Quantitation of aflatoxins in ground nut and ground nut-based products by high performance liquid chromatography

Sivakumar Sneha[1], Selvakumarasamy Saranya[2*] and Ghadevaru Sarathchandra[3]
1. SRM University, Kattankulathur, Kanchipuram-603203, Tamil Nadu, INDIA
2. Bannari Amman Institute of Technology, Sathyamangalam-638401, Tamilnadu, INDIA
3. Pharmacovigilance Laboratory for Animal feed and food safety (PLAFFS), Directorate of Centre for Animal Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600051, Tamil Nadu, INDIA
*saranselvakumar@gmail.com

Abstract

Aflatoxins (AFs) belong to the family of mycotoxins and are produced as secondary metabolites by various species of Aspergillus. Aflatoxins gave rise to major health issues and had an adverse impact on liver leading to malignancy. Aflatoxins are categorized into different types as B_1, B_2, G_1, G_2, M_1 and M_2. Contamination by Aflatoxin B_1 was highly identified in groundnut compared to other food produce.

The purpose of this study was to identify and evaluate the level of contamination in groundnut and its products which are consumed without proper awareness of their toxin content. This was done to emphasize the importance of tracking the aflatoxin levels in groundnuts. 40 samples were procured from local market and aflatoxins were extracted using Romar's all-purpose method. The samples were analyzed using HPLC. Out of 40, 25 samples showed positive for aflatoxin (42.5%, 32.5%, 32.5% and 17.5% of the samples showed positive results for G_1, G_2, B_1 and B_2 respectively). Contamination with G_1 was highly predominant in the samples.

Keywords: Aflatoxin, Groundnut, Romar's all-purpose method, HPLC.

Introduction

Historically, Aflatoxins were reported to be the cause for heavy loss of turkey poultry in UK during 1960. The disorder thus hesitantly takes its name from the incidence as Turkey-X-Disease. In turkey birds, this disease was associated with the appetite loss, lethargy and fragility of the wings succeeded by death in a week. The factors for this condition were examined and it was found that it was because of the incorporation of Brazilian groundnut meals in livestock and poultry feeds[3]. Aflatoxins are mycotoxins which are also potent mutagens produced by *Aspergillus* molds, primarily *flavus* and *parasiticus*[5]. The aflatoxins are majorly classified as AFB_1, AFB_2, AFG_1 and AFG_2. Aflatoxin B_1 was commonly seen among plants and showed high toxicity content. The IARC had declared AFB_1 as class 1A human carcinogen. Aflatoxins are commonly seen in many of the food commodities like corn, soybean and peanuts[11].

Aflatoxin B_1 was more frequently observed in groundnut in comparison to other crops[14]. This class of fungi prevails commonly in soil and on plants, causing deterioration of grains and food products on storage[11]. The incidence of these fungi was stimulated by two main factors viz. high moisture content and temperature[2].

As these were assessed to be inevitable toxins in food cycle, regulatory authorities like Food and Drug Administration (FDA) of United States had set a tolerable level of 20 ppb for aflatoxins, with regard to all food products and animal feeds[22].They pretense a potent public threat for many developing countries and act as a hindrance for the development of domestic and international markets for food and feed[8].

Aflatoxins are carcinogenic and mutagenic in their biological activity. Liver being the primarily affected organ, toxins accumulate into it and stimulate malignant hepato-cellular carcinomas. On the other hand, rare tumors in organs like kidney are also related to aflatoxins[12]. In 1974, aflatoxins outbreak of hepatitis was reported in the tribes of Gujarat and Rajasthan.

In India, Indian Childhood Cirrhosis was predominantly seen due to the intake of groundnut contaminated with aflatoxin. This disorder prevails in the initial 3 years of the newborns. It affects the liver leading to degeneration and fibrosis, which when untreated brings about jaundice and hepatic coma. Cirrhosis not only confines to India, it had also been prevalent in many tropical countries[26]. Nearly 4.5 billion people (64% of the total world population) in developing countries are prone to aflatoxin risks in their diets[25].

Aflatoxins are detected in a variety of food commodities like cereals, spices, maize, groundnuts (peanuts), chilies, pistachios and black pepper. Milk and milk products also revealed the presence of these mycotoxins[19]. Literature revealed that peanut and peanut products are the most vulnerable foods for aflatoxin contamination[4,7,23].

Contamination by aflatoxins was briefly investigated in the groundnut products of Malawi[20], Ethiopia[21] and Taiwan[18]. The study focusses on screening for the content of aflatoxin in groundnut and its products from Chennai, Tamil Nadu and also on monitoring the levels of aflatoxin in those products.

Material and Methods

Sample preparation: 40 random samples of groundnut and groundnut products were procured from different retail shops and traditional bazaars of Chennai, Tamil Nadu. Every sample was carefully homogenized prior to analysis. Analysis of these samples took place in Pharmacovigilance Laboratory for Animal Feed and Food Safety, Chennai.

Extraction: Extraction of aflatoxins from groundnut samples was carried as per AOAC Romer's all-purpose method[17]. HPLC grade chemicals were used for the extraction. Extraction and filtration followed by clean-up, partitioning and evaporation were carried out as per Romer's all-purpose method. On evaporation, the dried extract was reconstituted with methanol. It was then vortexed and filtered through 0.45 µm nylon membrane filter. The filtered sample was further analysed by HPLC.

Chromatographic conditions: The extracts were subjected to reversed-phase isocratic high performance liquid chromatography, from Shimazu LC 10A employing a platinum C18 column (250 mm × 4.6 mm id, 5 µm)[11]. Temperature was maintained at 40°C. The excitation and emission wavelength were specified as 375 nm and 440 nm respectively in a fluorescence detector. The mobile phase comprises of deionized water, acetonitrile and methanol, applied in the ratio of 60:20:20 at the flowrate of 1.0 mL/min. 20 µL injection volume was applied.

Validation of the methodology: The analytical method was evaluated based on their linearity in results, recovery percentage, accuracy and detection limits prior to the test sample analysis[17].

Estimation of aflatoxin concentrations by HPLC: Standard aflatoxin aliquots of G_1, G_2, B_1 and B_2 were run as per the adopted HPLC conditions. The same conditions were employed for all the sample aflatoxin extracts. The concentration of each aflatoxin was estimated by correlation of the peak area relative to the standards.

Results and Discussion

HPLC, being the most acclaimed procedure for isolation, identification and quantitation of compounds, had gained a lot of pharmacological importance[16]. The chromatographic conditions were adopted from Supelco Reporter[27] with modification. By varying the parameters like temperature, excitation and emission wavelength, peak areas were determined. Optimisation of these parameters also depended on the reduction in noise and background interference.

Based on the results, parameters like temperature, excitation and emission wavelength were optimised. 40°C temperature yielded good results. Similarly, excitation and emission wavelengths were confirmed in the range of 375 nm and 440 nm respectively based on the detection results of aflatoxins B_1 and B_2 as displayed in figures 1 and 2 respectively. This method proved to yield better sensitive results and detected toxin levels even in lower concentrations.

Baseline separation was accomplished for resolution of 1.5 as it caused complete recovery and elution of the appropriate peak without interference by other peaks. Depending on the percentage recovery and nature of chromatogram, Mycosep® #226 AflaZon clean-up cartridge was recommended due to its adequate removal of impurities and precise results in detecting the aflatoxins B_1 and B_2 in groundnut-based products[16]. This finalised method was then utilized for all groundnut and groundnut-based products which were taken for analysis.

Food regulatory authorities namely FDA, USA had established a permissible level of 20 ppb for aflatoxins whereas the European Union (EU) have laid a stringent tolerance limit on aflatoxins as 4 ppb[6]. In India, with regard to food safety and standards, the acceptable level for aflatoxins in foodstuffs was set to be 30 ppb. The outcome of the study was listed in the table 1. The findings report that out of the 40 analyzed samples, 13 samples were positive for both aflatoxins G_1 and G_2. 4 samples affirmed the presence of aflatoxin G_1. B_1 aflatoxin was identified in 13 of the overall 40 samples. Occurrence of aflatoxin B_2 was least predominant as it was seen in seven experimental samples.

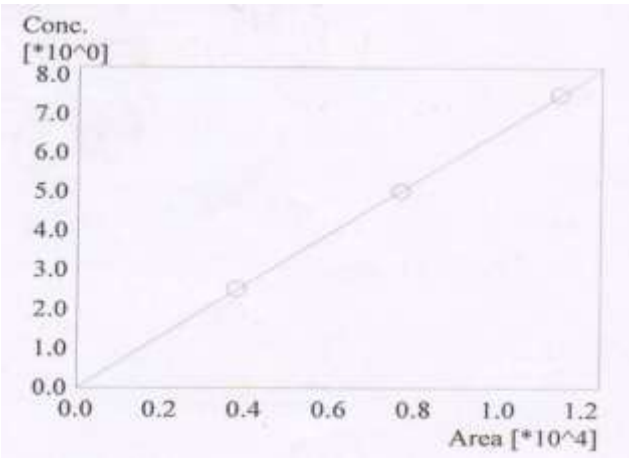

Fig. 1: Calibration curve of Aflatoxin B_1 (ppb)

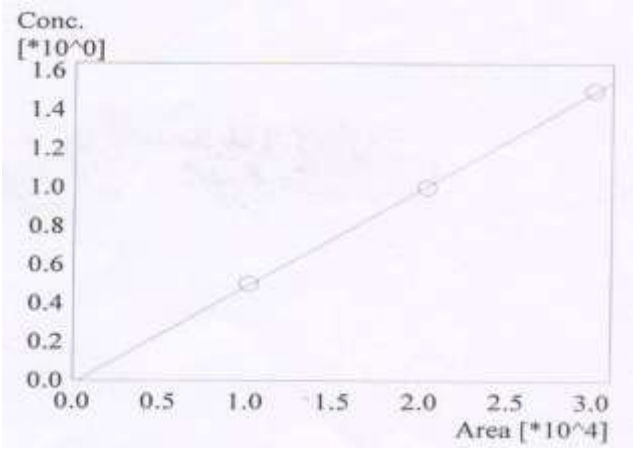

Fig. 2: Calibration curve of Aflatoxin B_2 (ppb)

Table 1
Analysis of the groundnut samples

S.N.	Aflatoxins	Number of positive samples	Percentage of contaminated samples (%)
1	G_1	17	42.5
2	G_2	13	32.5
3	B_1	13	32.5
4	B_2	7	17.5

Total number of samples – 40

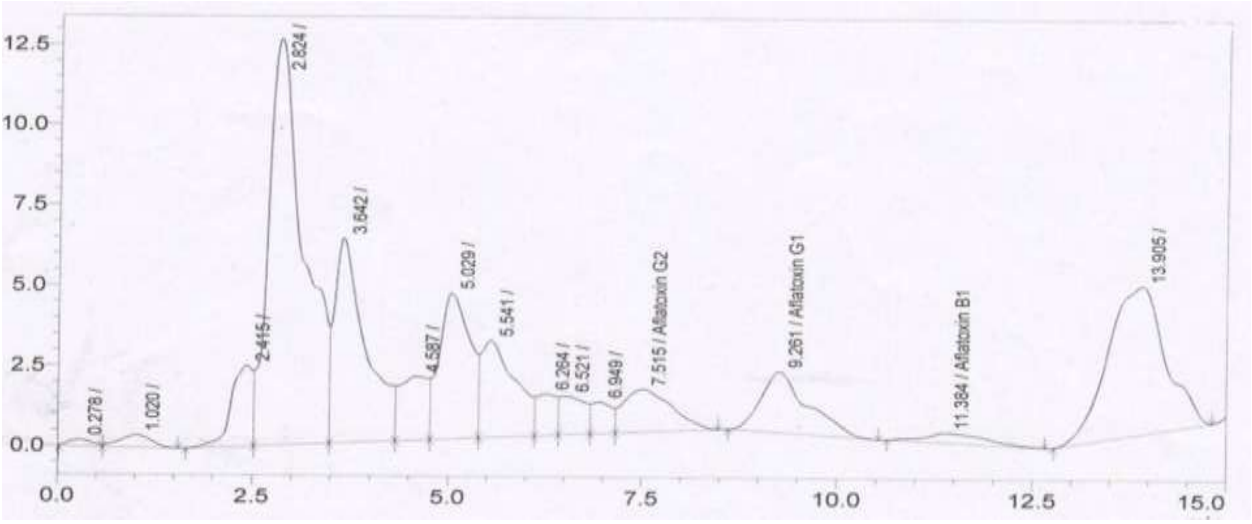

Fig. 3: HPLC Chromatogram of the contaminated groundnut sample

HPLC chromatogram of the sample is illustrated in figure 3. Two samples exceeded the limits of FDA but still pertained to the Indian standards. The concentrations of aflatoxin G_1 were in the range of 0.09-58.8 ppb whereas G_2 varied between 0.3-38.4 ppb. B_1 levels in the groundnut samples varied from 1.13-40.5 ppb. Aflatoxin B_2 was seen in the range of 0.04-31.7 ppb. All these positive samples have future date of expiry.

The ground nut products which were imported to Taiwan from different countries like China, Vietnam, Indonesia and Philippines were assessed for the mycotoxins[18]. The concentration of aflatoxins varied between 0.947-8.1 ppb for Chinese products, 4.18-258.3 ppb for Vietnam products, 11.8-412 ppb for those from Indonesia and 1.05-441 ppb for Philippines food samples. The magnitude of aflatoxins in groundnut and groundnut cake of Eastern Ethiopia was examined and it was found that around 22% and 41% of samples were contaminated. The overall aflatoxin levels were found to be 786 and 3135 ppb[21].

Increased prevalence of aflatoxin G_1 compared to aflatoxin B_1 was described in groundnuts and maize of Malawi[20]. Aflatoxin concentration in groundnut cakes from Sudan was in the range of from 7 to 10 ppb. Aflatoxins in groundnut butter varied from 32 to 54 ppb[28]. Higher concentrations of aflatoxin B1 and B2 (1041 ppb) were reported in the groundnut snack of Nigeria[15].

Aflatoxin contamination in groundnuts is attributed to a wide variety of factors. The high moisture content of groundnut seeds stimulated the distribution of *Aspergillus* on seeds. This might be due to the geographical distribution of the area, management practices by the farmers and also on the genetic diversity of the crops. Groundnuts, when dried up to 6.6% moisture levels, were devoid of fungal contamination for a period of 6 months irrespective of the storage methods and defended the previous findings[1]. Groundnut products are also prone to *Aspergillus* infections in the production and processing stages[13]. Mechanical damage caused as a result of threshing made them exposed for the attack of moulds thereby increasing the toxin levels[9].

Also, the insects induced damage on groundnut pods paved way for fungal entry, thereby leading to aflatoxin accumulation[24]. Investigations revealed a strong association between the inappropriate post-harvest storage and handling with the development of moulds as well as with the increase in aflatoxins[10]. This might be due to the lack of knowledge on groundnut post-harvest techniques like curing, drying and also on storage. Thus, aflatoxin management should begin from farms by provoking a good realization among the farmers on crop management, post-harvest storage and various processing conditions.

Conclusion

Our investigations admitted that HPLC in conjunction with Mycosep® #226 AflaZon clean-up cartridge ensures great product recovery and accuracy for the quantitation of aflatoxins B_1 and B_2 from groundnut and its products. This work revealed that 32.5% of the groundnut-based products evaluated with the recommended procedure showed positive results for aflatoxins and their concentration was beyond the permissible limits. This implies the importance of evaluation and eradication of mycotoxin in food products.

Acknowledgement

The project work was extensively carried out in Pharmacovigilance laboratory for animal feed and food safety, Directorate of Centre for Animal Health Studies, Chennai - 600051.

References

1. Awuah R.T. and Ellis W.O., Effects of some groundnut packaging methods and protection with *Ocimum* and *Syzygium* powders on kernel infection by fungi, *Mycopathologia*, **154(1)**, 29-36 **(2002)**

2. Aycicek H., Aksoy A. and Saygi S., Determination of aflatoxin levels in some dairy and food products which consumed in Ankara, Turkey, *Food Control*, **16(3)**, 263-266 **(2005)**

3. Blount W.P., Turkey "X" disease, *Turkeys*, **9(2)**, 52-55 **(1961)**

4. Chang H.H., Vries J.W.D. and Hobbs W.E., Comparative study of two methods for extraction of aflatoxin from peanut meal and peanut butter, *Journal of the Association of Official Analytical Chemists*, **62(6)**, 1281-1284 **(1979)**

5. Espenschied K.G. and Claus J.E., Improved reproducibility and reduced sample preparation time for the HPLC analysis of aflatoxins in raw peanut paste, *Reporter*, **31**, 16 **(2015)**

6. FAO, Worldwide regulations for mycotoxins in food and feed in 2003, FAO, Rome, Italy **(2004)**

7. Freitas V.P. and Brigido B.M., Occurrence of aflatoxins B1, B2, G1 and G2 in peanuts and their products marketed in the region of Campinas, Brazil in 1995 and 1996, *Food Additives and Contaminants*, **15(7)**, 807-811 **(1998)**

8. Golan E. and Unnevehr L., Food product composition, consumer health and public policy: Introduction and overview of special section, *Food Policy*, **33(6)**, 465-469 **(2008)**

9. Heathcote J.G. and Hibbert J.R., Aflatoxins: chemical and biological aspects, Elsevier Scientific Publishing Co **(1978)**

10. Hill R.A., Wilson D.M., Burg W.R. and Shotwell O.L., Viable fungi in corn dust, *Appl. Environ. Microbiol.*, **47(1)**, 84-87 **(1984)**

11. Hong L.S., Yusof N.I.M. and Ling H.M., Determination of aflatoxins B_1 and B_2 in peanuts and corn based products, *Sains Malaysiana*, **39(5)**, 731-735 **(2010)**

12. International Agency for Research on Cancer and World Health Organization, IARC Working Group on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Papers Presented at the Meeting Held in Geneva, 13-17 December 1971, 1, International Agency for Research on Cancer **(1972)**

13. Kaaya A.N., Harris C. and Eigel W., Peanut aflatoxin levels on farms and in markets of Uganda, *Peanut Science*, **33(1)**, 68-75 **(2006)**

14. Kabbashi E.B.M. and Ali S.E.E., Aflatoxins in groundnut paste in Khartoum State, Sudan, *US Open Food Science and Technology Journal*, **1(3)**, 1-8 **(2014)**

15. Kayode O.F., Sulyok M., Fapohunda S.O., Ezekiel C.N., Krska R. and Oguntona C.R.B., Mycotoxins and fungal metabolites in groundnut-and maize-based snacks from Nigeria, *Food Additives and Contaminants: Part B*, **6(4)**, 294-300 **(2013)**

16. Kazakevich Y.V. and Lobrutto R., HPLC for pharmaceutical scientists, John Wiley & Sons **(2007)**

17. Krishnan B., Ghadevaru S., Manimehalai N., Athmaselvi K. and Padmavati R., Determination of aflatoxin B1 in corn flour using high performance liquid chromatography, *Int J Adv Biol Res*, **5**, 172-176 **(2015)**

18. Lien K.W., Wang X., Pan M.H. and Ling M.P., Assessing aflatoxin exposure risk from peanuts and peanut products imported to Taiwan, *Toxins*, **11(2)**, 80 **(2019)**

19. Makun H.A., Anjorin S.T., Moronfoye B., Adejo F.O., Afolabi O.A., Fagbayibo G., Balogun B.O. and Surajudeen A.A., Fungal and aliatoxin contamination of some human food commodities in Nigeria, *African Journal of Food Science*, **4(4)**, 127-135 **(2010)**

20. Matumba L., Sulyok M., Njoroge S.M., Ediage E.N., Van Poucke C., De Saeger S. and Krska R., Uncommon occurrence

ratios of aflatoxin B1, B2, G1 and G2 in maize and groundnuts from Malawi, *Mycotoxin Research*, **31(1)**, 57-62 **(2015)**

21. Mohammed A., Chala A., Dejene M., Fininsa C., Hoisington D.A., Sobolev V.S. and Arias R.S., *Aspergillus* and aflatoxin in groundnut (*Arachis hypogaea* L.) and groundnut cake in Eastern Ethiopia, *Food Additives and Contaminants: Part B*, ***9*(4)**, 290-298 **(2016)**

22. Munkvold G.P., Hurburgh C.R., Meyer J.R., Loy D. and Robertson A., Aflatoxins in corn, Ames, IA: Iowa State University, University Extension **(2005)**

23. Njapau H., Muzungaile E.M. and Changa R.C., The effect of village processing techniques on the content of aflatoxins in corn and peanuts in Zambia, *Journal of the Science of Food and Agriculture*, **76(3)**, 450-456 **(1998)**

24. Waliyar F., Kumar P.L., Traoré A., Ntare B.R., Diarra B. and Kodio O., Pre-and post-harvest management of aflatoxin contamination in peanuts, Mycotoxins: detection methods, management, public health and Agricultural trade, CABI, Wallingford, UK, 209-218 **(2008)**

25. Williams J.H., Phillips T.D., Jolly P.E., Stiles J.K., Jolly C.M. and Aggarwal D., Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences and interventions, *The American Journal of Clinical Nutrition*, **80(5)**, 1106-1122 **(2004)**

26. Yadgiri B., Reddy V., Tulpule P.G., Srikantia S.G. and Gopalan C., Aflatoxin and Indian childhood cirrhosis, *The American Journal of Clinical Nutrition*, **23(1)**, 94-98 **(1970)**

27. Yearick V. and Doughty E., Aflatoxins standard available from Supelco, *Food Bev*, **16**, T297085 **(1997)**

28. Younis Y.M. and Malik K.M., TLC and HPLC assay of aflatoxin contamination in Sudanese peanuts and peanut products, *Kuwait J Sci Eng*, **30(1)**, 79-93 **(2003)**.

(Received 28th April 2020, accepted 06th July 2020)