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Aflatoxins B_1 in different grades of chillies (*Capsicum annum* L.) in India as determined by indirect competitive-ELISA

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Samples of the three grades of chilli pod (grades 1 to 3) were collected during surveys in 1998 and 1999 from the principal market yards and cold storage facilities of the major chilli-growing areas of Andhra Pradesh (AP), India. Chilli powders were collected from different supermarkets in Hyderabad, AP. They were analysed for aflatoxin B_1 (AFB_1) content by an indirect competitive ELISA. To avoid the influence of interfering substances present in chilli extracts, it was necessary to prepare the aflatoxin standards in methanol extracts of chillies free from aflatoxins. For nine representative samples there was good agreement between ELISA and HPLC estimations of AFB_1 and the results suggested that the ELISA procedure adopted was dependable. Of the 182 chilli samples tested, 59% of the samples were contaminated with AFB_1 and 18% contained the toxin at non-permissible levels. The highest AFB_1 concentration of 969 μg/kg was found in one sample representing grade 3. Overall the maximum percentage of chilli pods showing AFB_1 levels higher than 30 μg/kg (non-permissible levels) was in grade 3. Chilli pods stored in refrigerated rooms showed the lowest proportion of samples containing aflatoxin. Nearly 9% of the chilli powders sold in supermarkets contained non-permissible aflatoxin levels. This report highlights the importance of using grade 1 chilli pods to minimize aflatoxin contamination.

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Introduction

Aflatoxin contamination of agricultural commodities has gained global significance as a result of their deleterious effects on human as well as animal health and its importance to international trade. Aflatoxins are potent carcinogenic, mutagenic and immuno-suppressive agents, produced as secondary metabolites by the fungus *Aspergillus flavus* and *A. parasiticus* on a wide range of food products. Many commodities are contaminated by aflatoxins, including cereals, oilseeds and spices (Jelinek *et al.* 1989, Vasanthi and Bhat 1998). Chillies (*Capsicum annum* L.) occupy a prominent place among the spices and form an ingredient of a variety of food preparations in India consumed by the majority of people, including those below the poverty line. India produces 946 000 tonnes of chilli pods from 957 000 hectares. Over 50 000 tonnes valued at more than 40 million US dollars are exported to various countries, including the UK and USA.

Chilli samples representing all the three grades were collected from the major wholesale markets in Andhra Pradesh, India. Grade 3 samples contained over 25% of discoloured pods and were contaminated by *A. flavus*. Because of its low price poor people largely consume grade 3 pods. Chillies are also used extensively in the preparation of chilli powders, sold in the supermarkets as 'Ready to Use' chilli powder. In this paper we report on the estimation of aflatoxins in different grades of commercially available chilli pods as well as commercially available chilli powders by an indirect competitive ELISA. This paper also highlights the importance of using high quality chillies for minimizing aflatoxin exposure.

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Materials and methods

Antibody production

For the production of polyclonal antibodies to AFB_1, commercially available aflatoxin B_1–bovine serum albumin (AFB_1–BSA) from Sigma chemical company was used. Five hundred g of AFB_1–BSA in 0.25 ml of 0.9% NaCl was emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly at multiple sites into the hind leg of a New Zealand White inbred rabbit. For the subsequent four injections at weekly intervals, AFB_1–BSA was emulsified with Freund's incomplete adjuvant. The rabbit was bled and the titre of the antiserum was determined by an indirect competitive-ELISA.

Sample collection and processing for ELISA

During 1998 and 1999, dried graded chilli pod samples were collected randomly from regulated market yards, and cold storage facilities from the major chilli-growing areas of Andhra Pradesh State, India. Ripened chilli pods are normally dried under the sun, often on bare soils, after harvesting. They are sold in the markets after they are graded at commercial outlets into three categories. Grade 1 comprises early-developed reddish-coloured pods, 8–10 cm long without any visual damage and infection from fungi and insects. Grade 2 contains pods 6–8 cm long and can contain up to 20% discoloured pods. Grade 3 pods are 2–5 cm in length, contain more than 40% of discoloured pods, and insect damage and mould growth are apparent. They are packed in 35 kg quantities in loosely woven cloth bags. Three of the principal market yards in Andhra Pradesh were visited and chilli pods of all the three grades were collected. Samples of approximately 500 g were drawn from a 35 kg bag, and the pods were thoroughly mixed. From each 500 g sample, three 50 g sub-samples were drawn and dried at 40 °C for 2 days. Pods were cut into small portions, thoroughly mixed and a 15 g analytical sample was drawn from each of the 50 g sub-samples. The analytical sample was ground in a Waring blender and then utilized for analysis. In addition to pods, chilli powders were also analysed for aflatoxin B_1 content. They were purchased from 10 different supermarkets located in Hyderabad city, AP, and all of them were sold as 'Superior Quality' chilli powders. From each 200 g sample, three 15 g sub-samples were drawn and used for analysis. Each of the 15 g chilli powders (from pods as well as commercially prepared chilli powders) was extracted with 75 ml of a solvent containing 70 ml methanol + 30 ml water + 0.5 g KCl and blended for 2 min. This was followed by shaking in a rotary shaker for 30 min at 250 rpm. The extract was filtered through Whatman No. 41 filter papers and diluted to 1:10 with 0.2% bovine serum albumin (BSA) prepared in 0.05 m PBS-Tween, pH 7.4 (PBST–BSA). Depending on the concentration of aflatoxin, the sample was either used directly or diluted further at 10 fold intervals, prior to analysis by ELISA.

ELISA procedure

An indirect competitive ELISA was used, similar to that reported by Reddy *et al.* (1988), Devi *et al.* (1999) and Thirumala-Devi *et al.* (2000). Prior to utilizing this procedure concentrations of the various reagents required to give optimum results were determined. These include the concentrations of AFB_1–BSA and dilution of polyclonal antiserum and goat antirabbit IgGs labelled with alkaline phosphatase. Maxi-sorp (Nunc A/S, DK-4000 Roskilde, Denmark) ELISA plates were coated with 150 µl/well of AFB_1–BSA at a concentration of 125 µg/ml prepared in carbonate coating buffer (Hobbs *et al.* 1987). At each step plates were incubated at 37°C followed by three washes with PBS–Tween. In the second step plates were treated with PBST–BSA. AFB_1 standards ranging from 0.1 to 100 ng/ml were prepared in extracts (diluted to 10%) from chillies not containing any aflatoxin. Chilli pods (25 g) free of aflatoxin, as determined by HPLC (details given below), were dried at 40°C for 2 days, powdered and extracted with 125 ml of 70% methanol containing 0.5% KCl. The extract was filtered and diluted to 1:10 (= 10% extract) in PBST–BSA. This was used as a diluent for preparing aflatoxin standards. Concentrations of the standards used were 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.097 ng/ml and each concentration was duplicated in two wells. Similarly each test sample was duplicated in two wells. One hundred µl of each of the test sample extract or standards was mixed with 50 µl of antiserum diluted to 1:10 000 in 0.2% PBST–BSA. This step was followed by the addition of alkaline phosphatase labelled goat antirabbit IgG conjugate diluted to 1:2000 in PBST–BSA. The substrate was *p*-nitrophe-

nyl phosphate prepared in 10% diethanolamine. The plates were incubated at room temperature and then read in an ELISA reader (Multiskan plus Labsystems). A maximum interval of 3 h was allowed until optical densities from wells not containing any toxin reached 1.5–2.0 OD units at 405 nm. Log_{10} values of concentration for aflatoxin standards were plotted on the *x*-axis and optical density values were plotted on the *y*-axis. Aflatoxin concentration in the sample extract was determined using the formula: AFB_1 concentration (ng/ml) in sample extract $\times$ α dilution with buffer $\times$ extract solvent volume used (ml) $\div$ sample weight.

Recovery of AFB_1 from artificially-contaminated chilli powders

AFB_1 concentrations ranging from 1 to 250 μg/kg were added to finely ground chilli powders free from aflatoxins. For each aflatoxin concentration a 25 g quantity of chilli powder was used and then analysed by ELISA as described. The aqueous methanol extracts were divided into three aliquots and three samples of each of the aliquot were analysed by ELISA.

Comparison of AFB_1 estimations performed by ELISA and HPLC

To test the accuracy of estimations by ELISA, nine chilli pod samples representing all the three grades, were chosen. Each sample (200 g) was finely ground to a powder and three sub-samples of 25 g quantity were utilized for aflatoxin estimation by indirect competitive ELISA. For aflatoxin analysis by HPLC, the AOAC International (1995a) method was adopted with modifications for extraction and clean-up. Briefly, dried chilli pods were ground to fine powder in a Waring blender and two 25 g sub-samples were used for aflatoxin estimation. Each of the 25 g chilli powders (from pods) were extracted with 125 ml of 85% acetone followed by shaking for 30 min. The extract was filtered, to the filtrate was added 10 ml of 20% lead acetate prepared in 0.3% acetic acid and then it was filtered again. The sample extract was extracted with 50 ml of chloroform in a separatory funnel and the chloroform extract was evaporated on a steam bath. The dried residue was dissolved in 1–2 ml chloroform and further clean-up of the sample was achieved on a silica gel column. HPLC was done using a Shimadzu LC-6A instrument fitted with a fluorescence detector, as described by AOAC International (1995b).

Results and discussion

Determination of optimum conditions for indirect competitive ELISA

The three important components which influence indirect competitive ELISA are aflatoxin B_1–BSA concentration for coating the ELISA plates, antibody dilution required for neutralizing the toxin and dilution of antirabbit IgG labelled with alkaline phosphatase. Three ELISA plates were coated with AFB_1–BSA concentrations ranging from 31 to 1000 ng/ml were used. Two wells of each individual ELISA plate were used for each concentration and optimum AFB_1–BSA was determined by utilizing various concentrations of aflatoxin B_1 standards, from 0.1 to 100

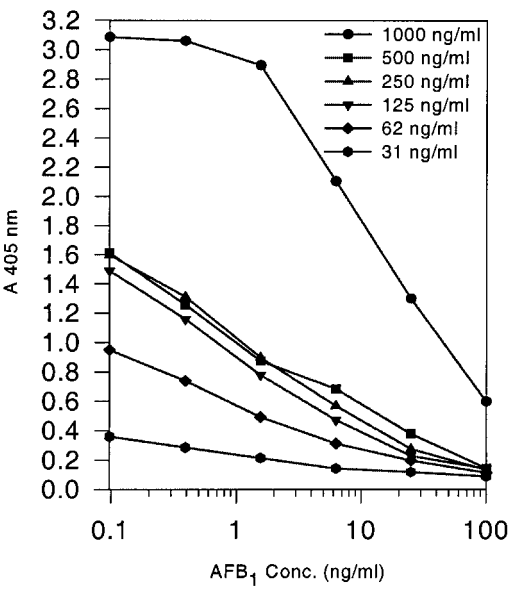

Figure 1. Standard curves for different AFB_1–BSA coating concentrations AFB_1–BSA coating at 1000 ng/ml (●) 500 ng/ml (■) 250 ng/ml (▲) 125 ng/ml (▼) 62 ng/ml (◆) 31 ng/ml (●). Antibodies as well as enzyme conjugates were used at optimum dilution.

ng/ml. Results are presented in figure 1. It is apparent that AFB_1–BSA at 125 ng/ml consistently gave a linear standard curve ($R = 0.99$) when AFB_1 standards of concentrations of 0.1–25 ng/ml were tested. Different workers have employed different AFB_1–BSA concentrations (Candlish *et al.* 1985, Ward *et al.* 1990, Ramakrishna and Mehan 1993); presumably conditions employed for performing ELISA influenced the coating concentration. Although not reported in this paper, we have noticed that two different batches of AFB_1–BSA from Sigma Chemical Company required different concentrations to coat the ELISA plates. Therefore prior to utilizing any AFB_1–BSA in ELISA it is essential to determine the optimum concentration for coating the plates. AFB_1–BSA used for coating the plates and for antibody production was derived from the same batch. As anticipated, polyclonal antiserum also contained antibodies to BSA. Therefore it was essential to block the influence of these antibodies by incubating the antiserum in BSA for 30 min, prior to its utilization.

Optimum dilution of antiserum required for neutralization is expected to depend on the titre of the aflatoxin-specific antibodies. Antiserum employed in this study gave optimum results when diluted to 1:5000 or 1:10 000. The poyclonal antibodies to AFB_1 had cross-reactivities of 10%, 8%, 0.2% and 0.1% to aflatoxin B_2, G_1, G_2 and M_1, respectively.

Effect of chilli extract on the aflatoxin content

It was found in the initial experiments that substances present in chilli extracts interfered in the analysis. This observation was not unexpected because substances that interfered with estimation of aflatoxin content have been reported to occur in chillies (Shantha 1999, Thirumala-Devi *et al.* 2000). In order to eliminate their influence, it was essential to prepare the standards in aqueous methanol extracts of aflatoxin-free chillies (figure 2). Extracts from three different chilli cultivars showed similar matrix effects. The procedure gave precise estimation of aflatoxins down to 10 μg/kg sample. For determining aflatoxin content below this level, it was essential to clean up the sample extract as described by Ramakrishna and Mehan (1993).

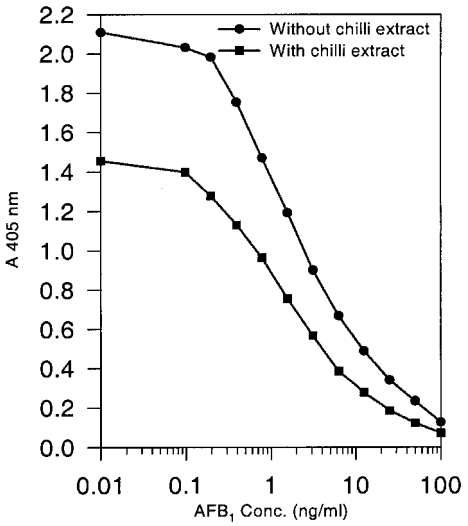

Figure 2. Standard curves for different dilution of AFB_1 standards prepared in the absence (●) and presence (■) of aflatoxin-free chilli extracts.

Recovery of AFB_1 from artificially-contaminated chilli

As determined by ELISA the mean recoveries of AFB_1 from the chilli samples spiked with 1–250 μg/kg of toxin ranged from 67 to 112%. The inter-assay CV was less than 10% for over 90% of the samples. More than 86% of the spiked toxin was recovered from the majority (82%) of the samples (table 1).

Comparison of AFB_1 estimations between ELISA and HPLC methods

In the HPLC analysis only aflatoxin B_1 was detected; other aflatoxins (B_2, G_1 and G_2) were not detectable in the samples tested. Results of comparison of nine samples by ELISA and HPLC methods are presented in table 2. The AFB_1 concentration in these samples ranged from 2 to 283 μg/kg. There was good agreement between ELISA and HPLC estimations in eight of the nine samples tested but significant variation ($P = 0.05$) between the two tests was noticed with one sample. Additionally the inter-assay CV was less than 10.6% with a mean of < 7.4%. In 78% of assays the AFB_1 estimations by ELISA were slightly higher than those by HPLC. Results suggest that the ELISA procedure adopted is dependable.

Table 1. Recovery of aflatoxin B_1 by indirect competitive ELISA from chilli samples spiked with different concentrations of toxin.

AFB_1 added, µg/kg	AFB_1 recovered, µg/kg ± SE (CV%)	Percentage recovery
1	0.67 ± 0.09 (13.23)	66.67
5	3.83 ± 0.34 (8.83)	76.67
10	11.23 ± 0.71 (6.34)	112.33
20	19.87 ± 1.17 (5.89)	99.39
30	27.70 ± 1.39 (5.01)	102.47
50	51.23 ± 0.95 (1.86)	102.47
75	66.63 ± 3.28 (4.93)	88.84
100	101.17 ± 5.67 (5.61)	101.17
150	135.49 ± 8.80 (6.50)	90.79
200	173.03 ± 7.01 (4.05)	86.56
250	225.03 ± 11.38 (5.06)	90.1
Mean of sub-sample CV (%)	6.16	
Mean recovery (%)		92.50

SE = Standard error; CV = coefficient of variation.

Analysis of chilli samples for AFB_1 contamination

The results of the aflatoxin B_1 analysis of chilli pods collected from market yards, cold storage units and chilli powders from supermarkets are shown in table 3. Aflatoxin contamination in chilli pods could be correlated with sample grades (50% in grade 1, 66% in grade 2, 93% in grade 3). Samples stored in cold storage showed the lowest proportion of samples containing aflatoxin. It is noteworthy that the same batch of chilli pods also did not contain detectable ochratoxin A (Thirumala-Devi *et al.* 2000). Of the 59% of the AFB_1-contaminated samples, 41% showed less than 30 µg/kg (permissible levels as reported by Vasanthi and Bhat (1998)) and 18% contained non-permissible levels. The highest concentration of 969 µg/kg AFB_1 was found in one sample, representing grade 3. Nearly 9% of the chilli powders contained more than 30 µg AFB_1/kg. It is likely that the source for preparing these powders may have been chilli pods of grade 3. The same chilli powders that showed high aflatoxin (> 30 µg/kg) content also contained ochratoxin A at levels exceeding 30 µg/kg (Thirumala-Devi *et al.* 2000). It is well known that growth of moulds and consequent mycotoxin production is dependent upon a number of factors such as temperature, humidity, handling during the harvesting and storage (Atanda *et al.* 1990, Mehan *et al.* 1991, Garrido *et al.* 1992). It is apparent that factors contributing to the aflatoxin contamination also favoured ochratoxin A contamination. Therefore grade 3 chillies and some chilli powders currently being marketed in India pose serious health hazards to human beings. High levels of aflatoxin contamination in chillies were reported from Thailand (Shank *et al.* 1972), India (Madyastha 1985) and the UK (MacDonald and Castle 1996). Cultural practices, especially those followed after harvest, which can lead to minimization of mycotoxin contamination, should be investigated. Chilli pods often are wetted by sprinkling with water prior to marketing them. This practice is likely to favour growth of moulds. Therefore guidance on post-harvest handling of chilli pods to farmers as well as traders can greatly help in

Table 2. Comparison of analysis of AFB_1 concentration in naturally-contaminated chilli samples by indirect competitive-ELISA and HPLC methods.

	AFB_1 (µg/kg) ± SE (CV%)		
Sample grades	ELISA ($n = 27$)	HPLC ($n = 18$)	*t*-test
Chilli grade 1	2.07 ± 0.22 (10.58)	1.95 ± 0.15 (7.69)	0.38 NS
Chilli grade 1	2.02 ± 0.31 (8.87)	2.98 ± 0.28 (6.71)	−3.48 S
Chilli grade 1	21.20 ± 1.53 (7.22)	19.88 ± 1.12 (5.63)	0.62 NS
Chilli grade 2	40.70 ± 3.26 (8.02)	37.28 ± 2.15 (4.08)	0.78 NS
Chilli grade 2	77.82 ± 6.51 (8.37)	70.01 ± 4.09 (5.76)	0.76 NS
Chilli grade 2	99.93 ± 7.55 (7.56)	92.87 ± 4.23 (4.54)	0.69 NS
Chilli grade 3	140.10 ± 6.65 (4.74)	129.06 ± 8.06 (6.39)	1.05 NS
Chilli grade 3	188.86 ± 10.73 (5.69)	204.85 ± 9.45 (4.61)	−1.03 NS
Chilli grade 3	283.00 ± 14.84 (5.24)	242.38 ± 11.62 (4.79)	1.93 NS
Mean of sub-sample CV (%)	(7.36)	(5.57)	

NS = Non-significant; S = significant ($p = 0.05$); SE = standard error; CV = coefficient of variation.

Table 3. Aflatoxin B_1 levels in different grades of chilli samples analysed by indirect competitive-ELISA.

Sample grade	Number analysed	No. of samples with aflatoxin B_1 in the range (μg/kg)					
		0	< 10	11–30	31–30	51–100	> 100
Chilli grade 1	42	21	16	3	1	1	0
Chilli grade 2	38	13	10	6	3	4	2
Chilli grade 3	44	3	21	4	3	2	11
Cold store	15	12	2	0	1	0	0
Powders	43	26	12	1	0	3	1

minimizing mould growth. Since chilli is an important high value export commodity, we expect farmers to respond to any improved processing methods which can result in a safe and quality product. Additionally, creation of awareness among the consumers about the presence of mycotoxins in chillies may induce producers as well as traders to market mycotoxin-free chilli pods as well as 'Ready to Use' powders.

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