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Research Article

ESTIMATION OF AFLATOXINS CONTAMINATION LEVELS IN INFANT PROCESSED FOODS BY RP-HPLC

Konda Ravi Kumar*[1], P.V. Suresh[2]

*[1]Department of Pharmaceutical Chemistry, Hindu college of Pharmacy, Guntur, Andhra Pradesh, India -522002.

[2] Department of Pharmaceutical Analysis, Chalapathi Institute of Pharmaceutical Sciences, Lam, Guntur, Andhra Pradesh, India -522034.

Abstract

Processed infant food samples (ready to eat) were analyzed for aflatoxins contamination using Reverse Phase High Performance Liquid Chromatography (RP-HPLC) with fluorescent detection. A solvent mixture of acetonitrile-water was used for the extraction followed by immunoaffinity clean-up to enhance sensitivity of the method. The limit of detection (LOD) (0.01–0.02 ng/g) and limit of quantification (LOQ) (0.02 ng/g) was established for aflatoxins based on signal to noise ratio of 3:1 and 10:1, respectively. T he processed food samples tested, 38% were contaminated with four types of aflatoxins, *i.e.*, AFB1 (0.02–1.24 µg/kg), AFB2 (0.02–0.37 µg/kg), AFG1 (0.25–2.7 µg/kg) and AFG2 (0.21–1.3 µg/kg). In addition, the results showed that 21% of the processed foods intended for infants contained AFB1 levels higher than the European Union permissible limits (0.1 µg/kg), while all of those intended for adult consumption had aflatoxin contamination levels within the permitted limits.

Keywords: aflatoxins contamination, cereals based products, immunoaffinity clean-up, Effective recovery, HPLC..

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***Corresponding Author**

Dr. K. Ravi Kumar

Hindu college of Pharmacy, Guntur, A.P.

Email: drkondaravikumar@gmail.com

INTRODUCTION

Aflatoxins are polyketide based potent liver carcinogenic, mutagenic and immunosuppressive compounds, primarily produced by food-borne fungi, mainly Aspergillus species such as flavus, parasiticus, niger, nomius, pseudotamari and bombycids, etc. These fungi can colonize a variety of products such as corn, maize, oilseeds, spices, groundnuts and tree nuts, etc., under favourable conditions, thus leading to food contamination and spoilage.

There are almost 20 different types of aflatoxins identified until now, among these B1, B2, G1 and G2 are more prominent while AFB1 is considered to be the most toxic. The health issues related to aflatoxins are equally complex and demand more research. The ingested aflatoxin undergoes various possible pathways depending on different parameters like dose quantity, type of species, age, diet, and immune system of host. Exposure of biological systems to harmful levels of aflatoxin results in the formation of epoxide, which reacts with proteins and DNA leading to DNA-adducts, thus causing liver cancer. Aflatoxins persist to some extent in food even after the inactivation of the fungi by food processing methods, such as ultra high temperature products, due to their significant chemical stability. Infants are at much higher risks of health problems compared to adults. The maximum legal limit allowed for AFB1 in infant food in the European Union is 0.1 µg/kg. In developing countries, the majority of the people survive largely on cereal based diets. Consequently, nutritional deficiencies are very prevalent in populations consuming high levels of cereals, particularly in children. Moreover, poor diet and multiple infectious hazards are associated with malnutrition and growth faltering in infancy and childhood. Various approaches exist for the determination of aflatoxin in food and feed commodities. Generally, all analytical methods follow the basic protocol of extraction, clean-up, separation, detection, identification and quantification. However, the most widely used techniques are those which include a chromatographic step to separate the mycotoxin of interest like mini column chromatography, thin layer chromatography, high performance liquid chromatography and gas liquid chromatography. Although immunoassay-based quantitative methods are fast and promising, for mycotoxin research they have the possibility of producing misleading results because of cross-reaction and interference in the complex matrixes. Therefore, a more selective treatment followed by specific purification is required before the analysis in such cases[1-3].Some purification, preconcentration and clean-up protocols have been used over the years to enhance the sensitivity and selectivity of HPLC methods for the determination of aflatoxins in different food commodities. However most of such sample preparation techniques are tedious and offer less sensitivity. There was no data available in the literature on the processed cerelac infant foods with which to compare the results of our present analysis. Currently, immunoaffinity column (IAC) clean-up followed by RP-HPLC with fluorescence detector has emerged as a promising technique for the reliable detection and quantification of aflatoxins in diversified foods. The aim of this study was, therefore, to provide information about aflatoxin levels in processed infant foods marketed in India by using IAC clean-up assisted RP-HPLC method with fluorescence detection[4-5].

CHEMICALS AND MATERIALS

Aflatoxin standards were purchased from Supelco (Bellefonte, PA, USA). All other chemicals and reagents used were of analytical and HPLC grade from Merck (Darmstadt, Germany). The stock and working standard solutions were prepared in acetonitrile according to the Association of Official Analytical Chemists (AOAC) method and stored at 20 ^{0}C in amber glass vials until analysis[6-11].

Collection of Samples

The samples of processed infant food samples were purchased from the local food stores of Guntur region of Andhrapradesh. The selected foods, derived from cereal grains, dairy and herbs, have been processed by the local and multinational manufacturers in India.

Extraction of Aflatoxins

Accurately weighed 5 g of representative sample was taken in a conical flask; mixed with 20 mL of extraction solvent (acetonitrile: Water 84:16) and shaken for 90 min in an orbital shaker at ambient conditions (average temperature 37^{0}C). The extract was filtered using Whatman filter paper No. 4 and the filtrate thus obtained was concentrated at 50 ^{0}C to a final volume of 2–5 mL by evaporation under reduced pressure [12-15].

Clean-Up

With the purpose to enhance the selectivity and

sensitivity, 2–5 mL of concentrated sample was diluted with 20 mL of deionized water and passed through Vicam (waters) Aflatest WB immunoaffinity column at a flow rate of 2 mL min−1 with the help of suction pump[16-19]. The immunoaffinity column was washed with a further 20 mL of deionized water and dried by air streaming for 1-2 min. The retained aflatoxins were eluted from the column by passing 2 mL of methanol in two steps (1 mL each). The samples thus obtained were dried under N2 gas.[20-21]

Derivatization

Pre-column derivatization enhances the detection and recoveries of aflatoxin, which was done as follows:

200 µL n-hexane was added to the dried vial containing aflatoxin residues and vortexed for 30 s to remove the fat, then 50 µL of TFA (trifluro acetic acid) was added and the sample mixture vortexed again for 30 s followed by addition of 1.95 µL of water:acetonitril (9:1). The sample mixture was finally vortexed for 20 s and used for HPLC analysis.

HPLC Analysis Conditions

For quantitative estimation of aflatoxins, measurements were performed on LC-system in the Central Instrumentation Lab, Department of Pharmaceutical Analysis, Chalapathi Institute of Pharmaceutical Sciences, Lam, Guntur, A.P, India.

An HPLC apparatus (Agilent1220 series) containing Ezichrome Elite software package designed for HPLC real time and post operative analysis operated through computer equipped with Mediterranae Sea 18® 5 µm; 25 × 0.46 cm column. Isocratic mobile phase consisting of acetonitrile, methanol and water ratio (22.5:22.5:55) was used at a flow rate of 1 mL/min. The elute was detected using spectrofluorometer detector set at emission 440 nm and excitation at 360 nm. Limit of detection (LOD) was estimated as signal to noise ratio (S/N) = 3 and limit of quantification (LOQ) as (S/N) = 10.

Sample Analysis

The results obtained in this study showed variable levels of aflatoxin contamination in a variety of processed food collected from January–September, 2015. Overall, 37% of the processed food samples were found to be contaminated with aflatoxins. The incidence of alfatoxins in processed foods intended for infant use was 35% as shown in Table 2. The data showed that 21% (11/40) of the contaminated samples contained AFB1 higher than the permissible limits (0.01 µg/kg) of the European Union (EC, 2006). The AFB1 and AFT level ranged 0.01–0.4 µg/kg and 0.02–3.8 µg/kg, respectively. Individually 40% Cerelac, 33% Powder Milk, 50% Noodles, 20% Biscuits, were found positive for aflatoxins contamination. These results depicted that difference in aflatoxins levels among the different types of food were significant ($p < 0.05$).

Cereal Infant Food Samples

A brand of cereal foods is frequently used for infants and as a snack for the whole family in India as well as in several countries around the world. The brands are available in different flavors and composition depending upon the age and needs of the infant. During this study typically 20 cerelac food samples having different flavour and were analyzed. The results obtained showed that aflatoxin contents of the foods varied depending upon their ingredients composition. On average 40% of the cerelac baby food samples were aflatoxin-contaminated, whereas rice and wheat-flavoured products contained average AFB1 level (0.2 ± 0.01 µg/kg) higher than the limits set by the European Union (EU).

Noodles

Noodles are used as food for children ranging between 1 and 6 years of age. This food product is usually derived from wheat, rice, legumes, or maize depending on their type and flavour. Among the analyzed 10 noodle food samples, 5 (50%) were positive for aflatoxin contamination with amounts of 0.36±0.01 µg/kg and 0.03±0.01 µg/kg for AFB1 and AFT, respectively. The reason for the high incidence of aflatoxin in noodles might be linked to the ingredients, especially, the corn flour. The aflatoxin levels in 40% of the noodle samples were higher than European Union permissible limits (0.01 µg/kg).

Baby Powder Milk

The US Federal Food, Drug, and Cosmetic Act (FFDCA) defines infant formula as "a food which purposes to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk". So the composition of infant milk formula should be roughly based on a mother's milk. The most commonly used infant formulas, as prescribed by manufacturers, contain purified protein, lactose, mixture of vitamins and minerals and other ingredients. If these ingredients are obtained from

cow's milk, the infant powder milk might be contaminated with AFM1 instead of AFB1. However, the results showed the aflatoxin range of 0.17 ± 0.05 µg kg−1, 0.03 ± 0.01 µg/kg, 0.06 µg/kg, 0.11 ± 0.03 µg/kg and 0.07 ± 0.001 µg/kg for AFB1, AFB2, AFG1 and AFG2, respectively which are indications of the fact that baby powder milk samples were not mostly manufactured from cow's milk.

Furthermore, the contamination level for AFB1 (0.2 ± 0.05 µg/kg) was also higher than the limits set by the European Union. A large number of infants are fed with powdered milk around the world and likewise in India, so occurrence of AFB1 in milk samples can exert potential health hazards for infants in India, as infants are more susceptible to aflatoxin attack than adults.

Biscuits

Sweet biscuits, commonly eaten as a snack food by children and adults, in general, are made with wheat flour, peanuts and oats, and sweetened with sugar or honey. There is usually a dedicated section for sweet biscuits in most Asian and European supermarkets. A variety of biscuits sold in India under different trade names were analyzed for their aflatoxin contamination. The trade name biscuits were found contamination level (0.31 ± 0.01 µg/kg, 0.38 ± 0.01 µg/kg, 1.13 ± 0.06 µg/kg and 0.68 ± 0.01 µg/kg of AFB1, AFB2, AFG1 and AFG2, respectively).

Statistical Analysis

Triplicate samples were prepared and data thus obtained was analyzed statistically to calculate the level of significance of various parameters using analysis of variance technique by Origin Software Package Version 13.0 and data were reported as mean ± SD. A probability level $p < 0.05$ was used to denote the statistically significant differences.

RESULTS AND DISCUSSION

Linearity

As is evident from the HPLC chromatogram in Figure 2, the standard calibration curves were linear over 0.05–150 ng mL−1, 0.02–20 ng mL−1, 0.05–20 ng mL−1 and 0.02–6.0 ng mL−1 for AFB1, AFB2, AFG1 and AFG2, respectively, presenting a concentration dependent response and linearity of the detector.

Repeatability and Reproducibility

A typical HPLC chromatogram showing the clear separation of 5 ppb standard mixture of four aflatoxins (AFB1, AFB2, AFG1 and AFG2) is depicted in

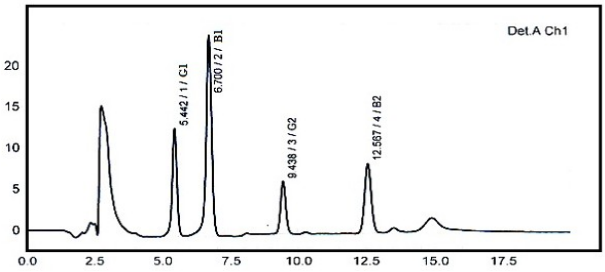

Fig 01: High Performance Liquid Chromatography (HPLC) Chromatogram of 5 ppb standard mixture of four aflatoxins.

Recovery

The percentage recoveries were found to be 97.6% for AFB1 and AFG1 and 91.2% for AFB2 and AFG2 as shown in Table 1. A reasonably high recovery of the most important aflatoxin components (AFB1 and AFG1), as high as 97.6%, through spiking diversified foods, depicts that the method used is efficient and can be employed successfully for the reliable analysis of aflatoxins in processed food products.

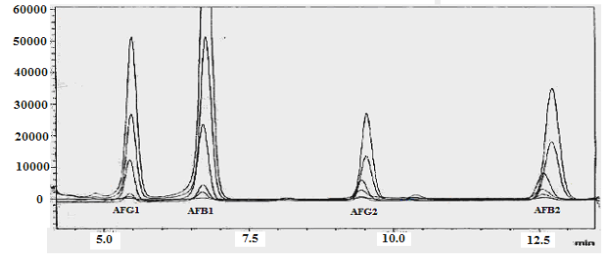

Fig 2: HPLC chromatogram showing the Linearity of AFGl, AFBl, AFGl and AFB2 standards.

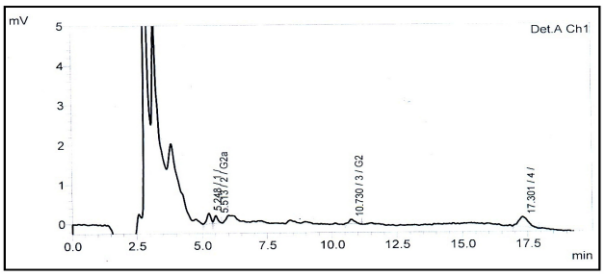

Fig 03: Typical chromatogram for detectable amounts of aflatoxins in Cereal processed food sample.

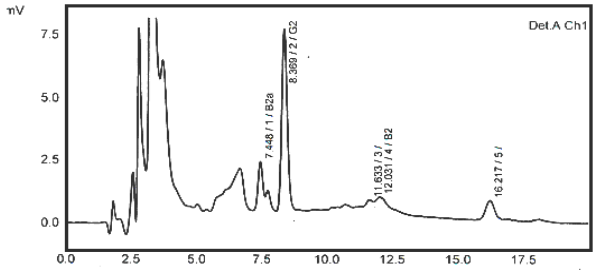

Fig 04: Typical chromatogram for detectable amounts of aflatoxins in milk powder processed food sample.

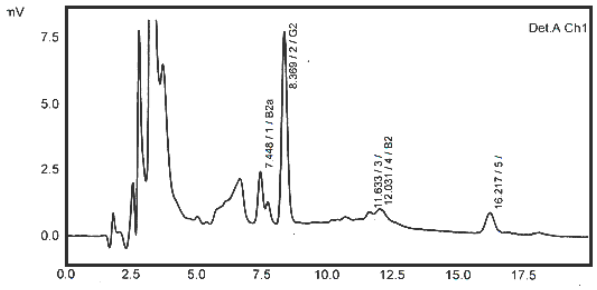

Fig 05: Typical chromatogram for detectable amounts of aflatoxins in n o o d l e s processed food sample.

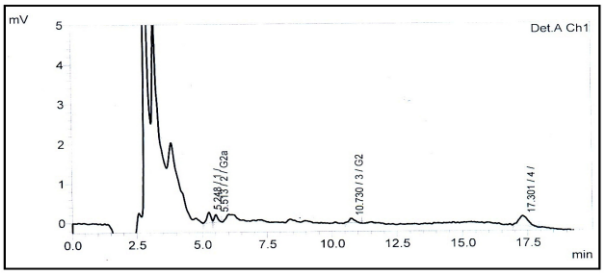

Fig 06: Typical chromatogram for detectable amounts of aflatoxins in b i s c u i t s processed food sample.

Tab 01: Linearity, LOD, LOQ and recovery of HPLC method used for aflatoxin determination

Aflatoxin	LOD[a] (ng/g)	LOQ[b] (ng/g)	r^2	%Recovery	Mean (µg/kg)± %RSD)[d]
AFB1	0.02	0.05	0.9997	97.6	125.3± **9.12**
AFB2	0.01	0.02	0.9995	91.2	15.3**±2.01**
AFG1	0.02	0.05	0.9996	97.6	15.3±1.44
AFG2	0.01	0.02	0.9991	91.2	6.3**±3.42**

[a]Limit of detection; [b]Limit of quantification; [c]Accuracy was determined by the determination of the recoveries of aflatoxins. By spiking 125.5 µg/kg aflatoxin B1, 15.3 µg/kg aflatoxin G1 and B2 and 6.3 µg/kg G2 to the samples (uninfected ground nuts); [d]Replicate analysis of each spiked sample was used to determine the accuracy, expressed as mean (µg/kg) ± relative standard deviation (%).

Tab 02:Level of AFB1 and total aflatoxin (AFT) in processed food intended for infants.

Sample type	No. of samples analysed (N)	Presence of Aflatoxin Samples n (%)	Samples having AFB1 >0.1µg/kg n (%)	Samples having AFB1< 0.1µg/kg n (%)	Total Aflatoxin (Means ±SD) mg/kg
Cereal food	10	6 (40)	4 (20)	2 (20)	0.052 ±0.06
Powder Milk	10	4 (33)	3 (20)	1 (13)	0.030 ±0.07
Noddels	10	4 (50)	3 (40)	1 (10)	0.025 ±0.09
Biscuits	10	3 (20)	1 (32)	2 (68)	0.041 ± 0.02
Total no of samples	40*	17 (35**)	11 (26**)	6 (25**)	0.0356 ± 0.006**

*Total number of samples
**Mean of % presence of Aflatoxins

CONCLUSION

The results obtained in this study showed that the magnitude of AFB1 contamination varied widely among processed infant and adult foods. The levels of aflatoxins in the processed foods intended for infant consumption was found to be higher than the permissible limits set by the European Union. This can be more hazardous for infants, who are more sensitive and prone to exposure and toxic effects of such highly carcinogenic food contaminants. In addition, the amount of aflatoxins found presently was lower while the magnitude of their incidence was higher as compared with those reported for the

unprocessed foods. This situation clearly demands wider national and international programs for the control of aflatoxin contamination in processed foods, especially in infant foods. The results of the present study may provide awareness regarding the aflatoxins in processed infant foods and adult food, from the point of view of food safety.

AUTHOR CONTRIBUTION

All authors Contributed Equally

REFERENCES

1. Ito, Y.; Peterson, S.W.; Wicklow, D.T.; Goto, T. Aspergillus pseudotamarii, a new aflatoxin producing species in Aspergillus section Flavi. Mycol. Res. 2001, 105, 233–239.
2. Turner, N.W.; Sreenath, S.; Piletsky, S.A. Analytical methods for determination of mycotoxins: A review. Anal. Chim. Act. 2009, 632, 168–180.
3. Strosnider, H.; Azziz-Baumgartner, E.; Banziger, M.; Bhat, R.V.; Breiman, R.; Brune, M.; DeCock, K.; Dilley, A.; Groopman, J.; Hell, K.; et al. Workgroup report: Public health strategies for reducing aflatoxin exposure in developing countries. Environ. Health Perspect. 2006, 114,1989–1903.
4. Pildain, M.B.; Frisvad, J.C.; Vaamonde, G.; Cabral, D.; Varga, J.; Samson, R.A. Two novel aflatoxin-producing Aspergillus species from Argentinean peanuts. Int. J. Syst. Evol. Microbiol.2008, 58, 725–735.
5. IARC monographs International Agency for Research on Cancer (IARC). Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; World Health Organization, International Agency for Research on Cancer: Lyon, France, 2002; Volume 82, pp. 1–556.
6. Groopman, J.D.; Kensler, T.W.; Wild, C.P. Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. Ann. Rev. Public Health 2008, 29, 187–203.
7. 7.Park, D.L.; Trucksess, M.W.; Nesheim, S.; Stack, M.E.; Newell, R.F. Solvent-efficient thin-layer chromatographic method for the determination of aflatoxins B1, B2, G1, and G2 in corn and peanut products: Collaborative study. J. Assoc. Off. Anal. Chem. 1994, 77, 637–646.
8. Yazdanpanah, H.; Mohammadi, T.; Abouhossain, G.; Cheraghali, A.M. Effect of roasting on degradation of aflatoxins in contaminated pistachio nuts. Food. Chem. Toxicol. 2005, 43, 1135–1139.
9. Sergent, T.; Ribonnet, L.; Kolosova, A.; Garsou, S.; Schaut, A.; de Saeger, S.; van Peteghem, C.; Larondelle, Y.; Pussemier, L.; Schneider, Y.J. Molecular and cellular effects of food contaminants and secondary plant components and their plausible interactions at the intestinal level. Food Chem. Toxicol. 2008, 46, 813–841.
10. European Commission. Commission regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union 2006, 1, 5–24.
11. Bankole, S.A.; Adebanjo, A. Mycotoxins in food in West Africa: Current situation and possibilities of controlling it: Review. Afr. J. Biotechnol. 2003, 2, 254–263.
12. Hu, Y.Y.; Zheng, P.; Zhang, Z.X.; He, Y.Z. Determination of aflatoxins in high-pigment contents samples by matrix solid-phase dispersion and high-performance liquid chromatography. J. Agric. Food Chem. 2006, 54, 4126–4130.
13. Nilu, F.D.; Boyaciog, L.D. Comparative study of three different methods for the determination of aflatoxins in Tahini. J. Agric. Food Chem. 2002, 50, 3375–3379.
14. Horwitz, W. Chapter 49: Natural Toxins. Official Methods of Analysis of AOAC International,17th ed.; AOAC International: Gaithersburg, MD, USA, 2000.
15. Sheibani, A.; Ghaziaskar, H. Pressurized fluid extraction for quantitative recovery of aflatoxins B1 and B2 from pistachio. Food Control 2009, 20, 124–128.
16. Wang, Y.; Chai, T.; Lu, G.; Quan, C.; Duan, H.; Yao, M.; Zucker, B.A.; Schlenker, G.Simultaneous detection of airborne aflatoxin, ochratoxin and zearalenone in a poultry house by immunoaffinity clean-up and high-performance liquid chromatography. Environ. Res. 2008, 107,139–144.
17. Beltran, E.M.I.; Yosa, V.; Felix, H. UHPLC-MS/MS highly sensitive determination of aflatoxins, the aflatoxin metabolite M1 and

ochratoxin A in baby food and milk. Food Chem. 2011, 126,737–744.
18. Sizoo, E.A.; Egmond, V. Analysis of duplicate 24-hour diet samples for aflatoxin B1, aflatoxin M1 and ochratoxin A. Food Addit. Contam. 2005, 22, 163–172.
19. Douglas, H. Noodle. Online Etymology Dictionary. Retrieved 14-10-2009. Available online:http://www.etymonline.com/index.php (accessed on 15 May 2011).
20. Seta, E.; Erkmen, O. The aflatoxin contamination of ground red pepper and pistachio nuts sold in Turkey. Food Chem. Toxicol. 2010, 48, 2532–2537.
21. Sirhan, A.Y.; Tan, G.H.; Wong, R.C.S. Method validation in the determination of aflatoxins in noodle samples using the QuEChERS method and high performance liquid chromatography coupled to a fluorescence detector (HPLC-FLD). Food Control 2011, 22, 1839–1843.