

Quantification of aflatoxins from chillies and chilli powder by H.P.L.C Method

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ABSTRACT:- Aflatoxins are highly toxic compounds and can cause both acute and chronic toxicity in humans and many other animals. Aflatoxins are toxic metabolites produced by different species of toxigenic fungi, called mycotoxins. Mycotoxins are secondary metabolites of fungi. Due to the widespread distribution of fungi in the environment, mycotoxins are considered to be one of the most important contaminants in foods and feeds. Aflatoxin types include B1, B2, G1, G2, B1 is most prevalent and toxic aflatoxin. **Aflatoxins** are poisonous and cancer-causing chemical that are produced by certain molds - *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. Other fungi have been described in the literature as aflatoxins' producers such as *A.bombycis*, *A. ochraceoroseus* and *A. pseudotamari* (Klich et al, 2000; Mishra & Das, 2003). The mycotoxin problem is most acute in developing countries where climate and lack of resources compromise production (Paterson, 2007).

KEYWORDS: - Aflatoxins, Mycotoxins, B1, B2, G1, G2, *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*

I. INTRODUCTION

The aflatoxins are a group of chemically similar toxic fungal metabolites (mycotoxins) produced by certain moulds of the genus *Aspergillus* growing on a number of raw food commodities. Aflatoxins are highly toxic compounds and can cause both acute and chronic toxicity in humans and many other animals. Their importance was first established in 1960 when 100,000 turkeys and other poultry in the UK died in a single event. The cause of this was eventually traced to a toxic contaminant in groundnut meal used in the bird's feed. The contaminant was later named aflatoxin.

Aflatoxins are toxic metabolites produced by different species of toxigenic fungi, called mycotoxins. Humans can be exposed to aflatoxins by the periodic consumption of contaminated food, contributing to an increase in nutritional deficiencies, immunosuppression and hepatocellular carcinoma (Wagacha & Muthomi, 2008). Mycotoxins are secondary metabolites of fungi. Due to the widespread distribution of fungi in the environment, mycotoxins are considered to be one of the most important contaminants in foods and feeds. According to the Food and Agriculture Organization (FAO), more than 25% of the world's agricultural production is contaminated with mycotoxins, resulting in economic losses in the grain industry (Cazzaniga and others 2001).

Toxic compounds can contaminate food and feedstuffs and these contaminated materials may be pathogenic for animals and humans; therefore, one of the most effective measures to protect the public health is to establish reasonable regulatory limits of these toxins. Consequently, guidelines regarding the allowed levels of mycotoxins present in food and feed products and in raw materials have been established by the FAO (FAO 1995) Toxins produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*

- Aflatoxins are secondary fungal metabolites.
- Aflatoxin types include B1, B2, G1, G2.
- B1 is most prevalent and toxic aflatoxin.
- Detection:
- Fluorescence can be of *Aspergillus* on crops

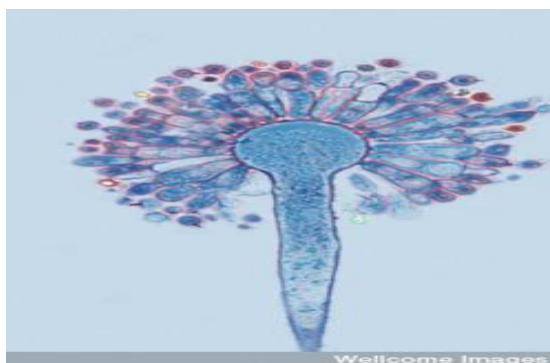
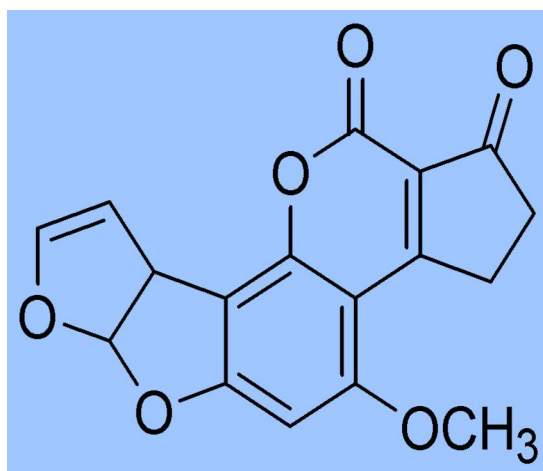

 FIG 1:-Microscopic view:pore formation of *Aspergillus*


FIG 2:- Chemical structure used to detect presence of aflatoxin AFB1

Aflatoxins (AFs) have a wide occurrence in different kind of matrices, such as spices, cereals, oils, fruits, vegetables, milk, meat, etc. Among the 18 different types of aflatoxins identified, the major members are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1) and M2 (AFM2) which are produced by *Aspergillus flavus* and/or *Aspergillus parasiticus*. Strains of *A. flavus* can vary from non-toxic to highly toxic and are more likely to produce AFB1 than AFG1. Strains of *A. parasiticus* generally have less variation in toxigenicity and produce AFB1 and varying amounts of AFB2, AFG1 and AFG2 (Coppock & Christian, 2007).

Aflatoxins are poisonous and cancer-causing chemical that are produced by certain molds

Aspergillus flavus

Aspergillus parasiticus

Aspergillus nomius

Causal organism: *Aspergillus flavus*

Family: Eurotiaceae

Order: Eurotiales

Class: Plectomycetes

Sub. Division: Ascomycotina

Other fungi have been described in the literature as aflatoxins' producers such as *A. bombycis*, *A. ochraceoroseus* and *A. pseudotamari* (Klich et al, 2000; Mishra & Das, 2003). *A. flavus* and *A. fumigatus* have also been identified as pathogens for animals and humans (Zain, 2011). commodities throughout the world that can significantly impact human and animal health. The mycotoxin problem is most acute in developing countries where climate and lack of resources compromise production (Paterson, 2007).



FIG 3:- Chilli fruits severely affected by Aflatoxin

In areas of the world where aflatoxin contamination and HBV are both high, liver cancer may be a significant source of all mortality (Wang et al., 1996). The national maximum tolerated levels for aflatoxins in human food varies from country to country, with some setting levels as low as 4 mg/kg and others setting levels as high as 50 mg/kg (Wu, 2004). Many analytical methods have been developed for aflatoxin B₁. These include chromatographic techniques such as thin-layer chromatography (TLC) (Aziz, Youssef, El-Fouly, & Moussa, 1998; Golinski and rabarkiewicz-Szczesna, 1984), high-performance liquid chromatography (HPLC) (Elshafie, Al-Rashdi, Al-Bahry, & Bakheit, 2002; O Riordan & Wilkinson, 2008), HPLC-FD LC-MS (Hernandez Hierro, Garcia-Villanova, Torrero, & Toruno Fonseca, 2008) and immune affinity-based methods, such as enzyme-linked immunosorbent assay (ELISA) (Aydin, Emin Erkan, Bas, kaya, & Ciftcioglu, 2007; Paterson, 2007) and immunoaffinity column (IAC) HPLC (Elshafie et al., 2002; Fazekas, Tar, & Kovacs, 2005; Shundo et al., 2009).

Chronic Aflatoxin Exposure and Human Health:-

1. Carcinogenicity

- Liver cancer is a serious consequence of long-term exposure to aflatoxins.
- Hepatitis B infection may exacerbate the effects of aflatoxin exposure and vaccination against Hepatitis B can help reduce carcinogenicity of the toxin.

2. Other consequences of chronic exposure include decreased immune and reproductive function.

3. Children chronically exposed may experience growth failure.

- Infants may be exposed through breast milk.
- The fetus may be exposed during pregnancy if the mother consumes aflatoxins.

II. MATERIALS AND METHODS

2.1 Collection of samples:- A total of 57 Chilies samples were collected from Agricultural market yard, Guntur and chili powder were collected from Sri Lakshmi Ganapathi Chilli powder Industries, Guntur. samples were collected between December 2011 to July 2012. These samples were selected randomly and purchased in amounts greater than 0.5 kg. The samples were maintained at 04^o C until arrival at the laboratory, where all samples were ground into powder and stored in plastic bags at 4^oC until the onset of the analysis. The collected samples were different varieties namely Teja (07), 334 (5), 341 (7), Mixed teja (04), 4884 (04), 4884 Mixed Talu (08), Dabhi (10), Byadgi (05), and 273 (07).

2.2 Preparation of samples:-

1. The case of spices, weigh 25 g of sample into a blender jar. For oleoresins, weigh 10 - 12 g.
2. Weigh 5 g of salt into blender jar.
3. Add 100 mL of 80% methanol/20% water mix.
4. Cap blender jar and seal with parafilm.
5. Blend at high speed for 1 minute.
6. Filter blender contents through a fluted filter into a 250 mL beaker.
7. Pipet 10 mL of filtrate into 50 mL graduated cylinder.

8. If the sample is nutmeg, oregano, or black pepper, add 40 mL of 20% Tween-20 solution to the graduated cylinder. If the sample is not one of the products mentioned, add 40 mL of DI Distilled water to the cylinder. Mix.

9. Filter the contents of the graduated cylinder through a glass fiber filter into a 250 mL beaker. This filtrate will be used for the aflatest column.

Immuno-Affinity Column Clean-Up:

a. Attach an Aflatest-P column to the pump stand

b. Pipet 10 mL of filtrate on the column and allow to absorb on column.

c. Once all the sample has passed through the column, rinse the column with 10 mL of HPLC water. Repeat HPLC water rinse.

d. Place 20 mL stoppered test tube under the tip of the column and add 1 mL of methanol to the column.

e. Collect all the methanol eluent in the test tube. The sample is now ready for injection into the HPLC.

Instrument Condition :

a Mobile Phase

For Kobra Cell:

63% DI water with 0.1 g/L KBr and 0.02% Nitric Acid/22% Methanol/ 15% Acetonitrile.

b. For PHRED Photochemical Reactor

a. 55% HPLC water, 45% HPLC Methanol

b. Flow Rate - 1.2 mL/minute.

c. Column - Symmetry C-18; 4.6x250 mm column (Part No. WAT 054275)

d. Injection Volume - 50 μ L

e. Detection - Excitation 365 nm, Emission 464 nm

f. Kobra Cell at 100 μ -amps / PHRED switched on

2.3 Standard Preparation :

a. Allow Supelco Aflatoxin Mix Kit-M standard to come to room temperature.

b. Put 5 mL of methanol into a 10 mL volumetric flask.

c. Add 25 μ L of undiluted Supelco standard to the methanol. Mix well.

d. Bring to volume with 1% Acetic Acid in HPLC water solution.

A slight modification of the Association of Analytical Communities (AOAC) Official Method of Analysis (991.31) for determination of aflatoxins in commercial spice preparations (AOAC, 1995) was used for analysis. Extraction and sample cleanup were done using the method described by Stroka, Anklam, Jorissen, & Gilbert (2000). The IAC was selected as the cleanup column for this project because it is more environmentally and user-friendly. The IAC has the capability to isolate and purify the aflatoxin B1 that was then separated and quantified by high-performance liquid chromatography (HPLC) with fluorescence detection. All reagents were of analytical grade. Solvents used for chromatography were of HPLC grade. Aflatoxin B1 standards were purchased from Sigma Chemicals Company. Immunoaffinity column (AflaTest-P) and phosphate-buffered saline (PBS) was supplied by VICAM (Watertown, MA, USA). Working standard solutions were prepared daily from these standards.

2.3. Aflatoxin analysis:-

The samples were taken in vial (waters company -2ml). this vial was kept in auto samplers.

The H.P.L.C Instruments contain 4 parts

1. Pump

2. Autosampler

3. Detectors (Fluorescence-RF-10A)

4. PHRED



FIG 4:- H.P.L.C Instruments and components (Shimadzu-10x)

First vial run blank, second vial standard and third vials on words starts samples. each vial was taken 18 minutes .after saw the peaks in Lc Solution software.

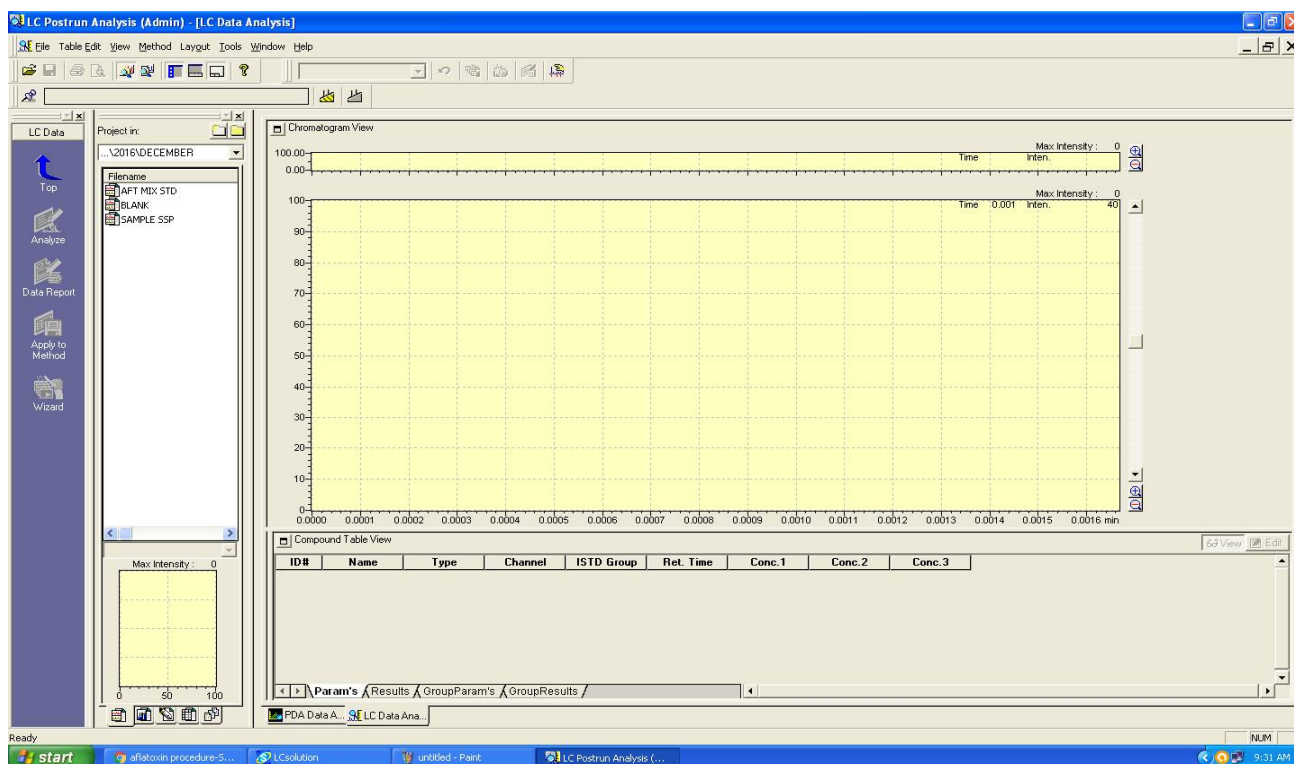


FIG 5:- LC Solution software

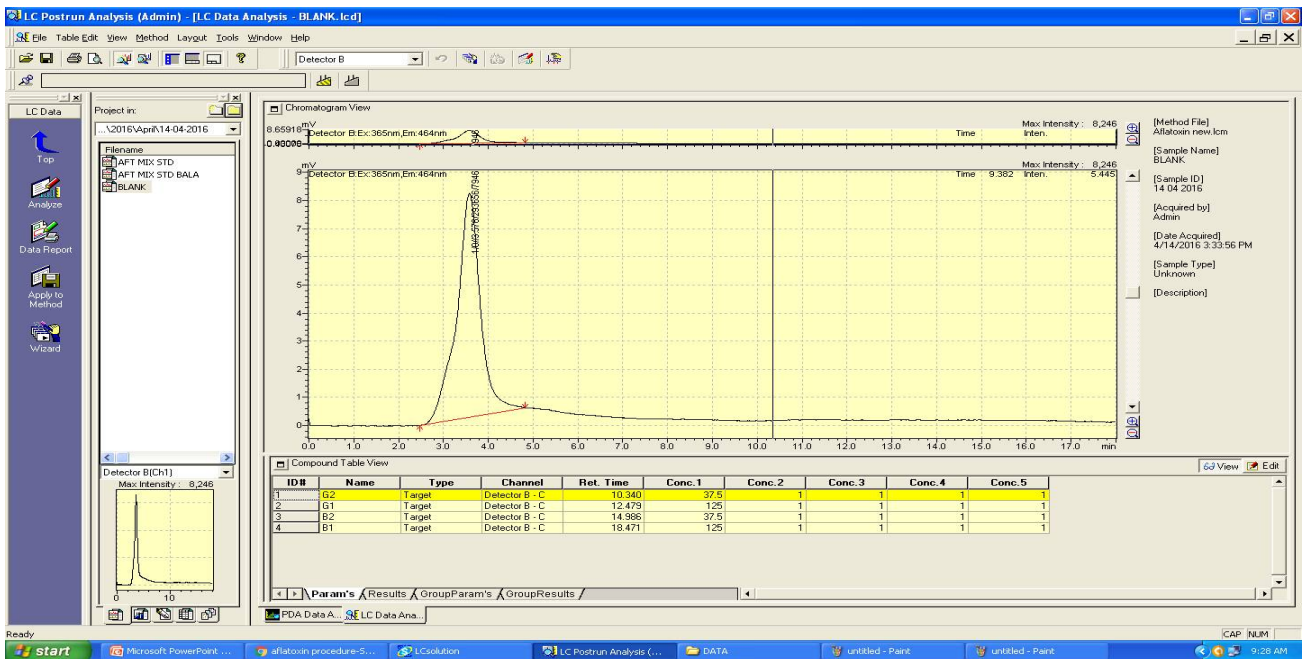


FIG 6:- Blank

Sample Information

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Acquired by      : Admin
Sample Name     : AFT MIX STD
Sample ID       :
Tray#           : 1
Vial#           : 2
Injection Volume: 50 uL
Data Filename   : AFT MIX STD.lcd
Method Filename : Aflatoxin Method new.lcm
Batch Filename  : 07-04-2016.lcb
Report Filename : Aflatoxin Report.lcr
Date Acquired   : 07/04/2016 11:06:46 AM
Data Processed  : 07/04/2016 11:37:41 AM
  
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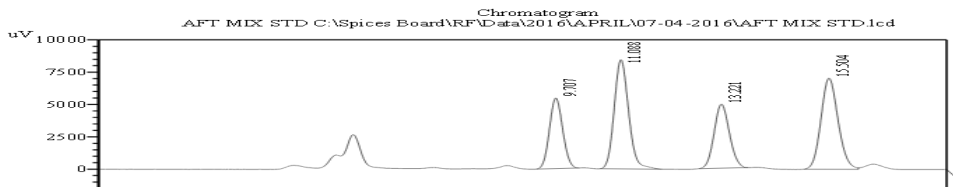


FIG 7:- Afla mix std

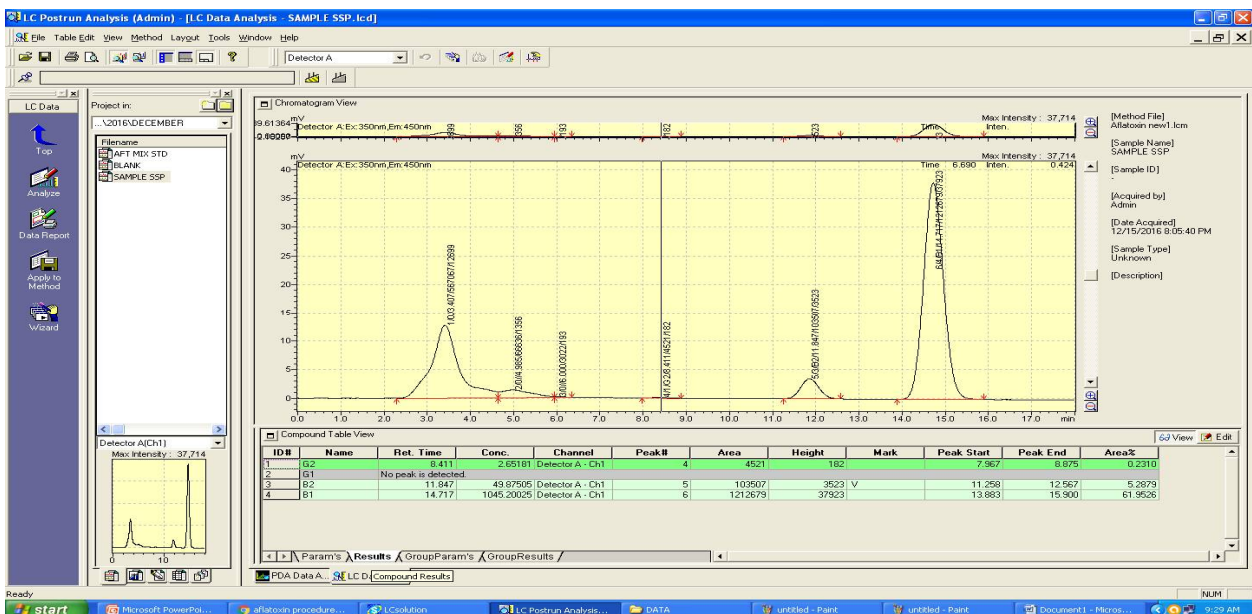


FIG 8:- Sample peak

D. Calculation :

Calculation of aflatoxin content in sample.

$$*Aflatoxin (ug/kg) = \frac{pG (conc) \times 1000 \mu L \times 50 mL \times 100 mL}{50\mu L \times 10 mL \times 10 mL \times 25 \times 10^3 mg}$$

E. Result and Reporting :

Report the value after rounding to the first decimal place

The analytical result is given as

Aflatoxin

- B1 (ug/kg) =
- B2 (ug/kg) =
- G1 (ug/kg) =
- G2 (ug/kg) =
- Total (ug/kg) =

If the level of aflatoxin is below 0.5 ug/kg, then the result can be given as < 0.5 ug/kg

III. RESULTS

3. Results and Discussion

During the present investigation, 57 samples of chillies and chili powder were taken in the agriculture market ,Guntur of Andhra Pradesh ,India were screened for the presence of B1and B2 contamination. Perusal of data presented in table 1 shows that 19.0 percent of the market samples that were sold loose were detected to be contaminated with aflatoxin B1 whereas more samples (29.0 percent) were positive for afla B2 contamination.

S.NO	DATE	NAME OF VARIETY	Aflatoxin (ppb)
1.	24/12/2011	4884	3.3
2	25/12/2011	DABHI	5.98
3	27/12/2011	4884 MIXED TALU	11.8
4	27/12/2011	341	0.5
5	29/12/2011	TEJA	0.5
6	29/12/2011	MIXED TEJA	0.5
7	29/12/2011	TEJA	0.5
8	29/12/2011	4884 MIXED TALU	10.5
9	02/01/2012	DABHI	4.36
10	02/01/2012	4884 MIXED TALU	10.5
11	02/01/2012	341	0.5
12	04/01/2012	DABHI	0.5
13	04/01/2012	334	0.5
14	04/01/2012	DABHI	0.5
15	04/01/2012	334	6.25
16	05/01/2012	341	16.8
17	12/01/2012	4884	0.5
18	22/01/2012	341	0.5
19	22/01/2012	TEJA	1.67
20	22/01/2012	4884	27.92
21	22/01/2012	334	4.7
22	23/01/2012	4884	3.68
23	23/01/2012	341	0.5

24	23/01/2012	BYADGI	7.0
25	02/02/2012	DABHI	0.5
26	05/02/2012	DABHI	26.0
27	08/02/2012	MIXED TEJA	0.8
28	09/02/2012	TEJA	1.0
29	10/02/2012	BYADGI	14.2
30	11/02/2012	MIXED TEJA	14.2
31	12/02/2012	BYADGI	0.5
32	14/02/2012	BYADGI	0.5
33	15/02/2012	341	0.56
34	20/02/2012	BYADGI	24.29
35	21/02/2012	DABHI	6.97
36	26/02/2012	4884 MIXED TALU	12.97
37	28/02/2012	DABHI	1.00
38	03/03/2012	334	2.45
39	14/03/2012	341	1.8
40	17/03/2012	DABHI	22.4
41	17/03/2012	341	3.8
42	23/03/2012	273	1.28
43	15/04/2012	TEJA	0.5
44	18/04/2012	341	1.5
45	18/04/2012	273	10.0
46	22/04/2012	4884 MIXED TALU	12.0
47	26/04/2012	273	0.5
48	27/04/2012	DABHI	10.0
49	27/04/2012	TEJA	0.5
50	02/05/2012	273	18.0
51	12/05/2012	4884 MIXED TALU	17.00
52	20/05/2012	334	8.0
53	27/06/2012	273	12.0
54	28/06/2012	273	18.0
55	11/07/2012	4884 MIXED TALU	18.0
56	18/07/2012	273	0.5
57	29/17/2012	4884 MIXED TALU	17.0

Table 1. Presence of aflatoxin in chillies

Survey of literature shows that chilli powder samples have earlier also been detected to be contaminated with aflatoxins (Abdulkadir et al., 2002; Fazekas et al., 2005; Ozbey and Kabak, 2012). *Aspergillus flavus* and *A. parasiticus* are the most important producers of aflatoxins in various agricultural commodities (Cotty and Garcia, 2007). Aflatoxins are the most potent of all the mycotoxins, as they are known to have hepatotoxic, carcinogenic, teratogenic and mutagenic effects (WHO, 1979; IARC, 1993).

Human foods are allowed 4-30 ppb aflatoxins, depending on the country involved (FDA, 2004). European Union, has imposed a maximum acceptable limit of 4 µg/kg in food for human consumption, the strictest in standard worldwide (EC, 2006), whereas Indian government under 57A of prevention of Food Adulteration Rules has imposed maximum permissible limit of 30 ppb for aflatoxin B1 in all the foods prone to aflatoxin contamination (Sinha, 1995). It has been estimated that more than 5 billion people in developing countries worldwide are at risk of chronic exposure to aflatoxins through contaminated foods (Strosnider et al. 2006). Postharvest conditions such as storage, transportation and food processing amongst others; have been implicated as predisposing factors that enables *A. flavus* and *A. parasiticus* to produce aflatoxins in contaminated food (Wu and Khlangwiset 2010).

5. Conclusion:- Our results show that about 11% of the 57 s samples tested contained detectible levels of aflatoxin B1, with the highest concentrations found in chillies.

In fact aflatoxin B1 is the most toxic member of the group and its tolerance level has been fixed by World Health Organization (1979) as 20 ppb. Detection of this toxin in contaminated chillies and contaminated chili powder is a serious issue as it can adversely affect the health of consumers. Therefore, its management is urgently required

“No level of aflatoxin exposure is considered safe for humans”

REFERENCES

1. Wagacha, J.M. & Muthomi, J.W. (2008). Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology*. Vol.124, No.1, pp. 1–12, ISSN 0168-1606
2. Coppock, W.R. & Christian, R.G. (2007). Aflatoxins, In: *Veterinary Toxicology – Basic and Clinical Principles*, R. C. Gupta; pp. 939-950, Academic Press, ISBN 0123704677, San Diego.
3. Klich, M. Mendoza, C. Mullaney, E. Keller, N. & Bennett, J.M. (2001). A new sterigmatocystin-producing *Emericella* Variant from agricultural desert soils. *Systematic and Applied Microbiology*, Vol.24, No.1, pp. 131-138, ISSN 0723-2020.
4. Mishra, H.N. & Das, C. A review on biological control and metabolism of aflatoxin. (2003) *Critical Reviews in Food Science and Nutrition*, Vol.43, No.3, pp. 245–264, INSS 1040-8398.
5. Zain, M.E. (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society*, Vol.15, No.2, pp. 129–144, ISSN 1319-6103.
6. Paterson, R. R. M. (2007). Aflatoxins contamination in chilli samples from Pakistan. *Food Control*, 18(7), 817e820.
7. Wogan, G. N., Kensler, T. W., & Groopman, J. D. (2012). Present and future directions of translational research on aflatoxin and hepatocellular carcinoma. A review. *Food Additives and Contaminants Part A e Chemistry Analysis Control Exposure & Risk Assessment*, 29(2), 249e257.
8. Wang, J. S., Qian, G. S., Zarba, A., He, X., Zhu, Y. R., Zhang, B. C., et al. (1996). Temporal patterns of aflatoxin-albumin adducts in hepatitis B surface antigen-positive and antigen-negative residents of Daxin, Qidong county, People's Republic of China. *Cancer Epidemiology Biomarkers & Prevention*, 5(4), 253e261.
9. FAO/WHO. (1998). Safety evaluation of certain food additives and contaminants in food: aflatoxins. In Forty-ninth meeting of the joint FAO/WHO expert committee on food additive, Vol. 40, (pp. 359e469). FAO/WHO Food Additives Series.
10. Wu, F. (2004). Mycotoxin risk assessment for the purpose of setting international regulatory standards. *Environmental Science & Technology*, 38(15), 4049e4055.
11. Aziz, N. H., Youssef, Y. A., El-Fouly, M. Z., & Moussa, L. A. (1998). Contamination of some common medicinal plant samples and spices by fungi and their mycotoxins. *Botanical Bulletin of Academia Sinica*, 39(4), 279e285.
12. Goliński, P., & Grabarkiewicz-Szczesna, J. (1984). Chemical confirmatory tests for ochratoxin A, citrinin, penicillic acid, sterigmatocystin, and zearalenone performed directly on thin layer chromatographic plates. *Journal of the Association of Official Analytical Chemists*, 67(6), 1108e1110.
13. Elshafie, A. E., Al-Rashdi, T. A., Al-Bahry, S. N., & Bakheit, C. S. (2002). Fungi and aflatoxins associated with spices in the Sultanate of Oman. *Mycopathologia*, 155(3), 155e160.
14. O' Riordan, M. J., & Wilkinson, M. G. (2008). A survey of the incidence and level of aflatoxin contamination in a range of imported spice preparations on the Irish retail market. *Food Chemistry*, 107(4), 1429e1435.
15. Hernandez Hierro, J. M., Garcia-Villanova, R. J., Torrero, P. R., & Toruno Fonseca, I. M. (2008). Aflatoxins and ochratoxin A in red paprika for retail sale in Spain: occurrence and evaluation of a simultaneous analytical method. *Journal of Agricultural and Food Chemistry*, 56(3), 751e756
16. Aydin, A., Emin Erkan, M., Bas, Kaya, R., & Ciftcioglu, G. (2007). Determination of Aflatoxin B1 levels in powdered red pepper. *Food Control*, 18(9), 1015e1018.
17. Fazekas, B., Tar, A., & Kovacs, M. (2005). Aflatoxin and ochratoxin A content of spices in Hungary. *Food Additives and Contaminants*, 22(9), 856e863.
18. Shundo, L., de Almeida, A. P., Alaburda, J., Lamardo, L. C. A., Navas, S. A., Ruvieri, V., et al. (2009). Aflatoxins and ochratoxin A in Brazilian paprika. *Food Control*, 20(12), 1099e1102.
19. AOAC. (1995). Official methods of Analysis. (15th ed. Vol. II.). Gaithersburg, Maryland, USA: AOAC. Natural toxins, method number 991.31.
20. Stroka, J., Anklam, E., Jorissen, U., & Gilbert, J. (2000). Immunoaffinity column cleanup with liquid chromatography using post-column bromination for determination of aflatoxins in peanut butter, pistachio paste, fig paste, and paprika powder: collaborative study. *Journal of AOAC International*, 83(2), 320e340.
21. Abdulkadir, E.E., Tahiya, A.A., Saif, N.A and Charles, S.B. 2002. Fungi and aflatoxins associated with spices in the Sultanate of Oman. *Mycopathol.* 155: 155-160.
22. Fazekas, B., Tar, A. and Kovacs, M. 2005. Aflatoxin and ochratoxin A content of spices in Hungary. *Food Addit. Contam.* 22: 856-863.
23. Ozbey, F. and Kabak, B. 2012. Natural co-occurrence of aflatoxins and ochratoxin A in spices. *Food Control* 28: 354–361
24. Cotty, P. J. and Garcia, R. J. 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. *Int. Jour. Food Microbiol* 119: 109–115
25. W.H.O. 1979. Environmental health criteria 11. Mycotoxins. World Health Organization, Geneva.
26. IARC. 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins, p. 397-444. In IARC monographs on the evaluation of carcinogenic risks to humans, Vol. 56 International Agency for Research on Cancer, Lyon, France.
27. EC (European Commission) 2006. The Commission Decision, 2006/504/EC Official Journal of the European Union L199, pp. 21-32.
28. FDA (Food and Drug Administration). 2004. Compliance guidance manual. <http://www.cfsan.fda.gov>.
29. Sinha, A.K. 1995. Mycotoxin- A health hazard. *Yojna*. June issue: 15.
30. Strosnider, H., Azziz-Baumgartner, E., Banziger, M., Bhat, R.V., Breiman, R., Brune, M., DeCock, K., Dilley, A., Groopman, J., Hell, K., Henry, S.H., Jeffers, D., Jolly, C., Jolly, P., Kibata, G.N., Lewis, L., Liu, X., Luber, G., McCoy, L., Mensah, P., Miraglia, M., Misore, A., Njapau, H., Ong, C., Onsongo, M.T.K., Page, S.W., Park, D., Patel, M., Phillips, T., Pineiro, M., Pronczuk, J., Schurz Rogers, H., Rubin, C., Sabino, M., Schaafsma, A., Shephard, G., Stroka, J., Wild, C., Williams, J.T., Wilson, D. 2006. Public health strategies for reducing aflatoxin exposure in developing countries. *Environ. Health Perspectives*. 114:1989-1903