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Development of a Robust Tandem Mass Liquid Spectroscopic Method for Aflatoxins in Cereals and Millets: Differentiating Aflatoxin Types

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ABSTRACT
Aflatoxins G1, G2, B1 and B2 are carcinogenic metabolic products of the fungus Aspergillus, that often contaminates cereals and millets, causing serious safety concerns for human consumption. A sensitive and selective analytical method for the determination of aflatoxins in Sorghum and Pearl millet has been developed using the Tandem LC/MS Triple Quadrupole Mass Spectrometer. The method uses a sample preparation technique involving dispersing the sample in acetonitrile water mixture (9:1 v/v), followed by centrifugation and cleanup using a Solid phase extraction (SPE) cartridge (MultiSep number 228). The cleaned-up extract of the sample is then analyzed using LC-MS/MS. The developed method showed linearitycorrelation coefficient > 0.997 in the concentration range of 0.1 µg/l- 50.0 µg/l for all four types of aflatoxins with a limit of detection of ≤ 0.02µg/kg. The lowered detection limit was achieved through concentrating the sample solution (5 times) compared to other conventional methods. Compared with the conventional methods, this new method has unique features such as (i) differentiate different types of aflatoxins quantitatively (ii) very low limit of detection. This method would be a boon for routine analysis of different types of aflatoxin residues in Sorghum and Pearl millet.
Key Words: Aflatoxins, Sorghum, Pearl millet, Liquid Chromatography with Mass Spectrometry, LC-MS/MS.

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INTRODUCTION

Generally, aflatoxins are found in tropical regions with high humidity and temperature and they accumulate post-harvest when food commodities are stored under conditions that promote fungal growth in the food products.Aflatoxins belong to the group of mycotoxins, which are secondary metabolites produced by the fungus Aspergillus (mainly the *Aspergillus flavus*, and *Aspergillus parasiticus*). They are of four types: Aflatoxin B1 (AF B1), Aflatoxin B2 (AF B2), Aflatoxin G1 (AF G1) and Aflatoxin G2 (AF G2); AF B1 being the most abundant, toxic and carcinogenic amongst them. Aflatoxins M1 and Aflatoxins M2 are the hydroxylation products of AF B1 and AF B2, respectively and they are found in milk and milk products.Aflatoxins are highly toxic; having genotoxic, mutagenic, teratogenic and carcinogenic effects and can cause both acute and chronic toxicity in humans. They act as hepatocarcinogenic in human body, mainly when conjugated with chronic hepatitis B virus

infection, and cause aflatoxicosis in episodic poisoning outbreaks. In animals, these toxins also impair growth and suppress the immunity in body.

EnzymeP450, in the liver, metabolizesespecially AFB1 into a reactive oxygen species (AFB1-8,9-epoxide), which may then bind either with proteins to cause acute toxicity (aflatoxicosis) or with DNA to induce liver cancer [1-7]. AF B1 8,9-exo-epoxide gets introduced more promptly into DNA, yielding higher levels of adducts for a given dose. AF B2 and AF G2 are normally considered to be far less biologically active due to the absence of an 8,9 double bond and subsequently 8,9-epoxide formation is not possible here cases.World Health Organization (WHO) and by the U.S. Environmental Protection Agency (USEPA) have classified aflatoxins as human liver carcinogens.To avoid excessive exposure to aflatoxins, therefore, maximum residual limits (MRLs) for various aflatoxins in different food products is fixed as 4µg/kg by WHO and many other regulatory authorities for total aflatoxins [8-14].

Several analytical methods have been reported for the determination of aflatoxins in different raw and processed food products [15-23]. Thin-layer chromatography (TLC)[22], High performance thin-layer chromatography (HPTLC) and liquid chromatography (LC) with fluorescence detection (FLD) in combination with both pre-column derivatization and post-column derivatization are the most commonly used techniques for this purpose [24-30]. While TLC is only a qualitative technique, HPTLC and LC with FLD, are both qualitative as well as quantitative techniques. Analysis of aflatoxins, at trace levels using HPTLC or LC-FLD, is known to suffer from interferences from complex food matrix. Hence, there is always a need to have a selective and sensitive method for this. A significant number of analytical methods involving hyphenated techniques of liquid chromatography such as LC coupled to mass spectroscopy have been developed and applied to the residual analysis of aflatoxins in food. However, there exist no method that is sensitive enough to meet the regulatory requirements as prescribed by USEPA and WHO [31-38].

Therefore, a selective and sensitive mass spectrometric detection method with the high resolution of LC is attempted for qualitative as well as quantitative method of analysis of different aflatoxins present at trace levels of ≤ 0.02µg/kg. A sensitive and selective analytical method for the determination of aflatoxins G1, G2, B1, and B2 in Sorghum and Pearl millet using the Tandem LC/MS Triple Quadrupole Mass Spectrometer has been developed. The suggested technique is not only highly sensitive but also shows a linearity in wide concentration range, 0.1 µg/l to 50.0 µg/l, besides being able to separate different types of aflatoxins.

MATERIAL AND METHODS

Chemicals:

The reference standards of: a) Aflatoxins G2 (AF G2), b) Aflatoxins G1 (AF G1), c) Aflatoxins B2 (AF B2), and d) Aflatoxins B1 (AF B1) were obtained from Sigma Aldrich, Mumbai India. The purity of these compounds was greater than 99%. HPLC grade Ammonium acetate, acetonitrile and methanol were procured from Merck Chemicals Delhi, India. Purified Milli-Q (Millipore, Tokyo, Japan)water of 18.2 MΩ resistivity.

Sorghum and Pearl millet grain samples were procured from local market. The grains were finely ground in an electric grinder and flour was used for all studies here.

Instrumentation/ Equipment:

LC-MS/MS system: An LC-MS/MS system consisting of an Agilent 1290 Series vacuum degasser, binary pump, well-plate auto sampler, thermo stated column compartment, the Agilent G 6460 Triple Quadrupole Mass Spectrometer with an electrospray ionization (ESI) source. The separation of the different aflatoxins was carried out on Agilent Zorbax Eclipse plus RP C18, 150 mm X 2.1 mm, 1.8 µm analytical column.The Mass hunter software was used for data acquisition and processing.

Procedure:

Sample preparation for Analysis by LC-MS/MS:

Approximately, 20 g of homogenized samples of flour(Sorghum and Pearl Millet) was taken in separate 250 ml Erlenmeyer flask and to this 20 ml of acetonitrile-water (9:1, v/v) was added. This mixture was shaken for 30 minutes, and centrifuged for 5 minutes at 2000 rpm. The supernatant was filtered through a glass microfiber GF/B grade filter (pore size: 0.2 µm, Whatman International Ltd, UK). The residue on the filter was discovered while the filtrate was used for further studies. The filtrate (10 ml) was passed through MultiSep

number 228 cartridge column for the cleanup at a flow rate 1 ml/ min. The elute was collected and evaporated to dryness at 40°C under the gentle stream of nitrogen using solvent Nitrogen evaporator (Rapid Vap, Labconco Corporation, Missouri, USA). The residue remaining in the evaporator was dissolved in 1 ml 5 mM ammonium acetate in methanol: water: acetic acid (10:89:1, v/v/v) and 5 mM ammonium acetate in methanol: water: acetic acid (97:2:1, v/v/v). To this way, the extracted residues were recovered after extraction followed by cleanup for further analysis. In the previous methods, normal SPE combined with normal absorbent has been used [32, 36, 38-40]. However, the process of clean up in our method is very simple, easy to handle and allows an effective as well as intensive solvent-matrices separation

Sample preparation for Recovery:

For recovery studies, 20 g of Sorghum and Pearl millet flour were spiked with 0.1µg/kg, 0.2 µg/kg and 0.5 µg/kg of aflatoxins reference standard and the procedure of extraction, cleanup and reconstitution was followed as per the same procedure as described above.

Reference Standard Preparation:

Preparation of calibration Standard and Matrix-matched calibration solutions:

Accurately weight ~ 10 mg of aflatoxins standards (AF G2, AF G1, AF B2 & AF B1) was dissolved in acetonitrile and made up the volume to 100 ml in volumetric flask. This was taken as the stock solution used for preparation of calibration standard and matrix match calibration solutions, was stored at 4 °C in the dark until use.

Appropriate aliquots were taken and further diluted with mobile phase so as to give a series of calibration standard solutions having AF G2, AF G1, AF B2 and AF B1 concentration range of 0.1 µg/l, 0.50 µg/l, 1.0 µg/l, 2.5 µg/l, 5.0 µg/l, 10.0 µg/l, 25.0 µg/land 50.0 µg/l respectively. Matrix-matched calibration standards were prepared by fortifying the extracted pre-analyzed sample solution (Sorghum and Pearl Millet sample) with known concentration of standard working solution at 8 levels in the range between0.1 µg/kg, 0.50µg/kg, 1.0µg/kg,2.5µg/kg, 5.0 µg/kg, 10.0 µg/kg, 25.0 µg/kg and 50.0 µg/kg and final concentration. A mixed standard of aflatoxins of 0.1 µg/l concentrations was prepared for estimation of Limit of detection (LOD) of the instrument. We spiked 0.02µg /l concentration level of aflatoxins mixed standard into the 20 g of Sorghum and Pearl Millet sample for determination of LOD for the method. Extraction procedure was followed as illustrated in and final concentration was made 0.1µg/kg. All solutions were stored at 2°C to 8°C until analysis.

LC-MS/MS conditions:

The analysis for determination of the different aflatoxins was carried with two mobile Phases, A = 5 mM ammonium acetate in Methanol: Water: Acetic acid (10:89:1, v/v/v) and B = 5 mM ammonium acetate in Methanol: Water: Acetic acid (97:2:1, v/v/v) were prepared whereas Methanol: Acetonitrile: Water mixture (25:50:25 v/v/v) was used for flushing. Flow rate was 200 µl / min and Mobile phase gradient for separation of AF G2, AF G1, AF B1 and AF B2 in Sorghum and Pearl millet sample is listed in Table 1.

Table 1: Mobile phase gradient for separation of AF G2, AF G1, AF B1 and AF B2 in Sorghum and Pearl millet sample

Sr. No.	Time (min)	%A	%B
1	0	100	0
2	1	100	0
3	2.5	50	50
4	7	0	100
5	9.25	0	100
6	9.3	100	0
7	10	100	0

Mass Spectrometry conditions:

Instrument: Agilent 6460 LC /MS Triple Quadrupole was used in present study and the concentration of aflatoxins was determined in Positive ESI mode. The Drying gas flow was 12 L/min with Nebulizer pressure of 35 psi. The analytical condition (Drying gas temp: 350

°C, Capillary (V) at 4000 V, Scan range between *m/z* 100 – 550 and Fragmentor variable 120 V) was maintained during the analysis.
Multi Reaction Monitoring mode transitions for the analysis are given below Table 2.

Table 2. Data Acquisition Parameters of MRM Transitions for Each Aflatoxin

Sr. No.	Mycotoxins	RT (min)	Molecular Weight	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)
1	Aflatoxin G2	6.655	330	331	245	30
2	Aflatoxin G1	6.835	328	329	243	30
3	Aflatoxin B2	7.055	314	315	259	30
4	Aflatoxin B1	7.235	312	313	241	30

RESULT AND DISCUSSION

Liquid chromatographic separation:
A comparatively simple, sensitive and an accurate method was developed for the determination of AF G2, AF G1, AF B1 and AF B2 residues in Sorghum and Pearl millet using positive ESI LC-MS/MS with optimum chromatographic conditions. In the previous methods, aflatoxin compounds were co -eluting with each other and therefore, the base to base peaks were not separated well [41-43].In order to develop the optimum condition for better peak separation in LC-ESI-MS/MS, we eluted aflatoxins in different proportions of mobile phases and with different flow rates. We also ensured that optimized chromatographic conditions provided short retention times, adequate peak shapes and good base to base separation of aflatoxins peak. A well resolved and separated peaks for AF G2, AF G1, AF B1 and AF B2 were obtained at retention time of 6.655, 6.835, 7.055, 7.235 minutes respectively (Figure 1). The optimum separation condition was achieved using 5 mM ammonium acetate in Methanol: Water: Acetic acid (10:89:1, v/v/v) (A) and 5 mM ammonium acetate in Methanol: Water: Acetic acid (97:2:1, v/v/v)(B) at a flow rate 200 µl / minin the ratio as given in LC program (Table 1).
Mass Spectrometric detection:
The reference standard solution of AF G2, AF G1, AF B1 and AF B2 were infused separately using both positive and negative ESI mode of the mass spectrometer detector for the purpose of evaluating the fragment ions and the intensity of the signals. The positive ionization mode, compared with negative ionization mode, produced a high intensity signal, which in turn allowed us to detect AF G2, AF G1, AF B1 and AF B2 residues up to the concentration levels of 0.1µg/l. In addition, MRM transition for each aflatoxin was carried out using single-MS (ESI-LC-MS) positive full scan mode followed by MRM mode using aflatoxin standard mixture at 0.5 µg/l. The mass spectrum of each aflatoxin in full-scan mode exhibited a protonated molecular ion $[M+H]^+$ as the base peak ion m/z331 for AF G1, m/z 329 for AF G2, m/z 315 for AF B2 andm/z 313 for AF B1 [15, 38, 39]. The mass spectra were obtained for the standard mixtures in full scan mode and MRM mode and are shown in Figure 2, 3, 4 and 5.
The optimum collision voltage is compound dependent; therefore, it was varied from 5 to 40 V using a step size of 5V to establish the optimum collision voltage for better identification. A distinct optimum intensity of the product ion of each aflatoxin was observed at 30 V in this study. The product ions that indicated the highest intensity were *m/z* 245 (AFG2), 243 (AFG1), 259 (AFB2), and 241 (AFB1), respectively. Table 2 shows the parameters of MRM mode of each aflatoxin.
Method Performance Characteristics:
The method was validated as per SANTE guide lines[44]to establish specificity, linearity, precision, accuracy, limit of detection and limit of quantitation for our new method.
Specificity: The chromatographic interferences from the Sorghum and Pearl millet samples were investigated by comparing the chromatograms of sample free from aflatoxin (Blank), sample with aflatoxins and the spiked aflatoxins into blank sample. It was found that the presence of interferences did not have any effect on the quantitative results of the analyte of interest (Figures 1-5) thus providing reliability of the LC-MS/MS method for determination of Aflatoxins.

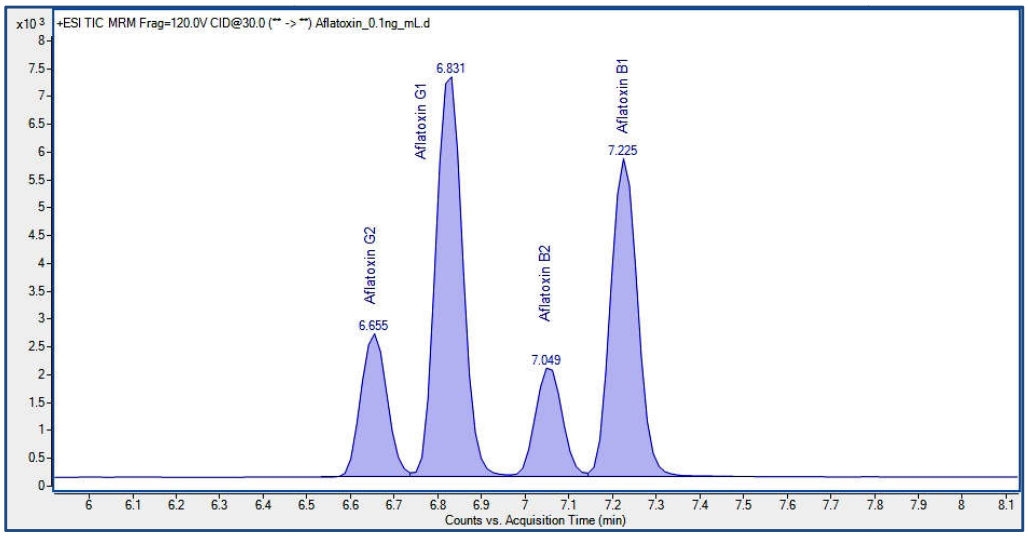

Figure 1: Total ion chromatogram of Aflatoxin G1, Aflatoxin G2, Aflatoxin B2and Aflatoxin B1

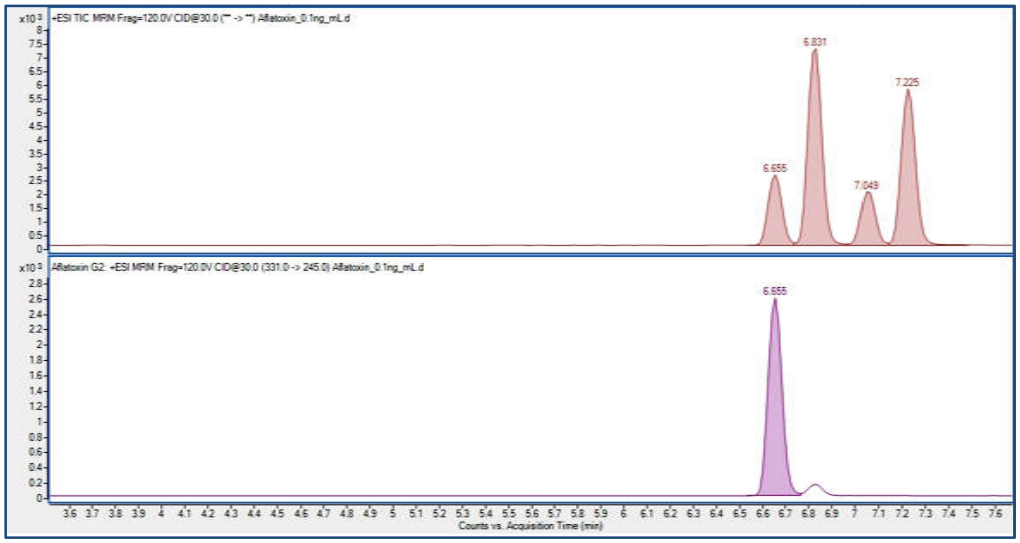

Figure 2: MRM transitions showing Aflatoxin G2 from analysis of spiked samples (Aflatoxins standard at 0.5µg/kg)

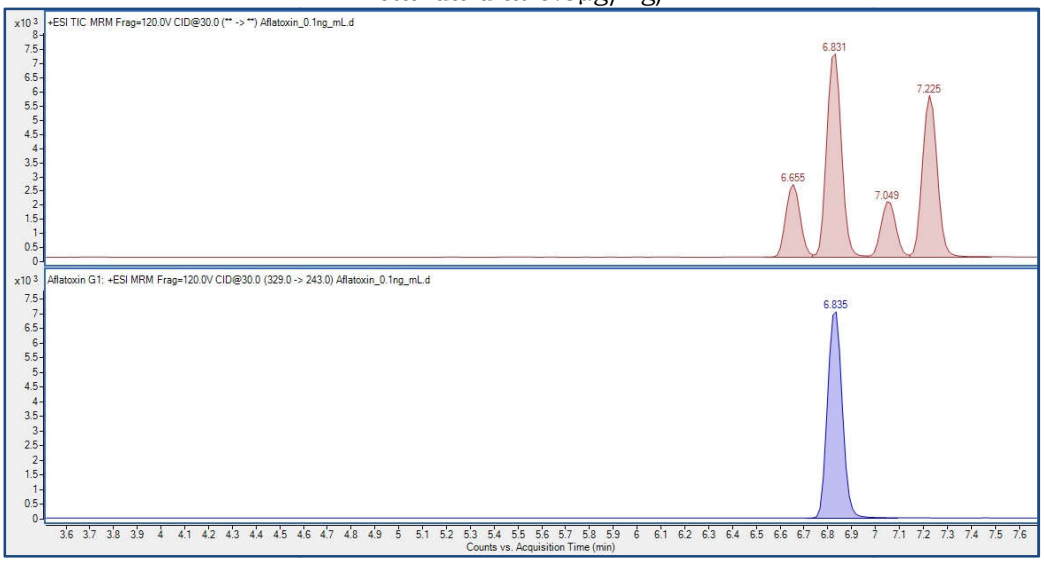

Figure3:MRM transitions showing Aflatoxin G1 from analysis of spiked samples (Aflatoxins standard at 0.5 µg/kg)

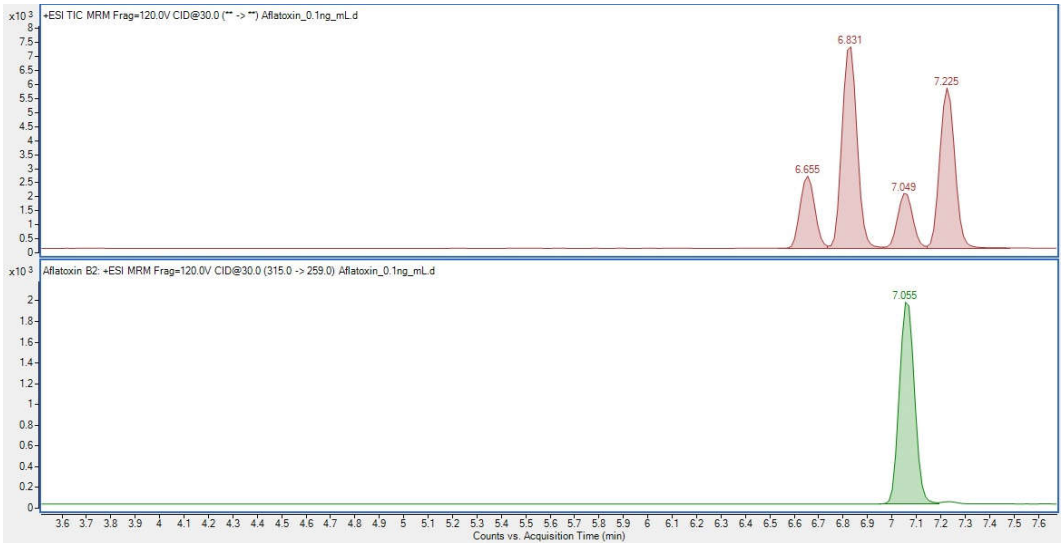

Figure 4: MRM transitions showing Aflatoxin B**2** from analysis of spiked samples (Aflatoxins standard at 0.5µg/kg)

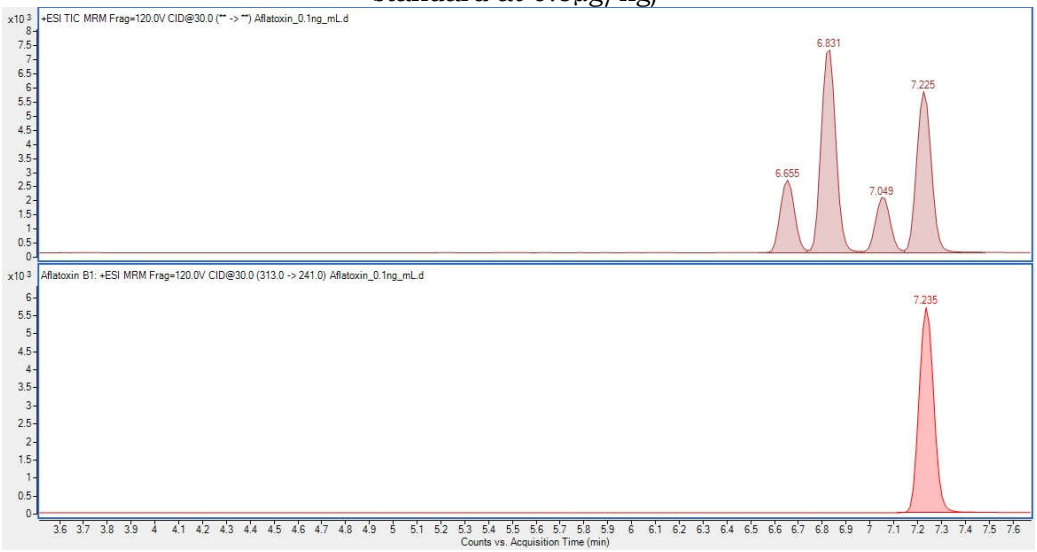

Figure 5: MRM transitions showing Aflatoxin B1 from analysis of spiked samples (Aflatoxins standard at 0.5µg/kg)

Linearity: Eight calibration standards evenly spread over the concentration range of interest and encompassing the concentration levels reflecting EU regulatory limits[11] were analyzed. The calibration standards were run in six replicates. The calibration curve prepared using the pure standards was found to be linear in the range of 0.1 µg/l to 50µg/l with correlation coefficient of $\geq$0.9985. Further, the linearity of the matrix-matched calibration standards for the concentration range of 0.1µg/kg to 50µg/kg, was checked in six replicates. The calibration curve for the matrix-matched standards was also found to be linear with correlation coefficient of $\geq$0.9974. The linear equations, correlation co-efficient and RSD values of calibration standard and matrix matched calibration for both Sorghum and Pearl millet samples are presented in Table 3.

Limit of detection (LOD) and Limit of quantitation (LOQ): LOD was determined by considering signal to noise (S/N) ratio of 3:1 for the strongest mass transition with respect to the background noise obtained from the blank sample whereas LOQ was determined similarly by considering signal to noise ratio (S/N) ratio of 10:1 (Table 2). Based on the mean noise level for the ten injections each for the matrix blank of Sorghum and Pearl millet samples, the detection limit of the instrument was calculated as 0.1µg/kg and lowest detection limit for the method was calculated 0.02 µg/ kg for both matrices (Sorghum and Pearl millet) and confirmed using standard solutions of aflatoxins mixed with concentration of 0.1µg/l. The lowest concentration level that could be quantified with reproducible values for Sorghum

and Pearl millet was determined as 0.5 µg/kg for the instrument and limit of quantification for the method was calculated 0.1 µg/ kg. The results were further confirmed by injecting matrix matched standard solution of having concentration of 0.5 µg/ml.

Precision: The data of measurements of precision for both intra-day and inter-day repeatability and reproducibility by measuring the concentrations in seven replicates are presented in (Table 4). Samples of Sorghum and Pearl millet were spiked at different concentration levels i.e. 0.1 µg/kg, 0.5 µg/kg and 1.0 µg/kg respectively and the solutions were injected on the same day and on three subsequent days by three different analysts. Relatively low relative standard deviation (%RSD value of < 4.2%) were obtained for both the samples. Overlay of chromatograms for different concentration of AF G2, AF G1, AF B1 and AF B2shown in Figure 6.

Accuracy: The recoveries of AF G2, AF G1, AF B1 and AF B2 in spiked samples were calculated to study the effect of matrix on the determination of aflatoxins. The recovery studies were carried out at spiked level 0.1 µg/kg, 0.5 µg/kg and 1.0 µg/kg concentrations of aflatoxins in Sorghum and Pearl millet sample respectively. The solutions were injected in seven replicates on three different days and then extracted and determined by the same method as mentioned earlier. The recoveries of aflatoxins from the Sorghum and Pearl millet samples were found in the range of 87% to 105% Table 5.

Table- 3: Fragment ions produced using + ve ESI mode, Selectivity, LOD & LOQ of Aflatoxins

Compounds	R.T (min)	Solvent Calibration (R^2)	Matrix (Sorghum) Calibration (R^2)	Matrix (Pearl millet) Calibration (R^2)	LOD (µg/kg)	LOQ (µg/kg)
Aflatoxin G2	0.937	0.9992	0.9989	0.9991	0.1	0.5
Aflatoxin G1	1.110	0.9987	0.9981	0.9985	0.1	0.5
Aflatoxin B2	1.324	0.9985	0.9974	0.9980	0.1	0.5
Aflatoxin B1	1.660	0.9994	0.9991	0.9992	0.1	0.5

Table- 4: Intra-day and Inter-day precision data for the proposed method for Aflatoxins G2, G1, B2 and B1 residues in samples of Sorghum and Pearl millet.

Concentration µg/kg	Sample	Day 1		Day 2		Day 3		Intra-assay	
		Concn*. obtained (µg/kg) n=7	% RSD	Concn obtained (µg/kg) n=7	% RSD	Concn obtained (µg/kg) n=7	% RSD	Concn obtained (µg/kg) n=7	% RSD
Aflatoxins G2									
0.1	Sorghum	0.0962	4.18	0.0957	2.89	0.0901	2.65	0.0889	2.69
	Pearl millet	0.0951	3.66	0.0925	3.71	0.0967	2.38	0.0958	3.55
0.5	Sorghum	0.4920	2.11	0.4890	2.55	0.4715	3.92	0.4780	2.71
	Pearl millet	0.4975	2.45	0.4945	2.87	0.5005	3.15	0.4915	2.62
1.0	Sorghum	0.9970	2.85	0.9980	3.42	1.0270	1.25	1.0020	3.35
	Pearl millet	0.9600	2.51	0.9330	2.64	0.9760	2.11	1.0110	2.16
Aflatoxins G1									
0.1	Sorghum	0.0982	3.82	0.0891	4.07	0.0953	2.39	0.0936	1.83
	Pearl millet	0.0949	3.75	0.0879	2.88	0.0991	3.17	0.0954	2.89
0.5	Sorghum	0.4535	1.88	0.4735	1.52	0.4880	2.22	0.4915	1.96
	Pearl millet	0.4770	2.97	0.4905	1.99	0.4975	2.86	0.4985	2.34
1.0	Sorghum	0.9930	2.83	0.9830	3.05	0.9890	2.34	1.0260	3.56
	Pearl millet	1.0290	3.59	0.9980	3.00	1.0150	2.45	0.9920	3.95
Aflatoxins B2									
0.1	Sorghum	0.0957	3.89	0.0978	3.09	0.0899	2.70	0.0951	3.32
	Pearl millet	0.0939	2.96	0.0903	3.19	0.0897	3.11	0.0949	2.13
0.5	Sorghum	0.4585	2.18	0.4850	2.61	0.4745	2.95	0.4895	2.64
	Pearl millet	0.4480	3.73	0.4725	3.26	0.4840	2.61	0.4785	3.16

1.0	Sorghum	1.0250	1.80	1.0200	2.97	0.9990	2.93	0.9890	2.77
	Pearl millet	1.0170	2.11	1.0130	3.58	1.0060	2.54	1.0080	3.06
Aflatoxins B1									
0.1	Sorghum	0.0959	2.81	0.0937	1.86	0.0981	3.87	0.0959	2.91
	Pearl millet	0.0946	3.19	0.0949	2.33	0.0997	3.19	0.0947	2.43
0.5	Sorghum	0.4995	2.47	0.4930	3.56	0.5105	2.54	0.4955	2.08
	Pearl millet	0.4930	2.86	0.4890	2.17	0.4920	2.93	0.4945	2.13
1.0	Sorghum	0.9980	1.95	0.9980	3.62	0.9960	1.09	1.0210	2.17
	Pearl millet	0.9860	1.87	0.9990	2.54	0.9740	1.12	1.0270	2.77

* Concentration

Table- 5: Percent recovery of aflatoxins G2, G1, B2 and B1 from Sorghum and Pearl millet samples analyzed on different days (n=7)

Concentration µg/kg	Sample	Day-1	Day-2	Day-3	Intra-assay
		% Recovery	% Recovery	% Recovery	% Recovery
Aflatoxins G2					
0.1	Sorghum	96.2	95.7	90.1	88.9
	Pearl millet	95.1	87.6	96.7	95.8
0.5	Sorghum	98.4	97.8	94.3	95.6
	Pearl millet	99.5	98.9	100.1	98.3
1.0	Sorghum	99.7	99.8	102.7	100.2
	Pearl millet	96.0	93.3	97.6	101.1
Aflatoxins G1					
0.1	Sorghum	98.2	89.1	95.3	93.6
	Pearl millet	94.9	87.9	99.1	95.4
0.5	Sorghum	90.7	94.7	97.6	98.3
	Pearl millet	95.4	98.1	99.5	99.7
1.0	Sorghum	99.3	98.3	98.9	102.6
	Pearl millet	102.9	99.8	101.5	99.2
Aflatoxins B2					
0.1	Sorghum	95.7	97.8	89.9	95.1
	Pearl millet	93.9	90.3	89.7	94.9
0.5	Sorghum	91.7	97.0	94.9	97.9
	Pearl millet	89.6	94.5	96.8	95.7
1.0	Sorghum	102.5	102.0	99.9	98.9
	Pearl millet	101.7	101.3	100.6	100.8
Aflatoxins B1					
0.1	Sorghum	95.9	93.7	98.1	95.9
	Pearl millet	94.6	94.9	99.7	94.7
0.5	Sorghum	99.9	98.6	102.1	99.1
	Pearl millet	98.6	97.8	98.4	98.9
1.0	Sorghum	99.8	99.8	99.6	102.1
	Pearl millet	98.6	99.9	97.4	102.7

CONCLUSIONS

From the studies presented here, following conclusion may be drawn: (i) Positive ESI LC-MS/MS method developed for AF G1, AF G2, AF B1 and AF B2 is found to be a rapid method with excellent chromatography separation, lowest limit of detection and limit of quantification reported so far in. (ii) The method yields high precision, accurate and sensitive quantification by using simple extraction procedure in a wide array of matrices and is advantageous. (iii) Aflatoxin G2, G1, B2 and B1 shows the precursor ions m/z 331, 329, 315 and 313 with product ions 245, 243, 259 and 241. (iv) In spite of using a simplified extraction procedure, no interferences were observed from the matrix components during the determination of aflatoxin residues reflecting the robustness of the method.

Although in the current study, the method has been demonstrated to be usefulfor routine analysis of aflatoxinresidues in Sorghum and pearl millet, the chromatographic separation and results indicate that the method can be used for measurement in other matrices food products like cereals, nuts etc.

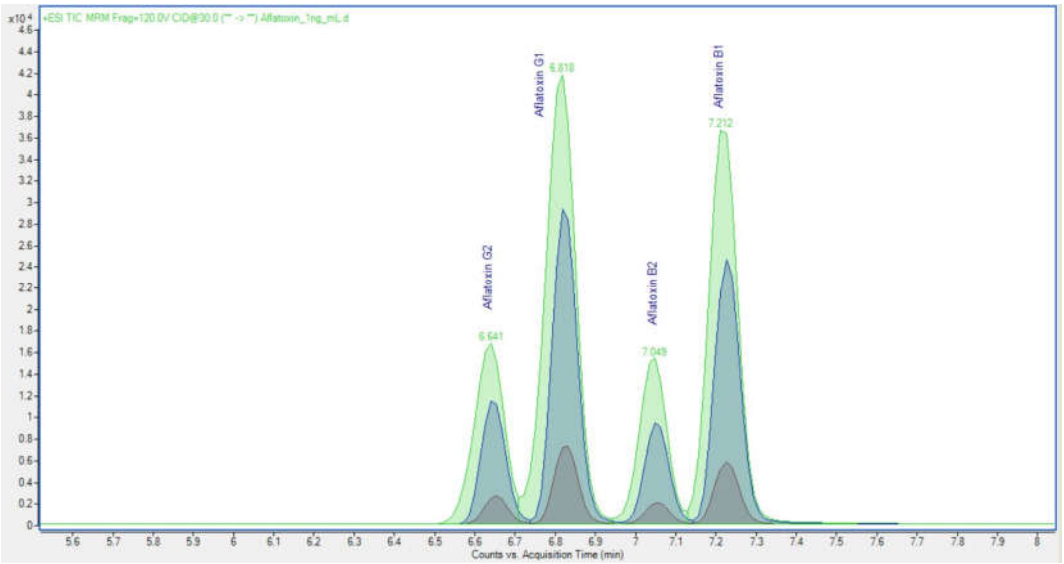

Figure 6: Overlay chromatograms for different concentration of Aflatoxin G2, Aflatoxin G1, Aflatoxin B1 and Aflatoxin B2

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