LC-MS/MS DETERMINATION OF ANTIBIOTIC RESIDUES IN DISTILLERS GRAINS: METHOD MODIFICATION

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Overview

Antibiotics are used in ethanol production to discourage bacterial growth that would lower the ethanol content.

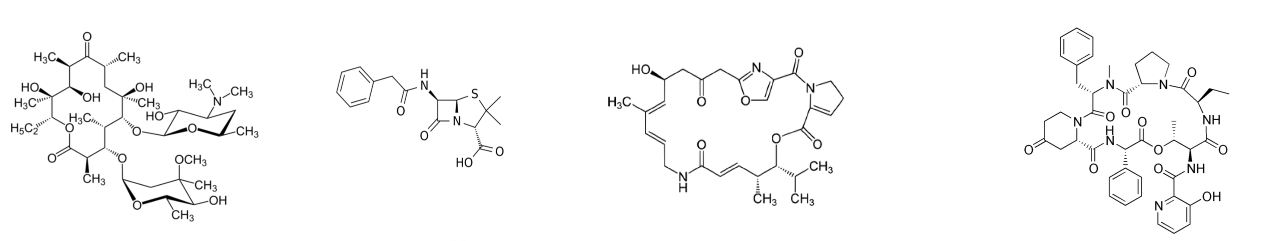
Residues of these antibiotics could remain in the distillers grain (DG) by-product, which is then used as an animal feed ingredient. Low levels of antibiotic residues in DG can lead to antimicrobial resistance development when animals consume them.

An FDA survey revealed several antibiotic residues, erythromycin A, penicillin G, virginiamycin M1 and virginiamycin S1, to be present in DG. To quantify these compounds, a liquid chromatography-tandem mass spectrometry method was developed, and multi-laboratory validated (1).

This method initially quantitated erythromycin and penicillin G using their corresponding isotopically labelled internal standards (ISTDs), which are considered optimal ISTDs for quantitation in mass spectrometry using the stable isotope dilution technique. Isotope ISTDs for virginiamycins were not available at the time.

Virginiamycin M1-d_2 (VIR M1-d2) has since become commercially available, and in this study, we sought to include it for better virginiamycin M1 quantitation. However, an ISTD that is only doubly-deuterated may complicate quantitation: the naturally occurring isotope of the analyte could interfere in the ISTD ion transition thereby falsely increasing the amount of ISTD detected and lowering the relative response for the analyte.

A feasibility study was carried out to test the suitability of VIR M1-d2 as an ISTD. We also explored the use of solvent calibration curves for all the analytes to replace the labor-intensive matrix-matched calibration curves currently used in the method.



Erythromycin   Penicillin G   Virginiamycin M1   Virginiamycin S1

(1) Hemakanthi G. De Alwis, Philip J. Kijak, Cristina Nochetto, An LC-MS/MS Method for the Determination of Antibiotic Residues in Distillers Grains: Collaborative Study, Journal of AOAC international, Volume 104, Issue 5, 2021, Pages 1213–1222

Disclaimer

How this work supports FDA's mission

This method enables FDA to conduct surveys on antibiotic residues in distillers grain and facilitate regulatory decision making, if deemed necessary.

Materials and Methods

Extraction method

Distillers grain

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Extract with acetate buffer & acetonitrile, centrifuge and transfer supernatant
Repeat extraction with ACN & centrifuge
Supernatants combined and diluted with water

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Clean-up of extract by hexane wash and solid phase extraction

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

LC-MS/MS

Shimadzu LC–20AD Prominence, Agilent Poroshell LC column AB Sciex 4000 MS, Flow rate: 0.4 mL/ min
Mobile phases: A - 0.1% aq. formic acid, B - 0.1% formic acid in acetonitrile

Time program

Time (min)	0	7	7.1	13	13.1	20
Mobile Phase A	78	25	0	0	78	78
Mobile Phase B	22	75	100	100	22	22

LC-MS parameters

Compound	RT (min)	Precursor ion (m/z)	Product ions[1] (m/z)	DP (V)	CE (V)[2]	CXP (V)
Erythromycin A	2.4	734.7	158.2, 576.5, 116.2	78	44, 28, 66	12, 16, 9
Penicillin G	2.67	335.1	160.0, 176.0, 141.1	46	16, 19, 45	10, 11, 7
Virginiamycin M1	3.89	526.4	355.2, 337.1, 133.1	70	25, 29, 42	9, 9, 13
Virginiamycin S1	5.47	824.7	205.1, 177.2, 134.3	100	65, 97, 83	14, 10, 9
Erythromycin-(N-methyl-^{13}C, d_3)[3]	2.4	738.6	162.1	89	43	10
Penicillin G-d_7[4]	2.67	342.2	160.0	43	17	10
Virginiamycin M1-d_2[5]	3.89	528.5	357.2	73	26	9

[1]Quantitation ion is underlined. [2]CE and CXP are listed in order corresponding to the product ions. [3-5]Internal standards.

Results and Discussion

VIR M1-d2 suitability as an internal standard

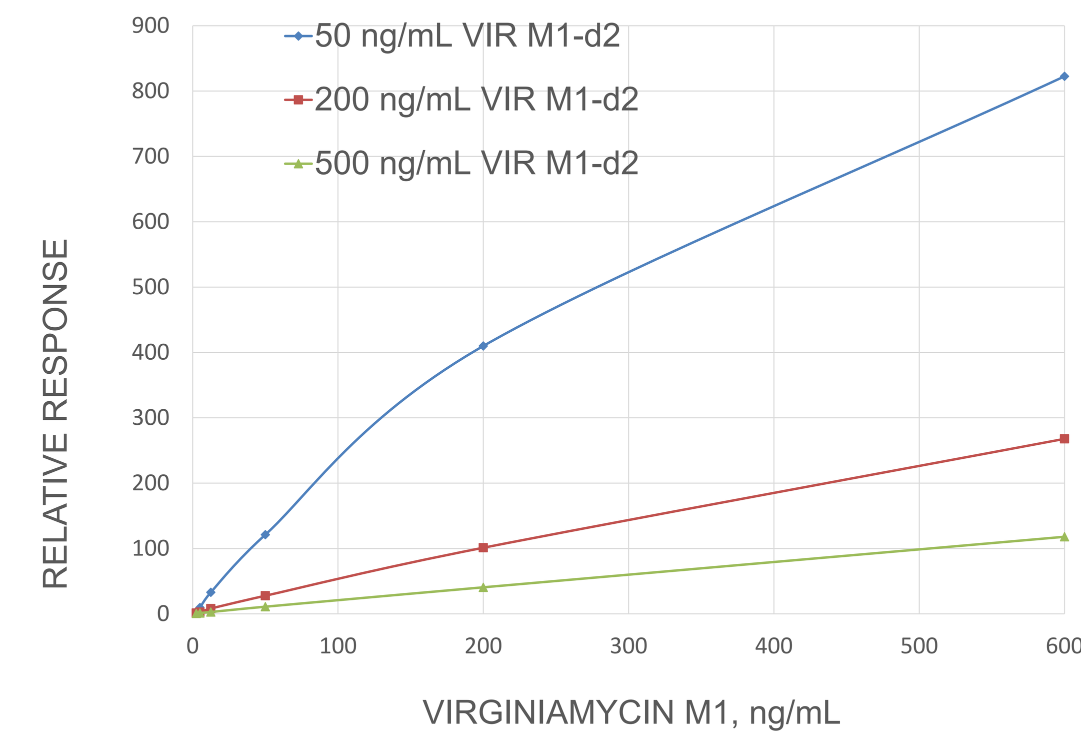

Figure 1. Calibration curves of peak area ratio between virginiamycin M1 and VIR M1-d2 against virginiamycin M1 concentration at different VIR M1-d2 levels (50, 200 & 500 ng/mL)

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VIR M1-d2 at 500 ng/mL level resulted in a good fit with a correlation coefficient 0.9999 (weighted (1/X) linear regression).

Quantitation of all analytes with solvent calibration curve vs. matrix calibration curve

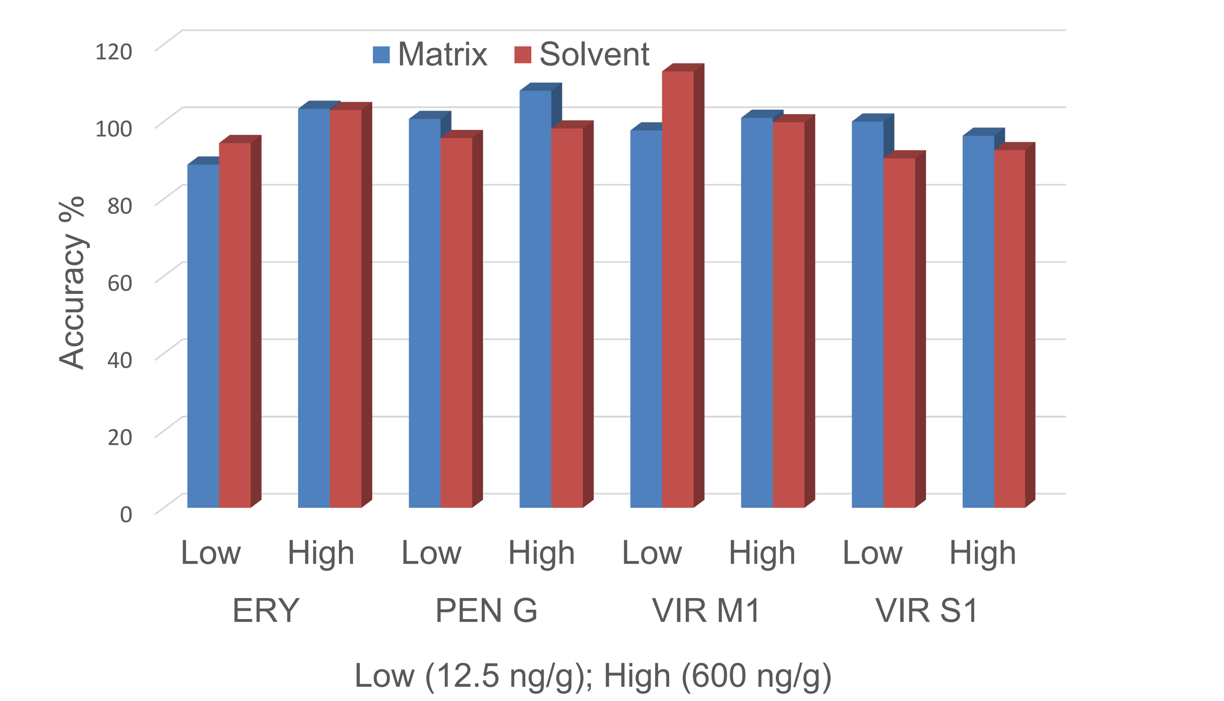

Figure 2. Accuracies of erythromycin, penicillin G, virginiamycin M1 and virginiamycin S1 fortified at 12.5 ng/g and 600 ng/g in DG and quantitated using calibration curves constructed in solvent (red) and in matrix (blue).

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For all compounds and fortification levels, recoveries obtained were comparable between solvent calibration curve and matrix calibration curve.

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After establishing, 1. applicability of 500 ng/mL VIR M1-d2 to quantitate virginiamycin M1 and, 2. accurate quantitation of all analytes using calibration curves constructed in solvent, we incorporated them in the method and validated it.

Method Attributes

Compound	ISTD	Accuracy & RSD[1] (in parenthesis)			
		0.01 µg/g, n= 6	0.10 µg/g, n= 6	1.0 µg/g, n= 6	All levels, n=18
Erythromycin A	Erythromycin-(N-methyl-^{13}C, d_3)	103 (5.3)	99 (3.6)	99 (3.3)	100 (4.4)
Penicillin G	Penicillin G-d_7	96 (5.6)	108 (2.9)	103 (3.6)	102 (6.3)
Virginiamycin M1	Virginiamycin M1-d_2	98 (3.4)	104 (1.6)	97 (1.7)	99 (3.8)
Virginiamycin S1	Virginiamycin M1-d_2	92 (7.5)	89 (6.2)	88 (7.5)	90 (6.8)

[1]Relative standard deviation

Compound	Inter-day accuracy and RSD[1] (in parentheses) for different matrices[2]		
	Matrix 1, n=6	Matrix 2, n=6	Matrix 3, n=6
Erythromycin A	99 (4.6)	99 (2.5)	103 (5.1)
Penicillin G	99 (5.9)	101 (7.1)	106 (4.7)
Virginiamycin M1	99 (4.6)	100 (3.1)	99 (4.3)
Virginiamycin S1	92 (7.8)	84 (2.7)	93 (1.6)

[1]Relative standard deviation. [2]Matrix 1, 2 and 3 are corn-based reduced-oil DG, corn DG and corn & milo DG, respectively, from different sources.

Conclusions

An optimal internal standard, virginiamycin M1-d2 , for virginiamycin M1 quantitation was successfully incorporated into the method.
It was established that, despite being only double-deuterated, virginiamycin M1-d2 can be used to quantitate virginiamycin M1 at an appropriate concentration.
This addition also allowed calibration curves for all analytes to be constructed in solvent rather than in matrix thereby simplifying the method.
Attributes of the method such as accuracy, precision, and correlation coefficient are all satisfactory for all analytes.
The enhanced method can support future surveillance studies to determine the drugs of interest in distillers grain.