

**Analysis of Antibiotics in Distillers Grains
Using Liquid Chromatography and Ion Trap Tandem Mass Spectrometry**

David N. Heller,* G.K. Hemakanthi de Alwis

Office of Research, Center for Veterinary Medicine, Food and Drug Administration

*corresponding author: david.heller@fda.hhs.gov, 301-210-4579

Administrative Note

The Laboratory Information Bulletin is a tool for the rapid dissemination of laboratory methods (or information) which appear to work. It may not report completed scientific work. The user must assure him/herself by appropriate validation procedures that LIB methods and techniques are reliable and accurate for his/her intended use. Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

Abstract

A method has been developed for the screening, confirmation and determination of thirteen antibiotics in distillers grains (DG): streptomycin, ampicillin, tetracyclines (oxytetracycline, tetracycline, chlortetracycline), bacitracin A, erythromycin, tylosin, chloramphenicol, clarithromycin, penicillin G, virginiamycin M1 and monensin. The method was validated with ion trap tandem mass spectrometry. Samples of dry DG (DDG) were used as negative controls and fortified controls to assess method performance. In this matrix, extraction efficiency ranged from 65 – 97%, and limits of quantitation ranged from 0.1 – 1.0 µg/g (ppm), depending on the compound. The method's quantitative range was assessed over the 0.1 – 10 ppm range using extracted matrix standards. Method accuracy (based on calibration with extracted matrix standards) ranged from 88 – 111% with CVs ranging from 4 – 30%, depending on compound. This method was also tested in distillers wet grains (DWG), and corn syrup residue from ethanol production (DG solubles, syrup, or stillage). Method performance in those matrices was comparable to DDG.

Introduction

The increased production of ethanol for fuel has led to large amounts of distillers grains becoming available for animal feed. Distillers grains (DG) are essentially the remainder of corn or other grains once the starch has been removed by fermentation. DG may be available wet, dried, or as the soluble fraction. Their use as animal feed ingredients has become commonplace because DG has significant nutritional content. However, any antimicrobial processing aids (i.e., antibiotics) remaining from the fermentation process could be a cause for regulatory concern if DG is fed to animals.

Antibiotics are sometimes used to prevent unwanted bacterial growth in the fermentation process. In this case, the compounds are referred to as antimicrobial processing aids. The Office of Research developed a new chemical method to test DG for residues of antibiotics, in order to provide better information on the types and amounts of such residues that might occur in DG. The method relies on analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The sample extraction is capable of recovering a wide range of compounds. However, due to the unique chemical properties of streptomycin, this aminoglycoside (out of the thirteen in the target list) has to be cleaned up and analyzed in a separate track from the other tested compounds.

Summary of Procedure

Samples are not dried before extraction. Distillers Grains (5 g) are extracted with an aqueous solution of EDTA and trichloroacetic acid followed by methanol. The combined extract is diluted with water and an aliquot is cleaned up on an Oasis HLB solid phase extraction (SPE) cartridge for ampicillin, tetracyclines, bacitracin, erythromycin, tylosin, chloramphenicol, clarithromycin, penicillin G, virginiamycin M1 and monensin. A second aliquot is basified with sodium hydroxide, and subjected to SPE cleanup on an Isolute CBA cartridge for streptomycin extraction. Extracts from the Oasis HLB method are analyzed by reverse phase liquid chromatography on a phenyl-bonded silica LC column. Detection is carried out by electrospray ionization and tandem mass spectrometry on an ion trap mass spectrometer. Extracts from the CBA method are analyzed by hydrophilic interaction liquid chromatography (HILIC) on a silica LC column. Detection is carried out by atmospheric pressure chemical ionization and tandem mass spectrometry on an ion trap mass spectrometer. Moisture analysis is carried out on a separate aliquot of sample, using AOAC Method 934.01 - Loss on drying (moisture) for Feeds (Vacuum oven, 95 - 100 C).

Equipment

- Liquid chromatograph – Agilent 1100 series binary pump and autosampler with refrigerated sample tray; or equivalent.
- Mass spectrometer – Thermo Finnigan LCQ Deca XP Plus (ion trap) equipped with electrospray and atmospheric pressure chemical ionization interfaces.
- Liquid chromatograph columns (Waters Corporation, Milford, MA.):
 - Phenyl cartridge column, 4.0 x 50 mm, 3 micron, 120 A, Cat. No. PH12S030504WTA, with end fittings, Cat. No. XPEF43WTI.
 - Atlantis HILIC Silica column, 2.1 x 100 mm, 5 micron, Cat. No. 186002014.
- Polypropylene Centrifuge tubes:
 - 15 mL and 50 mL, Cat. Nos. 21008089 and 21008240, (VWR Scientific),
 - 225 mL, Cat. No. 352075, (BD Falcon, Franklin Lakes, NJ).
- Solid phase extraction (SPE) cartridges:
 - Oasis HLB, 150 mg/6 mL (Waters Corporation Cat. No. 186003365),
 - Isolute CBA cartridges, 500mg/ 3mL (Biotage, Charlottesville, VA, Cat. No. 520-0050-B).

- 20 mL reservoirs –Varian Inc., Bond Elut, Cat # 12131011.
- Polyethylene frits - Supelco, Cat # 57183.
- TurboVap LV Concentration Workstation (Zymark).
- Volumetric flasks, glass, 10 and 100 mL, class A.
- Volumetric flasks, polypropylene, 25 and 50 mL.
- Calibrated variable pipettors, 500-5000 μ L, 100-1000 μ L and 10-100 μ L (Eppendorf).
- Autosampler vials, 2 mL capacity:
 - Amber glass, for Oasis HLB extracts
 - Polypropylene, for CBA extracts containing streptomycin
- Centrifuges - IEC Centra GP8R, with 218A rotor (International Equipment Co.). HermLe Z 230 A MKII benchtop for up to 5500 RPM.
- Vacuum manifold for SPE (Supelco Inc.)
- Horizontal laboratory shaker (HS501 digital, IKA Works, Inc., Wilmington, NC)
- Mini vortexer – VWR Scientific Products
- pH meter. – Fisher Scientific Accumet BASIC
- Syringe filters. – Cat. No. 09-911-8, PTFE, 0.45 μ m pore size, 13 mm (Whatman).
- Luer-slip plastic syringe, 3 mL – National Scientific Co.

Reagents

- Antibiotic Standards: Streptomycin sulfate, ampicillin sodium, bacitracin, erythromycin, tylosin tartrate, chloramphenicol, virginiamycin M₁ (Sigma-Aldrich, St. Louis, MO); Oxytetracycline, tetracycline hydrochloride, chlortetracycline hydrochloride, clarithromycin, penicillin G potassium (USP, Rockville, MD); Monensin sodium (Riedel-de Haem)
- EDTA, disodium salt dihydrate, ACS grade – Fisher Scientific
- Trichloroacetic acid, ACS grade, 99+% – Fisher Scientific
- Methanol, LC grade – Burdick & Jackson.
- Acetonitrile, LC grade – Burdick & Jackson.
- Water – Processed with the Milli-Q system to give resistivity > 18 M Ω -cm (Millipore). Use for all subsequent references to water.
- Formic Acid, 95%, Reagent grade – Cat. No. F0507, Sigma Aldrich.
- Sodium hydroxide, ACS grade – Sigma-Aldrich
- Glacial acetic acid – Fisher Scientific.

Preparation of Standards and Reagent Solutions:

Preparation of 1500 µg/mL stock standards.

Weigh about 15 mg of each drug into a 10 mL volumetric flask. Dissolve and make up to the mark with the given solvent (Table 1). Calculate the concentration of each standard, correcting for the purity and salt content. NOTE: streptomycin solutions must be stored in polypropylene, not glass. Stability period: one year under specified storage conditions.

Table 1: Stock Standard Solutions

	Solvent	Concentration, µg/mL	Storage
Ampicillin	water	1500	-15 °C
Bacitracin	water	1500	-15 °C
Chlortetracycline	methanol	1500	-15 °C
Erythromycin	water	1500	-15 °C
Oxytetracycline	methanol	1500	-15 °C
Penicillin G	water	1500	-80 °C
Tetracycline	methanol	1500	-15 °C
Streptomycin	water	1500	-15 °C
Clarithromycin	water	1500	-15 °C
Virginiamycin M ₁	methanol	1500	-80 °C
Chloramphenicol	methanol	1500	-15 °C
Tylosin	water	1500	-15 °C
Monensin	methanol	1500	-15 °C

Penicillin standards should be subdivided into 2 mL aliquots in 2 mL glass autosampler vials, and frozen at -80 °C. Vials should be removed as needed and discarded after use.

Preparation of 100 µg/mL mixed standard of 13 analytes from 1500 µg/mL stock standards:

Transfer a volume equivalent to 2500 µg of analyte from each individual stock solution into a 25 mL **polypropylene** volumetric flask and make up to volume with water. Subdivide in 5 mL aliquots into 15 mL polypropylene centrifuge tubes and store at -80 °C. Once thawed, the mixed standard should be immediately replaced in the -80 °C freezer. Once an aliquot has been thawed and refrozen five times, it should be discarded.

Preparation of calibration standards in polypropylene volumetric flasks: Use the 100 µg/mL mixed standard.

Table 2: Mixed Standard Dilutions

Mixed Standard to dilute ($\mu\text{g/mL}$)	Volume of the mixed standard to dilute, mL	water (mL)	Prepared calibration std ($\mu\text{g/mL}$)	ID
			100	S-8
100	37.5	12.5	75	S-7
75	33.3	16.7	50	S-6
50	25.0	25.0	25	S-5
25	20.0	30.0	10	S-4
10	25.0	25.0	5	S-3
5	25.0	25.0	2.5	S-2
2.5	20.0	30.0	1	S-1

Subdivide into 5 - 10 mL aliquots in 15 mL polypropylene centrifuge tubes and store at $-80\text{ }^{\circ}\text{C}$. Once thawed and used, the mixed standard should be immediately replaced in the $-80\text{ }^{\circ}\text{C}$ freezer. Once an aliquot has been thawed and refrozen five times, it should be discarded.

Preparation of Reagents and Mobile Phase Solutions

- 1.5 mM EDTA. Dissolve 0.56 g sodium EDTA dihydrate in 1000 mL water.
- 1% Trichloroacetic acid. Dissolve 10 g trichloroacetic acid in 1000 mL water.
- Trichloroacetic acid in water (pH 4.0). Add few milliliters of 1% trichloroacetic acid to water to get a pH of 4.0.
- 1 M NaOH. Dissolve 4 g NaOH in 100 mL water.
- Eluant for SPE on CBA cartridge. Mix 20 mL glacial acetic acid, 780 mL water and 200 mL ACN.
- Mobile phase: 0.1% Formic Acid in water (w/v). Dissolve 0.877 mL formic acid in 1000 mL water.

Instrument Parameters

Liquid chromatography, phenyl-bonded silica LC for Oasis HLB extract:

- Autosampler tray temperature: 5 °C
- Column temperature: 30 °C.

Table 3: Phenyl-Bonded LC Method

Time, min	Flow Rate, $\mu\text{L}/\text{min}$	% Mobile Phase A (0.1% aqueous formic acid)	% Mobile Phase B (ACN)
0	450	87	13
7.5	450	60	40
9.5	450	30	70
10.5	450	5	95
14	450	5	95
16	450	87	13
20	450	87	13

Electrospray Tandem Mass Spectrometry (Thermo LCQ Deca XP Plus)

- Divert flow from ion source from 0-2.75 min and 15-19 min.
- Time Segments are one minute wide and are keyed to retention time. For example, Segment 4 lasts from 3.0 – 4.0 min, Segment 7 from 6.0 – 7.0 min.
- Tuning conditions (use for all time segments): capillary temperature 325 °C, spray needle voltage 5 kV, Sheath gas 55, Auxiliary gas 20, maximum injection time 50 ms.
- Other acquisition parameters represent optimum conditions found by infusing each compound individually.
- Tuning optimization: Infuse 10 $\mu\text{g}/\text{mL}$ standard solution into mobile phase at 450 $\mu\text{L}/\text{min}$, post-column, as follows:
 - Time Segments 1 – 4, use conditions optimized by infusing ampicillin with Mobile Phase A:acetonitrile 87:13.
 - Time Segments 5 – 7, use conditions optimized by infusing tetracycline with Mobile Phase A:acetonitrile 80:20.
 - Time Segments 8 – 13, use conditions optimized by infusing virginiamycin M1 (positive ions) and chloramphenicol (negative ions) with Mobile Phase A:acetonitrile 60:40.
 - Time Segment 14, use conditions optimized by infusing monensin with Mobile Phase A:acetonitrile 30:70.

Table 4: Phenyl-Bonded LC Acquisition Parameters

Compound	RT, min	Precursor ions, m/z	CE	Isol'n Width	Diagnostic Product Ions, m/z	Scan range	Time Segments
Ampicillin	3.6	+ 350	26	5	160 ^a , 174, 106	100 - 200	3, 4, 5
Oxytetracycline	3.9	+ 461.1	44	4 w	398, 408, 426 ^a	300 - 450	4, 5
Tetracycline	4.8	+ 445.0	46	3 w	154, 392, 410 ^a	120 - 435	4, 5, 6
Chlortetracycline	6.8	+ 479.1	34	3 w	154, 444 ^a	130 - 460	6, 7, 8
Bacitracin	7.6	++ 712.1	30	3	554.2, 669.4 ^a , 869.4	300 -1000	7, 8
Chloramphenicol	8.2	- 321	34	4	152, 176, 194 ^a , 257	85 - 310	8, 9
Erythromycin	8.5	+ 734.2	28	5	522, 576 ^a	200 - 725	8, 9
Tylosin	9.4	+ 916.3	28	5	772.2 ^a , 407, 598.2	250 - 900	9, 10
Dehydro-Erythromycin	9.6	+ 716.2	28	5	558 ^a , 540, 522	195 - 710	10
Clarithromycin	9.8	+ 748.2	36	3	558, 590 ^a	205 - 735	10, 11
Penicillin	10.0	+ 335	38	5	114, 160 ^a , 176 ^a	100 - 200	10, 11
Virginiamycin M1	11.5	+ 526.1	40	4 w	260, 337, 355 ^a	140 - 515	11, 12, 13
Monensin	13.8	+ 693.4	44	2 w	461.2, 581.3, 657.3 ^a	400 - 680	14

Key + positive ion mode
 ++ doubly-charged ion
 - negative ion mode
 w wideband oscillation on
 CE collision energy (arbitrary units)
^a Required for "screening" identification. (NOTE: Every listed ion is required for "confirmed" identifications.)

Liquid chromatography, silica HILIC for CBA extract:

- Autosampler tray temperature: 5 °C
- Column temperature: 30 °C.

Table 5: HILIC Silica LC Method

Time, min	Flow Rate, $\mu\text{L}/\text{min}$	Mobile Phase A (0.1% aqueous formic acid)	Mobile Phase B (ACN)
0	325	30	70
6.5	325	95	5
7.5	325	95	5
8.5	325	30	70
11	500	30	70
14.5	500	30	70

Atmospheric pressure chemical ionization (APCI) Tandem Mass Spectrometry (Thermo LCQ Deca XP Plus)

- Divert flow from ion source from 0 - 3.5 min and 8.5 – 14.5 min.
- Tuning conditions: vaporizer temperature 475 °C, capillary temperature 175 °C, discharge current 5 µA, Sheath gas 60, Auxiliary gas 10, maximum injection time 100 ms.
- Tuning optimization: Infuse 10 µg/mL streptomycin standard solution, post-column, into a 50:50 mixture of Mobile Phase:acetonitrile at 325 µL/min.

Table 6: HILIC Silica Acquisition Parameters

Compound	RT, min	Precursor ions, m/z	CE	Isol'n Width	Diagnostic Product Ions, m/z	Scan range
Streptomycin	5.87	+582.2	35	1.6 w	263, 246, 407	160 - 550

Sample Preparations

Critical Step: Samples of DG should be kept stored at < -15 °C, whether dry DG, wet DG or DG solubles. Otherwise some antibiotics are susceptible to degradation prior to extraction.

Samples are not dried prior to extraction, but are used “as is.”

Each batch must include at least one negative control sample, quality control sample (fortified control), and appropriate matrix calibration standards. These are prepared from a dry DG sample which has been previously analyzed without yielding any interfering signals for any compound (used for all DG samples: wet, syrup, with solubles, etc.)

Preparation of blanks, matrix standards and quality control (QC) samples is given in Worksheet # 1. A typical Batch Preparation Worksheet is given in Worksheet # 2.

- Weigh 5 g of Distillers Grains into a 50 mL centrifuge tube.
- Add the appropriate volume of the relevant Mixed Standard to prepare calibration standards and fortified control samples in the range 0.1 - 10 µg/g. Refer to Table #2 and Worksheet #1 for the Mixed Standard required for a specified concentration of matrix standard or quality control sample, for example:
 - Use 500 µL standard S-4 to prepare matrix standard M-4, equivalent to 1.0 µg/g in DG.
 - Use 300 µL standard S-5 to prepare a mid-range quality control sample (QCM) equivalent to 1.5 µg/g in DG.
- Carefully vortex and let the samples stand for 10 min for equilibration.
- Add 20 mL 1.5 mM EDTA.
- Add 20 mL 1% TCA.

NOTE: When extracted Distillers Wet Grains or DG Solubles, volumes may be reduced to 10 mL to compensate for the smaller amount of dry matter.

- f. Shake for 15 min on a laboratory shaker.
- g. Centrifuge at 4000 RCF for 10 min.
- h. Transfer supernatant to a 225 mL centrifuge tube and dilute immediately to about 150 mL with water so acid concentration in the extract is low.
- i. Repeat extraction with 30 mL methanol, centrifuge and transfer supernatant to the 225 mL tube containing first extract.
- j. Dilute extract to 200 mL with water and vortex. Note: Use weight measurement for dilution since it is difficult to measure 200 mL accurately in the falcon tube. First weigh 200 mL extract diluted in a volumetric flask, and use this weight for dilution of samples.
- k. Use 10 mL for SPE with Oasis HLB.
- l. For SPE with Isolute CBA cartridges: Just before the SPE step, transfer 20 mL of extract to 50 mL centrifuge tube, adjust pH to 7-8 with 1 M NaOH (few drops), and centrifuge at 4000 RCF for 15 min. Use 10 mL supernatant.

Solid phase extraction with Oasis HLB cartridges:

- a. Condition the Oasis HLB column with 3 mL methanol and 3 mL dilute trichloroacetic acid (pH ~4.0).
- b. Attach 20 mL reservoirs fitted with frits on top of the SPE cartridges.
- c. Pass 10 mL of sample through the column slowly.
- d. Dry for 5 min under vacuum @~20 inHg.
- e. Wash with 5 mL water.
- f. Dry for 5 min under vacuum @~20 inHg.
- g. Elute with 2.5 mL x 2 methanol into 15 mL tube.
- h. Evaporate eluate down to about 1 mL on TurboVap under nitrogen (12 psi) at 35 °C.
NOTE: The bath temperature should not exceed 35 °C as degradation of some analytes may occur.
- i. Dilute to 2 mL with diluent (87:13 water:acetonitrile) and vortex for 1 min.
- j. If samples are cloudy, filter through PTFE syringe filter into autosampler vial.
- k. Prepare a comparison standard equivalent to a 1 µg/g DG sample by combining 25 µL standard S-4 and 1975 µL diluent (87:13 water:acetonitrile) in a 2 mL autosampler vial (final concentration 0.125 µg/mL).
- l. Inject 50 µL for LC/MS/MS analysis.
NOTE: There is a dilution factor of 8. That is, a 1 µg/g DG sample is equivalent to an injection solution at 0.125 µg/mL.

Solid phase extraction with Isolute CBA cartridges:

- a. Condition the CBA cartridge with 3 mL methanol and 3 mL water.
- b. Attach 20 mL reservoirs.
- c. Pass 10 mL sample slowly.
- d. Dry for 1 min.
- e. Wash with 4 mL water and 4 mL ACN.
- f. Dry for 1 min.

- g. Elute twice with 2.5 mL each time, using the CBA SPE eluant (1:4 acetonitrile: aqueous acetic acid, 2.5%).
- h. Evaporate extracts in TurboVap at 50 °C at 12 psi to < 1.5 mL (~80 min).
NOTE: The HILIC chromatography is sensitive to the water:acetonitrile balance in the injection solution. If water has not been consistently evaporated to < 1.5 mL before adding acetonitrile, the retention time may show significant sample-to-sample variability during batch analysis.
- i. Dilute to 5 mL with ACN.
- j. Vortex and transfer to autosampler vial.
- k. Prepare a comparison standard equivalent to a 1 µg/g DG sample by adding 25 µL standard S-4 to 5 mL acetonitrile (final concentration 0.050 µg/mL).
- l. Analyze on LCQ, injection volume 60 µL.
NOTE: There is a dilution factor of 20. That is, a 1 µg/g DG sample is equivalent to an injection solution at 0.050 µg/mL.

Procedure for Instrumental Analysis of Samples, Controls and Standards

- Extracts may be stored up to 48 hours in amber autosampler vials at 5 °C.
- Make several injections of comparison standard to equilibrate the LC/MS system and establish System Suitability: at least 2 for the phenyl LC method and at least 6 for the HILIC method.
- Inject the matrix standard curve from low to high, two solvent blanks, up to 24 extracts (including controls, QCs, and test samples), then, two solvent blanks and repeat the standard curve. Inject one solvent blank prior to a control extract.
- [For either column] After the run, flush for at least 20 minutes with acetonitrile and water (90+10). This mixture may also be used to store the column.

Data Treatment.

Data Treatment includes five stages: evaluate system suitability, prepare standard curves, prepare comparison chromatograms and mass spectra, evaluate qualitative acceptability, and evaluate quantitative acceptability. Specific steps are described below. The process is summarized in the Flow Charts (Figures 4a and 4b).

1. Evaluate system suitability.
 - a. Evaluate the last of the comparison standards injected before the batch. Signals for every diagnostic product ion should meet signal-to-noise (4.b) and peak shape (4.c.) criteria. Otherwise, the batch is not acceptable for that compound. The sequence may be reinjected after taking steps to improve instrument performance (eg, by cleaning source or retuning).
 - b. If the control sample meets screening or confirmation criteria for any compound, the batch is not acceptable for screening or confirming that compound, respectively.

- c. If the fortified control sample (Quality Control, or QC sample) does not meet screening or confirmation criteria, the batch is not acceptable for screening or confirming (respectively) that compound at that fortification level.
2. Prepare standard curves.
 - a. Create a reconstructed ion chromatogram (RIC) for each matrix standard by combining all the diagnostic products ions listed in Table 4, applying a five-point smooth, and integrating.
 - b. Prepare a calibration curve using a quadratic fit and 1/x weighting, excluding the origin, and including both bracketing standard curves and applying to all samples within the bracket.
 - c. Quantitative results are reported as the measured value in $\mu\text{g/g}$ or ppm sample, based on starting weight “as is” (without drying first).
 - d. Evaluate the calibration curve for linearity and make adjustments if necessary, as follows:
 - e. $R^2 > 0.99$. Compare back-calculated values with nominal values. Every point must fall within 20% of nominal. Otherwise, calibration points are excluded until all remaining points meet this criterion. The operational LOQ is adjusted upward to include only the acceptable points.
 - f. $R^2 < 0.99$. Compare back-calculated values with nominal values. The average of all points at each level must fall within 20% of nominal for that level. Otherwise, calibration points are excluded until all points meet this criterion. The operational LOQ is adjusted upward to include only the acceptable points. Report found value as an estimate. (Likely to be necessary for the more problematic compounds: bacitracin, chloramphenicol, penicillin, and erythromycin, see Table 7.)
 - g. If neither of the Standard Linearity criteria can be met, then the result is reported only as “> LOQ” and the operational LOQ is reported also.
 - h. For the quantitative data to be acceptable for that compound, at least one QC sample at each level tested in the batch must show deviation < 15% from nominal value.
 - i. The operational limits of quantitation and identification must be set > 3-fold higher than any autosampler or system carryover observed in control extracts (a potential issue for monensin, tylosin and clarithromycin).
 3. Prepare comparison chromatograms and mass spectra
 - a. Calculate the retention time comparison value to be used for every compound in each batch by averaging the retention times of corresponding solvent standards and high-level matrix standards bracketing the batch of test samples.
 - b. Create averaged mass spectra for every compound by averaging across each compound’s peak in the solvent standards at > 10% full height.

4. Evaluate qualitative acceptability

NOTE: Qualitative data may be found acceptable for either a “screening” identification or a “confirmed” identification. If criteria for neither form of identification are acceptable, the compound is reported as “not found (NF).”

- a. Create a reconstructed ion chromatogram (RIC) for each compound in each sample extract by combining all the diagnostic products ions listed in Table 4, and applying a five-point smooth.
- b. If no RIC peak appears with signal-to-noise (S:N) > 3:1 at the expected retention time, then the compound is reported “not found” (NF).
- c. If an RIC peak appears with signal-to-noise (S:N) > 3:1 at the expected retention time, then the peak is integrated. To be acceptable, each peak must be well-formed, with > 4 continuous points above baseline: not ragged spikes or lacking a clear peak top from which to assign a retention time.
- d. If the integrated RIC peak is not acceptable, the compound is “not found.”
- e. If the smoothed and integrated RIC peak is acceptable, then create individual diagnostic ion chromatograms for each ion specified for that compound in Table 4, and apply a five-point smooth to each one.
- f. Create an averaged mass spectrum across the RIC peak by averaging at > 10% full peak height.
- g. Criteria to assign a “screening” identification: Inspect the ion chromatogram(s) for the ion transitions marked “a” in Table 4. Note: for all compounds except penicillin, this is the strongest diagnostic ion. For penicillin, two diagnostic ions are required before a “screening” identification may be assigned. Evaluate the ion chromatogram(s) for the following:
 - i. Signal-to-noise (S:N) must be > 3:1.
 - ii. Retention time must be within 2.5% of the retention time comparison calculated above, except for penicillin, which requires that both peaks’ retention times be within 1.0% of the retention time comparison value, and for streptomycin (HILIC) which must be $\pm 5\%$ of the RT comparison.
 - iii. The diagnostic ion integrated for this test must also be the base peak in the averaged mass spectrum. For penicillin, the ion at m/z 160 must be the base peak and the ion at m/z 176 must appear at a relative abundance which corresponds to the relative abundance observed in the penicillin standard’s averaged mass spectrum, prepared earlier.
 - iv. If all these criteria are met, the compound is assigned a “screening” identification.
- h. Additional criteria to assign a “confirmed” identification: Inspect all the ion chromatogram(s) for ion transitions that are listed in Table 4. Evaluate the ion chromatogram(s) for the following criteria. For each compound, every ion chromatogram and averaged spectrum must satisfy every criterion for the identification to be “confirmed.”

- i. Signal-to-noise (S:N) must be $> 3:1$.
- ii. Retention time must be within 2.5% of the retention time comparison calculated above, except for penicillin, which requires that all peaks' retention times be within 1.0% of the retention time comparison value, and for streptomycin (HILIC) which must be $\pm 5\%$ of the RT comparison.
- iii. Retention times for the individual ions must be within 0.5% of the RIC peak top.
- iv. The ion marked with an "a" in Table 4 must also be the base peak in the averaged mass spectrum. For penicillin, the ion at m/z 160 must be the base peak. The relative abundances of the other ions must correspond to the relative abundance observed in the averaged standard mass spectrum. This correspondence may be established by visual comparison of the mass spectra, or by calculation of ion ratios. For ion ratio calculations, the ratio of each peak to the base peak must be within 20% of the same ratio for the comparison standard. The matching window is found by addition / subtraction, for example, the window for an ion ratio of 0.7 (70% relative abundance) is 0.5 – 0.9 (50% - 90%). If only two diagnostic product ions are specified in Table 4, then the matching window is reduced to 10% (60 – 80% in this example).
- v. If all these criteria are met, the compound is assigned a "confirmed" identification.

5. Evaluate quantitative acceptability

- a. If the integrated RIC peak (prepared above) was acceptable, it is quantified by processing against the accepted standard curve for that compound.
- b. If the found value is below the lower limit of quantitation shown in Table 4, then no quantitative value is reported.
- c. If the found value is below the operational limit of quantitation obtained after evaluating the standard curves, then no quantitative value is reported.
- d. If the qualitative data are not acceptable according to screening or confirmation criteria, the compound is reported "not found" even if the found value is above the LOQ.
- e. Depending on R^2 for each compound's standard curve, the found value is either reported as is ($R^2 > 0.99$), reported as an estimate ($R^2 > 0.99$), or not reported (or, reported as not quantifiable).
- f. Print the results for any findings so that all individual ion chromatograms, the RICs and the averaged spectra from samples, standards, QC samples and negative controls may be reviewed by an independent expert.
- g. Refer to CVM Guidance Document #118 (Reference 1) for a complete discussion of confirmation criteria in full scan MS/MS mode.

Penicillin G. Refer to the Notes Section for a Critical Note about Penicillin

- Results are not corrected for water content, so as to provide data for wet DG products on an as-is basis, not on a dry matter basis. However, if water content is measured separately, this value may be used as a correction factor for dry weight.

Table 7: Control Chart and Quantitation Limits.

Average R^2 values and slope coefficients obtained during method validation. R^2 values were poorer for the less sensitive compounds (marked with asterisk).

Quantitation limits shown in Table 7 represent the lowest concentration at which the relevant criteria could still be met, when applied to a set of control samples fortified at various fixed levels. Detection limits for screening criteria were not determined. Confirmation limits were \geq quantitation limits in all cases.

Analyte	R^2 n=4	Quantitation Limit, ppm	First order coefficient (slope), n=4	Comments
tylosin	0.9975	0.1	279412500	
oxytetracycline	0.9961	0.25	140436000	
chlortetracycline	0.9961	0.1	58118275	
dehydro- erythromycin (as erythromycin)	0.9956	0.1	122872000	Degradant (see Discussion)
ampicillin	0.9953	0.25	10997028	
Virginiamycin M1	0.9947	0.1	98441925	
clarithromycin	0.9942	0.1	149087000	
tetracycline	0.9915	0.5	32259300	Weak signals < 0.5 ppm
monensin	0.9900	0.1	346441750	Signal saturates detector at high end
streptomycin	0.9934	0.5	337241	Weaker signals
bacitracin*	0.9853	0.5	16015975	Weaker signals
chloramphenicol*	0.9763	0.5	952192	Weak signals, but free of interferences
penicillin*	0.9686	1.0	1136852	Weak signals, matrix suppression
erythromycin*	0.9381	0.5	19177500	Degrades steadily during analysis

* Indicates analytes for which quantitative performance was less than ideal.

Extraction Efficiency (Recovery)

Recovery was measured by comparing pre-fortified and post-fortified samples at three concentrations, 3 replicates at each level (one extraction batch).

Table 8: Extraction Efficiency

Analyte	1 µg/g		2.5 µg/g		7.5 µg/g	
	Recovery %	RSD ^a %	Recovery %	RSD%	Recovery %	RSD%
Ampicillin	97	5.6	94	5.2	99	4.9
Oxytetracycline	84	4.3	72	3.2	80	3.8
Tetracycline	71	6.0	68	4.6	73	4.2
Chlortetracycline	71	3.8	65	1.0	68	4.6
Bacitracin	89	7.0	93	13.4	76	5.3
Chloramphenicol	91	12.0	103	2.2	89	4.0
Erythromycin ^b	18	14.1	25	5.8	20	1.7
Tylosin	99	1.4	88	8.3	96	4.5
Dehydro-erythromycin ^b	122	6.8	108	3.9	107	9.9
Clarithromycin	108	3.4	87	2.0	96	6.7
Penicillin	64 ^c	30.4 ^c	70	20.5	63	11.3
Virginiamycin M1	84	6.3	86	9.9	87	17.4
Monensin	63	11.0	51	13.2	80	5.7
Streptomycin	79	4.4	77	5.7	73	1.3

KEY

^a Relative standard deviation

^b See Discussion section for comments on erythromycin degradation.

^c Indicates a concentration at the quantitative limit for that compound.

Validation Data.

Accuracy was measured by comparing pre-fortified samples at three concentrations, 2 replicates at each level, 4 extraction batches.

Table 9: Accuracy At Individual Levels

Analyte	QCL 0.4 µg/g		QCM 1.5 µg/g		QCH 4.0 µg/g	
	Accuracy ^b %	RSD ^c %	Accuracy %	RSD%	Accuracy %	RSD%
Ampicillin	92.5	8.6	96.3	4.4	97.6	5.4
OTC	91.6	5.4	96.9	4.5	99.1	5.1
TC	133.5	13.7	82.3	7.9	96.3	6.9
CTC	95.7	5.0	97.8	4.4	96.8	3.8
Bacitracin	109.8 ^a	22.9 ^a	93.3	21.5	102.2	8.8
Erythromycin ^d	98.9 ^a	25.2 ^a	81.6	28.5	93.4	21.9
Chloramphenicol	89.7 ^a	44.3 ^a	104.8	24.9	106.7	6.3
Tylosin	100.1	4.5	97.3	5.9	97.5	6.0
Dehydro-erythromycin ^d	101.4	6.2	101.2	4.4	94.3	5.5
Clarithromycin	98.9	13.8	100.8	9.4	96.8	7.9
Penicillin	162.1 ^a	29.9 ^a	106.8 ^a	25.9 ^a	96.5	10.3
Virginiamycin M1	82.9	13.3	95.2	5.2	92.4	11.1
Monensin A	129.8	15.5	115.1	28.3	89.4	38.6
Streptomycin	145.8 ^a	13.4 ^a	96.4	16.9	104.4	15.5

^a Indicates concentrations at or below the Quantitative Limit for that compound. Quantitative results were not considered acceptable at those levels.

^b Ratio of measured value and actual value.

^c Relative standard deviation.

^d See Discussion section for comments on erythromycin degradation.

Table 10: Overall Accuracy (N=24, Unless Otherwise Noted)

Refer to Table 9 for individual results at individual levels.

Analyte	Accuracy % ^a	RSD % ^b
Ampicillin	95	6.5
OTC	96	5.8
TC	89	11 ^c
CTC	97	4.3
Bacitracin	98	16 ^c
Erythromycin ^e	88	25 ^c
Chloramphenicol	106	17 ^c
Tylosin	98	5.4
Dehydro-erythromycin ^e	99	6.2
Clarithromycin	97	10
Penicillin	97	10 ^d
Virginiamycin M1	90	11
Monensin A	111	30
Streptomycin ^c	100	16

This table does not include quantitative values that were either below the lowest point of the calibration curve or the lowest concentration of fortified samples that still met the screening criteria.

KEY

^a Accuracy value reflects correction by use of matrix-matched, extracted standard curve

^b Fortification levels: 0.4, 1.5, and 4.0 µg/g, n = 24, unless otherwise noted

^c Fortification levels: 1.5, and 4.0 µg/g, n = 16

^d Fortification level: 4.0 µg/g, n = 8

^e See Discussion section for comments on erythromycin degradation.

Discussion

- Virginiamycin. Commercial formulations of virginiamycin combine two forms, called M1 and S1, which act synergistically. The major percentage of the active formulation is the M1 form. For purposes of this method, the M1 form is considered a surrogate for detection of the combination product. The measured concentration of Virginiamycin is for the M1 form only, not the combination product.
- Bacitracin. In pure standard, the singly-charged ion at m/z 1422.7 predominates, while in extracts the doubly- and triply-charged ions predominate. The doubly-charged ion at m/z 712.1 was used for method validation. However, the triply-

charged ion at m/z 474.9 may be added for additional sensitivity and selectivity. This ion yields the same singly-charged product ions as the doubly-charged parent ion.

- Penicillin G. When testing this method with samples of distillers grains, it was discovered that some samples yielded an interference for penicillin (using ion trap mass spectrometry). This ion appeared at m/z 160, but was separated by > 0.15 min from *bona fide* penicillin. Also, the interfering compound did not yield the penicillin confirmatory ions at m/z 114 or 176. Specific, penicillin-only criteria have been added to this procedure, to prevent mis-identifications, if based only on the ion at m/z 160. If the interference is not separated from penicillin by > 0.10 min it is recommended to install a new LC column. The quality of LC separation may be found by adding penicillin to an extract that shows this interference.

The relatively poor sensitivity for penicillin was found to be related to the use of ion trap mass spectrometry, for reasons that were not determined. Detection limits were at least five fold lower when extracts were analyzed on a linear tandem mass spectrometer (a quadrupole/ time-of-flight hybrid).

- Erythromycin. During the extraction, exposure to acid conditions causes erythromycin to slowly degrade to dehydro-erythromycin. Both compounds are monitored in the detection method, and each compound gives results in terms of erythromycin in the starting sample. If both compounds are present at measurable concentrations, the results may be averaged to report a single finding for erythromycin. It is important to note that if erythromycin was present in the sample but degraded to the dehydro- form prior to extraction, the dehydro- form will give a higher value compared to erythromycin.
- Carryover. High level solvent and matrix standards are prone to carryover, especially monensin. Inject two solvent blanks after high-level solvent and matrix standards.

Acknowledgments

The authors thank Cristina Nochetto of the CVM Office of Research for her assistance with sample handling, Nancy Thiex of South Dakota State University for DG samples used in method development, and Sherri Turnipseed of the FDA Animal Drugs Research Center for helpful discussions.

References

CVM Guidance 118: Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues, <http://www.fda.gov/cvm/Guidance/guide118.pdf> (accessed April 3, 2009).

Key words: Distillers grains, antibiotics, analysis, LC/MS, multiresidue, extraction, residues

Worksheet # 1. Example Validation Batch Worksheet

Preparation of blanks, matrix standards and quality control (QC) samples.^a

Use 5 g control DG in all cases.

Injection Level	Equiv. Conc. in DG, $\mu\text{g/g}$	Conc. Mixed Standard, $\mu\text{g/mL}$	Mixed Standard ID to use	Volume to add, μL
Control	--	--	--	--
M-1	0.1	1	S-1	500
M-2	0.25	2.5	S-2	500
M-3	0.5	5	S-3	500
M-4	1	10	S-4	500
M-5	2.5	25	S-5	500
M-6	5	50	S-6	500
M-7	7.5	75	S-7	500
M-8	10	100	S-8	500
QCL (Low)	0.4	5	S-3	400
QCM (Medium)	1.5	25	S-5	300
QCH (High)	4	50	S-6	400

^a This complete batch should be used for method validation. To reduce overhead for surveillance analyses of unknown samples, M-5, M-7, QCL, and QCH can be omitted from the batch.

Worksheet # 2. Example Test Batch Worksheet

Batch Preparation: _____ in DG

Study #:			Prep dates:
Batch#:		Analyte stds:	
Prep date:			
Matrix:			
Method			
Analyst:			
Storage after extraction:			

Injection Level	Conc. in DG, $\mu\text{g/g}$	DG ID	DG, g	Mixed Standard, $\mu\text{g/mL}$		Comments
				Conc.	μL to add	
Control	--			--	--	
M-1	0.1		S-1	1	500	
M-2	0.25		S-2	2.5	500	
M-3	0.5		S-3	5	500	
M-4	1		S-4	10	500	
M-5	2.5		S-5	25	500	
M-6	5		S-6	50	500	
M-7	7.5		S-7	75	500	
M-8	10		S-8	100	500	
QCL	0.4		S-3	5	400	
QCM	1.5		S-5	25	300	
QCH	4		S-6	50	400	
1	unknown					
2	unknown					
3	unknown					
4	unknown					
5	unknown					

Figure 1.
Reconstructed Ion Chromatograms for a DDG extract fortified at 1 $\mu\text{g/g}$.

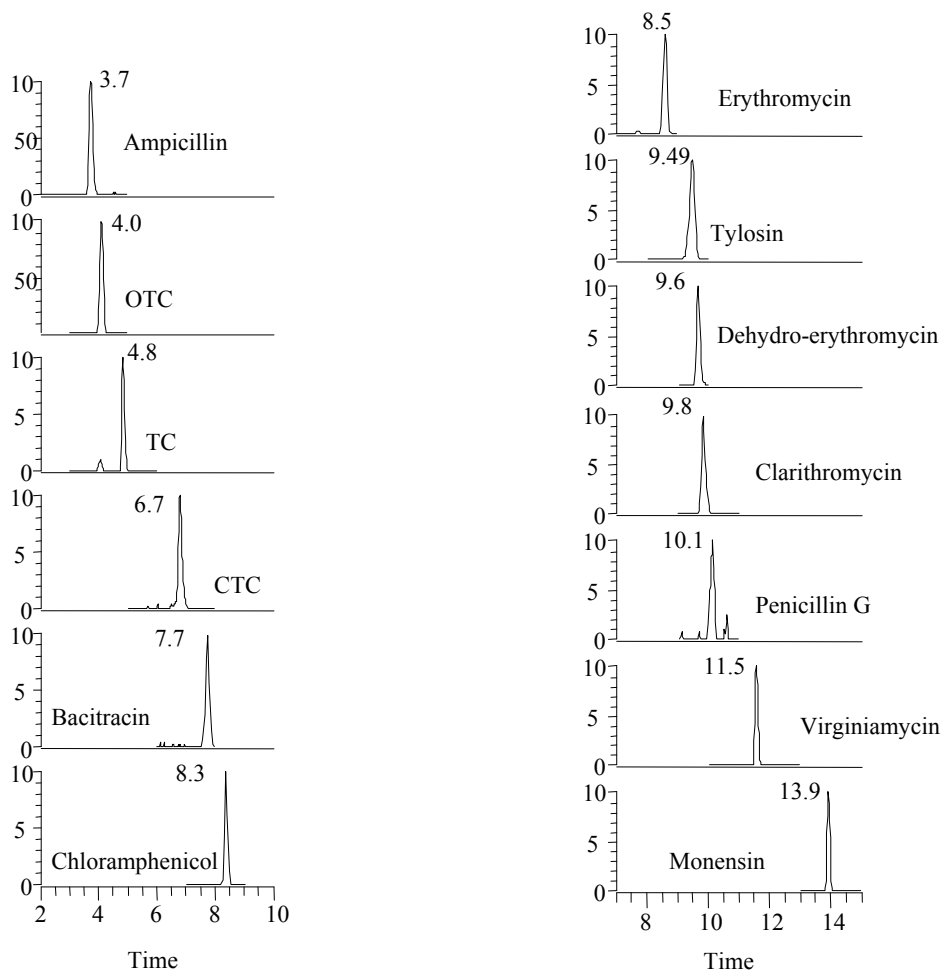


Figure 2.

Reconstructed ion Chromatogram of streptomycin for a DDG extract fortified at 1 $\mu\text{g/g}$.

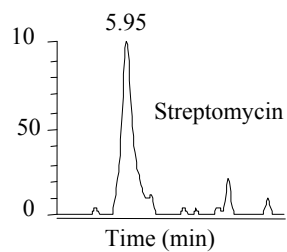
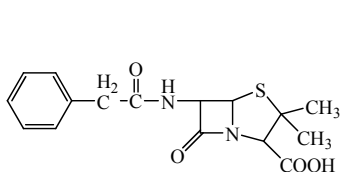
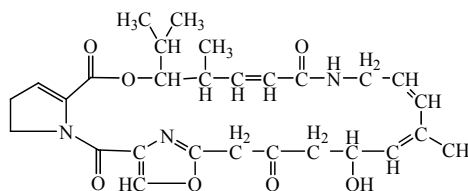


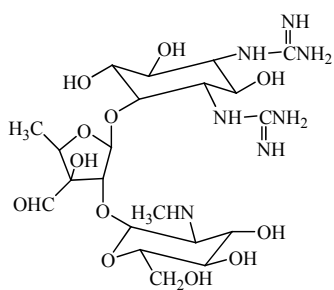
Figure 3.
Structures of representative antibiotics.



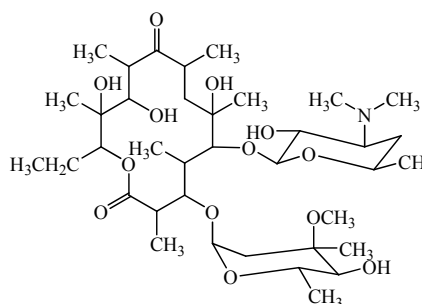
Penicillin G



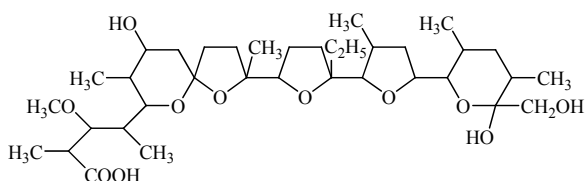
Virginiamycin M₁



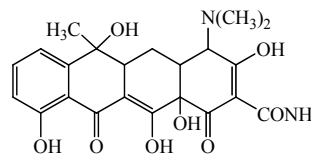
Streptomycin



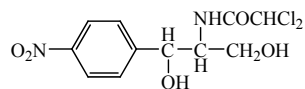
Erythromycin A



Monensin



Tetracycline



Chloramphenicol

Figure 4.a.
Flow Chart for Data Interpretation

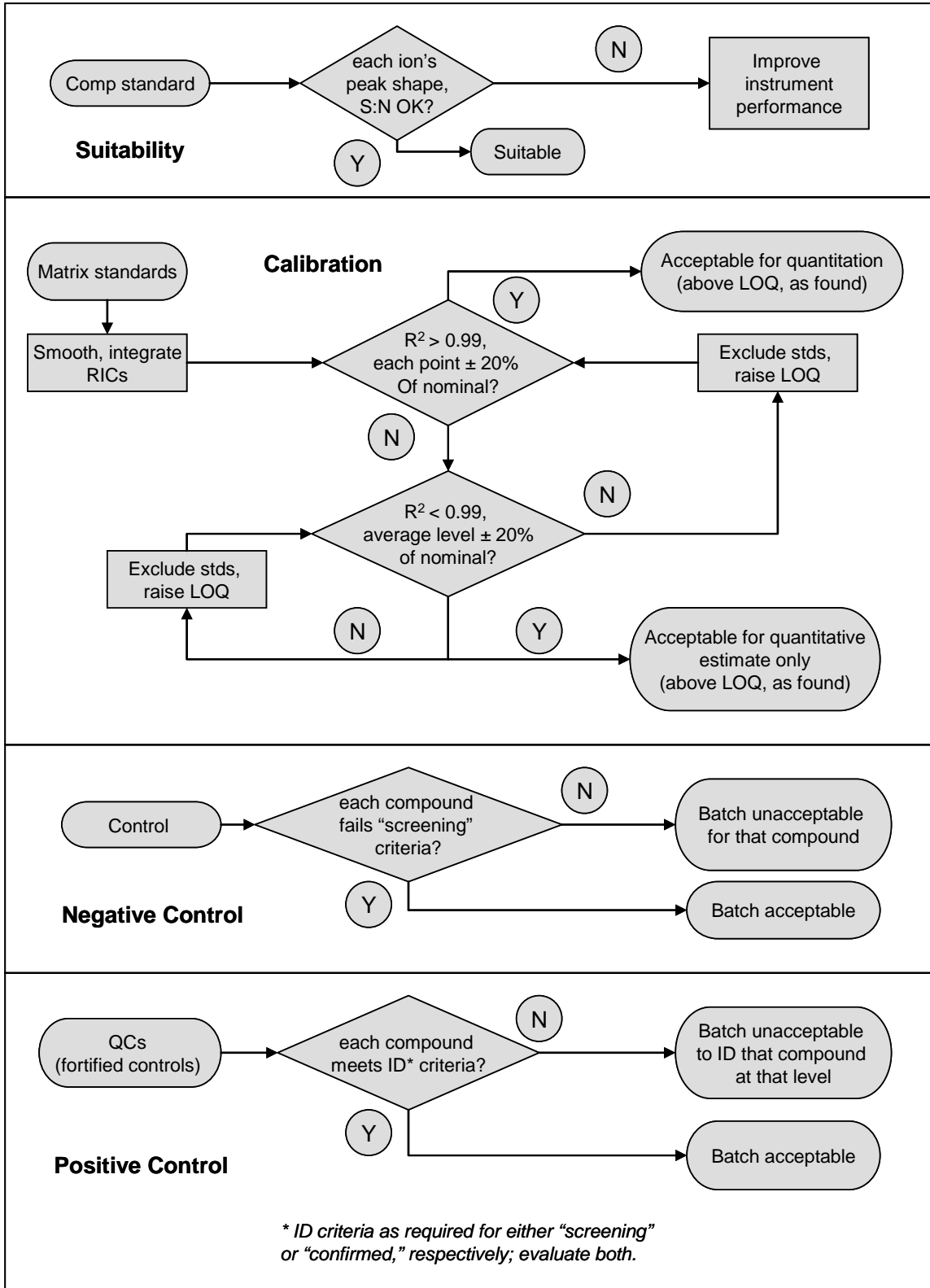


Figure 4.b.
Flow Chart for Data Interpretation

