Multi-Laboratory Collaborative Validation: An LC-MS/MS Method for Antibiotics in Distillers Grains

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Abstract

Antibiotic based antimicrobials are sometimes used in biofuel production to discourage the growth of bacteria that would result in lower ethanol production. Residues of antibiotics used could remain in the distillers grain (DG) co-product, which is used as an animal food ingredient. Low levels of antibiotic residues in DG could lead to antimicrobial resistance development.

In this method, antibiotic residues were extracted from DG with a mixture of acetonitrile (ACN) and buffer. The extract was diluted with water and washed with hexane. An aliquot was cleaned on an Oasis HLB solid phase extraction cartridge. The eluent was concentrated and reconstituted with water and ACN. After centrifugation the supernatant was analyzed by reverse phase LC-MS/MS.

Eight laboratories participated in the study. They used their available LC-MS/MS platforms and analyzed duplicate samples from three DG matrices fortified with the antibiotics at concentrations 0, 10, 100 and 1000 ng/g.

Average accuracies for combined three DG matrices for all four compounds at all fortification levels ranged from 83 to 109% with repeatability RSD_r (within laboratory) ≤17% and reproducibility RSD_R (between laboratory) ≤21%. The HorRat values ranged 0.4-1.0 indicating that method reproducibility is acceptable.

The results demonstrate that the method is fit-for-purpose to determine the antibiotics of interest in DG. The method could serve as a regulatory method capable of being used for compliance actions for DG containing these antibiotic contaminants.

*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

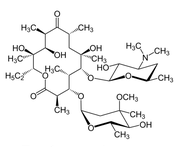
Introduction

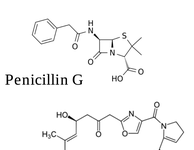
Distillers grain is a major co-product of the corn ethanol industry. It is rich in proteins, fats, and minerals making it an excellent feed supplement for livestock, especially for beef and dairy cattle, swine, and poultry.

Antibiotics are used to control bacterial contamination during the ethanol fermentation. FDA surveys have revealed antibiotic residues in the DG. Low levels of antibiotic residues in DG could lead to antimicrobial resistance and, therefore, could be a public health concern.

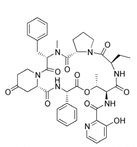
To enable quantitation of erythromycin, penicillin G, virginiamycin M1 and virginiamycin S1 at low ppb levels, we developed a sensitive LC-MS/MS method. A single laboratory validation was completed following FDA's Guidelines for the Validation of Chemical Methods for the FDA Foods Program. A variety of different matrices such as corn DG, corn & milo DG, and reduced-oil corn DG were used for the validation.

To ensure the method's robustness for use in regulatory settings, a multi-laboratory validation was also successfully completed.


Erythromycin


Penicillin G

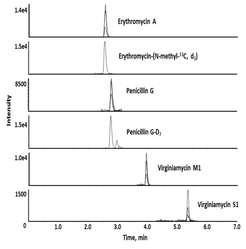
Virginiamycin S1


Virginiamycin M1

Materials and Methods

Method

Distillers grain
⇩
Extract with buffer & acetonitrile, centrifuge and transfer supernatant
Repeat extraction
Combine supernatants & dilute with water
⇩
Clean-up of extract by hexane wash and solid phase extraction
⇩
Analysis by LC-MS/MS: Shimadzu LC–20AD Prominence; AB Sciex 4000 MS; Agilent Poroshell LC column; Flow rate: 0.4 mL/ min; Mobile phases: A - 0.1% aq. formic acid, B- 0.1% formic acid in acetonitrile; Gradient started at 22% B and ramped to 75% by 7 min, 100% by 7.1 min, and was held at 100% until 13 min.



Collaborators

- FDA/CVM/Office of Research, Laurel, MD
- Eurofins Central Analytical Laboratories, New Orleans, LA
- OMIC USA Inc., Portland, OR
- ALS Marshfield, Marshfield, WI 54449
- NYS Dept. of Agriculture & Markets, Albany, NY
- FDA/Center for Food Safety & 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, CO

Validation Sample Plan*

	Fortification Level, ng/g		
	DG Source 1	DG Source 2	DG Source 3
Day 1	Blank	100	Blank
	100	1000	10
Day 2	1000	Blank	Blank
	10	10	1000
Day 3	10	1000	100
	100	Blank	100
Day 4	1000	100	1000
	Blank	10	10

*FDA Guidance: Guidelines for the Validation of Chemical Methods for the FDA Foods and Veterinary Medicine program, 2nd Edition, April 2015

Instrumentation

Lab	LC	MS
1	Agilent UHPLC 1290 Infinity II	Agilent 6470 Triple Quad
2	Waters Acquity UPLC I-Class	AB Sciex 6500 Q-trap
3	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

Results and Discussion

Invalid Data

Inspected chromatograms for peak resolution, peak shape, and integration. Assessed System suitability, calibration parameters, retention time, signal/noise, ion ratios etc. against acceptance criteria in the Method SOP. Removed invalid data points from the data set.
Outliers - Grubbs' Outlier test, α = 0.05

$$G = \frac{\bar{Y} - Y_{min}}{s} \quad or \quad \frac{Y_{max} - \bar{Y}}{s}$$

$\bar{Y}$ –Sample mean, s–standard deviation

If $G > G_{critical}$ (Grubbs' Table of Critical Values), the corresponding data point is an outlier. Outliers were removed from the data set.

Erythromycin	No outliers
Penicillin G:	No outliers
Virginiamycin M1:	<1% (1/124 data points)
Virginiamycin S1:	4% (5/114 data points)

Method Parameters & Method Acceptability

FDA Guidance - Quantitative Method Acceptability criteria:	**Level** / **10 ng/g** / **100 ng/g** / **1000 ng/g** **RSD_r** 22% 11% 8% **RSD_R** ≤ 44% ≤ 44% ≤ 32% **Recovery** 60%-115% 80%-110% 80%-110% **HorRat (acceptable method reproducibility) = ≤2**
Average Accuracy%:	The closeness of agreement between a test result and an accepted reference value
Repeatability Relative Standard Deviation (RSD_r, %):	Variation of the data within laboratories Accuracy (%) = (Experimental ng/g) x 100 / (Theoretical ng/g)
Reproducibility Relative Standard Deviation (RSD_R, %):	Total variation of the data including between- and within-laboratory variations
HorRat (Horwitz Ratio):	Measure of acceptability of methods with respect to among-laboratory precision (reproducibility) HorRat = $\frac{\%RSD_R}{\%PRSD_R}$ ($\%PRSD_R$ =Predicted $\%RSD_R$) $\%PRSDR = 2C^{-0.15}$ Horwitz Equation (C- concentration as a mass fraction)

Statistical Summary of Validation Data

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

Conclusion

Good method performance metrics point to an acceptable method.

Method is well-suited for regulatory use to determine low levels of penicillin G, erythromycin, virginiamycin M1 and virginiamycin S1 in distillers grain.

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