Food Additives and Contaminants: Part B
Vol. 3, No. 4, December 2010, 268–274

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Survey of aflatoxins in chillies from Pakistan produced in rural, semi-rural and urban environments

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(*Received 27 July 2010; final version received 30 August 2010*)

Chilli peppers from Pakistan are consumed locally and also exported. Their quality is compromised by aflatoxins (AF) contamination. AF in chillies from rural, semi-rural and urban areas of the Punjab region of Pakistan were determined. Twenty-three (52.3%), 22 (50%) and 29 (65.9%) samples from rural, semi-rural and urban areas respectively, contained levels of aflatoxins which exceeded the European Union limits of $>5\,\mu g\,kg^{-1}$ for AFB_1 and $>10\,\mu g\,kg^{-1}$ for total AF that apply to spices. Mean values for AFB_1 in ground samples were 23.8, 14.8 and $14.0\,\mu g\,kg^{-1}$ for rural, semi-rural and urban areas, respectively. Mean total AF in ground samples were 27.7, 17.7 and $16.2\,\mu g\,kg^{-1}$ from equivalent locations. Eleven (50%), 12 (54.5%) and 14 (63.6%) whole samples from rural, semi-rural and urban areas, respectively, contained total levels of AF that exceeded European Union limits. The data indicate that individual localities have particular problems. In conclusion, the concentrations were often greater than the statutory limits set by the European Union.

Keywords: ingredients; aflatoxins

Introduction

Aflatoxins (AF) are human liver carcinogens (Liu and Wu 2010). The mycotoxins pose a threat to human and animal health because they are toxigenic, carcinogenic, mutagenic and teratogenic (Paterson and Lima 2010). They are produced by the fungi *Aspergillus flavus*, *Aspergillus nominus* and *Aspergillus parasiticus* in, *inter alia*, maize, spices and nuts: the most active form is AFB_1. Furthermore, AF occur in foods in combinations that may exert a greater degree of damage to health than being present individually.

Liver cancer (hepatocellular carcinoma (HCC)) is the third highest cause of cancer deaths, with a frequency 16–32 times higher in developing countries. Approximately 25,200–155,000 new cases occur worldwide each year, which may be attributable to AF exposure from peanut and maize consumption: This has most often been observed in people infected with the hepatitis B virus (HBV). These problems are much more severe in Pakistan than in Western Europe and North America, although not so serious as in Sub-Saharan Africa. The estimated annual global burdens of HCC cases attributable to AF exposure in HBsAg-positive and -negative populations in Pakistan are 116–832 and 119–851, respectively, where HBsAg is a biomarker of chronic HBV infection (Liu and Wu 2010). In addition, there is a significant economic loss due to food being rejected from importing countries. Pakistani chillies were rejected for import into the European Union and Japan because of high AF concentrations (Iqbal et al. 2010), and there is a requirement to regain these markets.

Chilli peppers are particularly susceptible to AF contamination: surveys have indicated high concentrations from the UK (Garner et al. 1993; Macdonald and Castle 1996; Patel et al. 1996), Portugal (Martins et al. 2001), Belgium (Goryacheva et al. 2006), Spain (Santos et al. 2010), Japan (Tabata et al. 1993), Morocco (Zinedine et al. 2006), the Netherlands (Goryacheva et al. 2006), Australia (Klieber 2001), India (Scott and Kennedy 1973; Reddy et al. 2001), Turkey (Erdogan 2004; Bircan 2005; Colak et al. 2006; Aydin et al. 2007), Hungary (Sawinsky et al. 1988; Fazekas et al. 2005), Russia (Goryacheva et al. 2006), Canada (Scott and Kennedy 1973), China (Hu et al. 2006), Singapore (Scott and Kennedy 1973), Egypt (El-Kady et al. 1995), and Ireland (O'Riordan and Wilkinson 2008). Finally, Paterson (2007) and Iqbal et al. (2010) detected high AF in Pakistani chillies.

The European Union chilli legislative limit for AFB_1 and total AF are 5 and $10\,\mu g\,kg^{-1}$, respectively. However, Pakistan does not have statutory standards

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ISSN 1939–3210 print/ISSN 1939–3229 online

DOI: 10.1080/19393210.2010.520341
http://www.informaworld.com

or regulations for this commodity (Paterson 2007). In developing countries there are poor production practices (i.e. good agricultural practice (GAP) and good harvesting practices (GHP) are not adhered to). In addition, there is inadequate storage, transportation and marketing conditions that contribute to mould growth and increased risk of mycotoxin contamination. Moulds are distributed widely as environmental contaminants and under favourable conditions of temperature and humidity will grow on commodities, including spices (Brera et al. 1998), and produce mycotoxins. Quality also depends on drying: freshly picked chillies have a high moisture content which results in rapid biodeterioration. The moisture content in dried pods should not exceed 10% by weight to avoid fungal activity and AF production. The favourable conditions for *A. flavus* growth are $a_w = 0.85–0.90$ and temperatures = 27–37°C (Marín et al. 2009).

The commercial potential of chillies for developing countries such as Pakistan is compromised by contamination with AF which severely restricts exports. Contamination of chillies from Pakistan was discussed by Paterson (2007) and Iqbal et al. (2010), where concentrations were particularly high. The deficiencies in production methods for chillies lead to increased AF and growth of the relevant fungi (e.g. *A. flavus*); although Paterson (2007) indicated there was no relationship between concentrations of AF and the fungus in chillies from Pakistan. However, the Pakistani crop appears to be particularly susceptible. For example, 22 chilli powdered and whole samples demonstrated high concentrations of AFB_1 ranging from 0.00 to 89.5 $\mu g\,kg^{-1}$ and from 0.00 to 96.3 $\mu g\,kg^{-1}$, respectively, by using HPLC with fluorescence detection in Pakistan (Iqbal et al. 2010). There is a general requirement for more information about the level of contamination within Pakistan to obtain a clear impression of the severity of the problem.

In particular, about the contamination levels from different locations within the production system are required. Remedial action can be focused directly upon the areas of greatest need when such information is obtained. A prerequisite is for trained personal within the country to have the capacity to carry out appropriate methods for the collection of samples and undertaking of AF analyses. There are numerous chromatographic methods available (Turner et al. 2009). The most suitable are HPLC with florescence detection and HPLC mass spectrometry (LC-MS) (Blesaa et al. 2003; Ventura et al. 2004; Paterson 2007; Iqbal et al. 2010). HPLC with florescence remains the most used and is perhaps 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 and making control difficult.

This paper compares the presence of AF in chillies obtained from rural, semi-rural and rural locations of the Punjab region of Pakistan to indicate whether these factors contribute to contamination and to add to the weight of data on overall concentrations. Importantly, the work was wholly undertaken in that country.

Materials and methods

Chilli samples

Samples were obtained from rural, semi-rural and urban areas between April and December 2009 in the Punjab region of Pakistan. Samples were collected randomly, without biases towards good or poor quality, enabling them to be as representative as possible. The sample sizes of whole and ground chillies were 1 and 500 g, respectively. Most chillies grown in Punjab belong to *C. annuum* L. Samples were stored at −4°C in sealed plastic bags until analyses. Rural areas are those in open countryside where there are few facilities; farmers use conventional methods that have existed for decades. Those in urban areas are much more capable of adopting better strategies to improve their crops and yield, such as the use of (1) high-quality seeds, (2) the latest equipment for cultivation and (3) good storage practices (GSP). Semi-rural areas are where some farmers have more sophisticated facilities whilst others use the rural practices. In rural areas open air drying is employed, while in urban areas electric dryers are used.

Chemicals and reagents

Standard 2 $\mu g\,ml^{-1}$ solutions of AFB_1, AFG_1 and 0.5 $\mu g\,ml^{-1}$ of AFB_2 and AFG_2 and MycoSep column 226 (AflaZone) were purchased from Romers 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 of analytical grade.

Extraction and purification

Extraction and purification of samples were carried out using a modified method of Akiyama et al. (2001). The whole chilli samples were ground to uniform consistency in a coffee mill. Samples of these and the already ground chillies (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 9-ml portions of the filtrates were added 70 µl acetic acid, and each was 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.

Pre column derivatisation

Pre-column derivatisation increases the fluorescence response of AFB_1 and AFG_1 where the fluorescence is low or absent. A total of 100 µl of TFA were added to the residues or AF standards to derivatise AFB_1 and AFG_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. A 20 µl portion of the sample was subjected to LC analysis.

HPLC

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 Supelco C18 Column (Discovery HS) with a 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. A typical chromatogram of naturally contaminated Pakistani chilli is provided by Iqbal et al. (2010), and hence is not reproduced herein. The chromatogram is indicative of the general case that pigments from chilli samples did not interfere with the analyses.

Validation of HPLC

LOD and LOQ were 0.05 and 0.53 µg kg^{-1} for AFB_1 and AFG_1, and 0.10 and 0.60 for AFB_2 and AFG_2, respectively. LOD was calculated with a signal-to-noise ratio (S/N) = 3/1 and for LOQ was S/N = 10/1. The recoveries of the AF are provided in Table 1. The recovery study was performed by adding 2, 5 and 10 µg kg^{-1} of each AF standard to uncontaminated chillies. The spiked samples of control chillies provided high levels of recoveries of all AF. The standard graph of AFB_1 and AFG_1 were linear at seven concentration between 1 and 100 µg kg^{-1} using the equations:

$$y = 4103.3x - 2941.2 \quad \text{where } R^2 = 0.98$$
$$y = 15435.2x + 11317 \quad \text{where } R^2 = 0.99$$

where y is area; and x is concentration. The equivalents for AFB_2 and AFG_2 were linear for six concentrations between 0.5 and 12 µg kg^{-1} using the equations:

$$y = 3421.9x + 1798 \quad \text{where } R^2 = 0.99 \text{ and}$$
$$y = 14315.3x + 2014.7 \quad \text{where } R^2 = 0.98.$$

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 obtain the samples they stored. The samples were analysed as an individual batch. Standards curves were used for quantification. A standard commercial mixture of aflatoxins was run and the concentrations compared before and after analyses by HPLC. Blank samples were run three times before, during and after the experiment to ensure the results. Certified reference material (CRM) was not available to our laboratory in Pakistan. However, spiked samples with known concentrations of aflatoxins were tested (see 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

A Student's paired t-test was applied to analyse the differences between sampling regions and AF level and chilli 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).

Table 1. Recoveries of aflatoxins from spiked chillies.

	AFB_1		AFB_2		AFG_1		AFG_2	
Spiked level (µg kg^{-1})	Mean recovery (%)[a]	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)
2	87.5	2.23	85.5	1.34	83.2	3.14	85	2.3
5	86.6	2.14	87.3	1.65	85.4	1.86	89	2.34
10	89.4	1.94	88.8	3.23	87.3	1.65	87	2.84

Note: [a]Mean of three replicates.

Table 2. Mean concentrations of aflatoxin B_1 and total aflatoxins in chillies from rural, semi-rural and urban areas.

Region	Chillies	*n*	Number of AF-contaminated samples	Mean of AFB_1		Total AFs	
				Mean ($\mu g\,kg^{-1}$)[a]	SD	Mean ($\mu g\,kg^{-1}$)[a]	SD
Rural	Ground	22	12	23.83	1.26	27.68[b]	1.34
	Whole	22	11	18.39	1.07	23.02[c]	1.45
Semi-urban	Ground	22	10	14.84	1.14	17.86[d]	1.23
	Whole	22	12	11.00	1.11	14.89[e]	1.16
Urban	Ground	22	15	13.99	0.89	16.20[f]	1.24
	Whole	22	14	11.97	0.76	16.05[g]	1.31

Note: [a]Mean of positive samples.

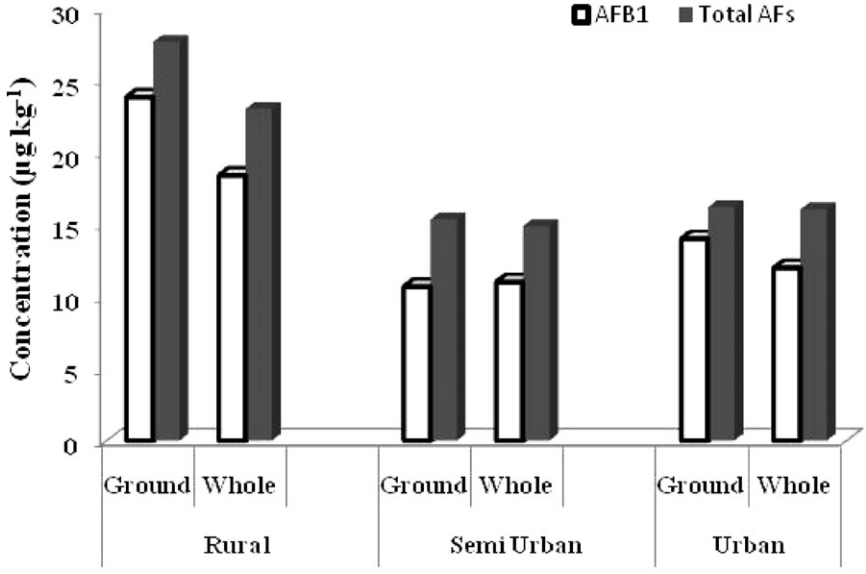

Figure 1. Mean concentrations of AFB_1 and total AF in the chilli samples.

Results and discussion

The mean concentrations of AFB_1 and total AF of the samples are presented in Table 2 and Figure 1. Statistically significant differences exist between sampling areas and AF level ($t=-9.8$ and $p<0.05$ at $\alpha=0.05$) and chilli preparation (i.e. powdered or whole) and AF levels ($t=-8.4$ and $p<0.05$ at $\alpha=0.05$). Twenty-three (52.3%), 22 (50%) and 29 (65.9%) samples from rural, semi-rural and urban areas, respectively, were higher than European Union limits ($>5\,\mu g\,kg^{-1}$ for AFB_1 and $>10\,\mu g\,kg^{-1}$ for total AF) for AF that apply to spices. The totals for ground samples that were higher than European Union regulation for AF were 12 (54.5%), 10 (45.4%) and 15 (68.2%) from the same areas. Mean values for AFB_1 in ground samples were 23.8, 14.8 and $14.0\,\mu g\,kg^{-1}$ for rural, semi-rural and urban areas, respectively. Total AF in ground samples were 27.7, 17.7 and $16.2\,\mu g\,kg^{-1}$ for these locations.

The numbers of samples higher than the European Union limit for AF in whole chillies were 11 (50%), 12 (54.5%) and 14 (63.6%) from rural, semi-rural and urban areas, respectively. Mean AFB_1 values for whole chillies were 18.4, 11.0 and $12.0\,\mu g\,kg^{-1}$ for these samples. Total AF for whole chillies were 23.2, 14.9 and $16.1\,\mu g\,kg^{-1}$ for the equivalent samples.

The frequency of occurrence and ranges of AFB_1 and total AF in rural, semi-rural and urban areas are presented in Table 3. Figure 2 demonstrates the number of samples above the European Union limit in ground and whole chillies, and hence indicates which would be rejected under European Union regulations. In ground chillies from rural areas, 45.5% and 40.9% of samples for AFB_1 and total AF respectively were above the European Union statutory limit. The whole chillies values were 27.3% and 27.3%, respectively. This represents a very large difference and is highly significant in terms of optimising production, i.e. the use of poor-quality whole chillies to produce the powders is a likely contributing factor to high AF in rural areas. The figures were 36.4% for AFB_1 and 27.3% for total AF for semi-rural samples in the case of ground chillies which are intermediate in value for AFB_1. The figures were 40.9% and 31.8% for whole

Table 3. Incidence of aflatoxin B1 and total aflatoxins in chillies from rural, semi-rural and urban areas.

Region	Chillies	*n*	AF-contaminated samples (%)	Range of AFB_1 ($\mu g\ kg^{-1}$)	Percentage age ($n > 5\ \mu g\ kg^{-1}$)	Range of total AFS ($\mu g\ kg^{-1}$)	Percentage age ($n > 10\ \mu g\ kg^{-1}$)
Rural	Ground	22	54.5	0.00–67.33	45.4	0.00–73.6	40.91
	Whole	22	50.0	0.00–60.00	27.3	0.00–70.67	27.3
Semi-urban	Ground	22	45.4	0.00–48.33	36.4	0.00–55.60	27.3
	Whole	22	54.5	0.00–39.00	40.9	0.00–45.67	31.8
Urban	Ground	22	68.2	0.00–45.17	59.0	0.00–55.00	50.0
	Whole	22	63.6	0.00–31.83	50.0	0.00–43.67	40.9

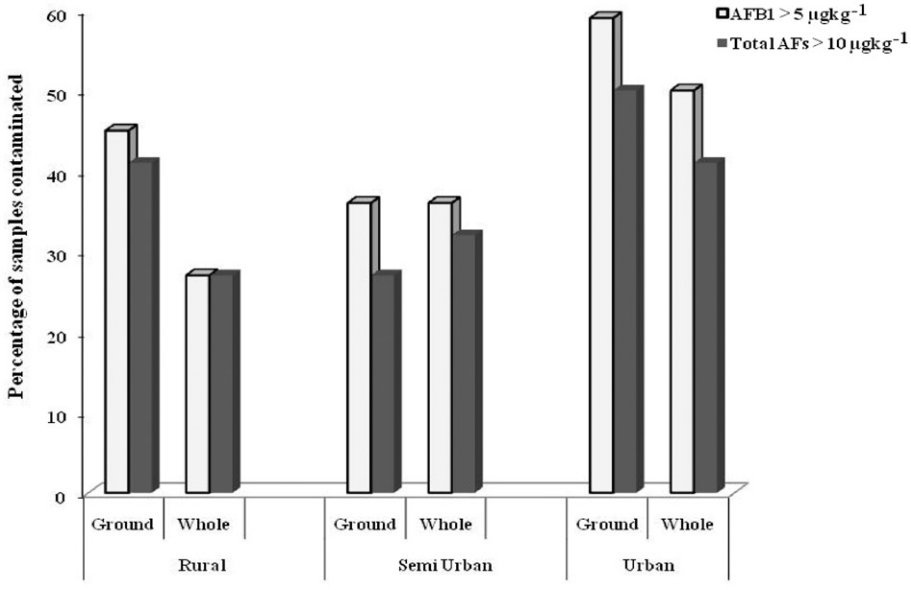

Figure 2. Percentage of chilli samples contaminated at above European Union statutory levels.

chillies, which again are somewhat higher than the rural situation. Interestingly, 59.0% of samples exceeded the European Union limit for AFB_1 and 50.0% were higher than European Union limits for total AF from urban ground samples, whereas 50.0% and 40.9% were the equivalent values for whole chillies. The frequencies of samples with higher than the European Union limit in the case of urban areas are significantly higher than the rural and semi-urban areas. These differences may be from mixing low- and high-quality chillies where the concentrations are kept low (e.g. below European Union statutory levels) at the expense of more frequent contamination of samples.

AF contamination is a serious constraint for effective chilli production in Pakistan. Very high levels of AF were also observed by Paterson (2007) and Iqbal et al. (2010) and further investigations are required to determine what caused these high concentrations. In rural areas farmers employ the direct heat of the sun for drying chillies, whereas solar dryers are more often used in urban locations. There are likely to be other factors that are detrimental to quality in low-technology rural areas that may account for the higher values in the rural samples.

There is a lack of infrastructure in developing countries to monitor and control fungal and AF concentrations. The situation in Pakistan may be improved by adopting GAP and GHP (Iqbal et al. 2010). Traders and exporters need to employ suitable methods for transportation and storage, with refrigeration and rapid transport being obvious options. Some basic steps are to use good-quality pods for the production of powder. Highly AF contaminated, or chillies with obvious fungal growth, should be discarded, although the relationship between the presence of AF producing fungi and AF contamination requires further investigation (Paterson 2007). Drying of chillies after harvesting is also an important factor.

Chillies upon harvesting have a moisture content of 65–80% depending on whether they are partially dried on the plant or harvested while still succulent. Drying the fruit on the plant would appear not to be

best practice purely from a commonsense point of view. Good drying practices need to be adopted that involve spreading chillies thinly and evenly, rather than in uneven clumps. Compacted soil is probably not good practice due the possible presence of aflatoxigenic fungi in the earth. Better would be the use of a concrete plinth if clean and well-swept. A sheet of polythene or a tarpaulin is also recommended, again if these are clean and they should be black to absorb heat and hence generate more heat to dry the chillies. A layer of empty sacks may be placed on the ground on which the chillies can be spread. While drying, the produce can be covered with polythene sheets during the night to avoid dew deposition and humidity. The chillies need to be packaged quickly after drying in a low-humidity environment within a 'moisture barrier', e.g. tea sacks or paper bags with a plastic bag attached to the exterior. Storage temperature below 13°C may decrease AF accumulation. However, this is not essential if the products are dried thoroughly and placed in air-tight packaging. Sound sanitation practices need to be implemented to minimise the level of fungal contamination. Finally, grinding of chillies should only be done shortly before shipment.

Conclusions

More comprehensive surveys of AF in chillies which are produced using various production methods are required. The effect of the harvesting season on chillies in storage should be undertaken, and work is required on whether particular varieties of chillies are more susceptible. A hazard analysis control point approach may be useful, as has been suggested for apple juice (Venâncio and Paterson 2006) and wine production (Martínez-Rodríguez and Carrascosa 2009), although this may be more difficult to implement in developing countries. However, having the analytical facilities in the country is a great advantage in this respect as the various factors can be tested readily in Pakistan and the segregation of contaminated batches undertaken. Importantly, the use of CRMs, obtaining ISO 17025 standards and proficiency testing will be introduced in the laboratory used in the present study as it builds towards full international accreditation.

Furthermore, in-country training courses for farmers are desirable that need to involve how best to grow, harvest, dry, store and transport chillies. Courses for scientists in Pakistan are also necessary concerning fungal identification and analysis of AF. Great care is required if mixing of good- and poor-quality chillies is to be undertaken to ensure that the problem is not simply exacerbated by producing more samples with (some) contamination. Finally, high standards are required to enhance exports and for the sake of the nation's health.

Acknowledgements

The authors acknowledge the financial support of the Higher Education Commission, Pakistan, under Indigenous PhD fellowship 5000 batch-III (Grant Number 063-00445-Ps3-047). R.R.M.P. is grateful for the FCT framework position: Commitment to Science (C2008-UMIN-HO-CEB-2).

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