A “Dilute-and-Shoot” UPLC/MS/MS Method for Simultaneous Determination and Confirmation of Eleven Mycotoxins in Distillers Dried Grains with Solubles

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Abstract

Background. Distillers dried grains with solubles (DDGS) is the by-product of the corn-based industrial ethanol production. DDGS contains rich nutrition for food animals and has become a mainstream feed ingredient since mid-2000’s. However, natural contamination of mycotoxins is a known and recurring problem that affects product quality and animal health. In the U.S., the FDA has set up systematic residue levels for selected mycotoxins of high animal health concerns in feeds or feed ingredients, depending on animal species, developmental stages and intended use. Currently there is no method that can simultaneously quantify the most important 11 mycotoxins (aflatoxins B1/B2/G1/G2, fumonisins B1/B2, deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 toxins) in DDGS. Purpose. To develop and validate an analytical method that can quantify eleven mycotoxins of interest in DDGS, and simultaneously confirm their chemical identities. Methodology. DDGS samples are extracted with acetonitrile/water under acidic condition. Two aliquots from the crude extract are further diluted by either 400-fold with solvent or 16-fold (pH adjusted to 7.5) with buffer for analysis of two sub-sets of these mycotoxins. Isotope-labeled analytes are added in the end for accurate quantitation. Ultra-High-Pressure Liquid Chromatography Tandem Mass Spectrometry is used to detect the 11 mycotoxins in the sample extracts. Results. The linear quantitation range for each mycotoxin has been set up to embrace the respective residue level of concern. Overall, accuracies for these analytes at each of three fortification levels range from 70.7% to 100%, with corresponding relative standard deviations between 1.4% and 10.5%. Conclusion. This method has been successfully validated in our lab to meet the performance criteria by pertinent FDA guidelines for regulatory quantitative method. Extensive robustness testing has also been conducted. Public Health Impact. FDA regulates harmful chemical substances in feed and feed ingredients including DDGS. This work provides a sensitive, selective, and high through-put analytical method to monitor 11 high-priority mycotoxins in DDGS, to enhance the agency's capacity to protect the health of food animals.

Introduction

In the U.S., the FDA has set action, guidance, or advisory levels for selected mycotoxins (or sum of the same kind) in feeds or feed ingredients, such as corn and corn by-products (including DDGS), cottonseed meal, and peanut products. These regulatory levels vary upon animal species, growth stages, and intended use, as well as feed (grain) or ingredient type, and percentage of finished diet. Currently there is no method that can simultaneously quantify and confirm all mycotoxins of high regulatory priority in DDGS.

- Aflatoxins (B1, B2, G1, G2), action level (sum of 4 AFs) 20 – 300 ppb
- Fumonisins (B1, B2, B3), guidance level (sum of 3 FBs) 5 – 100 ppm
- Deoxynivalenol (DON), advisory level 5 – 30 ppm
- Other mycotoxins, currently no regulatory level assigned

References

1. Guidance for the Industry and FDA: Advisory level for Deoxynivalenol (DON) in Finished Wheat products for Human Consumption and Grains and Grain By Products used for Animal Feed.
2. Guidance for the Industry: Fumonisin Levels in Human Foods and Animal Feeds
3. Compliance Policy Guide Sec. 683.100 Action Levels for Aflatoxins in Animal Feeds

Materials and Methods

Sample extraction:

One sample is extracted twice with acetonitrile/water 84:16 v/v under mildly acidic condition. Two portions of the combined crude extract was taken and then diluted with solvent by 15.7-folds for analysis of AFs, OTA, T2, HT2, ZON, and 400-folds for analysis of DON, FB1, FB2, respectively. Mixture of uniformly ^{13}C-labelled internal standards (ISTD) for all mycotoxins were added at the final dilution steps. See Figure 1.

Instrumentation:

- ❑ UPLC/MS/MS: Waters UPLC H-class, Xevo TQ-S, MRM mode, ESI (+)
- ❑ Column: Acquity UPLC BEH C18 1.7 µm (2.1 x 50 mm) with guard column
- ❑ Gradient flow rate 0.4 mL/min; total time 10 min (2 injections per sample for Extract Group A and B respectively; see Figure 1)
- ❑ Mobile Phase A: 0.1 % Formic acid (FA) in water
- ❑ Mobile Phase B: 0.1 % Formic acid in acetonitrile (ACN)
- ❑ Calibration curve: Standard mixture in solvent with uniformly ^{13}C-labelled mycotoxins as ISTD

Method Validation Plan:

- ❖ Unable to obtain one DDGS sample that is clean of all 11 targeted mycotoxins, two pre-screened lots of DDGS with “complementary profile” were used as (partial) negative controls
- ❖ Three fortification levels; 9 or 12 replicates per level
- ❖ Additional naturally contaminated samples to evaluate same-lab/inter-day repeatability

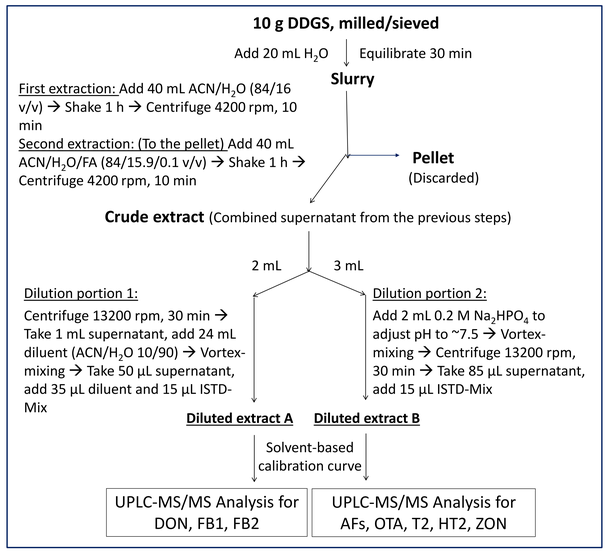

Figure 1. Sample extraction and dilution procedure

Results and Discussion

Typical ion chromatograms for the 11 mycotoxins (Figure 2), summary of key method performance characteristics (Tables 1), and the analytical results of five selected commercial DDGS samples (Table 2) are presented below. Systematic and quantitative evaluation of the other crucial aspects on analysis of each of the 11 mycotoxins included (results not shown): (1) true recoveries; (2) matrix suppression/enhancement factors; and (3) method robustness tests for critical operating parameters in the sample extraction procedure using fractional factorial design.

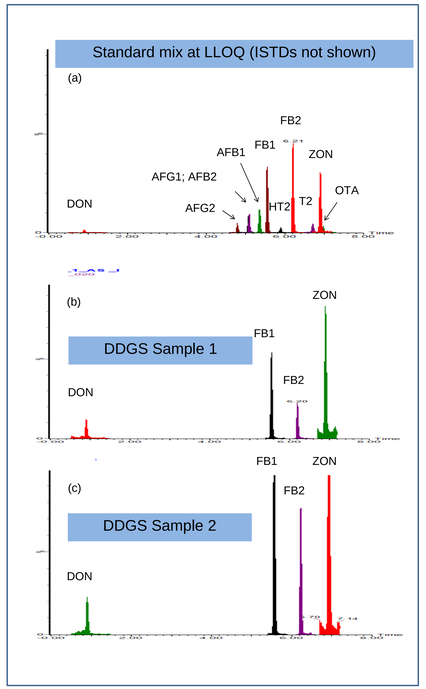

Figure 2. Typical ion chromatograms of the spiked or naturally contaminated mycotoxins in standard solution or sample extracts

Table 1. Method Validation Summary: calibration range, method detection limit (MDL), accuracy, and precision

Mycotoxin	Calibration Range (µg/kg)	MDL (µg/kg)	Accuracy%[c] and Precision (CV%)			
			Level 1[a] (N = 12)	Level 2[a] (N = 9)	Level 3[a] (N = 12)	Level 2[b] (N = 9)
AFB1	1.57 - 105	0.74	82.9 (9.3)	81.0 (2.8)	83.2 (7.8)	77.1 (1.4)
AFB2	1.57 - 105	0.10	84.0 (7.7)	85.2 (3.2)	87.5 (6.3)	84.7 (3.5)
AFG1	1.57 - 105	0.47	81.4 (4.4)	87.1 (3.0)	87.8 (5.4)	85.5 (2.3)
AFG2	1.57 - 105	0.50	80.3 (10.3)	72.2 (3.3)	80.0 (9.1)	70.7 (4.1)
ZON	16.3 – 1.09x10^3	2.91	74.5 (3.7)	79.6 (3.5)	75.6 (3.9)	77.5 (1.6)
T2	3.14 – 208	0.68	77.4 (5.8)	87.9 (6.3)	82.5 (4.5)	84.1 (3.8)
HT2	48.2 – 3.22x10^3	12.8	96.9 (5.4)	91.6 (3.1)	90.8 (4.1)	90.5 (4.0)
OTA	0.470 – 31.4	0.09	80.8 (6.4)	85.0 (3.0)	84.0 (6.0)	82.2 (9.2)
DON	240 – 1.60x10^3	215	99.3 (7.5)	93.7 (5.6)	96.2 (6.7)	94.6 (4.0)
FB1	320 – 2.12x10^3	33.7	90.2 (4.6)	86.7 (6.0)	91.0 (6.1)	85.1 (5.8)
FB2	320 – 2.12x10^3	60.3	100 (10.5)	92.1 (5.2)	98.9 (8.2)	92.4 (7.7)

[a] DDGS Lot# 30 was used; the 3 fortification levels (1-3) corresponded to 3.33x, 10.0x, and 33.3x of the lowest calibration concentration for each mycotoxin; all fortified samples were confirmed positive, except for AFG2 (2 of the 12 samples) fortified at Level 1 (below the 20-ppb target level) were not confirmable. [b] DDGS Lot#38 was used; [c] Accuracy was calculated with background subtraction as applicable

Table 2. Concentration of naturally contaminated mycotoxins detected in each of the five selected commercial DDGS samples (unit: µg/kg)

Lot #28 N = 8 (CV%)	Lot #31 N = 8 (CV%)	Lot #33 N = 6 (CV%)	Lot #38 N = 6 (CV%)	Lot #30 N = 14 (CV%)
ZON, 205 (1.2%)	T2, 5.80 (8.1%)	OTA, 3.51 (22%)	AFB1, 6.35 (2.3%)	ZON, 16.8 (11%)
DON, 2886 (11%)	ZON, 67.2 (2.8%)	ZON, 28.7 (5.7%)	OTA, 1.73 (20%)	DON, 618 (11%)
FB1, 772 (6.2%)	DON, 912 (3.1%)	DON, 399 (12%)	T2, 4.00 (8.1%)*	AFB1, < 1.57
T2, < 3.14	FB1 & FB2, < 320	T2, < 3.14	FB1, 5075 (4.2%)	T2, < 3.14
FB2, < 320		FB1 & FB2, < 320	FB2, 2032 (3.5%)	FB1 & FB2, < 320
			ZON, < 16.3	

* Only two of the six positive sub-samples were confirmed of T2’s chemical identity

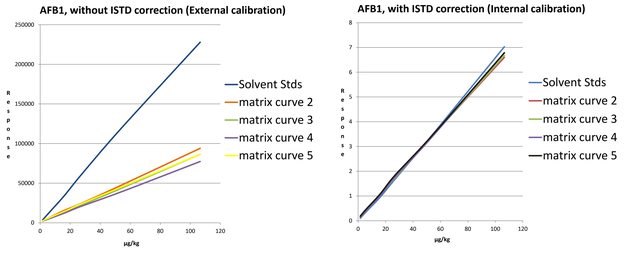

Figure 3. Representative regression curves for solvent-standards and post-extraction fortified matrix extracts

Conclusion

Under FDA guidelines, we validated a new LC-MS/MS method that is easy, quick, and reliable to simultaneously quantify and confirm the presence of eleven regulated mycotoxins in DDGS. Use of uniformly ^{13}C-labelled internal standards for each monitored mycotoxin effectively offset the bias due to matrix effects, some of which were significant, to meet the performance requirements on accuracy and precision. Taking advantage of high recoveries of these mycotoxins with the extraction procedure, these internal standards were added at the final dilution step to make this method affordable for routine use. Analysis of seventeen commercial DDGS samples revealed that DON, FB1, FB2, ZON, and T2 toxin were commonly present, while AFB2, AFG1, AFG2, and HT2 toxin were not detected.