APPLICATION NOTE

Liquid Chromatography/
Mass Spectrometry

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Determination of Antibiotics and Veterinary Drugs in Milk by QSight LC/MS/MS

Introduction

Antibiotics and veterinary drugs are often used in animal production to improve animal health. However, improper use of drugs in animal production can lead to residue violations in animal source foods (ASF) and possible health risks, especially the potential of developing antibiotics resistance effect. Regulatory agencies around the world have established maximum residue levels (MRLs) or tolerance limits (TLs) in ASF or animal-derived food products, including milk.[1-5] Food Safety and Standards Authority of India (FSSAI) recently published a revised list of antibiotics and veterinary drugs and their TLs, including over 30 compounds in milk.[5] The MRLs or TLs for milk are usually lower than those set for other animal food matrices (such as meat and tissues) and span a wide concentration range depending on the drug used (from 2 µg/kg for monensin to 1500 µg/kg for neomycin in milk as listed by FSSAI). To ensure food safety and enforce regulations, reliable analytical methods are needed for monitoring drugs in different food matrices. However, it is challenging to develop a method that can determine all the drugs in a single run due to the complexity of sample matrices, the diversity of target analytes from various classes of chemical properties, and the instability of certain drug classes (such as β-lactams, and tetracyclines) in mixed solutions.

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The objective of this work was to develop methods for the analysis of antibiotics and veterinary drugs in milk listed by FSSAI using a PerkinElmer QSight 420 LC/MS/MS system. Two methods were developed for the analysis of over 30 analytes in milk due to their different polarities. For polar analyte analysis, such as aminoglycoside antibiotics (apramycin, spectinomycin, and neomycin) in milk, a mixed mode LC method was used for analyte separation and a trichloroacetic acid/acetonitrile mixed solution was used for protein precipitation. For other less polar analytes, a reversed phase UHPLC method was used for analyte separation and an acidified acetonitrile extraction was applied for sample preparation. The methods were validated by spiking different concentrations of analytes in a milk sample matrix. The results demonstrated that the developed methods are sensitive and reliable for most of the analytes studied and can be applied to monitor antibiotics and veterinary drugs in milk samples.

Experimental

Hardware/Software

Chromatographic separations of different analytes were conducted by a PerkinElmer QSight LX 50™ ultra-high-performance liquid chromatography (UHPLC) system, and subsequent detection was achieved using a PerkinElmer QSight® 420 triple quadrupole mass spectrometer with a dual ionization source (ESI and APCI). All instrument control, data acquisition and data processing were performed using the Simplicity™ 3Q Software.

Materials and Methods

Chemicals and materials

LC-MS grade solvents methanol (MeOH), acetonitrile (ACN), and water, and other chemicals such as formic acid, ammonium formate, trichloroacetic acid (TCA) and ethylenediaminetetraacetic acid, disodium salt, dihydrate ($Na_2EDTA{\bullet}2H_2O$), were obtained from Sigma-Aldrich (Oakville, ON, Canada). Most of the analytical standards of antibiotics and veterinary drugs (including two isotope labeled internal standards: flunixin-d3 and sulfamethazine-(phenyl-$^{13}C_6$) hemihydrate) were purchased from Sigma-Aldrich. The rest standards were obtained from Toronto Research Chemicals (Toronto, ON, Canada), Disposable syringe (1 mL), and syringe filter (0.22 µm) were obtained from VWR. Polypropylene centrifuge tube (15 mL), plastic autosampler vials and caps were obtained from PerkinElmer Inc. The end capped C18 sorbent was obtained from UCT (Bristol, PA. USA). An organic whole milk sample (used as control blank) and three milk test samples were purchased from local stores (Toronto, ON, Canada).

Standard preparation

Stock solutions for individual standard (1mg/mL) were prepared by weighing a small amount (5 mg) of the standard into a 15 mL centrifuge tube and dissolving the standard into a suitable solvent (5 mL). For standards parbendazole, albendazole, fenbendazole, oxfendazole, since they are difficult to dissolve completely in either methanol or acetonitrile, 0.6 mL of DMSO (dimethyl sulfoxide) was added first to help dissolve the standard and then 4.4 mL of methanol or acetonitrile was added. Standards ampicilline trihydrate, apramycin sulfate and neomycin trisulfate could be dissolved in 5 mL of water and should be stored in plastic vials to avoid adsorption on glass surface. For all other standards, they were dissolved in 5 mL of methanol or acetonitrile. Two mixed standard solutions (Mix-1 and Mix-2) were prepared. Mix-1 contains all the analytes at 10 µg/mL by adding 100 µL each analyte stock in 15 mL tube and then diluted to 10 mL with a diluent (a mixture of methanol and water (1/1 in v/v)); Mix-2 contains only the polar analytes (10 µg/mL for apramycin, diminazene and spectinomycin, respectively, and 100 µg/mL for neomycin), prepared by adding 100 µL each stock of apramycin, diminazene and spectinomycin and 1 mL neomycin stock in 15 mL tube and then diluted to 10 mL with a diluent (a mixture of acetonitrile and water (1/1 in v/v)). An internal standard (IS) mix solution containing two internal standards (flunixin-d3 and $^{13}C_6$-sulfamethazine, each at 10 µg/mL) was prepared by appropriate dilution of IS stock solutions in a mixture of acetonitrile and water. All stock solutions and mixed standard solutions were stored in a freezer at – 22 °C after preparation.

To prepare calibration standards, matrix-matched calibration standards and quality control samples, two working standard (WS) mix solutions (WS-Mix1 and WS-Mix2) and one internal standard spiking solution (IS-Spike) were prepared from the corresponding mixed standard solutions by appropriate dilutions. WS-Mix1 contained all analytes (each 1 µg/mL) and WS-Mix2 consisted of the polar analytes (1 µg/mL for apramycin, diminazene and spectinomycin, respectively, and 10 µg/mL for neomycin); IS-Spike consisted of flunixin-d3 and $^{13}C_6$-sulfamethazine (each at 1 µg/mL). Nine levels of calibration standard solutions (each 1 mL) were prepared by a series of dilutions of the appropriate amount of WS-Mixes 1 and 2, respectively; and then IS-Spike solution (25 µL) was fortified into each calibration standard. The analyte concentrations in the standards are 0.1, 0.5, 1, 5, 10, 25, 50, 100, and 200 ng/mL (corresponding to µg/kg in milk) for all analytes except for neomycin (its concentrations are 1, 5, 10, 50, 100, 250, 500, 1000, 2000 ng/mL). Working solutions, IS spiking solution and Calibration standard solutions were prepared on the day of analysis and were stored in a dark place in a fridge.

Sample preparation

The following sample preparation procedures were followed for analysis of most antibiotics and veterinary drugs except for the polar analytes apramycin, neomycin, and spectinomycin.

(1). Weigh 1g of the homogenized milk into a 15 mL polypropylene centrifuge tube. (2). Add an amount of WS-Mix1 and IS-spike and vortex for 1 min. (3). Add 100 µL 0.1 M EDTA solution, vortex for 1 min and allow the sample solution to sit for 10 min. (4). Add 5 mL of acidified acetonitrile (1% formic acid) and vortex for 5 min for analyte extraction and protein precipitation. (5). Centrifuge for 10 min at 4 °C and 4000rpm. (6). Decant the supernatant to a 15 mL centrifuge tube containing 50 mg end-capped C18 sorbent and vortex 2 min for d-SPE clean up (defatting). (7). Centrifuge for 10 min at 4 °C and 4000rpm. (8) Transfer the supernatant to a 15 mL centrifuge tube and evaporate the solution to dryness at 40 °C with N_2 gas. (9). Add 1.0 mL 50% methanol and vortex 30 second. (10). Filter the solution through a 0.22 um filter to an autosampler vial for LC/MS/MS analysis.

For polar analytes and aminoglycoside antibiotics (apramycin, neomycin, and spectinomycin) analysis, milk samples were prepared by the following procedures:

(1). Weigh 1g of the homogenized milk into a 15 mL polypropylene centrifuge tube. (2). Add WS-Mix2 and vortex for 1 min. (3). Add 100 µL 0.1 M EDTA solution, vortex for 1 min and allow the sample solution to sit for 10 min. (4). Add 1 mL of extraction solution [5% TCA in a mixture of acetonitrile/water (50:50 in v/v)] and vortex for 5 min for analyte extraction and protein precipitation. (5). Centrifuge for 10 min at 4 °C and 4000rpm. (6). Decant the supernatant to a 15 mL centrifuge tube containing 50 mg end-capped C18 sorbent and vortex 2 min for d-SPE clean up (defatting). (7). Centrifuge for 10 min at 4 °C and 4000rpm. (8) Filter the supernatant through a 0.22 um filter to a plastic autosampler vial for LC-MS/MS analysis.

For method validation, a laboratory reagent blank (LRB) was prepared and tested first to ensure that there is no interference or contamination from reagents or materials used and from the sample preparation processes. Then, an organic whole milk was tested as the control matrix blank to check if there is any analyte peak or any interfering components. Sample matrix effects (ME%) were evaluated by comparing the slopes of matrix-matched calibration curves to those of neat-solution calibration curves. Due to matrix effects, analyte quantification in milk sample was carried out by matrix-matched calibration method. Finally, to evaluate analyte recovery from sample matrix, Laboratory Fortified Matrix samples (LFM) were prepared by following the same sample preparation procedures as described above, using the organic milk blank as a sample matrix spiked with analyte at different concentration levels. At each spiking level, LFM samples were prepared in triplicates.

LC methods and MS source conditions

The optimized LC methods and MS source parameters are shown in Table 1-2. The multiple reaction monitoring mode (MRM) transitions of all analytes and their optimized parameters are shown in Table 3. Multiple MRM transitions were monitored to evaluate potential interfering components for certain transitions in milk samples, which will help confidently identify analyte from milk sample matrices, reduce false positive and false negative in the results and increase the accuracy of analyte quantification. Optimization of MS/MS parameters, such as collision energies (CE), entrance

Table 1: LC Method and MS Source Conditions for Less Polar Analytes.

LC Conditions	
LC Column and Guard Column	PerkinElmer Brownlee SSP Phenyl-Hexyl (2.7µm, 2.1 x 100 mm, part number: N9308485) and guard column (2.1 x 5 mm, part number: N9308519)
Mobile Phase A	0.1% formic acid and 2 mM ammonium formate in water
Mobile Phase B	0.1% formic acid and 2 mM ammonium formate in MeOH/ACN (1:1 in v/v)
Mobile Phase Gradient	Start at 5% mobile phase B and hold at 5% B for 0.5 min, then increase B to 95% at 7 min and keep at 95% B for 1 min to clean the column, finally return to initial condition at 8.1 min and keep running at initial conditions till 12 min.
Flow Rate	0.3 mL/min
Column Oven Temperature	30 °C
Auto Sampler Temperature	8 °C
Injection Volume	10.0 µL
MS Source Conditions	
ESI Voltage (Positive)	5500 V
ESI Voltage (Negative)	-4000 V
Drying Gas	120
Nebulizer Gas	150
Source Temperature	350 °C
HSID Temperature	220 °C
Detection Mode	Time-managed MRM™

voltages (EV), and lens voltages (CCL2), was performed by infusion of standards and use of autotune feature in the simplicity software. MS source parameters including gas flows, temperature, and probe position settings, were optimized for maximum sensitivity by infusion of standard solution with a T-unit connected to mobile phase flow. Based on the optimized conditions, the MS acquisition method was generated using Simplicity software in the time-managed-MRM module with the retention times and corresponding retention time windows for all analytes.

Table 2: LC Method and MS Source Conditions for Polar Analytes.

LC Conditions	
LC Column and Guard Column	Obelisc R (5µm, 2.1 x 150 mm; 2.1 x 10 mm) from SIELC Technologies Inc.
Mobile Phase A	1% formic acid in water
Mobile Phase B	Acetonitrile (ACN)
Mobile Phase Gradient	Start at 85% mobile phase B and hold at 85% B for 0.5 min, then decrease B to 5% at 5 min and keep at 5% B for 3 min, finally return to initial condition at 8.1 min and keep running at initial conditions till 13 min.
Flow Rate	0.3 mL/min
Column Oven Temperature	30 °C
Auto Sampler Temperature	8 °C
Injection Volume	10.0 µL
MS Source Conditions	
ESI Voltage (Positive)	5500 V
Drying Gas	120
Nebulizer Gas	120
Source Temperature	400 °C
HSID Temperature	220 °C
Detection Mode	Time-managed MRM™

Results and Discussion

LC/MS/MS Method Optimization

To optimize mass detection conditions, both positive and negative electrospray ionization (ESI) modes were evaluated for all analytes. The results showed that higher signal intensity and better signal to noise ratio were observed for most analytes under positive mode except for oxyclozanide, which showed better signal to noise ratio in negative mode, and therefore, negative ESI mode for oxyclozanide and positive ESI mode for all other analytes were used in this study. For most analytes in the positive mode, the highest abundant precursor

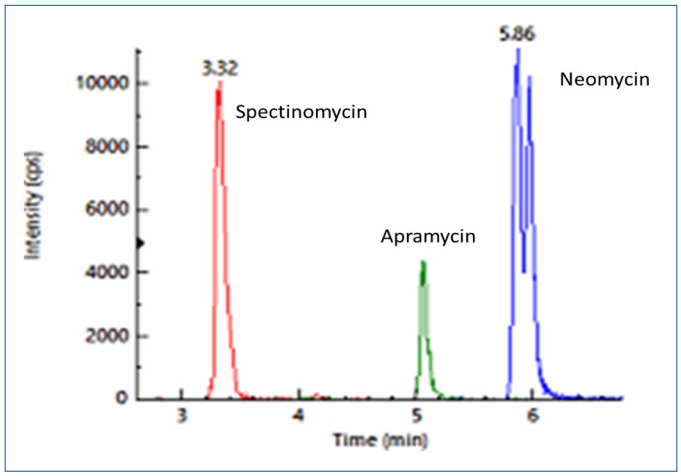

Figure 1: Extracted ion chromatograms of the three aminoglycoside antibiotics in a spiked milk sample.

ions were protonated $[M+H]^+$ species. But for doramectin and ivermectin, their ammonium adducts $[M+NH_4]^+$ showed higher abundance than their $[M+H]^+$ ions and therefore, their ammonium adducts $[M+NH_4]^+$ were used as precursors in the method. For spectinomycin, the water adduct $[M + H_2O + H]^+$ was predominant due to hydration of its carbonyl group in aqueous solution.[6] Multiple MS/MS transitions for each analyte were employed in this study to improve analyte identification and method's accuracy. The optimized MS/MS (or MRM) parameters were listed in Table 3.

Efforts were made to develop a LC/MS/MS method that could analyze all the compounds in a single run. However, polar analytes, such as diminazene, and aminoglycoside antibiotics (apramycin, neomycin, and spectinomycin), do not have good retention on reversed phase LC columns and thus, they are often analyzed by using ion-pair chromatography [6-8] or hydrophilic interaction chromatography (HILIC). [9-10] In this study, after evaluating different columns and mobile phase compositions, a mixed mode Obelisc R column (5 µm, 2.1 x 150 mm) was selected for separation of these polar compounds and a Brownlee SSP Phenyl-Hexyl column (2.7 µm, 2.1 x 100 mm) was used for separation of all other analytes with the mobile phases listed in Table 1-2. Obelisc R column has better retention for polar analytes with the increase of acetonitrile in mobile phases, indicating that it has HILIC separation mechanism. In addition, this column has cationic groups (quaternary amine) close to the silica surface linked by a hydrophobic chain to anionic groups (carboxyl type), offering multiple interactions with analytes (hydrophobic, ion exchange, zwitterionic and hydrophilic interactions) simultaneously. To optimize the separation conditions with this column, mobile phase pH and buffer (ammonium formate) concentration were studied. It was found that the use of acidic aqueous mobile

phase (1% formic acid) could improve analyte separation and peak shape. Figure 1 illustrates the separation of three aminoglycoside antibiotics in a spiked milk sample. Similar results were also reported in a previous application using this column for analysis of 13 aminoglycoside antibiotics in meat products. [11] For separation of other analytes, it was found that Phenyl-Hexyl column showed better analyte retention, separation and peak shape compared with C18 column. It was also found that 50% methanol in acetonitrile as organic mobile phase provided the overall best performance in terms of analyte peak shape, retention, and mass signal intensity. Figure 2 shows the chromatograms of 30 less polar analytes in a spiked milk sample.

Calibration Curves and Linearity

Nine concentration levels of calibration standards were prepared in both neat solution (solvent-only calibration) and milk sample matrix (matrix-matched calibration) to evaluate sample matrix effects (see next section). All the calibration curves show good linearity with correlation coefficients (R^2) greater than 0.99. Since there are significant sample matrix effects (>20%) for most of the analytes, matrix-matched calibration was used for analyte quantification. Example matrix-matched calibration curves are shown in Figure 3.

Sample Preparation, Sample Matrix Effects and Analyte Recovery

One of the challenges in milk analysis is the high fat, protein, and metal ions (such as calcium) content that can cause matrix effects (ion suppression/enhancement) and interference with analysis. The commonly used method for protein removal is protein precipitation using organic solvents (acetonitrile or methanol) or acids. In this study, the effects of different extraction conditions on extraction efficiency were evaluated and the results using tetracycline compounds as examples are shown in Table 4. Acetonitrile was used initially as an extraction solvent for analyte extraction and protein precipitation, then, it was found that the inclusion of EDTA during sample extraction increased extraction recovery because EDTA could prevent the complexation of analytes (such as tetracyclines) with metal ions (e.g. calcium). Finally, addition of 1% formic acid in acetonitrile could lead to more efficient protein precipitation and thus further improved the analyte extraction efficiency. However, further addition of salt ($MgSO_4$ and NaCl) in extraction solution could not benefit analyte recovery further, but it led to the formation of clumps in solution and liquid phases separation after centrifugation, which made it difficult to transfer the supernatant and could affect reproducibility of sample results. Therefore, acidified acetonitrile with small amount of EDTA was applied for extraction of most analytes from milk samples without adding salt.

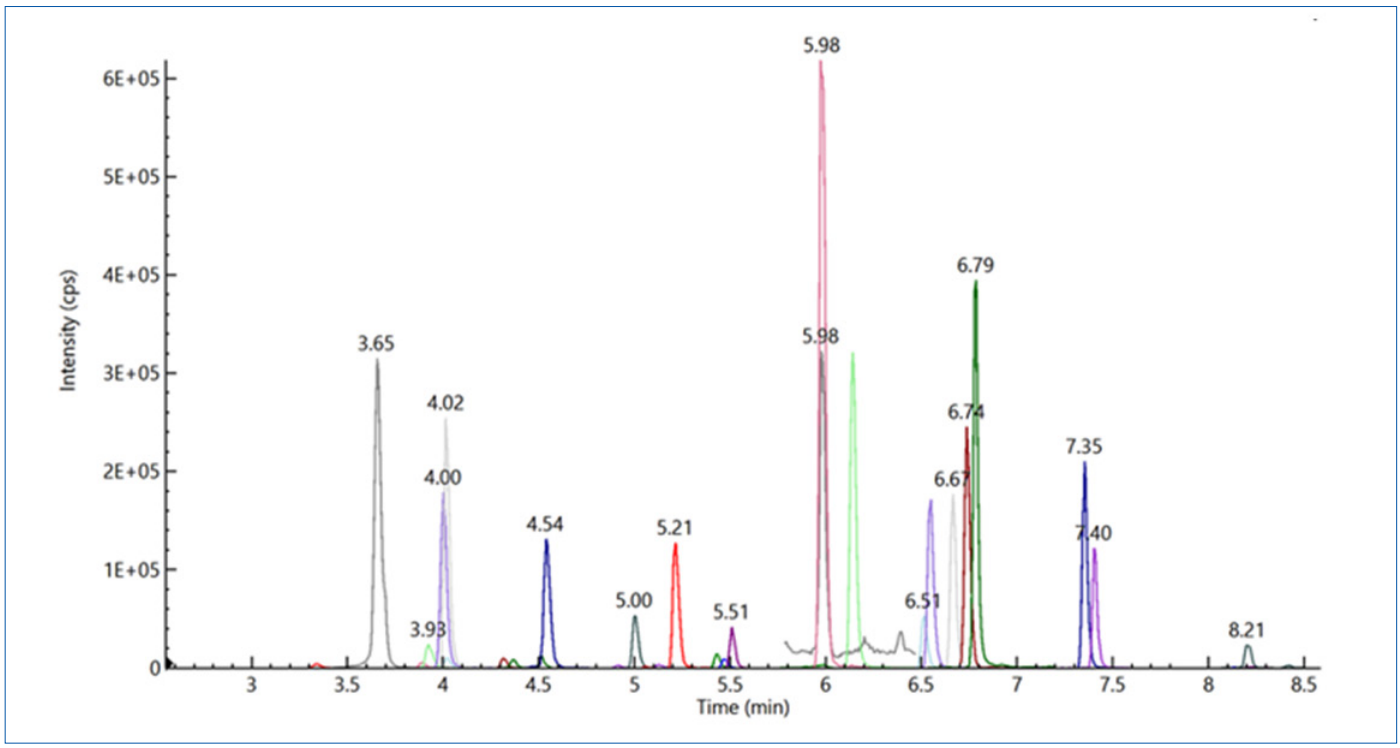

Figure 2: Extracted ion chromatograms of the less polar analytes in a spiked milk sample.

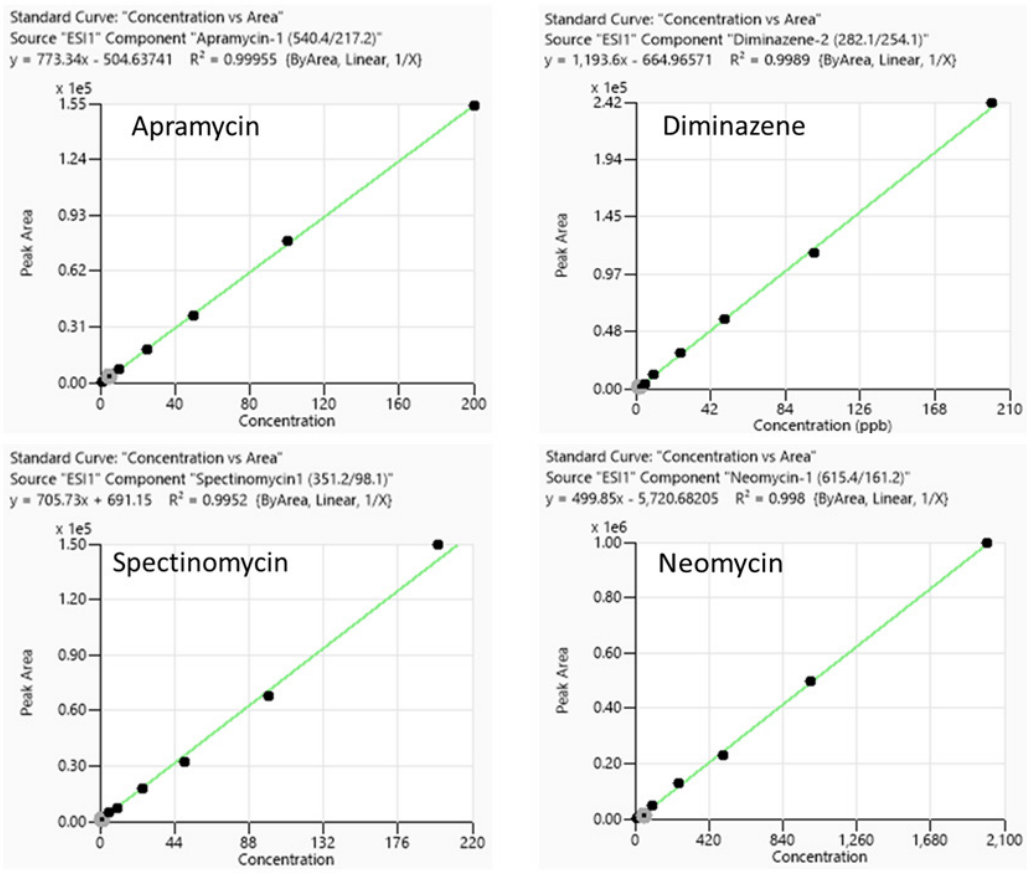

Figure 3: Example matrix-matched calibration curves.

Table 3: Optimized MRM Transitions and Parameters.

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Apramycin-1	Positive	540.4	217.2	-37	6	-192
Apramycin-2	Positive	540.4	378.3	-21	6	-260
Apramycin-3	Positive	540.4	199.1	-33	6	-212
Diminazene-1	Positive	282.1	119.1	-25	21	-128
Diminazene-2	Positive	282.1	254.1	-11	2	-128
Diminazene-3	Positive	282.1	102.1	-50	21	-132
Neomycin-1	Positive	615.4	161.2	-42	15	-252
Neomycin-2	Positive	615.4	163.2	-47	15	-232
Neomycin-3	Positive	615.4	293.3	-32	15	-232
Spectinomycin-1	Positive	351.2	98.1	-40	48	-204
Spectinomycin-2	Positive	351.2	100.1	-33	58	-204
Spectinomycin-3	Positive	351.2	116.1	-34	49	-204
Lincomycin-1	Positive	407.3	126.1	-39	1	-128
Lincomycin-2	Positive	407.3	70.1	-101	1	-136
Lincomycin-3	Positive	407.3	359.3	-23	1	-120

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Ampicillin-1	Positive	350.2	106.2	-23	35	-96
Ampicillin-2	Positive	350.2	192.2	-20	35	-100
Ampicillin-3	Positive	350.2	160.3	-18	35	-100
Sulfadiazine-1	Positive	251.2	156.1	-22	1	-112
Sulfadiazine-2	Positive	251.2	92.2	-39	1	-104
Sulfadiazine-3	Positive	251.2	108.1	-33	1	-92
Cefacetrile-1	Positive	357.2	280.2	-13	20	-180
Cefacetrile-2	Positive	357.2	252.1	-21	19	-180
Cefacetrile-3	Positive	357.2	156.1	-23	17	-180
Thiabendazole-1	Positive	202.2	175.1	-33	0	-156
Thiabendazole-2	Positive	202.2	131.2	-45	0	-152
Thiabendazole-3	Positive	202.2	92.1	-45	0	-152
Trimethoprim-1	Positive	291.2	230.1	-30	27	-136
Trimethoprim-2	Positive	291.2	123.1	-31	27	-112
Trimethoprim-3	Positive	291.2	261.2	-33	27	-120
Oxytetracycline-1	Positive	461.1	426.1	-25	39	-400
Oxytetracycline-2	Positive	461.1	443.1	-15	34	-400
Oxytetracycline-3	Positive	461.1	201.2	-53	36	-400
Tetracycline-1	Positive	445.3	410.2	-24	5	-128
Tetracycline-2	Positive	445.3	154.1	-35	5	-140
Tetracycline-3	Positive	445.3	427.1	-16	5	-140
Sulfadimidine-1	Positive	279.1	186.1	-22	0	-96
Sulfadimidine-2	Positive	279.1	92.1	-44	1	-88
Sulfadimidine-3	Positive	279.1	124.2	-30	0	-96
Sulfadimidine-C13-1	Positive	285.2	186.2	-22	0	-124
Sulfadimidine-C13-2	Positive	285.2	98.1	-40	1	-112
Sulfachloropyridazine-1	Positive	285.1	156.1	-19	30	-252
Sulfachloropyridazine-2	Positive	285.1	92.1	-44	30	-260
Sulfachloropyridazine-3	Positive	285.1	108.1	-36	30	-260
Chlortetracycline-1	Positive	479.2	154.2	-35	12	-184
Chlortetracycline-2	Positive	479.2	444.2	-29	12	-164
Chlortetracycline-3	Positive	479.2	98.2	-58	12	-200
Oxfendazole-1	Positive	316.2	159.1	-44	52	-112
Oxfendazole-2	Positive	316.2	191.1	-27	43	-124
Oxfendazole-3	Positive	316.2	284.1	-24	52	-136
Ceftiofur-1	Positive	524.3	241.2	-22	2	-128
Ceftiofur-2	Positive	524.3	125.2	-80	2	-132
Ceftiofur-3	Positive	524.3	210.1	-30	2	-128
Sulfachloropyrazine-1	Positive	285.1	156.1	-21	6	-96

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Sulfachloropyrazine-2	Positive	285.1	92.1	-45	6	-84
Sulfachloropyrazine-3	Positive	285.1	108.1	-40	6	-136
Sulfaquinoxaline-1	Positive	301.1	156.1	-24	35	-96
Sulfaquinoxaline-2	Positive	301.1	92.1	-48	35	-100
Sulfaquinoxaline-3	Positive	301.1	108.1	-38	35	-100
Tylosin-1	Positive	916.6	174.2	-47	75	-332
Tylosin-2	Positive	916.6	101.4	-65	62	-264
Tylosin-3	Positive	916.6	144.9	-51	80	-328
Parbendazole-1	Positive	248.1	216.1	-26	42	-220
Parbendazole-2	Positive	248.1	173.1	-43	42	-216
Parbendazole-3	Positive	248.1	145.1	-55	42	-224
Sulfanilamide-1	Positive	173.1	173.1	-7	98	-72
Sulfanilamide-2	Positive	173.1	92.1	-43	98	-244
Albendazole-1	Positive	266.1	234.1	-24	16	-96
Albendazole-2	Positive	266.1	191.2	-44	16	-128
Albendazole-3	Positive	266.1	159.1	-55	16	-104
Virginiamycin M1-1	Positive	526.3	508.3	-17	40	-152
Virginiamycin M1-2	Positive	526.3	109.1	-50	40	-152
Virginiamycin M1-3	Positive	526.3	355.3	-21	40	-152
Meloxicam-1	Positive	352.1	115.1	-27	10	-96
Meloxicam-2	Positive	352.1	141.1	-29	10	-100
Flunixin-1	Positive	297.2	279.2	-30	50	-120
Flunixin-2	Positive	297.2	264.3	-42	50	-132
Flunixin-3	Positive	297.2	109.1	-65	50	-136
Flunixin-d3	Positive	300.2	282.2	-28	50	-152
Praziquantel-1	Positive	313.3	203.3	-21	0	-96
Praziquantel-2	Positive	313.3	83.1	-37	0	-100
Praziquantel-3	Positive	313.3	174.1	-40	38	-88
Fenbendazole-1	Positive	300.1	268.2	-27	0	-364
Fenbendazole-2	Positive	300.1	159.1	-47	0	-388
Fenbendazole-3	Positive	300.1	104.1	-81	0	-380
Febantel-1	Positive	447.3	383.1	-24	35	-148
Febantel-2	Positive	447.3	415.1	-17	35	-152
Febantel-3	Positive	447.3	280.2	-43	35	-140
Oxyclozanide-1	Negative	399.8	175.8	36	-10	170
Oxyclozanide-2	Negative	399.8	363.8	24	-10	110
Oxyclozanide-3	Negative	399.8	201.8	35	-10	218
Doramectin-1	Positive	916.6	331.3	-30	35	-224
Doramectin-2	Positive	916.6	145.2	-41	33	-400

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Doramectin-3	Positive	916.6	219.1	-35	32	-235
Ivermectin-1	Positive	892.6	307.1	-32	38	-400
Ivermectin-2	Positive	892.6	145.2	-74	42	-292
Ivermectin-3	Positive	892.6	195.1	-37	16	-216
Monensin-1	Positive	688.5	125.1	-60	38	-248
Monensin-2	Positive	688.5	461.4	-31	38	-284
Monensin-3	Positive	688.5	635.5	-20	38	-228

Table 4: Recoveries (%) of Tetracyclines under Different Extraction Conditions.

Tetracycline	Chlortetracycline	Oxytetracycline	Extraction Conditions
33.8	28.6	28.5	1. ACN (no salt, no acid, no EDTA)
43.7	37.6	36.3	2. ACN + EDTA (no salt, no acid)
72.7	61.3	64.5	3. ACN + EDTA+ 1% acid (no salt)
72.4	63.2	61.0	4. ACN + EDTA+ 1% acid + salt

To reduce the fat content of the supernatant solution after centrifugation, two different sample cleanup steps were carried out: liquid-liquid (L-L) extraction with hexanes and d-SPE with end-capped C18 sorbent. As shown in Tables 5-6, sample matrix effects (MEs) were reduced by d-SPE (and similarly by L-L extraction with hexane, data not shown, but available upon request), but recoveries for some hydrophobic compounds (such as ivermectin and monensin) were also reduced compared to the results obtained without sample clean up. To reduce matrix effects, protect column lifetime and reduce instrument down time, it is recommended to use d-SPE for sample clean up when analyzing many samples in routine testing laboratories.

In this study, sample MEs were evaluated by comparing the slopes of matrix-matched calibration curves to slopes of reagent-only (RO) calibration curves. Sample ME (%) for each analyte was calculated by the percentage difference between the slopes. When the percentage difference is positive, there is a signal enhancement effect, whereas a negative value indicates signal suppression effect. As shown in Table 5-6, significant MEs (>20%) were observed for many analytes even after sample clean up, some showed enhancement such as tetracycline, chlortetracycline, and oxytetracycline, but most of them showed signal suppression effects. To overcome matrix effects and reduce variations in analytical results, matrix-matched calibrations (or isotope dilution assay) should be used for the quantification of all analytes.

Table 5: Matrix Effects (ME) and Analyte Recovery (%) from Milk Samples Spiked at Different Levels without Sample Clean-up.

Compound name	Recovery (5 µg/kg)	Recovery (10 µg/kg)	Recovery (25 µg/kg)	Recovery (50 µg/kg)	Recovery (100 µg/kg)	Recovery (200 µg/kg)	ME (%)
Lincomycin	110	106	111	104	96.1	88.5	-20.7
Ampicillin	72.0	70.6	64.7	58.9	62.7	57.9	-4.7
Sulfadiazine	88.4	85.5	73.2	69.7	67.6	58.5	-34.2
Cefacetrile	104	96.5	112	100	102	95.4	-1.9
Thiabendazole	98.0	93.0	88.7	75.2	71.7	64.5	-33.5
Trimethoprim	113	110	98.1	86.8	80.8	76.8	-25.2
Oxytetracycline	65.7	67.1	79.2	63.5	66.3	75.5	38.6
Tetracycline	81.1	81.7	84.2	73.8	79.8	81.4	13.5

Compound name	Recovery (5 µg/kg)	Recovery (10 µg/kg)	Recovery (25 µg/kg)	Recovery (50 µg/kg)	Recovery (100 µg/kg)	Recovery (200 µg/kg)	ME (%)
Sulfadimidine	107	88.6	85.8	76.2	73.0	62.7	-33.5
Sulfachloropyridazine	79.5	77.1	73.4	70.8	65.3	56.1	-25.8
Chlortetracycline	51.0	64.5	66.3	61.4	69.8	77.5	11.5
Oxfendazole	118	115	114	96.9	92.6	79.8	-28.1
Ceftiofur	84.6	82.4	89.6	83.3	85.8	84.3	-8.8
Sulfachloropyrazine	79.7	81.2	73.6	73.6	69.2	61.9	-24.3
Sulfaquinoxaline	83.1	82.1	76.3	72.7	64.2	52.5	-29.4
Tylosin	74.0	77.9	65.2	60.5	61.1	43.8	-57.5
Parbendazole	108	94.1	94.1	79.2	71.9	55.3	-65.6
Sulfanilamide	118	110	107	88.7	77.8	54.1	-85.7
Albendazole	100	103	102	88.1	88.4	86.4	-50.4
Virginiamycin M1	97.3	94.3	101	85.6	91.1	84.9	-28.4
Meloxicam	102	104	105	96.2	97.0	108	-29.3
Flunixin	114	103	102	92.2	85.3	82.3	-42.7
Praziquantel	100	111	102	96.1	89.2	87.2	-42.2
Fenbendazole	98.5	106	97.4	93.5	90.4	86.6	-45.8
Febantel	107	90.5	111	90.3	97.3	87.1	-35.1
Oxyclozanide	96.8	78.7	82.4	83.8	80.3	81.7	-18.0
Doramectin	76.1	107	67.2	84.4	81.3	76.5	-12.9
Ivermectin	N/A	102	93.7	105	79.7	128	-31.7
Monensin	91.2	81.3	97.8	87.9	103	97.5	-44.2

Table 6: Matrix Effects (ME), LOQ, Tolerance Limits (TLs) and Analyte Recovery (%) from Milk Samples Spiked at Different Levels with d-SPE Sample Clean-up

Compound name	Recovery (5 µg/kg)	Recovery (10 µg/kg)	Recovery (25 µg/kg)	Recovery (50 µg/kg)	Recovery (100 µg/kg)	Recovery (200 µg/kg)	ME (%)	LOQ (µg/kg)	TLs (µg/kg)
Lincomycin	120	109	119	102	94.8	77.8	-14.7	< 0.5	150
Ampicillin	60.6	49.2	55.3	48.8	53.4	50.9	-0.5	2.0	10
Sulfadiazine	78.8	69.2	70.2	69.3	65.8	53.2	-28.9	< 0.5	10
Cefacetrile	97.9	81.6	98.3	91.4	98.1	91.2	-2.5	2.0	10
Thiabendazole	80.6	79.6	89.2	83.2	75.7	61.5	-28.3	< 0.5	100
Trimethoprim	117	102	92.3	77.6	74.1	64.6	-18.0	< 0.5	10
Oxytetracycline	92.6	64.0	82.1	64.5	66.6	67.7	46.0	2.0	100
Tetracycline	87.7	70.8	78.0	72.7	73.2	70.3	22.5	2.0	100
Sulfadimidine	97.5	74.1	79.6	61.7	63.4	48.3	-26.4	< 0.5	10
Sulfachloropyridazine	70.0	65.6	75.3	72.5	67.3	54.1	-15.4	< 0.5	10
Chlortetracycline	64.3	52.4	63.4	61.3	71.9	61.5	26.1	2.0	100

Compound name	Recovery (5 µg/kg)	Recovery (10 µg/kg)	Recovery (25 µg/kg)	Recovery (50 µg/kg)	Recovery (100 µg/kg)	Recovery (200 µg/kg)	ME (%)	LOQ (µg/kg)	TLs (µg/kg)
Oxfendazole	88.9	93.1	108	105	100	81.3	-19.5	< 0.5	100
Ceftiofur	92.9	79.3	84.0	84.8	87.4	79.5	2.6	< 0.5	100
Sulfachloropyrazine	70.1	78.9	67.0	75.8	68.4	56.0	-14.0	< 0.5	10
Sulfaquinoxaline	76.2	73.0	71.7	70.1	64.0	48.8	-18.2	< 0.5	10
Tylosin	73.1	57.8	65.9	53.9	52.5	40.2	-49.4	5.0	100
Parbendazole	86.6	81.7	97.0	81.8	70.9	54.2	-57.3	< 0.5	10
Sulfanilamide	108	82.9	89.8	74.4	67.7	49.0	-82.5	5.0	10
Albendazole	96.9	79.4	95.5	89.8	84.6	75.7	-44.4	< 0.5	100
Virginiamycin M1	97.4	77.6	82.8	82.2	85.5	75.8	-26.7	< 0.5	10
Meloxicam	104	80.4	105	92.7	95.6	84.8	-21.9	< 0.5	10
Flunixin	100	90.1	92.6	88.0	84.1	77.9	-32.9	< 0.5	10
Praziquantel	90.4	103	94.8	102	72.5	72.5	-36.4	< 0.5	10
Fenbendazole	102	87.8	103	92.7	72.9	71.7	-38.4	< 0.5	100
Febantel	84.4	71.2	97.0	84.3	71.3	65.6	-21.5	< 0.5	100
Oxyclozanide	102	80.7	89.2	72.1	71.5	58.8	41.9	< 0.5	10
Doramectin	74.4	62.0	50.5	62.8	58.9	55.2	24.1	10.0	15
Ivermectin	N/A	26.9	54.9	87.1	65.2	63.7	-12.5	10.0	10
Monensin	59.4	51.5	65.0	56.6	60.6	51.7	-12.2	0.5	2

As shown in Tables 5-6, the recoveries of most analytes are within 70 to 120%. Although the recovery values are lower for some analytes, most of them are consistent and thus can be corrected during the result calculations. In addition, the low recoveries and variations in results can be further improved by applying stable isotope dilution assay (SIDA)[12] or standard additions method during sample preparation as demonstrated in Table 7 using sulfadimidine and flunixin as examples. In this study, SIDA was carried out by spiking stable isotope labeled internal standards flunixin-d3 and sulfadimidine-C^{13} in milk samples before extraction and the standards additions were performed by spiking different levels of standards in each milk sample before extraction to build a milk matrix-based calibration curve (different from the matrix-matched calibration in that standards were spiked to sample solution post extraction and cleanup).

Although the above acidified acetonitrile/EDTA extraction method worked well for most of the analytes, the recoveries for polar analytes are low, especially for aminoglycoside antibiotics (apramycin, neomycin, and spectinomycin). As listed in Table 8, the recoveries of diminazene and spectinomycin are only about 22% and 15%, respectively. The recoveries of apramycin and neomycin are even lower than 10% by this sample preparation method. Thus, a different sample preparation method is developed for extraction of the polar analytes.

Traditionally, aminoglycoside antibiotics were extracted first using a pH adjusted buffer solution containing 2% trichloroacetic acid (TCA), 10 mM ammonium acetate (NH_4OAc), 0.4 mM EDTA, and 1% NaCl. After centrifugation, the supernatant was pH-adjusted to 7 and then was cleaned up by SPE with HLB cartridge. Finally, the eluant was evaporated with N_2 gas, and the residue was reconstituted with a suitable solution, which was filtered before analysis by LC/MS/MS. These procedures are time-consuming and labor-intensive. To simplify the method, an extraction solution containing 5% TCA in a mixture of acetonitrile/water (50:50 in v/v) without pH adjustment was used in this study for polar analyte extraction and protein precipitation from milk samples. As in the method for less polar analytes, a small amount of EDTA (100 µL of 0.1M EDTA) was added to each sample before extraction to prevent the complexation of analytes with metal ions. After centrifugation to remove the precipitated proteins, the supernatant was treated with end-capped C18 sorbent for defatting/d-SPE clean up, and then the cleaned supernatant

Table 7: Comparison of The Recovery Results Obtained by Matrix-Matched Calibration, SIDA and Standard Additions Method.

Spike (µg/kg)	Recovery (%) without IS	Recovery (%) with IS	Recovery (%) with Std addition
	Sulfadimidine	Sulfadimidine	Sulfadimidine
1	66.3	103	88.4
5	45.6	103	96.8
10	74.3	102	107
25	41.0	85.2	105
50	55.2	98.2	106
100	35.3	104	94.9
	Flunixin	Flunixin	Flunixin
1	76.8	91.0	99.2
5	92.4	101	98.1
10	108	106	96.8
25	84.8	95.1	97.8
50	104	98.5	102
100	75.3	96.1	100

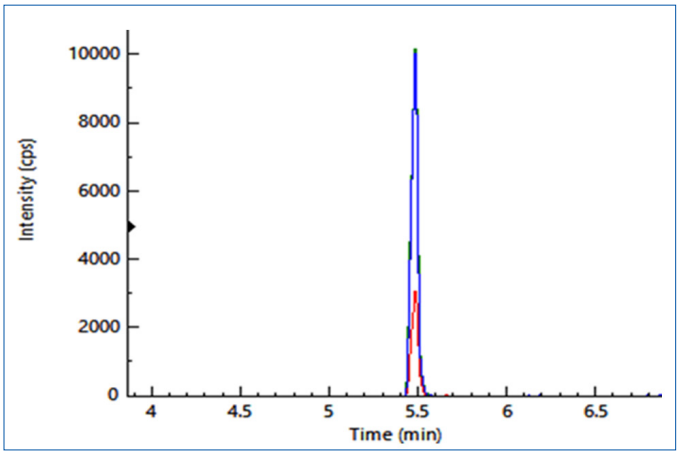

Figure 4: MS/MS chromatograms of diminazene in a spiked milk sample separated using the mixed mode Obelisc R column.

was filtered through a 0.22µm filter before LC/MS/MS analysis. As illustrated in Table 9, the recoveries of these aminoglycoside antibiotics from milk samples are between 84% and 114% using this simplified polar analyte sample preparation method. However, results for diminazene were not obtained due to heavy ion suppression matrix effects, and thus diminazene was analyzed by the less polar sample preparation method (Table 8) and separated by a polar HPLC method (Figure 4).

Table 8: Analyte Recovery (%), Tolerance Limits (TLs) and LOQ.

Spike Level (µg/kg)	Diminazene	Spectinomycin
50	22.2	15.3
100	24.0	15.3
200	21.4	15.2
TLs (µg/kg)	150	200
LOQ (µg/kg)	10	10

Table 9: Aminoglycoside Antibiotics Recovery (%), LOQ and Tolerance Limits (TLs).

Spike Level (µg/kg)	Spectinomycin	Apramycin	Neomycin
25 (250 for Neomycin)	84.0	114	106
50 (500 for Neomycin)	91.5	110	101
100 (1000 for Neomycin)	84.8	103	98.8
200 (2000 for Neomycin)	93.6	93.7	93.0
LOQ (µg/kg)	1	1	10
TLs (µg/kg)	200	10	1500

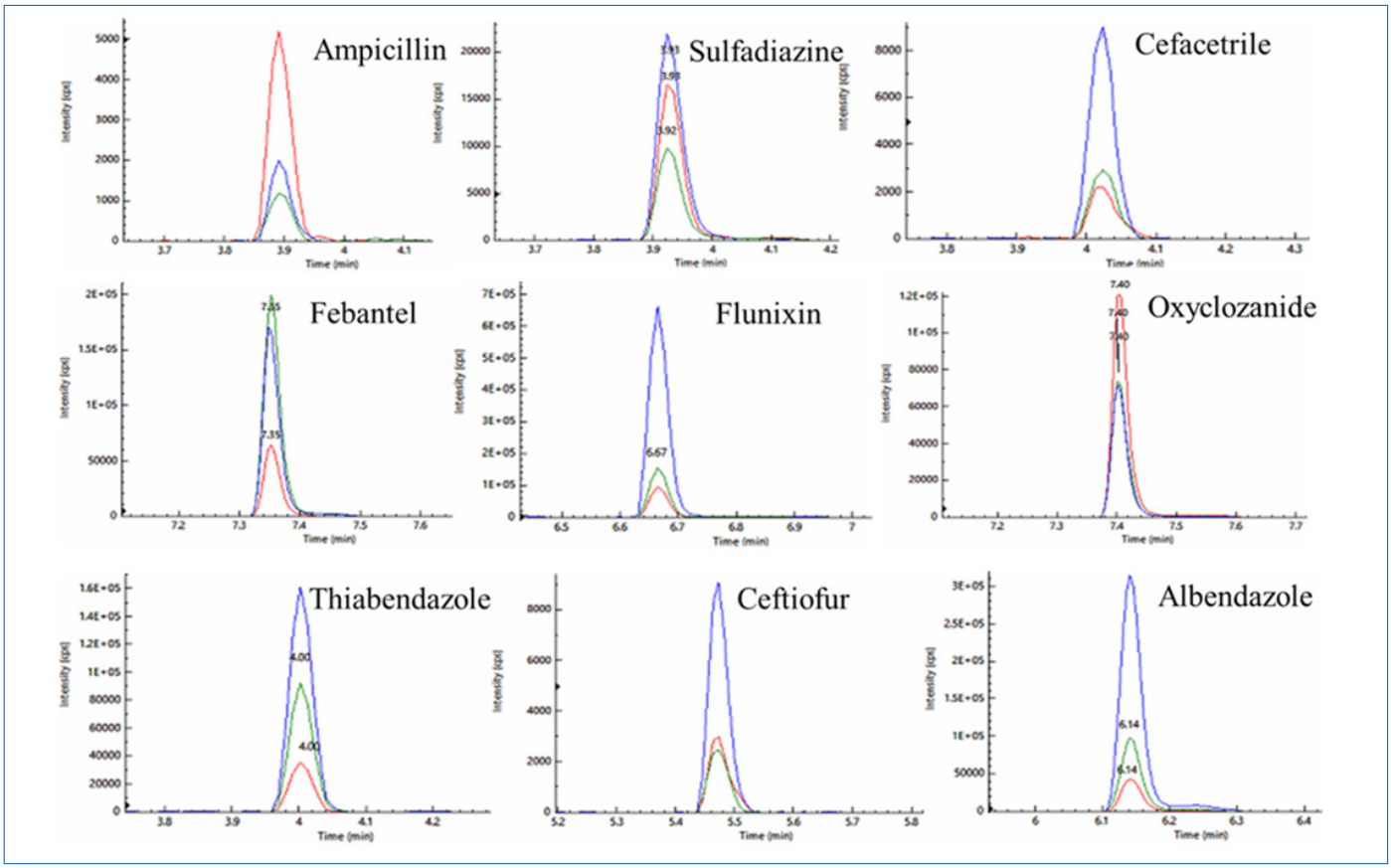

Figure 5: Overlapped three MS/MS chromatograms of some less polar analytes spiked at 5µg/kg in milk.

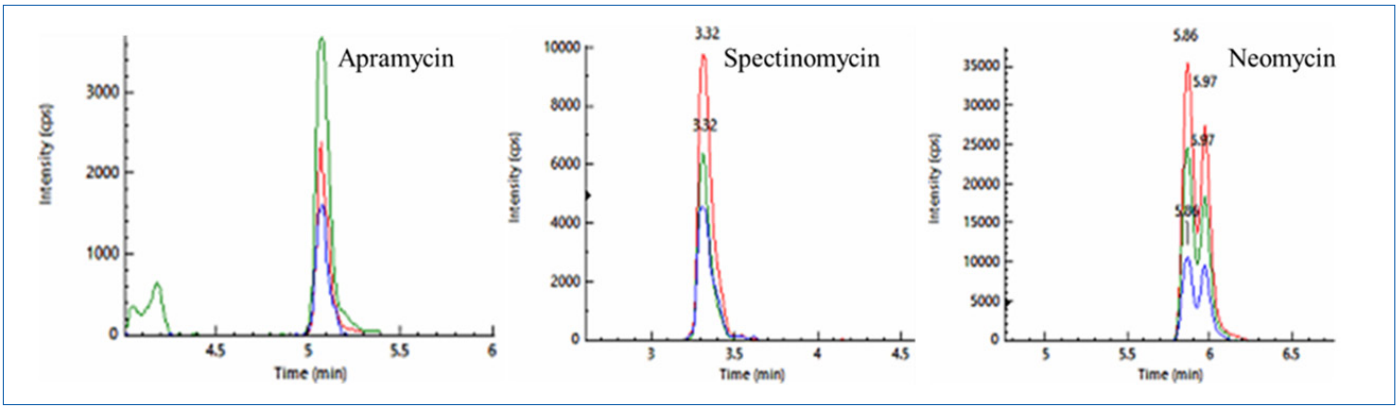

Figure 6: Overlapped three MS/MS chromatograms of aminoglycoside antibiotics spiked at 50µg/kg of apramycin, 100 µg/kg of spectinomycin, and 1000 µg/kg of neomycin in milk.

Method Performance and Application

The method's selectivity and analyte confirmation from milk samples can be evaluated by comparing the analyte retention time and mass spectrum information (such as the peak area ratios of qualifier to quantifier ions of the analyte) between reference standard and samples. According to regulatory guidance on analytical method validation, at least two structurally specific MS/MS transition ion pairs should be used in a LC/MS/MS method.[13-15] In this study, three MS/MS ion pairs were employed for most analytes in the method to identify the peaks of interest in the studied milk samples. For example, the overlapped three MS/MS chromatograms of some analytes in a spiked milk sample were illustrated in Figures 5 and 6. The stereoisomers of neomycin can be observed as the splitting peaks in Figure 6.

The sensitivity of a method depends on the instrument sensitivity, sample matrix effects (signal suppression or enhancement) and sample preparation methods (sample dilution factors). In this study, significant matrix effects were observed for many analytes (refer to Table 5-6). Therefore, the limit of quantification (LOQ) of the method was estimated by the signal to noise ratio (S/N = 10) of each analyte peak (quantifier) in the milk sample matrix. Overall, the LOQ values of the methods for all analytes are below the FSSAI regulated tolerance limits (TLs) for milk samples as shown in Tables 6,8-9, demonstrating good method's sensitivity for all the studied antibiotics and veterinary drugs.

No interference or contamination from reagents, glassware, and sample tubes was observed in this study (no analyte was detected in all LRB samples). Method precision was assessed based on replicate analyses of spiked samples (3 replicates) in the milk sample matrix. The precision was then calculated based on the coefficient of variation (RSD %) of the collected data. The RSDs were less than 20% for most of the analytes in the spiked samples. Method accuracy assesses how close the experimental value is to the expected value. Method's accuracy was evaluated by the recovery of a known amount of analyte spiked to a sample matrix (LFM samples). As shown in Tables 5-9, the recoveries for most analytes from the spiked LFM samples were between 70% and 120%. As discussed previously (and as shown in Table 7), analyte recoveries can be improved by applying stable isotope dilution assay (SIDA) or standard additions method during sample preparation.

Finally, three milk samples with different fat contents were analyzed by the developed methods and no analytes were found above the limit of quantification of the methods.

Stability of standards and samples

Individual standard and internal standard (IS) stock solutions are stable during the period of analysis (3 months) when stored in a freezer after preparation. The mixed standard solutions and IS solutions are stable for a month if kept in a freezer after preparation. Working standard solutions, IS spiking solution, calibration standards and sample extracts were prepared freshly on the day of analysis and they may be stable for a week when kept in a fridge. Take precaution that certain drug classes (such as β-lactams, and tetracyclines) may not be stable in the mixed solutions.

Conclusions

In this study, due to the very different physicochemical properties of analytes, two methods were developed for the analysis of over 30 antibiotics and veterinary drugs in milk regulated by the Food Safety and Standards Authority of India (FSSAI). For polar analyte analysis, such as aminoglycoside antibiotics (apramycin, spectinomycin, and neomycin) in milk, a mixed mode LC method was used to improve analyte retention and a trichloroacetic acid - acetonitrile extraction method was used to enhance analyte extraction efficiency. For less polar analytes, a reversed phase UHPLC method was used for analyte separation and an acidified acetonitrile extraction was applied for sample preparation. The methods were validated by spiking different concentrations of analytes in the milk sample matrix. Although the recoveries for some analytes are lower, they are consistent and can be corrected in calculation. In addition, the recoveries can be significantly improved by applying isotope dilution or standard additions method. All the analytes could be determined with LOQ below the tolerance limits set by FSSAI. The methodology can be extended to analysis of similar analytes in milk and milk products regulated by other regulatory bodies.

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