Reliable and direct quantitative analysis of multi-mycotoxins in animal feed by using Shimadzu LCMS-8050RX

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1. Introduction

Mycotoxins are toxic compounds produced by moulds that contaminate crops such as cereals, nuts, spices, dried fruits, apples, and coffee especially under warm, humid conditions. These chemically stable toxins can persist through food processing and pose serious health risks to humans and animals, ranging from acute poisoning to cancer and immunosuppressive. Among the many identified toxic compounds, Aflatoxins, Ochratoxin A, Fumonisins, T-2, Zearalenone, and Deoxynivalenol (DON) (Figure 1) are of particular concern. These are the most studied and regulated due to their significant impact on human and animal health, making their testing critically important.
While conventional testing methods (HPLC-UV/FLD) are still common, regulatory focus is shifting toward LC-MS/MS testing due to its sensitivity and specificity. The European Committee for Standardization (CEN), under the M/520 mandate, is developing standardized methods for this purpose. Mycotoxins are generally extracted via liquid–liquid (for milk, wine, juices) or solid–liquid extraction (for cereals, dried fruits, spices, feed), typically using acidic buffers. In this study, we developed a fast, high-throughput LC-MS/MS method for multi-mycotoxin detection in animal feed using the Shimadzu LCMS-8050RX (Figure 2). MRM based quantitative method was developed using Shim-pack GIST C18-AQ HPLC column with an aim to achieve improved peak separation and required sensitivities for the compounds under study. A matrix-matched calibration curve (0.5–50.0 ppb) showed accuracy between 80–120 %, and recoveries (at 2.5 and 5.0 ppb) were 70–120 %, complying with SANTE guidelines[1].

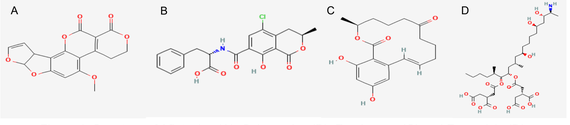

Figure 1. Structure of Aflatoxins (A), Ochratoxins (B), Zeralenone (C) and Fumonisin (D)

2. Materials and method

2.1. Sample preparation

Preparation of calibration curve standards and quality control (QC) samples

Nine mycotoxins namely Aflatoxin (B1,B2,G1& G2), Ochratoxin A, Fumonisins, T-2, Zearalenone, and Deoxynivalenol (DON) standards were sourced from a local vendor and used to prepare standard solutions. An LC method was developed using a Shim-pack GIST C18 AQ column to achieve superior peak separation, while MRM and MS parameter optimization resulted in finalized LC-MS conditions (Table 1). A blank animal feed matrix extract was spiked to obtain a 250.0 ppb working stock, which was further diluted to generate matrix-matched calibration standards ranging from 0.5 ppb to 50.0 ppb.

Sample extraction procedure

1) Commercially available animal feed (maize) sample was procured from local vendor.
2) This sample was weighed about 2.5 g in 50.0 mL centrifuge tube.
3) The sample was then spiked with mycotoxins standard solution to have spiking concentration of 2.5 ppb and 5.0 ppb.
4) 15.0 mL LC-MS grade water was added to the sample tubes to make a fine slurry.
5) Further, 15.0 mL of extraction solvent was added to the same tube and was vortexed for 2 min.
6) Added salts to maximize the extraction efficiency & the sample tube was again vortexed for 2 min..
7) The sample tubes were centrifuged at 7000 rpm at 4 ^{0}C for 10 min.
8) Withdraw the 3.0 mL supernatant for dSPE cleanup followed by evaporation and reconstitution in diluent.
9) The supernatant was collected and filtered through 0.22 µ nylon syringe filter in auto-sampler vial and analyzed on LCMS-8050RX.

Figure 2. Shimadzu LCMS-8050RX triple quadrupole with Nexera X3 liquid chromatography

2.2. LC-MS/MS analysis

Table 1. LC-MS/MS Instrument parameters

UHPLC condition (Nexera X3)	
Column	Shim-pack GIST C18-AQ, 3 µm, 4.6 mm x 150 mm (P/N: 227-30724-07)
Mobile phase	A: 0.1 % formic acid in LC-MS grade water
	B: 0.1 % formic acid in LC-MS grade methanol
Flow rate	0.75 mL/min
Column temperature	40 ^{0}C
Elution mode	Gradient
Gradient program (% B)	20 % (0.0 to 1.0 min); 98 % (8.0 to 12.0 min); 20 % (12.2 to 15.0 min)
Mass Spectrometry parameters	
MS interface	Electrospray Ionization (ESI)
Desolvation line temperature	150 ^{0}C
Heating block temperature	400 ^{0}C
Interface temperature	300 ^{0}C
Nebulizing gas flow	2 L/min
Drying gas flow	10 L/min

3. Results

3.1. Specificity

To ensure that the matrix blank is free from the target analytes, a specificity study was conducted. The extracted matrix blank sample was screened, and none of the target mycotoxins were detected. (Figure 3 & 4)

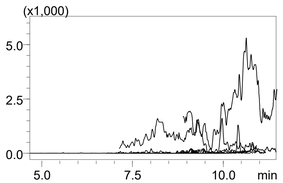

Figure 3. Chromatogram of animal feed sample matrix blank depicting absence of target mycotoxins

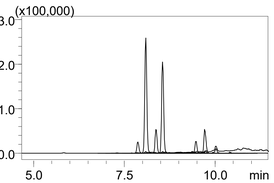

Figure 4. Chromatogram of pre-spiked animal feed sample depicting well resolved target mycotoxins

3.2. Linearity

A matrix match standard linearity ranging from 0.5 ppb to 50.0 ppb was plotted. All calibration levels showed linear response with the accuracy ranging from 80 to 120 %. (Figure 5 & 6).

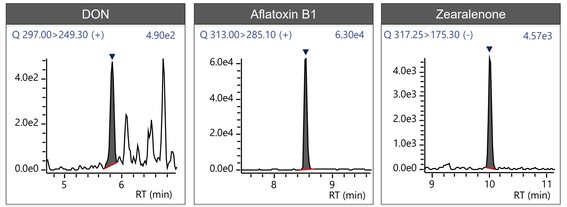

Figure 5. Chromatograms for representative compounds from pre-spiked animal feed samples

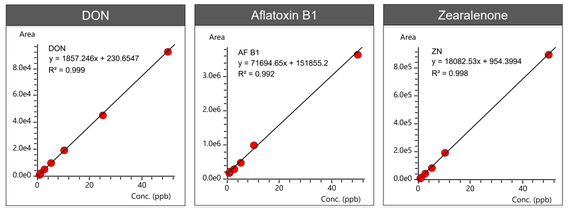

Figure 6. Calibration curve for representative compounds

3.3. Recovery

Recovery was evaluated by analyzing six pre-spiked samples at 2.5 ppb and 5.0 ppb against matrix match calibration curve mentioned in section 3.2. Average recovery values of mycotoxins were found to be within acceptance criteria as per SANTE guidelines (Figure 7).

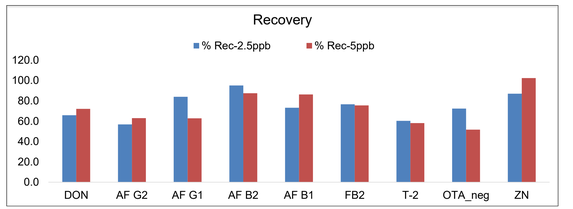

Figure 7. Graphical representation of mycotoxins recoveries in pre-spiked animal feed samples

3.4. Reproducibility (RSDr)

Six recovery samples at each level (2.5 ppb and 5.0 ppb) were analyzed to assess reproducibility. The mycotoxins demonstrated good precision, with RSDr values below 20 %, meeting the acceptance criteria outlined in the SANTE guidelines. (Figure 8).

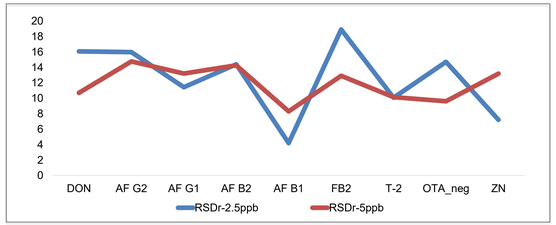

Figure 8. RSD of pre-spike samples analysed on LCMS-8050RX

4. Conclusion

- Average recovery values for multi-mycotoxins in animal feed sample were found to be within the acceptance criteria as per the SANTE/12682/2019 guidelines.
- The results obtained at 2.5 ppb and 5.0 ppb concentration level were accurate, repeatable and reproducible with RSD less than 20 %.
- A simple liquid-liquid extraction method has been successfully developed and validated for the simultaneous quantification of nine mycotoxins in a single run.

5. References

1. Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed. (SANTE/11312/2021).