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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: Multi-laboratory validation per the Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition.

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METHOD SUMMARY/SCOPE:
The method describes a procedure for measuring 12 mycotoxins in food using LC-MS/MS. A summary of method validation and extension work is below:

Matrices	Validation	Date	Laboratories[a]
corn, peanut butter, and wheat flour	multi-laboratory validation	2016	Study Organizer: HFP/OLOAS/OCT/DBC Participating Laboratories: 6
rice	matrix extension	2020	ATLHAFL
corn, peanut butter, rice, wheat flour	column extension	2021	ATLHAFL, DBC, KCHAFL, SEAHAFL

[a]ATLHAFL: Atlanta Human and Animal Food Laboratory; DBC: Division of Bioanalytical Chemistry; KCHAFL: Kansas City Human and Animal Food Laboratory; SEAHAFL: Seattle Human and Animal Food Laboratory.

Analyte(s): 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.

Matrices: Corn, peanut butter, rice, wheat flour.

REVISION HISTORY: Version C-003.02 replaces version C-003.01 (2017). The method text was updated to improve clarity. In addition, the scope table was updated to include additional method extensions that have since been validated.

Version C-003.03 replaces version C-003.02 (2023). The method reporting limit was updated to 2.5 ng/g for aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, and ochratoxin A.

OTHER NOTES:

- The guard and analytical columns were updated to Agilent InfinityLab Poroshell 120 Stablebond-C18, 2.1 x 5 mm, 2.7 µm (Part Number: 821725-912) and Agilent InfinityLab Poroshell 120 Stablebond-C18, 2.1 x 100 mm, 2.7 µm (Part Number: 685775-902). Update was supported by a column verification study performed by ATLHAFL, DBC, KCHAFL, SEAHAFL, to address carryover limitations for fumonisin B_1, fumonisin B_2, fumonisin B_3 using the original column chemistry.
- Matrix extension performed by ATLHAFL to include rice products (e.g., polished white rice, whole grain brown rice, and rice cereal) in method scope.
- MS/MS mass transitions updated to monoisotopic mass.
- For routine analysis, recommend five-point calibration curve. Ensure calibration range continues to meet program requirements for regulated mycotoxins and minimize influence of low (sub-STD1) concentrations of carryover.

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 3.0 (2024)
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Reviewers: Mycotoxin Technical Advisory Group

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2024.1. 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).

2024.2. 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 (AB1, AB2, AG1, AG2); deoxynivalenol (DON); fumonisin B_1, B_2, B_3 (FB1, FB2, FB3); HT-2 toxin (HT-2), ochratoxin A (OTA), T-2 toxin (T-2), and zearalenone (ZEA). The method has been validated in the following food matrices:

Table 2-1: Summary of method validation.

Matrices	Validation	Date	Laboratories
corn, peanut butter, and wheat flour	multi-laboratory validation	2016	Study Organizer: HFP/OLOAS/OCT/DBC Participating Laboratories: 6
rice	matrix extension	2020	ATLHAFL

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

2024.3. Principle

Samples are prepared by fortifying with ^{13}C uniformly labeled mycotoxins as internal standards (IS), followed by extraction using 50% acetonitrile: 50% water (v/v), 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. Quantitation is performed using native calibration standards in solvent and [^{13}C]-IS. The concentration of each target mycotoxin is determined using the peak area ratio of response of the mycotoxin quantitation transition to that of the corresponding [^{13}C]-IS, adjusted for dilution.

2024.4. Reagents

Reagents should be LC-MS grade or better, unless otherwise specified. Reagents should be purchased from an authorized vendor with a Certificate of Analysis (COA) including traceability, purity, storage conditions, and expiration.

(1) Acetonitrile
(2) Methanol
(3) Water
(4) Formic acid
(5) Ammonium formate (powder) or 10M ammonium formate (solution)

2024.5. Standards

Standards were procured from Romer Labs for method validation. Equivalent vendors also accepted. See Section 2024.16 for additional information on standard Source and Purity.

(1) Aflatoxin B_1, B_2, G_1, G_2 (Romer Labs)
(2) Deoxynivalenol (Romer Labs)
(3) Fumonisin B_1, B_2, B_3 (Romer Labs)
(4) Ochratoxin A (Romer Labs)
(5) HT-2 toxin (Romer Labs)
(6) T-2 toxin (Romer Labs)
(7) Zearalenone (Romer Labs)
(8) [$^{13}C_{17}$]-aflatoxin B_1, B_2, G_1, G_2 (Romer Labs)
(9) [$^{13}C_{34}$]-fumonisin B_1 (Romer Labs)
(10) [$^{13}C_{34}$]-fumonisin B_2 (Romer Labs)
(11) [$^{13}C_{34}$]-fumonisin B_3 (Romer Labs)
(12) [$^{13}C_{15}$]-deoxynivalenol (Romer Labs)
(13) [$^{13}C_{20}$]-ochratoxin A (Romer Labs)
(14) [$^{13}C_{22}$]-HT-2 toxin (Romer Labs)
(15) [$^{13}C_{24}$]-T-2 toxin (Romer Labs)
(16) [$^{13}C_{18}$]-zearalenone (Romer Labs)

2024.6 Equipment

The use of trade names in this method constitutes neither endorsement nor recommendation by the U. S. Food and Drug Administration (FDA). Citation of sample preparation equipment is based on the original method validation. Equipment of equivalent specifications also accepted, however, all chemistry (e.g., analytical column) must be maintained with the original method.

(1) Repeating pipettes and dispensers: 2 µL → 50 mL. Calibrated for organic and aqueous liquids.
(2) Analytical balance. Capable of weighing with a precision of 0.01 g.
(3) Volumetric glassware.
(4) IKA Tube Mill Control and IKA Tube Mill 100 Control.

(5) IKA MT 40 (40 mL) and MT 100 (100 mL) disposable grinding chambers.
(6) High-speed shaker with pulsation (e.g., Glas-Col, Spex Geno/Grinder).
(7) Vortex mixer.
(8) Centrifuge tubes. Polypropylene conical tubes with screw-on, plug seal caps, 15 mL.
(9) Benchtop centrifuge with sealing rotor cups. Capable of handling 15 mL centrifuge tubes containing sample extracts, 4200g.
(10) Plastic Syringes, disposable, general use and non-sterile, 5 mL, Luer-Loc tip (or equivalent).
(11) Syringe filters for filtering samples, disposable, 13 mm x 0.2 µm PTFE membrane, Luer-Loc inlet (or equivalent).
(12) Amicon Ultra-4 centrifugal filter with Ultracel-3 membrane (molecular weight cutoff value of 3 kDa), Millipore Sigma.
(13) Autosampler vials (2 mL) with screw-on caps.
(14) LC guard and analytical column: Agilent InfinityLab Poroshell 120 Stablebond-C18, 2.1 x 5 mm, 2.7 µm, guard column (Part Number: 821725-912) and Agilent InfinityLab Poroshell 120 Stablebond-C18, 2.1 x 100 mm, 2.7 µm, analytical column (Part Number: 685775-902).

2024.7. Standard and Solution Preparation

Standard concentrations and solution compositions provided with example protocol for preparation. Alternate procedures for preparation, including final volumes, are permitted within the scope of the method.

2024.7.1. Preparation of 50% acetonitrile: 50% water (v/v)

Example: Mix 125 mL water and 125 mL of acetonitrile. Invert to mix. Solution prepared for standard dilution and sample extraction.

2024.7.2. Preparation of native mycotoxin stock and working standards

When available, native mycotoxins may be purchased as ready-to-use ampoules (Table 7-1). Customized mixtures are also accepted. All standard solutions should be stored in amber vials at or below -18ºC or according to the manufacturer's recommendations and brought to room temperature prior to use. Standard solutions are recommended for use prior to manufacturer's expiration.

2024.7.2. Preparation of native mycotoxin stock and working standards (continued)

Table 7-1: Example concentrations for native mycotoxin stock standards[a]

Mycotoxin	Stock Standard (µg/mL)	Solvent
aflatoxin B_1 aflatoxin B_2 aflatoxin G_1 aflatoxin G_2	2.0	Acetonitrile
ochratoxin A	10	Acetonitrile
fumonisin B_1	50	50% acetonitrile: 50% water (v/v)
fumonisin B_2	50	50% acetonitrile: 50% water (v/v)
fumonisin B_3	50	50% acetonitrile: 50% water (v/v)
deoxynivalenol	100	Acetonitrile
HT-2 toxin	100	Acetonitrile
T-2 toxin	100	Acetonitrile
zearalenone	100	Acetonitrile

[a] Alternate concentrations for the preparation of stock standards are accepted

Prepare the following native working mycotoxin standards by mixing and diluting appropriate amounts of the stock standards using the defined dilution solution. Final volume of each working standard: 2000 µL (Table 7-2).

Table 7-2: Example concentrations for native mycotoxin working standards[a]

Working Standard	Mycotoxin	Stock Standard (µg/mL)	Volume Stock Standard (µL)	Volume Dilution Solution (µL)[b]	Working Standard (µg/mL)
A	aflatoxin B_1, B_2, G_1 G_2	2.0	1000.0	800.0[c]	1.0
	ochratoxin A	10	200.0		1.0
B	fumonisin B_1	50	400.0	800.0[d]	10
	fumonisin B_2	50	400.0		10
	fumonisin B_3	50	400.0		10
C	deoxynivalenol	100	200.0	1200.0[e]	10
	HT-2 toxin	100	200.0		10
	T-2 toxin	100	200.0		10
	zearalenone	100	200.0		10

[a] Alternate concentrations for the preparation of stock and working standard, including final volumes, are accepted

[b] Dilutions may be prepared to final volume (2000 µL) in volumetric flask

[c] Dilution solution: 100% acetonitrile

[d] Dilution solution: 50% acetonitrile: 50% water (v/v)

[e] Dilution solution: 100% acetonitrile

Alternatively, the native mycotoxin working standard may be prepared as a mixed standard in 50% acetonitrile: 50% water (v/v) This solution is recommended to be prepared daily. See Appendix I for preparation.

2024.7.3. Preparation of isotopically labeled mycotoxin stock and working standards

Isotopically labeled (^{13}C-IS) mycotoxins may be purchased as ready-to-use ampoules (Table 7-3). Customized mixtures are also accepted. All standard solutions should be stored in amber vials at or below -18ºC or according to the manufacturer's recommendations and brought to room temperature prior to use. Standard solutions are recommended for use prior to manufacturer's expiration.

Table 7-3: Example concentrations for [^{13}C]-IS mycotoxin stock standards[a]

Mycotoxin	Stock Standard (µg/mL)	Solvent
[$^{13}C_{17}$]-aflatoxin B_1 [$^{13}C_{17}$]-aflatoxin B_2 [$^{13}C_{17}$]-aflatoxin G_1 [$^{13}C_{17}$]-aflatoxin G_2	0.5	Acetonitrile
[$^{13}C_{20}$]-ochratoxin A	10	Acetonitrile
[$^{13}C_{34}$]-fumonisin B_1	25	50% acetonitrile: 50% water (v/v)
[$^{13}C_{34}$]-fumonisin B_2	10	50% acetonitrile: 50% water (v/v)
[$^{13}C_{34}$]- fumonisin B_3	10	50% acetonitrile: 50% water (v/v)
[$^{13}C_{15}$]-deoxynivalenol	25	Acetonitrile
[$^{13}C_{22}$]-HT-2 toxin	25	Acetonitrile
[$^{13}C_{24}$]-T-2 toxin	25	Acetonitrile
[$^{13}C_{18}$]-zearalenone	25	Acetonitrile

[a]Alternate concentrations for the preparation of stock standards are accepted

Prepare the following mixed [^{13}C]-IS working standard by mixing and diluting appropriate amounts of the stock standard(s) using the dilution solution, 50% acetonitrile: 50% water (v/v). Final volume of each working standard: 2000 µL (Table 7-4).

Table 7-4: Example concentrations for mixed [^{13}C]-IS mycotoxin working standard[a,b]

Mycotoxin	Stock Standard (µg/mL)	Volume Stock Standard (µL)[c]	Working Standard (µg/mL)
[$^{13}C_{17}$]-aflatoxin B_1 [$^{13}C_{17}$]-aflatoxin B_2 [$^{13}C_{17}$]-aflatoxin G_1 [$^{13}C_{17}$]-aflatoxin G_2	0.5	200.0	0.05
[$^{13}C_{20}$]-ochratoxin A	10	20.0	0.1
[$^{13}C_{34}$]-fumonisin B_1	25	160.0	2
[$^{13}C_{34}$]-fumonisin B_2	10	400.0	2
[$^{13}C_{34}$]- fumonisin B_3	10	400.0	2
[$^{13}C_{15}$]-deoxynivalenol	25	160.0	2
[$^{13}C_{22}$]-HT-2 toxin	25	160.0	2
[$^{13}C_{24}$]-T-2 toxin	25	160.0	2
[$^{13}C_{18}$]-zearalenone	25	160.0	2

[a] Alternate concentrations for the preparation of stock and working standards, including final volumes, are accepted

[b]Add dilution solution: 180.0 µL as 50% acetonitrile: 50% water (v/v)

[c]Dilutions may be prepared to final volume (2000 µL) in volumetric flask

2024.7.4. Preparation of calibration standards

Solvent-only calibration standards are prepared from dilutions of native working standard(s) and the mixed [^{13}C]-IS working standard. Solvent-only calibration standards are prepared in 50% acetonitrile: 50% water (v/v) and fortified with 20.0 µL of the mixed [^{13}C]-IS working standard. Final volume of each calibration standard: 1000 µL.

The original method validation evaluated a ten-point extended range calibration curve (Appendix II): AB1, AB2, AG1, AG2, and OTA (0.05 – 50 ng/mL); FB1, FB2, FB3, DON, HT-2, T-2, ZEA (0.5 – 500 ng/mL). For routine analysis, a five-point calibration curve is recommended (Table 7-5). Ensure calibration range meets program requirements for regulated mycotoxins and minimizes influence of low (sub-STD1) concentrations of carryover. Example: AB1, AB2, AG1, AG2, and OTA (0.25 – 25 ng/mL); FB1, FB2, FB3, DON, HT-2, T-2, ZEA (5 – 500 ng/mL). See Appendix III for preparation of individual calibration standards.

Table 7-5: Example concentrations for 5-point mycotoxin calibration curve[a,b]

Level	AB1, AB2, AG1, AG2 (ng/mL)	OTA (ng/mL)	FB1, FB2, FB3, DON, HT-2, T-2, ZEA (ng/mL)
STD1	0.25	0.25	5.0
STD2	1.0	1.0	25
STD3	2.5	2.5	50
STD4	10	10	250
STD5	25	25	500
[^{13}C]-IS[b]	1.0	2.0	40

[a]Dilution Solution: 50% Acetonitrile: 50% Water (v/v)
[b][^{13}C]-IS incorporated into all samples at fixed concentration

2024.7.5. Preparation of blanks

Solvent Blank: A solvent blank (50% acetonitrile: 50% water, v/v) is recommended prior to the start of batch and after high concentration calibration standard(s). See 2024.12.7 for alternate composition of solvent blank to mitigate carryover.

Method Blank: A method blank (50% acetonitrile: 50% water, v/v) is prepared and processed according to the method protocol. Extraction is performed concurrently with matrix samples and fortified with [^{13}C]-IS. A minimum of one method blank is recommended with every batch.

2024.7.6. Mobile Phase A: Water (10 mM ammonium formate, 0.1% formic acid)

Example:

(1) Using weigh paper and an analytical balance, weigh approximately 630.6 mg of ammonium formate (powder).
(2) Quantitatively transfer the ammonium formate to a 1-L volumetric flask.
(3) Add approximately 500 mL water. Swirl bottle slowly until the ammonium formate dissolves.
(4) Add 1.0 mL formic acid (LC/MS grade). Swirl bottle slowly to mix.
(5) Dilute to 1-L volume with water. Invert to mix. Transfer to bottle.

Alternatively,

(1) Add 1.0 mL ammonium formate (10 M) to a 1-L volumetric flask.
(2) Add approximately 500 mL water. Swirl bottle slowly.
(3) Add 1.0 mL formic acid (LC/MS grade). Swirl bottle slowly to mix.
(4) Dilute to 1-L volume with water. Invert to mix. Transfer to bottle.

2024.7.7. Mobile Phase B: Methanol (10 mM ammonium formate, 0.1% formic acid)

Example:

(1) Using weigh paper and an analytical balance, weigh approximately 630.6 mg of ammonium formate (powder).
(2) Quantitatively transfer the ammonium formate to a 1-L volumetric flask.
(3) Add methanol. Swirl bottle slowly until the ammonium formate dissolves.
(4) Add 1.0 mL formic acid (LC/MS grade). Swirl bottle slowly to mix.
(5) Dilute to 1-L volume with methanol. Invert to mix. Transfer to bottle.

Alternatively,

(1) Add 1.0 mL ammonium formate (10 M) to a 1-L volumetric flask.
(2) Add methanol. Swirl bottle slowly.
(3) Add 1.0 mL formic acid (LC/MS grade). Swirl bottle slowly to mix.
(4) Dilute to 1-L volume with methanol. Invert to mix. Transfer to bottle.

2024.8. Sample Preparation

2024.8.1. Sampling

Instructions on sampling may be found in the Mycotoxin Compliance Program Guidance Manual 7307.001, Part III or Investigations Operations Manual, Chapter 4, Sample Schedule Chart 6.

2024.8.2. Slurry procedure

A water-slurry procedure is used for food commodities (e.g., corn) that are difficult to homogenize using dry milling:

(1) Weigh 25.0 ± 0.5 g of a sample into a 40- or 100-mL disposable grinding chamber (IKA, NC, USA) and add 25.0 ± 0.5 mL of water. Record mass.
(2) Blend the sample and water for approximately 1.5 min at 25,000 rpm (nominal setting) using an IKA Tube Mill. Note: dispose grinding chamber after single use.
(3) Weigh a test portion (2.00 ± 0.05 g) from the blended sample into a 15 mL disposable screw-capped polypropylene centrifuge tube. Record mass. Calculate the adjusted sample weight without water (e.g., 2.00 g test portion × 25.0 g sample/50.0 g slurry).
(4) Fortify the test portion with 100.0 µL of the mixed [^{13}C]-IS working standard.

2024.8.2. Slurry procedure (continued)

Table 8-1: Example concentrations for [^{13}C]-IS: Slurry

Mycotoxin	**[^{13}C-IS] Concentration (ng/g)[a]**
[$^{13}C_{17}$]-aflatoxin B_1	5.0
[$^{13}C_{17}$]-aflatoxin B_2	5.0
[$^{13}C_{17}$]-aflatoxin G_1	5.0
[$^{13}C_{17}$]-aflatoxin G_2	5.0
[$^{13}C_{20}$]-ochratoxin A	10
[$^{13}C_{34}$]-fumonisin B_1	200
[$^{13}C_{34}$]-fumonisin B_2	200
[$^{13}C_{34}$]- fumonisin B_3	200
[$^{13}C_{15}$]-deoxynivalenol	200
[$^{13}C_{22}$]-HT-2 toxin	200
[$^{13}C_{24}$]-T-2 toxin	200
[$^{13}C_{18}$]-zearalenone	200

[a]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 approximately 30 seconds.
(6) Add 4.0 mL of extraction solvent (50% acetonitrile: 50% water, v/v) into the tube and cap tube securely. Invert or vortex to mix. Continue to Section 2024.8.4.

2024.8.3. Direct extraction

A direct extraction procedure is used for food commodities (e.g., infant rice cereal and peanut butter) that are generally considered homogenous and can be mixed using dry-milling:

(1) Weigh a test portion of homogenized sample (1.00 ± 0.05 g) into a 15 mL disposable screw-capped polypropylene centrifuge tube. Record mass.
(2) Fortify the test portion with 100.0 µL of the mixed [^{13}C]-IS working standard.

2024.8.3. Direct extraction (continued)

Table 8-2: Example concentrations for [^{13}C]-IS: Direct extraction

Mycotoxin	[^{13}C]-IS Concentration (ng/g)[a]
[$^{13}C_{17}$]-aflatoxin B_1	5.0
[$^{13}C_{17}$]-aflatoxin B_2	5.0
[$^{13}C_{17}$]-aflatoxin G_1	5.0
[$^{13}C_{17}$]-aflatoxin G_2	5.0
[$^{13}C_{20}$]-ochratoxin A	10
[$^{13}C_{34}$]-fumonisin B_1	200
[$^{13}C_{34}$]-fumonisin B_2	200
[$^{13}C_{34}$]- fumonisin B_3	200
[$^{13}C_{15}$]-deoxynivalenol	200
[$^{13}C_{22}$]-HT-2 toxin	200
[$^{13}C_{24}$]-T-2 toxin	200
[$^{13}C_{18}$]-zearalenone	200

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

(3) Re-cap the tube and vortex it for approximately 30 seconds.

(4) Add 5.0 mL of extraction solvent (50% acetonitrile: 50% water, v/v) into the tube and cap tube securely. Invert or vortex to mix. Continue to Section 2024.8.4.

2024.8.4. Sample extraction and cleanup

(1) Extract the samples prepared using a high-speed shaker with pulsation. If a Glas-Col shaker is used (Terre Haute, IN, USA), use a motor speed setting of 75 (1540−1560 rpm) and pulser frequency at 30−35 pulsations/min for approximately 30 minutes. If a Spex Geno/Grinder is used (Metuchen, NJ, USA), use a motor speed setting of 1500 rpm for approximately 3 minutes.

(2) Centrifuge sample for approximately 15 min at a relative centrifugal force of 4200g (approximate). Time and force parameters may be adjusted to accommodate different sample types.

(3) Filter the clarified sample supernatant through a 0.2 µm PTFE filter directly into an amber autosampler vial for LC-MS/MS analysis.

Alternatively, the clarified extract (~2.0 mL) can be transferred to an Amicon Ultra-4 centrifugal filter with Ultracel-3 membrane (molecular weight cutoff value of 3 kDa). Centrifuge for approximately 15 min at a relative centrifugal force of 4200g (approximate). Time and force parameters may be adjusted to accommodate different sample types. The resulting filtrate is transferred into autosampler vials for LC-MS/MS analysis.

2024.9. Quality Control Samples

2024.9.1. Matrix Spike (MS) and Matrix Spike Duplicate (MSD)

A matrix spike sample is prepared by fortifying the sample or a similar matrix-matched commodity with target mycotoxins at pre-defined concentrations. Fortifications are recommended at a final concentration that is above the method limit of quantitation (LOQ) and different from the unfortified concentration (e.g., 50-100%).

Spike recovery samples are prepared as matrix spike (MS) and matrix spike duplicate (MSD) samples and processed according to the method protocol. MS and MSD sample test portions are fortified with [^{13}C]-IS and native mycotoxin standards prior to processing. A non-fortified sample (i.e., matrix blank) is included and analyzed concurrently with the batch.

Table 9-1: Example native mycotoxin concentrations for spike recovery (direct extraction)[a,b]

Working Standard	Mycotoxin	Working Standard (µg/mL)	Volume Working Standard (µL)[c]	Spike Conc. (ng/g)[d]
A	aflatoxin B_1, B_2, G_1 G_2	1.0	10.0	10
	ochratoxin A	1.0		10
B	fumonisin B_1	10	10.0	100
	fumonisin B_2	10		100
	fumonisin B_3	10		100
C	deoxynivalenol	10	10.0	100
	HT-2 toxin	10		100
	T-2 toxin	10		100
	zearalenone	10		100

[a]Alternate concentrations for spike recovery preparation are accepted
[b]Assumption: 1.00 ± 0.05 g test portion
[c]Add extraction solution as 50% acetonitrile:50% water (v/v)
[d]Add mixed [^{13}C]-IS working solution: 100.0 µL

2024.9.2. Certified Reference Material (CRM)

A certified reference material may be used to demonstrate accuracy and precision in place of a MS and MSD for relevant matrices. The certified reference material sample will be processed in duplicate according to the method protocol.

Examples:

- National Institute of Standards and Technology
  SRM 2387: Aflatoxins in peanut butter
  SRM 1565: Multi-mycotoxins in corn
- 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
- FAPAS
  TET017RM: Mycotoxins in maize flour

2024.9.3. Method Blank

A method blank (5.0 mL) shall be prepared with every sample batch. The method blank (50% acetonitrile: 50% water, v/v) is processed according to the method protocol. The method blank is fortified with 100.0 µL of the mixed [^{13}C]-IS working standard.

2024.9.4. Initial Calibration Verification (ICV)

An initial calibration verification (ICV) is analyzed directly following the calibration standards. It is used to verify the calibration standards were prepared correctly. The ICV is prepared at a concentration approximately equivalent to the level 3 standard (STD3). The ICV standard is prepared from a second source (supplier or lot #). If a second source is not available, a second analyst may prepare the ICV from the purchased standard.

2024.9.5. Continuing Calibration Verification (CCV)

A continuing calibration verification (CCV) is recommended at least once for every ten injections (approximate), and at the conclusion of the injection sequence. The CCV is generally a reinjection of one of the calibration standards, and should be near the midpoint of the calibration curve (STD3). All samples will be bracketed by a passing CCV to ensure that the instrument was in calibration during the analytical run. The calibration check standard can be analyzed more often to evaluate instrument deviations.

2024.9.6. Batch Sequence

Samples analyzed concurrently are referred to as a batch.

(1) Solvent Blank
(2) Calibration Standards
(3) ICV
(4) Method Blank
(5) Sample(s), MS, MSD (order is not significant)
(6) CCV

Note: Solvent blank injections may be analyzed throughout a batch sequence to monitor carryover.

2024.10. Method of Analysis

The use of trade names in this method constitutes neither endorsement nor recommendation by the U. S. Food and Drug Administration (FDA). Citation of instrumentation is based on the original method validation and supporting method verification and platform extension studies.

A Shimadzu LC-30 system with a binary pump (LC-30AD), autosampler (SIL-30AC) and column oven (CTO-20AC) was used for method development and validation. A Sciex ExionLC and Shimadzu LC-40 have also been tested in method verification.

Mass spectrometric analysis is performed on a Sciex QTRAP® 4000, Sciex QTRAP® 6500/6500+, or Sciex Triple Quad 6500/6500+ platform in positive ionization mode. Source and compound dependent parameters are summarized below. MS/MS data are collected in a scheduled multiple reaction monitoring (MRM) mode using Analyst® or Sciex OS for instrument control (Sciex). Quantitative data analysis is performed using MultiQuant™ or Sciex OS Version 1.6 or higher (Sciex).

2024.10.1. Liquid Chromatography (LC)

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

Table 10-1: LC gradient timetable

Time (minutes)	Solvent B (%)	Flow (µL/minute)
0	5.0	300
2.0	40.0	300
10.0	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:[a]	200 µL
Rinse solvent:	50% Methanol
Needle stroke:[a]	52 mm
Rinsing speed:[a]	35 µL/s
Sampling speed:	5 µL/s
Rinse dip time:[a]	5 s
Controller temperature:	10 ºC
Column temperature:	40 ºC

[a]Autosamplers may have different parameters for sampling and rinsing. The settings listed are for a Shimadzu LC-30 system with SIL-30AC autosampler.

2024.10.2 Mass Spectrometry (MS)

Table 10-2: MS source parameters

Source Parameters	Sciex 4000	Sciex 6500/6500+
Collisionally Activated Dissociation (CAD) Gas	N_2, Medium (9)	N_2, Medium (9)
Curtain Gas	N_2 30 au	N_2 30 au
IonSpray Voltage	5000 Volts	5500 Volts
Interface Heater	ON	ON
TurboIon Source Temperature	400ºC	450ºC
Gas 1	50 au	60 au
Gas 2	50 au	60 au
Q1 Resolution	Unit	Unit
Q3 Resolution	Unit	Unit

Scheduled MRM transitions for target mycotoxins are listed in Table 10-3. Representative extracted ion chromatograms of target mycotoxins and [^{13}C]-IS displayed in Appendix IV.

*Note: Instruments of the same series from the same vendor may have performance differences which would require optimized source conditions or collision energies to meet the same performance specifications. Generally, these differences are small and therefore any changes should be minor adjustments to temperature, voltages and/or gas flows. Additionally, analysts should avoid making multiple changes that could have detrimental additive effects, such as reducing both temperature and gas flow. The new conditions should maintain (or improve) the calibration range of the method. (Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition, Appendix 6).[1]

Table 10-3: Scheduled MRM parameters for the target mycotoxins

Mycotoxins	**Molecular Formula**	**Molecular Weight**	**RT (min)**	**Ion**	**MRM transitions**	**6500/6500+ QTRAP/Triple Quadrupole Series**			**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	6.4	$[M+H]^+$	313.1→**241.0**/285.1	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	6.4	$[M+H]^+$	330.1→**255.1**/301.1	86/106	55/37	14/8	107/85	53/35	13/15
Aflatoxin B_2	$C_{17}H_{14}O_6$	314.1	6.2	$[M+H]^+$	315.1→**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	6.2	$[M+H]^+$	332.1→**303.1**/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	5.9	$[M+H]^+$	329.1→**243.1**/200.0	86/86	41/95	14/10	93/93	39/58	13/10
[$^{13}C_{17}$]-aflatoxin G_1	$^{13}C_{17}H_{12}O_7$	345.1	5.9	$[M+H]^+$	346.18→**257.1**/124.1	86/86	41/99	14/10	80/80	38/92	13.5/8
Aflatoxin G_2	$C_{17}H_{14}O_7$	330.1	5.6	$[M+H]^+$	331.1→**313.1**/245.1	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	5.6	$[M+H]^+$	348.1→**330.1**/259.1	111/111	36/49	18/20	88/100	36/42	18/13
Deoxynivalenol	$C_{15}H_{20}O_6$	296.1	3.9	$[M+H]^+$	297.1→**249.1**/231.1	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	3.9	$[M+H]^+$	312.2→**263.2**/245.1	71/61	17/21	44/22	65/64	17/20	20/13
Fumonisin B_1	$C_{34}H_{59}NO_{15}$	721.4	7.9	$[M+H]^+$	722.4→**352.3**/334.3	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	7.9	$[M+H]^+$	756.5→**374.4**/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	9.1	$[M+H]^+$	706.4→**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	9.1	$[M+H]^+$	740.5→**358.4**/340.4	106/106	55/59	10/20	125/92	51/55	20/26
Fumonisin B_3	$C_{34}H_{59}NO_{14}$	705.4	8.5	$[M+H]^+$	706.4→**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	8.5	$[M+H]^+$	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	8.4	$[M+H]^+$	404.1→**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	8.4	$[M+H]^+$	424.2→**250.0**/110.0	66/66	41/101	16/16	74/73	36/101	13/17
HT-2 toxin	$C_{22}H_{32}O_8$	424.2	7.3	$[M+NH_4]^+$	442.2→**215.1**/323.1	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	7.3	$[M+NH_4]^+$	464.3→**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	8.0	$[M+NH_4]^+$	484.3→**215.1**/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	8.0	$[M+NH_4]^+$	508.3→**229.2**/198.1	57/57	29/33	17/11	55/50	26/30	12/9
Zearalenone	$C_{18}H_{22}O_5$	318.1	8.5	$[M+H]^+$	319.2→**283.1**/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	8.5	$[M+H]^+$	337.2→**138.1**/124.1	71/81	79/87	10/10	45/35	70/77	10/21

[a]**Quantitation transition**

2024.11. Calculations

Quantitation is based on a linear least squares regression with 1/x weighting, plotting the peak area ratio (Native / [^{13}C]-IS) against mycotoxin concentration. In each sample, the calculated concentration of mycotoxin was determined from the equation:

$$C = \left[\frac{S - y\text{-}intercept}{slope} \times RF\right] \div m$$

Where *C* is the concentration in the sample (ng/g), *S* is the signal (peak area) of native mycotoxin/signal (peak area) of [^{13}C]-IS in the sample, and *m* (grams) is the mass of the sample. The *RF* defines the response factor or the total extraction volume added (mL) × dilution factor × [^{13}C]-IS concentration ratio ([^{13}C]-IS$_{\text{sample extract}}$/[^{13}C]-IS$_{\text{calibration standard}}$). The peak area ratio is calculated using the quantitation ions for each mycotoxin (native and [^{13}C]-IS) in accordance with Table 10-3. See Appendix V for step-by-step calculation.

2024.12. Method Performance and Quality Control Criteria

2024.12.1. Accuracy and precision

Method accuracy expressed as percent recovery, should be between 70-130% of the known value for acceptable performance. Precision expressed as relative standard deviation (RSD), should be ≤ 22%. These metrics are within the range of those reported for the matrices in the original validation[2] and consistent with recommendations in Appendix 2A of the Guidelines for the Validation of Chemical Methods for the FDA Foods Program.[1]

2024.12.2. Method limits of detection and quantitation

For trace analysis, the method detection limit (MDL) may be estimated following 40 CFR Part 136 Appendix B.[3] The LOQ for this approach may be subsequently calculated as LOQ = 3.33× MDL.

For regulatory analysis, reporting limits for mycotoxin quantitation were established at 2.5 ng/g AB1, AB2, AG1, AG2, OTA; 50 ng/g FB1, FB2, FB3, DON, HT-2, T-2, ZEA. These limits are above the lowest fortified concentration evaluated in the multi-laboratory validation[2] and below current FDA action/guidance/advisory levels for mycotoxins in food and feed.

At the limit of quantitation, all identification criteria shall be met for each mycotoxin target as defined in 2024.12.4.

2024.12.3. Measurement uncertainty

Measurement uncertainty was estimated using the formula:

$$U = k \times RSD$$

Where *U* is the expanded uncertainty, *k* is the coverage factor based on the level of confidence (e.g., t-value at 95% confidence interval, two-sided), and *RSD* is the relative standard deviation of reproducibility. For this analysis, the *RSD* was calculated from spike recovery data collected during the original multi-laboratory validation and column verification study. The uncertainty value was calculated for each mycotoxin and reported as 11-27% (range) or 17% (average) for the method.

2024.12.4. Specificity and selectivity

To be reportable, analyte concentration shall be within the operating range of the calibration curve and greater than or equal to the method's limit of quantitation. A summary of method identification criteria is below:

(1) Retention Time: Retention time shall match the comparison standard within ± 5%.[4]
(2) Ion Ratio: Relative abundances for two unique, structurally-specific ions shall coelute and match the comparison standard within ±10% absolute units.[4]
(3) All diagnostic ions shall have a signal-to-noise ratio greater than or equal to three.
(4) All mycotoxin analyses shall be conducted consistent with the requirements of the method and laboratory-defined quality measures.

2024.12.5. Linearity

Quantitation is based on a linear least squares regression with 1/x weighting, plotting the peak area ratio (Native / [^{13}C]-IS) against mycotoxin concentration. Linearity of all target mycotoxins within pre-defined calibration ranges shall be $r^2 \geq 0.990$. The dynamic linear range shall not exceed the dynamic linear range reported in the original validation without additional supporting evidence: 0.05-50 ng/mL for AB1, AB2 AG1, AG2, OTA and 0.5-500 ng/mL for FB1, FB2, FB3, DON, HT-2, T-2, ZEA. A sample shall be reprepared if reporting concentration values are above the validated quantitation range.

2024.12.6. Quality control specifications

(1) ICV should be within 80-120% of expected concentration.
(2) The CCV should be within 80-120% of expected concentration.
(3) Method Blank: Carryover mitigation is recommended in situations where a solvent blank injection exceeds 50% the peak area response of the lowest calibration standard (STD1).
(4) CRM: Analyte concentration values shall be within the uncertainty range for values provided in the certificate of analysis, and consistent with in-house statistical analysis/control charting.
(5) Spike Recovery: Method accuracy expressed as percent recovery for MS and MSD samples, should be between 70-130% of the known value for acceptable performance. Precision expressed as relative percent difference (RPD), should be ≤ 22%. Results for accuracy and precision values should be within acceptable limits for all analytes tested, unless otherwise specified (i.e., in-house statistical analysis/control charting).

(6) Check Analysis: The relative percentage difference (RPD) of the same analyte between the original and check analysis should be ≤ 44%.

(7) Failure of any quality control sample to meet method performance measures for one or more analytes requires the laboratory to investigate the cause(s) in accordance with its non-conformance investigation procedure.

2024.12.7. Troubleshooting: Carryover

(1) Characterize System:
   a. Analyze solvent and method blanks using selected reagents and instrumentation. Calculate percent carryover for each mycotoxin.
   b. Systematically identify, isolate, and mitigate potential sources of carryover and contamination prior to (and periodically throughout) analysis of samples.

(2) Persistent Carryover:
   a. Carryover mitigation is recommended in situations where a solvent blank injection exceeds 50% the peak area response of the lowest calibration standard (STD1).
   b. Solvent blanks or method blanks may be analyzed throughout a run to monitor contamination and carryover. For example, blanks may be added at the beginning of the batch, following the highest calibration standard, and/or interposed between subject samples(s).
   c. In situations of persistent carryover, mitigation strategies may include modification of solvent blank composition (e.g., 1% formic acid in water, isopropanol, acetonitrile, methanol [1+1+1+1 v/v]), autosampler washing procedures, and/or column flushing (e.g., saw-tooth gradient) at the end of sample gradient and prior to equilibration for the next injection.
   d. Discuss additional maintenance steps for remediation (based on isolated source of system carryover) with local service engineer.
   e. If carryover mitigation remains unresolved, recommend samples with concentrations of a single mycotoxin equivalent to 50% the guidance level (e.g., 2 µg/g fumonisins in human food), be reinjected preceded by at least one solvent blank, after completion of the batch. For check analyses, at least one solvent blank is recommended to be interposed between subject sample(s).

2024.13. Instrument Verification

Instruments must achieve the following performance criteria prior to implementation of the method for routine analysis. For initial method implementation, instrument performance may be evaluated concurrently with a method verification study.

(1) Prepare a matrix spike sample, in triplicate, at the established method reporting limit for each mycotoxin.
(2) Analyze the samples according to the method protocol.
(3) Confirm identification for each mycotoxin as defined in 2024.12.4.

2024.14. Method Validation, Verification, and Extension

2024.14.1. Multi-laboratory validation

The method specified in this document meets a Level 3 validation under Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition.[1] The method was validated for 12 mycotoxins in corn, peanut butter, and wheat flour at four fortification levels (5, 10, 25, 100 ng/g AB1, AB2, AG1, AG2, OTA; 50, 100, 250, 1000 ng/g FB1, FB2, FB3, DON, HT-2, T-2, ZEA), and prepared by each laboratory in quadruplicate. Matrix blanks were analyzed concurrently with fortified samples. The method demonstrated recoveries ranging between 80 – 120% for the majority of samples 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 (between laboratory) < 15%. Three certified reference materials, BCR-385R (peanut butter), BCRBC600 (wheat flour), and TET002 (corn) were also evaluated with Z scores between -2 and 2. More details regarding the method validation study can be found in Zhang et al., 2017.[2]

2024.14.2. Method verification

Method verification is recommended for laboratories performing a method that has been previously validated elsewhere. The minimum criteria for method verification are defined in the Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition, Appendix 5.[1] A successful method verification assumes all method parameters are to be performed in accordance with C-003 and all recoveries are within the range of those reported in the original validation.

2024.14.2.1. Column verification study

A column verification study was performed to evaluate column performance for all mycotoxins in four validated matrices (corn, peanut butter, rice, wheat flour) using different column lots of the Agilent InfinityLab Poroshell 120 Stablebond-C18 guard and analytical column. Method performance metrics were determined for two sample matrices at two fortification levels, performed in triplicate by four participating laboratories. Matrix blanks were analyzed concurrently with fortified samples. Across all laboratories, average recoveries ranged from 85–122% for all mycotoxins with relative standard deviations between 2–20% RSD_R (Table 12-1). Recoveries were determined to meet method acceptability criteria and are consistent with the multi-laboratory validated method. Based on these data, Compendial Laboratory Method C-003 was updated to replace original column chemistry with Agilent Poroshell 120 Stablebond C18 column.

2024.14.2.1 Column verification study (continued)

Table 14-1: Column verification study: Average recovery (%) and reproducibility (%RSD_R) in corn, peanut butter, rice, and wheat flour for each participating laboratory (n=6 for each concentration in each matrix)

Mycotoxin	Fortified Concentration (ng/g)	Corn %Recovery (%RSD_R)	Peanut Butter %Recovery (%RSD_R)	Rice %Recovery (%RSD_R)	Wheat Flour %Recovery (%RSD_R)
AB1	10	105 (3)	108 (4)	102 (10)	105 (6)
	100	101 (8)	96 (4)	102 (15)	101 (7)
AB2	10	108 (6)	113 (3)	110 (6)	111 (8)
	100	104 (8)	108 (7)	108 (8)	107 (4)
AG1	10	103 (6)	115 (4)	103 (7)	97 (6)
	100	97 (10)	109 (6)	105 (12)	95 (11)
AG2	10	100 (6)	105 (13)	104 (5)	104 (6)
	100	94 (9)	99 (7)	106 (7)	101 (4)
OTA	10	103 (6)	97 (4)	109 (16)	104 (7)
	100	100 (8)	96 (7)	108 (16)	105 (7)
FB1	200	102 (7)	99 (4)	86 (14)	96 (18)
	1000	99 (9)	99 (3)	91 (10)	93 (7)
FB2	200	100 (9)	95 (4)	89 (15)	91 (20)
	1000	97 (9)	95 (8)	91 (10)	90 (13)
FB3	200	96 (4)	102 (7)	93 (17)	88 (10)
	1000	95 (8)	99 (3)	91 (11)	88 (7)
DON	200	101 (4)	122 (9)	96 (3)	100 (8)
	1000	94 (6)	102 (4)	105 (8)	99 (8)
HT-2	200	100 (4)	104 (7)	93 (8)	101 (10)
	1000	95 (11)	104 (3)	96 (4)	98 (11)
T-2	200	102 (2)	96 (9)	85 (3)	97 (8)
	1000	96 (6)	97 (5)	94 (5)	96 (4)
ZEA	200	106 (6)	97 (7)	95 (3)	103 (9)
	1000	100 (7)	97 (5)	104 (12)	102 (3)

2024.14.3. Method extension

The minimum criteria for method extensions are defined in the Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition, Appendix 5.[1]

2024.14.3.1. Matrix extension

For commodities that are not closely grouped (i.e., carbohydrate, fat, moisture content, homogeneity) with a validated matrix or do not exhibit similar sample handling and performance, a matrix extension may be warranted. A successful matrix extension assumes all method parameters are to be performed in accordance with C-003.

Compendial Laboratory Method C-003 was originally validated in corn, peanut butter, and wheat flour (2016) and extended to rice in 2020. A summary of completed matrix extensions, to date, is below.

2024.14.3.1. Matrix extension (continued)

2020: Rice and rice products
A matrix extension was performed in polished white rice, whole grain brown rice, and rice cereal. Method performance metrics were determined for each sample matrix at one fortification level (40 ng/g for AB1, AB2, AG1, AG2, OTA; 400 ng/g for FB1, FB2, FB3, DON, HT-2, T-2, ZEA), performed in triplicate. Matrix blanks were analyzed concurrently with fortified samples. Average recoveries ranged from 81–96% for all mycotoxins with relative standard deviations between 1–9%. The matrix extension for rice successfully met the requirements of the mycotoxin program including all relevant regulatory limits and technical criteria for acceptance.

2024.14.3.2. Platform extension
For new platforms (not listed in Section 2024.10) that apply the same technique as used in the initially validated quantitative method, but retain the same parameters (e.g., transition ions) of the original method, a platform extension is recommended. Expanding the use of a validated method to include another significantly different instrument or platform requires further validation.

2024.15. Safety Considerations

Use appropriate personal protective equipment including safety glasses, gloves, and lab coats when handling concentrated solutions containing mycotoxins. Analysts shall be familiar with their laboratory's chemical hygiene, safety plan, and Safety Data Sheets (SDS) for all reagents and standards listed. Refer to the instrument manuals for safety precautions regarding use. All wastes generated must be handled appropriately.

Strict precautions need to be taken when working with mycotoxin standards. Standards should only be handled in clean, uncluttered hoods or bench spaces. 42 CFR Part 73 is recommended as best practice guidance for handling and disposing native T-2 toxin. Standard preparations should be performed in a separate space than sample extractions.

2024.16 Source and Purity

(1) Purity or concentration of mycotoxin standards are established using the manufacturer's COA.

(2) For accredited laboratories, standards shall be obtained from ISO Guide 34 compliant or ISO 17034 accredited producers when available, with a COA including concentration (with uncertainty), traceability, purity, storage conditions, expiration, and evidence of ISO Guide 34 compliance or ISO 17034 accreditation. If no source meeting these requirements can be identified, records of the search are retained by the laboratory.

(3) A stock standard is the first solvation/dilution of the primary standard. All dilutions of the stock standard are working standards and may be referenced as intermediate standards, LC standards, calibration standards, etc.

(4) Standards that exceed the duration of use are required to be properly discarded. Destruction of mycotoxin standards shall be done within the laboratory using known effective methods or methods advised by the SDS.

2024.17. Definitions

(1) Percent recovery is defined for each mycotoxin as the measured concentration (fortified-unfortified sample) divided by the fortified concentration and multiplied by 100.

(2) Ion Ratio: Peak Area of Confirmation Ion/Peak Area of Quantitation Ion] × 100.

(3) Percent carryover is defined as the peak area of an analyte in a blank divided by the peak area of the corresponding analyte in a solvent standard multiplied by 100.

2024.18. References

(1) Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition, 2019; https://www.fda.gov/media/81810/download.

(2) 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.

(3) 40 CFR Appendix B to Part 136 – Definition and Procedure for the Determination of the Method Detection Limit, Revision 2, 2016; https://www.epa.gov/sites/default/files/2016-12/documents/mdl-procedure_rev2_12-13-2016.pdf.

(4) Center for Veterinary Medicine Guidance for Industry #118: Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues, 2003; https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cvm-gfi-118-mass-spectrometry-confirmation-identity-animal-drug-residues.

Appendix I. Example concentrations for mixed native mycotoxin working standard

Mycotoxin	Stock Standard (μg/mL)	Volume Stock Standard (μL)	Working Standard (μg/mL)
aflatoxin B_1 aflatoxin B_2 aflatoxin G_1 aflatoxin G_2	2.0	100.0	0.1
ochratoxin A	10	20.0	0.1
fumonisin B_1	50	80.0	2.0
fumonisin B_2	50	80.0	2.0
fumonisin B_3	50	80.0	2.0
deoxynivalenol	100	40.0	2.0
HT-2 toxin	100	40.0	2.0
T-2 toxin	100	40.0	2.0
zearalenone	100	40.0	2.0

[a]Add dilution solution: 1480.0 μL as 50% acetonitrile: 50% water (v/v)

Appendix II. Example of solvent-only calibration standards (10-point calibration curve)

Mycotoxin/^{13}C-IS	Level 1 (ng/mL)	Level 2 (ng/mL)	Level 3 (ng/mL)	Level 4 (ng/mL)	Level 5 (ng/mL)	Level 6 (ng/mL)	Level 7 (ng/mL)	Level 8 (ng/mL)	Level 9 (ng/mL)	Level 10 (ng/mL)
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. Example of solvent-only calibration standards (5-point calibration curve)
Solvent-only calibration standards are prepared from dilutions of stock standard(s) A, B, and C and the [^{13}C]-IS working standard. Solvent-only calibration standards are prepared in 50% acetonitrile: 50% water (v/v) and fortified with 20.0 µL of the mixed [^{13}C]-IS working standard. Final volume of each calibration standard: 1000 µL.

STD5[a,b]:

Working Standard	Mycotoxins	Working Standard (µg/mL)	Volume Working Standard (µL)	Calibration Standard (ng/mL)
Native A	aflatoxin B_1, B_2, G_1 G_2	1	25.0	25
	ochratoxin A	1		25
Native B	fumonisin B_1	10	50.0	500
	fumonisin B_2	10		500
	fumonisin B_3	10		500
Native C	deoxynivalenol	10	50.0	500
	HT-2 toxin	10		500
	T-2 toxin	10		500
	zearalenone	10		500

[a]Add dilution solution: 855.0 µL as 50% acetonitrile: 50% water (v/v)
[b]Add mixed [^{13}C]-IS working solution: 20.0 µL

Duplicate STD5 (without [^{13}C-IS][a]:

Working Standard.	Mycotoxins	Working Standard (µg/mL)	Volume Working Standard (µL)	Calibration Standard (ng/mL)
Native A	aflatoxin B_1, B_2, G_1 G_2	1	25.0	25
	ochratoxin A	1		25
Native B	fumonisin B_1	10	50.0	500
	fumonisin B_2	10		500
	fumonisin B_3	10		500
Native C	deoxynivalenol	10	50.0	500
	HT-2 toxin	10		500
	T-2 toxin	10		500
	zearalenone	10		500

[a]Add dilution solution: 875.0 µL as 50% acetonitrile: 50% water (v/v)

Appendix III. Example of solvent-only calibration standards (5-point calibration curve) (continued)

STD4[a,b]:

Working Standard	Mycotoxins	Working Standard (µg/mL)	Volume Working Standard (µL)	Calibration Standard (ng/mL)
Native A	aflatoxin B_1, B_2, G_1 G_2	1	10.0	10
	ochratoxin A	1		10
Native B	fumonisin B_1	10	25.0	250
	fumonisin B_2	10		250
	fumonisin B_3	10		250
Native C	deoxynivalenol	10	25.0	250
	HT-2 toxin	10		250
	T-2 toxin	10		250
	zearalenone	10		250

[a]Add dilution solution: 920.0 µL as 50% acetonitrile: 50% water (v/v)
[b]Add mixed [^{13}C]-IS working solution: 20.0 µL

STD3[a,b]:

Working Standard	Mycotoxins	Working Standard (µg/mL)	Volume Working Standard (µL)	Calibration Standard (ng/mL)
Native A	aflatoxin B_1, B_2, G_1 G_2	1	2.5	2.5
	ochratoxin A	1		2.5
Native B	fumonisin B_1	10	5.0	50
	fumonisin B_2	10		50
	fumonisin B_3	10		50
Native C	deoxynivalenol	10	5.0	50
	HT-2 toxin	10		50
	T-2 toxin	10		50
	zearalenone	10		50

[a]Add dilution solution: 967.5 µL as 50% acetonitrile: 50% water (v/v)
[b]Add mixed [^{13}C]-IS working solution: 20.0 µL

Appendix III. Example of solvent-only calibration standards (5-point calibration curve) (continued)

STD2[a,b]:

Working Standard	Mycotoxins	Duplicate STD5 (µg/mL)	Volume Working Standard (µL)	Calibration Standard (ng/mL)
Native A	aflatoxin B_1, B_2, G_1 G_2	25	40.0	1.0
	ochratoxin A	25		1.0
Native B	fumonisin B_1	500	50.0	25
	fumonisin B_2	500		25
	fumonisin B_3	500		25
Native C	deoxynivalenol	500	50.0	25
	HT-2 toxin	500		25
	T-2 toxin	500		25
	zearalenone	500		25

[a]Add dilution solution: 840.0 µL as 50% acetonitrile: 50% water (v/v)
[b]Add mixed [^{13}C]-IS working solution: 20.0 µL

STD1[a,b]:

Working Standard	Mycotoxins	Duplicate STD5 (ng/mL)	Volume Working Standard (µL)	Calibration Standard (ng/mL)
Native A	aflatoxin B_1, B_2, G_1 G_2	25	10.0	0.25
	ochratoxin A	25		0.25
Native B	fumonisin B_1	500	10.0	5
	fumonisin B_2	500		5
	fumonisin B_3	500		5
Native C	deoxynivalenol	500	10.0	5
	HT-2 toxin	500		5
	T-2 toxin	500		5
	zearalenone	500		5

[a]Add dilution solution: 950.0 µL as 50% acetonitrile: 50% water (v/v)
[b]Add mixed [^{13}C]-IS working solution: 20.0 µL

Appendix III. Example of solvent-only calibration standards (5-point calibration curve) (continued)

Alternate preparation: Example of solvent-only calibration standards (5-point calibration curve) using mixed mycotoxin native standard working standard[a]

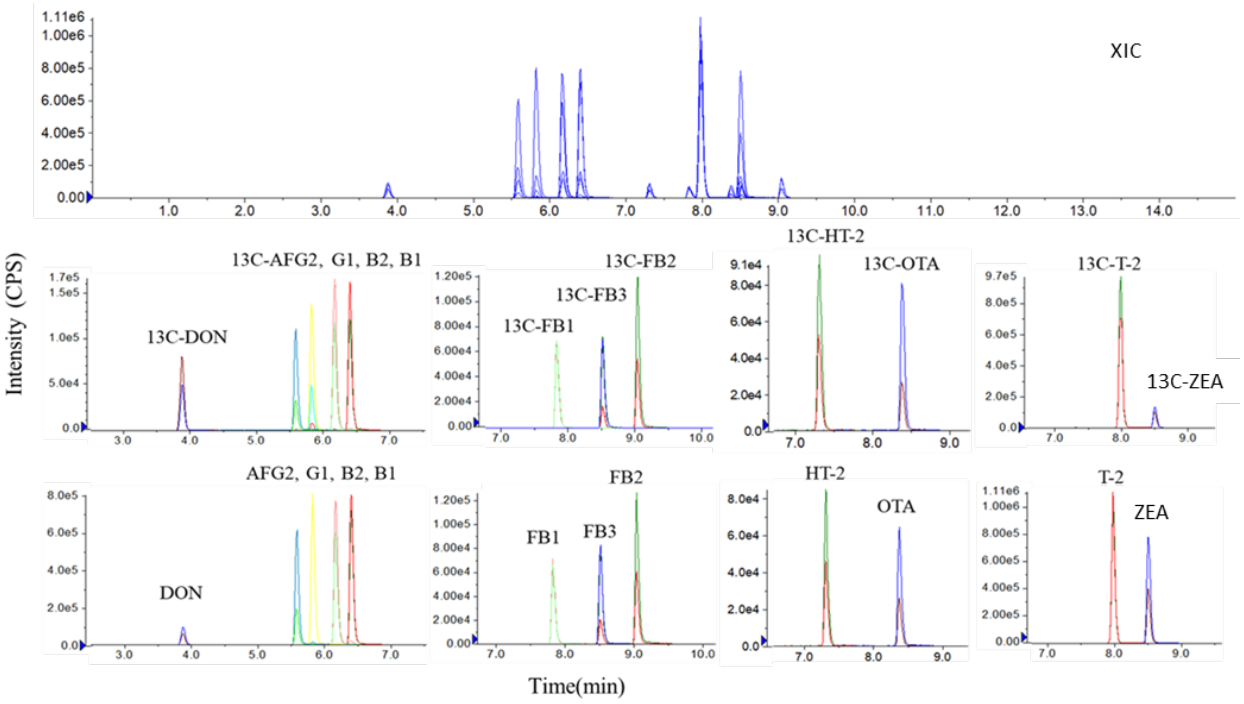
Level	AB1, AB2, AG1, AG2 (ng/mL)	OTA (ng/mL)	FB1, FB2, FB3, DON, HT-2, T-2, ZEA (ng/mL)
STD1	0.25	0.25	5.0
STD2	1.0	1.0	20
STD3	2.5	2.5	50
STD4	10	10	200
STD5	25	25	500
[^{13}C]-IS[b]	1.0	2.0	40

[a]Dilution Solution: 50% Acetonitrile: 50% Water (v/v)
[b][^{13}C]-IS incorporated into all samples at fixed concentration

Standard	Volume Mixed Native Working Standard (µL)	Volume Mixed [^{13}C]-IS Working Standard (µL)	Volume Dilution Solution (µL)[b]
STD 1	2.5	20.0	977.5
STD 2	10.0	20.0	970.0
STD 3	25.0	20.0	955.0
STD 4	100.0	20.0	880.0
STD 5	250.0	20.0	730.0

[a]Mixed mycotoxin native working standard prepared according to Appendix I
[b]Dilution solution: 50% acetonitrile: 50% water (v/v)

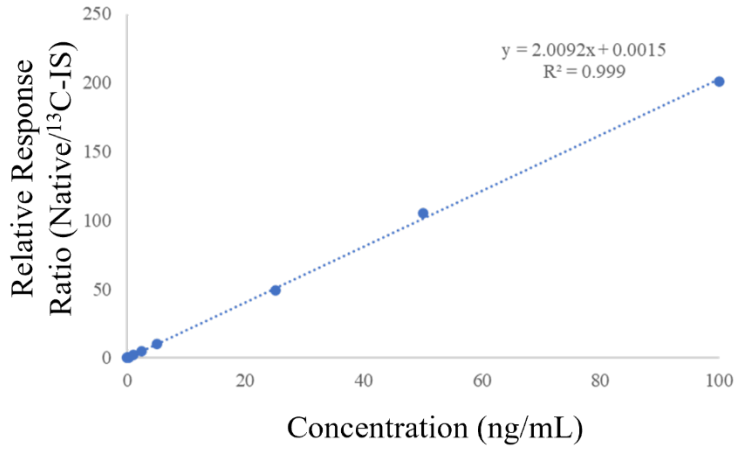
Appendix IV: Extracted ion chromatogram (XIC) of native and [^{13}C]-IS mycotoxin standards generated using conditions listed in Section 2024.10



Appendix V. Example Calculation

Step 1: Establish calibration curve for a target mycotoxin with 1/x weighting (e.g., AB1). Calibration curves are not fit through the origin

Concentration (ng/mL)	**AB1 Area**	**[^{13}C]-IS Area**	**AB1/[^{13}C]-IS 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: Calculate mycotoxin concentration in test sample.

Calibration Curve: S = 2.00925x + 0.0015
Sample 1: x = (1.04E+00-0.0015)/2.0092
x = 0.517 ng/mL
Sample 2: x = (1.99E+01-0.0015)/2.0092
x = 9.904 ng/mL

Where *x* is the concentration in the sample (ng/mL) and *S* is the signal (peak area) of native mycotoxin/signal (peak area) of [^{13}C]-IS in the sample.

Appendix V. Example Calculation (continued)

Concentration:

Sample 1: $C = (0.517\ ng/mL \times RF)/m$

$RF = 5\ mL \times 1 \times (1\ ng/mL / 1\ ng/mL)$

$m = 1.0\ g$

$C = (0.517\ ng/mL \times 5\ mL)/1.0\ g$

$C = 2.59\ ng/g$

Sample 2: $C = (9.904\ ng/mL \times RF)/m$

$RF = 5\ mL \times 1 \times (1\ ng/mL / 1\ ng/mL)$

$m = 1.0\ g$

$C = (9.904\ ng/mL \times 5\ mL)/1.0\ g$

$C = 49.5\ ng/g$

Where C is the concentration in the sample (ng/g), m (grams) is the mass of the sample, and RF defines the response factor or the total extraction volume added (mL) × dilution factor × [^{13}C]-IS concentration ratio ([^{13}C]-$IS_{sample\ extract}$/[^{13}C]-$IS_{calibration\ standard}$).