Application Note 1055

Determination of Virginiamycin, Erythromycin, and Penicillin in Dried Distillers Grains with Solubles

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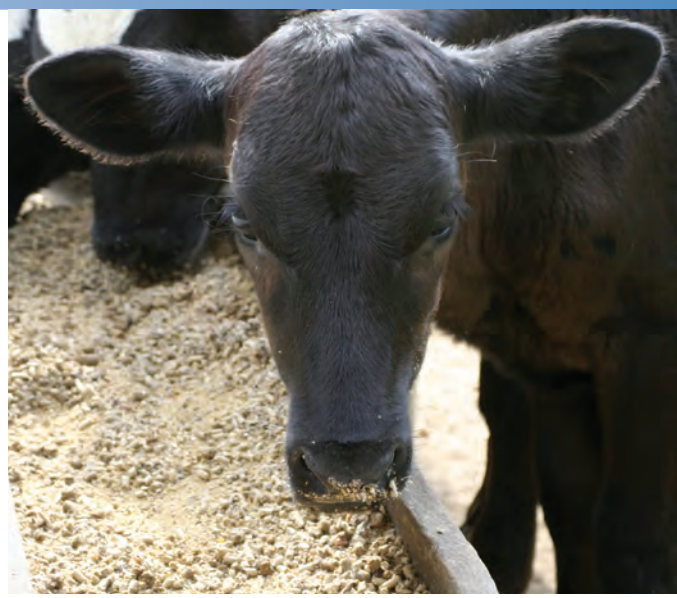
Key Words
Ethanol Coproduct, Bacterial Contamination, Animal Feed, Antibiotics, Acclaim 300 C18 Column, Charged Aerosol Detection



Introduction

Distillers grain (DG), a major coproduct of dry-grind ethanol processing, quadrupled in production during 2004–2005. It is currently the second largest category of processed feed in the U.S., with an estimated 35 million metric tons (tonnes) produced in 2011. Nearly 25% of U.S. DGs are exported and the primary markets are China, Mexico, and Canada. DG is a valuable product to the livestock industry because it is a rich source of protein, fat, minerals, and vitamins, thus making it an excellent feed supplement for livestock and poultry.[1–4] However, bacterial contamination from lactic acid-producing bacteria—such as *Lactibacillus*, *Lueconostoc*, and *Weissella*—is a concern for ethanol production facilities because bacteria compete with yeast for sugar and micronutrients. Antibiotics such as virginiamycin, penicillin, and erythromycin are commonly used during fermentation to inhibit bacterial growth.[5]

Although the U.S. Food and Drug Administration (FDA) is responsible for regulating all drugs and ingredients used for animal feed production, there is currently no active enforcement for antimicrobials used in DG products. The FDA has raised concern over food-producing animals consuming DGs with antibiotic residues, and how this may lead to increased antibiotic resistance in humans and animals. Therefore, to assess the amount of antibiotics in DGs and to meet possible future regulatory requirements, analytical methods are needed to determine residual antibiotics in DGs.

This study discusses the determination of penicillin G, erythromycin, and virginiamycin S_1 and M_1 in dried distillers grains with solubles (DDGS). These compounds (Figure 1) are the four major antibiotics used in ethanol production and belong to different antibiotic classes that include β-lactams, macrolides, and streptogramins. Their different physical and chemical properties increase the challenge to extract, separate, and detect these compounds in a single analysis.[6–8] For example, penicillin is hydrophilic in nature and therefore is easily extracted from DDGS with water. However, erythromycin and virginiamycin M_1 and S_1 are insoluble or only slightly soluble in water and therefore require an organic solvent for extraction.

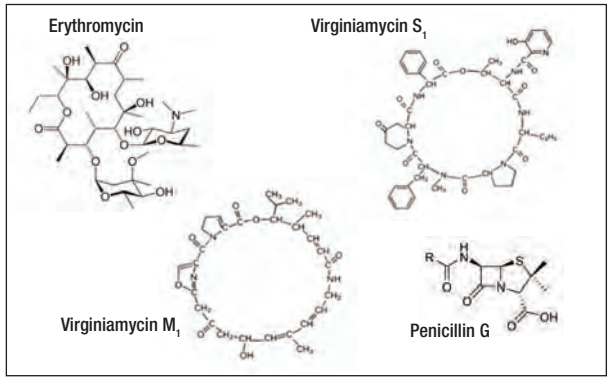


Figure 1. Structures of antibiotics used in the ethanol production process.

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The challenges are further magnified because UV detection lacks the sensitivity to detect some of these antibiotics because either they do not have a chromophore or have only a weak chromophore (e.g., virginiamycin and erythromycin). One approach to overcome this problem is to use a Thermo Scientific™ Dionex™ Corona™ ultra RS™ Charged Aerosol Detector. This mass-sensitive detector provides good sensitivity of nonvolatile and some semivolatile analytes that lack a strong chromophore.

After sample preparation, the four antibiotics are separated from the remaining components of the DDGS sample using a Thermo Scientific™ Acclaim™ 300 C18 column and then detected by charged aerosol detection. This method allows accurate determination of these antibiotics in DDGS.

Goal

To develop a method to determine erythromycin, penicillin, and virginiamycin S_1 and M_1 in DDGS using high-performance liquid chromatography (HPLC)

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system, including:
  - SRD-3600 Integrated Solvent and Degasser Rack, 6 Channels (P/N 5035.9230)
  - HGP-3400RS Binary Rapid Separation Pump with Solvent Selector Valves (P/N 5040.0046)
  - WPS-3000TRS Rapid Separation Wellplate Sampler, Thermostatted (P/N 5840.0020)
  - Sample Loop, 25 µL (P/N 6820.2415)
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- Corona ultra RS Charged Aerosol Detector (P/N 70-9406)
- Centrifuge equipped with a ten-place, aluminum fixed-angle rotor

Consumables

- Acclaim 300, C18, 3 µm, Analytical Column, 2.1 × 150 mm (P/N 060264)
- Thermo Scientific™ Dionex™ Viper™ UHPLC Fingertight Fitting incl. Capillary for 10-32 Fitting, i.d. 0.13 mm/0.005", Length 250 mm, SST (P/N 6040.2325)
- Viper UHPLC Fingertight Fitting incl. Capillary for 10-32 Fitting, i.d. 0.18 mm/0.007", Length 450 mm, SST (P/N 6040.2365)
- Mixer Kit to 400 µL Mixing Volume (P/N 6040.5310)
- Vial Kit, 1.5 mL, Glass with Caps and Septa (P/N 055427)
- Fisherbrand™ 20 mL Borosilicate Glass Scintillation Vials (Fisher Scientific P/N 03-337-14)
- Nitrogen, 4.5 grade (99.995%) or better, <5 ppm oxygen
- Serum Acrodisc® syringe filter, GF/0.2 µm, 37 mm, sterile (Pall Corporation P/N 4525)*

*Use the recommended filters only because the analytes of interest bind nonspecifically to syringe filters made from cellulose acetate.

Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistance or better
- Heptafluorobutyric Acid (HFBA), 99% (Fisher Scientific P/N AC17280-0250)
- Acetronitrile, LC/MS Grade (Fisher Scientific P/N AC61514-0025)
- Ethyl Acetate, HPLC Grade, 99.9% (Fisher Scientific P/N E195-1)
- Virginiamycin S_1, HPLC, ≥99% (Sigma-Aldrich® P/N V4140)
- Virginiamycin M_1, ~95% (Sigma-Aldrich P/N V2753)
- Penicillin-G Potassium Salt (Fisher Scientific P/N BP914-100)
- Erythromycin (Fisher Scientific P/N BP920-25)

Sample

The DDGS sample was provided as a generous gift from an industry source.

Conditions

Column:	Acclaim 300, C18, 3 µm, Analytical (2.1 × 150 mm)			
Mobile Phase A:	0.025:25:75 HFBA:Acetonitrile:Water			
Mobile Phase B:	Ethyl Acetate			
Mobile Phase C:	0.025:99.9 HFBA:Acetonitrile			
Gradient: Time (s)	% A	% B	% C	Curve
0.0	100	0.0	0.0	5
3.0	100	0.0	0.0	5
3.0	99.0	1.0	0.0	5
19.0	24.0	1.0	75.0	5
20.0	24.0	1.0	75.0	5
20.0	100	0.0	0.0	5
25.0	100	0.0	0.0	5
Flow Rate:	0.75 mL/min			
In. Volume:	10.0 µL			
Temperature:	25 °C			
Detection:	Charged aerosol, low filter, 60 Hz data collection rate, nebulizer temperature 15 °C			
Backpressure:	~450 bar (6750 psi)			
Baseline Noise:	~0.07 pA			
Run Time:	20 min			

Preparation of Solutions and Reagents

Acetonitrile, 25%

Carefully transfer 250 mL of HPLC-grade acetonitrile to approximately 500 mL of filtered and degassed DI water in a 1 L volumetric flask. Allow the solution to reach room temperature before bringing to volume with DI water. Invert the flask several times to mix.

Acetonitrile, 50%

Carefully transfer 500 mL of HPLC-grade acetonitrile to approximately 400 mL of filtered and degassed DI water in a 1 L volumetric flask. Allow the solution to reach room temperature before bringing to volume with DI water. Invert the flask several times to mix.

HFBA, 0.025%, in 25% Acetonitrile (Mobile Phase A)

Carefully transfer 250 mL of HPLC-grade acetonitrile to approximately 500 mL of filtered and degassed DI water in a 1 L volumetric flask. Transfer 250 µL of HFBA to the mix using a pipette. Allow the solution to reach room temperature before bringing to volume with DI water. Invert the flask several times to mix.

HFBA, 0.025%, in Acetonitrile (Mobile Phase C)

Carefully transfer 750 mL of HPLC-grade acetonitrile to a 1 L volumetric flask. Transfer 250 µL of HFBA to the acetonitrile using a pipette. Allow the solution to reach room temperature before bringing to volume using acetonitrile. Invert the flask several times to mix.

Stock Standard Solutions, 1 mg/mL

Prepare the penicillin stock solution by carefully weighing 20.0 mg of the solid into a preweighed glass vial. Add 20 mL of 25% acetonitrile to the penicillin to make a 1 mg/mL stock and mix the contents until the solid is dissolved. Follow the same procedure to prepare the erythromycin stock solution.

The virginiamycin standards were purchased in pre-weighed 5 mg amounts. Prepare the stocks for virginiamycin M_1 and S_1 by adding 2.5 mL of 50% acetonitrile to the preweighed vial directly, followed by vigorous mixing for 2 min. Add 2.5 mL of DI water to the mixture to make a 1 mg/mL stock in 25% acetonitrile. Diluted stock standards are stable for three months at -40 °C.

Working and Calibration Standards

To prepare working standards, use a calibrated pipette to deliver the appropriate volumes of the 1 mg/mL stock standard into a glass vial containing the appropriate volume of 25% acetonitrile. The working and mixed standards are stable for four weeks at 2–4 °C. Determine method linearity by diluting the stock solution to working standard solutions. Prepare calibration standards of 100, 50, 25, 10, 5, and 3 µg/mL for erythromycin, penicillin, and virginiamycin M_1 and S_1 as shown in Table 1. Prepare calibration standards daily.

Table 1. Preparation of calibration standards.

Antibiotic Concentration (µg/mL)	Volume of 100 µg/mL Penicillin Stock (µL)	Volume of 100 µg/mL Erythromycin Stock (µL)	Volume of 100 µg/mL Virginiamycin Stock, S_1 (µL)	Volume of 100 µg/mL Virginiamycin Stock, M_1 (µL)	Volume of 25% Acetonitrile (µL)	Total Volume (µL)
3.0	30	30	30	30	880	1000
5.0	50	50	50	50	800	1000
10	100	100	100	100	600	1000
Antibiotic Concentration (µg/mL)	**Volume of 1000 µg/mL Penicillin Stock (µL)**	**Volume of 1000 µg/mL Erythromycin Stock (µL)**	**Volume of 1000 µg/mL Virginiamycin Stock, S_1 (µL)**	**Volume of 1000 µg/mL Virginiamycin Stock, M_1 (µL)**	**Volume of 25% Acetonitrile (µL)**	**Total Volume (µL)**
25	25	25	25	25	900	1000
50	50	50	50	50	800	1000
100	100	100	100	100	600	1000

Sample Preparation (Spiked and Unspiked)

To prepare the DDGS sample, weigh 375 mg of solid, and then add 5 mL of DI water. Vortex the DDGS and water mixture for 1 min and then centrifuge at 7000 revolutions per min (rpm) for 10 min at 4 °C. Collect the supernatant in a glass vial.

Add 5 mL of 25% acetonitrile to the pellet and do a second extraction by vortexing for 1 min followed by centrifugation at 7000 rpm for 10 min at 4 °C. Add the supernatant to the first 5 mL to make a total volume of 10 mL.

To perform a third extraction, add 5 mL of 50% acetonitrile to the pellet. Vortex the mixture for 1 min followed by centrifugation at 7000 rpm for 10 min at 4 °C. Add the supernatant to the first 10 mL to make a total volume of 15 mL. Filter the samples using 0.2 µm glass sterile syringe filters and analyze within 24 h.

To prepare the spiked sample, weigh 375 mg of solid and then add the desired amount of each of the antibiotics directly onto the DDGS solid. Wait 2 min to allow the spiked antibiotic solution to be absorbed onto the DDGS sample. After approximately 2 min, perform three 5 mL extractions using water, 25% acetonitrile, and 50% acetonitrile as described above.

Precautions

After column installation, allow it to equilibrate at 49% A, 1% B, and 50% C for a minimum of 4 h prior to connecting it to the Corona ultra RS Charged Aerosol Detector. This will enable stable retention times and a low background.

Because the DDGS samples are a rich source of protein, fat, minerals, and vitamins, a column wash of 75% acetonitrile is necessary after every sample injection to ensure good run-to-run reproducibility.

Results and Discussion

Separation of Antibiotic Standards

The polar-embedded Acclaim PolarAdvantage II column and the Thermo Scientific™ Accucore™ PFP HPLC column were initially investigated for the separation of penicillin, erythromycin, and virginiamycin M_1 and S_1. These columns—while often excellent choices for the separation of polar compounds for which typical reversed-phase columns are poorly suited— demonstrated poor retention of penicillin, while erythromycin and virginiamycin S_1 were unresolved with poor peak shapes. However, the Acclaim 300 C18, 3 µm column—designed for fast, high-resolution separations of peptides and biological macromolecules—demonstrated that it could provide good resolution and peak efficiencies for these antibiotics.

The Corona ultra RS Charged Aerosol Detector, a universal detector capable of measuring nonvolatile and semivolatile compounds, was used for this study. When compared to other universal HPLC detectors such as refractive index (RI) and evaporative light scattering (ELSD), the Corona ultra RS Charged Aerosol Detector has greater sensitivity, has a larger linear calibration range, and can accommodate gradients, unlike the RI detector.

The Corona ultra RS Charged Aerosol Detector is a nebulizer-based detector that provides a consistent response for all nonvolatile and some semivolatile analytes. A major requirement for this detector is that the mobile phase be volatile. That generally means an aqueous/organic solvent mixture must be used and only volatile ion-pairing agents (e.g., HFBA and trifluoroacetic acid) can be added to the mobile phase. Because penicillin is poorly retained, a starting mobile phase concentration of 25% acetonitrile/0.025% HFBA was used to improve retention from the void. The mobile phase strength was then increased to a higher concentration of acetonitrile with 1% ethyl acetate added to the mobile phase to elute the more strongly retained erythromycin, virginiamycin S_1, and virginiamycin M_1.

Figure 2 shows a separation of the four antibiotics in the mixed standard using the Acclaim 300 column. The retention times of penicillin, erythromycin, virginiamycin M_1, and virginiamycin S_1 are 3.0, 5.3, 6.7, and 9.3 min, respectively. All antibiotics are well resolved with a total analysis time of 25 min.

Column:	Acclaim 300 C18, 3 µm, Analytical (2.1 × 150 mm)	Detection:	Charged aerosol, low filter, 60 Hz data collection rate, nebulizer temperature 15 °C
Eluent:	A: 0.025% HFBA in 25% CH_3CN B: Ethyl Acetate C: 0.025% HFBA in CH_3CN	Sample:	Mixed Antibiotic Standard
Gradient:	0.0–3.0 min 100% A, 3.0–19.0 min 1.0% B/75% C, hold at 1.0% B/75% C for 1 min, step change at 20.0 min 100% A, hold at 100% A for 5 min	Peaks: 1. Penicillin 2. Erythromycin 3. Virginiamycin M_1 4. Virginiamycin S_1	 50.0 µg/mL 50.0 50.0 50.0
Flow Rate:	0.75 mL/min		
Inj. Volume:	10.0 µL		
Temperature:	25 °C		

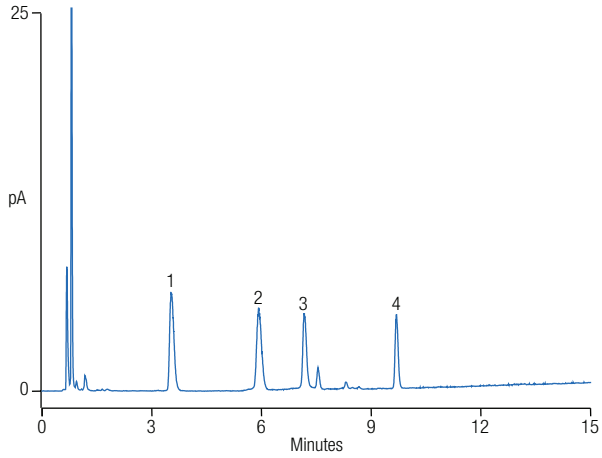

Figure 2. Chromatogram showing the separation of a mix of four antibiotics on the Acclaim 300 C18 column using charged aerosol detection.

Table 2. Calibration data, LODs, and LOQs of penicillin, erythromycin, virginiamycin M_1, and virginiamycin S_1.

Analyte	Range (µg/mL)	Coefficient of Determination (r^2)	LOD[a] (µg/mL)	LOQ[b] (µg/mL)	RSD		
					Retention Time (n = 7)	Peak Area (n = 7)	Peak Height (n = 7)
Penicillin	3.0–100	0.9993	1.0	3.0	0.19	0.98	0.73
Erythromycin	5.0–100	0.9998	1.8	5.2	0.27	0.87	0.91
Virginiamycin M_1	5.0–100	0.9996	1.6	5.1	0.15	0.68	0.95
Virginiamycin S_1	5.0–100	0.9997	1.5	5.0	0.11	0.96	0.74

[a]Estimated from 3× S/N
[b]Estimated from 10× S/N

System Suitability

The linearity, limits of detection (LODs), and limits of quantification (LOQs) were evaluated to establish the suitability of the method for determining the four antibiotics in DDGS. A literature search was conducted to determine the appropriate calibration ranges.[1] Penicillin, erythromycin, virginiamycin M_1, and virginiamycin S_1 exhibited linear peak area responses in the ranges summarized in Table 2.

According to *Ethanol Producer Magazine*, 3–5 pounds of antibiotics—usually virginiamycin—per 500,000 gallons of corn mash is added to the fermenter during ethanol production. The amount of antibiotic added is increased if the amount of lactic acid present in the fermentation broth is higher than normal.[9] This results in a final virginiamycin concentration of 0.72–1.2 µg/mL. During the ethanol production process, the antibiotic concentration is increased from 3× to 5× the starting concentration; thus, the amount of virginiamycin and other residual antibiotics in the distillers grain can range from 2.16 to 6.0 µg/mL.

The LODs for the antibiotics were determined based on the concentration of the analytes that provided a peak height of 3× the measured noise (signal-to-noise ratio [S/N] = 3). The LOQs were determined based on the concentration of the analyte that provided a peak height of 10× the measured noise (S/N = 10). The LODs ranged from 1.0 µg/mL for penicillin to 1.8 µg/mL for erythromycin, and the LOQs ranged from 3.0 µg/mL for penicillin to 5.2 µg/mL for erythromycin.

This method can quantify antibiotics at the levels expected in DDGS samples at the end of the ethanol production process. Drying the DDGS extract and resuspending it in a smaller volume will enable quantification of antibiotics in ranges lower than that described above.

Seven consecutive injections were performed using a mixed antibiotic standard at a concentration of 12.5 µg/mL for all four antibiotics. Retention time precisions of the antibiotics were excellent, with RSDs ranging from 0.11% for virginiamycin S_1 to 0.27% for erythromycin. This demonstrated good precision of the gradient delivered by the HPG-3400RS UltiMate 3000 pump. Peak area precision ranged from 0.68% for virginiamycin M_1 to 0.98% for penicillin, while peak height precision ranged from 0.73% for penicillin to 0.95% for virginiamycin S_1 for the replicate injections.

Sample Analysis

The four antibiotics have different solubilities, which had to be considered when developing the extraction method to ensure good recovery of each antibiotic from the sample. The first step of the extraction used water to facilitate the removal of penicillin from DDGS. The second and third extractions used increasing concentrations of acetonitrile to improve the recoveries of erythromycin and virginiamycin, respectively.

The spiked samples were prepared by adding a known amount of each of the antibiotics directly onto the DDGS solid, as described in the Sample Preparation section. The three successive 5 mL extractions using water, 25% acetonitrile, and 50% acetonitrile were performed after the DDGS sample was spiked. Figure 3A shows a chromatogram of an unspiked DDGS sample with no antibiotics detected. To verify that the matrix did not interfere with the separation and quantification of the antibiotics, the sample was spiked with 12.5 µg/mL of each of the four antibiotics. Figure 3B shows a chromatogram of the spiked DDGS sample with no observed matrix-related interferences.

Sample Precision and Accuracy

The DDGS sample was extracted three separate times per day over three days. For each extraction the sample was spiked directly on the solid material with known amounts of the antibiotics to evaluate the precision and accuracy. These data are summarized in Tables 3 and 4. For the extractions performed in this study, the intraday peak area RSDs ranged from 0.45% for virginiamycin M_1 to 0.78% for virginiamycin S_1. The between-day peak area RSDs ranged from 1.08% for virginiamycin M_1 to 1.45% for virginiamycin S_1. As shown in Table 4, the recoveries ranged from 91.8% for penicillin to 105% for virginiamycin S_1, demonstrating good method accuracy.

Column:	Acclaim 300 C18, 3 µm, Analytical (2.1 × 150 mm)	Detection:	Charged aerosol, low filter, 60 Hz data collection rate, nebulizer temperature 15 °C
Eluent:	A: 0.025% HFBA in 25% CH_3CN B: Ethyl Acetate C: 0.025% HFBA in CH_3CN	Samples:	(A) Unspiked DDGS (B) Spiked DDGS
Gradient:	0.0–3.0 min 100% A, 3.0–19.0 min 1.0% B/75% C, hold at 1.0% B/75% C for 1 min, step change at 20.0 min 100% A, hold at 100% A for 5 min	Peaks:	
		1. Penicillin	12.5 µg/mL
		2. Erythromycin	12.5
		3. Virginiamycin M_1	12.5
		4. Virginiamycin S_1	12.5
Flow Rate:	0.75 mL/min		
Inj. Volume:	10.0 µL		
Temperature:	25 °C		

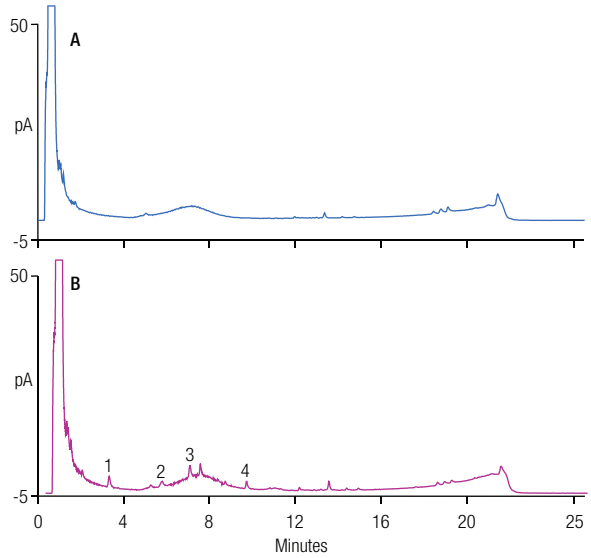

Figure 3. Chromatograms showing the separation of (A) an unspiked DDGS sample and (B) a DDGS sample spiked with a mix of the four antibiotics using the Acclaim 300 C18 column with charged aerosol detection.

Table 3. Spiked sample analysis for intraday and between-day precision.

Analyte	Amount (µg/mL)	Intraday Precision	Between-Day Precision
		Peak Area RSD (n = 3)	Peak Area RSD (n = 9)
Penicillin	12.5	0.65	1.32
Erythromycin	12.5	0.70	1.35
Virginiamycin M_1	12.5	0.45	1.08
Virginiamycin S_1	12.5	0.78	1.45

Table 4. Recovery of the antibiotics in DDGS.

Analyte	Amount Spiked (µg/mL)	Recovery (%)
Penicillin	12.5	91.8
Erythromycin	12.5	97.4
Virginiamycin M_1	12.5	103
Virginiamycin S_1	12.5	105

Conclusion

This study describes a simple and accurate method to separate and quantify antibiotics in DDGS using a multistep sample extraction. This method demonstrates the ability of the Acclaim 300 column to efficiently separate and resolve the target antibiotics within 15 min, followed by column cleanup and equilibration for a total analysis time of 25 min. The method is ideal for routine screening and quantification of erythromycin, penicillin, and virginiamycin M_1 and S_1 in DDGS.

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