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Ultratrace Analysis of Nine Macrolides, Including Tulathromycin A (Draxxin), in Edible Animal Tissues with Minicolumn Liquid Chromatography Tandem Mass Spectrometry

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The analysis of nine macrolides is presented, including tulathromycin A (Draxxin), in beef, poultry, and pork muscle with a simple multiresidue extraction and analysis method using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry. The sample preparation method involves extraction with acetonitrile and defatting with hexane followed by dilution of the extracts for analysis. Separation of the nine macrolides was performed using an Atlantis dC_{18}, 3 μm, 3.9 mm $\times$ 20 mm minicolumn (guard column). Detection was carried out with two multiple reaction monitoring experiments per macrolide. The method detection limits (MDLs) were based on three times standard deviation of eight repeat spikes at 3.0 ng/g of a mix of the nine macrolides in the various tissues. The MDLs and retention times for the macrolides were as follows: lincomycin, 0.19 ng/g (t_R = 5.00 min); tulathromycin, 0.46 ng/g (t_R = 5.63 min); spiramycin, 0.21 ng/g (t_R = 6.06 min); pirlimycin, 0.10 ng/g (t_R = 6.04 min); clindamycin, 0.16 ng/g (t_R = 6.20 min); tilmicosin, 0.29 ng/g (t_R = 6.38 min); erythromycin, 0.19 ng/g (t_R = 6.62 min); tylosin, 0.10 ng/g (t_R = 6.72 min); and josamycin, 0.09 ng/g (t_R = 6.98 min). Precision at 25 ng/g (n = 4) ranged from 2.3 to 9.4% for the compounds from beef muscle. Of interest is the detection of incurred residues of tulathromycin A in edible calf tissue at 0.10−7 μg/g, which is presented here for the first time.

KEYWORDS: Macrolides; tulathromycin A (Draxxin); LC-MS/MS; residue analysis; animal tissues; rapid separation

INTRODUCTION

Macrolides are a widely used group of antibiotics for the treatment of respiratory infections in food-producing animals, and they are also used as growth promoters, which increases their prevalence in the food supply (*1*). 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. As a result of the need for regulatory enforcement, liquid chromatography coupled to mass spectrometry and tandem mass spectrometry (LC-MS and LC-MS/MS) are the instruments of choice for their determination, particularly given the need for identification (*2*−*4*).

Commonly tested macrolides include lincomycin, tulathromycin, spiramycin, pirlimycin, clindamycin, tilmicosin, erythromycin, tylosin, and josamycin (*5*−*10*) (**Figure 1**). The Canadian Food Inspection Agency (CFIA) indicates the need to routinely test for these macrolides, with the more recently introduced and commercially used tulathromycin A currently not part of that testing requirement.

The mandated testing for a wide range of macrolides often requires the use of several methods for their complete determination; extractions generally make use of aqueous buffer systems, and sample cleanup generally requires solid phase extraction (SPE) (*11*−*13*). While SPE is common, it is not without difficulties, given that it can limit analytical throughput and is costly relative to methods based on solvent extractions alone. Variables such as lot-to-lot differences in manufacturing, loading rates, and solvent channelling can all lead to issues with precision and recovery. Although some multiresidue methods for macrolides have been described in the literature, there are no published LC-MS/MS methods making use of simple extraction methodologies for a wide range of commonly used macrolides that include the new macrolide tulathromycin A (Draxxin) and that do not include SPE.

The objectives of this work were to investigate an extraction technique without the need for SPE for lincomycin, tulathromycin A, pirlimycin, spiramycin, clindamycin, tilmicosin,

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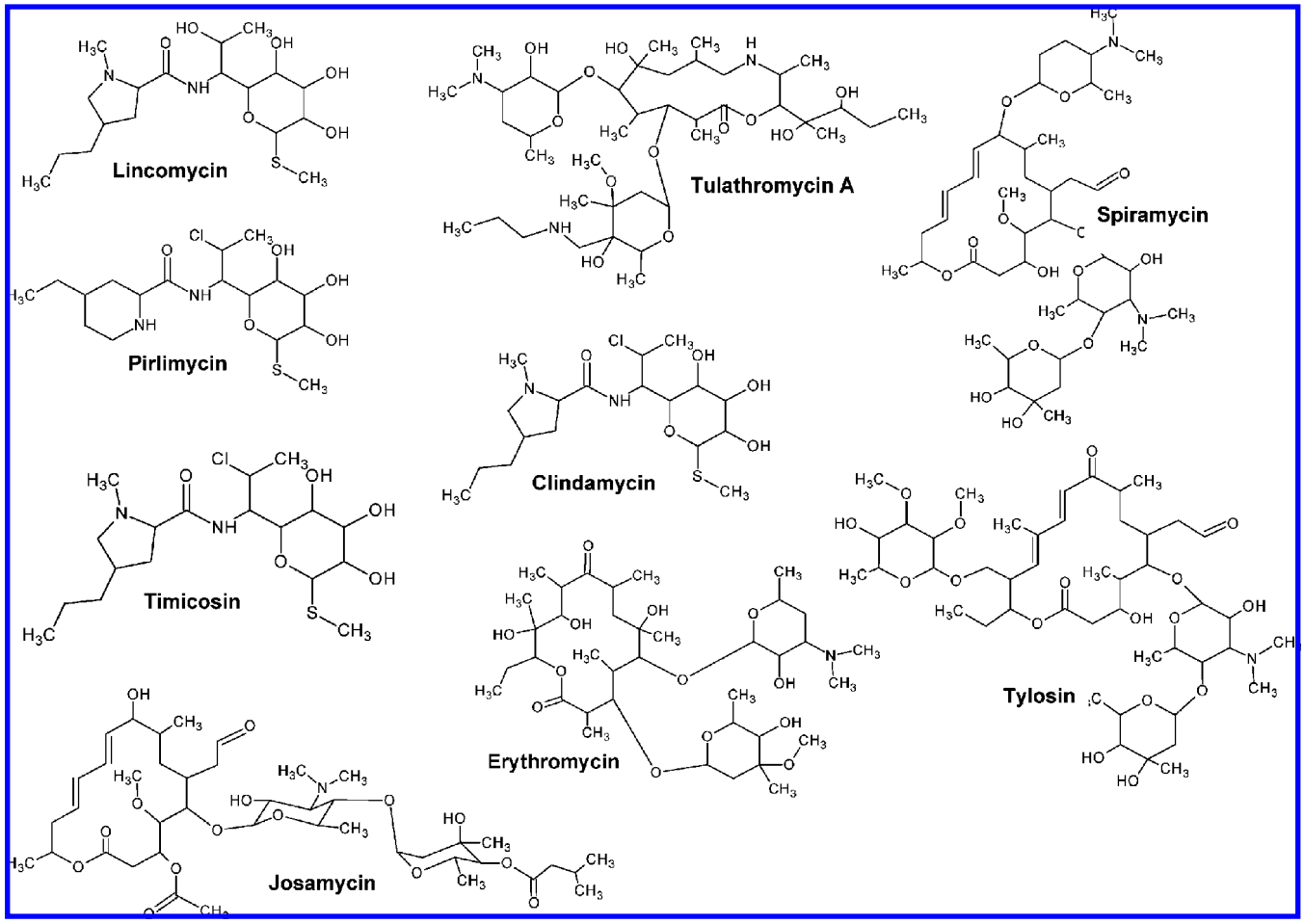

Figure 1. Structures of the macrolides.

erythromycin, tylosin, and josamycin in beef, poultry, and pork and to establish a fast chromatographic approach using mini-column high-performance liquid chromatography (HPLC) for their separation specifically from beef muscle, calf muscle, poultry muscle, and pork muscle.

MATERIALS AND METHODS

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

Chemicals. Clindamycin (99%), josamycin (100%), spiramycin (93.6%), tilmicosin (86.8%), and tylosin (91.7%) were purchased from Sigma (St. Louis, MO). Erythromycin (93.5%) and lincomycin (99.5%) were from Dr. Ehrenstorfer GmbH (EQ Laboratories, Atlanta, GA). Pirlimycin (87%) and tulathromycin A (80%) were obtained from Pfizer Animal Health (Montreal, QC, Canada). Formic acid (88%), acetonitrile (CH_3CN, distilled-in-glass), and hexane (99%) were purchased from Caledon Laboratories (Georgetown, ON, Canada).

HPLC. An Agilent 1200 series system included an autosampler, pump, degasser, and column heater (set to 20 °C) (Agilent Technologies, Santa Clara, CA), all controlled by Analyst 1.4.2 (Applied Biosystems, Foster City, CA). All chromatography was carried out on a Waters Atlantis guard dC_{18} 20 mm × 3.9 mm, 3 μm column in a guard column holder (Mississauga, ON, Canada). The mobile phases consisted of A, 0.1% formic acid in ultrapure water (in-house distilled water passed through a NANOPure system from Barnstead, Dubuque, IA), and B, CH_3CN. All injection volumes were 5 μL. The column flow rate was 1.0 mL/min. The gradient was 99% A for 1.0 min, linearly ramped to 65% until 5.0 min, then to 1.0% up to 8.0 min, where it was held for 4.0 min. At 12.1 min, the conditions were returned to 99% A for 4.4 min.

Tandem Mass Spectrometer (MS/MS). The tandem MS used in this work was an API 4000 (Applied Biosystems). Electrospray ionization in positive mode was used for each of the nine macrolides followed by multiple reaction monitoring (MRM) experiments with two transitions per ion yielding a total ion current (TIC) for all of the transitions and TICs for the two MRMs for each macrolide. Optimized MS conditions are provided in **Table 1**. The source conditions were, for all compounds, as follows: curtain gas, 25 psi; GS1 set to 65 psi (capillary pneumatic gas flow); GS2 set to 50 psi (thermo heaters); temperature, 600 °C; ionspray, 4900 V; interface heater on at 100 °C; and CAD gas at 8 psi. Air from a GA7FF air compressor (Atlas Copco Compressors, Dollard-des-Ormeaux, QC, Canada) was diverted to a Parker Balston model N2-2010 nitrogen generator (Haverhill, MA) at 120 psi, yielding 99% pure N_2.

Standard Solutions. Individual standards were weighed using a Mettler Toledo XS105 five-point calibrated balance (Columbus, OH). All volumetric apparati were class A, including pipettes. Stock solutions of each standard were accurately prepared by weighing out 5−14 mg of each, followed by a quantitative transfer to a 50 or 100 mL volumetric flask and filling to volume with CH_3CN. Individual final stock solution concentrations ranged from 71.13 to 214.5 μg/mL. A 10 μg/mL mixed working standard was prepared by transferring the appropriate volume of individual stock solutions into a 50 mL volumetric flask, filling to the line CH_3CN. One μg/mL and 0.1 μg/mL spiking solutions were prepared by serially diluting the 10 μg/mL working solution (5 mL into 50 mL volumetric flask filled to volume with CH_3CN).

Optimization. Infusion, for the purpose of characterizing preliminary conditions for target analyte ionization, was carried out using a model 11 Plus syringe pump (Harvard Apparatus, Holliston, MA). The ion currents fell within the acceptable limits of instrument operation. Infusion rates were set between 10 and 20 μL/min. For further optimization and verification of conditions, flow injection analysis (FIA) was carried out without a column between the injector and the MS

Table 1. Summary of Source (Electrospray Ionization Positive Mode) and MS/MS Parameters[a]

compound	DP	CE	CXP	transition
				$[M + H]^+$
clindamycin	81	39	10	425.3 > 126.0
	86	39	8	427.3 > 126.0
				$[M + H]^+$
erythromycin	81	27	34	734.6 > 576.6
	81	45	10	734.6 > 158.1
				$[M + H]^+$
josamycin	46	45	10	828.6 > 174.1
	46	71	18	828.6 > 109.1
				$[M + H]^+$
lincomycin	76	27	24	407.2 > 359.2
	76	55	22	407.2 > 126.1
				$[M + H]^+$
pirlimycin	81	38	18	411.4 > 112.2
	91	39	18	413.4 > 112.2
				$[M + H]^+$
spiramycin	131	49	16	843.6 > 174.1
	131	73	18	843.6 > 101.0
				$[M + 2H]^{2+}$
tilmicosin	76	61	16	435.5 > 88.1
	76	23	16	435.5 > 695.7
				$[M + H]^+$
tylosin	130	53	12	916.5 > 174.2
	130	41	12	916.5 > 772.6
				$[M + H]^+$
tulathromycin A	106	33	44	806.7 > 577.6
				$[M + 2H]^{2+}$
	71	31	10	404.0 > 158.2

[a] DP, declustering potential; CE, collision energy; and CXP, collision exit potential.

interface. Typically, 1 μL of a standard was injected, which would have permitted ion currents to fall within the acceptable limits of instrument operation.

Calibration. Mixtures of the nine macrolide matrix-matched standards (macrolide standards prepared in blank matrix extract) ranged in concentration from 0.1 to 7.5 ng/mL (five-point) in vial for the method detection limit (MDL) study and from 2 to 50 ng/mL (five-point) in vial for all other routine instrument operations. Equivalent sample concentrations (in vial) correspond to 0.5−37.5 and 10−250 ng/g.

Samples and Proficiency Samples. Samples were obtained from random abattoirs in Ontario and local grocery stores. Blank tissues that were free of the analytes were obtained from abattoirs and local grocery stores, and the tissues were used for preparation of matrix-matching calibration standards by adding the analytes after extraction of the blanks. The proficiency program is facilitated by the Centre for Veterinary Drug Residues, CFIA (Saskatoon, Saskatchewan, Canada). Beef muscle samples were tested as part of the proficiency testing program, with a focus on tilmicosin ranging from 0.2 to 0.35 μg/g.

Sample Preparation and Spiked Samples. Finely chopped (1−2 mm) muscle tissue samples of beef, calf, poultry, or pork (5.0 ± 0.1 g) were weighed into 50 mL polypropylene centrifuge tubes, and 5 mL of CH_3CN was added. In the case of spikes, final in-tissue concentrations were prepared in tissue prior to delivery of extraction solvents. The tissue slurries in CH_3CN were then sonicated for 15 min (Branson 2510 ultrasonic bath, Danbury, CT). The samples were shaken at 400 rpm for 10 min on a C2 Platform Shaker (New Brunswick Scientific, Edison, NJ), vortexed for 1 min using a Thermolyne Tyde 16700 vortex mixer (Fischer Scientific Company, Ottawa, ON, Canada), and then centrifuged at 2225*g* for 10 min at 5 °C with an Allegra 6R centrifuge with GH 3.8A Rotor (Beckman Coulter, Inc., Fullerton, CA). The supernatants were then transferred into new 50 mL centrifuge tubes. An additional 2.5 mL of CH_3CN was added to the tissue pellets, and the mixtures were again sonicated, shaken, vortexed, and centrifuged as described above. The clear supernatant was combined with the supernatant from the first extraction, and the final volume was made up to 25 mL using ultrapure water. The extract was defatted using 5 mL of hexane, with hand shaking for 1 min. The extracts were then centrifuged for 10 min at 2225*g* at 5 °C, and the hexane layer was discarded. (Analysis of hexane from spiked reagent extracts revealed that the macrolides were not extracted during this step.) An aliquot of the extract (~1 mL) was filtered through a 0.2 μm Teflon syringe filter (Chromatographic Specialties, Brockville, ON, Canada) and into a vial for analysis by LC-MS/MS. The 0.2 g/mL equivalent sample extracts were not concentrated for analysis, and typically, 1 mg of equivalent sample was injected onto the column. All spiked samples were prepared by adding appropriate aliquots of the mixed standard to 5.0 g of homogenized tissue, letting the sample settle for 30 min, then preparing the sample as indicated above. All final in vial concentrations were 5-fold dilutions of the samples.

MDL Study. Approximately 50 g of blank tissue (beef, poultry, or pork) was chopped as indicated in the previous section and thoroughly homogenized. Blank tissue was identified through analysis. Eight separate 5.0 ± 0.1 g subsamples from the homogenate were individually placed into eight 50 mL polypropylene tubes. The macrolides were spiked into each subsample to 3.0 ng/g, allowed to settle for 30 min, and then treated as samples. The standard deviations of the eight results for each macrolide, which were in units of concentration, were then multiplied by 3 (for 7 degrees of freedom and 99% confidence level), thereby providing an estimate of the MDL; this statistical approach (*14*) ensures that random noise distributions from the entire analysis, from spiking to data reduction, are considered in the estimations and also help 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.

RESULTS AND DISCUSSION

Chromatography and Detection of the Nine Macrolides. One of the novelties of this method is the use of a guard column as the analytical column, which we refer to as a "minicolumn," for the rapid separation of the macrolide analytes. Chromatography of lincomycin, tulathromycin, spiramycin, pirlimycin, clindamycin, tilmicosin, erythromycin, tylosin, and josamycin following a 5 μL injection of a 5 ng/g matrix-matched standard in beef muscle and detection with MS/MS in MRM mode are presented in **Figure 2**, in order of retention time. One of the transitions for tulathromycin A, in addition to tilmicosin, was monitored using doubly charged ions. Monitoring the doubly charged ions for tilmicosin and tulathromycin A has been previously reported (*7, 10*), and this strategy was also employed here.

One of our concerns was whether the minicolumns would give high reproducibility from lot to lot. **Table 2** provides a summary of the retention times (t_R), the observed range in t_R for each of the macrolides over the course of 13 injections for a group of calibration standards, the capacity factors (k'), and peak widths at half-height ($W_{1/2}$) for each of the nine macrolides studied in this work for two of four columns. The other identical columns were acquired from different production lots from the same manufacturer, and we observed no differences for practical purposes in results of the factors presented in **Table 2** among the minicolumns (data for other columns not shown).

Of particular interest are the $W_{1/2}$ with this column, which were all less than 3.0 s for the nine macrolides, and where all of the k' values ranged from 24 to 34. The narrow peak widths are significant because an increase in signal-to-noise, and therefore a reduction in limit of detection, is possible due to a greater rate of analyte mass loading per unit time to the ion source, which in turn yields an opportunity to dilute the extracts and/or inject smaller volumes, thereby minimizing column matrix loading. Diluting the extracts in turn further simplified the extraction method since SPE was not required to concentrate the target compounds. Additionally, reducing the on-column introduction of matrix is significant given that the matrix introduces one of the largest undesirable variables in the

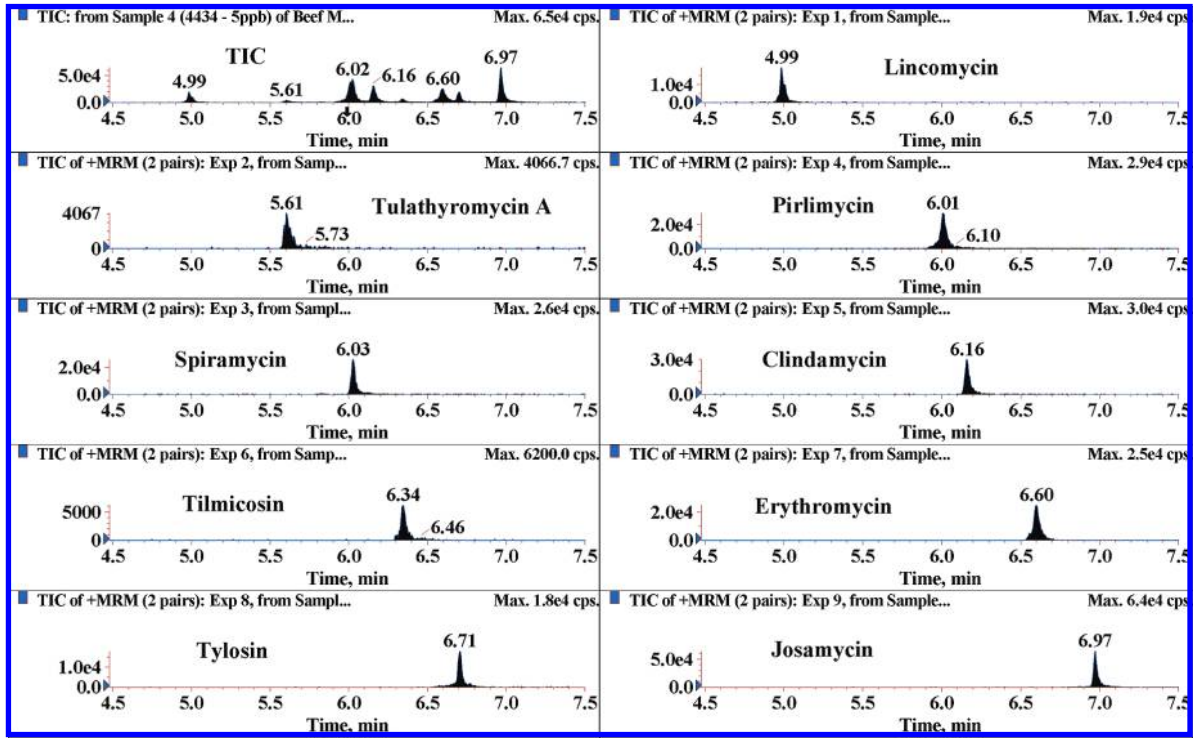

Figure 2. TICs of two MRMs for each of the macrolides at 5 pg/μL in vial, matrix-matched standard in beef tissue extracts.

Table 2. Summary of Peak Performance Characteristics, Column to Column Assessment of Retention Times, Signal to Noise (S/N) at 25 pg On-Column (for the TIC of Two MRMs)[a]

	column 1					column 2			
compound	t_R (min)	± (min) (n = 13)	K'	$W_{1/2}$ (s)	S/N (rms)	t_R(min)	± (min) (n = 13)	$W_{1/2}$ (s)	% Er
lincomycin	5.00	0.02	24	2.2	35	4.74	0.03	2.1	−5.1
tulathromycin A	5.62	0.03	27	2.8	14	5.50	0.03	2.7	−2.2
pirlimcyin	6.05	0.03	29	2.5	36	6.01	0.03	2.4	−0.5
spiramycin	6.07	0.03	29	1.9	46	6.02	0.03	1.7	−0.4
clindamycin	6.20	0.03	30	2.0	43	6.04	0.03	2.0	−2.5
tilmicosin	6.38	0.02	31	2.2	28	6.29	0.03	2.1	−1.4
erythromycin	6.62	0.03	32	2.3	52	6.56	0.03	2.2	−0.9
tylosin	6.72	0.03	33	1.9	57	6.67	0.03	1.9	−0.7
josamycin	6.99	0.03	34	1.7	64	6.93	0.03	1.7	−0.7

[a] Retention time ranges were based on standard injections.

Table 3. Percent Recovery, CV at 25 ng/g in Beef Muscle and 17 ng/g Spike in Pork and Poultry Muscle, and MDLs for Each Macrolide (ng/g)[a]

	beef		pork		poultry	
compound	recovery (CV)	MDL (ng/g)	recovery (CV)	MDL (ng/g)	recovery (CV)	MDL (ng/g)
lincomycin	78 (9.4)	0.19	103 (12)	0.44	72 (8.8)	0.61
tulathromycin A	102 (3.6)	0.46	117 (8.1)	0.70	137 (7.0)	0.90
pirlimcyin	62 (8.0)	0.10	88 (9.7)	0.40	66 (8.1)	0.52
spiramycin	74 (2.7)	0.21	68 (12)	0.65	67 (17)	0.35
clindamycin	91 (4.9)	0.16	99 (7.5)	0.55	88 (9.9)	0.62
tilmicosin	100 (5.2)	0.29	83 (9.9)	0.69	102 (7.8)	0.98
erythromycin	84 (4.4)	0.19	103 (3.9)	0.52	92 (4.5)	0.86
tylosin	73 (3.6)	0.10	83 (11)	0.38	68 (14)	0.52
josamycin	78 (2.3)	0.09	100 (2.4)	0.36	86 (4.5)	0.62

[a] Recovery (n = 4) from beef muscle and MDL results, in beef muscle, as estimated with eight replicates at 3.0 ng/g (95% confidence, 3 × SD of the measured concentrations).

chromatographic process, particularly with LC-MS/MS, by way of column lifetime and ionization suppression. With small injection volumes and dilute extracts, column 1 (**Table 2**) was used for approximately 600 injections over the course of a year just for macrolide determinations. Reduction in column performance for macrolides was only evident following substantial cross-utilization of the column for other methods where approximately 500 samples of fish, honey, liver, and kidney for other compounds were also analyzed. New columns dedicated to macrolides only using the method described in this work have not deteriorated in performance, even after 500 injections.

Short run times, for the purpose of sample throughput, are always important to achieve with any chromatographic method but cannot be at the expense of other key factors, particularly between-run repeatability of analyte t_R and peak shape characteristics such as $W_{1/2}$. Use of the 2 cm minicolumn necessitated additional investigation regarding the usefulness of this column for the macrolides by LC-MS/MS, especially for regulatory applications. The question of column to column variability was addressed using different production lots of columns from the one manufacturer over the course of a year. An example of this comparison is provided in **Table 2** for "column 1" and "column 2". The data indicate that while absolute t_R values changed, their differences were, except for lincomycin, all less than 3% between the two columns. The differences were well within t_R shifts observed among analytical LC columns.

While guard columns are not commonly thought practical for chromatographic purposes, perhaps due to the suggestion that they may be less stringently manufactured than conventional analytical columns, our data indicate that the guard columns that we tested can in fact provide reliable and rugged chromatography. Discarding the column is then significantly less costly following demonstration of poor column performance. It is also possible to study a large range of different stationary phases without substantial cost. While carbon loading tends to be larger with C18 guard columns, this actually presents an analytical advantage to the chromatographer for the retention of compounds with hydrophobic properties, such as the macrolides. Of interest is the common perspective that a guard column is used to protect the "analytical" column, when in fact in this

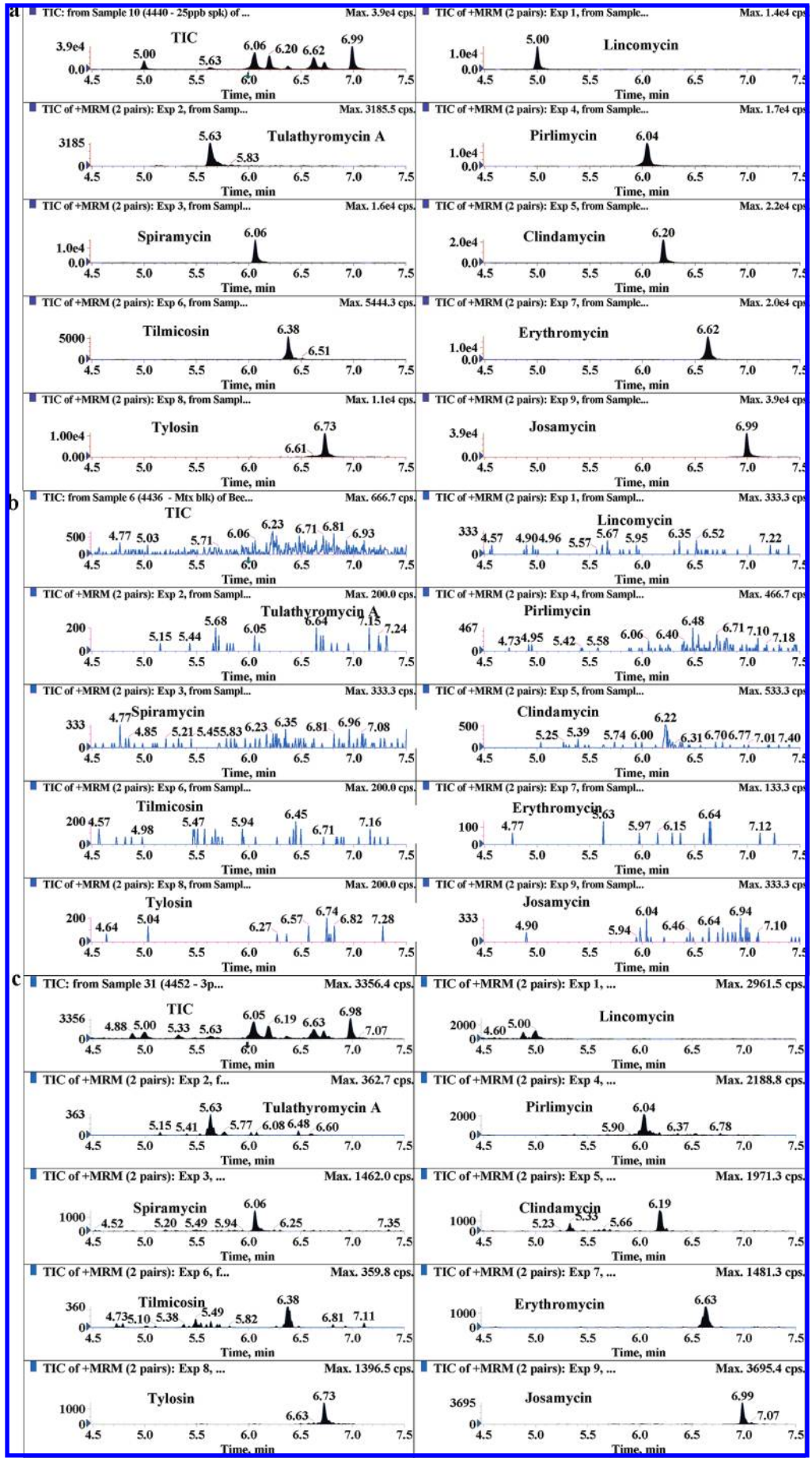

Figure 3. (**a**) TICs of two MRMs for each of the macrolides from 25 ng/g spiked beef muscle (≈5 pg/μL in vial). (**b**) TICs of two MRMs for each of the macrolides from blank beef muscle. (**c**) TICs of two MRMs for each of the macrolides from 3.0 ng/g spiked beef muscle (≈ 0.4 pg/μL in vial).

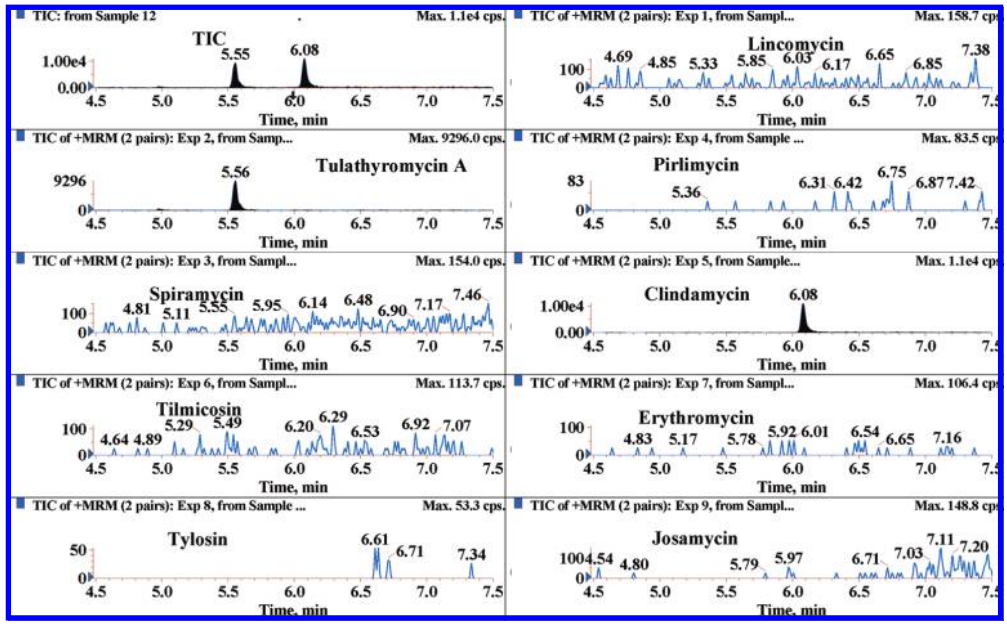

Figure 4. Calf muscle extracts showing tulathromycin A (~100 ng/g in tissue) and clindamycin (~2.5 ng/g in tissue).

example the guard column provided adequate separation of macrolides followed by MS/MS.

The disadvantage to using a guard column to protect a 10 or 15 cm "analytical" column is backpressure, which then requires using lower flow rates, and where in fact the "analytical" column contributes to large peak widths due to band broadening. LC systems delivering higher pressures assist in reducing those issues but require newer, dedicated technologies not readily present in most analytical laboratories.

With the low back-pressure in using the minicolumn, the use of higher flow rates, that is, 1.0 mL/min, was possible, which resulted in shorter run times as compared to a 15 cm column (0.4−0.5 mL/min flow rate with >25 min run times). Again, because the macrolides have a large affinity for the C18 stationary phase, as evidenced by the k' data, a higher flow rate was possible without compromise to chromatographic performance. The 2 cm minicolumn with 3.9 mm i.d. and flow rate of 1.0 mL/min (used in this work) gave a normalized mass flow rate delivery 77% narrower than a 15 cm column with a 2.1 mm i.d. and 0.5 mL/min flow rate.

For multiresidue methods of several macrolides, 25 min run times and 30 s peak widths are common (*15, 16*), and there is a method for six macrolides, which does not include tulathromycin A (*17*). While chromatography for tulathromycin A has been shown (*10, 18*), improvements in overall chromatography for it, along with the other (common) macrolides, were made in this work with the use of the minicolumn.

Calibration and Signal-to-Noise Ratios. Matrix-matched calibration was conducted using 2, 5, 10, 25, and 50 pg/μL concentrations (on-column from 10 to 250 pg). Recall that all in vial concentrations are 5-fold dilutions of sample extracts. Because of nonlinearity at the 250 pg level, quadratic regression was used for calibration. All calibration curves exhibited $R^2 \geq 0.999$ (square of the correlation coefficient) for the analytes, and back calculation of standards from the curves resulted in no more than 10% relative error from theoretical at each standard concentration. At 25 pg on-column (25 ng/g sample equivalent/5 pg/μL in vial), the signal-to-noise ratios ranged from 14:1 for tulathromycin to 64:1 for josamycin. **Table 2** summarizes the signal-to-noise ratios for all of the macrolides in the experiment.

Accuracy, Precision, and MDLs. The accuracy (recovery) at 25 ng/g sample spikes in beef muscle for four measurements and corresponding coefficients of variation (CVs) also appear in **Table 3**. Except for spiramycin, all other macrolides were recovered >70% and with less than 10% CV. Both poultry and pork were also spiked at 17 ng/g to test for recoveries (**Table 3**). No run to run carryover was observed. **Figure 3a−c** present chromatography for the 25 ng/g spike in beef muscle, a blank sample (a matrix blank), and spike at 3.0 ng/g, in that order. As the final in vial concentration of the macrolides entails a 5-fold dilution, the resulting in vial concentration from the 25 ng/g extract is expected to be 5 pg/μL.

A study was also carried out to estimate the MDLs of each macrolide in beef, pork, and poultry muscle (**Table 3**). Muscle is the regulatory target tissue. We first attempted the study at 10 ng/g in tissue, but the resulting CV for each compound was too low (generally much less than 10% CV), which prompted an additional study at 3.0 ng/g ($n = 8$, at 99% confidence) that resulted in up to ~40% CV for the compounds. This better indicated the closeness of the signal relative to overall noise for each set of transitions in the complete analysis. The advantage to the method is that these MDLs are all well below current Canadian maximum residue limits (MRLs). This sensitive method provides the opportunity to quantify macrolide usage in the food supply for research interests. Alternatively, depending on data quality objectives, the analyst could choose to increase the MDL by further dilution of the final extracts and/or reduced injection volume. This would further reduce matrix loading in the system, thereby extending column lifetime, and also reduce instrument downtime for cleaning. In this study, we felt that a 1 mg introduction of equivalent sample was appropriate for our purposes.

Field Samples. As part of a regulatory program for approximately 300 samples per year, selected samples of beef and/or calf muscle from abattoirs were tested for macrolides. Of those samples, four were found to contain tulathromycin A ranging in concentrations from 100 ng/g in tissue to 7000 ng/g. Of interest, **Figure 4** presents an example of a sample incurred with both tulathromycin A and clindamycin at approximately 100 and 2.5 ng/g in tissue, respectively. For tulathromycin A, the ratios of transitions 806.7 > 577.6 and 404.0 > 158.2 were

1.73 from a 125 pg on column standard and 1.50 for the sample. For clindamycin, the ratios of transitions 425.3 > 126.0 and 427.3 > 126.0 were 2.11 from a 25 pg on column standard and 2.38 for the sample. In both cases, the ratios of transitions in the samples were within ±20% of those in the matrix-matched standards.

Proficiency Testing. Successful participation in external proficiency testing rounds, where available, provided an excellent means of evaluating and demonstrating performance of an analytical method. The full method presented in this article was tested in national proficiency rounds for macrolides in beef muscle. The method was tested over a period of 16 months with four proficiency samples every 4 months. In all, there were 20 samples analyzed over 16 months, with typical concentrations of tilmicosin ranging from 0.2 to 0.3 μg/g. Of the 20 results, one was found questionable with a *Z* score (the *Z* score provides a statistical evaluation of the closeness of the reported result with those from a group of participating laboratories that analyze the same subsamples at approximately the same time) of +2.89; yet, it was still acceptable (> ±3.0 is unacceptable), with the other 19 samples at less than ±2.0. Example results from the last proficiency round were assigned values (μg/g) of 0.26, 0.25, 0.25, and 0.23, with this laboratory's results (μg/g) equal to 0.26, 0.25, 0.25, and 0.18 and with *Z* scores of 0.00, −0.36, 0.00, and −0.99, in that order.

ABBREVIATIONS USED

CE, collision energy; CV, coefficient of variation; CXP, collision exit potential; DP, declustering potential; k', capacity factor; (HP)LC, (high-performance)liquid chromatography; MDL, method detection limit; MRL, maximum residue limit; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometer; SPE, solid phase extraction; TIC, total ion current; t_R, analyte retention time (min); $W_{1/2}$, peak width at half height.

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