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Simultaneous Estimation of Aflatoxins (B_1, B_2, G_1 and G_2) by Liquid Chromatography Coupled with Mass Spectrometry (LC-MS) in Corn Samples

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Aflatoxins produced by *Aspergillus flavins* and *Aspergillus pasasiticus* have been investigated in the corn (maize) collected in different locations in Andhra Pradesh state, India. In the present study, 24 corn samples collected from different harvest, storage and dumping areas were analyzed for detection of aflatoxin contamination by liquid chromatography coupled with mass spectrometry (LC-MS). Mixed aflatoxin working standard solutions with a concentration of 10, 20, 30, 40, 50 and 60 ng/mL were prepared and followed by instrumental analysis and the retention times of the sample chromatograms were compared with the standard aflatoxins B_1, B_2, G_1 and G_2 retention times in order to determine the contamination. The findings revealed that among 24 collected samples, only 10 samples have been found positive for aflatoxins contamination when subjected to LC-MS analysis. Co-occurrence of aflatoxin was identified in the most of the samples. Even though 10 samples were tested positive total aflatoxin content in the most positive are found within the acceptable limits (30 µg/kg) and samples collected at dumping areas (S4, S8 and S10) show the presence of exceeding quantity than the limits described by FSSAI. Total aflatoxin content in the most positive were found within the acceptable limits (30 µg/kg) and the samples collected at dumping areas S4 (40.9 µg/kg), S8 (53.2 µg/kg) and S10 (71.86 µg/kg) had shown exceeding limits. The sample S7 collected from the storage was also found positive of presence of aflatoxin G2- 13.49 µg/kg and B2-21.14 µg/kg with total aflatoxin of 34.65 µg/kg. High contamination of these dumping areas may lead to contamination of storage points nearer to these areas. Present results showed that the corn samples at dumping areas were found to be contaminated with the *Aspergillus flavins* and *Aspergillus pasasiticus*.

Keywords: Corn, Aflatoxin, LC-MS, *Aspergillus flavins*, *Aspergillus pasasiticus*.

INTRODUCTION

Mycotoxins are the fungal metabolites emerging from the fungal growth process. They are a range of toxic and harmful substances and currently over 400 types of mycotoxins have been identified. Aflotoxins belongs to the mycotoxins produced primarily by the *Aspergillus flavins* and *Aspergillus pasasiticus*. World health organization (WHO) and U.S Environamental protection agency (USEPA) have classified that afltoxins are as human liver carcinogens [1,2]. Aflatoxins (B_1, B_2, G_1 and G_2) are the contaminants in food, feed and dairy products. The mycotoxins including aflatoxins produced by fungi can cause severe effects on animal and human health and recognized as potential threat to human and animals. Contamination of raw materials for food based supplements could leads to the contamination of food based supplements prepared with the raw materials. Moreover, the amount of aflatoxins increases under poor storage conditions which favour for the fungal growth. The Food safety standards (contaminants, toxins and residues) regulation, 2011 for all food are prescribed the 30 µg/kg tolerance limit of aflatoxins for human consumption [3]. Exposure to these aflatoxins has shown to cancer in humans and livestock and among all aflatoxins B_1 is most carcinogenic. The major commodities that are contaminated by the aflatoxins are peanut, corn, rice, dry fruit and spices. Since the animal food also uses these commodities and agricultural crops, contamination of aflatoxins also affects the animal health.

Strict regulations for aflatoxins have been set to prevent the consumption of aflatoxins. United States Food and Drug Administration (US-FDA) has established maximum level 20

ppb of aflatoxins in food and feed to protect human and animal health. The European Union (EU) limits the aflatoxin B_1 of 8 ppb and M_1 of 0.05 µg/kg. The detection of aflatoxins becomes an important topic for all over the world in order to monitor, provide safe grains and products for human and animals. Many of the established methods have been described for analysis of aflatoxins in complex matrices. The primary methods for analysis of aflatoxins include thin layer chromato-graphy (TLC), flurospectrometry, ELISA, liquid chromato-graphy (LC) and liquid chromatography with mass spectrometry (LC/MS) [4-9]. Among them liquid chromatography with mass spectrometry (LC/MS) and liquid chromatography tandam mass spectrometry have been proved to be promising and efficient technique for the detection of multiple mycotoxins in different samples with high sensitivity and selectivity that provides qualitative and quantitative data.

The maize has emerged as an important crop in the non-traditional regions in India. Maize is being used in different sectors and activities across in India and among them; poultry industry (47%) is the biggest of them all. Other uses of maize are cattle feed (14%) and starch (14%) followed by food and beverage industry (7%). Andhra Pradesh state has recorded the highest production (4.14 Mt) and productivity (5.26 t ha^{-1}) of maize in India although the productivity in some of the districts of Andhra Pradesh is more or equal to the USA [10,11]. Andhra Pradesh is the top of the states that produce maize, the predominant maize growing states that contributes more than 80% of the total maize in India are Andhra Pradesh (20.9%), Karnataka (16.5%), Rajasthan (9.9%), Maharashtra (9.1%), Bihar (8.9%), Uttar Pradesh (6.1%), Madhya Pradesh (5.7%) and Himachal Pradesh (4.4%). Among aflatoxins B_1 is most lethal and classified as group one carcinogenic to humans [12]. It is known for causing the hepatocellular carcinoma due to the synergic action with hepatitis B or with fumonisins and ochratoxins [13,14]. Other aflatoxins B_2, G_1 and G_2 are less carcinogenic than the B1 [15]. The present work is aimed to determine the contamination of different maize (corn) samples collected from different storage areas by using liquid-liquid extraction followed by liquid chromatography-mass spectrometry (LC/MS) analysis.

EXPERIMENTAL

The maize (corn) samples studied for aflatoxin are collected from harvesting area, storage area godowns and dumping areas located at the godowns and agricultural markets situated in different locations in Karnool, Kadapa, Anatapur and Guntur Districts in Andhra Pradesh, India. Total 24 samples have been collected and among them 10 at dumping area, 11 at storage area and 3 at harvesting area.

The liquid chromatography instrument Agilent 1100 series HPLC is used for separation of aflatoxins. The instrument is equipped with Quaternary G1311 A pump, COLCOM G1316A thermostat column temperature control, Thermostatic auto sampler G 1329A with sample volume capacity of 0.1-1500 µL and variable programmable UV detector G 1314 A. The software used for operation of the instrument is integrated Agilent chem. station LC software. The Agilent HPLC was coupled with Waters ZQ Mass Detector (model LAA 1369) with quadrapole analyzer with Waters Empower software. The mass spectra was taken in ESI (Turbo Ion Spray) positive mode in mass range of 40-1000 amu and analyzed in the triple quadrupole analyzer.

All the solvents used for chromatography separation were HPLC grade and the chemicals used for extraction and preparation of samples were of analytical grade. HPLC grade methanol and water used for LC was purchased from the Thermo-Fisher Scientific India Pvt. Ltd., Mumbai, India. The HPLC grade acetonitrile was purchased from the Merck chemicals private limited, Mumbai, India. The other chemicals sodium chloride, anhydrous sodium sulfate, ammonium formate, formic acid are purchased from Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India. The standard aflatoxins (B_1, B_2, G_1 and G_2) were purchased from the Sigma Aldrich Inc., Bangaluru, India.

Preparation of aflatoxin standard solutions: The stock solution of mixture aflatoxin B_1, B_2, G_1 and G_2 was prepared by weighing 10 mg of the mixture standard and dissolved in 100 mL of methanol. This standard stock solution was kept at -20 ºC. A series of volume 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mL of stock solution was pipette in to 10 mL volumetric flask and diluted with methanol and make up to 10 mL to prepare the six-calibration series from 10, 20, 30, 40, 50 and 60 ng/mL. All these working standard solutions were injected in to LC-MS in order to prepare standard aflatoxins calibration curve.

Extraction of aflatoxins: The method adopted for the extraction of aflatoxins from the maize samples is simultaneous extraction and purification of aflatoxins modified method of Sirhan *et al.* [16] was used. The maize sample was grounded with pulverizer and passed through a test sieve with a 1mm pore size then mixed well. About 0.5 g of sample and 0.2 g of sodium chloride were weighed into a 250 mL conical flask and 40 mL of methanol/water (80:20, v/v) was added. The mixture was stirred for 3 min at a high speed and filtered through a Whatman No.1 filter paper. The filtrate was rinsed twice with 5 mL methanol. After that the extracts were dried with anhydrous sodium sulfate and evaporated until dryness using a rotary evaporator at 45 ºC under vacuum. Finally, the residue was reconstituted with 0.5 mL methanol and diluted 10 times with the mobile phase (water containing 2 mM ammonium formate and 1% formic acid: methanol containing 1% formic acid, 40%:60%, v/v) and passed through a 0.2 mm disposable membrane filter prior to the LC-MS analysis.

LC-MS analysis: For the quantitative estimation of aflatoxins using HPLC, modified method [12] was adopted and summarized beneath. Chromatographic separation was achieved using symmetry Zorbax Ecilipse XBD C18 column (2.1 mm × 100 mm × 1.8 mm) at 40 ºC. The mobile phase was consisting of eluents 1% formic acid and 2 mM ammonium formate in water (A) and 1% formic acid in methanol (B), was employed in the isocratic mode with 40% of solvent A and 60% solvent B (v/v). The flow rate of the eluents is 0.3 mL/min. Sample volume was set at 10 µL in auto sampler. Mass spectrometer analysis was as follows: ESI source block and desolvation temperatures 150 and 300, respectively. Capillary

voltage was set at 3.2 Kv, cone voltage at 40 V, extractor voltage 3 V, Rf lens at 0, respectively. Cone nitrogen and desolvation gas flow was operated at 300 Psi. The mass of the aflatoxins was determined by the MS finger print data of Mass LYNX V4 was built up firstly based on the MS spectra solutions of standards. Initially calibration standard solutions of aflatoxins were injected to plot the calibration curve. The retention time of the standard aflatoxins (B_1, B_2, G_1 and G_2) were compared with the retention times of adopted method for conformation. The mass of analyte peaks also compared with their original mass value and confirmed the successful implementation of Sirhan *et al.* method [16]. The individual samples prepared after extraction from the maize samples were injected and retention times of the eluted compounds were compared to identify the aflatoxins present in the samples.

RESULTS AND DISCUSSION

The results of moisture analysis and physical condition of the maize samples are presented in the Table-1 and images of the collected corn samples are presented in Fig. 1. Mixed aflatoxin working standard solutions with a concentration of 10, 20, 30, 40, 50 and 60 ng/mL were prepared and followed by instrumental analysis. The calibration results were drawn in to calibration graph in order to found the linearity of the standard solution and the calibration graph with prepared working standard solutions were found to be linear with good correlation coefficient (0.999) (Fig. 2). All the aflatoxins were separated within 10 min under method conditions and the retention times of the sample chromatograms were compared with the standard aflatoxins B_1, B_2, G_1 and G_2 retention times in order to determine the contamination. The regression equation and correlation coefficient of the standard calibration curves of G_2, G_1, B_2 and B_1 were found G_2-y = 23420x – 270.79, R^2 = 0.9995, G_1-y = 17336x – 1044.1, R^2 = 0.9991, B_2-y = 17641x – 96.76, R^2 = 0.9997 and B_1-y = 20670x – 1229.6, R^2 = 0.9989, respectively. The chromatogram of aflatoxin standard solution and mass spectra of positive ion mode are presented in Fig. 3. A total 24 samples have been collected and studied for presence of aflatoxins in maize samples. The moisture of the samples collected and conditions of the sample also observed initially.

TABLE-1
REPRESENTING THE LIST OF SAMPLES COLLECTED IN DIFFERENT AREA AND THEIR MOISTURE VALUE WITH PHYSICAL CONDITION

Sample	Location	Moisture	Physical condition
S1	Dumping area	15.3	Damaged
S2	Storage area	14.3	Slightly damaged
S3	Harvesting area	14.0	Good
S4	Dumping area	14.9	Damaged
S5	Storage area	13.2	Good
S6	Storage area	13.3	Damaged
S7	Storage area	14.7	Damaged
S8	Dumping area	15.0	Damaged
S9	Dumping area	17.5	Damaged
S10	Dumping area	21.2	Damaged
S11	Dumping area	16.4	Damaged
S12	Harvesting area	14.6	Slightly damaged
S13	Storage area	13.0	Good
S14	Dumping area	13.8	Damaged
S15	Storage area	14.9	Slightly damaged
S16	Harvesting area	14.2	Good
S17	Storage area	17.6	Damaged
S18	Storage area	17.9	Damaged
S19	Dumping area	22.6	Damaged
S20	Dumping area	20.3	Slightly damaged
S21	Dumping area	18.3	Slightly damaged
S22	Storage area	19.7	Damaged
S23	Storage area	22.4	Slightly damaged
S24	Storage area	15.1	Good

The molecular weights of the peaks in mass spectra identified in samples were also compared with the molecular weight of the standard aflatoxins. The findings revealed that among all collected samples only 10 samples have been found positive for aflatoxins contamination when subjected LC-MS analysis.

Fig. 1. Images of collected corn samples

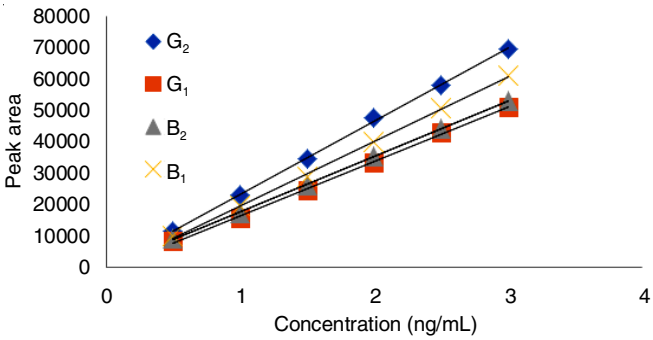

Fig. 2. Calibration curve of standard aflatoxins

Most of the aflatoxin positive samples were identified with presence of one or more aflatoxins. Four of them are contaminated with aflatoxin G_1, five of them are contaminated with aflatoxin G_2, six of them are contaminated with B_2 and four of them are contaminated with aflatoxin B_1 (Fig. 4). The presence of the individual aflatoxins and their concentration are presented in Table-2. Even though 10 samples were tested positive for aflatoxins only three of them are found showed levels of over the permissible limits.

The quantitative results of the positive samples collected at dumping areas (S4, S8 and S10) shows the presence of exceeding quantity than the limits described by FSSAI. Total aflatoxin content in the most positive are found within the permittable limits (30 µg/kg) and the samples collected at dumping areas S4 (40.9 µg/kg), S8 (53.2 µg/kg) and S10 (71.86 µg/kg) has shown exceeding limits. The sample S7 collected from the storage are also found positive of presence of aflatoxin G_2 13.49 µg/kg and B_2 21.14 µg/kg with total aflatoxin of

TABLE-2
RESULTS OF IDENTIFIED AFLATOXINS IN COLLECTED SAMPLES AND TOTAL AFLATOXINS CONTENT

Sample	Compound	Amount present (µg/kg)	Total aflatoxin content (µg/kg)
S1	G_1	22.18	31.69
	B_1	9.50	
S2	B_2	19.0	19.4
S4	G_2	14.76	40.9
	B_1	26.14	
S7	G_2	13.49	34.65
	B_2	21.14	
S8	G_2	7.53	53.2
	G_1	45.66	
S10	G_2	18.95	71.86
	B_2	29.47	
	B_1	23.44	
S14	G_1	18.40	18.4
S17	B_2	10.17	29.18
	B_1	19.01	
S20	G_1	11.04	16.01
	B_2	4.97	
S23	B_2	5.21	9.62
	G_2	4.41	

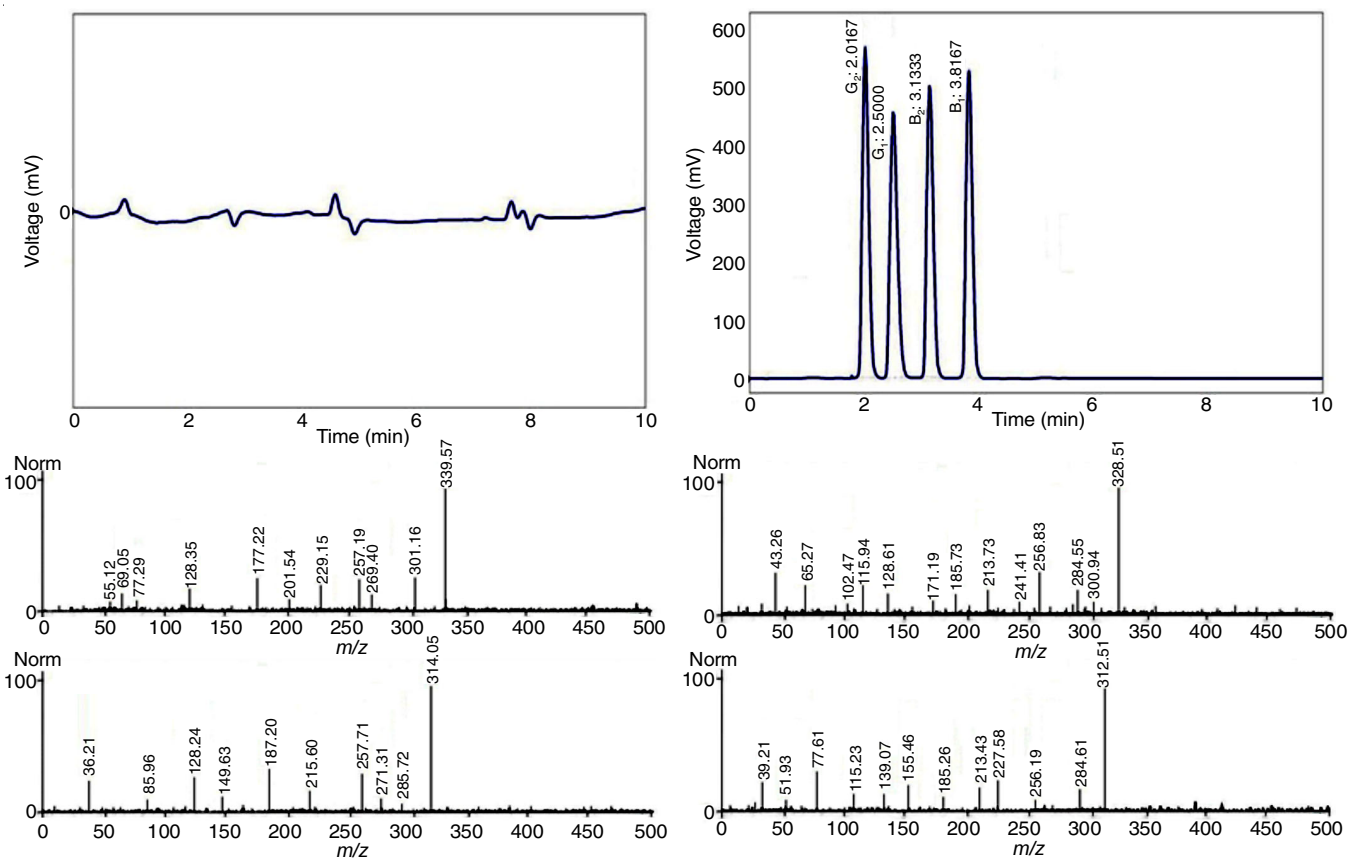

Fig. 3. Chromatograms of the blank and standard aflatoxin solution using LC/MS and mass spectra of identified aflatoxin G_2, G_1, B_2 and B_1, respectively. The retention time of the peaks corresponding to the each aflotoxin are as follows : G_1 - 2.06, G_1 - 2.50, B_2 - 3.13 and B_1 - 3.81

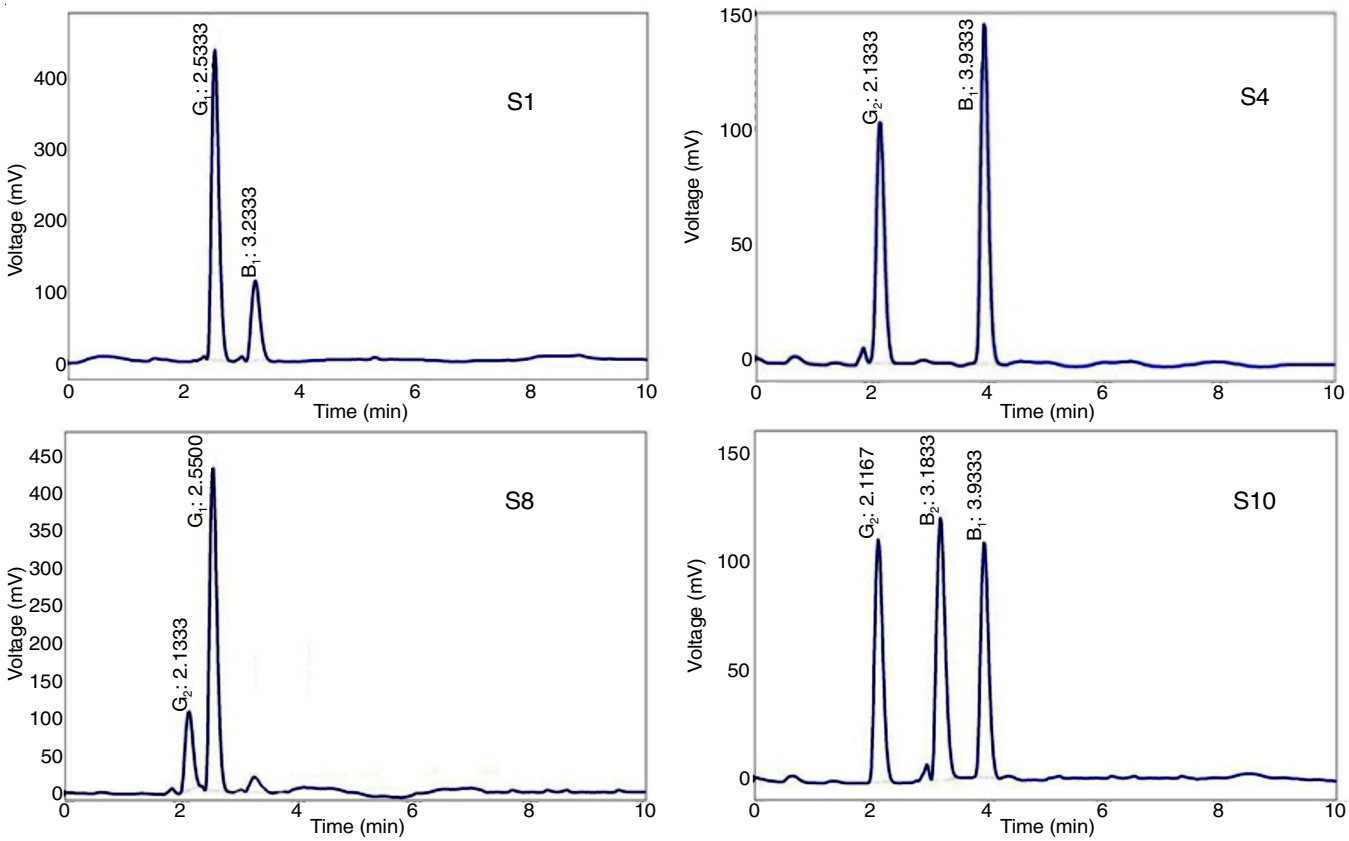

Fig. 4. Chromatograms of the samples detected with aflatoxins LC/MS in corn

34.65 µg/kg. The quantitative result of the positive corn samples and total aflatoxins content in the samples are presented in Table-2.

Co-occurrence of the aflatoxins in the positive detected samples was found in most of the samples. Out of 10 positive samples eight of them have found co-occurrence of two or more aflatoxins. Among them S10 found consist of aflatoxin B_1, B_2 and G_2 represents occurrence of three types in one maize sample. Total 80% of the samples consist of the co-occurrence of two types of aflatoxins. Aflatoxin B_2 and G_2 were frequently detected in positive corn samples than the B_1 and G_1. The total percentage of the B_2 content in all samples was found 33% and G_2 was found 29% and together shares 62% in total aflatoxin content. The relation between the moisture of the collected samples and aflatoxin content also studied and found that moisture which supports the growth of the moulds generally enhances the growth and subsequently the quantity of the aflatoxins produced by the fungi. Similar results were identified and presented in Fig. 5. Thus, the levels of B_2 and G_2 were found more frequent and high percentage in positive corn samples. They should have been much lower than the detected levels because low exposure of the dietary toxins could pose a carcinogenic risk to human and toxic to the animal also. Similar results of aflatoxin contamination 77.3% of B_1 and 28% of B_2 were reported with 80-110 µg/kg by the studies of Fareed *et al.* [17] in poultry feed and finished feed samples. Reddy & Saleha [18] also studied the aflatoxin contamination in corn samples and 22.5% positive contamination for B_1 and 20.6 to 135 µg/kg in poultry feed samples. Shah *et al.* [19] also reported 77.8% contamination of B_1 and 88.9% contamination of B_2 aflatoxins in corn samples.

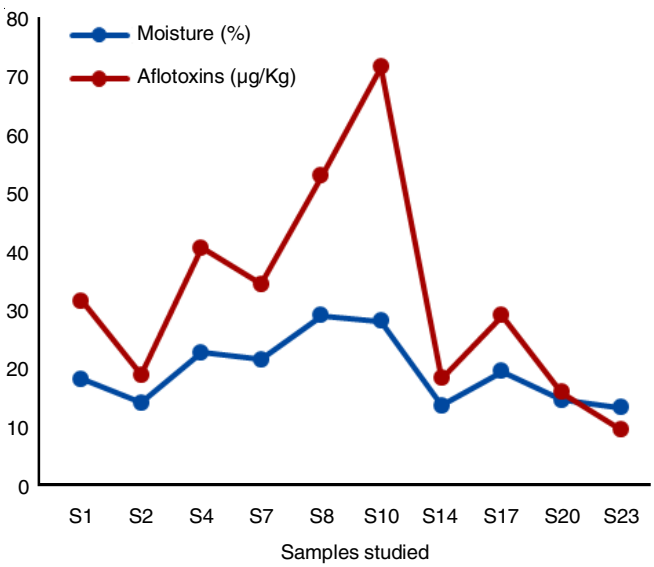

Fig. 5. Comparative graphs of the relation between the moisture of the samples and estimated aflatoxin content in µg/kg

The results show that the corn samples at dumping areas are found contaminated with the *Aspergillus flavins* and *Aspergillus pasasiticus*. Overall, total aflatoxin levels in 10 positive corn samples were analyzed and 6 of them were below the maximum allowable limit. The dumping area of the storage

areas found more contaminated by the fungi where more damaged samples are present and the content of the aflatoxins also found high than the allowable limit. Therefore, monitoring of the aflatoxins in corn samples should be continued and maintenance of the dumping areas at different storage points with control methods are recommended.

Conclusion

Aflatoxins contamination of maize (corn) in different storage conditions were analyzed and also investigated the co-occurrence. Liquid chromatography coupled with mass spectrometry (LC-MS) technique was adopted for determination of aflatoxin contamination. The results of the present show that the corn samples at dumping areas were found to be contaminated with *Aspergillus flavins* and *Aspergillus pasasiticus*. Overall, total aflatoxin levels in 10 positive corn samples were analyzed and 6 of them were below the maximum allowable limit. The co-occurrence of the aflatoxins also found in most of the samples and can cause synergetic effects to human and animals. It is necessary that efficient control methods to prevent and monitor contamination of aflatoxin in corn and other food grains.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. World Health Organization, WHO Traditional Medicine Strategy 2002-2005; WHO: Geneva, Switzerland (2002).
2. Action Levels for Poisonous or Deleterious Substances in Human Food and Animal Feed, Industry Activities Staff Booklet, U.S. Food and Drug Administration, Washington, DC (2000).
3. Food Safety and Standards (Contaminants, Toxins and Residues) Regulations F.No. 2-15015/30/2010; Food Safety and Standards Authority of India, Ministry of Health and Family Welfare: New Delhi: (2011).
4. V. Betina, *J. Chromatogr. Lib.*, **54**, 141 (1993); https://doi.org/10.1016/S0301-4770(08)60567-9
5. R. Krska, S. Baumgartner and R. Josephs, *Fresenius J. Anal. Chem.*, **371**, 285 (2001); https://doi.org/10.1007/s002160100992
6. R. Krska and R. Josephs, *Fresenius J. Anal. Chem.*, **369**, 469 (2001); https://doi.org/10.1007/s002160100715
7. R. Krska, P. Schubert-Ullrich, A. Molinelli, M. Sulyok, S. Macdonald and C. Crews, *Food Addit. Contam.*, **25**, 152 (2008); https://doi.org/10.1080/02652030701765723
8. J.C. Young and P. Lafontaine, *Rapid Commun. Mass Spectrom.*, **7**, 352 (1993); https://doi.org/10.1002/rcm.1290070509
9. S. Ommi, M. Medikonda and G. Krishnaveni, *Int. J. Pharma Bio Sci.*, **9**, 371 (2019).
10. https://farmer.gov.in/M_cropstaticsmaize.aspx.
11. https://www.kleffmann.com/en/kleffmann-group/news—press/press-releases/india—maize-productivity-and-crop-potection.
12. G.N. Wogan, *Bacteriol. Rev.*, **30**, 461 (1996).
13. I. Kamika and L.L. Takoy, *Food Control*, **22**, 1760 (2011); https://doi.org/10.1016/j.foodcont.2011.04.010
14. E.E. Golli-Bennour, B. Kouidhi, A. Bouslimi, S. Abid-Essefi, W. Hassen and H. Bacha, *J. Biochem. Mol. Toxicol.*, **24**, 42 (2010); https://doi.org/10.1002/jbt.20310
15. X. Ding, P. Li, Y. Bai and H. Zhou, *Food Control*, **23**, 143 (2012); https://doi.org/10.1016/j.foodcont.2011.06.026
16. A. Sirhan, G. Tan, A. Al-Shunnaq, L.B. Abdulra'uf and R.C.S. Wong, *J. Liq. Chromatogr. Rel. Technol.*, **37**, 321 (2014); https://doi.org/10.1080/10826076.2012.745138
17. G. Fareed, S. Khan, M. Anjum and N. Ahmed, *J. Adv. Vet. Anim. Res.*, **1**, 201 (2014); https://doi.org/10.5455/javar.2014.a38
18. K.R.N. Reddy and B. Salleh, *J. Anim. Vet. Adv.*, **10**, 668 (2011); https://doi.org/10.3923/javaa.2011.668.673
19. H.U. Shah, T.J. Simpson, S. Alam, K.F. Khattak and S. Perveen, *Food Chem. Toxicol.*, **48**, 1111 (2010); https://doi.org/10.1016/j.fct.2010.02.004