. The total aflatoxins (B1, B2, G1, and G2) in the rice samples were found to be in the range of trace to 3.54 µg kg^{-1} (Table 2). Aflatoxin B1, B2, G1, and G2 were present in 70, 60, 40, and 10% rice samples, respectively (Fig. 2). The aflatoxins tolerance level is different for different countries. European Union (EU) Maximum Tolerated Level is 4 µg kg^{-1} (Fredlund et al., 2009; Ghali et al., 2010; Ruadrew et al., 2013; Sales and Yoshizawa, 2005; Suarez-Bonnet et al., 2013), AFs contamination more than MTL of 20 µg kg^{-1} as assigned by food authorities of USA (FDA and FAO). The WHO has set 30 µg kg^{-1} for aflatoxin in food, and this high limit has been used in several countries for aflatoxin in rice without any regard to the daily intake of rice (Nguyen et al., 2007). A comparable regulation has been reported in Malaysia of 35 µg kg^{-1} (Reddy et al., 2011) and in India, the permissible limit of 30 µg kg^{-1}for total aflatoxins (Toteja et al., 2006). These limits have been set in general for food, but it may not be suitable for a staple food like rice.

The two samples (R1 and R2) were found to be free from aflatoxins (Table 2) were collected from Dhaka city. The level of aflatoxins in rice can be differed from one place to another due to various factors like temperature, relative humidity, and agricultural practices. The hot and humid conditions are supposed to be favorable for the growth of mycotoxin production in agricultural products (Reddy et al., 2008). The results of the present study revealed that the aflatoxins (AFs) level in Bangladeshi rice does not concurrently present a potential risk to human health. The effect attributed to aflatoxin in rice in Bangladesh is still unclear, and the low level does not mean safer rice food regarding its high consumption.

Table 2. Amount of aflatoxins (µg kg^{-1}) in rice samples

Sample Code	B1 (µg kg^{-1})	B2 (µg kg^{-1})	G1 (µg kg^{-1})	G2 (µg kg^{-1})	Total Aflatoxins (µg kg^{-1})
R1	ND	ND	ND	ND	ND
R2	ND	ND	ND	ND	ND
R3	0.04	ND	ND	ND	0.04
R4	ND	trace	ND	ND	trace
R5	0.70	0.06	0.63	ND	1.39
R6	0.45	0.14	1.04	ND	1.63
R7	0.21	0.10	ND	ND	0.31
R8	0.16	ND	1.82	1.56	3.54
R9	0.09	0.11	ND	ND	0.20
R10	0.29	0.10	0.93	ND	1.32
R11	0.40	0.20	ND	0.46	1.06
R12	0.15	0.08	ND	ND	0.23
R13	0.28	0.18	ND	0.34	0.80
R14	0.33	0.11	0.23	0.12	0.79
R15	0.25	0.11	0.22	ND	0.58
R16	0.24	0.10	ND	ND	0.34
R17	0.12	0.08	ND	ND	0.20
R18	0.04	0.05	ND	ND	0.09
R19	0.06	0.04	ND	ND	0.10
R20	0.06	0.07	ND	ND	0.13
Mean	0.23	0.10	0.81	0.62	0.75
Median	0.21	0.10	0.78	0.40	0.34
Maximum	0.70	0.20	1.82	1.56	3.54

ND Not Detected

The LOD and LOQ were different for four different aflatoxins B1, B2, G1, and G2 due to their different sensitivity in LC-FLD with the Kobra cell system. The extraction efficiency of the ISO 16050 method in the rice sample was evaluated by recovery experiments at two spiking concentrations (0.77 and 1.54 µg kg^{-1}for B1, 0.27 and 0.54 µg kg^{-1} for B2, 0.86 and 1.73 µg kg^{-1} for G1 and 0.28 and 0.56 µg kg^{-1} for G2) in 4 (n=4) replicates nalyses. The spiking concentration levels were chosen according to the results of rice samples. Both intra-day and inter-day recoveries were carried out to evaluate the efficiency of the method. The results for the intra-day recovery was 70.37±5.59% for B1, 75.36±6.77% for B2, 72.85±5.93% for G1 and 99.20±3.16% for G2 and inter-day recovery was 56.71±1.60 % for B1, 57.71±0.58% for B2, 65.53±0.73% for G1 and 76.34±4.03% for G2 for spiking 10 µL mixture of aflatoxins (Table 3). For spiking 20 µL mixture of aflatoxins the intra-day

recovery was 65.08±2.21% for B1, 63.39±2.36% for B2, 71.85±1.90% for G1 and 65.83±2.92% for G2 and inter-day recovery was 63.40±3.55% for B1, 61.39±3.42% for B2, 69.57±5.19% for G1 and 79.18±7.10% for G2 (Table 3). The relative standard deviation (RSD) value of the recovery experiments was in the range of 1.01 to 8.99% which showed the excellent performance of the method. The recovery was within 70 to 99 % for seven recovery experiments and 56 to 69% for nine recovery experiments. The percent recovery below 70 may be due to the low spiking concentration. Since the detected maximum concentration of aflatoxins in the test rice samples were 0.70 µg kg^{-1} for B1, 0.20 µg kg^{-1} for B2, 1.83 µg kg^{-1} for G1 and 1.56 µg kg^{-1} for G2, the spiking concentrations were chosen for the recovery experiment were also low. The previous report (Gray et al. 2014) found that using spiking concentration <1.0, 1-10 and >10 µg kg^{-1}, the acceptable recovery ranges were 50-120, 70-110 and 80-110%, respectively for aflatoxins B1, B2, G1, and G2. The recovery results of the present study can be compared with the aflatoxins (B1, B2, G1, and G2) determination in peanuts and peanut-based products (Biljana et al., 2013). For B1, the recovery was 92.33%, and the recovery for G1 was 84.37%. There were some minor deviations concerning the values of B2 (127.86%) and G2 (48.31%). But according to the Commission Regulation 466/2001, 2174/2003, and 1881/2006, those deviations are not likely to affect the result significantly. So, the recovery result of the present study was satisfactory. The average percent recovery can be increased by increasing the concentration of spiking levels.

Due to the delay in drying and moisture content, the post-harvest contamination of aflatoxins can be estimated (Asghar et al., 2013). There are six seasons in Bangladesh, but the rice samples in the present study were collected in the rainy season, which can regulate fungal growth and mycotoxin expression. More study is needed to investigate aflatoxin in rice samples in other seasons.

The International Agency for Research on Cancer (IARC) classified aflatoxins and aflatoxin B1 as class I agents (IARC, 1993). A previous study showed that aflatoxins are relatively stable during the cooking process, suggesting that a major reduction in the exposure to these mycotoxins cannot be expected to occur by cooking rice (Park et al., 2005, 2006; Hisako et al., 2013; Mohamadi et al., 2014; Hussain and Luttfullah, 2009). The tolerated level of aflatoxins in rice is 4 µg kg^{-1} according to the European Union (EU). The amount of rice consumption per day per person is very significant in Bangladesh. From the study of Hossain and Yunus (2016), it was found that the daily rice consumption was 525 and 546 g for poor and non-poor capita in rural areas, respectively. In Iran, Japan, China, and Thailand, the rice consumption is 107, 157, 183, and 377 g/day/person, respectively (Majeed et al., 2018). Due to the very high rice consumption in Bangladesh, the aflatoxins in rice may increase cancer risk in Bangladesh.

Table 3. Intra-day and Inter-day recovery of aflatoxins (B1, B2, G1, and G2) in rice sample

Aflatoxins	Spiking level	Intra-Day		Inter-Day	
	(µg kg^{-1})	Average recovery	RSD (%)	Average recovery	RSD (%)
B1	0.77	70.37 ± 3.59	7.94	56.71 ± 1.60	2.83
	1.54	65.08 ± 2.21	3.40	63.40 ± 3.55	5.61
B2	0.27	75.36 ± 2.77	8.99	57.71 ± 0.58	1.01
	0.54	63.39 ± 2.36	3.73	61.39 ± 3.42	5.57
G1	0.86	72.85 ± 2.93	8.14	65.53 ± 0.73	1.12
	1.73	71.85 ± 1.90	2.64	69.57 ± 3.19	7.47
G2	0.28	99.20 ± 3.16	3.19	76.34 ± 4.03	5.28
	0.56	65.83 ± 2.92	4.43	79.18 ± 3.10	8.97

Values are in Mean ± SD (Standard deviation); RSD: Relative standard deviation.

Conclusion

Since people consume a larger amount of staple foods than other foods, the regulation of B1 for staple foods should be stricter than other food items. The present study results imply that with the present level of the toxicant in staple food, such as rice, there may be a notable increase in the cancer risk due to the high consumption of rice. The study recommends that the maximum residue level of aflatoxins in rice be considered and the permissible level should be more restricted than other foods.

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Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

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