Development and Value Assignment of an Incurred Multi-Mycotoxin Reference Material

Melissa Phillips[1], Jennifer Ness[2], Tomás López Seal[4], Carolyn Q. Burdette[1], Kai Zhang[3]

[1]*National Institute of Standards & Technology, Gaithersburg, MD, USA and* [2]*Charleston, SC, USA*

[3]*US Food and Drug Administration (FDA), College Park, MD USA*

[4]*Instituto Nacional de Tecnología Industrial (INTI), Buenos Aires, Argentina*

Why a Multi-Mycotoxin Material?

Fungi of the genera *Aspergillus*, *Fusarium*, and *Penicillium* produce mycotoxins such as aflatoxins, fumonisins, deoxynivalenol, zearalenone, ochratoxin A, HT-2 toxin, and T-2 toxin onto foods and feeds during harvests, storage, or onto finished products under warm or humid conditions. Although mycotoxin contamination can be minimized with the implementation of good agricultural and manufacturing practices, dietary exposure to humans and animals is unavoidable as these toxins are resistant to degradation by current food-processing procedures. Due to the numerous negative health impacts associated with the consumption of foodstuffs contaminated with mycotoxins, accurate determination is an international concern. Many commercially-available certified RMs for mycotoxins mainly address a single mycotoxin or class of mycotoxins, requiring the use of multiple RMs for multi-target methods. In addition, none of these RMs contain all of the FDA-regulated mycotoxins and mycotoxins of health significance. To address the increasing needs of laboratories moving toward LC-MS-based multimycotoxin analysis, the U.S. National Institute of Standards and Technology (NIST) collaborated with the U.S. Food and Drug Administration (FDA) to produce a naturally incurred RM for multiple mycotoxins in corn.

Material Design and Preparation

Collected from 14 different batches of naturally contaminated corn.

Incurred Corn (14 kg)

Contains mycotoxin concentrations near the FDA lowest action levels.

Blank Corn (70 kg)

Contains low natural levels of FB1 and DON at 100x and 10x below the FDA lowest action levels (resp).

Corn collected from various markets in Maryland including 7 brands and various types.

Cryomilling

Particle Size Analysis

Incurred Corn (14 kg)

Homogenized

Blank Corn (70 kg)

Corn cryomilled at NIST Cryogenic Reference Material Production Facility

14 g of contaminated corn and 28 kg of homogenized corn were mixed.

Screening by LC-MS/MS

Irradiation

Blank Corn (28 kg)

Bottling

Homogeneity Testing

Value Assignment

Interlaboratory Comparison

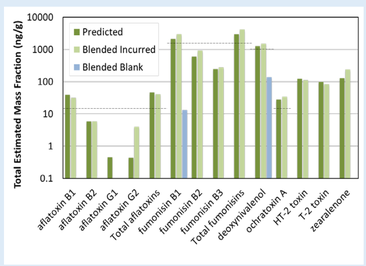
600 units x 60 g

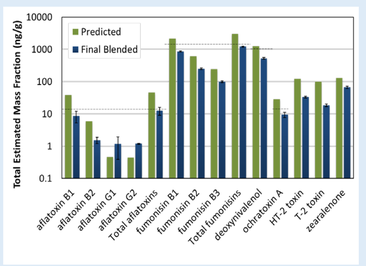
SRM 1565 Myctoxins in Corn (Incurred)

600 units x 60 g

SRM 1565 Mycotoxins in Corn (Blank)

Preliminary Screening by LC-MS/MS

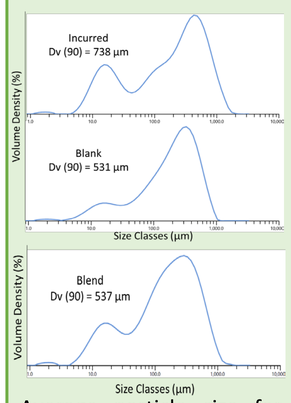




Estimated levels of mycotoxins in the blended incurred and blank corn materials determined by FDA/CFSAN using ID-LC-MS/MS (1) after first round of blending (**left**) and in final mixture for incurred corn (**right**).

Screened mass fraction values were used as target values and provided to participating laboratories in interlaboratory comparison.

Particle Size Analysis

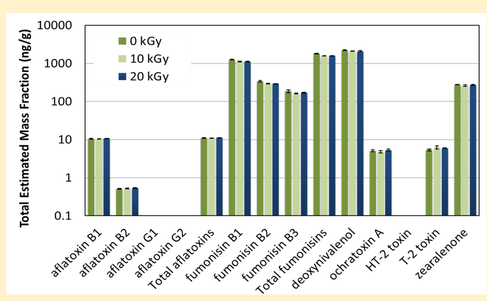


Target particle size for SRM 1565 was ≤ 841 µm (#20 sieve; based on guidance from AOAC).

Samples of blank (**top**), incurred (**center**), and blended (**bottom**) corn (≈ 0.5 g) were dispersed in ethanol, allowed to disperse for 10 s before 10 individual measurements were recorded at an obscuration of 10 % and 20 % using a Malvern Mastersizer 3000.

Average particle size for SRM 1565 (Blank) was 588 µm (SD = 17 µm, n = 10). For SRM 1565 (Incurred), the average particle size was 676 µm (SD = 92 µm, n = 10).

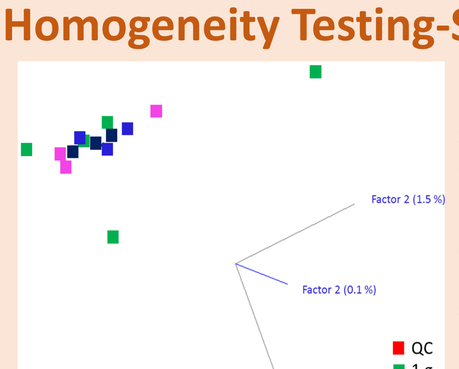
Effects of Irradiation



Mass fractions of mycotoxins in SRM 1565 were not affected significantly by exposure to γ-radiation at 10 and 20 kGy absorbed doses.

Final packaged units of SRM 1565 were irradiated to an absorbed dose of 11.9 kGy to 18.7 kGy to reduce the probability of microbial growth and additional mycotoxin production.

Homogeneity Testing-Sample Size



PCA scores plot of 1 g to 10 g sample sizes of SRM 1565 (Incurred) and QC material (FAPAS).

Clustering of data points indicated that sample sizes of SRM 1565 should be ≥ 3 g to ensure test portion homogeneity and reproducibility of analytical results.

Value Assignment by NIST and FDA/CFSAN

- A stratified random sampling scheme was used by NIST and FDA to select 10 bottles of SRM 1565 (Incurred) and SRM 1565 (Blank) to prepare in duplicates.
- A QC material with know levels of mycotoxins was purchased from FAPAS to use as a control and was prepared in duplicates.
- Recovery studies revealed automated shaking was optimal for extraction and no significant increase in extraction yield was observed from additional extractions.
- NIST used a relative response factor approach to calibration.
- Homogeneity was evaluated for each determined mycotoxin as a function of packaging order, sample preparation order, and chromatographic run order.
- FDA evaluated sample size variability between 1 g and 5 g sample sizes.

Analysis of mycotoxins by NIST ID-LC-MS/MS

Instrumentation: Agilent Series 1260 LC / Applied Biosystems SCIEX API 5000 LC-MS/MS system, electrospray ionization in positive ion mode.

MRM transitions and Compound MS Parameters:

Mycotoxins	Molecular formula	Molecular weight	RT (min)	Adduct ion	MRM Transitions	DP (eV)	CE (eV)	CXP (eV)
Aflatoxin B_1	$C_{17}H_{12}O_6$	312.1	9.2	$[M+H]^+$	313.1→241.0/285.0	107/85	53/35	13/15
$[^{13}C_{17}]$-aflatoxin B_1	$^{13}C_{17}H_{12}O_6$	329.1	9.2	$[M+H]^+$	330.1→255.2/301.0	107/85	53/35	13/15
Aflatoxin B_2	$C_{17}H_{14}O_6$	314.1	9.0	$[M+H]^+$	315.2→287.1/259.1	107/110	38/43	16/14
$[^{13}C_{17}]$-aflatoxin B_2	$^{13}C_{17}H_{14}O_6$	331.1	9.0	$[M+H]^+$	332.0→303.2/273.1	105/110	38/43	16/14
Aflatoxin G_1	$C_{17}H_{12}O_7$	328.1	8.7	$[M+H]^+$	329.0→242.2/200.0	93/93	39/58	13/10
$[^{13}C_{17}]$-aflatoxin G_1	$^{13}C_{17}H_{12}O_7$	345.1	8.7	$[M+H]^+$	345.8→257.1/124.2	80/80	38/92	13/8
Aflatoxin G_2	$C_{17}H_{14}O_7$	330.1	8.5	$[M+H]^+$	331.2→313.0/245.0	88/100	36/42	18/13
$[^{13}C_{17}]$-aflatoxin G_2	$^{13}C_{17}H_{14}O_7$	347.1	8.5	$[M+H]^+$	348.0→330.0/259.0	88/100	36/42	18/13
Deoxynivalenol	$C_{15}H_{20}O_6$	296.1	5.5	$[M+H]^+$	297.0→249.0/231.2	65/64	17/20	20/13
$[^{13}C_{15}]$-deoxynivalenol	$^{13}C_{15}H_{20}O_6$	311.2	5.5	$[M+H]^+$	312.0→263.0/245.2	65/64	17/20	20/13
Fumonisin B_1	$C_{34}H_{59}NO_{15}$	721.4	10.3	$[M+H]^+$	722.5→352.5/334.5	150/100	45/45	15/15
$[^{13}C_{34}]$-fumonisin B_1	$^{13}C_{34}H_{59}NO_{15}$	755.5	10.3	$[M+H]^+$	756.4→374.5/356.4	107/110	52/55	20/20
Ochratoxin A	$C_{20}H_{18}ClNO_6$	403.1	11.0	$[M+H]^+$	404.0→239.0/102.0	74/73	36/101	13/17
$[^{13}C_{20}]$-ochratoxin A	$^{13}C_{20}H_{18}ClNO_6$	423.1	11.0	$[M+H]^+$	424.1→250.1/110.1	74/73	36/101	13/17
T-2 toxin	$C_{24}H_{34}O_9$	466.2	10.6	$[M+NH_4]^+$	484.3→215.2/185.1	55/50	26/30	12/9
$[^{13}C_{24}]$-T-2 toxin	$^{13}C_{24}H_{34}O_9$	490.3	10.6	$[M+NH_4]^+$	508.3→229.2/198.2	55/50	26/30	12/9
Zearalenone	$C_{18}H_{22}O_5$	318.1	11.0	$[M+H]^+$	319.2→283.2/187.1	50/50	19/29	16/10
$[^{13}C_{18}]$-Zearalenone	$^{13}C_{18}H_{22}O_5$	336.2	11.0	$[M+H]^+$	337.2→138.2/124.0	45/35	70/77	10/21

MS Source Parameters:

Collision Gas (CAD)	10
Curtain Gas (CUR)	30
Ion Source Gas 1 (GS1)	50
Ion Source Gas (GS2)	50
IonSpray Voltage (IS)	5000
Temperature (TEM)	500
Entrance Potential	4

Injection Volume: 3 µL

Column: Phenomenex Kinetex XBC18 LC column (100 × 2.1 mm i.d., 2.6 µm particles) with a 10 × 2.1 mm guard cartridge, 40 °C

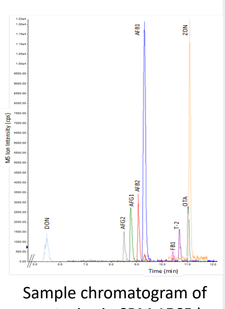
Mobile Phase A: 10 mmol/L ammonium formate in water containing 0.1 % formic acid (v/v)

Mobile Phase B: 10 mmol/L ammonium formate in methanol containing 0.1 % formic acid (v/v)

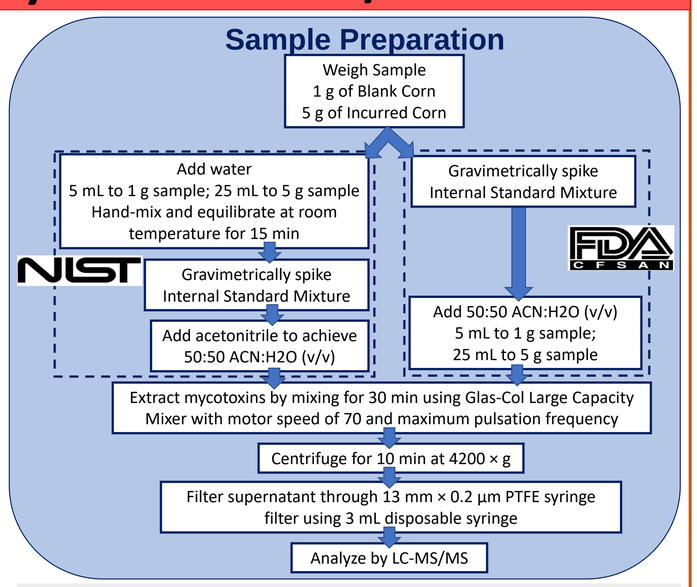
Flow Rate: 0.3 mL/min

LC Gradient:

Time (min)	%B
0	5
2.0	40
10.0	100
11.5	100
12.0	5
20.0	5



Sample chromatogram of mycotoxins in SRM 1565 by NIST ID-LC-MS/MS.

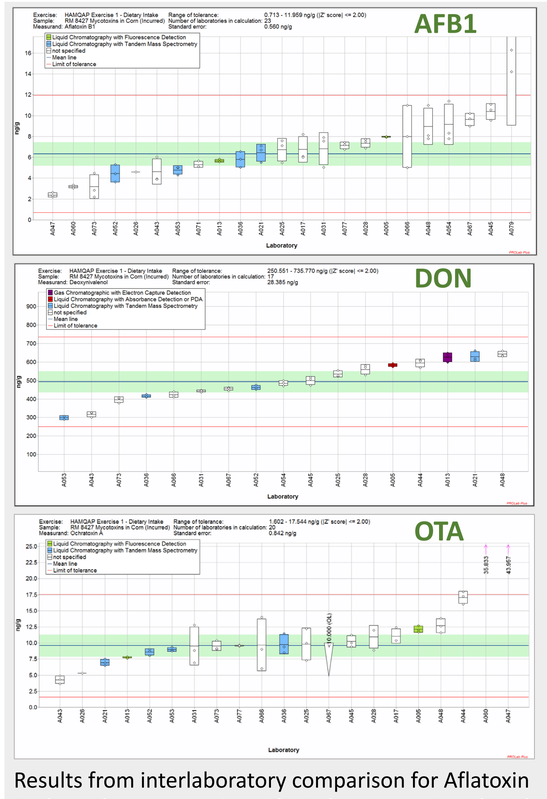


Summary of ID-LC-MS/MS Measurements by NIST and FDA/CFSAN

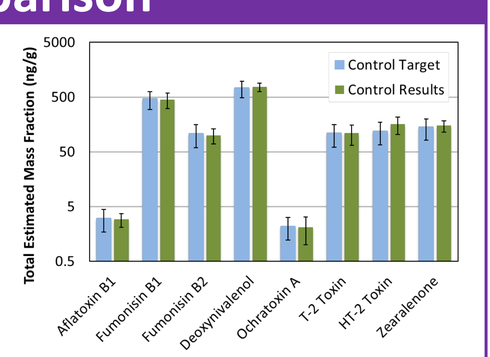
- All values obtained for the QC material using the ID-LC-MS/MS method were within the value range assigned by the manufacturer.
- NIST ID-LC-MS/MS measurement precision varied between 7.5 % and 41 % RSD in duplicate preparations of 10 samples of incurred and blank SRM 1565.
- Based on FDA ID-LC-MS/MS measurement data (1), 1 g sample size is recommended for SRM 1565 (Blank). Using 5 g aliquots decreased measurement precision for deoxynivalenol (17 % to 35 % RSD) and fumonisin B1 (11 % to 23 % RSD). 5 g sample size is recommended for the remaining mycotoxins.
- NIST did not provide data for fumonisin B2, fumonisin B3, total fumonisins, and HT-2 toxin.
- FDA data for ochratoxin A was not used for value assignment.
- FDA did not provide data for aflatoxin G2 based on failed identity criteria.

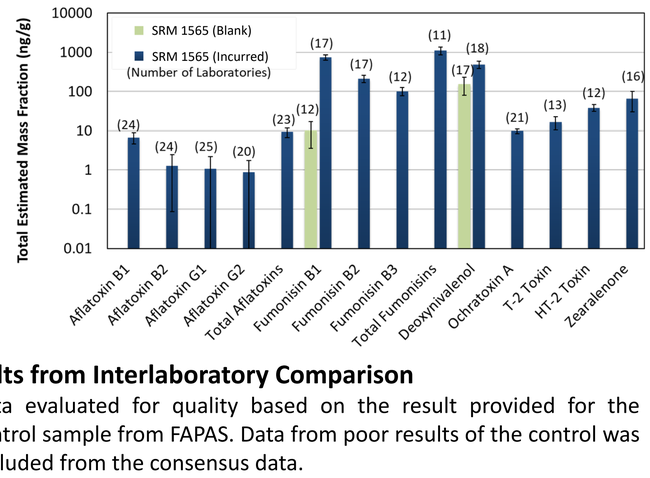
Interlaboratory Comparison

- 38 participating laboratories for NIST Health Assessment Measurements Quality Assurance Program (HAMQAP) Exercise 1.
- Participants were provided with blinded samples of SRM 1565 (Blank), SRM 1565 (Incurred), and a QC material (FAPAS).
- Asked to prepare 3 samples and report 3 results for each mycotoxin or group of mycotoxins in each sample, using their routine methods of analysis.



Results from interlaboratory comparison for Aflatoxin B1 **(AFB1)**, Deoxynivalenol **(DON)**, Ochratoxin A **(OTA)** in SRM 1565 Mycotoxins in Corn (Incurred).



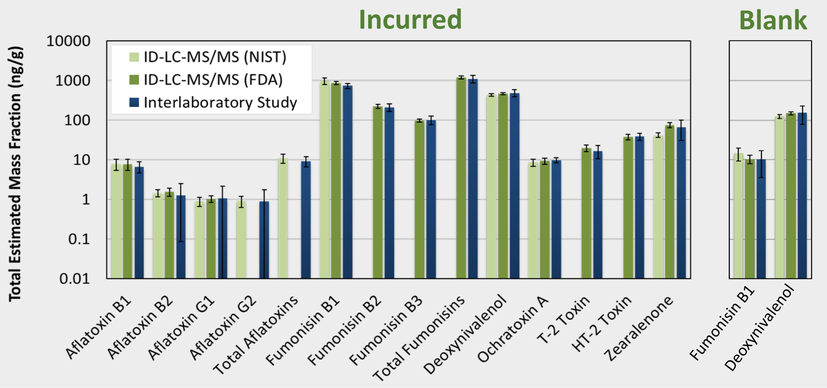


Results from Interlaboratory Comparison

- Data evaluated for quality based on the result provided for the control sample from FAPAS. Data from poor results of the control was excluded from the consensus data.
- Overall performance was good with between laboratory variabilities were comparable and ranged from 16 % to 49% for most mycotoxins in SRM 1565 (Incurred).
- Higher between-laboratory variability was observed for lower-level mycotoxins in the materials: aflatoxin G2 (82 %) in SRM 1565 (Incurred), and deoxynivalenol (46 %) and fumonisin B1 (84 %) in SRM 1565 (Blank).
- Consensus means were within the target ranges for all analytes in all samples, except for total fumonisins in SRM 1565 (Blank). Low levels of the fumonisins in the blank material may have resulted in measurement challenges for some laboratories.

Combined Results

Assigned values for mycotoxins in SRM 1565 were determined by combination of the means from NIST ID-LC-MS/MS and FDA ID-LC-MS/MS measurements and the median from qualified laboratories from the interlaboratory comparison using a linear pool method (2). The uncertainty represents the SD of the aggregate probability distribution.



Mycotoxin	Mass Fraction (ng/g) SRM 1565 (Blank)	SRM 1565 (Incurred)
Aflatoxin B1	ND	7.5 ± 1.7
Aflatoxin B2	ND	1.43 ± 0.34
Aflatoxin G1	ND	0.98 ± 0.19
Aflatoxin G2	ND	0.87 ± 0.24
Total Aflatoxins	ND	10.2 ± 2.9
Deoxynivalenol	142 ± 36	467 ± 67
Fumonisin B1	10.4 ± 3.9	805 ± 190
Fumonisin B2	ND	217 ± 30
Fumonisin B3	ND	99.3 ± 8.4
Total Fumonisins	ND	1150 ± 170
Ochratoxin A	ND	9.4 ± 1.2
HT-2 Toxin	ND	38.2 ± 6.0
T-2 Toxin	ND	18.4 ± 4.2
Zearalenone	ND	61 ± 36

Future Work

- With the availability of reference standards
  - Values for DON and ZON will be upgraded to certified with SI traceability
  - Values will be assigned for fumonisin B2, fumonisin B3, HT-2 toxin, and T-2 toxin
- Development of other matrix-matched foods contaminated with mycotoxins

References:

(1) Zhang, K., et al. (2017) J. Agric. Food Chem. 65, 7138–7152. doi:10.1021/acs.jafc.6b04872

(2) Koepke, A., Lafarge, T., Possolo, A., & Toman, B. (2017) Metrologia 54, S34–S62

(3) Phillips, M.M., et al. (2019) J. AOAC Int. 102, doi:https://doi.org/10.5740/jaoacint.19-0109

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