Original Articles
Toxicology International, *May-Aug 2015 / Vol-22 / Issue-2, 42-45*
ISSN (Print) : 0971-6580
ISSN (Online) : 0976-5131
DOI: 10.22506/ti/2015/v22/i2/137652

Detection of Mycotoxins using Liquid Chromatography-Mass Spectrometry/Mass Spectrometry in Fungal Infected Meadow Grass Fodder

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Abstract

The worldwide contamination of food and feed with mycotoxins is a significant problem. The present study is aimed to detect the mycotoxin present in fungal infected meadow grass fodder from the Uttara Kannada district, where the cattle and buffaloes were exhibiting clinical signs such as posterior paralysis, downer cow syndrome, rumen impaction, and keratomalacia of tail and even mortality. The infected meadow grass fodder samples were collected and were subjected to the multimycotoxin analysis using 1200 series high-performance liquid chromatography (LC) (Agilent Technologies, Waldbronn, Germany) coupled to MDS Sciex 3200 Q Trap LC-mass spectrometry (MS)/MS system (Applied Biosystems, USA). Calibration curves were built individually using five or six concentration levels in the wheat matrix (mycotoxin free) were injected into LC-MS/MS system for each mycotoxin to be analyzed. The peak area of the mycotoxins was plotted against the concentration to generate matrix matched calibration curve. The analysis revealed the presence various mycotoxins in the sample, mainly Fusarial toxins such as zearalenone and beauvericin along with traces of aflatoxins, ochratoxin, citrinin, and trichothecenes. Hence, it could be concluded that the mycotoxins got detected in fungal infected meadow grass using LCMS/MS technique, could be the causatives for the major livestock disorders mentioned.

Keywords: Fusarial Toxins, Liquid Chromatography Mass Spectrometry/Mass Spectrometry, Meadow Grass, Mycotoxicosis

Received: 13-05-2015 **Revised:** 19-07-2015 **Accepted:** 14-08-2015

1. Introduction

Fungi are ubiquitous, and formation of mycotoxins can occur in all agricultural commodities under appropriate field or storage conditions throughout the animal feed supply chain.[1] In one of the disease investigation process in Karnataka state, it was observed that cattle and buffaloes which were stall-fed with meadow grass fodder were exhibiting the clinical signs such as anorexia, rumen impaction, adduction of hock, posterior paralysis, downer cow syndrome, and also even death. Further investigations revealed that the meadow grass fodder was containing various fungi spp. In this study, the infected meadow grass sample was subjected to the liquid chromatography-mass spectrometry (LC-MS)/MS analysis to detect the presence of various mycotoxins.

2. Materials and Methods

Fungal contaminated dry meadow grass was collected from the suspected areas, where the cattle and buffaloes were exhibiting clinical signs such as posterior paralysis, downer cow syndrome, rumen impaction, and keratomalacia of tail. Mortality of buffalo calves also reported in places where the grass was being served as major roughage. The fungal contaminated meadow grass that had caused the illness was collected from the above said suspected area and used for further study.

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2.1 Chemicals and Reagents

High-performance LC (HPLC) gradient grade (Merck, India) acetonitrile, formic acid, ammonium acetate, and methanol were used. HPLC grade water (Spectrochem, India) was further purified using Thermo Scientific RO Water Purification System (Barnstead Nanopure, USA). The mycotoxin standards such as aflatoxins (B_1, B_2, G_1, and G_2), ochratoxin A, HT-2 toxin, diacetoxyscirpenol, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, beauvericin, apicidin, and citrinin were used (Sigma-Aldrich Chemicals, USA). T-2 toxin, fumonisin B1, fumonisin B2, and zearalenone were also used (Fermentek, Israel).

2.2 Sample Preparation – Extraction

The fungal infected dry meadow grass samples were ground to fine powder in a laboratory blender. After that, 5 g of sample was extracted for 2 h with a mixture of acetonitrile/water/formic acid at 25°C under constant agitation. Extract was centrifuged at 15.000 ×*g* for 15 min and 4 ml of extract was dried in a water bath under nitrogen streaming. The sample was dispersed in 4 ml ammonium acetate buffer (pH 8.5)/methanol mixture. Two milliliter of the sample was cleaned up using cleanup SPE cartridge (OASIS® - HLB). The purified samples were dried and reconstituted in 0.5 ml of HPLC mobile phase (water/methanol/acetic acid/ammonium acetate mixture) and filtered through PTFE membrane syringe filters (0.22 µm) to autosampler vial.

2.3 Screening and Quantitation using Liquid Chromatography-Mass Spectrometry/ Mass Spectrometry

HPLC, 1200 series (Agilent Technologies, Waldbronn, Germany) coupled to, MDS Sciex 3200 Q Trap (Applied Biosystems, USA) LC-MS/MS system was used. HPLC column was of Zorbax, Eclipse XDB - C18, rapid resolution HT 4.6 mm × 50 mm, 1.8 micron, and 600 Bar column (Agilent Technologies, Waldbronn, Germany).

The presence of 17 different mycotoxins was monitored using LC-MS/MS. Mycotoxin identity confirmation was performed by monitoring of at least 2 transition ions per analyte. The HPLC system utilized a binary gradient solvent system consisting of nanopure grade RO water buffered by ammonium acetate, acetic acid, and methanol buffered by ammonium acetate and acetic acid. The total run time was 28 min per sample analyzed. Calibration curves were built individually using five or six concentration levels in the wheat matrix (mycotoxin free) were injected into LC-MS/MS system for each mycotoxin analyzed. The peak area of the mycotoxins was plotted against the concentration to generate matrix matched calibration curve (Fig. 1).

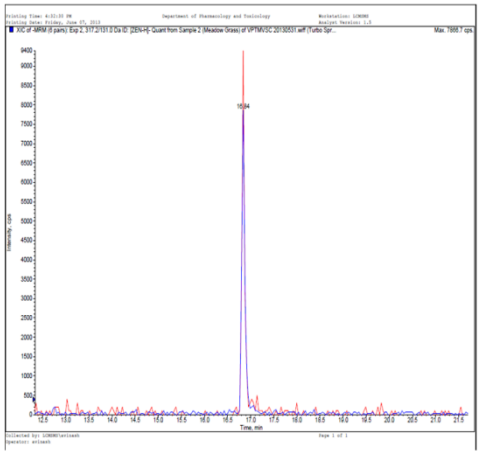


Figure 1. The extracted ion chromatogram of zearalenone showing the eluting.

3. Results and Discussion

Each year a million tons of forage consumed by animals are contaminated with fungal species invading forage plants prior to harvest or during storage as hay, straw, or silage.[2] The fungal infected meadow was analyzed for the presence of aflatoxins (B_1, B_2, G_1, and G_2), ochratoxin, trichothecenes, fumonisins, beauvericin, apicidin, zearalenone, and citrinin by LC-MS/MS method at Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bengaluru. Sterilized blank wheat sample was kept as control. The major mycotoxins got detected through the LCMS/MS analysis were mainly Fusarial toxins such as zearalenone and beauvericin along with traces of aflatoxins ochratoxin, trichothecenes, and penicillin. The results are given in Table 1.

<insert table 1 here>

When ingested, mycotoxins may cause a mycotoxicosis that can result in an acute or chronic disease episode.[3] Several assay techniques such as enzyme-linked immunosorbent assay, thin layer chromatography, and

Table 1. Data of Multimycotoxin analysis of Meadow grass

Mycotoxins	Sample name			
	LOD in corn matrix (ng/g)	LOQ in corn matrix (ng/g)	Blank Wheat matrix (ng/g)	Meadow grass (ng/g)
Aflatoxin B1	0.10	0.50	No Peak	< 0
Aflatoxin B2	0.20	0.50	No Peak	4.77
Aflatoxin G1	0.20	0.50	No Peak	4.93
Aflatoxin G2	0.20	0.50	No Peak	5.90
Ochratoxin A	ND	1.00	No Peak	0.52
T-2 Toxin	0.30	1.00	No Peak	2.26
HT-2 Toxin	2.00	5.00	No Peak	5.74
DAS	0.10	1.00	No Peak	No Peak
DON	ND	10.00	No Peak	< 0
3Ac-DON	ND	10.00	No Peak	No Peak
15-AcDON	ND	10.00	No Peak	< 0
Fumonisin B1	ND	2.00	No Peak	No Peak
Fumonisin B2	ND	2.00	No Peak	No Peak
Zearalenone	0.30	1.00	No Peak	218.88
Citrinin	ND	1.00	No Peak	8.84
Beauvericin	ND	2.00	No Peak	27.20
Apicidin	ND	5.00	No Peak	No Peak

HPLC have been employed in the previous analysis for the determination of mycotoxins.[4] However, LC/electrospray ionization (ESI)-tandem MS/MS based techniques are more and more applied to mycotoxin analysis in the recent years due to the alleged co-occurrence of different classes of mycotoxins in food and feedstuffs.[5]

HPLC and gas chromatography have traditionally been the favored choices for the analyst when sensitive, reliable results with minimum variability are required. Developing an LC-MS/MS method for the analysis of multiple mycotoxins is challenging due to the different physicochemical properties that they possess. Both ESI and atmospheric pressure chemical ionization (APCI) have been used in single- and multi-class mycotoxin methods.[6] ESI is more commonly used in multiclass multiresidue mycotoxins methods, although APCI has been reported to produce better results for the problematic trichothecene mycotoxins.[7] Acetonitrile is the preferred extraction solvent, as it extracts the widest range of mycotoxins and least amount of matrix components. The use of buffered extraction salts (acetate or citrate) was avoided, as this would raise the sample pH or require the use of higher amounts of acid to maintain a low pH of the extraction solvent.

Mycotoxins are secondary fungal metabolites that can be produced in crops and other food commodities both preharvest and postharvest. When ingested, mycotoxins may cause a mycotoxicosis that can result in an acute or chronic disease episode. Reduced growth and development, immunosuppression, and cancer are chronic conditions that have a higher prevalence in communities that have continual exposure to low-level mycotoxin ingestion as may occur in developing countries.[3] Today, even though, there is great experimental evidence characterizing the potential of mycotoxins and mouldy feedstuffs to cause animal disease, definitively linking a disease outbreak in the field to a specific mycotoxin can be very difficult. Briefly, the problem is that mycotoxins often do not cause acute disease, and when they do, there are often multiple interacting factors that can modify the expression of toxicity.[8]

4. Conclusion

The present study was undertaken to detect the mycotoxins from the fungal infected meadow grass. The fungal infested grass sample was subjected to LCMS/MS multimycotoxin analysis and the major mycotoxins got detected were mainly Fusarial toxins such as zearalenone

and beauvericin along with traces of aflatoxins, ochratoxin, citrinin, and trichothecenes. A number of compounds within these groups have been implicated in spontaneous worldwide cases of mycotoxicoses in livestock. Hence, it could be concluded that the mycotoxins got detected in fungal infected meadow grass using LCMS/MS technique, could be the causatives for the major livestock disorders mentioned.

5. References

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Source of Support: Nil. **Conflicts of Interest:** None declared.