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ORIGINAL PAPER

Aflatoxin B_1 in chilies from the Punjab region, Pakistan

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Abstract The occurrence of aflatoxin B1 (AFB1) in chilies from Pakistan was determined by using HPLC in work undertaken in Pakistan. Whole (n=22) and powdered (n=22) chilies were analyzed. Sixteen (73.0%) and 19 (86.4%) samples of whole and ground chilies, respectively, were contaminated. The mean concentration in powdered chilies (32.20 μg/kg) was higher statistically than in whole chilies (24.69 μg/kg). Concentrations ranged from 0.00 to 89.56 μg/kg for powdered chilies, compared with 0.00–96.3 μg/kg for whole chilies. The limits of detection and quantification were 0.05 μg/kg and 0.53 μg/kg, respectively. The concentrations were high in general and greater than the statutory limit set by the European Union. There is considerable scope for improvements in chili production in Pakistan.

Keywords Aflatoxins · Chilies · HPLC · Pakistan

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Introduction

Chilies account for 16% of the world spice trade, placing the commodity second after black pepper. The cash crop is one of the most valuable in Pakistan, where two varieties are grown, *Capsicum annum* and *Capsicum frutescens*. The share of chilies in the country's GDP is 1.5%. Pakistan was reported as the sixth largest exporter of chilies in the world (Abrar et al. 2009). However, other data indicated that India is the major exporter of the crop and the levels of other exporting countries were China (24%), Spain (17%), Mexico (8%), Pakistan (7.2%), Morocco (7%) and Turkey (4.5%), making Pakistan fifth (Arifeen 2009). Chili was grown in Pakistan in 473,000 hectares, with a production of 70,000 tonnes, and a yield of 1.5 tonnes per hectare until 7 years ago. Sindh province contributed 82%, Punjab 10.6% and Balochistan 6.1% of the total production. However, the areas producing chilies and their production levels have fallen by 9.2 and 14.2%, respectively; average yield has dropped 5.5%. It is possible to speculate that this is from an inability to compete with countries producing higher quality material (Arifeen 2009). The European Union and Japan banned the import of chilies from Pakistan due to aflatoxin (AF) problems. Chili peppers are an essential spice used as an enormously popular, basic ingredient of cuisines worldwide, which add tang, taste and color to food. *Capsicum* species are employed whole or ground, and alone or in combination, with other flavor ingredients (Kothari et al. 2010). There are approximately 27 species, of which five are cultivated worldwide, i.e. *C. annuum L*, *C. frutescens Mill*, *Capsicum baccatum L.*, *Capsicum chinense* and *Capsicum pubescens* (Csillléry 2006).

Chilies are subject to various pest and disease constraints to optimal production. The contamination of the crops with AF from the growth of *Aspergillus flavus* and/or *Aspergillus nominus* is one of the most serious problems (Paterson

2007). AFs are the most dangerous mycotoxins and are extremely carcinogenic (Paterson and Lima 2010). The chili crop appears particularly to be susceptible to this problem. For example, chili powder had the highest mean concentration of nine spices, with one chili sample having the highest value of all at 27.5 μg/kg (O'Riordan and Wilkinson 2008). This particular problem may be from a lack of infrastructure in developing countries to monitor and control fungal and mycotoxin concentrations. Reduced implementation of good harvesting practices (GHP), improper storage, inadequate transportation and marketing conditions can contribute to *Aspergillus* growth and increased risk of AF contamination. These problems are more likely to occur in developing countries (e.g., Pakistan). Generally, tropical conditions, such as high temperatures and moisture, unseasonal rains, monsoons, and flash floods, lead to fungal propagation and production of AFs (Turner et al. 2009; Bhat and Vasanthi 2003). Temperatures suitable for growth of *A. flavus* vary from minima of 10.0–12.8°C to maxima of 43.0–48.8°C, with an optimum of approximately 33.8°C (Pitt and Hocking 2009). AF production is "permitted" at 28°C and completely inhibited at 37°C, which is close to the growth optimum (Paterson and Lima 2009). Marín et al. (2009) determined that a water activity (a_w) of between 0.82 and 0.88 was the minimum required for growth of *A. flavus* on chili powder, hence obtaining moisture levels that would result in an a_w below these figures is desirable.

The EU has a stipulated maximum residual level (MRL) of AF for spices at 5 μg/kg for AFB_1 and 10 μg/kg for total AFs ($B_1 + B_2 + G_1 + G_2$) (Commission Regulation 2002). However, Pakistan and the United States of America do not have statutory standards or regulations for this commodity (Paterson 2007). Surprisingly little information exists on the extent of AF contamination in commercial chili powder (O'Riordan and Wilkinson 2008). What information exists is provided in Table 2. There is even less information on AF in chilies from Pakistan.

Chili samples were contaminated with *A. flavus* from Karachi, Pakistan (Shamshad et al. 1985). Paterson (2007) demonstrated the presence of *A. flavus*, AFB_1 and AFB_2 in chili samples bought randomly from the market place in Pakistan in the first such report from that country. The concentrations of AF were often beyond the EU's maximum limit for spices, and, interestingly, the number of *A flavus* strains isolated was not related to the concentration of AF. The highest value was 93.00 μg/kg of AFB_1 in powdered samples with a mean value of 32.11 μg/kg for powder and pods.

The samples from Pakistan in the report by Paterson (2007) were obtained more than 10 years ago and more recent information is required. In addition, the analysis was undertaken in the United Kingdom and there is an urgent requirement for this capability to be available in Pakistan. Hence, an assessment of the current situation is provided in the present report focusing on the concentrations of the most dangerous example, AFB_1. The chilies were from and analyzed in, Pakistan. In addition, considerably more samples were analyzed than by Paterson (2007).

Materials and methods

Samples

Powdered and whole samples (total 44) of predominantly *C. annuum L* were collected randomly from markets, herbal shops and chili-growing areas between June and December 2008 in Faisalabad, Punjab, Pakistan. Samples were stored at −4°C in sealed plastic bags until analysis.

Chemicals and regents

Standard solutions of 50 μg/ml AFB_1 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). All other chemicals and organic solvents were at least analytical grade.

Extraction and purification

Extraction and purification of samples were carried out using a slightly modified method of Hiroshi et al. (2001). The whole chili samples were ground to uniform consistency in a coffee mill. Samples of these and the 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 a stoppers. The solutions were filtered through Whatman No.5 papers. To 9-ml portions of the filtrates, 70 μl acetic acid was added ; the mixture was then transferred to MycoSep columns (product code 226) and passed through at a flow rate of 2 ml/min. The AF passed through the column. A 2-ml portion of each eluate was taken and evaporated to dryness at 40°C in a centrifuge glass tube for pre column derivatization.

Pre column derivatization

A 100-μl volume of TFA was added to the residues or AF standards to derivatize AFB_1. The sample was allowed to stand at room temperature for 20 min in the dark. A 0.4-ml sample of acetonitrile/water (1:9 v/v) was added to the tube.

Table 1 Recoveries of AFB_1 from spiked chilies

AF	Spiked level (μg/kg)	Mean recovery (%)[a]	RSD (%)
AFB_1	2	89.2	2.91
	5	93.8	2.95
	10	95.3	3.94

[a] Mean recoveries were calculated by analyzing three replicate samples at each spiked level

A 20-μl portion of the sample was subjected to HPLC analysis.

LC conditions

The mobile phase was acetonitrile/methanol/water (20:20:60 v/v/v), which was degassed by sonication. The HPLC (Shimadzu, Kyoto, Japan) was fitted with a Supelco C_{18} Column (Discovery HS) with a fluorescence detector (RF-530). Excitation and emission wavelengths were 360 nm 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

Validation of the HPLC procedure was carried out by determining the limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were 0.05 and 0.53 μg/kg, respectively. While LOD was calculated as signal-to-noise ratio (S/N)=3 and LOQ as S/N=10. The precision and accuracy of this procedure was examined with the recoveries of AFB_1 as shown in Table 1. The recovery study was performed by adding 2, 5 and 10 μg/kg AFB_1 to unaffected chilies. The spiked samples of control chilies shown good recoveries of AFB_1. The standard curve

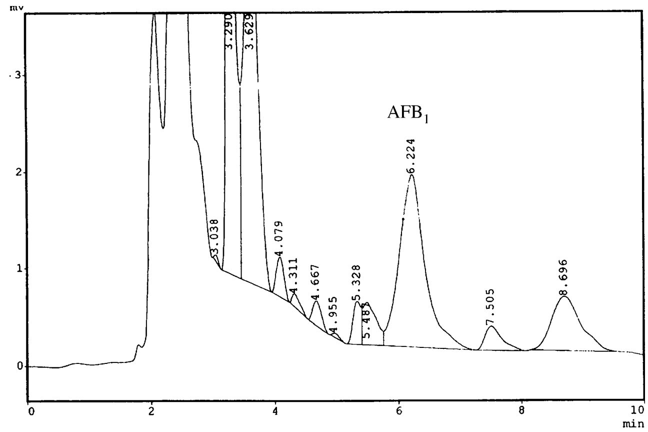

Fig. 2 Chromatogram of sample extract of naturally contaminated whole chilies showing AFB_1 concentration (8 μg/kg)

was linear at five concentrations between 5 and 100 μg/kg using the equation $Y = 2281.1X - 7535.1$, where R^2 is 0.991, Y = area and X = concentration. This method showed good repeatability and intra-laboratory reproducibility as 5 and 9% RSD. Chromatograms of AF standard, whole chilies and ground chilies are shown in Figs. 1, 2 and 3 respectively.

Results and discussion

The recoveries of control standard chili samples at 2, 5 and 10 μg/kg were 89.2±2.91, 93.8±2.95 and 95.3±3.94%, respectively. A comparison of the concentration of AFB_1 in whole and ground chilies is presented in Fig. 4. The values are high and somewhat similar. Table 2 summarizes the AFB_1 data in all samples. There is significant difference of variance $p<0.05$ ($\alpha=0.05$) by applying paired Student *t*-test. Ground chili samples showed the highest mean concentration of AFB_1 (32.20±9.15 μg/kg) compared with

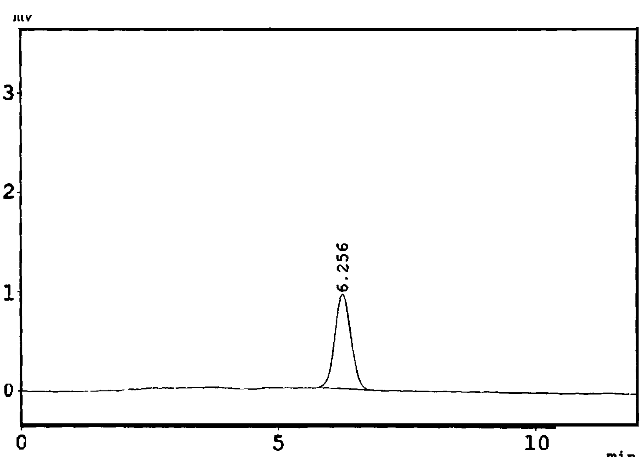

Fig. 1 Chromatogram of AFB_1 standard showing concentration (5 μg/kg)

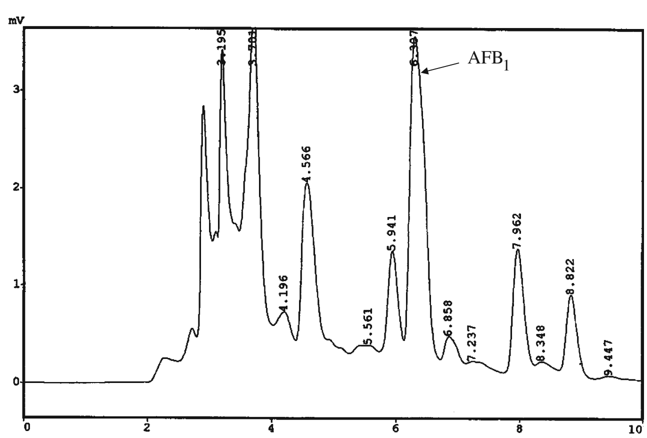

Fig. 3 Chromatogram of a sample extract of naturally contaminated ground chilies showing AFB_1 concentration (11.5 μg/kg)

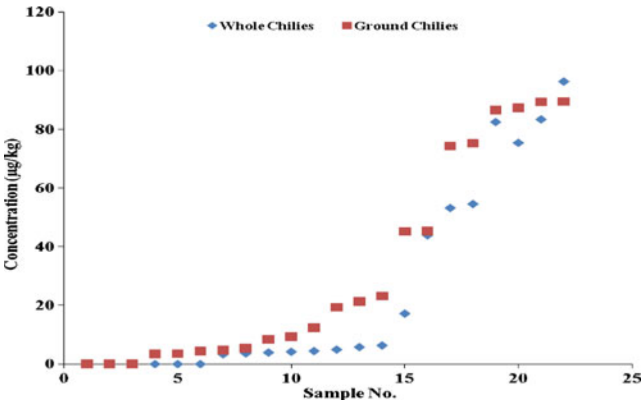

Fig. 4 Comparison of AFB_1 contamination in whole and ground chilies

whole chilies (24.69±8.19 µg/kg). The number of whole chilies with at least 5 µg/kg AFB_1 (the EU limit) was 16 (73%) with a range of 0.00–96.3 µg/kg; 19 (86.4%) of the ground chili samples (range from 0.00 to 89.56 µg/kg) were contaminated with AFB_1. The mean contamination of AFB_1 has highest in ground chilies. The highest concentration in ground chilies was 89.56 µg/kg, which is 18-times higher than the EU permissible limit.

AFB_1 contamination in whole and ground chilies was very high in the present findings and when compared with the other studies (Table 2). Pakistan has not yet established permissible limit for this important spice (Manual of Food Law in Pakistan 2004). Paterson (2007) analyzed 13 chili samples from Pakistan and found that eight (62%) had an AF level above the permissible limit of EU. The highest concentration of AF was found to be 96.2 µg/kg in ground chili samples collected from Karachi, Pakistan, which is in agreement with the present study of chilies from the Punjab.

The high AFB_1 level represents a significant constraint on exports to countries such as those in the European Union if this is found to be the general situation for Pakistan. Similarly, the levels represent a threat to the health of the indigenous population as AFB_1 is very toxic (Paterson and Lima 2010). The conditions and methods for chili production in Pakistan appear to be favorable to AF contamination (see Paterson 2007) and the situation could be improved by adopting good agricultural practices. Traders and exporters require to consider using more suitable methods for transporting and storage of this commodity, with refrigeration and rapid transport being obvious options. Some basic steps to avoid AF contamination are to use good quality pods. Highly contaminated, or chilies with obvious fungal growth, should be discarded. Humidity levels need to be controlled. Finally, a more extensive survey of Pakistani chilies for AF is merited.

There is a great potential for boosting production and export of chilies by Pakistan if they can be produced with low AF contamination. Abrar et al. (2009) indicated that gamma irradiation decreased total AF by 11% in Pakistani chilies, although such decreases were not observed by previous authors using other commodities and that pure AF was destroyed only at high doses of irradiation. Preventative measures require to be adopted. These may be picking and drying the fruit with the pedicel (fruit stalk), avoiding direct contact of fruit with soil, properly drying fruit and maintaining storage of powder at low humidity and temperature. Only high quality manure and water should be used on plants. There is a need to use local hybrids which may be more adapted to local conditions than imported ones. There exists currently a dehydration system which helps maintain the quality of the commodity in India

Table 2 AFs in chilies taken in the present and previous studies (*n* sample size)

Type	Form of AF	*n*	Positive samples	*n* (%) ≥ 5µg/kg	*n* (%) > 10µg/kg	Max µg/kg	Range	Reference	Place
Chilies 1	Total AF	50	50	2 (4)	9 (18)	14.8	0.05–14.8	Macdonald and Castle (1996)	UK
Chilies 2		14	14	1 (7)	3 (21)	50	0.05–50		
Chilies	AFB_1	8	3	–	–	2.5	1.00–2.50	Martins et al. (2001)	Portugal
Chili powder	AFB_1	43	17	12 (28)	5 (12)	283	0.00–283	Reddy et al. (2001)	India
Whole chilies		124	87	47 (38)	40 (33)	969	969		
Cold-store chilies		15	3	2 (13)	1 (7)	–	0.00–30		
Chilies	AFB_1	5	2	1 (20)	1 (20)	8.1	0.00–8.1	Fazekas et al. (2005)	Hungary
Chili powder	Total AF	28	8	–	–	13.8	0.00–13.8	UK Food Standards Agency (2005)	UK
Ground chilies	AFB_1 & AFB_2	9	9	1 (11)	8 (73)	96.2	6.8–96.2	Paterson (2007)	Pakistan
Chili pods		4	4	3 (75)	1 (25)	6.6	0.1–6.6		
Chili powder	AFB_1	30	10	–	–	27.5	0.00–27.5	O'Riordan and Wilkinson (2008)	Ireland
Whole chilies	AFB_1	22	16	6 (38)	8 (36)	96.3	0.00–96.3	Present data	Pakistan
Ground chilies		22	19	4 (21)	12 (55)	89.6	0.00–89.6		

and such a system may be suitable for Pakistan. Arifeen (2009) reports that Pakistani farmers have installed 588 units of solar dryers for chilies on an experimental basis. According to the chili growers, the quality of the solar dried chili has improved immensely, especially after taking the technical advice from the Agribusiness Support Fund with respect to harvest and post-harvest practices, including the washing of the produce before drying. The price of their produce has risen from Rs 1,800 to Rs 2,800 per 40 kg on the open market. The drying time has been reduced from 12 to only 5 days, resulting in a large reduction in labor costs. More units of solar driers require to be tested. If possible, the farmers' associations need to arrange meetings with their Indian equivalents to understand how India (or other countries) manage to obtain high quality chilies.

Conclusions

The contamination with AFB_1 of Pakistani chilies was found to be 18-times higher than that recommended by the EU in some cases. This is unacceptable if the country is to compete in the modern open market place, and moreover, has negative health implications for the population. More careful production of chili products in Pakistan is required. It is necessary now that a more comprehensive survey is undertaken to assist the farmers, traders and exporters in controlling AF in Pakistan. Having the analytical facilities in the country is a great advantage in this respect. High standards are required to be attained to enable exports to be accepted abroad as indicated in the recent UK Food Standards Agency (2005) report and for health reasons in general.

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