

## Aflatoxin concentrations in chilies vary depending on variety

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**Abstract** Aflatoxin (AF) contamination was determined in 40 chili samples, representing 3 varieties from Punjab, Pakistan, by HPLC with fluorescence detection. Total AF were determined in varieties Longi ( $n = 15$ ), Wonder hot ( $n = 12$ ), and Skyline 1 ( $n = 13$ ), and the concentrations were high in some samples. AF were detected in 9 (60%), 8 (67%), and 7 (54%) of Longi, Wonder hot, and Skyline 1 samples, respectively; concentration ranged from 4.7 to 34.6, 1.0 to 14.3, and 7.8 to 15.6  $\mu\text{g}/\text{kg}$ , respectively. The percentage of samples greater than the European Union statutory limit for AFB<sub>1</sub> and total AF in spices were 53% and 53%, 53% and 8%, and 53% and 38% for Longi, Wonder hot, and Skyline 1, respectively. Significant differences at  $\alpha < 0.05$  between mean concentration of AF in Longi ( $15.9 \pm 0.6 \mu\text{g}/\text{kg}$ ) and Wonder hot ( $5.8 \pm 0.3 \mu\text{g}/\text{kg}$ ) were determined. In conclusion, choosing the variety demonstrating the lowest AF concentration from various chili samples will assist in improving product quality. This preferred variety is Wonder Hot as described herein.

**Keywords** Aflatoxins · Chilies · HPLC · Wonder hot

### Introduction

Mycotoxins are toxic chemical substances that are produced by a variety of fungi and which contaminate food (Garner et al. 1993; Paterson and Lima 2010). Aflatoxins (AF) are dangerous natural carcinogens, and aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is the most potent natural carcinogen for animals and humans. The most common AF are B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>, which often co-occur in food and represent total AF. Species within the genus *Aspergillus*, specifically *Aspergillus flavus* and *Aspergillus nominus*, produce these mycotoxins (Bennett and Klich 2003; Aydin et al. 2007).

Chilies are susceptible to AF contamination (Macdonald and Castle 1995; Martins et al. 2001; Reddy et al. 2001; O’Riordan and Wilkinson 2008) and as discussed in (Paterson 2007) and (Iqbal et al. 2010a, b, c, d). Briefly, the tolerance limit of AFB<sub>1</sub> in chilies is 5  $\mu\text{g}/\text{kg}$  and for total AF is 10  $\mu\text{g}/\text{kg}$  (Commission Regulation 2010). However, Pakistan does not have statutory standards or regulations for this commodity. Consumption of the contaminated commodity is detrimental to the health of the indigenous population, and the commercial potential of chilies is compromised, which severely restricts exports. Indeed, the European Union (EU) and Japan have banned recently the import of chilies from Pakistan because of the high AF content.

The high concentration of AF in Pakistani chilies was confirmed (Paterson 2007; Iqbal et al. 2010a), and the effect of location of production (Iqbal et al. 2010b) and seasonal effect on AF contamination (Iqbal et al. 2010c) were investigated by the authors of the present paper. In general, samples from urban settings had lower AF

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concentrations. Most importantly, the analysis of the chilies was undertaken in Pakistan rather than elsewhere (e.g., the United Kingdom) in a recently established laboratory where a higher level of control of the analyses is obtained. Information about the effect of other factors on the level of AF contamination is required if the chilies are to be produced to acceptable standards. The objective of the present work was to determine the relationship between AF contamination and chili variety as part of continuing studies.

## Materials and methods

### Samples

Chili samples (40) of three varieties were collected from different areas between April and December 2009 in Punjab, Pakistan. The varieties were Longi, Wonder hot, and Skyline 1, belonging to *Capsicum annuum* L. In the present work, these three chili varieties were analyzed because they are grown frequently in different areas of the Punjab and Sindh provinces of Pakistan. Longi, Wonder hot, and Skyline 1 are renowned for their pungent odor. Wonder hot is the most pungent with a pungency of 40,000–50,000 Scoville heat units. The varietal status of each was confirmed by Dr. Muhammad Rafique Asi, Department of Plant Protection Division, Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan. Samples were stored at  $-4^{\circ}\text{C}$  in sealed plastic bags until analysis.

### Chemicals and reagents

Standard 2  $\mu\text{g}/\text{ml}$  solutions of AFB<sub>1</sub> and AFG<sub>1</sub>, 0.5  $\mu\text{g}/\text{ml}$  of AFB<sub>2</sub> and AFG<sub>2</sub>, and MycoSep column 226 (AflaZone) were purchased from Romers Labs, USA. High pressure liquid chromatography (HPLC) grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany), and trifluoroacetic acid (TFA) was obtained from Sigma–Aldrich Chemical USA. All other reagents and chemicals used were of analytical grade unless otherwise stated.

### Extraction and purification

Extraction and purification of samples were carried out using the method of Iqbal et al. (2010a, b, c, d). Briefly, 25 g whole or ground chilies was extracted with 100 ml 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 was added 70  $\mu\text{l}$  acetic acid, and each was transferred to MycoSep columns and passed through at 2 ml/min. A 2-ml aliquot was evaporated

to dryness at  $40^{\circ}\text{C}$  in a glass centrifuge tube for precolumn derivatization. Precolumn derivatization was carried out by adding 100  $\mu\text{l}$  TFA to the residues or AF standards to derivatize AFB<sub>1</sub> and AFG<sub>1</sub> and convert them into hemiacetals of AFB<sub>2a</sub> and AFG<sub>2a</sub> by adding a water molecule to the lactone ring (Iqbal et al. 2010c). The samples were allowed to stand at room temperature for 20 min in the dark. Then, 0.4 ml acetonitrile:water (1:9, v/v) was added to the tube. A 20- $\mu\text{l}$  portion of the solution was subjected to LC analysis.

### Liquid chromatography conditions

The mobile phase was acetonitrile:methanol:water (20:20:60, v/v/v), which was degassed by sonication. The samples were analyzed on reverse-phase HPLC (Shimadzu, Kyoto, Japan) with a Supelco C18 column (Discovery HS, Bellefonte, PA, USA) equipped with a fluorescence detector (RF-530). Excitation and emission wavelengths were 360 and 440 nm, respectively. The flow rate was 1 ml/min, and the column was maintained at  $40^{\circ}\text{C}$ . The injection volume was 20  $\mu\text{l}$ .

### Statistical analysis

All the data were presented as mean  $\pm$  SD and analyzed in triplicate; one-way analysis of variance (ANOVA) was applied to the data from the three varieties to determine the significance of the results. Scheffe and Tamhane tests were used for significant variance between the varieties, and regression and correlation analysis was used to determine  $R^2$  using SPSS software (IBM SPSS; PASW Statistics 18).

## Results and discussion

Limits of detection (LOD) and limits of quantification (LOQ) were 0.05 and 0.50  $\mu\text{g}/\text{kg}$  for AFB<sub>1</sub> and AFG<sub>1</sub>, respectively, and 0.10 and 0.60  $\mu\text{g}/\text{kg}$  for AFB<sub>2</sub> and AFG<sub>2</sub>, respectively. LOD was calculated with a signal-to-noise ratio of 3:1 ( $S/N = 3:1$ ) and LOQ at  $S/N = 10:1$ . The standard graph of AFB<sub>1</sub> and AFG<sub>1</sub> was linear at seven concentrations between 1 and 100  $\mu\text{g}/\text{kg}$  using the equations  $y = 3,902.3x - 2,524.2$  where  $R^2$  is 0.99, and  $y = 16,367.9x + 13,456$  where  $R^2$  is 0.99, respectively. The equivalents for AFB<sub>2</sub> and AFG<sub>2</sub> were linear for six concentrations between 0.5 and 12  $\mu\text{g}/\text{kg}$  using the equations  $y = 3,824.6x + 1,234$  where  $R^2$  is 0.99 and  $y = 19,234.5x + 1,512.9$  where  $R^2$  is 0.98, respectively ( $y = \text{area}$  and  $x = \text{concentration}$ ). The recovery study was performed on three separate days by adding 2, 5, and 10  $\mu\text{g}/\text{kg}$  of each AF standard to uncontaminated chilies. The spiked samples of control chilies provided high levels

**Table 1** Recoveries of aflatoxins with spiked concentration and precision data in chilies

	Spiked level ( $\mu\text{g}/\text{kg}$ )	Recovery (%)	RSD (%)	Linearity ( $\mu\text{g}/\text{kg}$ )	LOD ( $\mu\text{g}/\text{kg}$ )	LOQ ( $\mu\text{g}/\text{kg}$ )	Precision (%RSD)	
							Repeatability <sup>a</sup>	Reproducibility <sup>a</sup>
AFB <sub>1</sub>	2	89.7	2.5	1–100	0.05	0.50	4	6
	5	91.0	3.1	–	–	–		
	10	86.7	1.9	–	–	–		
AFB <sub>2</sub>	2	89.0	1.4	0.5–12	0.10	0.60	3	7
	5	92.3	2.1	–	–	–		
	10	90.0	1.1	–	–	–		
AFG <sub>1</sub>	2	90.0	1.7	1–100	0.05	0.50	4	5
	5	87.7	1.7	–	–	–		
	10	88.5	2.5	–	–	–		
AFG <sub>2</sub>	2	87.4	2.3	0.5–12	0.10	0.60	4	8
	5	90.4	2.4	–	–	–		
	10	89.9	3.5	–	–	–		

AF aflatoxins, RSD relative standard deviation, LOD limit of detection, LOQ limit of quantification

<sup>a</sup> Repeatability and reproducibility are given as mean percent RSD at 2, 5, and 10  $\mu\text{g}/\text{kg}$  levels

of recoveries of all AF. When spiking with 2, 5, and 10  $\mu\text{g}/\text{kg}$  of AF, the recoveries showed 89.7%, 91.0%, and 86.7% for AFB<sub>1</sub> and 89.0%, 92.3%, and 90.0% for AFB<sub>2</sub>, respectively, whereas AFG<sub>1</sub> and AFG<sub>2</sub> were 90.0%, 87.7%, and 88.5% and 87.4%, 90.4%, and 89.9%, respectively (Table 1). This method demonstrated good repeatability and intralaboratory reproducibility.

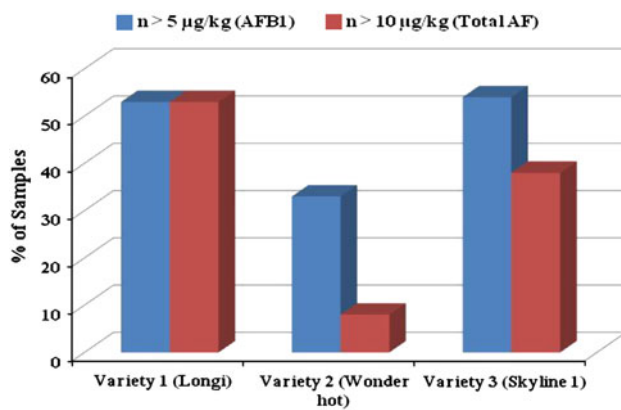
Table 2 summarizes the AFB<sub>1</sub> and total AF concentrations in the three varieties. In Longi, these concentrations were 4.7–30.8 and 4.7–34.6  $\mu\text{g}/\text{kg}$ , respectively; in Wonder hot, 1.00–14.3 and 1.00–14.3  $\mu\text{g}/\text{kg}$ , respectively; and in Skyline 1, 7.5–13.4 and 7.80–15.60  $\mu\text{g}/\text{kg}$ , respectively. There was a significant difference between Longi and Wonder hot ( $P = 0.009$ ;  $P < 0.05$ ), but there was no significant difference of variance between Longi and Skyline 1 ( $P = 0.287$ ;  $P > 0.05$ ). Tamhane analysis gave similar results to the Scheffe test.

Figure 1 compares the samples for AFB<sub>1</sub> and total AF that were higher than the EU and United States Department of Agriculture maximum limits for spices. Sixty percent of samples from Longi were positive for AF and 53% were higher than the permissible limits for AFB<sub>1</sub> and total AF. For Wonder hot, 67% of samples were positive; 33% and 8% of samples were higher than the limits for AFB<sub>1</sub> and total AF, respectively. Fifty-four percent of samples of Skyline 1 were positive; 54% and 38%, respectively, of samples were higher than the limits.

AF contamination is a serious constraint for effective chili production in Pakistan. Very high levels of AF were also observed by Paterson (2007) and Iqbal et al. (2010a), and further investigations are required to determine what

**Table 2** Total and individual concentration ( $\mu\text{g}/\text{kg}$ ) of aflatoxins in three varieties of chilies

	Concentration ( $\mu\text{g}/\text{kg}$ )					
	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFG <sub>2</sub>	Total AFs	
Variety 1	11.9	n.d.	2.30	n.d.	14.2 $\pm$ 0.76	
	9.80	n.d.	2.90	n.d.	12.7 $\pm$ 0.92	
	7.50	1.50	2.50	n.d.	11.5 $\pm$ 0.56	
	30.8	1.40	2.40	<LOQ	34.6 $\pm$ 1.12	
	4.70	n.d.	<LOQ	n.d.	4.70 $\pm$ 0.11	
	12.8	n.d.	n.d.	n.d.	12.8 $\pm$ 0.34	
	24.5	n.d.	n.d.	n.d.	24.5 $\pm$ 0.98	
	14.1	<LOQ	1.50	<LOQ	15.6 $\pm$ 0.78	
	12.6	n.d.	n.d.	n.d.	12.6 $\pm$ 0.23	
	Variety 2	9.00	n.d.	n.d.	n.d.	9.00 $\pm$ 0.21
5.60		n.d.	2.90	n.d.	8.50 $\pm$ 0.34	
2.00		n.d.	<LOQ	<LOQ	2.00 $\pm$ 0.31	
14.3		n.d.	n.d.	n.d.	14.3 $\pm$ 0.79	
1.00		n.d.	n.d.	n.d.	1.00 $\pm$ 0.09	
5.00		n.d.	n.d.	n.d.	5.00 $\pm$ 0.15	
3.40		n.d.	n.d.	n.d.	3.40 $\pm$ 0.39	
3.00		n.d.	n.d.	n.d.	3.00 $\pm$ 0.21	
Variety 3		11.2	1.50	2.90	n.d.	15.6 $\pm$ 0.67
		13.4	n.d.	1.10	n.d.	14.5 $\pm$ 0.62
	12.5	n.d.	<LOQ	n.d.	12.5 $\pm$ 0.89	
	7.50	1.10	3.60	1.20	13.4 $\pm$ 0.52	
	9.60	n.d.	n.d.	n.d.	9.60 $\pm$ 0.31	
7.80	n.d.	n.d.	n.d.	7.80 $\pm$ 0.49		
11.7	n.d.	n.d.	n.d.	11.7 $\pm$ 0.53		



**Fig. 1** Comparison of percent (%) in samples of three chili varieties with aflatoxins (AF) higher than United States Department of Agriculture (USDA) and European Union (EU) and permissible limits (5 µg/kg for AFB<sub>1</sub> and 10 µg/kg for total AFs)

caused these high concentrations. In the present findings, Wonder hot has significantly lower AF contamination compared with the other varieties. Wonder Hot is the most pungent of the chilies: the pungency of chilies results from the presence of capsaicin. Further studies involving the determination and isolation of capsaicin from chilies would be helpful to obtain and use good-quality seeds. In the present study, the results show that Wonder hot is more resistant against AF contamination compared to Longi or Skyline 1, which will be informative for farmers wishing to cultivate a better crop and to prevent AF contamination. Moreover, Santos et al. (2010a, b) documented that chilies with high concentrations of capsaicin had lower AFs and OTA contamination.

In conclusion, Iqbal et al. (2010b) observed that chilies from urban production sites had lower AF concentrations. The present work establishes that the Wonder hot variety had reduced AF. In another study, seasons may affect AF contamination and favors fungal growth. The chili samples collected in the summer had a higher level of AF contamination compared to samples in the winter season (Iqbal et al. 2010c). Hence, these factors, established by empirical research, can be employed in the production of chilies. The parameters will also be relevant to other countries that produce chilies, and should be considered, together with the more intuitive protocols described in Paterson (2007) and Iqbal et al. (2010a, b, c, d), when attempting to reduce AF in this commodity. Work is continuing by us to establish scientifically other factors that may be involved in producing chilies with reduced AF concentrations.

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