



**U.S. FOOD & DRUG  
ADMINISTRATION**

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**METHOD TITLE:** An LC-MS/MS Method for the Determination of Antibiotic Residues in Distillers Grains

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**METHOD SUMMARY/SCOPE:**

**Analyte(s):** Erythromycin A, Penicillin G, Virginiamycin M1 & Virginiamycin S1

**Matrices:** Dry Distillers Grains with Solubles

**REVISION HISTORY:**

**OTHER NOTES:**

# Title: An LC-MS/MS Method for the Determination of Antibiotic Residues in Distillers Grains

Version 2020

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## 2020.1 METHOD TITLE: An LC-MS/MS Method for the Determination of Antibiotic Residues in Distillers Grains

### 2020.2 SCOPE OF APPLICATION

Confirmation and determination of residues of erythromycin A, penicillin G, virginiamycin M1 and virginiamycin S1 (Figure 1.) in distillers grains (DG) in the 0.005 – 1.2 µg/g range. This method is applicable to regular and reduced-oil distillers dry grains with solubles. DG may originate from corn alone, or a mix of corn and milo.

In this study, an LC-MS/MS method was developed as requested by the CVM\Office of surveillance & Compliance to determine residues of antibiotics, erythromycin A, penicillin G, and virginiamycin M1 and virginiamycin S1, in distillers grains. The single-laboratory validated method subsequently underwent multi-laboratory validation. The lower and upper levels of interest were, respectively, 0.05 µg/g & 0.5 µg/g for erythromycin A, 0.01 µg/g & 1.00 µg/g for penicillin G, and 0.10 µg/g & 0.50 µg/g for virginiamycins.

The method was developed to support the determination of the above four drugs in DG at levels that may confer antibiotic resistance in bacterial pathogens, enabling the FDA to ensure that DG does not serve as a vector for antimicrobial resistance through animal feed.

## 2020.3 PRINCIPLE

DG is extracted with a mixture of acetonitrile and buffer followed by acetonitrile. The combined extract is diluted with water and washed with hexane. An aliquot is cleaned up on an Oasis HLB solid phase extraction (SPE) cartridge. The eluent is concentrated and reconstituted with water and acetonitrile. After centrifugation the supernatant is analyzed by reverse phase liquid chromatography. Detection is carried out by electrospray ionization and tandem mass spectrometry on a triple quadrupole mass spectrometer. Quantification is performed using extracted matrix curve with isotopically labeled internal standards.

## 2020.4 REAGENTS

Acetonitrile, HPLC grade – Burdick & Jackson, or equivalent.

Sodium acetate trihydrate, 99.5% – Acros, or equivalent.

Glacial acetic acid, 99.9% – Fisher Scientific, or equivalent.

Milli-Q Water – Processed with the Milli-Q system to give resistivity > 18 MΩ•cm (Millipore). Use for all subsequent references to water.

Hexane, HPLC grade – Fisher Scientific, or equivalent.

Ethanol, 99.9% – Sigma Aldrich, or equivalent.

Formic acid, 95% – Sigma Aldrich, or equivalent.

### 2020.4.1 Preparation of mobile phases

- (1) Formic acid in water (0.1%): With a micropipette, add 1 mL formic acid to a 1000 mL volumetric flask. Dilute to a final volume of 1000 mL with water and mix well. Store at room temperature. Expires in 6 months.
- (2) Formic acid in acetonitrile (0.1%): With a micropipette, add 1 mL formic acid to a 1000 mL volumetric flask. Dilute to a final volume of 1000 mL with acetonitrile and mix well. Store at room temperature. Expires in 6 months.

### 2020.4.2 Acetate buffer (1M, pH 5.0 ± 0.1):

Dissolve 53.2 g of sodium acetate trihydrate and 10.9 g (10.4 mL) glacial acetic acid in ~200 mL water in a 500 mL volumetric flask. Make up to volume with water. Check pH with pH meter, and adjust if necessary, by adding 1N sodium hydroxide or acetic acid. Store under refrigeration. Expires in 3 months.

### 2020.4.3 5% Ethanol in water (v/v) (SPE wash)

With a micropipette, add 2.5 mL of ethanol to a 50 mL volumetric flask. Dilute to volume with water. Mix well. Store in a tightly capped container. Store at room temperature.

#### 2020.4.4 50:50 Ethanol:ACN (v/v) (SPE elution solvent)

Combine 100 mL of ethanol and 100 mL of Acetonitrile. Mix well. Store in a tightly capped container. Store at room temperature.

#### 2020.4.5 80:20 Water: ACN (v/v) (Extraction diluent & solvent blank)

Combine 80 mL of water and 20 mL of Acetonitrile. Store in a tightly capped container. Store at room temperature.

### 2020.5 STANDARDS

#### 2020.5.1 Preparation of 100 µg/mL stock standards:

Erythromycin A dihydrate, CAS 59319-72-1 (Sigma-Aldrich Inc., St. Louis, MO, Cat. No. 46256), 95.4% purity, or equivalent.

Penicillin G potassium salt, CAS 113-98-41 (Sigma-Aldrich Inc., St. Louis, MO, Cat. No. 46609), 99% purity, or equivalent.

Virginiamycin M1, CAS 21411-53-0 (Sigma-Aldrich Inc. St. Louis, MO, Cat. No. V2753), 97% purity, or equivalent.

Virginiamycin S1, CAS 23152-29-6 (Sigma-Aldrich Inc., St. Louis, MO, Cat. No. V4140), 99% purity, or equivalent.

Weigh approximately 10 mg of each standard individually and transfer to 100 mL volumetric flasks and fill to volume with the appropriate solvent: Water for penicillin G potassium and ACN for the rest. Calculate concentration of each standard, correcting for the purity and salt content. Aliquot into cryovials and store at -80°C. Stability period: one year under specified storage conditions.

Example calculation for penicillin G potassium salt of 99% purity:

$$\begin{aligned} \text{Weight of salt} &= W \text{ mg} \\ \text{Net weight of penicillin G potassium} &= (W \times 0.99) \text{ mg} \\ \text{Net weight of penicillin G, mg} &= \frac{(W \times 0.99) \times \text{MW of penicillin G}}{\text{MW of salt}} \\ &= W_{\text{corrected}}, \text{ mg} \\ \text{Concentration} &= \frac{W_{\text{corrected}} \times 1000}{100} \mu\text{g/mL} \end{aligned}$$

#### 2020.5.2 Preparation of 25 µg/mL mixed standard from 100 µg/mL stock standards:

Transfer a volume equivalent to 500 µg of analyte from each stock solution to a 20 mL volumetric flask and fill to volume with 20:80 ACN: Milli-Q water. Store at -80°C. Expires in one year.

#### 2020.5.3 Preparation of fortification standards using 25 µg/mL mixed standard:

As given in table 1, add the given volume of the appropriate standard to a 25 mL volumetric flask and make up to volume with 20:80 ACN: Milli-Q water. Aliquot and store at -80°C. Do not use more than three freeze-thaw cycles. Expires in six months.

Table 1. Standard dilutions

Standard to use for dilution		Prepared fortification standard	
Concentration (µg/mL)	Volume to Dilute (mL)	Concentration (µg/mL)	ID
25.0	12.00	12.0	F6
12.0	8.33	4.0	F5
4.0	6.25	1.0	F4
1.0	6.25	0.25	F3
0.25	10.00	0.1	F2
0.1	12.50	0.05	F1

#### 2020.5.4 Preparation of 100 µg/mL individual stock internal standards:

Benzyl penicillinate-D7, potassium salt, (Toronto Research Chemicals, Inc., Toronto, ON, Cat. No. B288600), 95% purity, , or equivalent.

Erythromycin – (N-methyl-<sup>13</sup>C,<sub>3</sub>), (Sigma-Aldrich Inc. St. Louis, MO, Cat. No. 663506), 98% purity, or equivalent.

Weigh accurately about 2 mg of each internal standard individually and dissolve in 20 mL volumetric flasks using ACN for erythromycin-(N-methyl-<sup>13</sup>C, d<sub>3</sub>) and 50:50 ACN: Milli-Q water for penicillin G-D<sub>7</sub>. Aliquot and store at -80°C. Expires in one year.

#### 2020.5.5 Preparation of mixed internal standard (2.5 µg/mL penicillin G-D<sub>7</sub> and erythromycin-(N-methyl-<sup>13</sup>C, d<sub>3</sub>) from 100 µg/mL stock internal standard:

Transfer 500 µL each of Penicillin G-D<sub>7</sub> and erythromycin-(N-methyl-<sup>13</sup>C, d<sub>3</sub>) to a 20 mL volumetric flask and fill with 20:80 ACN: Milli-Q water. Aliquot and store at -80°C. Expires in six months.

### 2020.6 PREPARATION OF LABORATORY SAMPLE FOR ANALYSIS

Transfer bulk sample to a big container (e.g. bucket) and mix thoroughly. Homogenize ~500 g sample using a food processor or grain grinder (analogous to coffee grinder). Sieve using 1.18 mm pore size. Save the grain that does not fit through the sieve and regrind. Repeat the process up to three times.

### 2020.7 APPARATUS/INSTRUMENTATION

- (1) Liquid chromatograph – Shimadzu LC-20AD Prominence binary pump and autosampler with refrigerated sample tray, or equivalent.
- (2) Mass spectrometer – AB Sciex API 4000 equipped with Turbo V source with electro-spray ionization. Analyst 1.6.2, or equivalent.

- (3) Liquid chromatograph column—Agilent Poroshell 120 EC-C18, 2.1 x 100 mm, 2.7 $\mu$  column (Part # 695575-902); Poroshell 120 EC-C18, 2.1 x 5 mm, 2.7 $\mu$  guard column (Part #821725-911).
- (4) Polypropylene Centrifuge tubes—50 mL (Part # 352070 Falcon), 15 mL (Part # 352096 Falcon), or equivalent.
- (5) SPE cartridges – Oasis HLB, 60 mg/3 mL (Part # WAT094226, Waters Corporation).
- (6) 10 mL SPE reservoirs (Part # RFV0010P, UCT), or equivalent.
- (7) SPE vacuum adapter- (Part # 57020U, Sigma Aldrich), or equivalent.
- (8) Volumetric flasks, class A, glass, assorted volumes.
- (9) Calibrated micropipettes, Eppendorf Xplorer 0.5–10  $\mu$ L, Eppendorf 10-100  $\mu$ L, Eppendorf Research 20–200  $\mu$ L, 100–1000  $\mu$ L, and 500–5000  $\mu$ L, or equivalent.
- (10)Eppendorf Repeater<sup>®</sup> M4 pipette (Part # 4982000322), or equivalent (optional).
- (11)Eppendorf Combitips (Part # 0030089480) for the Repeater<sup>®</sup> M4 pipette, 50 mL, or equivalent.
- (12)Pasteur pipets, 9 in.
- (13)Microcentrifuge tubes, 2.0 mL (Part # 02-681-284, Fisher), or equivalent.
- (14)Glass autosampler vials with insertions, 1.5 mL (Part # 50-014-52371, Fisher Scientific), or equivalent. Vials without insertions (Part # 03-246-324, Fisher Scientific) may be used for solvent blanks.
- (15)Vacuum manifold for SPE (Visiprep 24), or equivalent.
- (16)Centrifuge— IEC Centra GP8R, with 218A rotor (International Equipment Co.), or equivalent.
- (17)Refrigerated microcentrifuge: Eppendorf Microcentrifuge 5417R, with F45-30-11 rotor, or equivalent.
- (18)Vortexers –Vortex-Genie 2 (Scientific Industries); Mini vortexer – VWR Scientific Products, or equivalent.
- (19)pH meter – Fisher Scientific Accumet BASIC, or equivalent.
- (20)Horizontal shaker – Eberbach 6010, or Reciprocating Shaker HS 501 digital (IKA Works), or equivalent.
- (21)Sonicator – Branson 3800, or equivalent.
- (22)Food processor – Robot Coupe RSI 2Y1, or equivalent.
- (23)Grain grinder – IKA A11 Basic, or equivalent.
- (24)Cryovials – Simport Cryovial T309 assorted sizes, or equivalent.
- (25)Freezer -80°C (or -20°C for short-term storage).

## 2020.8 METHOD

### 2020.8.1 Extraction

Critical point: Carry out the complete extraction procedure on the same day.

- (1) Weigh  $5.0 \pm 0.1$  g ground DG into individual 50 mL tubes.  
Note: Samples can be weighed on the previous day and refrigerated overnight.
- (2) For system suitability, prepare a solvent standard (Mix 50  $\mu$ L F2, 5  $\mu$ L internal standard and 945  $\mu$ L of 80:20 mixture of water:ACN) and a solvent blank (80:20 mixture of water:ACN)
- (3) Prepare six matrix calibration standards (M1-M6) at 0.005, 0.01, 0.25, 0.10, 0.40, 1.20  $\mu$ g/g by fortifying control matrix (5 g) with appropriate amounts of F1-F6. (For example, fortifying with 500  $\mu$ L of F1 yields M1 at 0.005  $\mu$ g/g; 500  $\mu$ L of F2 yields M2 at 0.01  $\mu$ g/g)

- (4) Add relevant amounts of fortification standards to control matrix to prepare quality control samples at 2-3 concentration levels.
- (5) Add internal standard mix (50  $\mu$ L) to all samples including M1-M6 and quality controls.
- (6) Cap and let samples sit for 5 minutes for equilibration.
- (7) Add 5 mL of acetate buffer and 20 mL ACN. Vortex 15 s to mix.
- (8) Shake on the horizontal shaker for 7 min, sonicate for 7 min, and centrifuge 3700 rcf 10 min.
- (9) Decant supernatant to new 50 mL centrifuge tube.
- (10) Add 10 mL ACN to original tube, vortex to break the feed pellet, shake on the horizontal shaker for 7 min, and centrifuge for 7 min.
- (11) Combine supernatants by decanting into the 2nd 50 mL centrifuge tube.
- (12) Add water up to 40 mL mark as accurately as possible and vortex for 15 s to mix.
- (13) Pipet 2 mL to a 15 mL centrifuge tube.
- (14) Fill with water to 10 mL mark as accurately as possible.
- (15) Add 1.5 mL of hexane and vortex for 30 sec.
- (16) Centrifuge 2500 rcf for 3 min.
- (17) Aspirate and remove hexane layer.

Important: Make sure no hexane remains by aspirating some of aqueous layer as well

Note: This is a stopping point for a short break.

#### 2020.8.2 Solid Phase Extraction on Oasis HLB (60 mg, 3 mL)

- (1) Conditioning: 2 mL ACN, 2 mL water.
- (2) Close cartridge and attach 10 mL reservoirs on top of SPE cartridges.
- (3) Pipet 5 mL of sample to the reservoir. Open cartridge and adjust flow to  $\sim$ 1 drop/sec.  
Note: Initially, for a brief moment, you may need to loosen the reservoir to start flow to cartridge.
- (4) After liquid flow has stopped, slowly apply vacuum to pass last bit of sample.
- (5) Dry for 5 min at  $\sim$ 10 inHg.
- (6) To rinse cartridge, add 2 mL water\*, followed by 2 mL 5% ethanol.  
\*To start flow, carefully apply a slight vacuum.
- (7) Dry for 5 min @  $\sim$ 10 inHg.
- (8) Place 15 mL collection tubes inside the manifold.
- (9) Elute with 8 mL 50:50 EtOH:ACN. Apply slight vacuum to start elution. Stop vacuum and close cartridge for 1 min to facilitate desorption of analytes. Open cartridge and continue elution slowly at  $\sim$  1 drop/s. Finally, apply vacuum carefully to elute the last drops of eluate.

#### 2020.8.3 Evaporation and reconstitution

- (1) Evaporate eluates in the TurboVap at 40 C to  $\sim$ 50  $\mu$ L ( $\sim$ 60 minutes) and reconstitute with 200  $\mu$ L of 80:20 mixture of water:ACN.
- (2) Vortex for 1 min, transfer to microcentrifuge tube, and centrifuge at 15,000 rcf for 15 min at 4°C.
- (3) Immediately transfer 75  $\mu$ L of supernatant to auto-sampler vials with insertion without disturbing the precipitate.  
The sample extracts can be stored at - 20°C overnight before analysis
- (4) Analyze by LC-MS/MS. Injection volume 5  $\mu$ L.

Note: There is a dilution factor of 2 during the procedure. A 0.01  $\mu$ g/g matrix sample is equivalent to an injection solution of 0.005  $\mu$ g/mL.

## 2020.8.4 LC-MS/MS Analysis

The following parameters were used. Minor adjustments may be necessary depending on other systems used.

- (1) Liquid chromatography, autosampler tray temperature: 10°C, Column temperature: 40°C. Flow Rate: 0.4 mL/min.

Table 2. LC gradient

Time (min)	Mobile Phase A <sup>1</sup>	Mobile Phase B <sup>2</sup>
0	78	22
7	25	75
7.1	0	100
13	0	100
13.1	78	22
20	78	22

<sup>1</sup>0.1% Formic acid in water. <sup>2</sup>0.1% Formic acid in ACN.

- (2) Electrospray tandem mass spectrometry

Divert flow from ion source for 0-2 min and 7-20 min.

Tuning optimization: Use positive ion electrospray mode. Tuning conditions: the entrance potential is 10 V, collision gas is 4, curtain gas is 35, ion source gas 1 is 80, ion source gas 2 is 50, ion spray voltage is 5500 V, and source temperature is 600°C. Scheduled MRM was used. Target scan time 1 s, MRM detection window 40 s.

Acquisition parameters are given in Table 3. Figure 2 displays representative ion chromatograms for a DG extract fortified at 0.01 µg/g.

Table 3. Acquisition parameters

Compound	Retention time (min)	Precursor ion (m/z)	Product ions <sup>1</sup> (m/z)	DP (V)	CE <sup>2</sup> (V)	CXP <sup>2</sup> (V)
Erythromycin A	2.55	734.7	158.21, 576.5, 116.2	78	44, 28, 66	12, 16, 9
Penicillin G	2.80	335.1	160.0, 176.0, 114.1	46	16, 19, 45	10, 11, 7
Virginiamycin M1	3.95	526.4	355.2, 337.1, 133.1	70	25, 29, 42	9, 9, 13
Virginiamycin S1	5.35	824.7	205.1, 177.2, 134.3	100	65, 97, 83	14, 10, 9
Erythromycin-(N-methyl- <sup>13</sup> C, d <sub>3</sub> ) <sup>3</sup>	2.55	738.6	162.1	89	43	10
Penicillin G-D <sub>7</sub> <sup>4</sup>	2.80	342.2	160.0	43	17	10

<sup>1</sup>Product ions 1, 2 & 3, respectively. Ion 1 is used for quantitation, and ions 2 & 3 are used for confirmation.

<sup>2</sup>CE and CXP are listed in order corresponding to the product ions. <sup>3,4</sup>Internal standards.

- (3) Procedure for Instrumental Analysis of Samples, Controls and Standards

Make at least 4 injections of solvent standard to establish system suitability. Inject solvent blank, standard curve from low to high concentration, solvent blank, matrix blanks, QCs, unknown samples, solvent blank and repeat the standard curve. After the



run, flush the column for at least 20 minutes with mobile phase A and B (10+90) or water and acetonitrile (10+90). Acetonitrile and water mixture may be used to store the column.

## 2020.9 CALCULATIONS

### 2020.9.1 Calculations for Determinative Analysis

- (1) Peak areas of analyte quantitation ion and internal standard ion are used for quantitation.
- (2) Erythromycin-(N-methyl-<sup>13</sup>C,<sub>3</sub>) is used as internal standard for erythromycin A. Penicillin G-d<sub>7</sub> is the internal standard for all others: Penicillin G, virginiamycin M1 and virginiamycin S1.
- (3) Construct calibration curve by plotting the peak area ratio between the analyte and the internal standard as the y-axis against the initial concentration (µg/g) in DG matrix. Calibration curve includes both bracketing standard curves and is applied to all samples within the bracket.
- (4) Determine sample concentrations by a weighted (1/X) linear regression that excludes the origin.
- (5) Care should be taken with integration of chromatographic peaks. The baseline determined by automated data systems should be individually inspected for each chromatogram. Manually adjust any baselines that are grossly incorrect, and force integrate very small peaks at the correct retention time in blanks.
- (6) The resulting final sample concentration (except for control and fortified samples) is corrected by taking into account the mass difference of the individual samples to the nominal 5 g sample used to calculate the concentrations of analyte in the calibration standards.

$$\text{Concentration in DG (}\mu\text{g/g)} = \frac{\text{Experimental } \mu\text{g/g} \times 5}{\text{Weight of sample in grams}}$$

- (7) Recovery (ref 1): The proportion of analyte (incurred or added) remaining at the point of the final determination from the analytical portion of the sample measured)

$$\text{Recovery (\%)} = \frac{(\text{Experimental } \mu\text{g/g of pre-fortified sample}) \times 100}{(\text{Experimental } \mu\text{g/g of post-fortified sample})}$$

- (8) Accuracy (ref 1): The closeness of agreement between a test result and an accepted reference value). It is calculated by dividing the concentration found in fortified samples by their theoretical concentration and expressing the result as a percentage.

$$\text{Accuracy (\%)} = \frac{(\text{Experimental } \mu\text{g/g}) \times 100}{(\text{Theoretical } \mu\text{g/g})}$$

- (9) Limit of quantitation (LOQ) was calculated by extracting fortified matrix, five replicates each, at three concentrations near the lowest calibration point. The concentration that resulted in a RSD% (n=5) <22% (ref 1) was taken as the LOQ.

- (10) The acceptance criteria for quantitation are described in Section 2020.10.

### 2020.9.2 Calculations for Confirmatory Analysis

- (1) Calculate ion ratios as percent relative abundances: Calculate the relative abundance of product ions and multiply by 100. Use product ion 1 as the denominator for both ratios.
- (2) Calculate any interference response as a percent of the lowest standard.

## 2020.10 SYSTEM SUITABILITY AND QUALITY CONTROL

### (1) Instrument Performance Specifications

Perform the following system suitability at initial set-up or after a major change on the system (e.g. replacement of analytical column or major repairs). Inject at least four replicates of a solvent standard (e.g. 5 ppb) and determine the following parameters. If this test fails, check for leaks/clogs, and/or re-equilibrate the system and/or prepare new mobile-phase solutions. The relative standard deviation of the ratio of the analyte peak area to the internal standard peak area should not be greater than 15%. The retention time repeatability should be within 2%.

### (2) Critical Points and Stopping Points

The critical points and stopping points are described in the procedure in section 8.2. Especially, since erythromycin and penicillin G are acid sensitive, once extracted in to acidic medium, the samples need to be processed within the same day. If severe peak tailing (tailing factor >2.0) is observed, replace the guard column with a new one.

### (3) Stability of Analyte in Solvent Standards and Extracts

Solvent standards: Stable for two weeks at 4°C and eight weeks at -20°C.

Final extracts may be stored for 7 days at -20°C or -80°C.

### (4) Acceptance Criteria for Quantitation Results

Matrix calibration curves should give a correlation coefficient ( $r$ )  $\geq 0.990$ . Back calculated accuracy should be within  $\pm 25\%$  of the theoretical concentration for all levels.

Quality control samples: Accuracy should be 80-120% with relative standard deviation of  $\leq 20\%$ .

### (5) Confirmation Criteria

The retention time of each analyte must agree within 5%, relative to that of the calibration standards. The three product ions associated with the analyte must be present and exceed a signal-to-noise (S/N) threshold of 3:1. The product ion ratios must match arithmetically within 20% of those of standards. If interference is observed in the negative control at the retention time of the analyte, the response (i.e., area) of the interference must be <10% the average response of the lowest calibration standard.

## 2020.11 VALIDATION INFORMATION/STATUS

A single-laboratory validation (ref 2) of the method was followed by a multi-laboratory validation conducted in accordance with FDA Guidance (ref 1). Eight laboratories participated in the study: FDA/Center for Veterinary Medicine, Laurel, MD; Eurofins Central Analytical Laboratories, New Orleans, LA; OMIC USA Inc., Portland, OR; ALS Marshfield, Marshfield, WI; NYS Dept. of Agriculture and Markets, Albany, NY; FDA/Center for Food Safety and Applied Nutrition, College Park, MD; FDA/Office of Regulatory Affairs, Southeast Food and Feed Laboratory, Atlanta, GA; FDA/Office of Regulatory Affairs, Denver Laboratory, Denver.

The validation plan model recommended in the FDA Guidance was used, as depicted in Table 4. The type of DG used in the study was dried distillers grains with solubles. Depending on the source of the DG, the matrices were slightly variable with respect to the raw materials used and some differences in the processing method applied. The matrices were corn-based reduced-oil DG (Source 1), corn-based DG (Source 2), and corn- and milo-based DG (Source 3, 70% Milo, 30% Corn). Each laboratory analyzed 24 samples: duplicate samples from each of the three matrices fortified at the

concentration levels, 0 ng/g (blank samples), 10 ng/g, 100 ng/g and 1000 ng/g. Analysts were given a choice of four to six separate days for analysis.

Table 4. Validation plan

Day of Analysis	Matrix <sup>1</sup>		
	Source 1	Source 2	Source 3
Day 1	Blank	Fortified (ML)	Blank
	Fortified (ML)	Fortified (HL)	Fortified (LL)
Day 2	Fortified (HL)	Blank	Blank
	Fortified (LL)	Fortified (LL)	Fortified (HL)
Day 3	Fortified (LL)	Fortified (HL)	Fortified (ML)
	Fortified (ML)	Blank	Fortified (ML)
Day 4	Fortified (HL)	Fortified (ML)	Fortified (HL)
	Blank	Fortified (LL)	Fortified (LL)

<sup>1</sup>LL, ML and HL are low (10 ng/g), medium (100 ng/g) and high (1000 ng/g) fortification levels, respectively.

Table 5. LC/MS platforms used by the participating laboratories

Laboratory No.	LC	MS
1	Agilent UHPLC 1290 Infinity II	Agilent 6470 Triple Quad
2	Waters Acquity UPLC I-Class	AB Sciex 6500 Q-trap
3 <sup>a</sup>	Shimadzu LC-30AD Nexera	Shimadzu 8050 Triple Quad
4	Waters Acquity Ultra Performance	Waters Premier XE
5	Shimadzu LC-20AD XR	AB Sciex 5500 QTrap
6	Shimadzu LC-20AD XR	AB Sciex 5500 QTrap
7	Agilent 1260	AB Sciex 5500 Q-Trap
8	Shimadzu LC-20AD	AB Sciex 4000 Triple Quad

<sup>a</sup>Laboratory No. 3 data were subsequently excluded due to some analytes not passing system suitability criteria.

Table 5 gives the various LC-MS platforms used by the laboratories. All participants used the same LC column, ion transitions and LC conditions as reported above.

Laboratory #s 2, 5, 6 and 7, which used a Sciex platform as the coordinating laboratory (#8), used same MS parameters as well. For Laboratory #1, the optimized MS parameters were as follows: nitrogen drying gas temperature and flow rate were 150 °C and 13 L/min, respectively. Nebulizer pressure was 35 psi. Ultrapure nitrogen sheath gas temperature and flow were 300 °C and 11 L/min, respectively. Capillary and nozzle voltages were 4 V and 0.5 V, respectively. Parameters for Laboratory 3 is not given as data were removed from evaluation due to some analytes not passing system suitability criteria. For Laboratory 4, the optimized conditions were desolvation gas 700 L/h at a temperature of 250 °C, cone gas 150 L/h, source temperature 100 °C, capillary and cone voltages 3 kV and 45 V, respectively. The collision energies (CE) used by Laboratory #1 and #4 in the order of the product ions are as follows: For Ery (m/z) 158, 576 and 116, the CE are 32V, 32V, 32V (Lab #1) and 30V, 22V, 45V (Lab #4); For Pen G (m/z) 160, 176 and 114, the CE are 8V, 12V and 40V (Lab #1) and 18V, 13V and 35V (Lab #4); For Vir M1 (m/z) 355, 337 and 133, the CE are 18V, 22V, 18V (Lab #1) and 20V, 23V and 30V (Lab #4); For Vir S1 (m/z) 205, 177 and 134, the CE are 54V, 18V and 18V (Lab #1) and 50V, 75V and 75V (Lab #4); For Erythromycin-(N-methyl-<sup>13</sup>C, d<sub>3</sub>) (m/z) 162, the

CE is 32V (Lab #1) and 45V (Lab #4); for Penicillin G-D7 m/z) 160, the CE is 8V (Lab #1) and 16V (Lab #4).

Validation data:

Raw data were evaluated against the appropriate determinative and confirmatory acceptance criteria. Data that were determined to be not valid based on the evaluation were removed. Outliers were then determined and excluded for each set of data for a given concentration level using a single-sample outlier test (Grubb's test,  $\alpha = 0.05$ ). Method parameters, average drug concentrations, percent accuracy, standard deviations of repeatability ( $S_r$ ) and reproducibility ( $S_R$ ), relative standard deviations of repeatability ( $RSD_r$ ) and reproducibility ( $RSD_R$ ), and HorRat values, were calculated for comparison with the recommended values (ref 1).

All laboratories produced correlation coefficients higher than the minimum acceptance criteria of 0.990 for all four analytes. A statistical summary of the data is given in table 6. Each data point represents 35-42 replicates originating from 7 laboratories. Average accuracies for all four drugs at all three fortification levels, 10 ng/g, 100 ng/g and 1000 ng/g, ranged 86-103%, 83-109% and 91-101%, respectively. Within-laboratory repeatability,  $RSD_r$ , for all drugs at 10 ng/g, 100 ng/g and 1000 ng/g ranged 13-17%, 8-15% and 7-14%, respectively. Between- and within-laboratory reproducibility,  $RSD_R$ , for all drugs at 10 ng/g, 100 ng/g and 1000 ng/g ranged 13-21%, 10-18% and 7-16%, respectively. HorRat values ranged from 0.4-1.0 for all compounds at all concentration levels.

Table 6. Statistical summary of drug recovery data encompassing all laboratories: Average Accuracy%, Repeatability Standard Deviation ( $S_r$ ), Reproducibility Standard Deviation ( $S_R$ ), Repeatability Relative Standard Deviation ( $RSD_r$ , %), Reproducibility Relative Standard Deviation ( $RSD_R$ , %), and HorRat

Fortified Conc., ng/g	Drug	No. of replicates	Average Accuracy%	$S_r$	$S_R$	% $RSD_r$	% $RSD_R$	HorRat
10	Ery	42	86	1.3	1.3	15	15	0.5
	Pen G	41	96	1.3	1.3	13	13	0.4
	Vir M1	41	103	1.4	1.8	14	17	0.5
	Vir S1	39	103	1.8	2.2	17	21	0.7
100	Ery	42	83	11	12	13	15	0.6
	Pen G	42	95	7.3	9.8	7.7	10	0.5
	Vir M1	42	109	16	20	15	18	0.8
	Vir S1	35	93	11	14	12	14	0.6
1000	Ery	42	91	86	86	9.4	9.4	0.6
	Pen G	42	97	71	71	7.3	7.3	0.5
	Vir M1	41	101	93	136	9.2	14	0.8
	Vir S1	40	97	135	152	14	16	1.0

The multi-laboratory validation was approved as a Level 4 multi-laboratory validation by the FDA Foods Program Chemistry Methods Validation Subcommittee and ratified by the Chemistry Research Coordination Group in May 2020.

## 2020.12 REFERENCES

- (1) Guidelines for the Validation of Chemical Methods for the FDA Foods and Veterinary Medicine program, 3rd Edition, October 2019
- (2) Kaleb J. Duelge, Upul Nishshanka, Hemakanthi G. De Alwis, "An LC-MS/MS method for the determination of antibiotic residues in distillers grains at levels of concern for antimicrobial resistance development", J. Chrom. B., vol 1053, 81-86, 2017.

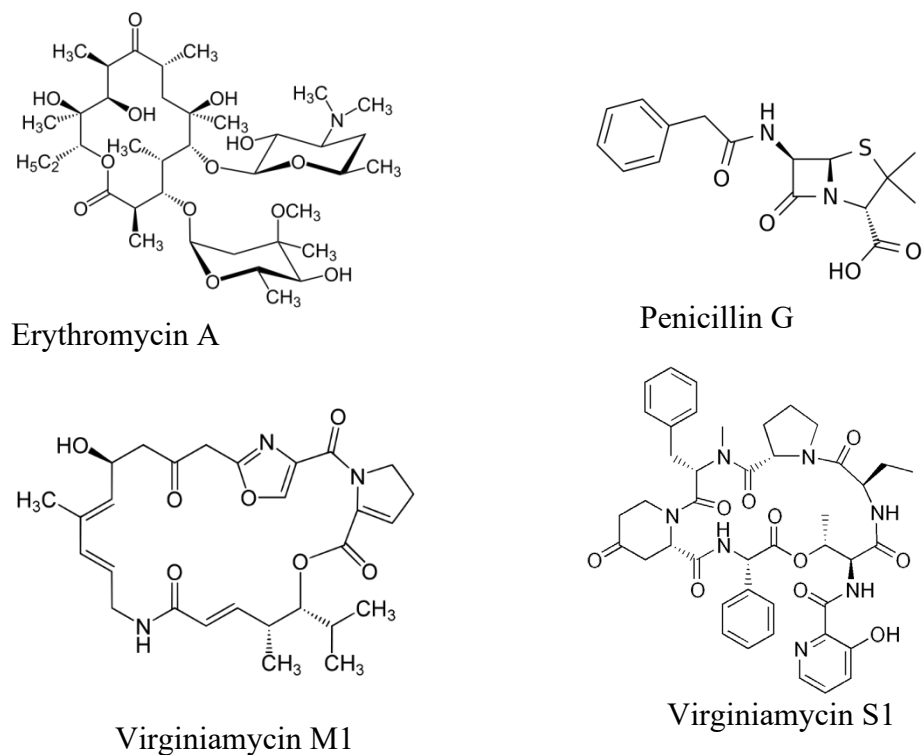


Figure 1. Chemical structures

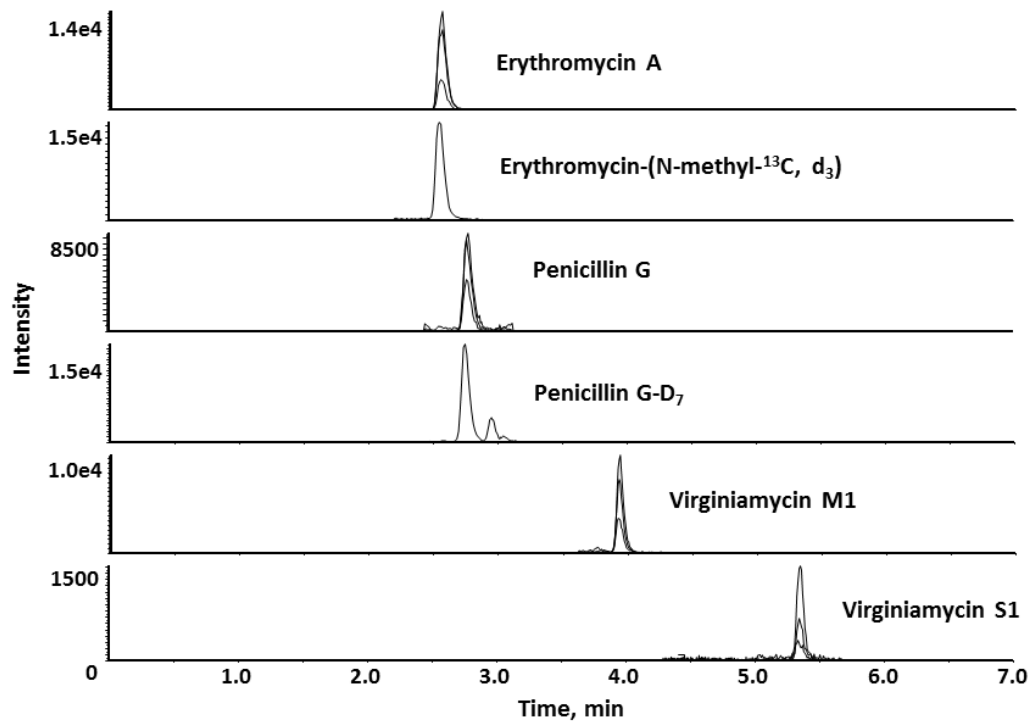


Figure 2. Representative ion chromatograms for a DG extract fortified at 0.01  $\mu\text{g/g}$ .