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QUANTIFICATION OF AFLATOXINS IN MAIZE SAMPLES COLLECTED FROM VARIOUS PARTS OF THE PUNJAB, PAKISTAN

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ABSTRACT

Maize, one of the main cash crops of Pakistan is cultivated under various ecological conditions. Maize quality deteriorates due to many factors like rainy season, adverse temperature, and traditional practices of harvesting and insufficient storage facilities that stimulate fungal infection (aflatoxins). The present study was designed to quantify the aflatoxins (AFB1, AFB2, AFG1 and AFG2) in maize grains collected from various districts of the Punjab. Seventy two samples of maize grains were collected from 12 districts of Punjab. Toxins were extracted by MycoSep columns (product code 226) and analyzed by High-Performance Liquid Chromatography (HPLC) for the quantification of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2). The study showed that AFB1 & AFB2 are the primary contaminants. Out of the analyzed samples, 77.7% of yellow maize grains were contaminated with AFB1, 61.1% AFB2 and 38.8% with high levels of AFG2. The positive samples showed higher than the legal limits as set by the European Union (EU), United States Food and Drug Administration (USFDA) Department and Pakistan Standards and Quality Control Authority (PSQCA). Study further found out that aflatoxin production was mainly caused due to inappropriate storage conditions. Variation in agro-ecological conditions and inappropriate storage cause aflatoxin contamination in maize grains which can be controlled by improvement in the storage conditions and use of proper detoxifying technologies.

Key words: Aflatoxins, Maize grains, HPLC, Food safety.

INTRODUCTION

Maize (*Zea mays*) is the vital cereal crop of Pakistan after wheat and rice and generally used as a source of food and feed. The average production of maize crop in Pakistan is 2850 kg ha^{-1} (Tariq & Iqbal 2010) out of which about 60% is grown by irrigation and 36% in rain fed areas (Abuzar *et al*. 2011). It has big contribution as a food in the form of cob, parched, boiled, fried, roasted, ground and fermented maize used for the manufacturing of breads, porridges, cakes, alcoholic beverages, corn protein, corn sugar, corn oil and syrup (Akmal *et al*. 2013; Ahmad *et al.* 2007). It provides 15 to 56% of the total calories in the diets of people where protein from animal source is deficient and costly and not available to majority of the population (Prasanna *et al.* 2001).

Cereal grains especially maize grains get easily contaminated by fungus in the field and during storage condition. Usually these fungi are capable of producing mycotoxins which are stable compounds. These mycotoxins are not destroyed during processing of food, which may lead to contamination of cereal based foods. Maize crops are easily attacked by the mycotoxins producing fungi in field as well as during poor storage conditions. Generally, these mycotoxins are produced by the filamentous fungi more specifically, *Aspergillus, Fusarium* and *Penicillium.* Contamination of maize with *Aspergillus flavus* and *Aspergillus parasiticus* and production of aflatoxins during storage is one of the serious problems all over the world (Kaaya and Kyamuhangire. 2006). These two types of fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) are responsible for spoilage of stored grains (Paster. 1995). Long-term storage under un-hygienic, hot and humid condition is responsible for fungal growth (Williams and McDonald. 1983; Hell *et al.* 2000). The main aflatoxins naturally occurring in foods are designated as AFB1, AFB2, AFG1, and AFG2. Aflatoxin B1 is the most toxic and usually predominant (FAO. 1997). These fungi survive in a broad range of environments and can be found in soil, in plant and animal remains, and in grains and seeds such as maize, peanuts, and tree nuts (Pitt. 2000). *Aspergillus flavus* is the main fungus that causes pre harvest aflatoxins contamination in field crops.

Aflatoxins have been identified in the early 1960s. They are secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* and target a variety of staple foods, especially maize and groundnuts, in low income countries (Wild and Gong. 2010). Williams *et al.* (2004) have reported a probability that 4.5 billion of the world's population is exposed to aflatoxins. The Food and Agriculture Organization of the United Nations (FAO) estimated that up to 25% of the world's cereal grains are contaminated by aflatoxins (Liu *et al.* 2006).

Aflatoxins occur usually in tropical regions with high temperature and humidity and they mount up to post-harvest stage when food commodities are stored under conditions that promote fungal growth. Tropical conditions such as high temperatures and moisture, monsoons, unseasonal rains during harvest and flash floods lead to fungal proliferation and production of mycotoxins. Poor harvesting techniques, inappropriate storage and less optimal conditions during transportation, marketing and processing can also contribute to fungal growth and increase the risk of aflatoxins production (Wagacha and Muthomi. 2008). The variations in climatic conditions as well as the food cultivation and production chains are characteristic in most parts of Pakistan. Consequently, the threat of aflatoxin contamination of foods and feeds resulting in human and livestock poisoning is real and of major concern (Wagacha and Muthomi. 2008).

Aflatoxins have carcinogenic and hepatotoxic actions, depending upon the extent and level of exposure. Chronic exposure of aflatoxins in dietary practices is a major risk factor for hepatocellular carcinoma, predominantly in the areas where hepatitis B virus infection is endemic. Ingestion of higher quantity of aflatoxins can produce acute aflatoxicosis, which is manifested as hepatotoxicity and in severe cases, fulminant liver failure (Fung and Clark 2004). Contamination in varieties of foods by these naturally occurring toxins is a great concern in rural communities of developing countries (Lewis *et al.* 2005). Aflatoxin causes illness and even death when consumed in large quantities. Low-level, chronic exposure is carcinogenic and has been linked to growth retardation in children.

The present study was designed to evaluate the occurrence of aflatoxins in maize, grown in different regions of Punjab, Pakistan and stored under local storage conditions.

MATERIALS AND METHODS

Twelve districts from the three regions of Punjab province were selected for sampling purpose according to climatic conditions. 72 samples of yellow and white variety of stored maize grains were procured from the whole sale markets of Southern Punjab (Multan, Vehari, Bahawalpur, Rahim Yar Khan) Central Punjab (Lahore, Faisalabad, Sargodha, Gojar Khan) and Northern Punjab (Rawalpindi, Chakwal, Khushab, Attock) of Pakistan. Samples of maize grains (yellow maize, white maize) were randomly collected from each district in triplicates and stored at 4°C in polythene bags with proper codes and identification marks for analysis.

Chemicals and Regents: Standard solutions of Aflatoxins (AFB1, AFB2, AFG1 and AFG2) were prepared in 50 µg mL^{-1} in acetonitrile. Aflatoxin standards were purchased from Sigma-Aldrich (St. Louis, Mo., USA). MycoSep® column 226 (AflaZone) was purchased from Romer Labs (Union, Mo., USA). HPLC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany) and Trifluoroacetic Acid (TFA) was obtained from Sigma-Aldrich (St. Louis, Mo., USA).

Extraction and purification: Extraction and purification of samples were carried out by using a slightly modified method of Iqbal *et al.* (2012). The maize samples were ground to uniform consistency by laboratory grinding mill (ZM 200. Germany). A portion from each homogenous ground samples (25g) was used for extraction with 100 ml of acetonitrile/water (80:20 v/v) and 5 gram of sodium chloride (NaCl) by shaking in water bath for 60 min at 50 rpm at room temperature in 250 ml Erlenmeyer flask fitted with a stopper. The solutions were filtered through Whatman No.5 papers. Then 9 ml of the filtrates were acidified with 70µl acetic acid and the mixture was transferred to MycoSep columns (product code 226) and passed through at a flow rate of 2 ml/min. The aflatoxins were passed through the column and 2 ml portion of each eluate was taken and evaporated to dryness by nitrogen evaporator in a centrifuge glass tube. After drying the mixture 200µl n-hexane was added and vortex mixed the sample for 1-2 min. Then 50µl Trifluoroacetic Acid (TFA) was added and mixed by vortex mixer. Then 1.95 ml of water and acetonitrile (1:0.95) were added and mixed again. The 1 ml of sample filtrate obtained from syringe filter was collected in HPLC vials. A 20µl portion of each sample was subjected to HPLC analysis.

Mobile phase: For aflatoxins analysis, mobile phase of acetonitrile/methanol/water (20:20:60 v/v/v) was applied which was degassed by sonication. The HPLC (Shimadzu, Kyoto, Japan) was fitted with a Supelco C18 Column (Discovery HS) with a fluorescence detector (RF-530). Aflatoxins were identified at excitation and emission wavelengths of 360 nm and 440 nm, respectively. The flow rate was 1 ml/min and the column was maintained at 40°C. The injection volume was 20µl. Resultant Chromatographs showed quality resolutions of peaks. For the validation of this method, calibration curves were drawn using a series of calibration solutions of aflatoxins in methanol. Standard solutions were chromatographed in duplicate.

Statistical Analysis: Data was analyzed by using SPSS software version 16. Independent sample T-test was applied to analyze data. P-value < 0.05 was taken as significant.

RESULTS AND DISCUSSION

Linearity of HPLC system was checked to inject different concentrations of reference standards (Figure No. 1). The system was calibrated using the working

solution of aflatoxins in the range of 0.5-10 µg/ml in acetonitrile. HPLC method was validated by testing linearity, recovery, Limit of Detection (LOD) and Limit of Quantification (LOQ). Results for recovery are given in (Table No. 1). The data for the calibration curves are given in (Table No. 2). LOD and LOQ were 0.5 and 1µg/kg for AFB1

Table 1. Recovery (%) of aflatoxins in maize samples (n = 6).

Aflatoxins	Spike level (µg/kg)	Recovery (%)	RSD (%)
AFB1	10	90 ± 0.17	0.17
AFB2	5	88 ± 0.12	0.14
AFG1	10	89 ± 0.17	0.19
AFG2	5	86 ± 0.12	0.15

Table 2. Parameters of linear regression for aflatoxins

Aflatoxins	Concentration (µg/kg)	Slope	Intercept	R^2
AFB1	0.5 - 10	1.0907	0.042	0.9996
AFB2	0.05 - 5	2.6638	0.0388	0.9993
AFG1	0.5 - 10	1.0907	0.042	0.9996
AFG2	0.05 - 5	2.6638	0.0388	0.9993

Pakistan is an agricultural country and maize is cultivated under different ecological conditions. Rainy season, adverse temperature, traditional practices of harvesting and insufficient storage facilities provoke fungal infection. Storage conditions of maize grains and marketing practices are also contributing factors for aflatoxins contamination (Hell *et al.* 2003). In this study an attempt has been made to find out the occurrence of aflatoxins in maize samples collected from 12 different districts of Punjab.

Present study showed that aflatoxins B1 & aflatoxins B2 are the primary contaminants. Forty-eight of total maize samples (66.6%) were contaminated with aflatoxins B1 and twenty-two (35) samples (48.6%) out of total (72) maize grains were found to be contaminated with aflatoxins B2. (Table no. 3) showed that 77.7% (28) samples from the yellow maize grains were contaminated with aflatoxin B1, out of which 47.22% (17) were at higher limits than acceptable levels and 30.55% (11) of yellow maize grains showed up to acceptable levels. Contamination of AFB1 in yellow maize was confirmed through HPLC chromatogram (Figure No. 2). The acceptable level for AFB1, as defined by EU regulations are 2 µg/kg, and for total aflatoxins the acceptable levels are 4 µg/kg (Cheli *et al.* 2014). But on the other side Food and Drug Administration (FDA) Authority, Food and Agriculture Organization (FAO) and Pakistan Standards and Quality Control Authority (PSQCA) fixed acceptable limit for AFB1 is 10 µg/kg and 20 µg/kg for total aflatoxins and AFG1, respectively, and 0.05 and 0.1 µg/kg for AFB2 and AFG2, respectively. Shah *et al*. (2010) found AFB1 contamination in 77.78% of total samples collected from Upper Swat and 88.89% samples from Lower Swat. Out of total 36 yellow maize samples collected, 19 samples were found to have AFB1 contamination > 20 µg/kg.

A total of 61.11% (22) yellow maize grains were contaminated with aflatoxin B2, out of which 36.11% (13) were at higher limits then acceptable levels and 25% (09) showed up to the acceptable levels (Table 3). Ghiasian *et al*. (2011) reported the incidence of AFB1 80% and AFB2 60% recovered from maize grain in Mazandaran which was also similar to those reported by *Yazadanpanah et al. (2001).* Pearson *et al.* (2011) also observed almost same type of results in his study. The highest level of AFB1 was 134 µg/kg observed in the samples of Chakwal and Rawalpindi and the highest of AFB2 was 33.13 µg/kg from the samples of Chakwal. Ghiasian *et al.* (2011) studied that in Iran maize grains collected from different districts AFB1 and AFB2 were in the range from not detected to 276.3µg/kg and not detected to 30.4µg/kg respectively. 33.3% of the yellow maize grains showed the results of higher levels of both B1 and B2. Lutfullah and Hussain (2012) observed 40% of maize grains were contaminated with high level of AFB1 and AFB2 in Khyber Pakhtun Khan (KPK), Pakistan. AFG1 was not detected in the present study. Yazadanpanah *et al*. (2001) reported no contamination of AFG1 and AFG2 from maize grains of high incidence area of Iran. Out of total 36 samples of yellow maize grains, 14 (38.8%) samples were found to be contaminated with high levels of AFG2 (Table No. 3). Samples of yellow maize collected from Lahore were found to have highest levels (20 µg/kg) of AFG2. Results of the presence of AFB1 and AFG2 were further confirmed through HPLC chromatogram (Figure No. 3). Ghiasian *et al.* (2011) observed two samples of maize grains from Kermanshah province, contained detectable concentrations ranged from not detected to 9.1 µg/kg of AFG1and AFG2 in Iran.

Out of 36 samples of white maize grains 55.5% (20) were contaminated with aflatoxins B1, out of which 19.4% (07) were at higher limits than acceptable levels and 36.1% (13) of white maize grains showed under the acceptable levels. 36.11% (13) out of total samples of white maize grains were contaminated with aflatoxins

AFB2, out of which 16.6% (06) were at higher limits than acceptable levels and 5.5% (02) of white maize grains showed up to the acceptable levels and 19.4% (07) were contaminated by AFG2 and 16.6% (06) showed upper limits (Table No. 3). Lewis *et al.* (2005) observed similar results of aflatoxins contamination in Eastern and Central Kenya during an outbreak of Acute Aflatoxicosis where high proportion (55%) of maize samples from the local markets in all four study districts had aflatoxin levels greater than the regulatory standards. 16.7% of the white maize grains showed the results of higher levels of both AFB1 and AFB2. Hell *et al.* (2003) observed similar results of aflatoxins contaminations of the maize grains which were improperly stored more than six months. Hell *et al.* (2003) observed that aflatoxins were produced by the unhygienic and dissimilar conditions of storage of maize grains for prolong time period. Overall, aflatoxins were detected with variable levels of infestations in 28 and 22 samples of yellow and white maize respectively, (Table No. 4). Data related to aflatoxins quantification were statistically analyzed by using independent t-test (Shah *et al.* 2010). For aflatoxins quantification AFB1 in two different varieties of maize (yellow and white), the independent sample "t" test was applied to confirm the mean difference between samples. Independent "t" test was also applied to check the significance levels of total aflatoxins in maize grains. $P < 0.05$ showed the significant results (Table 5).

Table 3. Aflatoxins contamination in yellow and white variety of maize

Product	Aflatoxins	No. of samples	Positive samples	Upper limit	Lower limit	Contamination detected (%)
Yellow maize	**AFB_1**	36	28	17	11	77.7
	AFB_2	36	22	13	09	61.11
	AFG2	36	14	05	09	38.8
White maize	**AFB_1**	36	20	07	13	55.5
	AFB_2	36	13	06	02	36.1
	AFG2	36	07	06	01	19.4

Table 4. Aflatoxins level (µg/kg) in maize samples collected from various districts of Punjab

Maize variety	No of samples	Aflatoxins detected	Aflatoxins ≥ 4 µg/kg		
			0 − 20 µg/kg	**21 − 40µg/kg**	**≤ 40µg/kg**
Yellow maize	36	28	9	11	8
White maize	36	22	8	8	6

Table 5. Independent sample T test for total aflatoxins

Aflatoxins	Maize type	No of samples	Mean	P value
Total Aflatoxins	Yellow maize	36	70.77 ± 18.6	*0.029
	White maize	36	25.96 ± 6.30	*0.033

Conclusion: The discussion concluded that the maize grains of yellow and white variety contains good nutritional value but the main concern is the contamination of AFB1, AFB2, AFG1 and AFG2 presence in the maize grains. Variation in agro- ecological conditions and poor storage condition directly influence the aflatoxins contamination so there is need to control it and properly monitor the storage conditions and storage time period and use of proper detoxifying technologies to prevent the maize crop from the fungal attack which could help in reduction of aflatoxins contamination.

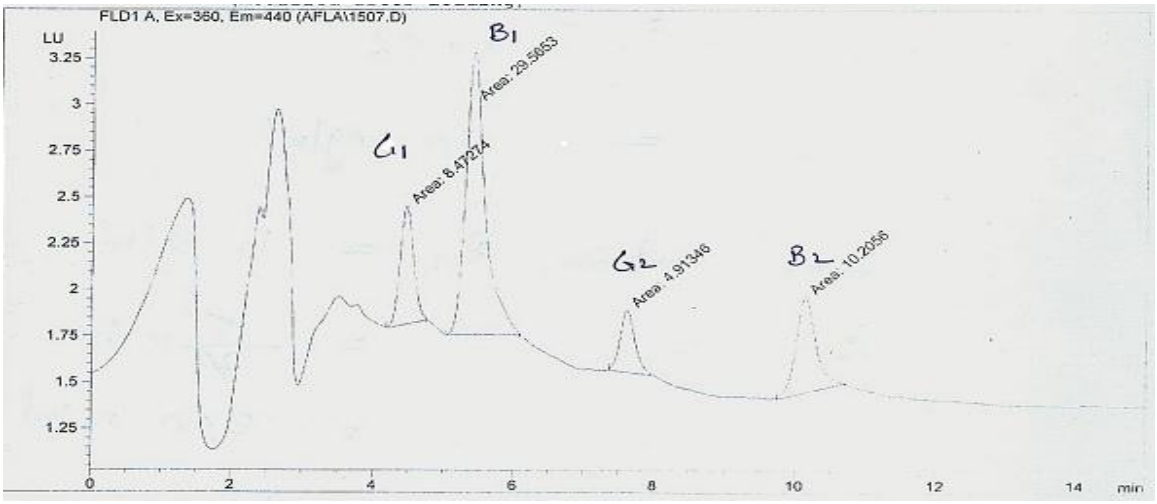

Fig: 1 HPLC chromatogram showing Aflatoxins standard.

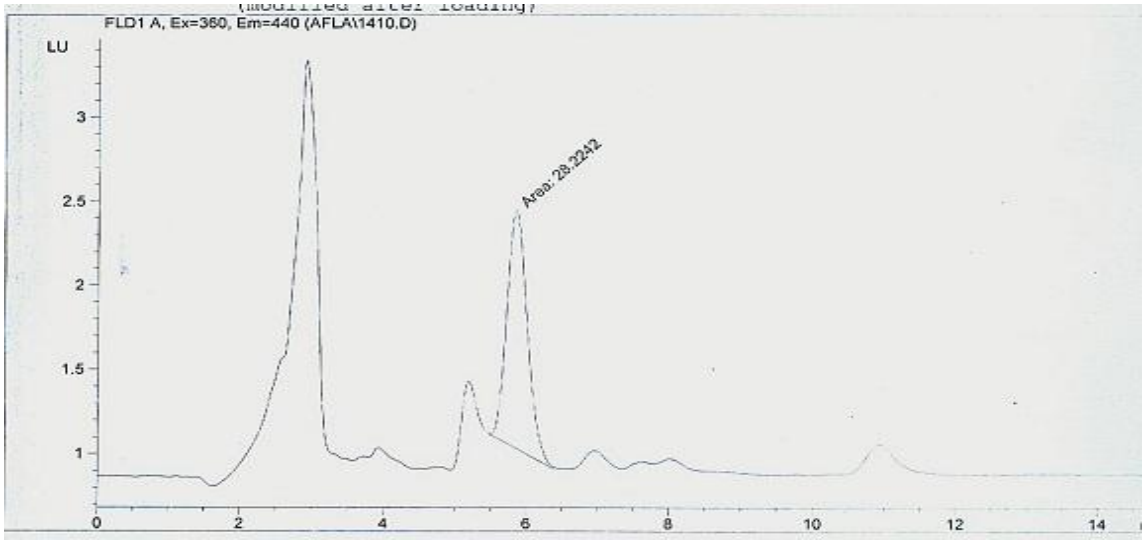

Fig. 2 HPLC chromatogram showing AFB1 in yellow maize

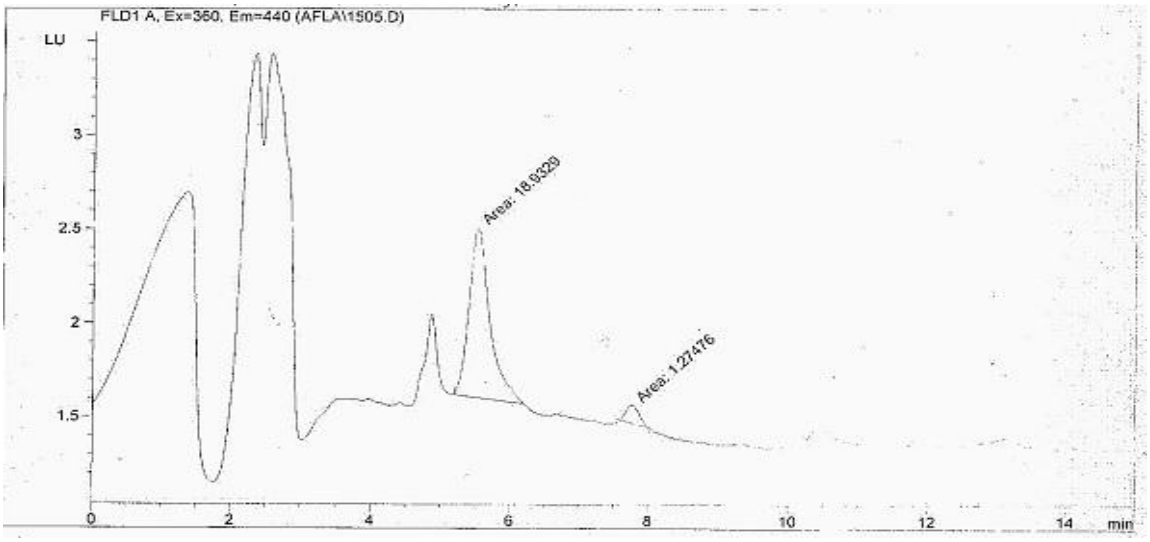

Fig. 3 HPLC chromatogram showing AFB1 and AFG2 in yellow maize

REFERENCES

Abuzar, M.R., G.U. Sadozai, M.S. Baloch, A.A. Baloch, I.H. Shah, T. Javaid and N. Hussain (2011). Effect of plant population densities on yield of maize. J Anim and Plant Sci. 21(4): 692-695.

Ahmad, Z. I., M. Ansar, M. Iqbal and N.M. Minhas (2007). Effect of planting geometry and mulching on moisture conservation, weed control and wheat growth under rain fed conditions. Pak J Bot. 39(4): 1189-1195.

Akmal, M., M. Ali and D.H. Putnam (2013). Row orientation and plant space in relation to nitrogen fertilizer application response on maize production. Int J Agron Plant Prod. 4(6): 1393-1408.

Cheli, F., D. Battaglia, R. Gallo and V.D. Orto (2014). EU legislation on cereal safety: An update with a focus on mycotoxins. Food Control. 37: 315-325.

F.A.O. (1997). World-wide regulations for mycotoxins. Food and Nutrition Paper 64, Rome.

Fung, F., and R.F. Clark (2004). Health effects of mycotoxins: a toxicological overview. J Toxicol Clin Toxicol. 42:217–234.

Ghiasian, S.A., G.S. Shephard and H. Yazdanpanah (2011). Natural of aflatoxins from maize in Iran. Mycopathologia. 172:153–160.

Hell, K., K.F. Cardwell and H.M. Poehling (2003). Distribution of fungal species and aflatoxin contamination in stored maize in four agro ecological zones in Benin, West-Africa. J Phyto. 151:690–698.

Iqbal, S.Z., M.R. Asi, A. Arino, N. Akram and M. Zuber (2012). Aflatoxin contamination in different fractions of rice from Pakistan and estimation of dietary intakes. Mycotoxin Res, 28(3): 175–180.

Kaaya, A.N., and W. Kyamuhangire (2006). The effect of storage time and agroecological zone on mould incidence and aflatoxin contamination of maize from traders in Uganda. Int J Food Microbiol. 110: 217–223.

Lewis, L., M. Onsongo, H. Njapau, S. H. Rogers, G. Luber, S. Kieszak, J. Nyamongo, L. Backer, A.M. Dahiye, A. Misore, K.D. Cock, C. Rubin and the Kenya Aflatoxicosis Investigation Group (2005). Aflatoxin Contamination of Commercial Maize Products during an Outbreak of Acute Aflatoxicosis in Eastern and Central Kenya. Environ Health Perspect. 113: 1763-1767.

Liu, Z., J. Gao and J. Yu (2006). Aflatoxins in stored maize and rice grains in Liaoning Province, China. J Stored Prods Res. 42: 468–479.

Lutfullah, G. and A. Hussain (2012). Studies on contamination level of aflatoxins in some cereals and beans of Pakistan. Food Cont. 23: 32-36.

Paster, N (1995). Fungi in stored grain and animal feeds: their occurrence and harm caused to animals. Israel J Vet Med. 50(2): 49–53.

Pearson, T.C., D.T. Wicklow, E.B. Maghirang, F. Xie and F.E. Dowell (2011). Detecting Aflatoxin in single corn kernels by transmittance and reflectance spectroscopy. Trans ASAE (Am Soc Agric Eng). 44(5): 1247–1254.

Pitt, J.I (2000). Toxigenic fungi & mycotoxins. Br Mel Bul. 56: 184-192.

Prasanna, B.M., S.K. Vasal, B. Kassahun and N.N Singh (2001). Quality protein maize. Current Sci. 81(10, 25): 1308-1319.

Shah, H.U., T.J. Simpson, S. Alam, K.F. Khattak and S. Perveen (2010). Mould incidence and mycotoxin contamination in maize kernels from Swat Valley, North West Frontier Province of Pakistan. Food Chem Toxicol. 48: 1111–1116.

Tariq, M and M. Iqbal 2010. Maize in Pakistan – an overview. Kasetsart J. (Nat. Sci.) 44: 757- 763.

Wagacha, J.M and J.W. Mothomi (2008). Mycotoxin problem in Africa: current status, implementations to food safety and health and possible management strategies. Int J Food Microbiol. 124: 1-12.

Wild, C.P and Y.Y. Gong (2010). Mycotoxins and human disease: a largely ignored global health issue. Carcin. 31(1): 71–82.

Williams, R.J and D. Macdonald (1983). “Grain molds in the tropics: Problems and importance”. Annual Reviews of Phytopathology 21(1): 153-178.

Williams, J.H., T.D. Phillips, P.E. Jolly, J.K. Stiles, C.M. Jolly and D. Aggarwal (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr. 80(5): 1106–1122.

Yazdanpanah, H., M. Miraglia, F.R. Calfapietra and C. Brera (2001). Natural occurrence of aflatoxins and ochratoxin an in corn and barley from mazandaran and golestan in north provinces of Iran. Mycotoxin Res. 17:21–30.