U.S. FOOD & DRUG ADMINISTRATION

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

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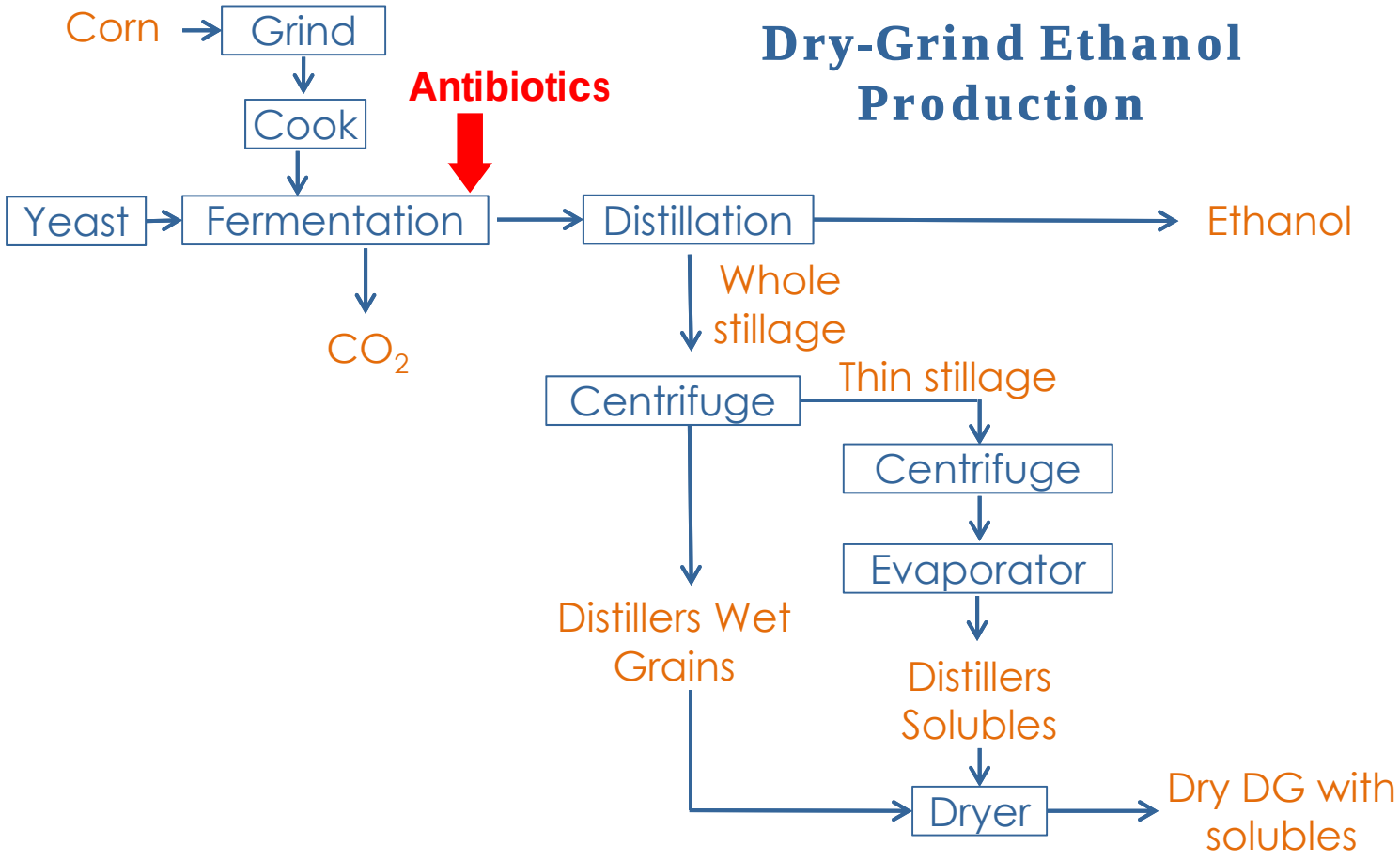
Distillers Grain (DG)

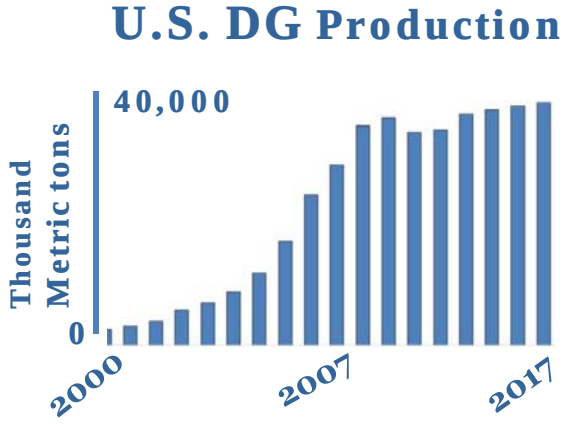
– DG is a major co-product of the dry-grind milling process in the corn ethanol industry.



Ref.: U.S. Grains Council, DDGS User Handbook, 4th Edition

DG as Animal Feed

- US DG production has increased rapidly over the last decade.
- DG is rich in proteins, fats and minerals. Hence, an excellent feed supplement for livestock.



- About 70% of DG produced is used domestically as animal feed. Remainder is exported.
- Majority is fed to beef and dairy cattle, swine, and poultry.

Reference: U.S. Grains Council, DDGS User Handbook, 4th Edition

Antibiotics in DG?

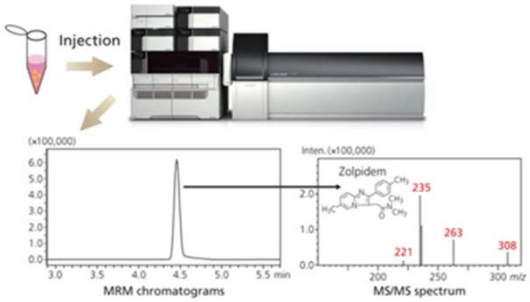
- Antibiotics are used to control bacterial contamination during the ethanol fermentation process

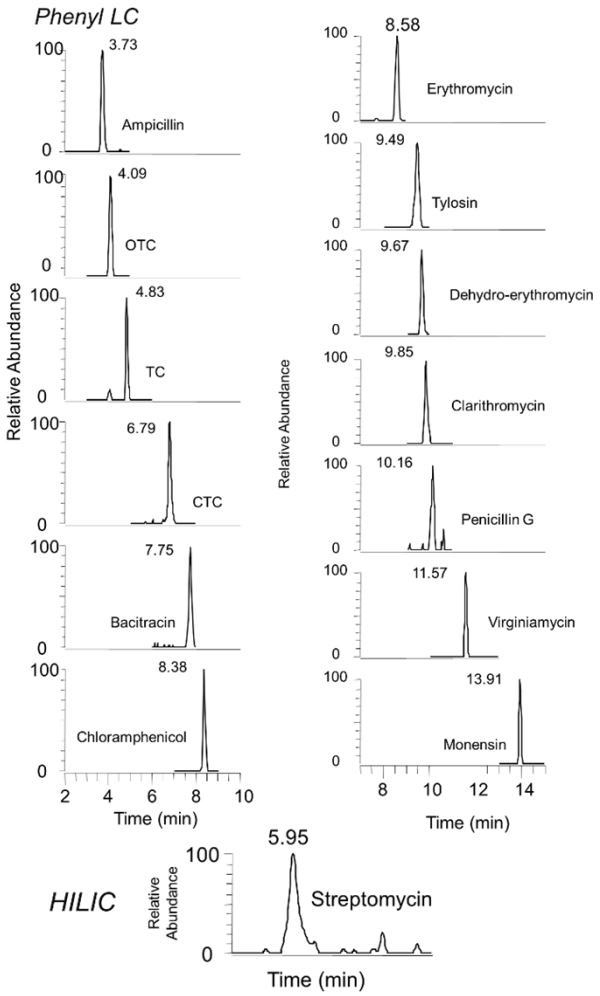
- FDA/CVM's concerns:
  - Any residues in DG?
  - What antibiotics are they?
  - Risk to animals and humans

Needed:

Analytical method to measure antibiotics in DG

LC-MS/MS Method - First





- Developed in 2008
- Used an ion trap tandem mass spectrometer
- Antibiotics analyzed: Penicillin G, ampicillin, virginiamycin M1, streptomycin, erythromycin, chloramphenicol, tetracycline, oxytetracycline, tylosin, chlortetracycline, bacitracin A, clarithromycin and monensin

De Alwis, H., Heller, D.N., "Multiclass, Multi-residue Method for the Determination of Antibiotic Residues in Distillers Grains by Liquid Chromatography and Ion Trap Tandem Mass Spectrometry", Journal of Chromatography A., vol. 1217, 3076-3084, 2010

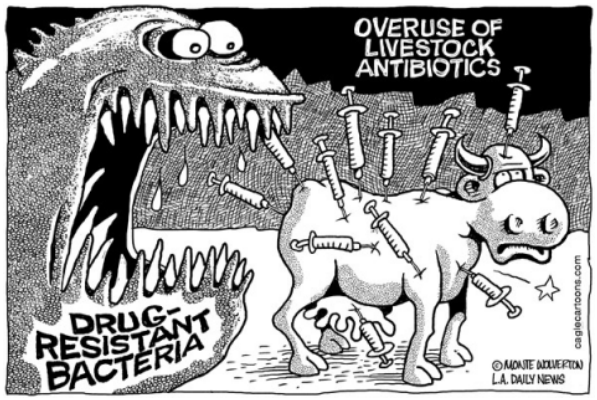
Nationwide Surveys

- In 2008 & 2010, FDA/CVM coordinated two nationwide surveys using this method.

Ref.: www.fda.gov

Development of Antimicrobial Resistance (AMR)?

- Surveys reported detecting several residues in DG: erythromycin, virginiamycin M1 & penicillin G
- CVM's concerns:
  - Possible rise of antimicrobial-resistant bacteria due to exposure to these antibiotics
  - What antibiotic levels would be relevant for any AMR development?



AMR -Microbiological Work

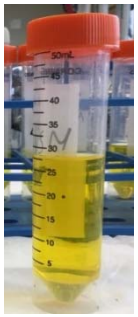
- FDA/CVM microbiologists examined the effects of these antibiotics on bacterial resistance development *in vitro.*
- **Low ppm** concentrations of erythromycin, penicillin, and virginiamycin may induce resistance development.

"Effects of low concentrations of erythromycin, penicillin, and virginiamycin on bacterial resistance development in vitro", B. Ge, K. J. Domesle, Q. Yang, S. R. Young, C. L. Rice-Trujillo, S. M. Bodeis-Jones, S. A. Gaines, M. W. Keller, X. Li, S. A. Piñeiro, B. M. Whitney, H. C. Harbottle & J. M. Gilbert, Scientific Reports, Vol 7, p 1-11 (2017)

Need a more sensitive method for residues at low levels

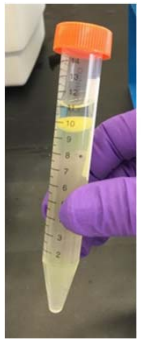
LC-MS/MS Method -2nd

Distillers grain

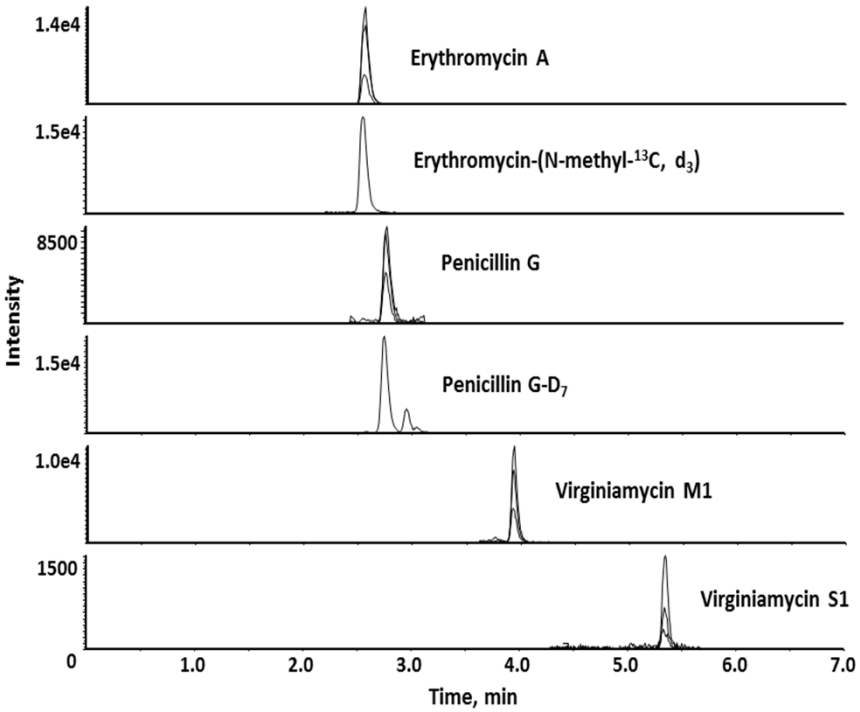


1. Extract with buffer & acetonitrile, centrifuge & transfer supernatant
2. Repeat extraction with ACN & centrifuge
3. Combine supernatants & dilute with water

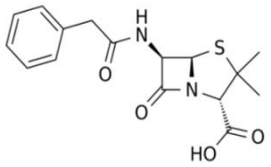
Clean-up extract: Hexane wash & solid phase extraction



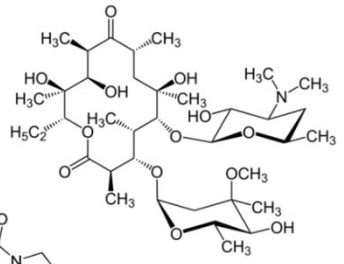
Analysis: Liquid chromatography -Tandem mass spectrometry (LC-MS/MS)



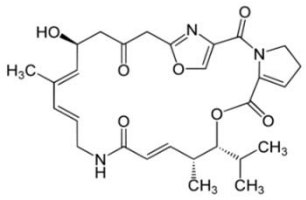
A representative chromatogram of a DG sample fortified with the drugs at 10.0 ng/g (ppb)



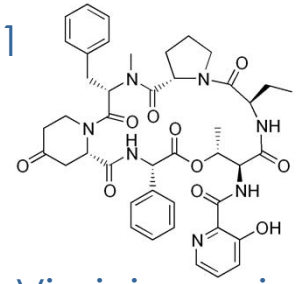
Penicillin G



Erythromycin



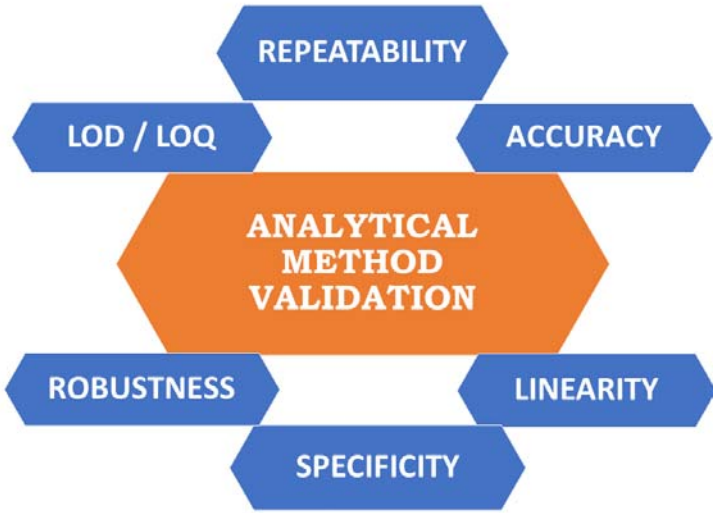
Virginiamycin M1



Virginiamycin S1

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", Journal of Chromatography B., vol 1053, 81-86, 2017

Multi-Laboratory Validation (MLV)



– To be able to use method for regulatory purposes, need to ensure method's robustness

Private Lab Joining

- Typically, our MLVs are with Federal and State laboratories
- FDA collaboration with private laboratories:
  - As mandated in the FDA Food Safety Modernization Act, FDA and the Independent Laboratories Institute (ILI) looked into collaboration
  - In order to promote scientific progress through exchange of scientific capital in analytical methods development and validation, as well as in educational initiatives
- DG method was the first one selected
- MoU and Research Collaboration Agreement (FDA & ILI)
- Private lab selection
  - 3 labs participated

Laboratories

FDA/Center for Veterinary Medicine/Office of Research (OR)
Laurel, Maryland 20708

Eurofins Central
Analytical Laboratories
New Orleans, LA 70122 USA

OMIC USA Inc.
Portland, OR 97210

ALS Marshfield
Marshfield, WI 54449

NYS Dept. of Agriculture & Markets
Albany, NY 12206

FDA/Center for Food Safety &
Applied Nutrition
College Park, MD 20770

FDA/Office of Regulatory Affairs,
Southeast Food and Feed Laboratory,
Atlanta, GA 30309

FDA/Office of Regulatory Affairs,
Denver Laboratory
Denver, CO 80225

Study Design

- **Phase A**:
  - Familiarization & proficiency sample analysis
  - Data processing & evaluation
  - Feedback to labs
- **Phase B**:
  - Validation sample analysis
  - Data processing, statistical analysis, evaluation of acceptability of method, report writing, review and approval

Phase A: Familiarization & Proficiency Sample Analysis

- Laboratory work
  - 5 weeks
  - CVM/Office of Research (OR) ship samples & other materials
  - Using Method SOP provided, labs set up LC-MS/MS
  - Familiarize with method by multiple analysis of samples
  - Analyze three non-blinded samples provided & submit data report
- Data evaluation-CVM/OR
  - 4 weeks
  - WebEx: Discuss results, feedback on improving

Phase A: Results

- Three samples per lab, 100 ng/g, concentration given, n=24 (8 labs)
- Results

	Erythromycin	Penicillin G	Virginiamycin M1	Virginiamycin S1
% accuracy	81	99	107	97
%RSD	9.1	12	18	20

  - **Pen G**: Performed quite well across all the labs
  - **Erythromycin**: There were several low values for accuracy, but for the most part, data were good
  - **Virginiamycins**: High variability (=high %RSD). Several values quite high or quite low
- Discussed results via WebEx
- Identified issues and made recommendations

Phase B: Validation Sample Analysis

- Laboratory work
  - 5 weeks
  - Ship samples
  - Analyze 24 blinded samples on 4 or 5 separate days
  - Submit data report
- Data evaluation-CVM/Office of Research
  - Data processing & statistical analysis
  - Evaluation of acceptability of method
  - Report writing
  - Review & approval

Phase B: Validation Sample Plan

- Followed FDA Guidance*

	Fortification Level, ng/g		
	Matrix Source 1	Matrix Source 2	Matrix 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

- DG matrix from 3 sources
- Samples fortified with drugs in duplicate at 4 levels, 0 ng/g (Blank), 10 ng/g, 100 ng/g, 1000 ng/g = 24 samples

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

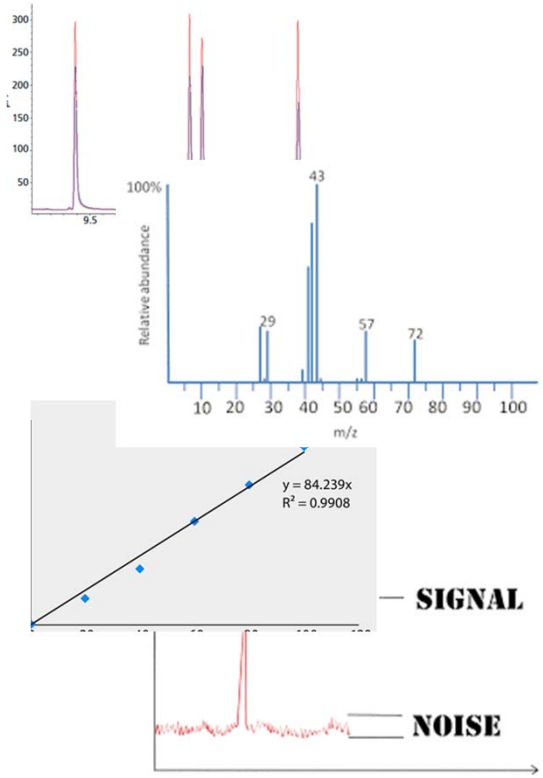
LC-MS Platforms

Lab 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	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**

- Labs with a Sciex MS used identical MS parameters as CVM/OR lab. Others did minor modifications to produce equivalent performance
- Identical LC column
- Same ion transitions & LC parameters as in the Method SOP

Data Evaluation

- 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.
- **Based on above evaluation, removed invalid data points.**



Raw Data*

		10 ng/g						100 ng/g						1000 ng/g					
		Source 1		Source 2		Source 3		Source 1		Source 2		Source 3		Source 1		Source 2		Source 3	
Drug	Lab No.	rep1	rep2	rep3	rep4	rep5	rep6	rep1	rep2	rep3	rep4	rep5	rep6	rep1	rep2	rep3	rep4	rep5	rep6
Ery	Lab 1	8.6	8.5	6.8	7.4	9.6	10	81	83	67	70	95	97	1002	922	861	843	1000	1016
	Lab 2	8.7	8.9	8.1	6.6	7.6	8.4	76	79	71	65	76	91	847	824	787	892	929	1015
	Lab 3	7.4	9.2	7.7	6.6	11	8.3	94	98	78	72	118	97	796	1031	1001	1012	1069	987
	Lab 4	7.9	9.7	7.0	6.8	11	9.2	91	80	76	67	92	89	937	999	978	770	841	995
	Lab 5	7.3	10	6.9	8.7	9.9	8.2	87	74	82	69	92	89	939	943	900	868	935	878
	Lab 6	8.7	8.5	7.4	7.8	10	10	98	92	78	86	99	106	800	902	892	888	884	1018
	Lab 7	8.1	8.0	7.5	6.4	10	10	70	72	59	55	93	91	814	839	806	744	965	1150
	Lab 8	9.4	10	7.8	8.3	9.5	9.7	91	93	87	84	99	101	968	947	914	868	1010	963
Pen G	Lab 1	8.7	-	11	6.2	9.0	10	100	109	80	84	84	95	872	905	954	938	1036	943
	Lab 2	10	10	10	10	5.9	10	105	93	90	85	87	100	979	937	962	938	1016	921
	Lab 3	10	8.8	6.4	7.4	12	9.1	99	93	100	82	105	103	941	1013	901	1078	1068	1078
	Lab 4	8.2	12	9.7	8.8	8.0	9.5	74	94	90	83	81	85	1048	909	896	907	988	1000
	Lab 5	9.1	11	8.0	9.7	9.6	8.5	91	84	96	89	94	99	902	978	964	886	977	941
	Lab 6	9.1	11	8.7	9.9	11	9.9	111	99	91	98	106	113	985	1061	900	838	1054	1095
	Lab 7	10	9.9	10	9.0	11	10	96	94	91	90	97	96	923	970	896	845	967	1169
	Lab 8	9.9	9.5	9.7	9.0	11	11	102	105	100	101	112	113	1010	975	953	1010	1090	1030
Vir M1	Lab 1	11	14	13	14	75	13	139	153	114	116	114	130	1149	1255	1146	1270	1047	1292
	Lab 2	11	12	11.1	12	7.4	11	167	123	101	105	96	108	1053	1027	1001	1131	1036	1006
	Lab 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lab 4	5.2	12	8.1	12	9.2	9.1	81	115	69	87	73	90	1021	833	1059	787	949	824
	Lab 5	9.0	10	9.1	10	9.8	8.5	116	80	119	92	88	95	874	-	813	913	1010	961
	Lab 6	10	10	11	11	7.2	10	117	118	99	106	115	122	766	1060	920	780	827	1054
	Lab 7	12	11	11	10	10	9.2	109	111	100	118	96	100	1003	1103	1007	936	895	1076
	Lab 8	8.7	10	10	9.6	11	11	94	109	109	112	124	131	1010	969	1100	1130	1170	1030
Vir S1	Lab 1	14	-	12	16	52	19	156	-	115	103	-	-	1248	1208	1315	-	785	1688
	Lab 2	11	11	11	12	6.4	11	161	120	84	97	96	105	1059	1060	902	1155	935	991
	Lab 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lab 4	4.9	11	6.3	10	9.1	10	81	100	74	63	57	81	988	801	1060	678	805	776
	Lab 5	11	11	6.9	8.9	9.9	5.3	-	79	-	99	85	101	902	982	592	878	1010	1110
	Lab 6	8.6	11	9.5	10	12	9.9	99	91	77	80	102	106	894	965	910	711	957	984
	Lab 7	12	11	12	11	11	10	103	106	82	100	92	96	999	1067	992	913	938	1035
	Lab 8	9.9	10	10	9.6	12	11	94	98	98	91	98	102	1030	948	880	1050	1190	1000

*Invalid data not included

Data Evaluation: Statistical Analysis

- Grubbs' Outlier test, a = 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

- G compared with $G_{critical}$

- Outliers excluded from the data set:

Erythromycin	– none
Penicillin G	– none
Virginiamycin M1	– <1% (1/124 data points)
Virginiamycin S1	– 4% (5/114 data points)

FRANK E. GRUBBS

TABLE 1

Table of Critical Values for T (One-sided Test) When Standard Deviation is Calculated from the Same Sample

Number of Observations n	5% Significance Level	2.5% Significance Level	1% Significance Level
3	1.15	1.15	1.1
4	1.46	1.48	1.4
5	1.67	1.71	1.7
6	1.82	1.89	1.9
7	1.94	2.02	2.1
8	2.03	2.13	2.2
9	2.11	2.21	2.3
10	2.18	2.29	2.4
11	2.23	2.36	2.4
12	2.29	2.41	2.5
13	2.33	2.46	2.6
14	2.37	2.51	2.6
15	2.41	2.55	2.7
16	2.44	2.59	2
17	2.47	2.62	
18	2.50	2.65	
19	2.53	2.68	
20	2.56	2.71	
21	2.58	2.73	
22	2.60	2.76	
23	2.62	2.78	
	2.64		

Data Evaluation: Calculating Method Parameters*

- **Average Accuracy%:** The closeness of agreement between a test result and an accepted reference value

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

- **Repeatability Relative Standard Deviation (RSD_r, %):** Variation of the data within laboratories
- **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)

$$\text{HorRat} = \frac{\%RSD_R}{\%PRSD_R} \qquad (\%PRSD_R = \text{Predicted } \%RSD_R)$$

$$\%PRSD_R = 2C^{-0.15}$$ Horwitz Equation

(C- concentration as a mass fraction)

**FDA Guidance*

Validation Data: Statistical Summary

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

Method Acceptability

Validation data encompassing all drugs

	10 ng/g	100 ng/g	1000 ng/g
No. of replicates	163	161	165
Average Accuracy%	86-103	83-109	91-101
%RSD_r	13-17	8-15	7-14
%RSD_R	13-21	10-18	7-16
HorRat	0.4-0.7	0.5-0.8	0.5-1.0

FDA Guidance: Method criteria

Alternative ML* unit	1 ppb	10 ppb	100 ppb	1 ppm	pp
RSD_r**	22%	22%	11%	8%	6%
$PRSD_R$#	22%	22%	22%	16%	
RSD_R##	≤ 44%	≤ 44%	≤ 44%	≤ 32%	≤
Recovery	40%-120%	60%-115%	80%-110%	80%-110%	80% 11

HorRat (acceptable method reproducibility) = ≤2

Good method performance metrics point to an acceptable method

Review and Approval

- Review by Veterinary Drug Residue and Animal Feeds Technical Advisory Group
- Review by Chemical Method Validation Subcommittee (CMVS)
- Final review by FDA/Chemistry Research Coordination Group (CRCG)
- Approved
- Posting to the FDA Methods Compendium public website

Conclusion

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

Acknowledgments

- Cristina Nochetto, staff scientist, for help with planning and laboratory work
- For all participant laboratories for their work
- Marla Keller, FDA study sponsor, for providing DG matrix
- Sonya Bodeis-Jones and Jake Guag of CVM/OR for help with shipping
- Michael Tai and Virginia Recta of CVM statistical team for statistical help
- Philip Kijak, my supervisor, for support throughout

Thank You!

Questions?