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ARTICLE

Multiclass, Multiresidue Drug Analysis, Including Aminoglycosides, in Animal Tissue Using Liquid Chromatography Coupled to Tandem Mass Spectrometry†

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A multiresidue, multiclass semiquantitative screening analysis of 39 drug residues covering 8 drug classes, including aminoglycosides in veal muscle, based on a single multiresidue extraction routine and using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), is presented. Sample preparation involves extraction of a 5 g diced tissue sample with 10 mL of acetonitrile/ water (86:14), incubated at 60 °C for 1 h, and then cooled for 10 min in ice. Formic acid is added to the suspension, then mixed, and centrifuged. The supernatant is retained, and the pellet is extracted with 10 mL of water for aminoglycosides and again centrifuged. Approximately 9.5 mL of each of the supernatants from both extracts is combined and diluted with water to 25 mL. The final solution is then defatted with 20 mL of hexane prior to analysis. Liquid chromatography for the aminoglycosides is carried out with ZIC-HILIC and for the remainder of the compounds with an Atlantis dC18 minicolumn. LC-ESI-MS/MS in positive and negative ionization modes (three injections total) is carried out, and two ion transitions per analyte are monitored. The method provides semiquantitative analysis to identify incurred positive drug classes in a rapid and cost-effective manner. Of particular interest is the detection of numerous compounds in the low nanograms per gram concentration range, which are not typically detected using receptor-based screening methods. All identified drugs were confirmed using internationally recognized regulatory methods, with no apparent false positives.

KEYWORDS: Multiresidue drug analysis; aminoglycosides; amphenicols; β-lactams; NSAIDS; macrolides; quinolones; sulfonamides; tetracyclines; LC-MS/MS; veal muscle

INTRODUCTION

The management and enforcement of a regulatory program aimed at identifying chemicals used in livestock for the purposes of disease control and growth promotion are significantly dependent on the analytical chemistry of the screening, identification, and confirmation of detected drug residues. Regulatory compliance is met when residues are below acceptable maximum residue limits (MRL) or when the compound is not detectable with the best available analytical methodology. In those cases when drugs are banned or if they have not been approved for use, the lowest level of detection with the screening method is used to highlight the need for additional testing to confirm a suspect positive. When a sample is suspect positive for a drug residue with a screening method and if there is an MRL, then confirmation of the drug and estimation of its concentration are required to satisfy regulatory demands. Additionally, baseline monitoring information on what drugs are being used regardless of an MRL is of interest to some regulators.

The use of screening methods, such as receptor based or microbial inhibition, has been generally considered an acceptable and economical approach to sorting out suspect positives from negatives for the target drugs of concern (*1*−*7*). Certainly, screening to "establish the presence or absence of residues of veterinary drugs" (*7*) using various approaches is required, and indeed some screening methods can provide low method detection limits for selected compounds; however, for some drug classes from animal tissue there is a high rate of false positives and, more importantly, poor sensitivity or no sensitivity for certain drugs within a class. This makes it difficult to expand the compound list for analysis for a drug class. In addition, inclusion of additional drug classes necessitates the use of additional screening test kits. Furthermore, deployment of a confirmatory method is always required following a suspect positive with a screening method, but in those cases of a high rate of false positives, for example, 40%, it is actually more practical to directly analyze all of the test samples in question with the confirmation method in a batch rather than a few samples per batch over time. Screening methods developed in the 1990s filled a need to discern suspect positive drug residues from tissues that do not contain residues. Today, screening methods are still used to provide an indication of a positive drug residue (*8*, *9*), but issues around false positives remain. Additionally, there is ongoing discussion whether the receptor-based methods also respond to metabolites or endogenous compounds,

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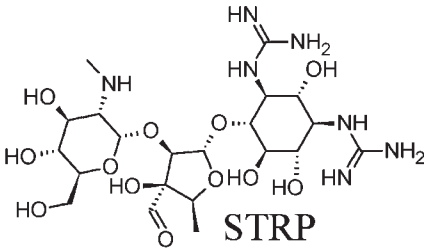
Aminoglycosides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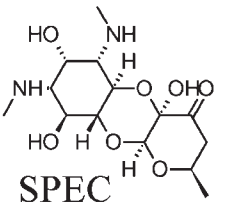
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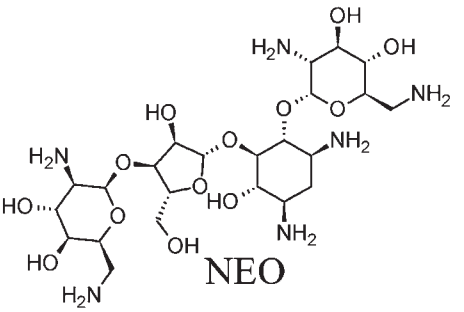
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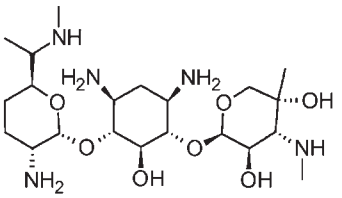
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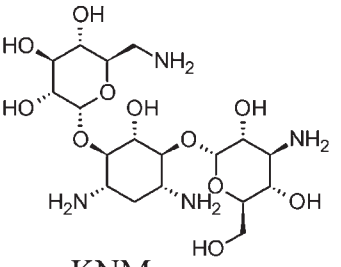
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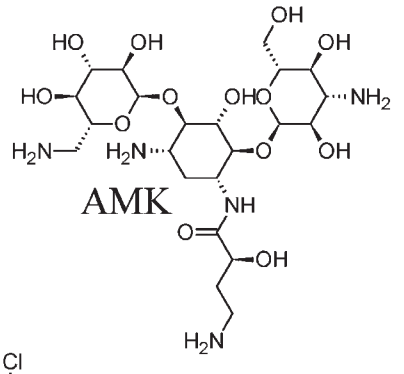
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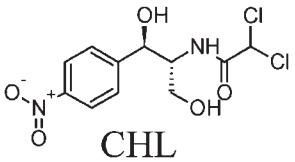
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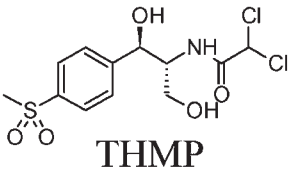
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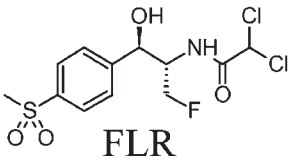
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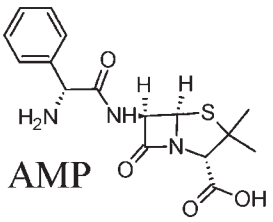
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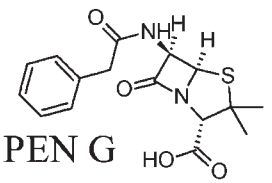
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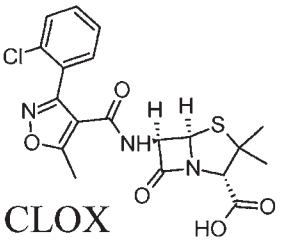
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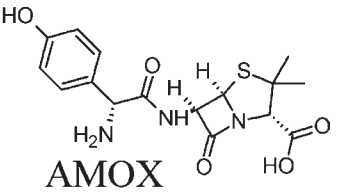
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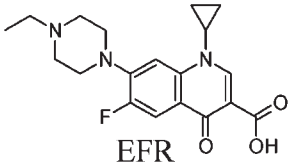
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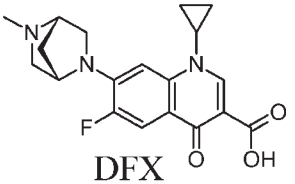
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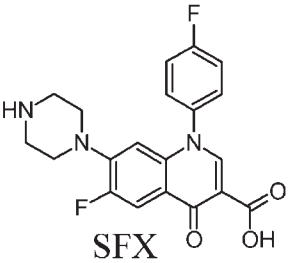
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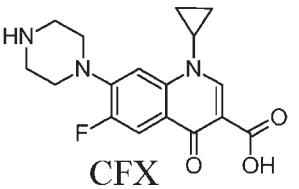
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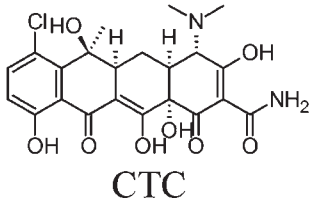
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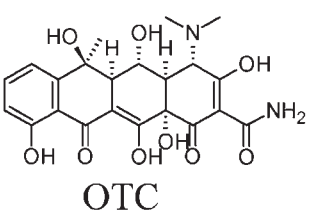
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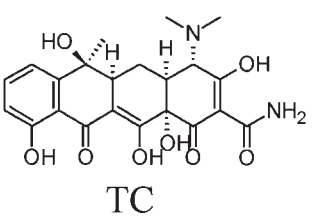
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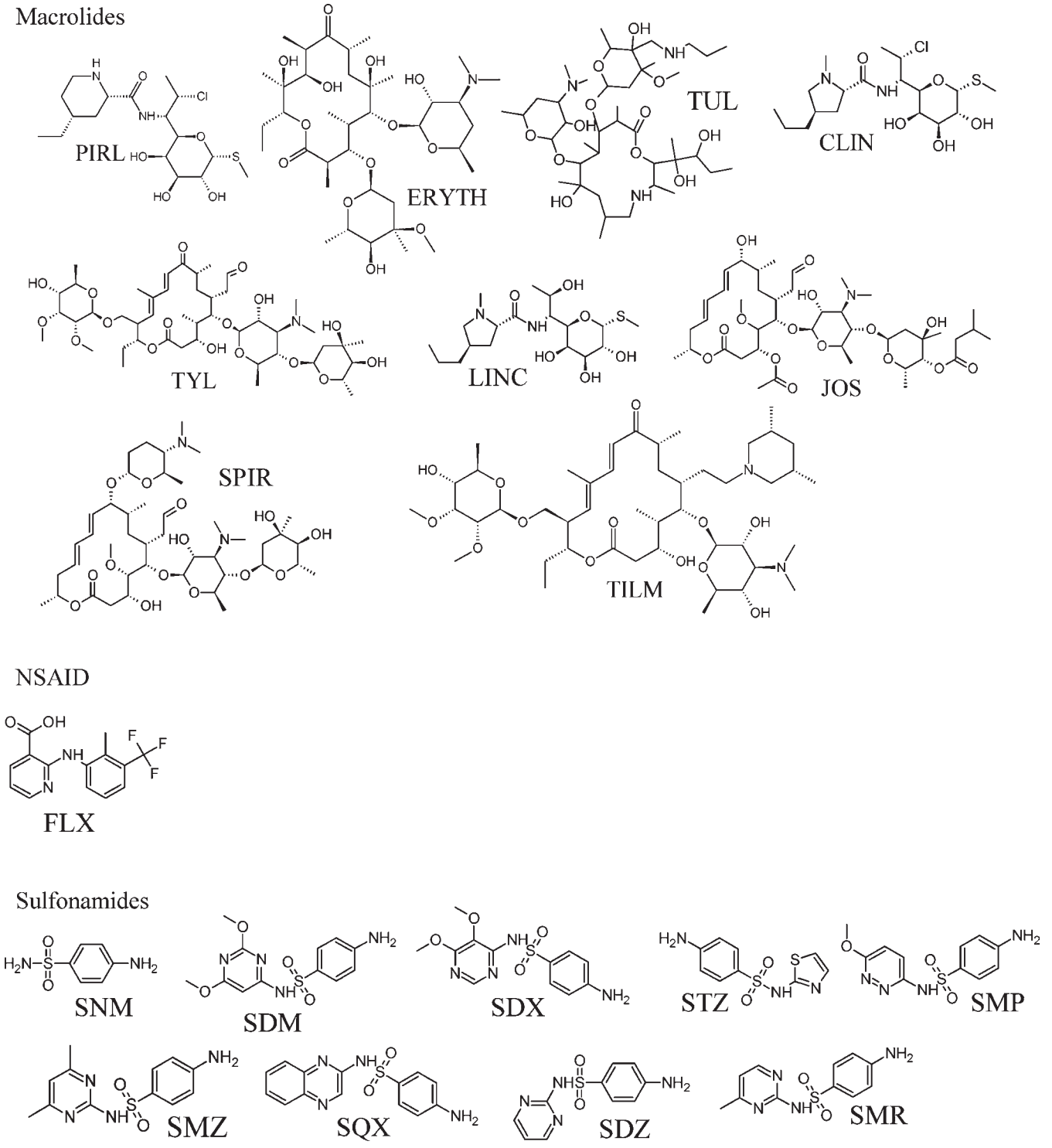


Figure 1. Structures of target drugs and the drug classes.

which create artifacts of positive responses that typically cannot be confirmed without authentic standards and which cannot be regulated. Given each suspect positive from a screening method often requires the retention of the suspect contaminated carcass, a high rate of false positives actually creates issues for the regulatory body and producers of those edible animal tissues.

At this time, mass spectrometric techniques have advanced considerably, which has resulted in rugged mass spectrometers that have been shown to be a powerful analytical tool for veterinary drug residue determinations; for food safety and regulatory enforcement purposes, regulatory chemists perform routine testing of a large number of animals and animal products destined for human consumption with mass spectrometric techniques. As a result of the need for regulatory enforcement, liquid chromatographs coupled to various mass spectrometers are the instruments of choice for veterinary drug determination, given the need for identification and particularly confirmation (*10*−*25*). Concurrently, there is increasing interest in developing complete methods that cover a wide range of drug classes and compounds to maximize a laboratory's efficiency and throughput.

As with all analytical residue methods, sample preparation and extraction routines can create issues for the residue chemist,

particularly if the analyses require considerable homogenization techniques that can be time-consuming and potentially lead to cross-contamination. In most of the methods referenced herein for the determination of multiclass drug residues from animal tissues, solid phase extraction (SPE) was used. The selectivity of SPE can provide the advantage of a cleaner sample extract and, therefore, less interference and suppression from matrix components in LC-MS/MS analysis; however, in the design of multiclass drug residue analyses, the selectivity of SPE can be a disadvantage due to the differences in chemical and physical properties of the drugs between different classes or even within the same drug class which needs to be simultaneously extracted. This is especially true with the aminoglycoside class. Recently, we showed that the extraction and analysis of macrolides from edible animal tissues are possible without aggressive tissue homogenization such as blending and without the need for SPE cleanup (*26*).

The primary objective of the present study was to develop a simple, efficient, semiquantitative method for fast screening of multiclass drug residues including aminoglycosides in veal muscle, without the need for blending and sample cleanup. The drug classes (**Figure 1**) were chosen on the basis of their potential use or recognized use for therapeutic purposes in livestock.

MATERIALS AND METHODS

Safety. Acetonitrile, formic acid, hexane, and methanol must all be handled with care. Avoid inhalation of vapors, spills, and contact with skin and mucous membranes.

Chemicals. Methanol, acetonitrile, and hexane were all high-purity grade, distilled in glass, and purchased from Caledon (Georgetown, Canada). Formic acid (minimum 98%), reagent grade, was purchased from VWR (Mississauga, ON, Canada). All water was purified by an in-house NANOPure Deionization system with charcoal polish and 0.20 μm filtration (Barnstead, Dubuque, IA).

Penicillin G potassium salt (PEN G), ciprofloxacin (CFX), and sulfadoxine (SDX) were obtained from US Pharmacopeia. Pirlimycin hydrochloride (PIRL) and tulathromycin (TUL) (salt-free base) were obtained from Pfizer Animal Health (Montreal, QC, Canada). Sulfamethoxypyridazine (SMP) was from Dr. Ehrenstorfer (Germany). Chlorampenicol (CHL), florfenicol (FLR), thiamphenicol (THMP), clindamycin hydrochloride (CLIN), erythromycin (ERYTH), josamycin (JOS), lincomycin hydrochloride (LINC), spiramycin (SPIR), tilmicosin (mixture of isomers) (TILM), tylosin (TYL), enrofloxacin (EFR), sarafloxacin hydrochloride (SFX), danofloxacin (DFX), sulfadiazine (SDZ), sulfadimethoxine (SDM), sulfamerazine (SMR), sulfamethazine (SMZ), sulfanilamide (SNM), sulfaquinoxaline (SQX), sulfathiazole (STZ), chlorotetracycline hydrochloride (CTC), oxytetracycline dehydrate (OTC), tetracycline (TC), gentamicin sulfate salt (GEN), neomycin trisulfate salt (NEO), streptomycin sulfate salt (STRP), amikacin disulfate salt (AMK), kanamycin disulfate salt (KNM) from *Streptomyces kanamyceticus*, spectinomycin dihydrochloride pentahydrate (SPEC), streptomycin sulfate salt (STRP), amoxicillin (AMOX), ampicillin (AMP), cloxacillin sodium salt (CLOX), and flunixin (FLX) were obtained from Sigma-Aldrich (Oakville, ON, Canada). The structure for each of these compounds is presented in **Figure 1**.

Analytical Standard Solutions. All standards were weighed using a Mettler Toledo XS105 five-point calibrated balance (Columbus, OH). Stock solutions were prepared by weighing 10−50 mg of each standard, followed by quantitative transfer to a 100 or 50 mL volumetric flask and filling to volume with methanol, acetonitrile, water, or acetonitrile/water (50:50). A balance uncertainty check was carried out prior to use with standard weights at or the near the weight of concern. The standards CHL, FLR, THMP, FLX, CLIN, ERYTH, JOS, LINC, PIRL, SPIR, TILM, TUL, TYL, CFX, EFR, SFX, DFX, CTC, OTC, and TC were dissolved in methanol, whereas SNM, SDZ, SMR, STZ, SMZ, SDX, SQX, SDM, and SMP were all dissolved in acetonitrile. The aminoglycosides GEN, AMK, KNM, SPEC, NEO, and STRP were dissolved in ultrapure water and stored in plastic tubes, whereas the β-lactam standards PEN G, AMP, AMOX, and CLO were prepared in acetonitrile/water (50:50 v/v). All standards were sonicated to ensure complete dissolution.

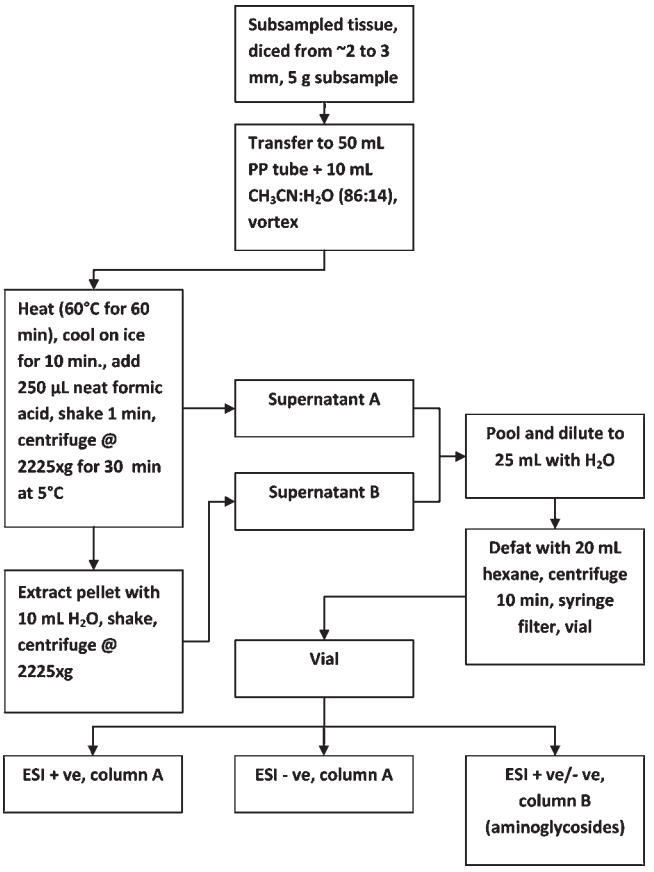


Figure 2. Schematic of the sampling and analysis routine.

Intermediate and Working Standards. Three intermediate standard solutions (1.0 μg/mL) containing several analytes grouped together, depending on the drug group, were prepared by dilution of the stock solutions with either methanol, water, or acetonitrile/water (50:50 v/v) (all solutions were stored in cryogenic vials and kept at −80 °C). Four working standard solutions (0.5−25 μg/mL) containing several analytes grouped together were prepared by dilution of the stock solutions with either methanol, water, or acetonitrile/water (50:50 v/v) for routine analysis. These solutions were prepared by using 25−1250 μL of 1000 μg/mL standards transferred to 50 mL volumetric flasks with class A pipets. All solutions were stored in cryogenic vials and kept at −80 °C.

Sample Preparation and Extraction. A schematic of the sample preparation, extraction, and analysis process for veal muscle is presented in **Figure 2**. Blank tissues free of the target compounds were obtained from local abattoirs and grocery stores, and the tissues were used for the preparation of spikes and matrix-matched calibration standards by the addition of analytes to blank extracts. Finely diced (2−3 mm) tissue samples of veal muscle (5.0 ± 0.1 g) were weighed into 50 mL polypropylene centrifuge tubes and fortified appropriately. All fortified samples were vortex-mixed for 30 s. Blank control tissue was extracted and run with each analytical run/batch. Ten milliliters of CH_3CN/H_2O (86:14 v/v) was added to each sample and vortex-mixed for 30 s on a Thermolyne Tyde 16700 vortex mixer (Fischer Scientific Co., Ottawa, ON, Canada). The sample set was placed in a circulatory hot water bath at 60 °C for 60 min. The tubes were then placed in an ice water bath for 10 min, followed by the addition of 250 μL of neat formic acid, vortexed for 1 min, and then centrifuged at 2225*g* for 30 min at 5 °C with an Allegra 6R centrifuge with a GH 3.8A rotor (Beckman Coulter, Inc., Fullerton, CA). The supernatant (supernatant A) was then transferred into a new 50 mL centrifuge tube. Ten milliliters of H_2O was added to the pellet, shaken at 400 rpm for 1 min on a C2 Platform Shaker (New Brunswick Scientific, Edison, NJ), and then centrifuged at 2225*g* for 10 min at 5 °C.

Table 1. Summary of Key Ionization Parameters for Each Compound, with Chromatographic Retention Time and Column Used

class	drug	monoisotopic mass	ionization mode	MRM 1	DP	CE	CXP	MRM 2	DP	CE	CXP	t_R(min)	column
amphenicols	THMP	355.0048	NEG	354.0 > 240.1	80	22	1	354.0 > 185.1	80	26	3	2.95	A
	FLR	357.0005	NEG	355.9 > 335.9	75	28	11	355.9 > 184.9	75	28	11	3.37	A
	CHL	322.0123	NEG	321.1 > 256.7	65	18	3	321.1 > 193.8	65	20	17	3.50	A
β-lactams	AMOX	365.1045	POS	366.1 > 349.0	61	13	8	366.1 > 114.0	61	33	8	1.99	A
	AMP	349.1096	NEG	348.0 > 206.9	65	16	1	348.0 > 303.9	65	12	19	2.91	A
	PEN G	334.0987	NEG	333.0 > 192.6	55	18	1	333.0 > 289.0	55	10	1	3.84	A
	CLOX	435.0656	NEG	434.1 > 293.1	65	14	5	434.1 > 390.1	65	30	15	4.25	A
macrolides	LINC	406.2138	POS	407.3 > 126.1	66	49	8	407.3 > 359.1	66	27	10	2.84	A
	TUL	805.5664	POS	404.0 > 577.5	61	19	14	404.0 > 158.2	61	29	12	3.09	A
	PIRL	410.1642	POS	411.2 > 112.0	81	43	8	411.2 > 363.1	81	25	10	3.33	A
	SPIR	842.5140	POS	422.5 > 174.1	51	29	14	422.5 > 101.1	51	27	6	3.35	A
	CLIN	424.1799	POS	425.2 > 126.1	76	41	8	425.2 > 82.0	76	129	4	3.40	A
	TILM	868.5660	POS	435.5 > 695.4	66	23	16	435.5 > 174.1	66	35	12	3.55	A
	ERYTH	733.4612	POS	734.5 > 158.1	56	39	12	734.5 > 576.3	56	31	14	3.74	A
	TYL	915.5192	POS	916.5 > 174.0	191	57	12	916.5 > 101.1	191	73	6	3.83	A
	JOS	827.4667	POS	828.6 > 109.0	111	67	6	828.6 > 174.1	111	47	14	4.10	A
NSAID	FLX	296.0773	NEG	295.0 > 251.0	55	24	15	295.0 > 190.9	55	44	11	4.28	A
sulfonamides	SNM	172.0306	POS	173.1 > 156.1	26	9	12	173.1 > 108.1	23	21	8	0.46	A
	SDZ	250.0524	POS	251.1 > 156.1	66	23	10	251.1 > 92.2	66	41	6	2.43	A
	STZ	255.0136	POS	256.0 > 156.0	66	23	12	256.0 > 92.0	66	37	6	2.71	A
	SMR	264.0681	POS	265.1 > 92.0	66	41	6	265.1 > 156.0	66	27	8	2.85	A
	SMZ	278.0837	POS	279.1 > 204.0	91	25	14	279.1 > 124.1	91	39	8	3.07	A
	SMP	280.0630	POS	281.1 > 156.1	81	27	10	281.1 > 92.0	81	41	6	3.07	A
	SDX	310.0736	POS	311.1 > 156.2	101	33	14	311.1 > 92.0	101	55	6	3.40	A
	SDM	310.0736	POS	311.1 > 156.2	101	33	14	311.1 > 92.0	101	55	6	3.68	A
	SQX	300.0681	POS	301.2 > 156.1	91	25	14	301.2 > 92.1	91	49	4	3.70	A
tetracyclines	OTC	460.1482	POS	461.0 > 442.8	66	19	16	461.0 > 426.0	66	29	12	3.02	A
	TC	444.1533	POS	445.2 > 154.1	51	39	12	445.2 > 410.1	51	29	12	3.09	A
	CTC	478.1143	POS	479.1 > 154.1	66	41	10	479.1 > 444.1	66	31	12	3.37	A
quinolones	CFX	331.1332	POS	332.2 > 231.2	81	51	18	332.2 > 314.0	81	33	8	3.10	A
	DFX	357.1489	POS	358.2 > 340.2	91	35	24	358.2 > 314.2	91	29	18	3.11	A
	EFR	359.1645	POS	360.3 > 316.1	46	31	16	360.3 > 245.1	46	37	20	3.18	A
	SFX	385.1238	POS	386.2 > 342.1	66	29	10	386.2 > 299.1	66	39	8	3.28	A
aminoglycosides	STRP	581.2657	POS	582.0 > 263.2	152	47	18	582.0 > 246.1	152	53	18	0.26	B
	GEN	477.3162	POS	478.1 > 322.3	80	21	8	478.1 > 157.1	80	31	12	0.97	B
	NEO	614.3123	NEG	613.4 > 321.0	115	36	7	613.4 > 112.8	115	42	7	2.22	B
	SPEC	332.1584	POS	333.2 > 189.2	96	29	14	333.2 > 140.1	96	31	8	3.29	B
	AMK	585.2857	POS	586.4 > 163.1	106	55	10	586.4 > 425.2	106	29	32	4.14	B
	KNM	484.2381	POS	485.3 > 324.3	76	23	8	485.3 > 163.1	76	35	12	4.30	B

This supernatant (supernatant B) was combined with supernatant A, and the final volume was made up to 25 mL using ultrapure water. The extract was defatted using 20 mL of hexane, shaken at 400 rpm for 1 min, and then centrifuged for 10 min at 2225*g* at 5 °C. Residual hexane was aspirated to waste. An aliquot of the extract (~1 mL) was filtered through a 0.2 μm Teflon syringe filter (Chromatographic Specialties, Brockville, ON, Canada) into a glass vial for analysis of all the compounds except for the aminoglycosides. Due to the high sorption affinity of the aminoglycosides to polar surfaces (e.g., glass), an aliquot of the extract was filtered through a 0.2 μm Teflon syringe filter into a polypropylene vial. The 0.2 g/mL equivalent sample extracts were not concentrated for analysis, and typically a 1 mg equivalent sample was injected onto the column for all analytes except for the aminoglycosides, for which 2 mg equivalents were injected. All incurred positives were analyzed on the day of receipt.

Instrumentation and Analysis. An Agilent 1200 series system was used for all chromatography. It included an autosampler, pump, degasser, and column heater (Agilent Technologies, Santa Clara, CA). All analyses were carried out with an API 4000 (Applied Biosystems, Foster City, CA).

Table 1 summarizes the acquisition conditions for the eight drug classes discussed in this work. Note that each sample was injected twice on column A: once in electrospray ionization positive mode and once in ESI negative mode. Polarity switching was employed for the analysis of the aminoglycosides with LC column B (one injection).

Chromatography (column A) was carried out on a Waters Atlantis guard dC18 20 × 3.9 mm, 3 μm, column in a guard column holder (Mississauga, ON, Canada) for all compounds except the aminoglycosides, which was carried out using a (column B) ZIC-HILIC column, 50 mm × 2.1 mm, 5 μm, 200 Å (SeQuant, Umea, Sweden).

For column A, the Atlantis dC18, the mobile phases were (A) 0.1% formic acid in ultrapure water (in-house distilled water passed through a NANOPure system from Barnstead) and (B) CH_3CN at a flow rate of 1.0 mL/min. The mobile phase starting conditions were 99% A for 1.0 min and then linearly ramped to 65% A in 4.0 min (8.5% B/min), then to 1.0% A in 3.0 min (21.3% B/min), and held for 4.0 min to ensure the column was rinsed of any residual organics. At 12.1 min, the conditions were returned to 99% A for 4.4 min, for a total run time of 16.5 min. Two separate injections were used with this gradient, one for those compounds requiring

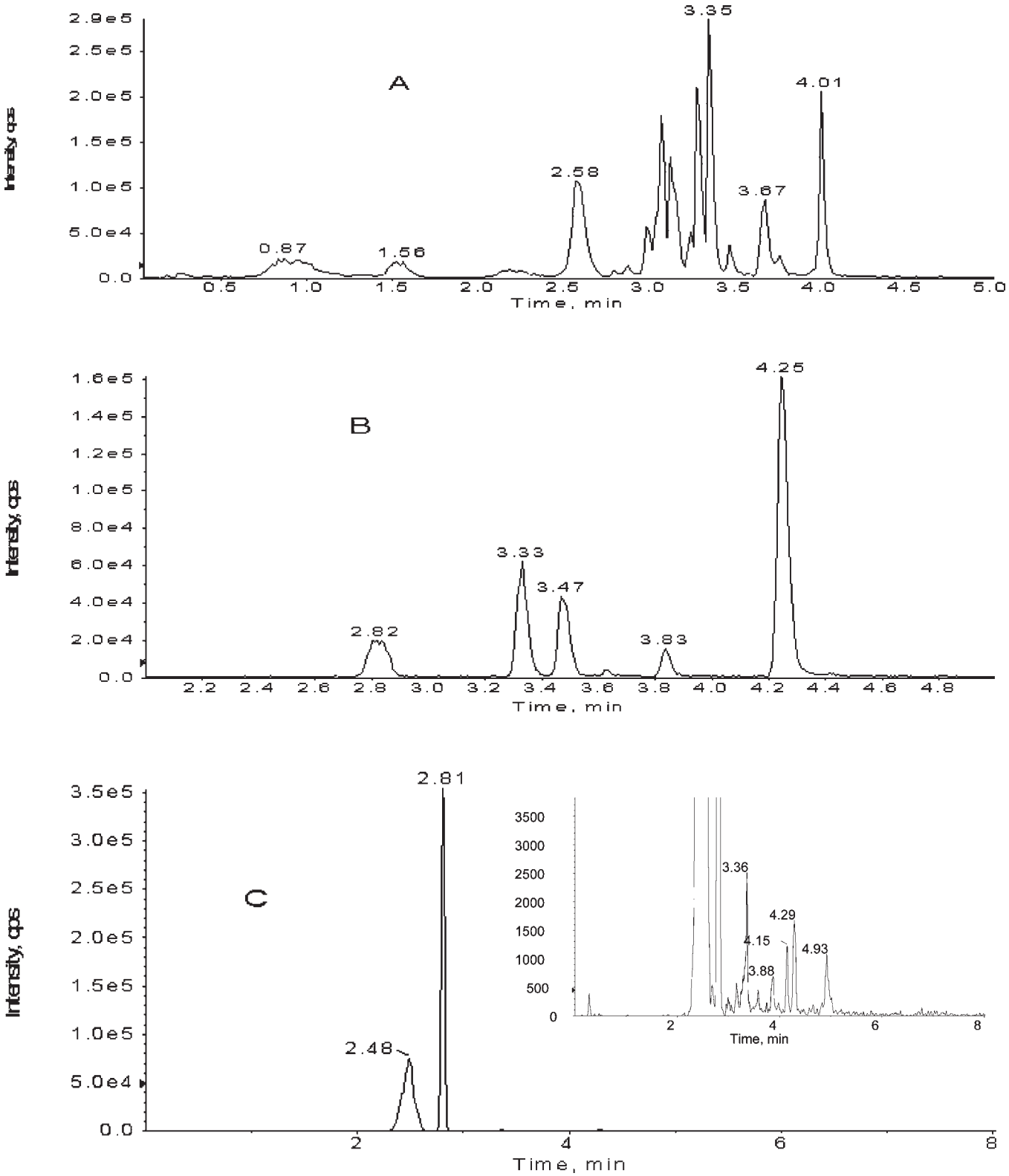
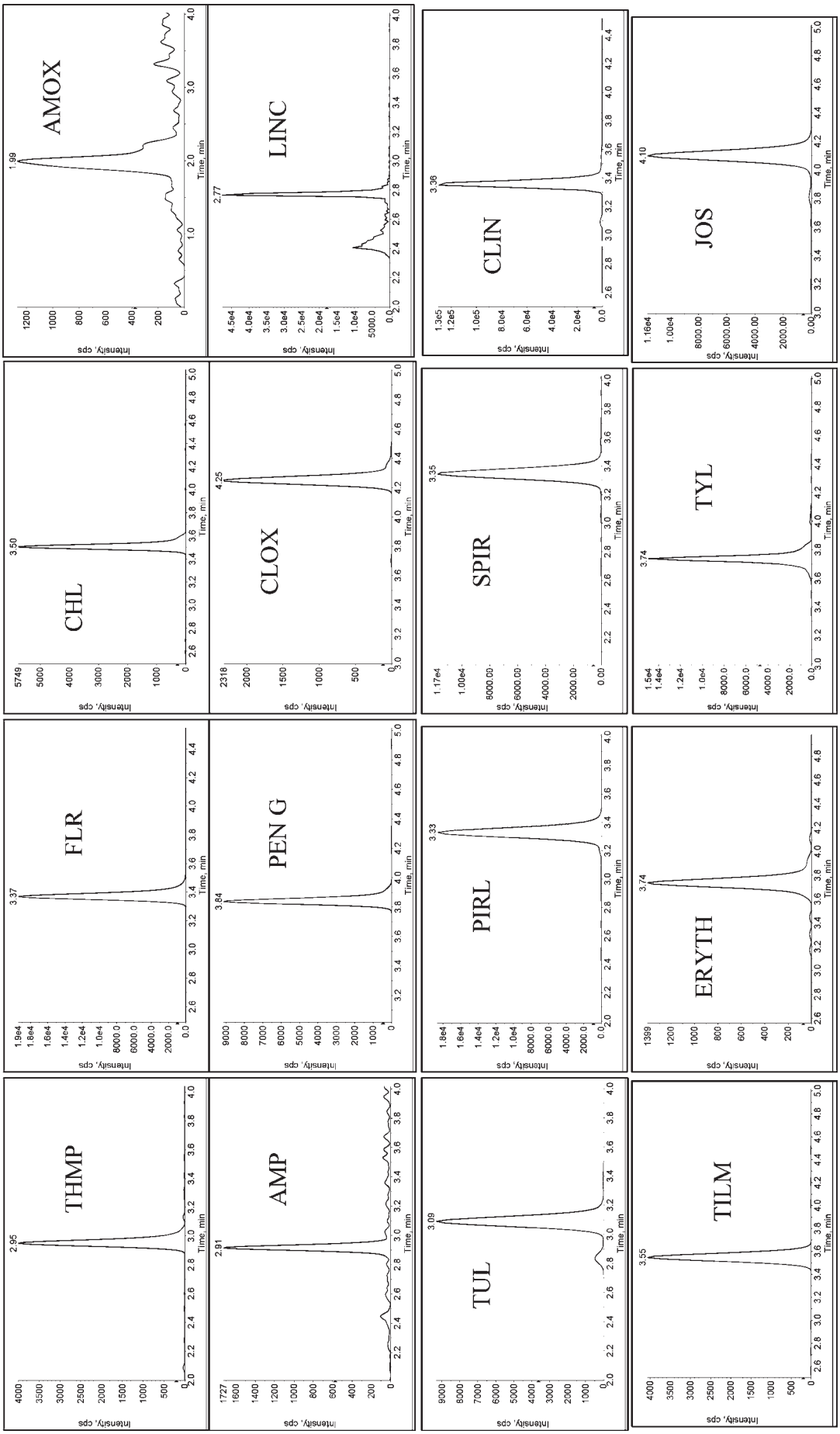


Figure 3. TICs of 50 ng/g spikes in veal muscle: (**A**) ESI positive using column A; (**B**) ESI negative using column A; (**C**) aminoglycosides using column B, gradient 2 (see Materials and Methods). The inset in **C** is a 10× magnification.

positive ionization and one for those requiring negative ionization (**Table 1**). Importantly, given that β-lactams break down in acid, extracts were immediately stored at 4 °C in the autosampler tray and analyzed on the day they were prepared. Injection volumes were 5 μL.

For column B, the ZIC HILIC column, two gradient options were used (gradient 1 or gradient 2) depending on the aminoglycosides of interest. In both cases, the mobile phases were (A) 0.4% formic acid in water and (B) CH_3CN at flow rate of 1.0 mL/min. For the determination of GEN, NEO, and STRP (gradient 1), the mobile phase starting conditions were 85% A for 0.1 min, then ramped to 95% A in 0.2 min, held at 95% for 3.2 min, then ramped to 85% A in 0.5 min, and held there for 4 min, for a total run time of 8.0 min. Gradient 2 was used to separate a broader range of aminoglycosides in addition to GEN, NEO, and STRP, specifically AMK, KNM, and SPEC. For gradient 2, the mobile phase starting conditions were 3% A for 1.0 min, then ramped to 85% A in 1.0 min, held at 85% A for 3.0 min, then ramped to 97% A in 0.5 min, held there for 3.0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LINC
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CLIN
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JOS
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CHL
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SPIR
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TYL
3.74
THMP
2.95
FLR
3.37
PEN G
3.84
PIRL
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ERYTH
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AMP
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TUL
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Intensity, cps
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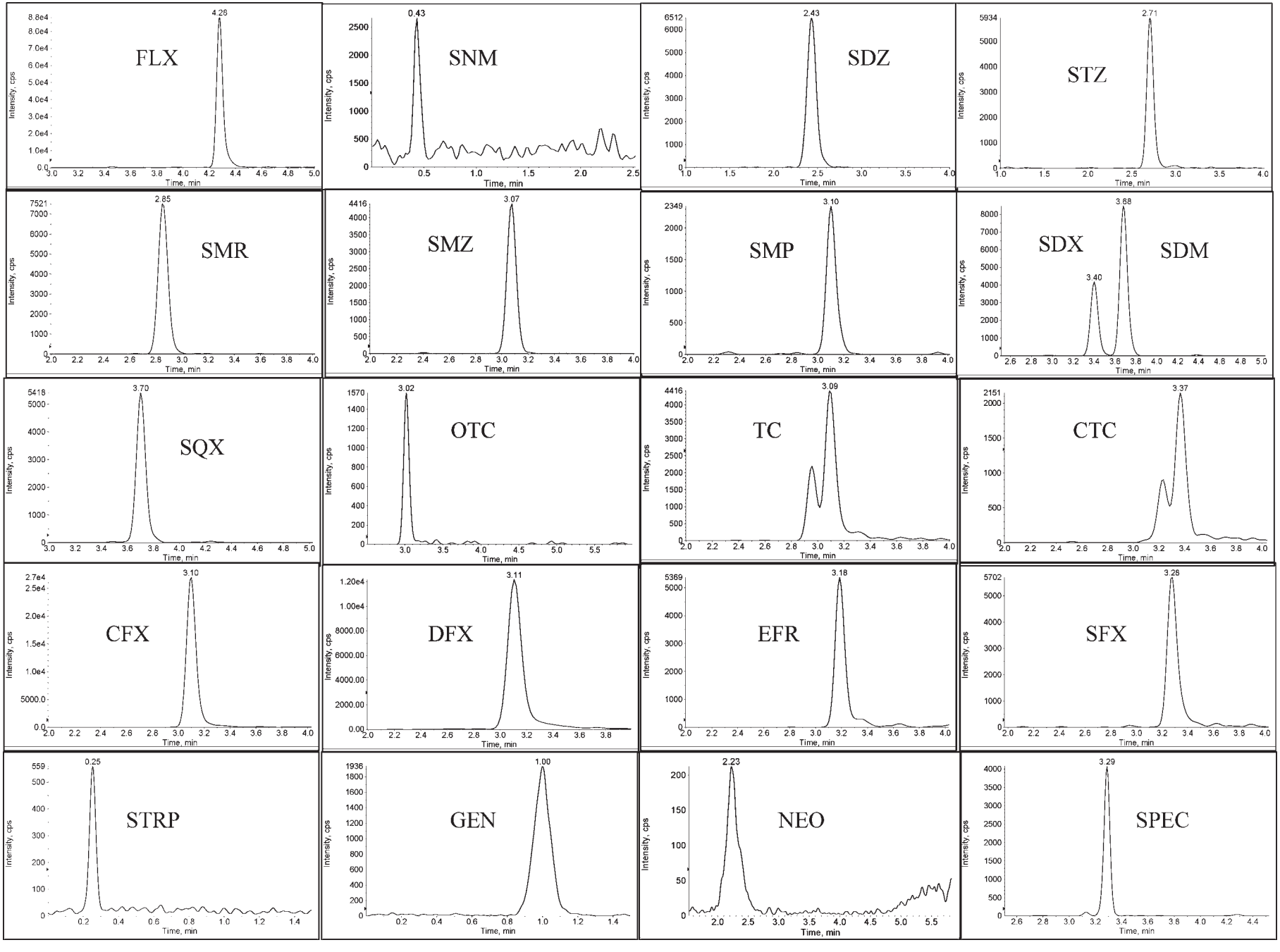
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Intensity, cps
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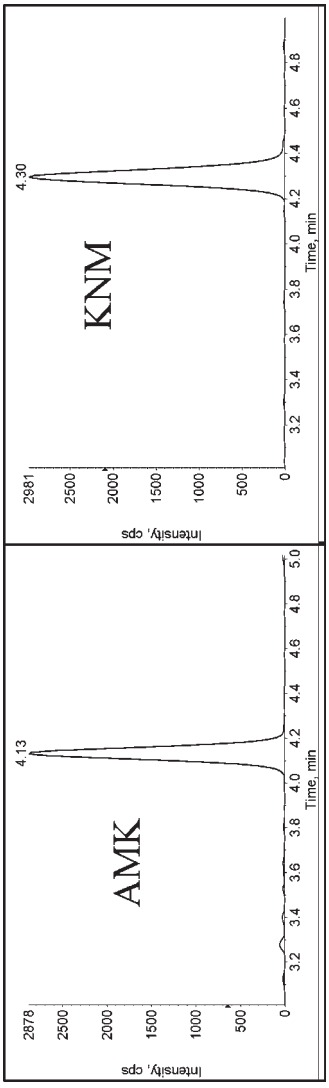

Figure 4. Example chromatograms of spikes, for MRM1, for each of the 39 target drugs. STRP, GEN, and NEO were separated using gradient 1, and SPEC, AMK, and KNM were separated using gradient 2.

Table 2. Summary of Accuracy, Standard Deviation (SD; n = 8 Spikes at 50 ng/g), and Method Detection Limit (MDL)

compound	accuracy (%)	SD (%)	MDL (ng/g)
AMK	78	9	4
AMOX	74	6	22
AMP	71	7	1
CFX	83	7	2
CHL	83	2	2
CLIN	85	4	2
CLOX	62	3	3
CTC	50	7	3
DANO	85	4	4
EFR	81	8	4
ERYTH	68	14	3
FLR	84	2	2
FLX	70	2	1
GEN	68	4	2
JOS	77	6	3
KAN	72	4	6
LINC	85	5	5
NEO	45	2	2
OTC	51	7	8
PenG	56	7	1
PIRL	81	4	1
SDM	78	3	3
SDX	78	5	6
SDZ	80	7	2
SFX	77	8	4
SMP	64	18	10
SMR	78	1	2
SMZ	77	6	3
SNM	106	15	41
SPEC	83	12	4
SPIR	72	2	2
SQX	70	3	4
STREP	54	3	1
STZ	78	8	2
TC	64	5	2
THMP	90	3	2
TILM	79	6	5
TUL	84	4	5
TYL	67	4	10

min, then ramped to 3% in 0.5 min, and held at 3% A for 5 for a total run time of 15.0 min. Injection volumes were 10 μL.

Calibration and Method Detection Limit Studies. Matrix-matched calibration standards were prepared with each run for veal muscle using the mixtures of the standards in blank extracts. For the method detection limit (MDL) studies, the standard deviation of eight replicate spikes at both 10 and 100 ng/g for each analyte were calculated in units of concentration and multiplied by 3 (for 7 degrees of freedom and 99% confidence level), thereby providing an estimate of the MDL (40 CFR, Appendix B to Part 136 revision 1.11, U.S.). This statistical approach ensures that random noise distributions from the entire analysis, from spiking to data reduction, are considered in the estimations and also helps to avoid potential difficulties in dealing with instances of essentially noiseless MRM transitions at the analyte retention times often observed in LC-MS/MS, which could result in artificially low MDLs.

Incurred Samples. Veal muscle samples were received from random abattoirs in Ontario as part of a routine monitoring program. Blank tissues that were tested to be free of the analytes from these samples were used for the preparation of matrix-matched calibration standard by adding the analytes after extraction of the blank tissue. Samples identified as positive using the semiquantitative screen were then reanalyzed for confirmation and quantitative analysis using one of the following standard methods: aminoglycosides (*27*), amphenicols (*28*), β-lactams (*29*, *30*), macrolides (*26*, *31*), flunixin, a nonsteroidal anti-inflammatory (NSAID) (*32*), fluoroquinolones (*33*, *34*), sulfonamides (*35*, *36*), and tetracyclines (*37*, *38*). The method performance for each utilized

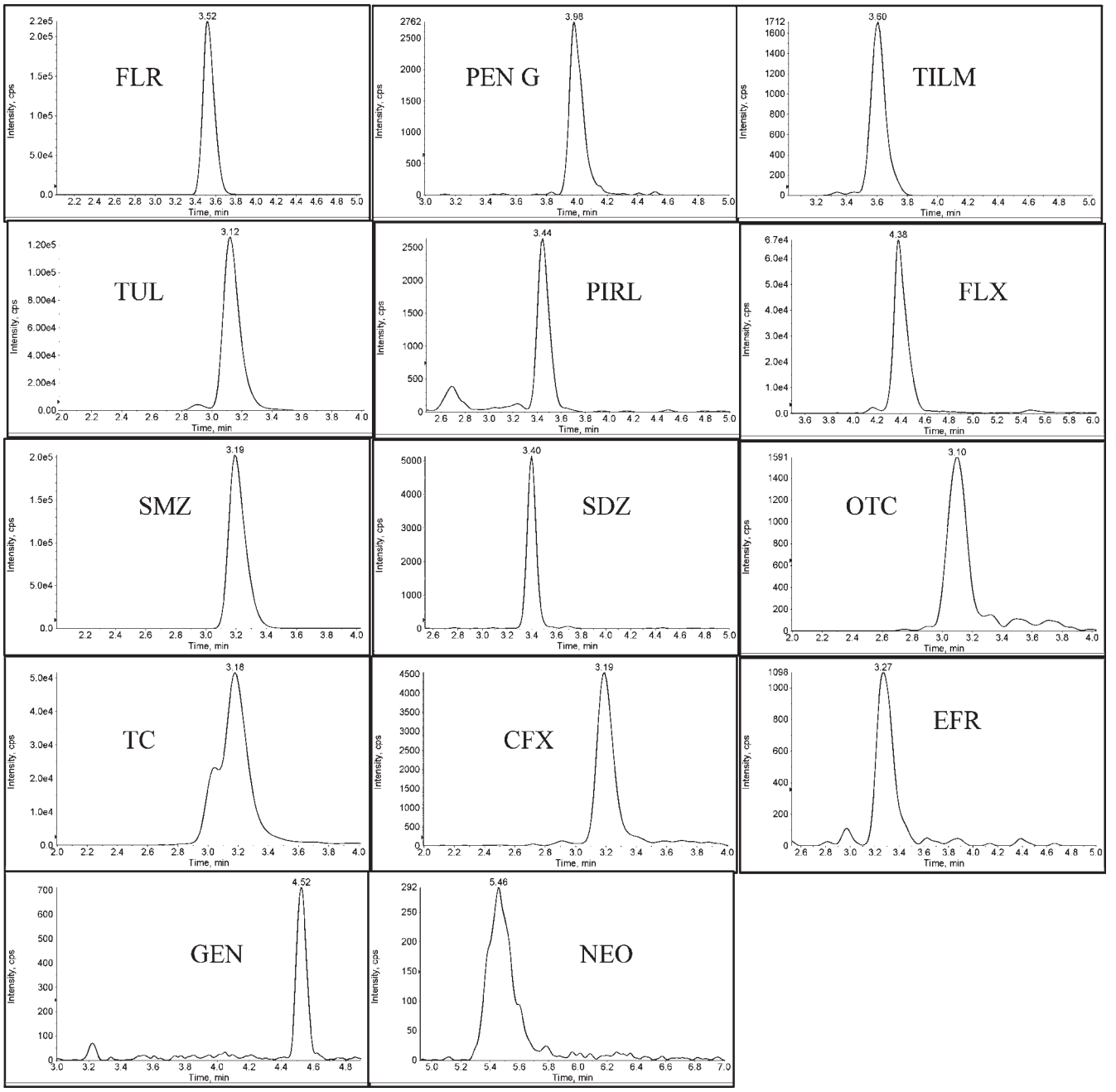

Figure 5. Example chromatograms of incurred positives. Gradient 2 was used to separate the aminoglycosides.

standard method was in part assessed using ongoing proficiency tests (data not shown).

RESULTS AND DISCUSSION

The method described in this work was developed to reduce the number of false positives realized from receptor and inhibitor assay-based drug screening systems for the routine screening of a large number of drug classes including aminoglycoside, macrolide, and tetracycline residues in tissue. For one of several programs with 300 samples of veal muscle alone over the course of a 12 month period, ~44% (132 samples) of those samples were suspect false positive for an aminoglycoside, ~25% (75 samples) false positive for a macrolide, and ~20% (60 samples) false positive for a tetracycline. Suspect positives required detention of the suspect positive carcass and triggered the need for confirmation with a confirmatory method specific to each of the drug classes. Confirmation results were required within 48 h for regulators to decide whether to condemn or release the suspect carcass. Typically, inefficient batch sizes of one or two samples were realized and, at times, for more than one drug class. Elimination of false positives resulting from the screening of these carcasses and increasing the confidence of rapidly identifying target drug residues during the screening led to the development of a multiresidue/multiclass drug determination using LC-MS/MS.

Sampling and Extraction. Screening samples using LC-MS/MS required alternate sampling and extraction routines for the various drug classes. Subsampling from bulk tissue was modified by introducing hand chopping and/or dicing the veal muscle to 2−3-mm-sized subsamples. This proved to be more efficient than mechanical homogenization techniques that were readily plugged with connective tissue. Hand chopping significantly reduced the possibility of cross-contamination from mechanical systems as well as the need to manage them.

Table 3. Summary of Estimated Concentrations Using the Semiquantitative Screening and Standard Methods

class	compound	screening estimate (ng/g)	standard method (ng/g)	method
aminoglycosides	gentamicin	50	120	ref *27*
	streptomycin	140	68	
amphenicols	florfenicol	200	130	ref *28*
		1700	1500	
		2900	3100	
		6.0	5.9	
		810	390	
		940	770	
β-lactams	penicillin G	150	140	ref *30*
		5500	3400	
		38	27	
fluoroquinolones	ciprofloxacin	1070	620	ref *34*
		2200	1400	
		35	37	
	enroloxacin	350	300	
		290	210	
		600	460	
		20	17	
macrolides	pirlimycin	6	7.4	ref *26*
		12	7.8	
		24	24	
	tulathromycin	490	570	
		35000	39000	
		140	85	
		28	28	
		32	47	
		26	16	
		730	640	
		770	630	
NSAID	flunixin	120	190	ref *32*
		9.5	14	
		47	69	
		870	560	
sulfonamides	sulfadoxine	100	61	ref *36*
tetracyclines	oxytetracycline	52	58	ref *38*
		89	140	
	tetracycline	170	89	
		1000	960	
		570	400	
		1500	1500	
		14	10	

Disposable polytron heads were considered, but in addition to the cost barrier, they proved to be unable to homogenize routine laboratory samples.

Extraction avoiding the use of organic solvent, which focused on using hot water or buffered water at temperatures ranging from 60 to 90 °C, yielded unacceptable to no recoveries for various drugs among all eight classes, such as the β-lactams. Interestingly, swelling and expansion of the tissue were realized when the tissue was heated in water after 30 min. This was considered to be a potential benefit for analyte extraction given the apparent enhancement in porosity of the tissue. The increase in chemical potential for solute migration to the extracting phase is expected with a large volume extraction of finely chopped, large surface area tissue at above ambient temperatures. Given the issues with water as the extracting phase, CH_3CN/water was substituted as the extracting phase. When heated above ambient, CH_3CN/water produced the same phenomenon of tissue swelling as was observed in water. Extraction temperatures above 70 °C were avoided given the boiling point of CH_3CN/water (86:14). With increasing extraction times at 60 °C using CH_3CN/water, an increase in the recovery of all drugs was realized with an optimal balance of recoveries realized at 60 min.; however, as expected, spiked aminoglycosides in tissue prior to this CH_3CN/water extraction demonstrated no recovery for the aminoglycosides. As described, this suspension was cooled and centrifuged, but importantly the aminoglycosides were observed adsorbed to the pellet, for example, denatured protein and other precipitated components of the tissue. It is generally understood that the aminoglycosides form strong complexes with various biopolymers, and their detection in the pellet versus the supernatant was consistent with that understanding (*39*).

Pooling the supernatants from both extractions resulted in a solution of the eight drug classes, which was semiquantitatively analyzed by LC-MS/MS. It should be noted that plastic LC vials were used for the aminoglycosides given their adsorption to glass.

Analysis. Table 1 summarizes the key analytical conditions used for the various drug classes. Building on previous work with macrolides (*26*), all of the target compounds demonstrated retention with the Atlantis minicolumn; however, the aminoglycosides were separated using a ZIC-HILIC column, which this laboratory has used for aminoglycoside analyses over the course of three years. Note that a number of compounds, such as the amphenicols, several lactams, and flunixin, which were separated using column A, were analyzed by ESI in positive mode. For column B, NEO was also ionized using ESI in negative mode. In all cases where ESI negative was used, the analytical advantage of greater signal-to-noise ratios and/or larger signal intensities was realized. This necessitated the requirement for two separate injections for those compounds with column A: one in positive ionization and one in negative ionization, given polarity switching was not an option with the closely eluting compounds (**Table 1**).

Chromatographic and Source Optimization for Gentamicin and Neomycin. Traditionally, mobile phase modifiers such as ion-pairing agents and acetate are used to provide optimum chromatographic conditions for GEN, NEO, and other aminoglycosides; however, eliminating the use of ion-pairing agents given their negative influence on ionization (*40*) and removal of acetate as a mobile phase modifier, because it was also observed to have a negative influence on ionization, resulted in enhanced signal-to-noise ratios, but with capacity factors of <2. The introduction of 0.4% v/v formic acid was found to provide retention of the target aminoglycosides (**Figure 4**). The removal of ion-pairing agents and acetate from the mobile phase necessitated additional source optimization for GEN and NEO. Quite interestingly, optimum source voltages for GEN realized following infusion, flow injection analysis, and tee-infusion with matrix were substantially higher than those realized following elution of GEN from the ZIC-HILIC column. Because the purchased GEN standard was prepared as a sulfate complex, it was hypothesized that the ZIC-HILIC column was providing an in-line separation of GEN from its complex with sulfate. Therefore, source voltages realized from GEN infusion were actually higher than required for its optimum ionization following separation with the ZIC-HILIC column. The result was excessive fragmentation with a minimal abundance of parent ion. Source optimization of GEN following elution from the ZIC-HILIC column resulted in a 35% decrease in the optimum declustering potential for GEN. The result was a decrease in the GEN MDL from approximately 100 to 2 ppb.

Conversely, this was not observed for NEO, presumably due to the sorption affinity of NEO for polar species, which is significantly greater than that for GEN (*39*); further work is required to investigate this. It was also observed that NEO was optimally ionized by ESI negative, yielding approximately 4 times greater response, which has not been previously reported. Work is currently under way to establish the mechanism for its ionization.

Figure 3 presents the total ion currents (TICs) from the chromatography realized using columns A and B. Note two multiple reaction monitoring (MRM) experiments were captured per target analyte.

Method Performance. Table 2 summarizes the accuracy for each of the 39 compounds established at 50 ng/g and the MDL estimated as indicated under Materials and Methods. The typical accuracy ranged from 45%to 106%, with only erythromycin, sulfanilamide, and spectinomycin with >10% RSD. Note that precision data were calculated for eight replicate measurements. All estimated MDLs were significantly better than those from in-house receptor-based screening methods and ranged from 1 to 22 ng/g in tissue. **Figure 4** demonstrates typical chromatography from the three injections for each of the 39 compounds (MRM1 is presented).

Incurred Positives. The method was tested as part of a routine regulatory monitoring program for edible veal tissue. **Figure 5** presents typical chromatography of positive drug residues from the analysis of routine samples, and **Table 3** summarizes the concentrations estimated with this semiquantitative method compared to the results using standard methods; note that sample preparation and extraction for the standard methods were carried out as prescribed in the method, that is, with blenders or polytrons. The drug residue concentration in tissue was estimated against control spikes, of blank tissue samples, at or near the level of concern. Of interest is the detection of a wide range of target drug residues and in some cases substantially above the MDL for those compounds, which went largely undetected with our in-house receptor-based screening method. The discovery of gentamicin at low levels was significant given it was not permitted for use in Canada.

Combined with the retention time, and two transitions, enhanced confidence with identification of these incurred positives was realized (*40*). Confirmation of the identified drug residues was carried out using standard methods and, given the use of orthogonal methods, demonstrated the ongoing ruggedness of this semiquantitative screening method. Expansion to include other matrices, such as kidney, and to include other species such as porcine, poultry, and caprine are underway. Additional compounds from the various drug classes will be included as well as metabolites from β-lactams not discussed. Finally, single laboratory validation of the method is also currently underway.

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