FDA Foods Program Compendium of Analytical Laboratory Methods: Chemical Analytical Manual (CAM)

METHOD NUMBER: C-003.01

POSTING DATE: 10/5/2017

POSTING EXPIRATION DATE: none

PROGRAM AREA: Mycotoxins

METHOD TITLE: Determination of Mycotoxins in Corn, Peanut Butter, and Wheat Flour Using Stable Isotope Dilution Assay (SIDA) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

VALIDATION STATUS: Level 3 Multilaboratory validation per the Guidelines for the Validation of Chemical Methods for the FDA Foods Program 3rd Edition

AUTHOR(S): Kai Zhang, CFSAN/ORS/DBC/Bioanalytical Methods Branch

METHOD SUMMARY/SCOPE:

Analyte(s): Aflatoxin B1, B2, G1, G2; deoxynivalenol; fumonisin B1, B2, B3; HT-2 toxin, ochratoxin A, T-2 toxin, zearalenone

Matrices: Corn, peanut butter and wheat flour

REVISION HISTORY:

OTHER NOTES:

Title: Determination of Mycotoxins in Corn, Peanut Butter, and Wheat Flour Using Stable Isotope Dilution Assay (SIDA) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Version: 1.0 (2017)

Author: Kai Zhang

CFSAN/ORS reviewers: Christine Parker, Gregory Noonan
Chemistry Research Coordination Group Concurrence: John Callahan, Chair, 10/5/2017

Table of Contents

2020.1 METHOD TITLE

2020.2 SCOPE OF APPLICATION

2020.3 PRINCIPLE

2020.4 REAGENTS

2020.5 STANDARDS

2020.6 PREPARATION OF SAMPLES OR TEST PORTIONS

2020.7 APPARATUS/INSTRUMENTATION

2020.8 METHOD

2020.9 CALCULATIONS

2020.10 VALIDATION INFORMATION/STATUS

2020.11 REFERENCES

2020.1 METHOD TITLE: Determination of mycotoxins in corn, peanut butter, and wheat flour using stable isotope dilution assay (SIDA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS)

2020.2 SCOPE OF APPLICATION

This method describes a procedure for using stable isotope dilution assay (SIDA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine 12 mycotoxins of regulatory and health significance. The 12 mycotoxins are aflatoxin B_1, B_2, G_1, G_2; deoxynivalenol; fumonisin B_1, B_2, B_3; HT-2 toxin, ochratoxin A, T-2 toxin and zearalenone. The method has been validated in the following food matrices: corn, peanut butter and wheat flour.

It is generally assumed that the more closely related a new food matrix is to a previously validated matrix for a defined analyte, the greater the probability that the new matrix will behave similarly. It is also usually the case that the regulatory chemical methods employed by FDA are used to analyze a diversity of products representing a large spectrum of matrices. It becomes unfeasible to carry out a matrix extension validation for each single matrix in order to expand the scope of the method. A more reasonable approach to demonstrate the applicability of a method to a set of product matrices is to validate the method for different "categories" of products. For instance, a multi-residue pesticide method can be validated for "high-sugar", "high-fat", "high-water", "dry" and "high-protein" matrices. Food and Drug Administration (FDA) Guidelines for the Validation of Chemical Methods for the FDA Foods Program Appendix 4 provides [1] guidance on commodity categories and gives examples of representative matrices in each category.

This method should be used by analysts experienced in the use of SIDA and LC-MS/MS, including but not limited to sample preparation, operation of LC-MS/MS, data analysis and reporting results. Analysts also should be able to identify chromatographic and mass spectrometric interferences in the course of sample analysis and take necessary actions following validated procedures correct instrument/method performance issues. The method should be used only by personnel thoroughly trained in the handling and analysis of samples for the determination of mycotoxins in food and feed products.

2020.3 PRINCIPLE

Samples are prepared by fortifying with ^{13}C uniformly labelled mycotoxins as internal standards (IS), followed by extraction using 50% acetonitrile (50/50, v/v, acetonitrile/water), centrifugation and filtration. The target mycotoxins are analyzed by LC-MS/MS and identified by retention time alignment and product ion transition confirmation with calibration standards. The identification criteria are detailed in FDA Guidelines for the Validation of Chemical Methods for the FDA Foods Program and FDA Center for Veterinary Medicine. Guidance for Industry 118, 2003, specifically, all transitions must be present, with appropriate relative abundances. [1, 2]

Quantitation was performed using solvent-only calibration standards. The concentration of each target mycotoxin was determined using the peak area ratio of response of the mycotoxin to that of the corresponding [^{13}C]-IS, and calculating the concentration by preparing a calibration curve using the peak area ratios of solvent-only calibration standards to that of the same [^{13}C]-IS.

2020.4 REAGENTS

(1) Acetonitrile, LC grade (Thermo Fisher Scientific, Part # 85188)
(2) Methanol, LC grade (Thermo Fisher Scientific, Part# A452SK-4)
(3) Water, LC grade (Thermo Fisher Scientific, Part# 85189)
(4) Formic acid, MS grade (Thermo Fisher Scientific, Part# 85178)
(5) Ammonium formate, MS grade (Thermo Fisher Scientific, Part# A11550)

NOTE: Reagents should be purchased from vendors that can provide certificates of analysis (CoAs) to show traceability, purity, storage condition and duration.

2020.5 STANDARDS

(1) Aflatoxin B_1, B_2, G_1, G_2 (Romer Labs, Part# 002050)
(2) Deoxynivalenol (Romer Labs, Part# 002050)
(3) Fumonisin B_1, B_2, B_3 (Romer Labs, Part# 002050)
(4) Ochratoxin A (Romer Labs, Part# 002050)
(5) HT-2 toxin ((Romer Labs, Part# 002050)
(6) T-2 toxin (Romer Labs, Part# 002050)
(7) Zearalenone (Romer Labs, Part# 002050)
(8) [$^{13}C_{17}$]-aflatoxin B_1 (Romer Labs, Part # ILM010)
(9) [$^{13}C_{17}$]-aflatoxin B_2 (Romer Labs, Part # ILM011)
(10) [$^{13}C_{17}$]-aflatoxin G_1(Romer Labs, Part # ILM012)
(11) [$^{13}C_{17}$]-aflatoxin G_2 (Romer Labs, Part # ILM013)
(12) [$^{13}C_{34}$]-fumonisin B_1 (Romer Labs, Part# ILM003)
(13) [$^{13}C_{34}$]-fumonisin B_2(Romer Labs, Part# ILM004)
(14) [$^{13}C_{34}$]-fumonisin B_3(Romer Labs, Part# ILM005)
(15) [$^{13}C_{15}$]-deoxynivalenol (Romer Labs, Part# 002005)
(16) [$^{13}C_{20}$]-ochratoxin A (Romer Labs, Part# ILM007)
(17) [$^{13}C_{22}$]-HT-2 toxin (Romer Labs, Part# ILM 008)
(18) [$^{13}C_{24}$]-T-2 toxin (Romer Labs, Part# 002004)
(19) [$^{13}C_{18}$]-zearalenone (Romer Labs, Part# ILM009)

NOTE: Standards should be purchased from vendors that can provide CoAs to show traceability, purity, storage condition and duration.

Standard preparation
Prepare or purchase the following three mycotoxin stock solutions A, B, and C (e.g., 5.0 mL/vial) from Romer Labs (Union, MO):

(1) Solution A: Aflatoxin B_1 (1.0 µg/mL), aflatoxin B_2 (1.0 µg/mL), aflatoxin G_1 (1.0 µg/mL), aflatoxin G_2, (1.0 µg/mL) and ochratoxin A (1.0 µg/mL) in methanol
(2) Solution B: Fumonisin B_1 (10 µg/mL), fumonisin B_2 (10 µg/mL), and fumonisin B_3 (10 µg/mL) in acetonitrile/water (50:50, v/v)
(3) Solution C: Deoxynivalenol (10 µg/mL), HT-2 toxin (10 µg/mL), T-2 toxin (10 µg/mL), and zearalenone (10 µg/mL) in acetonitrile.

The following [^{13}C]-IS stock solutions can be purchased from Romer Labs:[$^{13}C_{17}$]-aflatoxin B_1 + B_2 + G_1 + G_2, (0.5 µg/mL), [$^{13}C_{17}$]-aflatoxin M_1 (0.5 µg/mL), [$^{13}C_{20}$]-ochratoxin A (10 µg/mL), [$^{13}C_7$]-patulin (25 µg/mL), [$^{13}C_{34}$]-fumonisin B_1 (25 µg/mL), [$^{13}C_{34}$]-fumonisin B_2 (10 µg/mL), [$^{13}C_{34}$]- fumonisin B_3 (10 µg/mL), [$^{13}C_{15}$]-deoxynivalenol (25 µg/mL), [$^{13}C_{22}$]-HT-2 toxin (25 µg/mL), [$^{13}C_{24}$]-T-2 toxin (25 µg/mL), [$^{13}C_{18}$]-zearalenone (25 µg/mL). The working solution of [^{13}C]-IS can be prepared by mixing and diluting appropriate amounts of the stock solutions using the extraction solution, acetonitrile/water (v/v, 50/50).

The concentration of each [^{13}C]-IS in the working solution is as follows: [$^{13}C_{17}$]-aflatoxin B_1, B_2, G_1, and G_2 (0.05 µg/mL), [$^{13}C_{17}$]-ochratoxin A (0.2 µg/mL), [$^{13}C_{34}$]-fumonisin B_1 (2.0 µg/mL), [$^{13}C_{34}$]-fumonisin B_2 (2.0 µg/mL), [$^{13}C_{34}$]- fumonisin B_3 (2.0 µg/mL), [$^{13}C_{15}$]-deoxynivalenol (2.0 µg/mL), [$^{13}C_{22}$]-HT-2 toxin (2.0 µg/mL), [$^{13}C_{24}$]-T-2 toxin (2.0 µg/mL), and [$^{13}C_{18}$]-zearalenone (2.0 µg/mL).

Solvent-only calibration standards are prepared from dilutions of stock solutions A, B, and C and the corresponding [^{13}C]-IS working solution, respectively. Multiple levels of solvent-only calibration standards (0.5 mL for each level) can be prepared in acetonitrile/water (50/50, v/v) and each standard is fortified with 10 µL of the [^{13}C]-IS working solution. Concentration ranges may be adjusted for quantitation. The fortification volume of the 13C-IS working solution may be adjusted according to the sensitivity of LC-MS.

2020.6 PREPARATION OF SAMPLES OR TEST PORTIONS

2020.6.1 Water-slurry procedure used for food commodities (e.g., corn) that are difficult to homogenize using dry milling.

(1) Weigh 25.0 ± 0.5g of a sample into a 40 or 100 mL disposable grinding chamber (IKA, NC, USA) and add 25.0 ± 0.5 of water (HPLC grade).
(2) Blend the sample and water for 1.5 min at 25,000 rpm using an IKA Tube Mill.
(3) Weigh a test portion (2.00 ± 0.05 g) from the blended sample into a 15 mL disposable screw-capped polypropylene centrifuge tube.
(4) Fortify the test portion with 100 µL of the [^{13}C]-IS working solution, making the concentrations of [^{13}C]-IS in the sample as follows, [$^{13}C_{17}$]-aflatoxin B_1 (0.005 µg/g), [$^{13}C_{17}$]-aflatoxin B_2 (0.005 µg/g),[$^{13}C_{17}$]-aflatoxin G_1 (0.005 µg/g), [$^{13}C_{17}$]-aflatoxin G_2, (0.005 µg/g) and [$^{13}C_{17}$]-ochratoxin A (0.02 µg/g), [$^{13}C_{34}$]-fumonisin B_1 (0.20 µg/g), [$^{13}C_{34}$]-fumonisin B_2 (0.20 µg/g), and [$^{13}C_{34}$]- fumonisin B_3 (0.20 µg/g), [$^{13}C_{15}$]-deoxynivalenol (0.20 µg/g), [$^{13}C_{22}$]-HT-2 toxin (0.20 µg/g), [$^{13}C_{24}$]-T-2 toxin (0.20 µg/g), and [$^{13}C_{18}$]-zearalenone (0.20 µg/g). The concentrations of

[^{13}C]-IS fortified in each test portion were calculated based on mass of the sample (1.0 g).

(5) Re-cap the tube and vortex it for 30 sec, followed by the addition of 4.0 mL of extraction solvent (acetonitrile/water, 50/50, v/v) into the tube.

(6) Method blank (negative control QC) can be prepared using matrices of interest containing non-detectable target mycotoxins following steps 1-5.

(7) Method spike (positive control QC) can be prepared by fortifying blank matrices with target mycotoxins at pre-defined concentrations then following steps 1-5. Alternatively, certified reference materials may be used as QC.

2020.6.2 For food commodities, such as infant rice cereal and peanut butter that are generally considered homogenous, but can be homogenized using dry-milling.

(1) Weigh a test portion of homogenized sample (1.00 ± 0.05 g each) into a 15 mL disposable screw-capped polypropylene centrifuge tube.

(2) Fortify the test portion with 100 µL of the [13C]-IS working solution, making the final concentrations of [13C]-IS in the sample as follows, [13C17]-aflatoxin B1 (0.005 µg/g), [13C17]-aflatoxin B2 (0.005 µg/g), [13C17]-aflatoxin G1 (0.005 µg/g), [13C17]-aflatoxin G2, (0.005 µg/g) and [13C17]-ochratoxin A (0.02 µg/g), [13C34]-fumonisin B1 (0.20 µg/g), [13C34]-fumonisin B2 (0.20 µg/g), and [13C34]- fumonisin B3 (0.20 µg/g), [13C15]-deoxynivalenol (0.20 µg/g), [13C22]-HT-2 toxin (0.20 µg/g), [13C24]-T-2 toxin (0.20 µg/g), and [13C18]-zearalenone (0.10 µg/g)

(3) Re-cap the tube and vortex it for 30 sec, followed by the addition of 5.0 mL of extraction solvent (acetonitrile/water, 50/50, v/v) into the tube.

2020.6.3 Sample extraction and cleanup

(1) Extract the samples prepared above for 30 min using a high-speed shaker with pulsation (Glas-Col, Terre Haute, IN, USA) using a motor speed setting of 75 (1540−1560 rpm as measured by a DPM5 digital photo tachometer, Universal Enterprises, Inc., Beaverton, OR, USA) and pulser frequency set at the middle mark of the dial (~30−35 pulsations/min), followed by subsequent centrifugation for 15 min at 4200g (ThermoElectro Corp., Milford, MA, USA).

(2) Filter the supernatant of samples through a 13 mm × 0.2 µm PTFE syringe filter (Pall Life Sciences), using a 3 mL disposable syringe, directly into an amber autosampler vial (National Scientific, Rockwood, TN, USA) for LC-MS/MS analysis.

(3) Alternatively, the supernatant (~2.0 mL) of samples can be transferred to an Amicon Ultra-4 centrifugal filter with Ultracel-3 membrane (molecular weight cutoff value of 3 kDa) and centrifuged for 15 min at 4200g. The resulting filtrates are transferred into autosampler vials for LC-MS/MS analysis.

2020.7 APPARATUS/INSTRUMENTATION

Disclaimer: The use of trade names in this method constitutes neither endorsement nor recommendation by the U. S. Food and Drug Administration (FDA). Equivalent performance may be achievable using apparatus and materials other than those cited here.

(1) Liquid chromatography-coupled with triple quadrupole mass spectrometry that can meet instrument performance specifications defined Section 2017.13.

(2) HPLC guard and analytical columns –with a Phenomenex SecurityGuard ULTRA 10 mm × 2.1 mm i.d. guard cartridge coupled with a Phenomenex Kinetex XB-C18 (100 mm × 2.1 mm i.d., 2.6 µm).

(3) High-speed shaker with pulsation, Glas-Col.
(4) Centrifuge tubes—polypropylene conical tubes with caps, 15 mL.
(5) Vortex mixer
(6) Amicon Ultra-4 centrifugal filter with Ultracel-3 membrane (molecular weight cutoff value of 3 kDa), Millipore.
(7) Plastic Syringes, disposable, general use and non-sterile, 5 mL, Luer-Loc tip.
(8) Syringe filters—used in filtering samples, disposable, 0.45 µm PTFE membrane with polypropylene housing and Luer-Loc inlet.
(9) Analytical balance—precision of 0.01 g.
(10) Repeating pipettes and dispensers – 2 µL → 50 mL calibrated for organic and aqueous liquids, i.e., Drummond Pipet Aid XP (Drummond Scientific Co) or equivalent for dispensing 10 and 20 mL liquid volumes, Gilson pipettes M10, M100, M1000 for dispensing 1-10 µL, 10-100 µL, 100 – 1000 µL range and appropriate CP10, CP100, and CP1000 pipet tips (or equivalent brands)
(11) Benchtop Centrifuge (Thermo Scientific CR4i centrifuge or equivalent) for centrifuging 15 mL centrifuge tubes containing sample extracts.
(12) IKA Tube Mill and IKA Tube Mill 100.
(13) IKA disposable 40 mL and 100 mL grinding chambers.
(14)
(15)

2020.8 METHOD

The following instrument parameters are specific to two LC-MS/MS instruments listed below used in the multi-laboratory validation of this method These parameters may change according to the manufacturer, subtle variation in chemical composition of mobile phase, purity and type of ESI source and collision gases used. Depending on LC columns and origin of mobile phases, minor variations may occur.

High performance liquid chromatography (HPLC) – Shimadzu Prominence equipped with a binary pump (LC-20ADXR), autosampler (SIL-20ACXR) and column oven (CTO-20AC).

Mass spectrometer (MS) – Sciex 4000 and 6500 QTRAP with Analyst^{TM}, version 1.6 or higher, for instrument control software. LC-MS/MS data is processed using MultiQuan^{TM}, version 2.0 or higher (Sciex)

HPLC Binary Pump, autosampler and MS/MS, Positive Mode, parameters for FDA Mycotoxin Analysis

HPLC:
Mobile phase A: Water (10 mM ammonium formate, 0.1% formic acid)
Mobile phase B: Methanol (10 mM ammonium formate, 0.1% formic acid)

Stop time	15 min
Pressure Limits	≥ 9000 Psi
Minimum Pressure	0 Psi
Maximum Pressure	≥ 9000 Psi

Timetable

Time (min)	Solvent B (%)	Flow (µL/min)
0.0	5.0	300
2.0	40.0	300
10	100.0	300
11.5	100.0	300
12.0	5.0	300
15.0	5.0	300

Autosampler:

Injection volume:	3 µL
Rinsing volume:	200 µL
Needle stroke:	52 mm
Rinsing speed:	35 µL/s
Sampling speed:	5 µL/s
Rinse dip time:	5 s
Controller temperature:	10 ºC
Column temperature:	40 ºC

Mass Spectrometry:

Sciex 4000

Collision gas:	N_2, medium
Curtain gas:	N_2, 30
IonSpray Voltage:	5000 Volts
Interface heater:	ON
TurboIon source temperature:	500 °C
Gas 1:	50
Gas 2:	50
Q1 Resolution:	Unit
Q3 Resolution:	Unit

Scheduled MRM schemes for the target compounds are listed in the following table.

Sciex 6500

Collision gas:	N_2, medium
Curtain gas:	N_2, 30
IonSpray Voltage:	5500 Volts
Interface heater:	ON
TurboIon source temperature:	600 °C
Gas 1:	60
Gas 2:	60
Q1 Resolution:	Unit
Q3 Resolution:	Unit

Table 8-1: Scheduled MRM parameters for the target mycotoxins.

Mycotoxins	Molecular formula	Molecular weight	RT (min)	Adduct ion	MRM transitions*	6500 QTRAP			4000 QTRAP		
						DP (eV)	CE (eV)	CXP (eV)	DP (eV)	CE (eV)	CXP (eV)
Aflatoxin B_1	$C_{17}H_{12}O_6$	312.1	6.1	$[M+H]^+$	313.1→**241.0**/285.0	86/106	55/37	14/8	107/85	53/35	13/15
$[^{13}C_{17}]$-aflatoxin B_1	$^{13}C_{17}H_{12}O_6$	329.1	6.1	$[M+H]^+$	330.1→**255.2**/301.0	86/106	55/37	14/8	107/85	53/35	13/15
Aflatoxin B_2	$C_{17}H_{14}O_6$	314.1	5.8	$[M+H]^+$	315.2→**287.1**/259.1	91/91	39/45	16/14	105/110	38/43	16/14
$[^{13}C_{17}]$-aflatoxin B_2	$^{13}C_{17}H_{14}O_6$	331.1	5.8	$[M+H]^+$	332.0→**303.2**/273.1	91/91	39/45	16/14	105/110	38/43	16/14
Aflatoxin G_1	$C_{17}H_{12}O_7$	328.1	5.5	$[M+H]^+$	328.8→**243.2**/200.0	86/86	41/99	14/10	93/93	39/58	13/10
$[^{13}C_{17}]$-aflatoxin G_1	$^{13}C_{17}H_{12}O_7$	345.1	5.5	$[M+H]^+$	345.8→**257.1**/124.2	86/86	41/99	14/10	80/80	38/92	13.5/8
Aflatoxin G_2	$C_{17}H_{14}O_7$	330.1	5.3	$[M+H]^+$	331.2→**313.0**/245.0	111/111	36/49	18/20	88/100	36/42	18/13
$[^{13}C_{17}]$-aflatoxin G_2	$^{13}C_{17}H_{14}O_7$	347.1	5.3	$[M+H]^+$	348.0→**330.0**/259.0	111/111	36/49	18/20	88/100	36/42	18/13
Deoxynivalenol	$C_{15}H_{20}O_6$	296.1	3.6	$[M+H]^+$	297.0→**249.0**/231.2	71/61	17/21	44/22	65/64	17/20	20/13
$[^{13}C_{15}]$-deoxynivalenol	$^{13}C_{15}H_{20}O_6$	311.2	3.6	$[M+H]^+$	312.0→**263.0**/345.2	71/61	17/21	44/22	65/64	17/20	20/13
Fumonisin B_1	$C_{34}H_{59}NO_{15}$	721.4	7.5	$[M+H]^+$	722.5→**352.5**/334.5	111/111	53/57	10/54	107/110	52/55	20/20
$[^{13}C_{34}]$-fumonisin B_1	$^{13}C_{34}H_{59}NO_{15}$	755.5	7.5	$[M+H]^+$	756.4→**374.5**/356.4	111/111	53/57	10/54	107/110	52/55	20/20
Fumonisin B_2	$C_{34}H_{59}NO_{14}$	705.4	8.7	$[M+H]^+$	706.3→**336.3**/318.3	106/106	55/59	10/20	115/105	51/54	20/18
$[^{13}C_{34}]$ fumonisin B_2	$^{13}C_{34}H_{59}NO_{14}$	739.5	8.7	$[M+H]^+$	740.3→**358.4**/340.5	106/106	55/59	10/20	125/92	51/55	20/26
Fumonisin B_3	$C_{34}H_{59}NO_{14}$	705.4	8.2	$[M+H]^+$	706.3→**336.3**/318.3	106/106	55/59	10/20	105/115	51/53	19/17
$[^{13}C_{34}]$- fumonisin B_3	$^{13}C_{34}H_{59}NO_{14}$	739.5	8.2	$[M+H]^+$	740.5→**358.4**/340.4	106/106	55/59	10/20	105/115	51/53	19/18
Ochratoxin A	$C_{20}H_{18}ClNO_6$	403.1	7.6	$[M+H]^+$	404.0→**239.0**/102.0	66/66	41/101	16/16	74/73	36/101	13/17
$[^{13}C_{20}]$-ochratoxin A	$^{13}C_{20}H_{18}ClNO_6$	423.1	7.6	$[M+H]^+$	424.1→**250.1**/110.1	66/66	41/101	16/16	74/73	36/101	13/17
HT-2 toxin	$C_{22}H_{32}O_8$	424.2	7.1	$[M+NH_4]^+$	442.2→**215.3**/323..2	50/50	20/15	16/16	58/55	20/13	11/17
$[^{13}C_{22}]$-HT-2 toxin	$^{13}C_{22}H_{32}O_9$	446.3	7.1	$[M+NH_4]^+$	464.2→**229.2**/340.2	50/50	20/15	16/16	58/55	20/13	11/17
T-2 toxin	$C_{24}H_{34}O_9$	466.2	7.7	$[M+NH_4]^+$	484.3→**215.2**/185.1	57/57	29/33	17/11	55/50	26/30	12/9
$[^{13}C_{24}]$-T-2 toxin	$^{13}C_{24}H_{34}O_9$	490.3	7.7	$[M+NH_4]^+$	508.3→**229.2**/198.2	57/57	29/33	17/11	55/50	26/30	12/9
Zearalenone	$C_{18}H_{22}O_5$	318.1	8.2	$[M+H]^+$	319.2→**283.2**/187.1	101/86	17/31	10/10	50/50	19/29	16/10
$[^{13}C_{18}]$-Zearalenone	$^{13}C_{18}H_{22}O_5$	336.2	8.2	$[M+H]^+$	337.2→**138.2**/124.0	71/81	79/87	10/10	45/35	70/77	10/21

2020.9 CALCULATIONS

Quantitation was based on linear least squares calibration of the relative response ratio of a mycotoxin and its ^{13}C-IS plotted versus mycotoxin concentration. In each sample, the concentrations of mycotoxins were determined from the equation

C = ^{13}C-IS concentration ratio × (S − y-intercept)/slope

Where C is the concentration in the sample, ^{13}C-IS concentration ratio is the concentration of ^{13}C-IS in the sample/concentration of ^{13}C-IS in each calibration standard, and S is the signal (peak area) of native mycotoxin/signal (peak area) of ^{13}C-IS in the sample (using the quantitation ions of isotope and native mycotoxin). See Appendix IV for step-by-step calculation.

If necessary, the method LOQ of a target mycotoxin may be determined following the protocol for determining a method detection limit (MDL) and subsequently an LOQ (LOQ = 3.33 × MDL) detailed in 40 CFR Part 136 App B.[3] Briefly, the procedures include:

(1) Estimate an MDL (ng/g in matrix). This estimate can be based on previous work with the method, response from low end calibration standards, or limited spiking studies.

(2) Fortify the matrix at between one and five times the Estimated MDL. In all cases a minimum of 8 replicates should be processed and analyzed, but the fortification will vary slightly depending on the matrix. For dry matrices that use the water slurry method, we recommend separately fortifying each 25 g sample prior to the addition of water. For more homogeneous matrices, it is appropriate to separately fortify each 1 g sample or to fortify a larger sample, homogenize and then subsample 8 aliquots. For liquid samples, fortifying a larger volume, mixing and then subsampling 8 aliquots is appropriate.

(3) Process and analyze the samples and corresponding blanks using the method protocol.

(4) Determine the variance and standard deviation to calculate the MDL and LOQ using the results from the replicate analyses. In addition to the determination of these parameters, the quantitative results used in the calculations should meet the identification and confirmation criteria described in FDA Guidelines for the Validation of Chemical Methods for the FDA Foods Program; specifically, all transitions must be present, with appropriate relative abundances, at the MDL.

(5) Report estimated LOD and LOQ. Report quantitative results only when quality control criteria for a batch have been satisfactorily met. Report results for identified and quantitated mycotoxins ≥LOQ as the final concentrations (ng/g or ppb) in samples. Report results that are ≥LOD and <LOQ as "<LOQ", indicating mycotoxin is present at a trace level that is below the limit of quantification. Report results that are <LOD as "not detected". Due to variability between laboratories and instrumentation, values for LOD and LOQ should be determined in each instrument system at each laboratory.

2020.10 VALIDATION INFORMATION/STATUS

The method specified in this document was validated to level 3 under FDA Guidelines for the Validation of Chemical Methods for the FDA Foods Program. [1] In addition to three certified reference materials, the participating laboratories -analyzed corn, peanut butter, and wheat flour fortified with the 12 mycotoxins at concentrations ranging from 1.0 to 1000 ng/g. The majority of recoveries ranged between 80 ─ 120% with relative standard derivations (RSDs) <20%.

Greater than 90% of the average recoveries of the participating laboratories were in the range of 90-110%, with repeatability RSD_r (within laboratory) < 10% and reproducibility RSD_R (among laboratory) < 15%. All Z scores of the results of certified reference materials were between -2 and 2. More details regarding the method validation study can be found in Zhang et al., 2017.[4]

2020.11 REFERENCES

1. FDA Guidelines for the Validation of Chemical Methods for the FDA Foods Program;http://www.fda.gov/downloads/ScienceResearch/FieldScience/UCM298730.pdf (accessed May 1, 2017).

2. FDA Center for Veterinary Medicine. Guidance for Industry 118, 2003. Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues; http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM052658.pdf (accessed May 1, 2017)

3. 40 CFR Appendix B to Part 136 - Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11 https://www.gpo.gov/fdsys/granule/CFR-2011-title40-vol23/CFR-2011-title40-vol23-part136-appB/content-detail.html (accessed May 1, 2017)

4. Zhang, K., Schaab, M.R., Southwood, G., Tor, E.R., Aston, L.S., Song, W., Eitzer, B., Majumdar, S., Lapainis, T., Mai, H., Tran, K., El-Demerdash, A., Vega, V., Cai, Y., Wong, J.W., Krynitsky, A.J., Begley, T.H. (2017) A Collaborative Study: Determination of Mycotoxins in Corn, Peanut Butter, and Wheat Flour Using Stable Isotope Dilution Assay (SIDA) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). *J. Agric. Food Chem*. 2017, 65, 7138-7152.

Appendix I. Example of procedures used to prepare 5 mL of working solution of 13C-IS

1. Mix 100 mL HPLC or Millipore-grade water and 100 mL of HPLC-grade acetonitrile in a 250 mL flask.
2. Manually shake the flask for 2 min.
3. Pipette appropriate amount of each 13C-IS from the corresponding stock solution vials into a 5 mL volumetric flask (as shown in the table below)
4. Bring the volume to the mark (5.0 mL) by adding prepared extraction solution (v/v, 50/50, acetonitrile/water)
5. Transfer prepared working solution to a 15 mL amber glass vial and stored at -20ºC in

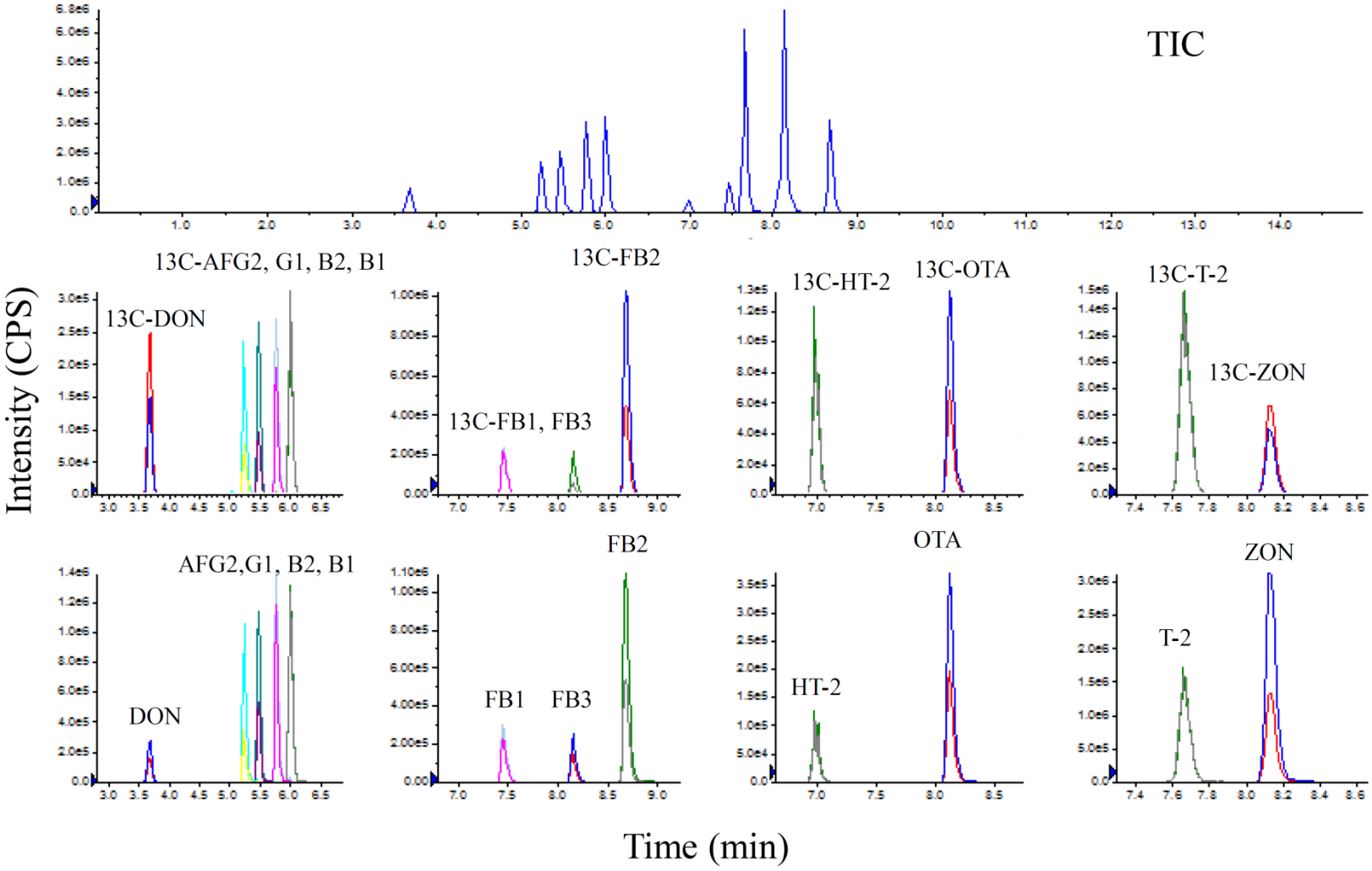
13C-IS	Stock solution Conc. (µg/mL)	Pipetted volume of stock solution (µL)	Working solution volume (µL)	Working solution conc. (µg/mL)
[^{13}C]-aflatoxin B_1+B_2+G_1+G2	0.5	500	5000	0.05
[^{13}C]-deoxynivalenol	25	400	5000	2
[^{13}C]-fumonisin B_1	25	400	5000	2
[^{13}C]-fumonisin B_2	10	1000	5000	2
[^{13}C]-fumonisin B_3	10	1000	5000	2
[^{13}C]-HT-2 toxin	25	400	5000	2
[^{13}C]-ochratoxin A	10	100	5000	0.2
[^{13}C]-T-2 toxin	25	400	5000	2
[^{13}C]-zearalenone	25	400	5000	2

Appendix II. Example of solvent-only calibration standards

Mycotoxin/^{13}C-IS	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7	Level 8	Level 9	Level 10
Aflatoxin B_1	0.05	0.1	0.25	0.5	1.0	2.5	5.0	10	25	50
[^{13}C]-aflatoxin B_1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Aflatoxin B_2	0.05	0.1	0.25	0.5	1.0	2.5	5.0	10	25	50
[^{13}C]-aflatoxin B_2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Aflatoxin G_1	0.05	0.1	0.25	0.5	1.0	2.5	5.0	10	25	50
[^{13}C]-aflatoxin G_2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Aflatoxin G_2	0.05	0.1	0.25	0.5	1.0	2.5	5.0	10	25	50
[^{13}C]-aflatoxin G_2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Ochratoxin A	0.05	0.1	0.25	0.5	1.0	2.5	5.0	10	25	50
[^{13}C]-ochratoxin A	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Deoxynivalenol	0.5	1.0	2.5	5.0	10	25	50	100	250	500
[^{13}C]-deoxynivalenol	40	40	40	40	40	40	40	40	40	40
Fumonisin B_1	0.5	1.0	2.5	5.0	10	25	50	100	250	500
[^{13}C]-fumonisin B_1	40	40	40	40	40	40	40	40	40	40
Fumonisin B_2	0.5	1.0	2.5	5.0	10	25	50	100	250	500
[^{13}C]-fumonisin B_2	40	40	40	40	40	40	40	40	40	40
Fumonisin B_3	0.5	1.0	2.5	5.0	10	25	50	100	250	500
[^{13}C]-fumonisin B_3	40	40	40	40	40	40	40	40	40	40
HT-2 Toxin	0.5	1.0	2.5	5.0	10	25	50	100	250	500
[^{13}C]-HT-2 Toxin	40	40	40	40	40	40	40	40	40	40

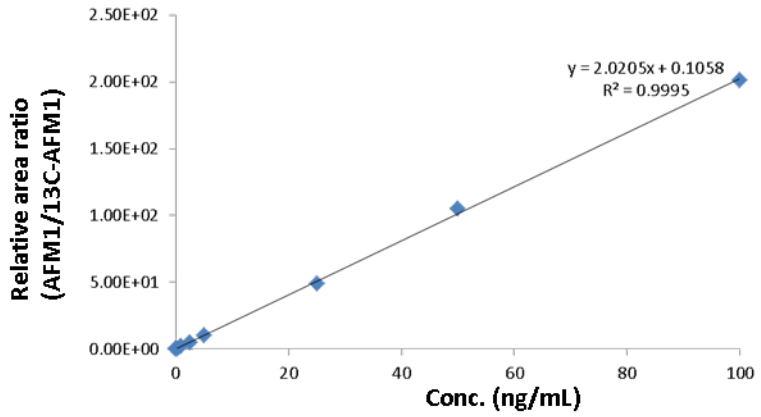
T-2 Toxin	0.5	1.0	2.5	5.0	10	25	50	100	250	500
[^{13}C]-T-2 Toxin	40	40	40	40	40	40	40	40	40	40
Zearalenone	0.5	1.0	2.5	5.0	10	25	50	100	250	500
[^{13}C]-zearalenone	40	40	40	40	40	40	40	40	40	40

Appendix III: Total ion chromatogram and extracted ion chromatograms of target mycotoxins and 13C-mycotoxins generetaed using conditions listed in Section 2017.8



Appendix IV. Example for calculation
Step 1: establish calibration curve for a target mycotoxin (e.g., AFM1)

	AFM1	13C-IS	AFM1/13C-IS
Conc. (ng/mL)	Area	Area	Area ratio
0.01	8.08E+03	3.71E+05	2.18E-02
0.05	3.59E+04	3.68E+05	9.78E-02
0.1	7.45E+04	3.74E+05	1.99E-01
0.25	1.82E+05	3.76E+05	4.85E-01
1	7.62E+05	3.68E+05	2.07E+00
2.5	1.87E+06	3.63E+05	5.14E+00
5	3.70E+06	3.63E+05	1.02E+01
25	1.86E+07	3.80E+05	4.88E+01
50	3.63E+07	3.46E+05	1.05E+02
100	7.16E+07	3.57E+05	2.01E+02
Sample 1	3.77E+05	3.62E+05	1.04E+00
Sample 2	7.43E+06	3.74E+05	1.99E+01



Step 2

Calibration curve: y = 2.0205x + 0.1058
Use x =R*(y-b)/m to calculate the concentration of a detected mycotoxin:
. Where x is the calculated concentration, R is the relative concentration ratio of the [13C]-IS fortified in the sample and calibration standards, which can be determined based on sample preparation, y is the relative peak area ratio of the mycotoxin and the [13C]-IS in the sample, b is the intercept on the Y axis, and m is the slope. In this case, R =5, m = 2.0205 and b = 0.1058

Step 3
Concentrations (ng/mL) of AFM1 in the two sample:

Sample 1 5*(A-0.1058)/2.205 = 2.12 (ng/g)
Sample 2 5*(B-0.1058)/2.205 = 44.80 (ng/g)

Appendix V. Sources of certified reference materials used for method verification

1. National Institute of Standards and Technology
    - SRM 2387 (aflatoxins in peanut butter)

2. European Commission Joint Research Institute, Institute for Reference materials and Measurements
    - BCR-385R (aflatoxins in peanut butter)
    - BCR-401 (aflatoxins in peanut butter)
    - ERM-BC600 (fusarium mycotoxins in wheat flour)

3. FAPAS
    - TET017RM (mycotoxins in maize flour)