



Determination of aflatoxin level in peanut using immunoaffinity column combined with ELISA

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Abstract

Peanut (*Arachis hypogaea* L.) is a largest source of edible oil in India, extensively consumed in the central and western parts of the country. The variability in the total aflatoxin and aflatoxin B₁ levels in the different peanut samples collected was investigated. Quantitative analysis of total aflatoxin and aflatoxin B₁ (AFB₁) content was performed by competitive ELISA micro plate reader using total aflatoxin and aflatoxin B₁ test kit. All the seed samples investigated were found positive for aflatoxin. The total aflatoxin content ranged from 24.53 to 250.34 ppb, whereas the concentration of AFB₁ was in the range of 18.55 to 234.50 ppb. More than 86% of samples showed aflatoxin content above regulatory limits. 40% of the samples showed high levels (>100 ppb) indicating high health risk of exposure to aflatoxin. Aflatoxin contamination of peanut seeds and oil is therefore an important public health concern. More precaution should be taken for proper storage of peanut seeds in order to prevent microbiological and chemical hazards.

Keywords: Peanut, *Arachis hypogaea*, aflatoxins, aflatoxin B₁, ELISA

Introduction

The problem of food and feed contamination with toxigenic moulds especially *Aspergillus* species has received a great deal of attention during the last three decades (Ardic *et al.*, 2008; Rustom, 1997). These fungi are capable of growing on a great variety of food commodities and animal feed materials when the conditions of temperature, relative humidity and moisture are favorable (Iqbal *et al.*, 2006; Rosi *et al.*, 2007). Mycotoxins are highly toxic, mutagenic and carcinogenic compounds contaminating a wide variety of agricultural commodities (Bilotti *et al.*, 2000; Abdulkadar *et al.*, 2000; Shenasi *et al.*, 2002; Arrus *et al.*, 2005). Aflatoxins are a group of extremely toxic metabolites produced by some *Aspergillus* species namely *Aspergillus flavus*, *Aspergillus parasiticus* and the rare *A. nomius*, during the growth on food and feeds. *A. flavus* produce only

aflatoxin B, while other species produce both B and G aflatoxins (Sweeney and Dobson, 1998; Creppy, 2002). There are four major aflatoxins referred to as B₁, B₂, G₁ and G₂, which are often found in tropical and subtropical climates (Shenasi *et al.*, 2002). Aflatoxin B₁ (AFB₁) is the most potent of these naturally occurring aflatoxins (Leontopoulos *et al.*, 2003). The peanut (*Arachis hypogaea* L.) which is also popularly known as groundnut is one of the world's most popular and universal crops, cultivated in more than 100 countries of six continents (Patil *et al.*, 2009). Aflatoxin contamination in peanut has been reported in Nigeria (Thomas *et al.*, 2005) and India (Bhat *et al.*, 1996). Earlier workers (Blesa *et al.* 2003; Yentur *et al.*, 2006) have reported a high incidence of occurrence of aflatoxins in peanuts and peanut products such as peanut butter.

Due to their frequent occurrence and toxicity, regulatory agencies are imposing uniformly rigorous standards on the level of acceptance in imported commodities. Acceptable levels regulatory limit of aflatoxins in different countries is shown in Fig. 1 (Anonymous, 2009). Despite lots of studies on aflatoxin in agricultural products, only a few are concerned with peanut that is more

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commonly used in cooking and play an important role in the economy of the country. The result of this study can contribute to the evaluation of peanut consumed by a lot of people in different states of India, from the point of view of food safety. Our aim was to determine the presence and levels of aflatoxins in peanut seed samples and to evaluate whether aflatoxin levels were within the Indian regulatory values or not.

Materials and Methods

Collection of Samples and Fungal counts

Peanut samples (5 kg each lot) were purchased from store merchants and open market vendors of Lucknow. Fifty grams seeds from each lot were drawn in triplicate and dried in an oven at 35 °C till complete removal of moisture content. Dried seeds were ground to fine powder using laboratory grinder and analysed for their aflatoxin content. Fungal count (C.F.U. g⁻¹) from seed samples were made by dilution method on potato dextrose agar using serial dilution following the method of Sidhu *et al.* (2009).

Estimation of aflatoxin

Five grams ground powder of peanut kernel was extracted with 25 mL of 70% aqueous methanol using a laboratory homogenizer and filtered through Whatman #1 filter paper. One hundred microliters of each filtrate was diluted with 600 µL of dilution buffer and 50 µL of diluted sample employed to immunoaffinity column for cleaning the samples. Aflatoxin fraction was finally eluted with 0.5 mL of HPLC grade methanol and total aflatoxin content and aflatoxin B₁ were determined using aflatoxin detection kit obtained from R-Biopharm AG, Darmstadt, Germany. Fifty microliters of standard solution of aflatoxin and eluted samples (in duplicate) were added to the wells of microtiter plate. Further, 50 µL of peroxidase enzyme conjugate and 50 µL of mouse monoclonal anti-aflatoxin antibodies were added to each well. The plates were incubated at room temperature in the dark for 30 min. After washing thoroughly with 250 µL distilled water three times, 50 µL of urea peroxidase (substrate) and 50 µL of tetramethylbenzidine (chromogen) were added to each well, mixed thoroughly and incubated for 30

min at room temperature in the dark. Reaction was stopped by adding 100 µL 1 M sulphuric acid (stop reagent) and the absorbance was measured at 450 nm using Bio-Rad ELISA microplate reader Model 680. There were three replicates for each seed lot investigated in the present study. Kernels of peanut were extracted by cold press expeller (Komet, IB6 Monforts, Germany) to determine the total aflatoxin and aflatoxin B₁ contamination in peanut kernels, cake and oil samples.

Results and Discussion

Quantitation of aflatoxins

Quantitative analysis of total aflatoxin and aflatoxin B₁ (AFB₁) was performed by competitive ELISA Microplate reader using total aflatoxin and aflatoxin B₁ test kit (RIDASCREEN, Darmstadt, Germany). Aflatoxins were separated and purified by immunoaffinity columns and purified fractions were analysed for total aflatoxin content and AFB₁ by antigen-antibody reactions using ELISA. A calibration curve was drawn using a wide range of total aflatoxin standards with concentration of 0–4050 ppt and for aflatoxin B₁ (AFB₁) with concentration of 250–4000 ppt. A plot in between the percentage absorbance and concentration of both the total aflatoxin and AFB₁ for a set of standard indicated a linear relationship (Sidhu *et al.*, 2009). Several methods have been reported for the determination of aflatoxins in a number of food commodities (Gilbert and Anklam, 2002). Methods based on thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) or enzyme-linked immunosorbent assays (ELISA) are mainly used in routine analysis. Nowadays, these methods involve immunoaffinity column cleanup procedures, which offer the extraction of aflatoxins from most food matrices with simple aqueous solvent mixtures (Stroka *et al.*, 2000).

Occurrence of aflatoxin in peanut seeds

Peanut samples were purchased from local markets of Lucknow in triplicate and analyzed for variability in their fungal counts, total aflatoxin and aflatoxin B₁ content. Fungal count, total aflatoxin and aflatoxin B₁ content of the investigated samples are presented in Table 1. Fungal counts ranged from 2.9 x 10³ to 2.5 x 10⁶ C.F.U. g⁻¹ (Table 1). All the seed samples investigated were found positive



for aflatoxin. Out of fifteen seed lots screened for their aflatoxin content, six were found to be contaminated with >100 ppb of total aflatoxin and aflatoxin B₁ content. Total aflatoxin content ranged from 24.53 to 250.34 ppb, the lowest being in Rajajipuram and the highest in Daliganj. The concentration of aflatoxin B₁ was in the range of 18.55 ppb in Rajajipuram to 234.50 ppb in Daliganj (Table 1). Peanut oil was extracted using cold press expeller and the aflatoxin content was determined in peanut oil and cake samples. The concentration of total aflatoxin content was 250.34 ppb in kernels, 205.6 ppb in oil and 40.77 ppb in cake whereas aflatoxin B₁ content was 207.55, 175.6 and 25.88 ppb in kernel, oil and cake, respectively (Fig. 2). by Loosmore *et al.* (1964) and Wagon (1965) have reported aflatoxins contamination in peanut

products, cotton seed cake and other nuts and oilseeds samples. A number of survey and monitoring programs have been carried out in several countries for aflatoxin contamination in food products (Yndestad and Underdal, 1975; Girgis *et al.*, 1977; Tabata and Kamimura, 1988). India has been reported to be a World leader in peanut farming (FAO, 2004). Peanut is the single largest source of edible oils in India and constitutes roughly about fifty percent of the total oilseeds production and extensively used in cooking in India. Whole kernels are used for table purpose by frying, soaking, roasting and boiling and in different types of *namkeens*. Roasted peanut is the most popular way of eating (Patil *et al.*, 2009). Aflatoxin contamination of market peanut, therefore, is an important public health concern.

Table 1 : Fungal count, total aflatoxin, aflatoxin B₁ and total aflatoxin/aflatoxin B₁ ratio in peanut grain samples collected from different markets of Lucknow, India

S.N.	Site of Collection	Fungal count (C.F.U. g ⁻¹)	Total Aflatoxin (ppb)	Aflatoxin B ₁ (ppb)	Total/AFB ₁ Ratio
1	Rajajipuram	2.9 x 10 ³	24.53 ± 0.76	18.55 ± 0.22	1.32
2	Chattarmanzil	2.8 x 10 ⁴	34.40 ± 1.15	28.65 ± 0.26	1.20
3	Rajajipuram	3.4 x 10 ⁵	34.83 ± 0.52	26.65 ± 0.48	1.30
4	Talkatora	6.0 x 10 ⁴	40.26 ± 0.55	32.60 ± 0.65	1.23
5	Hussainganj-I	3.2 x 10 ⁴	45.73 ± 1.73	32.48 ± 0.45	1.40
6	Sikandarbag	3.1 x 10 ⁶	62.30 ± 1.02	54.19 ± 1.05	1.15
7	Rakabganj	2.8 x 10 ⁴	72.40 ± 0.97	66.50 ± 1.10	1.09
8	Hazartganj-I	3.2 x 10 ³	80.64 ± 1.02	64.29 ± 0.78	1.25
9	Hussainganj-II	5.0 x 10 ⁵	83.27 ± 1.95	68.56 ± 1.08	1.21
10	Banthra-II	4.2 x 10 ⁴	126.35 ± 1.11	118.62 ± 1.03	1.06
11	Nishatganj-I	4.2 x 10 ⁵	126.62 ± 2.62	115.65 ± 1.02	1.09
12	Banthra-I	2.5 x 10 ⁶	144.27 ± 1.62	126.58 ± 1.64	1.14
13	Nishatganj-II	5.7 x 10 ⁵	162.18 ± 2.03	144.74 ± 1.07	1.12
14	Gomti Nagar	3.6 x 10 ⁶	164.10 ± 2.04	141.35 ± 1.95	1.16
15	Daliganj	2.2 x 10 ⁶	250.34 ± 2.50	234.50 ± 2.35	1.06

± = standard error

All the samples investigated had aflatoxin levels greater than the Indian regulatory standard of 30 ppb. Forty percent samples had exceedingly high levels (>100 ppb) indicating consumers risk for exposure to high levels of aflatoxin. Bhat *et al.* (1996) reported exceeded permissible Indian regulatory limit of 30 µg/kg aflatoxin in peanut samples. Peanut is prone to attack by various pests and diseases (Ghewande *et al.*, 1987; Subrahmanyam and Ravindranath, 1988). In the

assembling markets, decorticating factories and oil mills, the produce is generally stored in the form of nuts, either loose or in gunny bags in India. The period of storage may be varying from short or it may be stored for several months in anticipation of better prices. Aflatoxin contamination in peanut seed samples screened in the present study may be due to long time under improper storage conditions. High rate of fungal infection in maize (65%), peanut (70%) and soybean (66%) during long time



storage (8-9 months) have been reported by Bhattacharya and Raha (2002). Iqbal *et al.* (2006) have investigated aflatoxin contents of stored cereals and nuts and reported that *Aspergillus* growth and toxin production are directly correlated with increase in humidity. More than 40 ppb of total aflatoxin content in the cake sample indicates a risk for animal health concern using as feed. The regulatory level of aflatoxin B₁ has been set at 5 µg kg⁻¹ for complete feedstuffs for dairy animals by European Union (Delmulle *et al.*, 2005).

Fig. 1. Regulatory limits of aflatoxins.

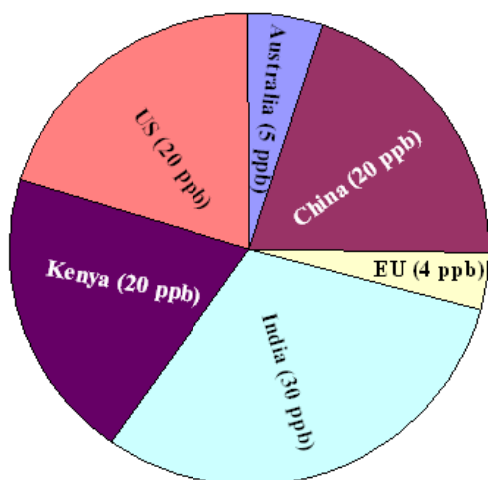
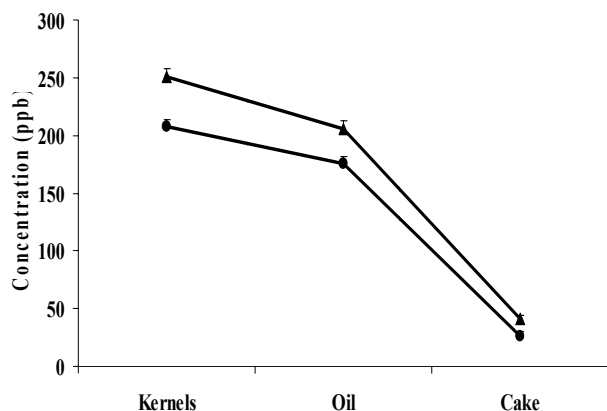


Fig. 2. Total aflatoxin (▲) and aflatoxin B₁ (●) in peanut seeds, oil and cake samples.



Conclusion

All the seed samples investigated were found positive for aflatoxin. More than 86% samples showed aflatoxin content above the regulatory limits used in the European Union and in India. Forty percent samples had exceedingly high levels (> 100 ppb) indicating consumers risk for exposure to high levels of aflatoxin. Aflatoxin contamination of market peanut, therefore, is an important public health concern. Precautions should be taken for proper storage of groundnut seeds in order to prevent microbiological and chemical hazards.

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