INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH

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DETERMINATION OF AFLATOXIN B_1 IN CORN FLOUR USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Baranitharan krishnan, *Sarathchandra Ghadevaru, Manimehalai, N. Athmaselvi, K.A. & Padmavati, R.
Department of Food Process Engineering, SRM University, Kattankulathur, Kanchipuram-603203, Tamil Nadu, India.
*Pharmaco-Vigilance Laboratory for Animal feed and food safety (PLAFFS). Directorate of Centre for Animal Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony,Chennai-600051, Tamil Nadu, India.
Corresponding author's email: sarathchandrag@tanuvas.org.in

ABSTRACT
Aflatoxins are a group of mycotoxins and secondary metabolites of various species of Aspergillus. Aflatoxins cause important health problems and have high potential effect on liver cancer. There are various forms of aflatoxins including B_1, B_2, G_1, G_2, M_1 and M_2 types. Aflatoxin B_1 is most commonly seen in corn, groundnut than in other crops. The aim of this work is to determine the contamination levels in the local and branded corn flour that are marketed in Chennai, Tamil Nadu, India. A total of 40 samples were collected from retail outlets and super markets located in the study area. The Romar's all-purpose method were used for the extraction of total aflatoxins. HPLC was used for the separation process. Out of 40 samples 23 samples (57.5%) have shown positive for aflatoxin (35% samples have shown positive for G_1, 5% samples have shown positive for G_2, 50% samples have shown positive for B_1, 32.5% samples have shown positives for B_2). The overall results of tested samples indicated that contamination of corn flour with B_1 type aflatoxin was very high.

KEYWORDS: Mycotoxin, *Aspergillus*, aflatoxin B_1, HPLC, corn flour.

INTRODUCTION

In the recent years, the agrifood sectors, due to the globalization and development of the new technologies, is undergoing radical changes that require a deeper characterization of food chain, starting from raw material up to the final products. In addition, the consumers are concerned about the food that they eat and ask for more assurances of the quality, the safety and the geographical origin of the products that they consume (Stefano *et al.*, 2012). Mycotoxins occurring in food commodities are secondary metabolites of filamentous fungi, which can contaminate many types of food crops throughout the food chain. Although hundreds of fungal toxins are known, a limited number of toxins are generally considered to play important roles in food safety (Shephard, 2008). The FAO has estimated that up to 25% of the world's food crops are significantly contaminated with mycotoxins (World Health Organization (WHO, 1999). Aflatoxin, perhaps the most famous of all mycotoxins, remains one of the most potent carcinogens of natural origin known to man. In 1952, an outbreak of 'moldy corn toxicosis' was caused by the consumption of mouldy corn by swine in southern USA. Another outbreak in 1960, Turkey 'X' disease, caused the death of 100,000 poults in England. Aflatoxins are potent hepatocarcinogens produced by *Aspergillus flavus, A. parasiticus* and *A. nomius.* Symptoms include anorexia, lethargy, muscle weakness, liver haemorrhages and necrosis, engorged kidneys and liver cancer. There is at least one human case of acute aflatoxicosis (severe hepatitis) in India. Long term effects of diets containing aflatoxin are correlated with high incidence of liver disease in certain regions. While acceptable levels of aflatoxin in food are about 15 ppm, samples of contaminated food in Nigeria measured 100 ppm, maize in India 15,000 ppm and corn in USA reached 320,000 ppm (WHO, 2006; Sean and Abbot, 2002). Aflatoxins occur naturally in most of the commodities; including corn, soybean and peanut which are consumed by human and animal (Hong, 2010). In general, the crops in tropical and subtropical areas with high humidity and temperature are susceptible to contamination by the most dangerous mycotoxins (Thomson and Henke, 2000). These toxins are frequently found in corn and corn products in several parts of the world (Miller 1995, Lazzari 1997). Several outbreaks of mycotoxicoses diseases in human and animals caused by various mycotoxins have been reported after the consumption of mycotoxin-contaminated food and feed (Peraica and Domijan, 2001; Reddy and Raghavender, 2007). Human aflatoxicosis continues to be an accasional serious problem. For example, a severe outbreak was reported in Kenya in 2002 (centers for Disease Control and prevention [CDC], 2004). Maize and groundnuts continue to be the major source of aflatoxin, particularly in India (Sinha, 1990). Sinha 1990, had done survey for three consecutive years in some state of Bihar and revealed heavy infestations of mycotoxin-producing fungi with different maize samples. Aflatoxin-producing fungi had the highest frequency of occurance in all the cases and aflatoxin were the most common mycotoxins. Maize samples of the kharif crop had a greater incidence of aflatoxins (47%) than the samples of Rabi crop (17%). Stored maize grains also had a high incidence of aflatoxins (43%). Most of the contaminated samples contained aflatoxins at levels above 20 ppb. In this background the present study was formulated with the aim of investigating

the toxin level of corn flour marketed in Chennai city, Tamilnadu, India.

MATERIALS & METHODS

Sample preparation

40 samples of corn flour were randomly collected from the super markets and retail outlets of Chennai, Tamil Nadu, India. Each sample was homogenized gently before taking part of it for analysis. Analyses of these samples were carried out in Pharmacovigilance Laboratory for Animal Feed and Food Safety, Madhavaram Milk Colony, Chennai, Tamil Nadu, India.

Aflatoxin analysis

Aflatoxin in corn flour was estimated by AOAC Romar's All Purpose Method (1990). All the chemicals used were of HPLC grade. All the corn flour samples were analyzed by HPLC technique for the presence of aflatoxin. Extraction, filtration, cleanup, partitioning and evaporation were done for each sample as required by Romar's All Purpose Method. After the evaporation, the concentrated extract was reconstituted with 200µl of methanol, vortexed and filtered through 0.45 µm nylon membrane filter prior to HPLC analysis.

Chemicals, Reagents and Chromatographic conditions

All solvents and chemicals used were of analytical grades. Aflatoxin standard were obtained from M/S Sigma Aldrich, U.S.A. Purified extracts were analyzed by reversed-phase isocratic high performance liquid chromatography (HPLC) from Shimazu LC 10A using platinum C18 column (250 × 4.6 mm id, 5 µm) maintained at 40°C. A fluorescence detector was set at 375 nm (excitation) and 440 nm (emission). The mobile phase applied was deionized water/ acetonitrile/ methanol (60:20:20) with flow rate of 1.0 ml/min and injection volume of 20 µL.

Validation of the analytical method

The analytical method was assessed for linearity, recovery, precision and limit of detection before sample analysis.

Estimation of aflatoxins by Hplc

Aliquots of standard aflatoxins G_1, G_2, B_1, and B2 were run first, adopting suitable HPLC conditions. The area under each peak was determined to ascertain concentration versus area. The sample aflatoxin extract was then run under the same HPLC conditions. The concentration of each aflatoxinwas estimated from the area under peaks, relative to those of standards.

RESULTS & DISCUSSION

The Food and Drug Administration (FDA) has established an"Action Level" of 20 ppb for aflatoxin in corn in interstate commerce (Table No.1). In India the regulatory level are set at 30 ppb for all foods. This is the action at which federal agencies may take action including seizure of the corn or prohibition of its sale. Elevators do not accept corn with 20 ppb or more of aflatoxin unless they have a known alternative use (CAST, 2003). The aflatoxin results of the current study were depicted in Table No.2. The results revealed that out of 40 sample, 23 samples (57.5%) were contaminated with aflatoxins either of the aflatoxin (G_1, G_2, B_1, B_2) and out of 23 sample, 13 samples was exceeding the minimum limit set by FDA (Table No.1). In specificaflatoxin G_1 was found in 14 samples (35%) and aflatoxin $G_2$was found in 2 samples (5%). Aflatoxin B_1 was found in 20 samples (50%) and aflatoxin B_2 was found in 16 samples (40%). 57.5% of samples were found to be contaminated with total aflatoxins that might contribute to health hazards for humans. Aflatoxins were detected in corn flour at concentrations ranging from 0.03-119.3 ppb. AFG_1 was detected in 14 samples ranging from 0.90-46 ppb. AFG_2 was detected in 2 samples ranging from 0.68-22 ppb. AFB_1 was detected in 20 samples ranging from 0.25-119.3 ppb. AFB_2 was detected in 16 samples ranging from 0.15-25 ppb. The study results indicated that 30% of tested samples contain aflatoxin level exceeded in the limit set by FDA.

TABLE 1: FDA Regulatory Levels for Total Aflatoxins in Livestock Feeds and Human Food

Commodity	Concentration ppb
All products, except milk, designated for humans	20
Corn for immature animals and dairy cattle	20
Corn and peanut products for breeding beef cattle, swine, and mature poultry	100
Corn and peanut products for finishing swine	200
Corn and peanut products for finishing beef cattle	300
Cottonseed meal (as a feed ingredient)	300
All other feed stuffs	20
Milk	0.5*

Food and Drug Administration (FDA) Compliance Policy Guides 7120.26, 7106.10, 7126.33 (revised 1994); *=Aflatoxin M1

TABLE 2: Number and percent of negative and positive samples

Total Number of samples = 40

S.No	Aflatoxins	% of sample contaminated	% of sample not contaminated
1	AFG_1	14 (35%)	26 (65%)
2	AFG_2	2 (5%)	38 (95%)
3	AFB_1	20 (50%)	20 (50%)
4	AFB_2	16 (40%)	24 (60%)

TABLE 3: Level of Aflatoxins in corn flour collected in Chennai

S.No.	Aflatoxins	Detection limit (ppb)	Obtained range in ppb	Mean	Std.Dev
1	AFG_1	1	0.90-46	7.881	12.124
2	AFG_2	2	0.62-22	11.34	15.075
3	AFB_1	5	0.25-119.3	21.392	25.832
4	AFB_2	5	0.15-25	1.946	6.278

Linearity graph for aflatoxin G_1, G_2, B_1, B_2 are depicted in Figure 1, 2, 3 and 4, respectively. The average retention time for AFG_1, AFG_2 peak was 7.448 min and 8.979 min, respectively. While for AFB_1, AFB_2 peak the retention time was 9.658 min and 11.847 min, respectively as projected in chromatogram of standard aflatoxins (Figure No. 5). HPLC chromatogram of a contaminated sample is projected in Figure No.6. The present findings were in confirmation with the earlier studies by(Zidedine *et al*., 2007) investigation was carried out for 37 samples of cereals used for the human consumption (20 corn flour, 17 wheat flour) and 21 samples of poultry food purchased from market of Rabat in Morocco by HPLC with immunoaffinity column (IAC) cleanup and Flourometrc determination. The results revealed thataflatoxin in corn flour, wheat flour and poultry feed was about 80, 17.6 and 66.6% respectively. Contamination of 10% sample of corn was higher than that of limit set by EU regulation for aflatoxin B_1 and Total aflatoxin. Similar study conducted in maize by (Reddy et al. 2002) analysed 41 sample out of 95 (43.16%) were found to be contaminated with aflatoxin (ranges from 10 to > 100 ppb). Various surveys conducted reveal that Indian Food and Feed of corn and corn products have been found to contain toxic metabolite products which are produced by certain strains of mold *Aspergillus sp.* or by other mold species.

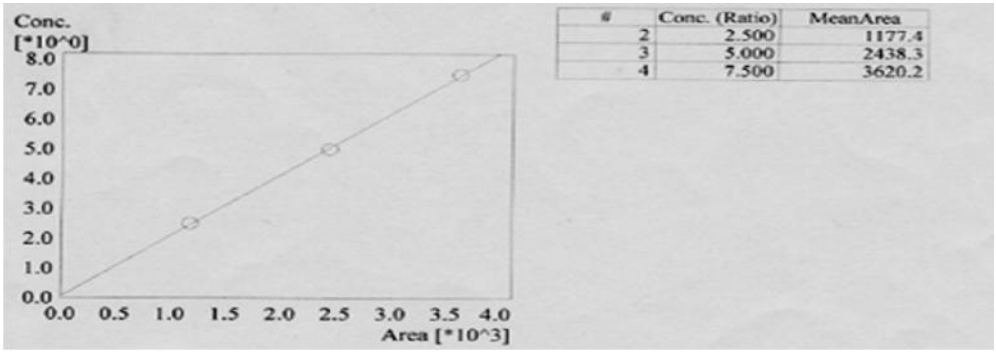

FIGURE 1: Calibration curve for aflatoxin G_1 in HPLC method

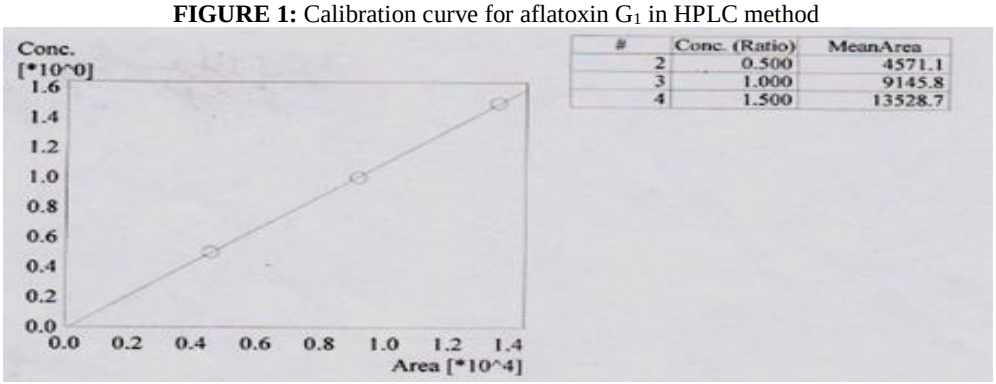

FIGURE 2: Calibration curve for aflatoxin G_2 in HPLC method

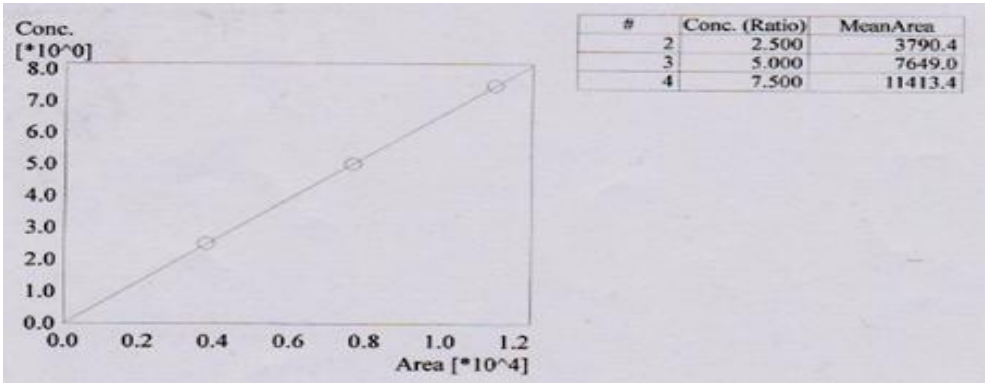

FIGURE 3: Calibration curve for aflatoxin B_1 in HPLC method

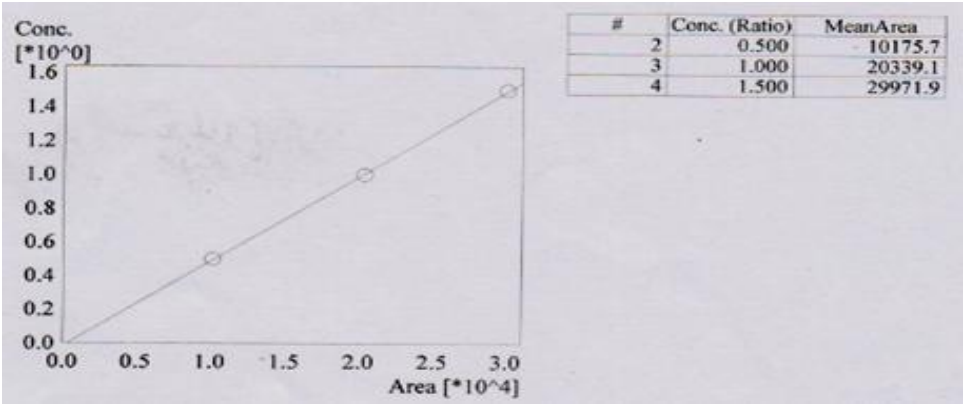

FIGURE 4: Calibration curve for aflatoxin B_2 in HPLC method

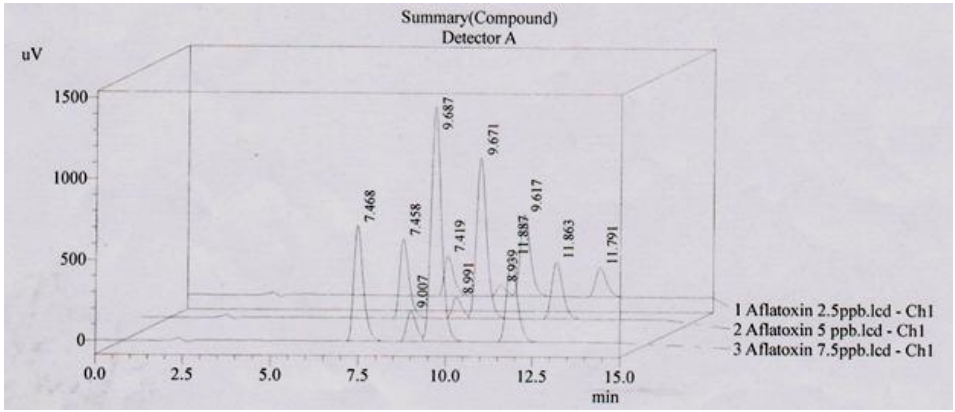

FIGURE 5: HPLC chromatogram of standard aflatoxins G_1, G_2, B_1 and B_2

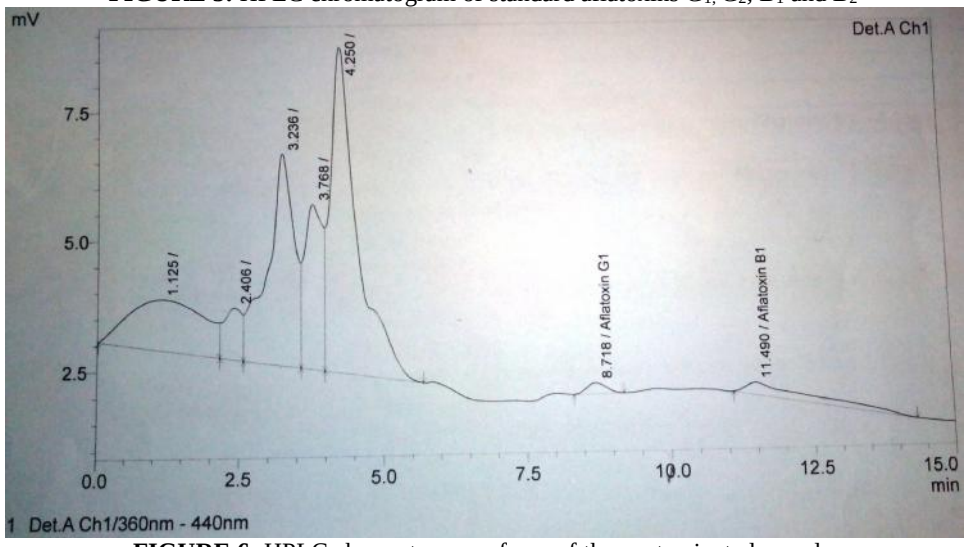

FIGURE 6: HPLC chromatogram of one of the contaminated sample

CONCLUSION

The present study showed that aflatoxins were detected in corn flour at concentrations ranging from 0.03-119.3 ppb. AFG_1 was detected in 14 samples ranging from 0.90-46 ppb. AFG_2 was detected in 2 samples ranging from 0.68-22 ppb. AFB_1 was detected in 20 samples ranging from 0.25-119.3 ppb. AFB_2 was detected in 16 samples ranging from 0.15-25 ppb. It emphasizes the need for regular monitoring and a more stringent food safety system in order to control the aflatoxins at the lowest possible level. Precautions must be taken in the storage of feed commodities. Low moisture content, low temperature and low humidity conditions should be maintained during storage because these depress the fungus growth and thus eliminate Aflatoxin contamination. Analysis of Aflatoxin at µg/ L or kg level needs high tech. laboratories equipped with highly sophisticated instrumentation. However, a practical approach may be the use of a food safety management system based on HACCP, GAP, or Hazard Analysis and Critical Control Point methodology, in which contamination is controlled throughout production and post-production operations. Continued research is required in these areas to provide more effective management of the risks posed by mycotoxin contamination.

ACKNOWLEDGMENTS
The authors gratefully acknowledge Dr. M. Vairamani, Dean, School of Bioengineering, SRM University, Kattankulathur, Tamil Nadu and Pharmacovigilance Laboratory for Animal Feed and Food Safety (PLAFFS) Directorate of Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu.

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