METHOD DEVELOPMENT

Development of a UPLC-MS/MS method for determination of mycotoxins in animal Feed

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Abstract

Mycotoxins, secondary metabolites of moulds, are natural compounds produced on vegetable raw materials, food and feed. These compounds are toxic to humans and animals. It is therefore necessary to test animal feed for contamination by mycotoxins. This testing is implemented as part of national programs for the official control of feed. The aim of this research work was to develop a multi-method for the determination of mycotoxins in feed by liquid chromatography coupled with tandem mass spectrometry.

Keywords

- Animal feed
- Determination
- Method development
- Mycotoxins
- UPLC-MS/MS

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METHOD DEVELOPMENT

Introduction

Mycotoxins, secondary metabolites of moulds, are natural compounds produced on vegetable raw materials, food and feed. They are toxic to humans and animals. More than 300 mycotoxins, synthesised by about 350 species of moulds, have been discovered. Initially, natural occurrence of mycotoxins was observed in foods that are most susceptible to mould growth. It has been demonstrated that mycotoxins also contaminate raw feed materials and feed [Zachariasova *et al.*, 2014]. Mycotoxin contamination of food and feed is recognised as one of the most important challenges in animal breeding and food production today [Adamse *et al.*, 2012].

It is therefore necessary to test for contamination of animal feed by mycotoxins. This testing is implemented as part of national programs for the official control of feed. Guidance values or maximum levels for mycotoxins (aflatoxins (B1, B2, G1 and G2), deoxynivalenol, fumonisins (B1 and B2), ochratoxin A, zearalenone, and T-2 and HT-2 toxin) in food and feed have been established in European Union legislation [EU, 2006].

Coexistence of even a few mycotoxins in samples and low levels in different matrixes indicate that only a multi-method based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) can provide reliable data on the contamination of feed ingredients and feed. Currently, ultra-performance liquid chromatography (UPLC) coupled with tandem mass spectrometry has the additional benefits of short analysis time, lower influence of the matrix effect, and lower detection limits.

The aim of this research work was to develop a multi-method for the determination of mycotoxins in feed using UPLC-MS/MS.

Materials and methods

■ Reagents and chemicals

Solvents: acetonitrile (analytical grade) and magnesium sulphate were obtained from POCh (Poland). Methanol (LC-MS grade) and C18 bulk sorbent were purchased from J.T. Baker (the Netherlands). Acetic acid was obtained from Sigma-Aldrich (Germany) and purified water was obtained with a Milli-Q apparatus (USA). AflaTest® immunoaffinity columns were obtained from Vicam (USA). Standards of nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), HT-2 toxin (HT-2), T-2 toxin (T-2), ochratoxin A (OTA), fumonisin B1 (FB1) and B2 (FB2), aflatoxin B1 (AFL B1), B2 (AFL B2), G1(AFL G1), and G2 (AFL G2), sterigmatocystin (STG) and zearalenone (ZEN), as well as internal standards of deoxynivalenol -13C15, zearalenone -13C18, T-2-13C24, HT-2-13C22, and ochratoxin -13C20, were purchased from Sigma-Aldrich (Germany). All standards were kept according to the recommendations of the certificates. Primary standard stock solution and working solutions were prepared in acetonitrile and the mobile phase at a concentration of 1000 µg/mL to 1 ng/mL at 2–8°C.

■ Sample preparation and extraction

Each sample was ground in a laboratory mill (Glen Mills Inc., USA), sieved (mesh size 1 × 1 mm), mixed to homogenise, and stored at ≤ -18ºC until analysis. Mycotoxins were extracted from the animal feed sample (5 g) with a mixture (20 mL) of acetonitrile:water:acetic acid (79:20:1, v/v/v) in 30 minutes (vertical shaker, 200 cycles/min). Next, the sample was centrifuged and the supernatant was divided into two aliquots. The first aliquot (used in the aflatoxins analysis) was cleaned-up with immunoaffinity columns (Aflatest, Vicam). The second, used for analysis of other mycotoxins, was cleaned-up with C18 sorbent (50 mg) and magnesium sulphate (150 mg). Extracts were evaporated, mixed with labelled internal standard solution (used for quantitation) and determined with a UPLC-MS/MS technique.

METHOD DEVELOPMENT

UPLC-MS/MS analysis

A Nexera X2 system with LCMS-8050 triple quadrupole mass spectrometer (Shimadzu, Japan) and Lab Solution 1.5.2 software were used for the analysis. The mass spectrometer was operated in electrospray positive (ESI+) and negative (ESI–) ionisation mode, and two multiple reaction monitoring (MRM) transitions for each analyte were monitored. The following mass spectrometer parameters were used: resolution Q1 and Q3 - unit, nebulising gas flow – 2 L/min, heating gas flow – 10 L/min, drying gas flow – 10 L/min, interface temperature - 300°C, desolvation line temperature 250°C, heat block temperature 400°C. The chromatographic separation of mycotoxins was performed with the Luna Phenyl column (150 × 2.1 mm; particle size 3.0 µm, Phenomenex, USA) using 0.3 mL/min of constant flow and oven temperature of 40°C. The mobile phase for analysis consisted of 0.01M ammonium acetate-methanol in gradient elution. The injection volume was 10 µL.

Validation

LOD and LOQ were obtained by the analysis of background noise of 20 different blank samples (feed samples produced for different animal species and collected in different parts of Poland). The results were checked by analysis of 10 samples spiked on LOD and LOQ levels. The 20 feed samples (for different animal species and from different parts of Poland) were spiked with mycotoxins at the maximum levels (Table 2) and processed through the extraction procedure. The internal standards were used for quantitation after spiking with DON-13C15, ZEN-13C18, T-2-13C24, HT-2-13C22, and OTA-13C20. The recoveries of mycotoxins were evaluated by comparing with the concentrations found in the standard solutions. The precision of the method was measured using the same samples.

Results

During the development of the detection method, the following conditions of MS/MS were optimised for each analyte: fragmentation reactions and ionisation mode (Table 1). For most mycotoxins (except NIV, DON, 3-AcDON and ZEN), positive ionisation was applied. In the case of DON, adduct with acetic ions yielded higher intensity. For HT-2 and T-2 toxins, sodium and ammonium adducts were the options for detection, but due to lower matrix interferences ammonium adducts were preferable.

The chromatographic conditions enabled sufficient separation of mycotoxins in 18 minutes. A combination of the mobile phase and phenyl column provided acceptable peak-shapes for highly polar compounds, such as nivalenol or deoxynivalenol. The retention time for hydrophobic compounds, such as sterigmatocystin, was shorter in comparison to C18 columns (Figure 1).

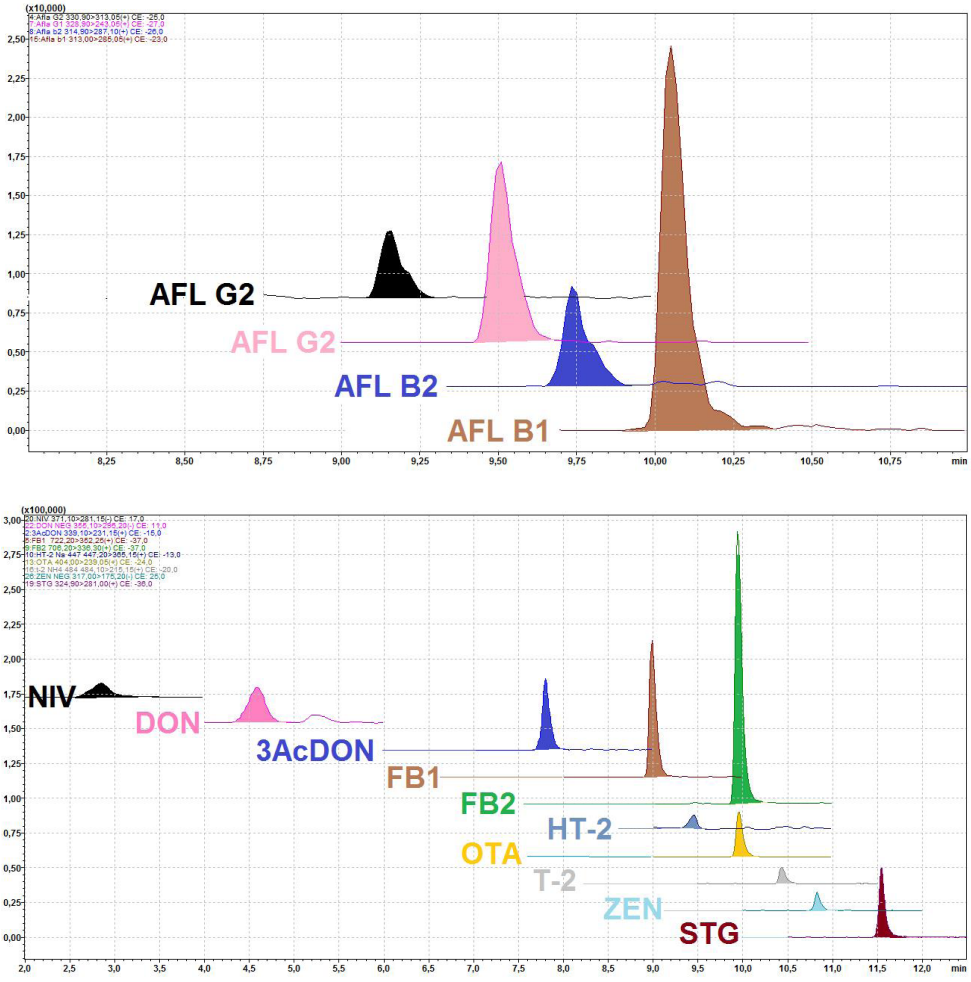
Sample preparation required use of different clean-up techniques. For most mycotoxins (except aflatoxins), the extract was cleaned-up with dispersive solid phase extraction with C18 sorbent and MgSO4 salt. Due to low signal and low required maximum level (ML) for aflatoxins, immunoaffinity clean-up was necessary.

METHOD DEVELOPMENT

TABLE 1 / Parameters of tandem mass spectrometry detection.

Analyte	Ionisation	Parent ion	1st production	2nd production
Nivalenol	[M+CH3COO]-	371	281	223
Deoxynivalenol	[M+CH3COO]-	355	295	265
3-acetyldeoxynivalenol	[M+CH3COO]-	397	307	59
Fumonisin B1	[M+H]+	722	352	334
Fumonisin B2	[M+H]+	706	336	318
T-2	[M+NH4]+	484	305	215
Ht-2	[M+NH4]+	442	263	215
Ochratoxin A	[M+H]+	404	239	193
Zearalenone	[M-H]-	317	131	175
Sterigmatocystin	[M+H]+	325	310	281
Aflatoxin B1	[M+H]+	313	285	241
Aflatoxin B2	[M+H]+	315	287	259
Aflatoxin G1	[M+H]+	329	200	243
Aflatoxin G2	[M+H]+	331	285	245

FIGURE 1 / Ion-chromatogram of spiked feed sample (ML).



METHOD DEVELOPMENT

The results of validation (Table 2) show good recovery, precision (within-laboratory reproducibility), and limit of detection, limit of quantification and usefulness of the developed procedure for multi-mycotoxin determination in animal feed.

TABLE 2 / Validation results.

	ML (·g/kg)	CVR	Recovery	LOD (·g/kg)	LOQ (·g/kg)
Nivalenol	900	18	78	50	100
Deoxynivalenol	900	15	62	50	100
3-acetyldeoxynivalenol	900	19	99	10	50
Fumonisin B1	500	17	57	10	50
Fumonisin B2	500	10	82	10	50
T-2	50	19	85	5.0	25
Ht-2	50	19	58	10	25
Ochratoxin	50	14	92	1.0	5.0
Zearalenone	100	13	96	5.0	10
Sterigmatocystin	100	18	84	5.0	20
Aflatoxin B1	5	12	95	0.50	1.0
Aflatoxin B2	5	19	85	0.50	1.0
Aflatoxin G1	5	26	75	0.50	1.0
Aflatoxin G2	5	28	86	0.50	1.0

ML – maximum level, CVR – within-laboratory reproducibility, LOD – limit of detection, LOQ – limit of quantification.

Discussion & conclusion

The issue of feed contamination by mycotoxins is well recognised. In Poland, researchers have carried out a study on the presence of 5 mycotoxins in feed materials and feed (1,255 samples over four years) [Grajewski *et al.*, 2012]. The authors found deoxynivalenol in most of the analysed samples in the concentration range 409-14470 µg/kg, and fumonisin (B1 and B2) in the range 435- 9409 µg/kg. Furthermore, they found toxins from the trichothecenes group (nivalenol, T-2 toxin and HT-2 toxin), as well as zearalenone and ochratoxin A. Authors from Belgium have developed an LC-MS/MS multi-method for the determination of mycotoxins for simultaneous determination of 23 mycotoxins in feed. Among the 82 samples of feed and feed materials, 82% were contaminated with toxins from the trichothecene and fumonisin groups. Most of the samples (75%) were contaminated with more than one mycotoxin [Monbaliu *et al.*, 2010]. Research on the occurrence of mycotoxins has also been carried out in ruminant feed (silage and hay) [Tsiplakou *et al.*, 2014]. The results showed that 13 samples tested contained aflatoxin B1 and 7 at levels higher than the limit set by European Union legislation (5 µg/kg). The problem of mycotoxins also occurs in pet nutrition (dogs and cats). In Poland, 25 dry dog foods and 24 dog foods were examined for mycotoxin contamination. In addition to commonly occurring deoxynivalenol and zearalenone, in relatively low concentrations (<120 µg/kg), the authors detected T-2 and HT-2 toxins in more than 80% of samples. The presence of these toxins in the feed is especially dangerous because of their high toxicity in cats [Błajet-Kosicka *et al.*, 2014].

As a result, a comprehensive and sensitive method for determination of mycotoxins in feed is still required. At present, only liquid chromatography coupled with tandem mass spectrometry enables control of dozens of toxic compounds in food and feed [Desmarchelier *et al.*, 2014; Jackson *et al.*, 2012; Shephard *et al.*, 2011]. The LC-MS/MS conditions used in the study

METHOD DEVELOPMENT

were based on the experience of other authors [Hickert *et al.*, 2015; Malachová *et al.*, 2014].

Analysis of mycotoxins in feed samples, due to their complexity and heterogeneity, required suitable sample preparation. In our method, we used acetonitrile mixed with acetic acid solution [Dzuman *et al.*, 2014]. The real challenge was to develop a single sample preparation and effective sample clean-up method for a wide range of chemically different analytes. For the aflatoxin analysis, we had to use immunoaffinity columns, due to the low abundance of aflatoxin peaks and the low value of maximum level in feed (5.0 µg/kg). However, most authors published procedures in which this step was not necessary. For the other mycotoxins, extracts were purified with C18 sorbent, which was sufficient to reach ML levels.

The validation results show acceptable values for LOD, LOQ, recovery, and precision. Significantly lower recoveries for DON, FB1 and HT-2 (62%, 57%, and 58%, respectively) were probably caused by the matrix effect. The results of validation are highly consistent with previously published methods. The developed method is an efficient tool for the determination of mycotoxins in animal feed.

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METHOD DEVELOPMENT

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