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Aflatoxin Contamination in Chilies from Punjab Pakistan with Reference to Climate Change

SHAHZAD ZAFAR IQBAL[1], IJAZ AHMAD BHATTI, MUHAMMAD RAFIQUE ASI†, HAQ NAWAZ BHATTI AND MUNIR AHMAD SHEIKH
Department of Chemistry and Biochemistry, University of Agriculture Faisalabad, 38040, Faisalabad, Pakistan
†Nuclear Institute for Agriculture and Biology (NIAB) Faisalabad, 38000, Pakistan
[1]Corresponding author's e-mail: shahzad10542005@yahoo.com

ABSTRACT

High prevalence of liver cancer is caused by aflatoxins in developing countries. The consumption of AFs contaminated foodstuff by humans or animals results in several carcinogenic diseases including liver cancer. Also, there is a need to know base line levels of aflatoxin in crops to know when abnormal concentrations may occur, for example, from climate change. Total aflatoxins were determined in 156 chili samples from Pakistan by reverse phase HPLC with florescence detection in work undertaken in that country. The limits of detection and quantification for aflatoxin B_1 and aflatoxin G_1 were 0.05 and 0.5 μg kg^{-1}, while for aflatoxin G_2 and aflatoxin B_2 they were 0.1 and 0.60 μg kg^{-1}. Total aflatoxins were determined in whole (n = 78) and ground (n = 78) chilies and the concentration were high in many cases. Aflatoxins were detected in 26 (33%) of whole chilies: concentration range was from 0.00 to 81.5 μg kg^{-1}. The equivalent values for ground chilies were 31 (40%) and 0.00 to 84.8 μg kg^{-1}. The percentage of samples greater than the European Union statutory limit for AFB_1 and total aflatoxins were 26 and 19%, respectively.

Key Words: Aflatoxins; Chilies; Liver cancer; Climate change

INTRODUCTION

Aflatoxins (AFs) are a group of mycotoxins formed principally by *Aspergillus flavus* and *A. parasiticus* (Paterson, 2007; Paterson & Lima, 2010a, b). Fungal spices like *A. bombycis*, *A. ochraceoroseus*, *A. nomius*, *A. niger*, *A. ruber*, *A. wentii*, *Penicillum citrinum* and *P. frequentans* and *A. pseudotamari* are less commonly encountered production of aflatoxins (Hussein & Brasel, 2001: Peterson *et al.*, 2007). AF due to their carcinogenic, teratogenic, genotoxic, immunotoxic and mutagenic properties are toxic to animals and human (Creppy, 2002). The major AFs are characterized as B_1, B_2, G_1 and G_2 (based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography (Paterson 2007; Iqbal *et al*. 2010a, b; Zain, 2010).

AFs are major concern with respect to public health, due to their potential as powerful hepatotoxins and carcinogens in humans and their proven toxicity to animals, birds. Hepatitis is a toxic form of Aflatoxicosis and in severe cases, leads to jaundice or even death. Worldwide each year, approximately 25,200–155,000 new HCC cases occur, which may be attributable to aflatoxin exposure. It has been observed that HCC and AFs have synergistic effect on liver and thus, cause liver cancer (Iqbal *et al*., 2010b).

Approximately, 5–10% of agricultural products worldwide are spoiled by fungi, to the extent that crops cannot be consumed by human or animals (Topal, 1993). The situation is serious particularly in developing countries such as Pakistan, where there are poor production practices [i.e., good agricultural practice (GAP) & good harvesting practices (GHP) are not adhered to]. In addition, there is inadequate storage, transportation and marketing conditions, which contribute to mould growth and increase the risk of mycotoxin contamination. Moulds are distributed widely as environmental contaminants and under favorable conditions of temperature and humidity, grow on commodities including spices (Brera *et al*., 1998) and produce mycotoxins.

The effects of climate change on crops are well recognized, but on mycotoxins such effects are only recently being considered (Sultan & Hanif, 2009; Khan *et al.*, 2010; Paterson & Lima, 2010b). The effect of climate change on mycotoxins is extremely complex, which involves many factors. The consequences in countries with hot climates, such as Pakistan, will be serious as increases in temperatures are predicted. However, at high temperatures, it is possible that mycotoxin concentrations will decrease due to lack of fungal growth. The situation requires to be monitored and a prerequisite is to obtain base line data of aflatoxin concentrations in crops to enable to determine the effect of future alterations in climate.

There are numerous chromatographic methods for the

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analysis of AFs (Turner *et al*., 2009). However, the most suitable ones are HPLC with florescence detection and HPLC mass spectrometry (LC-MS) (Blesaa *at al*., 2003; Ventura *et al*., 2004; Paterson, 2007; Iqbal *et al*., 2010). However, HPLC with florescence remains the most widely used method and in various food products. It is probably the minimum level of equipment required to provide confidence in the data obtained. However, even these methods are beyond the capabilities of many developing countries, where the mycotoxin problem is more severe.

Chili (*Capsicum annuum* L.) is one of the most valuable crops in Pakistan. Issues relating to local production practices are available in (Paterson, 2007; Iqbal *et al.,* 2010a, b & c). Chilies are particularly susceptible to aflatoxin contamination surveys have been reported from the UK (MacDonald & Castle, 1996), Portugal (Martins *et al*., 2001), Spain (Santos *et al*., 2010), India (Reddy *et al*., 2001), Hungary (Fazekas *et al.,* 2005), Ireland (O'Riordan & Wilkinson, 2008) and Pakistan (Paterson, 2007; Iqbal *et al*., 2010a, b & c).

The European Union has set strict limitations on AFs levels in various foodstuffs, such as groundnuts, nuts, dried fruits, cereals, milk and spices, including paprika and chili (Commission Regulation, 2010). The chili crop appears to be susceptible to aflatoxin contamination particularly in Pakistan. For example, 22 chili powder and whole samples demonstrated high concentrations of AFB_1 in some cases, ranging from 0.00 to 89.5 µg kg^{-1} and 0.00 to 96.3 µg kg^{-1}, respectively (Iqbal *et al*., 2010a). In previous finding, sample of chilies from rural semi-urban and urban areas have shown high contamination of AFs from Punjab Pakistan (Iqbal *et al*., 2010b). The deficiencies in production methods described above for mycotoxins, certainly lead to increased aflatoxins and growth of the relevant fungi (e.g., *A. flavus*). It has been observed that seasons may provide favorable conditions for AFs contamination in chilies during storage or selling (Iqbal *et al.,* 2010c). There is a general requirement for more information about the level of contamination within various regions of Pakistan to obtain a clear impression of the severity of the problem leading to the undertaking of remedial action. An essential step is for trained personnel within the country to carry out sample collection and aflatoxin analysis at a satisfactory level of competence.

The situation concerning AFs contamination of chilies in Pakistan is being understood increasingly from work as described in a series of recent papers (Paterson, 2007; Iqbal *et al.,* 2010 a, b & c). However, there is a requirement for information regarding the analysis of total aflatoxins in different regions. The present study describes the analysis of total aflatoxins in different regions of Punjab with large number of samples.

MATERIALS AND METHODS

Samples: Powdered and whole samples (total 156) were collected randomly from markets, herbal shops and chili growing areas during June 2008 to January 2010 from central cities of Punjab, Pakistan. It is well known that aflatoxins are heterogeneously distributed. Samples of ground chilies were taken as 0.5 kg each and due to uneven distribution of fungi in whole chilies, sample size were taken as 1kg each. Samples were stored at -4°C in sealed plastic bags until analyzed.

Chemicals and regents: Standard 2 µg mL^{-1} solutions of AFB_1, AFG_1 and 0.5µgml^{-1} of AFB_2 and AFG_2 and MycoSep column 226 (AflaZone) were purchased from Romers Labs, USA. HPLC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich Chemical USA. All other chemicals and organic solvents were at least analytical grade.

Extraction and purification: Extraction and purification of samples were carried as described (Iqbal *et al*., 2010a, b & c). Briefly, whole chili samples were ground to uniform consistency and already ground chilies (25 g) were extracted with 100 mL of acetonitrile-water (86:14; v/v) by shaking for 35 min at 50 rpm in 250 mL glass flasks fitted with stoppers. The solutions were filtered through Whatman No.5 papers. To each of 9 mL portions of the filtrates, 70 µL acetic acid was added, transferred to MycoSep columns and passed through at 2 mL min^{-1}. A 2 mL aliquot was evaporated to dryness at 40°C in a centrifuge glass tube for pre column derivatisation. For pre column derivatisation TFA (100 µL) was added to the residues or aflatoxin standards to derivatize AFB_1 and AFG_1 however, AFB_2 and AFG_2 samples were not derivatised as this is un-necessary. The samples were left at room temperature for 20 min in the dark. Acetonitrile:water (1:9, v/v) (0.4 mL) was added to each tube. The injection volume for the HPLC was 20 µL.

HPLC conditions: The mobile phase was acetonitrile-methanol-water (20:20:60; v/v/v), which was degassed by sonication. The HPLC was a Shimadzu (Keyto) Japan product with a Supelco C_{18} column (Discovery HS) with fluorescence detector (RF- 530). Excitation and emission wavelengths were 360 and 440 nm, respectively. The flow rate was 1 mL min^{-1} and the column was maintained at 40°C. The injection volume was 20 µL.

Validation of HPLC: LOD and LOQ were 0.05 and 0.53 µg kg^{-1} for AFB_1 and AFG_1, and 0.1 and 0.60 for AFB_2 and AFG_2. LOD was calculated with a signal to noise ratio (S/N=3) and LOQ (used S/N=10). The recoveries of the AFs are provided in Table I. The recovery study was performed by adding 2, 5 and 10 µg kg^{-1} of each AF standard to uncontaminated chilies. The spiked samples of control chilies provided high levels of recoveries of all AFs. The standard graph of AFB_1 and AFG_1 were linear at seven concentration between 1-100 µg/kg using the equations $y = 4305.9 x - 8321.7$, where R^2 is 0.99 and $y = 2165.5 x + 25885$, where R^2 is 0.98, respectively. The equivalent for AFB_2 and AFG_2 were linear for six concentrations between 0.5-12 µg/kg using the equations $y = 5475.2 x + 3362$,

where R^2 is 0.98 and y = 3944.5 x + 3220.5, where R^2 is 0.99, respectively. (y = area & x = concentration). This method demonstrated good repeatability and intra-laboratory reproducibility.

Analytical quality assurance: To ensure the accuracy of our data samples were collected by trained personal from the various locations and in rural areas, farmers were visited and interacted with directly to the obtain samples, which they stored. The samples were analyzed as an individual batch. Standards curves were used for quantification. Standard commercial mixtures of aflatoxins were run and the concentrations compared, before and after analyses by HPLC. Blank samples were run three times before, during and after the experiment to assure the results. Certified reference material (CRM) was not available to our laboratory in Pakistan. However, spiked samples with known concentrations of aflatoxins were tested (as given above). The laboratory has no ISO 17025 accreditation status and it has not participated regularly in proficiency testing at present. CRMs, ISO 17025 and proficiency testing will be introduced as the laboratory seeks accreditation.

Statistical analysis: The student paired t test was applied to analyze the differences between sampling regions and AF level and chilies type. Regression analysis was applied to calculate R^2 data and was expressed with mean standard deviation using SPSS software IBM SPSS (PASW Statistics 18).

RESULTS AND DISCUSSION

Twenty six (33%) of samples were positive in whole chilies with concentrations ranging from 0.00 to 81.5 µg kg^{-1}, compared to 31 (40%) for ground chilies and ranging from 0.00 to 84.8 µg kg^{-1} (Table II). However, 18 (23%) and 23 (30%) of whole and ground chilies, respectively had levels of AFB_1 above the EU statutory permissible limit. As presented in Table III, the mean total AFs in whole and ground chilies were 19.4 ± 0.78 and 21.1 ± 1.2 µg kg^{-1}, respectively. Total AFs concentration were found to be higher than the permissible EU limit in 12 (15%) and 18 (23%) of wholes and ground chilies, respectively implying that the problem is more severe in ground chilies.

These high concentrations of aflatoxins represent a health risk for the population of Pakistan and to countries to where the chilies are exported. Chilies represent another source of aflatoxins and liver cancer as it is very popular in various foods, although perhaps not consumed at equal rates in Pakistan to maize or, in particular, peanuts (Liu & Wu, 2010). In addition, the presence of AFs in chilies is problematic for the successful export of chilies to the EU and other countries. Chilies from Pakistan have been banned for export to Europe and Japan precisely, because of high aflatoxin concentrations. This lack of export revenue is likely to have detrimental effects on the health of the population through having less money to purchase food, housing essentials, medicines (Paterson, 2007). On the other hand, exporting good quality chilies would imply that the remaining crop would be of a higher aflatoxin concentration and so it is important to decrease concentration throughout the system. An increased ability to undertake aflatoxin analysis in Pakistan would be very beneficial, where there is currently limited information and capability.

Fortunately, the amount of information concerning aflatoxins in Pakistani chilies is beginning to increase through various initiatives. Paterson (2007) analyzed 13 chili samples from Pakistan and found that 8 (73%) of ground chilies had a higher concentration of AFs than the EU permissible limit of 10 µg kg^{-1}: the highest concentration was 96. 2 µg kg^{-1}. Furthermore, Iqbal *et al*. (2010) analyzed 22 ground and 22 whole chili samples in Pakistan from which 8 (36%) had a higher concentration than EU limits. The highest concentration was 96.3 µg kg^{-1}. In ground chilies 12 (55%) samples were at a higher concentration than EU limits, with the highest concentration of 89.6 µg kg^{-1}. In the present work, the presence of positive samples was lower than those of Iqbal *et al.* (2010a) or Paterson (2007). However, the highest concentration of total AFs was similar to those in the other reports (Ahmad & Ahmad, 1995; Fufa & Urga, 1996; Reddy *et al.*, 2001; Bircan, 2005; Colak *et al.*, 2006; Aydin *et al.*, 2007).

There is a general requirement to know what the base line levels of aflatoxin in crops are to enable assessments as to what represents abnormal concentrations under changing climate effect (Paterson, 2010; Paterson & Lima, 2010). Paterson and Lima (2010 b, c) suggested that aflatoxin levels may decrease and that the presences of the relevant *Aspergillus* species may decrease, or even become extinct, as the temperatures will simply become too high. Recently, nearly 54°C has caused a heat wave in the country (UK daily Guardian, http://www.guardian.co.uk/). When such temperatures increase a near Pasteurization effect may occur in some areas reducing the contamination levels (Paterson & Lima, 2010). Obviously, there will be many more problems apart from aflatoxin to contend with in these weather conditions. Nevertheless, the situation required to be monitored.

The situation in Pakistan may be improved by adopting GAP and GHP. Trader and exporters can usefully consider using more suitable methods for transporting and storage of this commodity, with refrigeration and rapid transport obvious options. Some basic steps to avoid AF contamination are to use good quality fruits and seeds. Also highly contaminated chilies and those with obvious fungal growth should be discarded. Humidity levels need to be controlled. There is a requirement to investigate AFs contamination in varieties of chilies and those varieties which resistant to the fungi should be tested as possible producing crops. Related to this, Santos *et al*. (2010) noted that chilies with a high concentration of capsaicin (the pungent component of chilies) had lower concentrations of AFs and further studies are needed to confirm this effect.

Table I: Recoveries of aflatoxins from spiked chilies

Spiked Level (µg kg^{-1})	AFB_1		AFB_2		AFG_1		AFG_2	
	[a]Mean Recovery (%)	RSD (%)	[a]Mean Recovery (%)	RSD (%)	[a]Mean Recovery (%)	RSD (%)	[a]Mean Recovery (%)	RSD (%)
2	90.7	7.1	87.0	9.15	90.0	6.7	91.5	7.5
5	90.0	8.9	91.8	7.82	87.7	5.7	91.8	5.6
10	90.7	6.3	90.0	8.11	91.3	8.3	90.0	8.9

Table II: Incidence and range of total aflatoxins level in whole and ground chilies from different regions of Punjab (*n*= sample size)

Sample Category	n	Positive Samples	AFB_1		Total AFs		Range of AFs	RSD
		n (%)	*n* > 5 (µg kg^{-1}) %	*n* > 5 (µg kg^{-1}) %	*n* > 10 (µg kg^{-1})%	*n* > 10 (µg kg^{-1}) %	(µg kg^{-1})	%
Whole Chilies	78	26 (33)	8 (10)	18 (23)	14 (18)	12 (15)	0.0- 81.5	4.0
Ground chilies	78	31 (40)	8 (10)	23 (30)	13 (17)	18 (23)	0.0- 84.8	5.3

Table III: Mean of total aflatoxin levels and individual aflatoxin levels in positive samples of whole and ground chilies from different regions of Punjab

Sample Category	n	Positive samples	[a]Mean AFs (µg kg^{-1} ± SD	[a]Mean of individual Aflatoxins (µg kg^{-1}) ± SD			
				AFB_1	AFB_2	AFG_1	AFG_2
Whole chilies	78	26	19.4 ± 0.8[b]	17.4 ± 0.5	0.5 ± 0.1	1.2 ± 0.2	0.4 ± 0.1
Ground Chilies	78	31	21.1 ± 1.1[c]	18.5 ± 0.6	0.5± 0.2	1.7 ± 0.3	0.3 ± 0.1

[a]Mean of positive samples, [b,c] Values in columns not sharing a common superscript alphabet differ significantly, $p < 0.05$

Interestingly, Paterson (2007) indicated no relationship between the number of *A. flavus* colonies isolated from chilies and the concentration of AFs and this relationship also requires further investigation.

CONCLUSION

AFs contamination in Pakistani chilies was 8 times higher than that recommended by the EU in some cases. This poses a considerable health threat to people in Pakistan. High standards are required to be attained to enable exports to be accepted abroad and for the health of nation.

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