Veterinary Drug Analysis in Animal Origin Food and Feed and Their Relevant Products: A Modern Multi-Class, Multi-Residue Method Using UHPLC-MS/MS

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Introduction

Veterinary drugs are a complex group of different chemical classes and therapeutic agents. They are used within animal husbandry to treat and prevent disease and ensure animal health and growth. Residues of such drugs in animal edible tissues are not desirable because they could pose a potential threat to consumer health and promote antibiotic resistant bacteria strains. Therefore, these substances are strictly regulated and monitored in food products to ensure food safety and prevent the unnecessary exposure of consumers to veterinary drugs. For that purpose multi-class, multi-residue methods are becoming increasingly popular in regulatory monitoring programs globally because of their extended analytical scope and laboratory efficiency.

Modern Multi-Class, Multi-Residue Method Using LC-MS

Benefits

- Cost-effective
- Time-effective
- Selective detection of individual analytes
- Improved sensitivity for low LODs/LOQs
- Identification/confirmation

Challenges

- A large spectrum of drug classes
- Parent drugs and metabolites
- Different physical/chemical properties
  - – Hydrophilic to hydrophobic
  - – Acidic, neutral and basic
  - – Stability
  - – Interaction with matrix components
- Compromise between analyte scope and performance characteristics
- Matrix effects and potential interference from co-extractives

Analytes in Positive Mode (~150) Divided into 9 Groups

Group (# of Analytes)	Veterinary Drug Classes
Mix A (22)	Anthelmintics
Mix B (20)	Antibiotics - Beta-lactams (cephalosporins and penicillins)
Mix C (13)	Antibiotics - Macrolides and lincosamides
Mix D (23)	Antibiotics - Quinolones and others
Mix E (24)	Antibiotics - Sulfonamides
Mix F (9)	Antibiotics - Tetracyclines
Mix G (22)	Beta-agonists, coccidiostats and antimicrobial growth promoters
Mix H (12)	Tranquilizers, dyes and pesticide
Mix I (4)	Antibiotics - Other

LC-MS/MS Analysis

UHPLC: Agilent Fast LC 1290
Column: Agilent C18 Zorbax Eclipse Plus, 2.1x100 mm, 1.8 µm
Column Oven Temperature: 40°C
Injection Volume: 5 µL
Flow Rate: 0.5 mL/min
Mobile Phase A: 0.1% Formic Acid in Water
Mobile Phase B: 0.1% Formic Acid in Methanol

Gradient:

Time (min)	%A	%B
0	98	2
0.75	98	2
7.0	60	40
11.0	0	100
13.0	0	100
13.1	98	2
17.0	98	2

Mass Spectrometer: Agilent Triple Quadrupole MS/MS 6495A
MS Acquisition: Dynamic MRM
Up to 10 MRMs per analyte were optimized, from which 3 MRMs were chosen for quantitation and identification
Cycle Time: 600 ms
Ion Source Type: AJS ESI+
Collision Energy: Optimized for individual MRM
Cell Accelerator Voltage: Optimized for individual MRM

Sample Preparation General Procedure

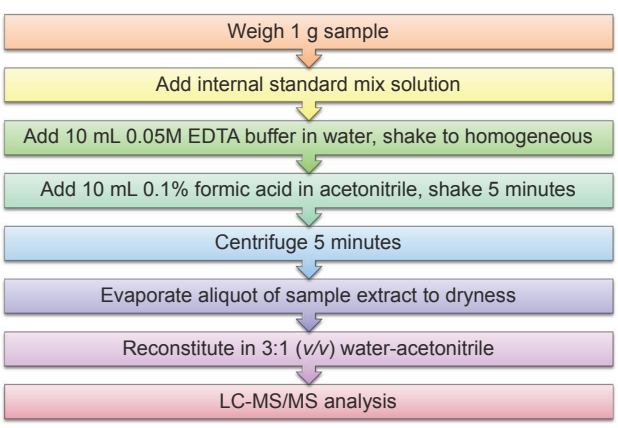

Table 1. Accuracy (corrected spike recovery, CR in %) and Precision (% CV) at 0.5, 50, 100, (*n* = 5 from one day), 1, 5, 10 ng/g (*n* = 10 from two days) in Infant Formula Powder

Fortified Level on Sample	LOQ ng/g	0.5 ng/g		1.0 ng/g		5.0 ng/g		10 ng/g		50 ng/g		100 ng/g	
Class/Compound Name		CR	CV	CR	CV	CR	CV	CR	CV	CR	CV	CR	CV
Anthelmintics													
Albendazole	2	-	-	111	20.2	98.7	14.2	101	9.3	97.0	7.9	102	15.1
Albendazole amino	5	96.5	13.3	102	15.7	101	7.4	97.6	7.1	92.2	10.9	104	11.6
Albendazole sulfone	1	102	18.8	105	9.9	95.1	5.9	99.0	5.4	94.9	4.1	103	5.4
Albendazole sulfoxide	1	108	11.6	104	14.2	98.1	7.1	97.1	8.2	90.6	8.8	105	15.4
Albendazole-2 aminosulfone	1	99.8	5.9	101	6.4	102	4.2	97.3	8.2	96.2	7.5	102	11.2
Cambendazole	1	106	7.6	103	9.4	99.1	6.2	98.7	8.5	88.6	3.2	106	10.1
Febantel	1	95.7	1.9	117	11.9	92.4	9.5	95.6	6.7	87.3	4.2	108	7.8
Fenbendazole	1	120	26.6	89.7	15.1	99.4	12.3	103	7.6	94.0	19.0	103	10.1
Fenbendazole sulfoxide	1	102	8.1	103	11.3	100	9.8	97.2	8.2	97.7	11.1	102	11.7
Fenbendazole sulfone	1	99.6	10.3	105	9.5	97.0	9.0	99.0	8.2	94.5	3.6	103	7.6
Flubendazole	1	112	13.7	107	17.0	88.7	14.6	99.3	20.7	92.9	10.6	105	12.6
Flubendazole-amine	1	104	6.6	102	12.3	100	9.9	97.2	4.1	91.6	9.6	105	6.9
Levamisole	1	96.0	12.9	101	9.7	103	11.1	97.2	9.7	96.0	12.8	102	13.2
Mebendazole	1	105	17.9	97.8	17.4	104	13.4	95.3	10.9	93.0	7.0	104	14.3
Mebendazole-5 hydroxy	1	100	9.4	103	9.6	100	8.0	97.1	8.2	101	3.7	100	8.9
Mebendazole-amine	5	-	-	107	8.5	102	9.5	97.5	7.4	94.3	2.4	104	13.8
Oxibendazole	1	103	2.2	99.3	8.6	101	8.9	98.2	9.4	97.9	6.7	101	9.8
Thiabendazole	1	105	25.5	100	12.3	101	11.2	98.9	8.7	101	13.6	99.5	10.7
Thiabendazole-5 hydroxy	1	104	11.9	95.1	12.6	99.1	7.0	103	9.0	102	10.9	98.6	5.1
Triclabendazole	1	108	4.0	97.5	10.1	99.3	10.3	101	3.3	102	5.0	99.2	15.7
Triclabendazole-sulfone	5	94.8	9.7	95.9	12.0	104	5.4	103	9.7	94.8	6.2	102	14.4
Triclabendazole-sulfoxide	5	110	27.3	98.1	23.3	96.5	7.4	97.8	15.6	97.9	3.9	102	12.5
Beta Lactams													
Cefadroxil	10	-	-	-	-	102	38.0	83.2	17.7	102	7.6	104	13.3
Cefazolin	10	-	-	-	-	113	24.0	92.5	17.1	98.4	7.0	101	15.9
Cefoperazone	10	-	-	-	-	94.0	15.2	109	11.5	97.0	7.0	101	11.6
Cefquinome	10	-	-	-	-	-	-	96.0	29.7	88.6	19.2	106	18.4
Ceftiofur	4	82.7	30.0	114	14.1	94.9	7.6	97.0	8.2	94.9	4.9	103	7.5
DCCD	10	-	-	-	-	109	15.6	96.5	22.7	98.8	8.7	103	9.4
Desfuroylceftiofur	100	-	-	-	-	-	-	-	-	-	-	97.8	12.8
Cefacetrile	10	-	-	-	-	-	-	101	14.2	104	12.5	103	5.1
Cephalexin	5	-	-	128	26.6	97.7	21.0	88.6	16.2	95.1	8.1	105	5.9
Cephalonium	10	-	-	-	-	110	19.0	96.3	8.4	91.9	4.8	104	9.5
Cephapirin	10	-	-	-	-	110	10.5	91.6	7.7	98.8	7.3	102	9.5
Desacetyl Cephapirin	5	-	-	-	-	80.4	23.1	105	18.5	103	8.2	98.7	6.6
Amoxicillin	4	-	-	112	23.4	105	7.2	97.8	6.4	90.3	3.6	105	7.4
Ampicillin	4	-	-	107	22.5	102	11.5	95.7	8.0	98.0	10.0	102	9.0
Cloxacillin	50	-	-	-	-	-	-	107	20.5	101	17.4	100	16.8
Dicloxacillin	50	-	-	-	-	122	29.6	90.6	42.2	100	21.4	103	15.6
Nafcillin	5	-	-	-	-	106	21.1	95.8	12.4	96.8	7.3	102	2.0
Oxacillin	10	-	-	-	-	-	-	113	64.6	102	11.3	103	26.0
Penicillin G	4	-	-	-	-	122	24.1	105	26.9	105	13.7	95.4	13.9
Penicillin V	10	-	-	-	-	-	-	89.8	33.0	94.2	16.2	103	10.2
Macrolides and Lincosamides													
Clarithromycin	10	106	25.6	110	25.6	90.0	14.3	96.1	13.9	102	9.6	100	8.5
Clindamycin	1	-	-	113	14.2	96.6	13.8	96.8	6.8	94.2	13.7	104	13.9
Desmycosin	5	-	-	113	24.6	96.4	15.1	96.7	9.3	87.9	4.8	107	11.1
Erythromycin A	50	-	-	-	-	-	-	109	13.9	111	15.1	99.3	14.7
Josamycin	10	-	-	-	-	97.1	19.7	106	10.1	96.3	13.4	101	15.1
Lincomycin	1	99.3	5.0	104	10.7	103	12.9	98.1	3.5	90.5	10.2	105	7.8
Oleandomycin	5	116	16.2	101	17.5	96.7	5.6	99.2	8.5	87.8	10.5	107	9.2
Roxithromycin	5	101	33.7	108	20.8	92.3	21.3	102	12.5	99.3	20.2	101	7.4
Spiramycin I	1	101	13.3	104	15.4	98.6	11.7	97.2	5.6	94.1	4.8	104	6.4
Tilmicosin	10	-	-	114	23.7	97.9	9.4	97.4	7.4	94.6	9.4	103	14.1
Tulathromycin A	100	-	-	-	-	-	-	-	-	72.6	24.5	105	18.2
Tylosin A	50	-	-	-	-	105	31.9	94.7	17.4	90.3	16.3	106	12.9
Quinolones													
Cinoxacin	5	99.8	27.6	102	14.3	101	8.0	97.3	8.7	95.5	10.9	103	8.5
Ciprofloxacin	1	104	18.4	101	15.8	96.7	11.3	97.3	12.7	100	18.0	101	4.6
Danofloxacin	5	95.8	7.7	114	13.4	96.9	14.1	94.7	7.9	95.6	15.0	103	12.4
Difloxacin	1	113	24.7	99.3	19.4	100	11.1	94.2	10.6	101	9.8	101	13.9
Enoxacin	5	-	-	122	19.2	89.5	16.9	96.0	9.7	91.5	16.6	106	8.4
Enrofloxacin	1	111	9.6	95.9	16.8	101	8.9	101	11.7	94.1	11.8	103	6.7
Flumequine	1	89.5	13.1	102	9.7	113	10.1	91.1	9.5	92.9	5.6	104	14.7
Levofloxacin	1	105	7.4	98.3	12.0	100	9.7	100	6.4	96.8	10.4	102	8.4
Lomefloxacin	1	-	-	104	18.7	94.5	13.7	96.9	11.1	99.7	5.9	101	9.9
Marbofloxacin	1	110	10.9	98.2	11.2	96.3	13.6	103	7.4	92.8	9.2	103	8.3
Nalidixic Acid	1	101	15.2	98.9	12.5	102	15.7	97.1	17.7	93.6	11.2	103	17.4
Norfloxacin	1	106	11.6	107	20.4	97.1	13.3	92.8	9.8	98.9	4.1	102	15.4
Ofloxacin	1	104	8.6	98.6	12.3	99.5	9.6	100	6.8	96.8	10.4	102	8.4
Oxolinic Acid	1	98.5	9.0	102	7.9	98.3	7.1	100	7.3	97.0	4.1	101	13.5
Sarafloxacin	5	96.8	4.4	114	11.1	95.9	6.1	94.9	11.5	92.2	6.4	105	21.1
Sparfloxacin	5	-	-	106	24.9	92.8	13.6	103	7.0	96.8	7.6	102	8.0
Sulfonamides													
Sulfabenzamide	5	112	27.6	106	22.1	96.5	15.6	96.3	12.9	98.6	5.7	102	15.1
Sulfacetamide	1	103	25.1	93.9	12.8	108	10.9	94.6	13.9	98.0	10.6	101	11.5
Sulfachloropyridazine	5	-	-	-	-	101	13.6	98.3	18.2	101	19.8	100	14.0
Sulfaclozine	5	-	-	-	-	118	6.5	98.5	9.1	104	11.2	97.1	15.7
Sulfadiazine	5	-	-	113	16.2	103	14.5	96.4	11.6	98.0	18.1	102	13.3
Sulfadimethoxine	1	112	17.4	100	16.4	97.8	8.8	95.7	10.6	97.3	7.2	103	7.9

Fortified Level on Sample	LOQ ng/g	0.5 ng/g		1.0 ng/g		5.0 ng/g		10 ng/g		50 ng/g		100 ng/g	
Class/Compound Name		CR	CV	CR	CV	CR	CV	CR	CV	CR	CV	CR	CV
Sulfonamides *(continued)*													
Sulfadoxine	1	102	9.6	104	12.2	102	8.9	92.4	14.2	100	10.3	101	12.1
Sulfaguanidine	5	-	-	109	15.4	96.1	5.2	98.1	13.4	87.7	9.8	107	18.3
Sulfamerazine	1	103	24.2	101	12.2	102	15.9	97.1	14.0	97.4	19.2	102	9.9
Sulfameter	1	-	-	89.8	23.9	104	6.7	94.8	18.2	104	10.4	99.0	13.1
Sulfamethazine	5	-	-	109	40.2	98.7	19.3	96.6	16.4	93.2	19.9	104	18.8
Sulfamethizole	5	116	10.3	94.3	32.0	98.9	15.1	94.3	15.9	99.7	25.2	101	14.6
Sulfamethoxazole	5	113	25.0	99.1	30.5	103	16.7	97.3	16.1	91.6	14.1	105	15.8
Sulfamethoxypyridazine	5	-	-	120	38.9	105	12.1	95.0	17.4	97.9	12.7	102	12.4
Sulfamonomethoxine	5	128	7.1	91.9	42.0	104	6.6	93.0	14.3	96.8	8.7	103	8.8
Sulfamoxole	1	116	17.5	87.8	16.1	110	13.2	95.9	17.1	104	16.3	98.4	20.2
Sulfanilamide	100	-	-	-	-	-	-	-	-	85.9	3.8	76.4	10.0
Sulfaphenazole	1	103	16.6	98.0	10.2	104	7.7	96.8	8.5	100	13.4	100	7.0
Sulfapyridine	5	-	-	112	18.4	110	9.0	96.7	14.0	88.8	12.3	106	9.1
Sulfaquinoxaline	1	94.6	22.1	105	6.5	103	8.4	95.7	10.2	95.8	10.2	103	3.8
Sulfathiazole	5	-	-	100	21.3	107	16.1	93.3	5.8	90.6	12.2	105	23.3
Sulfatroxazole	5	109	21.9	86.3	13.5	113	9.5	95.8	8.8	95.5	11.1	102	4.9
Sulfisomidine	5	-	-	94.2	15.0	107	12.1	93.5	10.8	99.6	18.2	101	12.8
Sulfisoxazole	5	-	-	97.1	26.0	103	9.7	99.0	15.6	97.1	17.0	101	14.4
Tetracyclines													
4-Epichlortetracycline	10	-	-	-	-	95.2	18.2	99.1	8.3	96.7	3.7	103	6.0
4-Epioxytetracycline	5	-	-	115	22.3	101	14.9	90.6	9.8	94.4	9.9	105	12.5
4-Epitetracycline	1	-	-	98.0	15.6	101	9.0	97.2	6.1	97.0	3.9	102	9.7
Chlortetracycline	5	108	8.1	102	14.1	98.8	9.6	99.2	6.1	94.5	7.3	103	5.6
Doxycycline	5	97.7	28.4	110	15.2	98.5	15.5	94.6	8.4	93.8	7.6	104	9.2
Isochlorotetracycline	5	99.7	8.8	106	14.4	94.9	10.6	99.2	10.1	93.5	6.4	104	7.2
Oxytetracycline	5	-	-	102	12.9	101	10.1	96.9	6.1	98.4	3.5	101	6.5
Tetracycline	1	106	10.6	101	7.8	99.0	11.5	100	8.8	96.9	4.6	102	4.5
Antibiotics - Other													
Dapson	5	101	18.7	105	35.2	98.1	8.1	95.3	13.1	97.2	14.4	102	15.8
Nifurstyrenate Sodium	50	-	-	-	-	120	3.6	105	2.1	110	20.1	111	15.7
Novobiocin	5	-	-	-	-	109	8.4	101	7.1	105	5.2	97.9	18.1
Ormetoprim	1	103	14.0	107	12.4	93.7	9.5	100	7.6	90.5	8.3	105	14.5
Rifaximin	1	97.5	34.4	106	8.7	100	6.4	94.7	6.2	102	1.2	100	5.1
Tiamulin	1	107	11.2	104	13.2	96.6	16.9	98.2	12.8	95.6	15.9	103	5.4
Trimethoprim	1	101	11.0	97.1	8.8	102	9.9	101	6.5	94.8	7.2	102	16.3
Chloramphenicol Succinate	50	-	-	-	-	112	25.1	100	32.4	95.2	13.7	102	10.1
Florfenicol Amine*	100	-	-	-	-	-	-	-	-	-	-	97.0	18.2
Streptomycin*	100	-	-	-	-	-	-	-	-	-	-	115	25.8
Antimicrobial Growth Promoters													
Bacitracin A	50	-	-	-	-	-	-	98.3	29.1	108	9.1	98.6	5.0
Nitrovin	5	-	-	87.5	33.6	110	18.0	104	21.0	91.8	5.7	103	13.1
Virginiamycin (M1)	50	-	-	-	-	-	-	101	16.9	99.6	16.5	99.7	9.3
Antiprotozoal													
Isometamidium	10	-	-	121	19.9	86.8	11.4	97.2	17.5	99.4	1.7	102	10.5
Beta-Agonists													
Cimaterol	1	113	8.9	97.5	12.9	101	9.4	98.6	7.4	91.1	3.9	105	7.2
Clenbuterol	1	106	26.6	101	16.4	102	12.7	96.5	8.9	93.9	11.5	104	7.9
Isoxsuprine	1	100	9.4	107	17.0	101	9.1	94.0	8.4	91.2	10.9	105	11.5
Mabuterol	1	-	-	96.5	15.0	101	4.6	97.2	7.0	97.7	9.4	102	6.1
Ractopamine	1	93.7	12.2	97.9	15.3	104	11.4	99.7	23.0	96.6	8.4	101	14.7
Salbutamol	1	84.6	17.8	102	7.9	101	7.2	97.7	7.4	94.2	3.1	103	8.8
Terbutaline	1	-	-	113	8.9	103	9.1	97.5	6.0	96.7	6.9	102	6.9
Zilpaterol	1	96.8	14.7	106	26.8	98.9	14.6	97.1	6.7	97.1	4.0	102	8.6
Coccidiostats													
Clopidol	5	-	-	119	25.0	96.0	10.9	94.6	8.9	91.0	3.2	106	10.3
Dimetridazole	5	94.3	4.9	101	8.3	103	6.6	98.7	5.4	96.5	8.7	102	11.0
Dimetridazole-hydroxy	10	-	-	-	-	99.7	9.0	100	10.9	96.9	7.9	102	9.1
Ipronidazole	1	102	28.2	95.3	14.4	107	9.9	100	7.1	94.8	10.2	102	9.1
Ipronidazole-hydroxy	1	112	11.3	97.8	17.2	100	12.1	98.5	4.2	93.7	17.1	103	11.2
Metronidazole	1	103	7.8	99.4	9.5	102	5.7	97.6	5.3	96.3	6.9	102	10.7
Metronidazole-hydroxy	1	96.6	11.4	100	6.5	102	5.9	100	5.1	99.4	5.3	100	10.1
Ronidazole	1	107	23.4	100	13.5	101	6.7	97.5	8.8	94.9	7.0	103	8.5
Dyes													
Brilliant green	1	103	16.5	103	9.0	101	13.7	98.3	12.1	97.2	20.4	102	13.3
Crystal violet	5	-	-	-	-	83.7	23.2	115	13.4	91.5	21.3	104	9.5
Leuco Crystal violet	10	-	-	-	-	110	24.7	104	13.5	95.5	12.9	103	12.9
Leuco Malachite green	5	116	17.7	112	22.4	97.7	17.9	96.3	11.4	78.1	8.8	112	7.0
Malachite green	5	108	19.0	103	23.8	93.1	10.6	97.1	13.0	106	7.4	98.2	7.9
Tranquilizers													
Chlorpromazine	1	108	20.3	90.2	26.4	111	20.5	95.3	16.7	103	17.7	98.6	7.0
Diazepam	1	-	-	102	22.5	97.5	10.8	105	11.3	87.7	8.1	106	4.5
Methaqualone	5	107	18.9	104	17.1	101	14.2	95.7	9.6	90.3	13.3	106	16.6
Procaine	1	117	21.8	91.0	12.8	102	9.3	98.5	17.6	98.1	15.5	101	10.4
Xylazine	1	102	19.5	104	20.5	101	12.2	92.1	9.0	93.6	4.8	105	10.2
Others													
Colchicine	5	-	-	109	18.7	96.5	11.2	103	10.5	98.1	13.9	100	8.9
Strychnine	1	105	28.2	101	16.6	103	13.8	96.1	17.5	94.5	17.3	103	12.1

- Data was not listed due to the recovery and/or CV being out of range at the level below the LOQ of the compound.
* There were not enough data points to establish the good linearity for quantitation, thus the absolute recoveries were listed for florfenicol amine and streptomycin.

Results and Discussion

Sample Preparation Optimization

- Extraction
  - – Ratio between aqueous and organic solvents: The 50:50 acetonitrile:water (*v/v*) mixture was selected over 75:25 acetonitrile: water (*v/v*) mixture to improve the recoveries of beta-lactams
  - – Addition of EDTA buffer: EDTA (0.05M) was added to the water to prevent chelation of tetracyclines and quinolones with metals
- Clean-up - evaluated options
  - – EMR (dSPE): enhanced matrix removal-lipid
  - – SLE: supported liquid extraction
  - – C18 SPE cartridge format
  - – PLD+: phospholipid removal
  - – Hexane defat + C18 cartridge SPE
- Factors used to evaluate clean-up efficiency:
  - – Recovery and precision
  - – Matrix co-extractive removal efficiency by a gravimetric test
  - – Matrix suppression/enhancement evaluation using post-column infusion
- From gravimetric test and post-column infusion, EMR and SLE provided the best co-extractive removal efficiency.
- Considering the lower recoveries observed for some critical compounds (e.g. tetracyclines, beta-lactams) when applying the various clean-up procedures, clean-up was omitted from the final method.

Method Validation Process

- Evaluation of accuracy and precision at 0.5, 1, 5, 10, 50 and 100 ng/g (*n* = 5) in the infant formula powder and intermediate precision (ruggedness) at 1, 5, and 10 ng/g (*n* = 10; 2 different analysts on 2 different days)
- To test the linearity of the method and compare quantification results, the following three sets of standards were prepared:
  - – Extracted matrix match standards to quantify the potential veterinary drug residues in the samples (mimicking the standard addition procedure) and determine corrected analyte recoveries presented in Table 1
  - – Post-extracted matrix matched standards to determine absolute analyte recoveries
  - – Standards in solvent to monitor matrix effects

Method Validation Results

- Method performance was evaluated according to CAC/GL 71-2009 guideline

Concentration, ng/g	CV within the lab, %	Accuracy, range of mean recovery, %
≤1	35	50-120
1 to 10	30	60-120
10 to 100	20	70-120

- Acceptable analyte corrected recoveries (CR, within the 70-120% range) and CVs (≤30%) were obtained for all analytes at and above their LOQs, except for 4-epidemeclocycline, decoquinate, diminazine and colistin A and B, which were excluded from the final method used for the routine analysis.
- The method validated LOQ was determined for each analyte as the lowest spiking level that met the validation criteria for recoveries and CVs. The typical analyte reporting limits in infant formula powder are between 1 and 10 ng/g.
- The coefficient of determination (r^2) values of matrix extracted calibration curves were >0.99 for the majority of analytes ranging from 1 (or 5 or 10) to 100 ng/g.

Conclusions

- The developed method was validated in infant formula powder, showing satisfactory validation results, including identification, selectivity, matrix effects, linearity, LOQs, accuracy and precision.
- This method can be used in routine analysis for the simultaneous detection and quantification of a large number of veterinary drug residues in infant formula.
- In the future, the method will also be validated for screening/quantitation of veterinary drugs in other relevant matrices, such as dairy products, seafood, meat, eggs, honey, pet food and animal feed.

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