ORIGINAL ARTICLE

Evaluation of Fumonisins Contamination using Liquid Chromatography Mass Spectrometry in Corn Samples Collected from Different Locations

Sailaja Ommi[1,2]*, Manoranjani Medikonda[3], Krishnaveni Gudela[2]

[1]Department of Chemistry, Krishna University, Machilipatnam, Andhra Pradesh, India, [2]Department of Chemistry, KBN College, Vijayawada, Andhra Pradesh, India, [3]Department of Chemistry, PB Siddhartha College, Vijayawada, Andhra Pradesh, India

Abstract

Aim: The aim of the study was to determine fumonisins (FMS) using liquid chromatography coupled with mass spectrometry in maize. **Materials and Methods:** FMSs are mycotoxins frequently found in contaminated corn samples. The presence of FMSs in corn samples should be continuously controlled to protect the population form risks associated with its proven toxicity. About 24 corn samples were collected in different locations of Andhra Pradesh such as harvesting area, storage area, and dumping area of godown for determination of contamination by FMSs. **Results and Discussion:** The retention time of the well-defined peak of FMS B1 and B2 was found 1.15 min and 2.59 min which is similar to the obtained method. Among 24 samples, about 7 corn samples were found positive contamination by FMS when subjected to liquid chromatography-mass spectrometry analysis. The high levels of FMS contamination were found in sample number 7(s-7) which is collected from the dumping area of storage godown. The fungal damaged sample was found B1concentration of 242.11 µg/kg and FB2 of 117.15 µg/kg. The concentration of FB1 is very high in the sample compared to the regulatory guidelines limit. The low amount of FMS is identified in sample number 8(s-8) which is collected from the dumping area and in damaged condition. FB1 concentration was found 15.0 µg/kg. The sample 4(s-4) is found contaminated with FB2 with 15.780 µg/kg concentration. **Conclusion:** Based on the results, it can conclude that corn samples collected from the dumping area of godown are found more contaminated with mycotoxin (FMS) than the other sample collecting area such as storage area and harvesting area.

Key words: Corn, fumonisins, liquid chromatography and mass spectrometry, mycotoxins

INTRODUCTION

Mycotoxins are secondary toxic metabolites released by some fungi. Different types of mycotoxins in the world most frequently observed and mostly affects health of humans and livestock are ochratoxins A, aflatoxins, and fumonisins.[1] Fumonisins are majorly produced by *Fusarium verticillioides* and *Fusarium proliferatum* strains. This fumonisins (FMS) group contains FMS Type A1 and A2, these are produced in very small amounts and nontoxic. The other type contains B1, B2, B3, and B4,[2] among them, B1 is the most toxic and abundant toxic substance produced by certain fungal strains. The B1 and B2 are majorly found contaminants in corn (maize).[3] Chemically, FMSs are made of two methyl groups and single amine groups (-NH2), 1-4 (-OH-), and bistricarboxylic ester groups present at different positions along with the (c-18) polyketide backbone. FMSs are having structural similarity to sphingosine and these are involved in the process of cell signaling, communication, and growth. FMS B1 chemically called as: 1, 2, 3-propanetricarboxylic acid, 1, 1′-[1-(12-amino-4,9,11-trihydroxy-2-methyltridecyl)-2- (1-methylpentyl)-1,2-ethanediyl] ester whose molecular formula is: C34H59NO15

Address for correspondence:
Sailaja Ommi, Department of Chemistry, KBN College, Vijayawada, Andhra Pradesh, India.
Phone: +91-9985019882.
E-mail: sailajabotta@gmail.com

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and molecular weight is: 721.[4,5] The contamination of crops or stored foods by FMS is due to agro climatic conditions.[6]

Dietary utilization of FMS causes many abnormal health conditions in both humans and animals. It increases risk factors to increase in esophageal cancers. This compound is also showing effects on the liver and also acts as nephrotoxic in many animal species, these toxins are also responsible for, pulmonary edema syndrome in pigs, and apoptosis in many other types of cells.[7] Every year 25% of harvested crops are affected due to mycotoxins contamination, which causes a huge economic loss to the agriculture and industrial sector. The aggregation of FMSs observed in different developmental stages of kernel. Due to Lower humidity, kernels lead to destruction by insects and also causes the growth of FMSs in plants.[4] These FMSs are stable in food items and will not eliminate during the processing, cooking, backing, pasteurization, and roasting. Grains contaminated with moderate amounts of FMS are known to be toxic to either horses or swine.[8] Hence, it is necessary to detect the FMS and their contamination levels in various foods items in their processing stages. The most accurate methods to detect the contamination of FMS s are high-performance liquid chromatography (HPLC) with fluorescence detection and/or mass spectrometry and/or enzyme-linked immunosorbent assays. Liquid chromatography mass spectrometry (LC-MS) has capability to multiplex several analytes with in a single analytical run. It provides superior specificity, sensitivity, and accurate compared to other analytical techniques. In recent years, HPLC-MS applications in agri sector are among the fastest developing fields. The present study is aimed to LC-MS determination of FMSs contamination in maize samples collected at different storage and harvest conditions.

MATERIALS AND METHODS

Samples

Total 24 Maize (Corn) samples have been collected and among them 10 samples at dumpig area, 11 samples at storage area, and 3 samples at harvesting area. The sampling points for FMSs 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.

Chemicals and solvents

All the solvents used for LC-MS analysis are HPLC grade chemicals and chemicals used for the extraction and preparation of samples are analytical grade. Methanol and water (HPLC-grade) were acquired commercially from Thermo Fisher Scientific India Private Limited, Mumbai. The HPLC grade acetonitrile was purchased from the Merck Chemicals Private Limited, Mumbai. The other chemicals such as formic acid purchased from Thermo Fisher Scientific India Private Limited, Mumbai. The standard FMSs (B1 and B2) and the extracting solvent Supelclean® primary secondary amine (PSA) were purchased from Sigma Aldrich, Bangaluru, India.

Standard solutions

The standard FMS B1 and FMS B2 stock solutions were prepared by dissolving standard FMSs in mixture of acetonitrile and water (50:50 v/v) as a diluent solvent. About 100 mg of FMS B1 and FMS B2 are weighed and taken in to 100 mL volumetric flask. About 10 ml of diluent added and sonicated for 15 min to prepare 1000 µg/mL each FMS B1 and FMS B2 as combined standard stock solution. This standard stock solution was further diluted with diluent to prepare 2, 5, 20, 50, 100, and 400 µg/mL of calibration solutions.

Instrumentation

The instrument was worked and incorporated with Waters 2695 Alliance HPLC system (waters Corporation, Milford, MA, USA) consisting of quaternary pump, column chamber with temperature control, auto injector, online degasser, and ultra violet detector (water 487 model). The liquid chromatography is interfaced with a mass spectrometer coupled with an electrospray ionization source operated in the positive mode. Mass spectrometry analysis was carried out with Micro mass ZQ mass detector model LAA 1369 (Micromass Ltd, UK). The chromatography and mass data interpretation were carried out with masslynx software.

Sample preparation

Extraction of mycotoxins FMNs for LC-MS analysis was performed by method described by.[9] The corn samples picked from different areas (Dumping area, harvesting area, and storage area,) are made to dried and grind to a fine meal with laboratory mill. 2 g of the sample was accurately weighed and taken into a 50 mL polypropylene tube. 20 mL of methanol/water (3:1 v/v) added and placed in ultrasonic water bath for 10 min at room temperature for sample extraction. 25 mg PSA of 5 ml plastic centrifuge with aliquot of the extract (2 ml) was mixed. This solution was shacked vigorously and tube was centrifuged for 5 min at 5000 rpm. Supernatant was discarded and 2 mL of 1.0% formic acid in methanol was added in tube. The supernatant was diluted with water in 1:1 (v/v) proportion. Preceding final instrumental examination, test solution was shifted to 0.2 µm nylon layer filter.

The estimation of FMS by HPLC-MS analysis

Determination and estimation of mycotoxins FMSs by LC-MS analysis were performed by method described by Yang and Wu.[9] HPLC carried out with stationary phase

of Zorbax Eclipse XDB-C18 column (150 mm × 2.1 mm, 3.5 µm) with temperature (column temperature) is 30°. Mobile phase of methanol: water: formic acid in the ratio of 75:25:0.2(v/v) has been used as eluent with flow rate of 0.2 mL/min. The injection volume was 10 µL. The observation was carried out onESI (Turbo Ion Spray) positive ion mode in the range of 40–1000 amu. The following FMS B1 and B2 mass operating conditions are: The nitrogen gas flow 3.8 bar; desolation gas temperature 350°C, temperature of source lamp 120°C; capillary voltage, 3.5 kV; cone; 40V, and extractor voltage 3V. The detection was carried out in multiple reaction monitoring mode.

RESULTS AND DISCUSSION

Agricultural commodities, products, and foodstuffs consist of variety of molecules with diverse structural characteristics, biological properties, and nutritional values. Among them, some of the compounds such as starch, triglycerides, and fatty acids are the large molecules and some of them such as flavonoids and phenolic compounds are less in minor components. Some of the undesirable compounds such as pesticides and mycotoxins are very less components. Various studies have been reported for contamination of mycotoxins in agricultural, food, and feed samples especially aflatoxins in India.[10-12] FMSs are the mycotoxins produced by *F. verticillioides* and *F. proliferatum* strains. Among all other sub group compounds of FMSs, Type B1 and B2 Figure 1 are found very frequently found toxic compounds and B3, B4, A1, and A2 are produced in very small amount and non-toxic. Dietary utilization of FMS causes many abnormal health conditions such as esophageal cancers and nephrotoxic in both humans and animals. It increases risk factors to increase in esophageal cancers. Hence, it is necessary to detect the FMS and contamination levels in various foods items in their processing stages. Liquid chromatography coupled with mass chromatography is used in many industries such as pharmaceuticals, biopharmaceuticals, forensic, industrial, food, and environmental sector. LC-MS technique has always been desirable due its sensitive and highly specific nature of MS compared to other chromatographic detector.

About 24 corn samples were collected in different locations such as harvesting area, storage area, and dumping are of godown for determination of contamination by FMSs. The images of collected samples are presented in Figure 2 and the condition of the samples is reported in Table 1. The comparison of FMSs contamination with respect to the collected area also done. Before analysis, a simple extraction step is required to concentrate the analytes of interest and eliminate non-desirable matrix components. This is particularly important for analysis of FMSs because low regulatory limits established for FMSs and the large sample size required to obtain necessary method sensitivity. The retention time of the well-defined peak of FMS B1 and B2 was found 1.15 min and 2.59 min which is similar to the obtained method. Standard calibration curve was plotted for quantification of FMS [Figures 3 and 4]. Figure 5 shows the standard chromatogram of FMS B1 and B2 standard mixture solution and individual injections are run for conformation of FMS B1 and B2. The blank chromatogram is presented in Figure 6. The precursor ion of the FMS B1 obtained from ESI was the adduct [M+H]+, m/z 706 for FMS B2, and m/z 723 for B2 which is similar to the theoretical mass value of m/z 706 for FMS B2 and m/z 722 for B2, respectively. The mass spectra of standard chromatogram of FMS B1 and B2 are

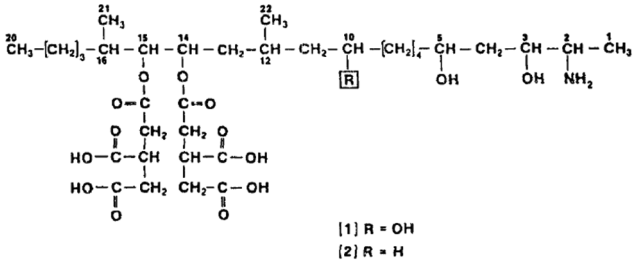

Figure 1: Chemical structures of FMS B1 and FMS B2[6]

Figure 2: Images of corn samples collected in different locations

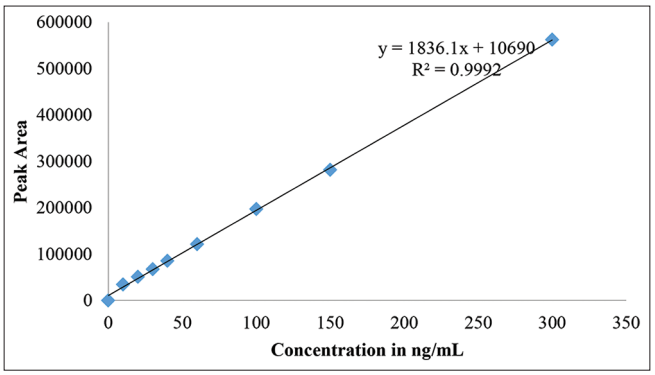

Figure 3: Standard calibration curve of FMS B1

Table 1: The 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

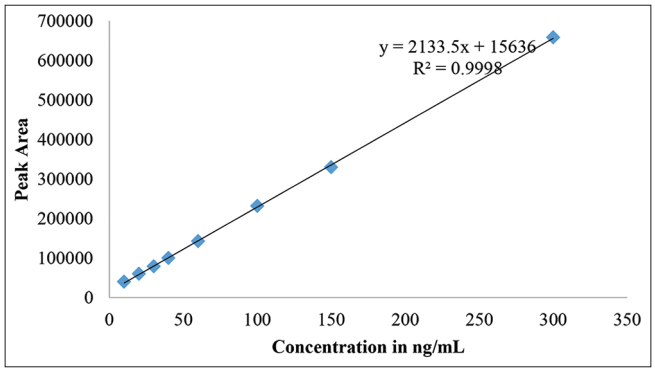

Figure 4: Standard calibration curve of FMS B2

represented in Figure 7. The calibration curves of FMS B1 and B2 are retrieved by plotting the concentrations in µg/ml (10, 20, 30, 40, 60, 100, 150, and 500) versus peak area of two compounds. The regression equation is y = 1836.1x + 10690 for FMS B1 where y = 2133.5x + 15636 for FMS B2 with the correlation coefficients(r) of FB1 and FB2 is 0.999 and 0.999, respectively. The retention time and product ion spectra of FMS B1 and B2 in the corn sample were similar to those of the standard solutions. Figures 8 and 9 represent the chromatograms of sample 7 and 20. The contamination of FMSs study is evaluated by analyzing 24 corn samples which are collected from the various types of locations such as harvesting area, storage area, and dumping area. Among 24 samples, about seven corn samples were found positive contamination by FMSs when subjected to LC-MS analysis. The list of corn samples contaminated by the FMSs and their concentrations is presented in Table 2. A high amount of FMS identified in sample number 7(s-7) which is collected from the dumping area of storage godown. The fungal damaged sample was found B1concetration of 242.11 µg/kg and FB2 of 117.15µg/kg. The concentration of FB1 is very high in the sample compared to the regulatory guidelines limit. The low amount of FMS is identified in sample number 8(s-8) which is collected from the dumping area and in damaged condition. FB1 concentration was found 15.0 µg/kg. The sample 4(s-4) is found contaminated with FB2 with 15.780 µg/kg concentration. FB1 and FB2 were not found in remaining other 17 samples as no peak was found similar to the standard retention times of FB1 and FB2. Based on the results, it can conclude that corn samples collected from the dumping area of godown are found more contaminated with mycotoxin (FMS) than the other sample collecting area such as storage area and harvesting area. The abundance of B1 and B2 compared and presented in Figure 10. The impact of moisture content in the FMSs contamination also assessed and presented in Figure 11. The damage of the corn is found high in samples with high moisture content and similar fungal contamination was observed.

Similar contamination studies are reported by various authors for determination of FMS in feed and corn samples. The study of[13] reported feed contamination by FMS B1 up to 8 mg/kg affected about 9700 hens with sticky diarrhea, egg production, rapid decrease in take-feed and body weight highly reduced, and death in Andhra Pradesh state, India. Similarly, Jindal *et al*., (1998)[14] were reported the FMS B1 contamination in mazie and poultry feed samples collected eight districts of Haryana state in India. He reported 91% of maize samples ranged from 0.1 + 87.0 ppm and 84% of poultry feed samples ranged from 0.02 to 28 ppm were contaminated with FMS B1. Asrani *et al*.[15] studied the effects of feeding Japanese quail with *F. verticillioides* cultures containing known amount of FMS B1. The quails supplemented with 300 ppm of FMS B1 and found symptoms such as ruffled feathers, reduced feed and water intake, poor body growth, and greenish mucus diarrhea with 59% mortality. About 30% birds have showed the nervous signs and increase in aspartate transaminase and alanine transaminase, concentration of total serum and albumin, serum calcium and cholesterol levels, and creatinine. Waliyar *et al*.[16] have studied the mycotoxins

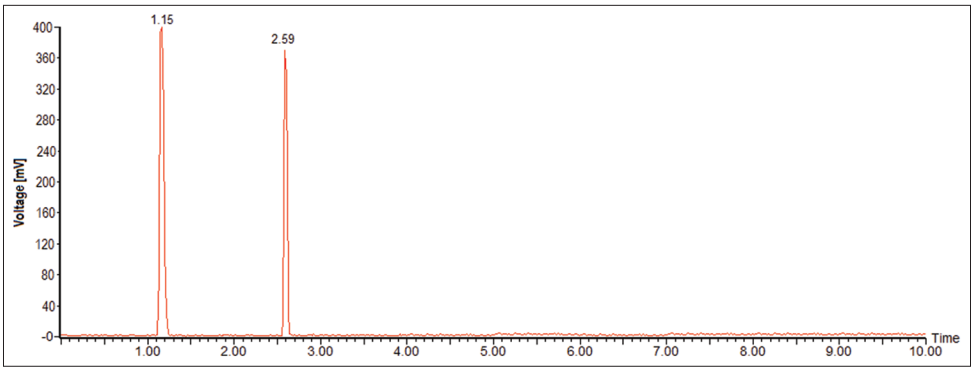

Figure 5: Standard chromatogram of FMS B1 and B2 (Rt 1.15 represents the B1 and Rt 2.59 represents the B2 FMS)

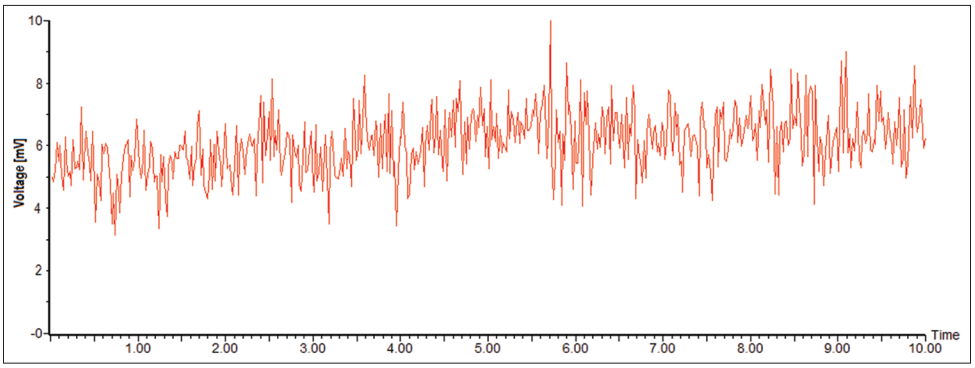

Figure 6: Blank chromatogram

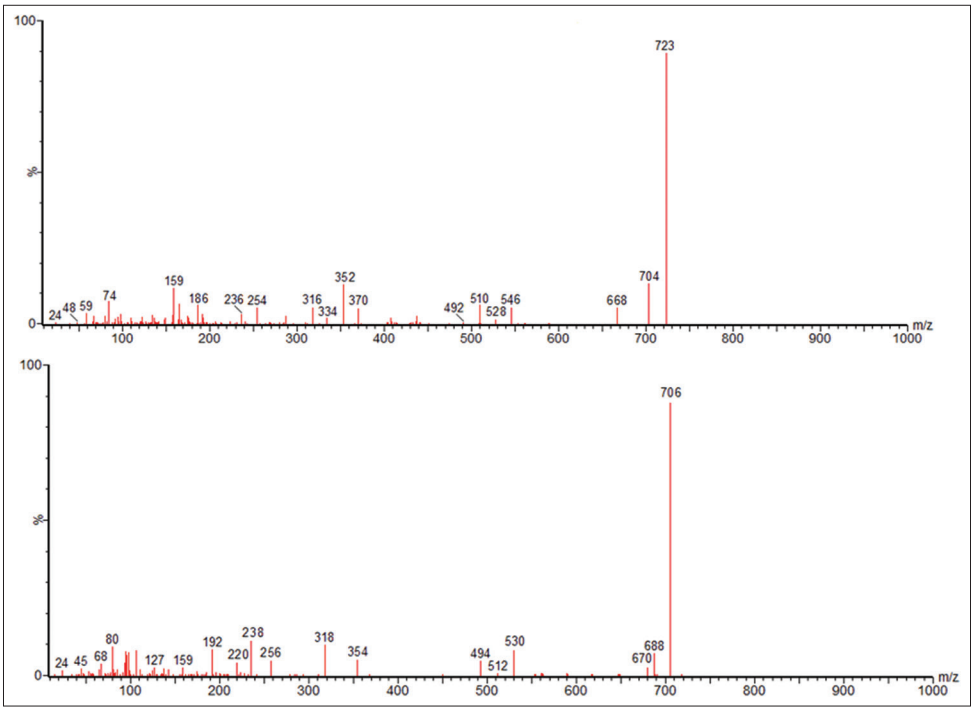

Figure 7: Mass spectra of FMS B1 and B2 (m/z 723 represents the B1 and m/z 709 represents the B2 FMS)

contamination (aflatoxins and FMSs) in sorghum grain samples in Andhra Pradesh and Maharashtra samples. The higher aflatoxins levels in Andhra Pradesh samples were found 0–362 µg/kg and lower levels of FMS contamination were found in Maharashtra samples. The European commission has recommended the FMSs contamination levels range 5 ppm for pets and 50 ppm for adults. Maize is the essential for protecting the livestock and animal feed.

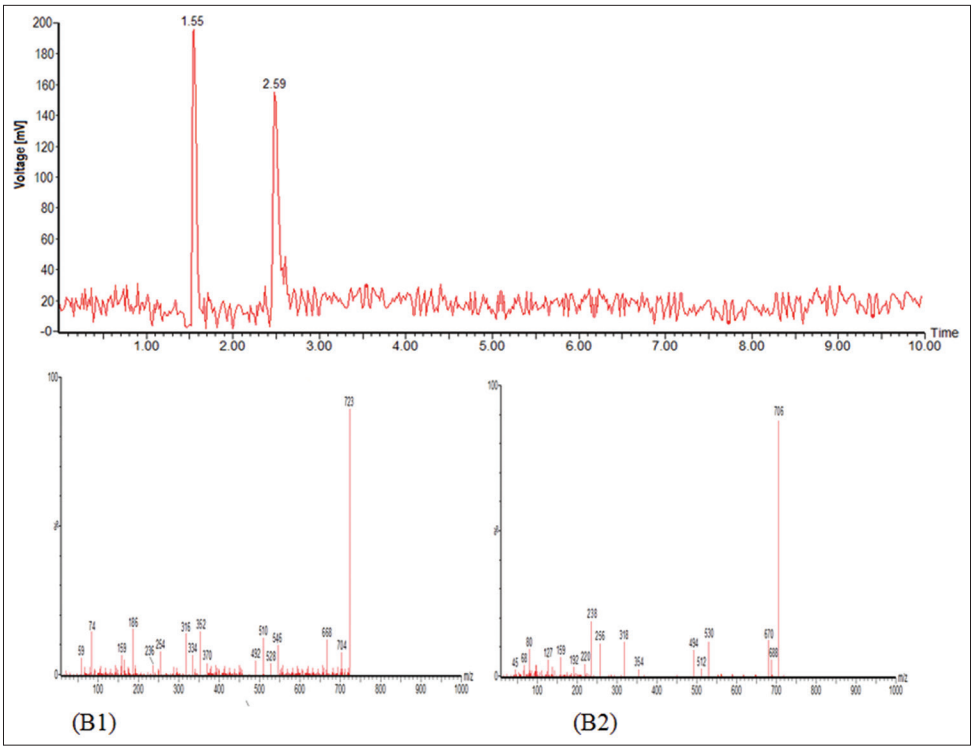

Figure 8: Chromatogram and mass spectra of Corn sample number 7

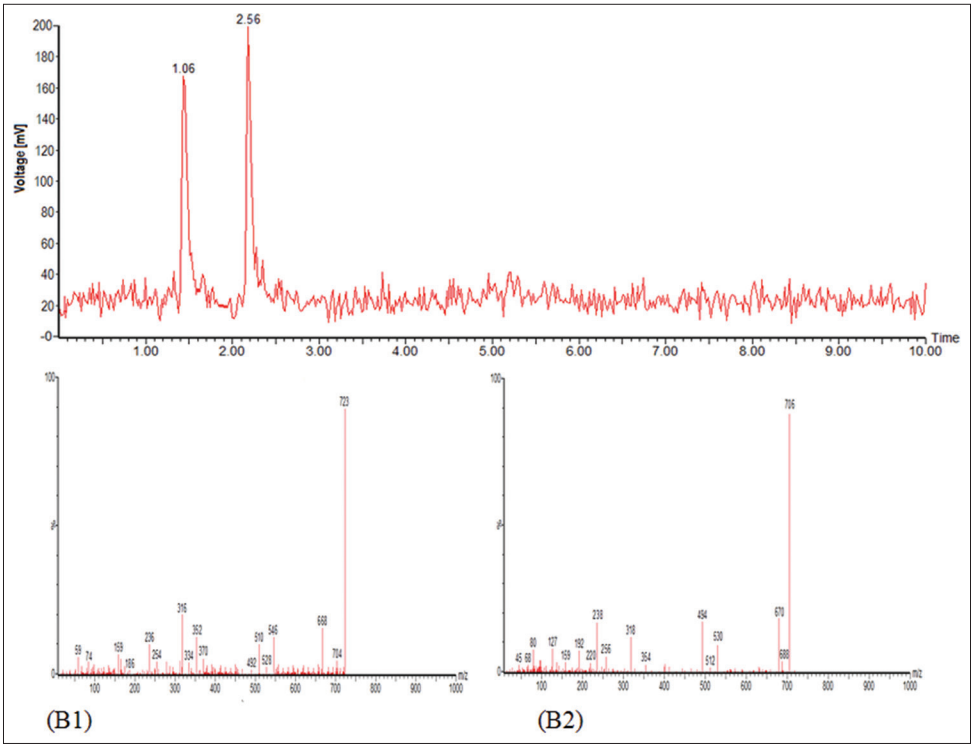

Figure 9: Chromatogram and mass spectra of Corn sample number 20

The FMNs are the common mycotoxins that contaminates maize. The acceptable limit of FMSs in feed products is not more than 5 ppm for horse and 100 ppm for other animals as the US FDA guidance levels.[17] The dumping area of the storage points found more contaminated by the fungi where more damaged samples are present and the content of the FMS also found high than the allowable limit. Therefore, monitoring these FMS in corn samples should be continued and maintenance of the dumping areas at different storage points with control methods is recommended.

Table 2: Results of FMS estimation in positive corn samples

Sample	Fumonisins compound	Amount estimated in µg/kg
S2	B1	42.25
S4	B2	15.78
S7	B1	242.11
	B2	117.15
S8	B1	17.14
S10	B1	47.69
S17	B2	72.18
S20	B1	106.67
	B2	38.06

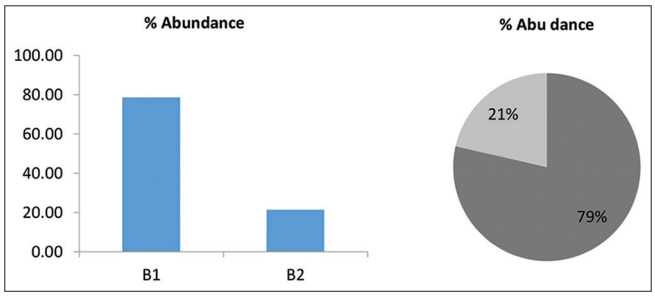

Figure 10: Comparative graph of the abundance of B1 and B2 in positive corn samples

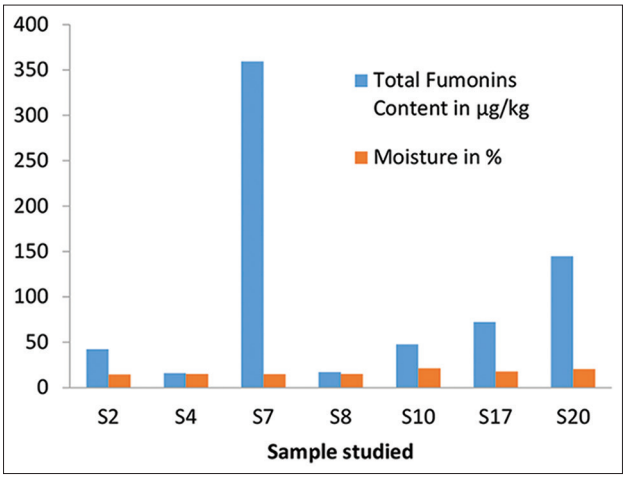

Figure 11: Comparison graph of impact of moisture content in the total FMS concentration in positive corn samples

CONCLUSION

The present study investigated the FMS contamination in corn samples collected from different locations. The study results confirm the fungal contamination and exceeding amounts of FMSs presence in the collected samples. Among 24 samples, about seven corn samples were found positive contamination by FMS when subjected to LC-MS analysis. Based on the results, it can conclude that corn samples collected from the dumping area of godown are found more contaminated with mycotoxin (FMS) than the other sample collecting area such as storage area and harvesting area. As the exceeding FMS concentration are observed in the dumping area of storage godown, it is not an alarming situation for FMS contamination in corn. The assessment of corn samples during the storage helps the processing chain by cautioning the usage of contaminated corn. The exceeding of FMS concentration than regulatory levels can cause synergetic effects to human and animals. It is necessary that efficient control methods are to prevent and monitor contamination of FMS in corn and other food grains.

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