Multiclass Residue Analysis of Veterinary Drugs in Animal Tissues and Eggs Using Liquid Chromatography Coupled With Tandem Mass Spectrometry (LC/MS/MS)

Application Note

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Abstract

This application note describes a workflow solution that was developed for the screening and quantification of multiclass veterinary drugs in different animal-derived food samples, from sample preparation to quantification of results. These veterinary drugs, belonging to 32 different chemical classes, included macrolides, sulfonamides, tetracyclines, β-lactams, β-agonists, chloramphenicols, nitroimidazoles, cephems, avermectins, benzimidazoles, glucocorticosteroids, nonsteroid anti-inflammatory drugs (NSAIDs), peptides, anticoccidiosis drugs, quinolones, imidazoles, androgens, polyethers, triphenylmethanes, phenothiazines, quinoxalines, trematocides, antivirus, pesticides, dapsones, organic acids, nitros, tranquilizers, and so forth. The animal-derived food samples included muscle and liver of swine, cow, and chicken, as well as fish and hen eggs. The sample preparation involved a rapid and efficient protein precipitation extraction by acidified acetonitrile, followed by Agilent EMR—Lipid dSPE (p/n 5982-1010) and a polish kit (p/n 5982-0102) for further cleanup. The workflow solution supports the mid-to-high-end of Agilent tandem mass spectrometers (Agilent 6460 Triple Quadrupole LC/MS, Agilent 6470 Triple Quadrupole LC/MS, and Agilent 6495 Triple Quadrupole LC/MS) coupled with the Agilent Jet Stream Ionization Source.

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Introduction

Veterinary drugs (VDs) have been used widely in veterinary practice to treat and prevent diseases, or to enhance growth and feed efficiency. If veterinary drugs are not administered correctly, it can lead to the presence of VD residues in foods of animal origin for human consumption, which can pose a health risk. With prolonged exposure, some antimicrobials, even at very low levels in foodstuff, may lead to an increase in antimicrobial resistance in human populations.

To better control the use of VDs and protect public health, the residue levels of VDs in meat and other foods are regulated by federal regulatory agencies. In China, Regulation No. 235, issued by the ministry of agriculture (MOA), separates veterinary drugs into four categories: Drugs in category I are permitted without maximum residue levels (MRLs) defined. Drugs in Category II are permitted for disease treatment with MRLs required. Drugs in Category III are permitted for disease treatment, however, no detectable levels are allowed in samples for human consumption. Drugs in Category IV are prohibited with no detection permitted in samples. The Chinese government annually sets up a series of monitoring plans to control the use of Category II, III, and IV veterinary drugs. Currently, the published veterinary drug residue analysis standard methods in China focus on only one chemical class or several chemical classes of drugs (for example, sulfonamides, quinolones, tetracyclines, and so forth). It can take days to finish testing and get the reports. Such a workflow is not only time-consuming, labor-intensive, and costly, but is also inefficient when dealing with a large number of samples. The aim of our workflow solution is to enable laboratories to analyze >180 commonly monitored VDs across multiple chemical classes simultaneously. The list of VDs in our method were based on general notice No. 235 (MOA, China).

Animal-derived food samples contain high amounts of proteins and lipids, and pose significant challenges with regards to sample preparation. In this study, acidified acetonitrile (5 % formic acid) was selected as an extraction solvent capable of efficient protein precipitation to extract analytes into the organic phase. Agilent Bond Elut EMR—Lipid dispersive SPE (EMR—Lipid) was used for cleanup with a polishing step. EMR—Lipid is a novel sorbent material that selectively removes major lipid classes from the sample without affecting analyte recoveries.

Experimental

Standards and reagents

Veterinary drug standards were purchased from Dr. Ehrenstorfer GmbH, WITEGA laboratorien Berlin-Adlershof GmbH, Toronto Research Chemicals (TRC) or AccuStandard, Inc. Ultrapure water (>18.2 MΩ, ELGA VEOLIA PureLab Chrous system), acetonitrile (LC/MS grade, Fluka), and formic acid (~98 %, for mass spectrometry, Fluka) were used for mobile phase preparation. Acetonitrile (ACN, HPLC grade, Sigma-Aldrich), dimethyl sulfoxide (DMSO, >99.9 %, Aldrich), ammonium acetate (≥98 %, Sigma-Aldrich), and formic acid (~98 %, HPLC grade, Fluka) were used during sample preparation.

Sample preparation

A quick and simple sample preparation method was developed. The homogenized sample was extracted using acidified acetonitrile, which in essence served as a protein precipitation step. The extract was further cleaned using Agilent EMR—Lipid to selectively remove lipids while not trapping contaminants of interest, followed by a final polishing step for further cleanup.

The sample preparation method suits many types of animal-derived food samples, including muscle and liver of swine, cow, and chicken, as well as fish and hen eggs.

Instrumentation

Analysis was performed on an Agilent 1290 Infinity II LC system consisting of:

- Agilent 1290 Infinity II Binary Pump with a 35 µL Jet Weaver (G7120A)
- Agilent1290 Multisampler with a 20-µL loop (G7167B)
- Agilent1290 Infinity II Multicolumn Thermostat (G7116B)

Three models of tandem mass spectrometers were tested:

- Agilent 6460 Triple Quadrupole LC/MS with Jet Stream technology (G6460CA)
- Agilent 6470 Triple Quadrupole LC/MS with Jet Stream technology (G6470AA)
- Agilent 6495 Triple Quadrupole LC/MS with the iFunnel and Jet Stream technology (G6495A)

Method development and validation

The following performance characteristics were considered during method development and validation: dynamic range, limits of detection (LODs), limits of quantification (LOQs), recovery, and repeatability.

The full validations were carried out on an Agilent 6495 Triple Quadrupole LC/MS with five matrices (pork, swine liver, eel, chicken, and egg) at three spiking levels (1 ng/g, 5 ng/g, and 20 ng/g). The full validation was carried out on an Agilent 6470 Triple Quadrupole LC/MS with pork matrix at three spiking levels (1 ng/g, 5 ng/g, and 20 ng/g). The full validations were carried out on an Agilent 6460 Triple Quadrupole LC/MS with pork and swine liver matrices at three spiking levels (1 ng/g, 5 ng/g, and 20 ng/g).

Results and Discussion

Table 1 lists the compounds covered in this solution.

Table 1. Veterinary Drugs Analyzed

Classification	English name	CAS
β-Agonists	Cimaterol	54239-37-1
	Clenbuterol	21898-19-1
	Clorprenaline	3811-25-4
	Penbutolol	38363-40-5
	Propanolol	318-98-9
	Ractopamine	90274-24-1
	Salbutamol	18559-94-9
	Terbutaline	23031-32-5
	Tulobuterol	56776-01-3
Androgens	Mengestrol Acetate	595-33-5
	Methyltestosterone	58-18-4
	Nadrolone/19-Nortestosterone	434-22-0
	Testosterone	58-22-0
	β-trenbolone	10161-33-8
Avermectins	Avermectin B1a	65195-55-3
	Doramectin	117704-25-3
	Eprinomectin	123997-26-2
	Ivermectin	70288-86-7
Benzimidazoles	2-Aminoflubendazole	82050-13-3
	5-Hydroxymebendazole	60254-95-7
	Albendazole	54965-21-8
	Albendazole sulfone	75184-71-3
	Albendazole sulfoxide	54029-12-8
	Albendazole-2-aminosulfone	80983-34-2
	Cambendazole	26097-80-3
	Fenbantel	58306-30-2
	Fenbendazole	43210-67-9
	Flubendazole	31430-15-6
	Mebendazole	31431-39-7
	Mebendazole-amine	52329-60-9
	Oxfendazole	53716-50-0
	Oxfendazole sulfone/ Fenbendazole sulfone	54029-20-8
	Oxibendazole	20559-55-1
	Thiabendazole	148-79-8
	Thiabendazole-5-hydroxy	948-71-0
	Triclabendazole	68786-66-3
Cephems	Cefaclor	53994-73-3
	Cefamandole	34444-01-4/ 58648-57-0
	Cefapirin	21593-23-7
	Cefazolin	25953-19-9
	Cefetamet pivoxyl	65243-33-6
	Cefoperazone	62893-19-0/ 62893-20-3
	Cefotaxime	63527-52-6
	Ceftiofur	80370-57-6
	Cephalexin	15686-71-2
	Cephalonium	5575-21-3
	Cephradine	38821-53-3
Chloramphenicols	Chloramphenicol	56-75-7
	Florfenicol	73231-34-2
	Thiamphenicol	15318-45-3
Glucocorticosteroids	Beclomethasone	4419-39-0
	Betamethasone	378-44-9
	Cortisone	53-06-5
	Dexamethasone	50-02-2
	Flumethasone	2135-17-3
	Hydrocortisone	50-23-7
	Methylprednisolone	83-43-2
	Prednisolone	50-24-8
	Prednisone	53-03-2
	Triamcinolone	124-94-7
	Triamcinolone acetonide	76-25-5
Macrolides	Acetylisovaleryltylosin/Tylvalosin	63409-12-1
	Erythromycin	59319-72-1
	Kitasamycin/Leucomycin	1392-21-8
	Oleandomycin	7060-74-4
	Tilmicosin	108050-54-0
	Tylosin	74610-55-2

Table 1. Veterinary Drugs Analyzed (continued)

Classification	English name	CAS
Nitroimidazoles	Dimetridazole	551-92-8
	Dimetridazole- OH (HMMNI)	936-05-0
	Metronidazole	443-48-1
	Metronidazole-OH	4812-40-2
	Ronidazole	7681-76-7
Peptides	Bacitracin A	1405-87-4
	Virginiamycin M1	211411-53-0
Polyethers	Lasalocid A	25999-20-6
	Maduramicin	61991-54-6
	Monensin	22373-78-0
Quinolones	Ciprofloxacin	85721-33-1
	Danofloxacin	112398-08-0
	Difloxacin	98106-17-3
	Enoxacin	74011-58-8
	Enrofloxacin	93106-60-6
	Fleroxacin	79660-72-3
	Flumequine	42835-25-6
	Lomefloxacin	98079-51-7
	Nalidixic acid	389-08-2
	Norfloxacin	70458-96-7
	Ofloxacin	82419-36-1
	Orbifloxacin	113617-63-3
	Oxolinic acid	14698-29-4
	Pefloxacin	70458-92-3
	Sarafloxacin	98105-99-8
	Sparfloxacin	110871-86-8
Sulfonamides	Sulfabenzamide	127-71-9
	Sulfacetamide	144-80-9
	Sulfachloropyridazine	80-32-0
	Sulfaclozine	102-65-8
	Sulfadiazine	68-35-9
	Sulfadimethoxine	122-11-2
	Sulfadoxine	2447-57-6
	Sulfaguanidine	57-67-0
	Sulfamerazine	127-79-7
	Sulfameter/Sulfamethoxydiazine	651-06-9
	Sulfamethazine/Sulfadimidine	57-68-1
	Sulfamethizole	144-82-1
	Sulfamethoxazole	723-46-6
	Sulfamethoxypyridazine	80-35-3
	Sulfamonomethoxine	1220-83-3
	Sulfamoxole	729-99-7
	Sulfanitran	122-16-7
	Sulfaphenazole	526-08-9
	Sulfapyridine	144-83-2
	Sulfaquinoxaline	59-40-5
	Sulfathiazole	72-14-0
	Sulfisomidine	515-64-0
	Sulfisoxazole/Sulfafurazole	127-69-5
	Trimethoprim	738-70-5
Tetracyclines	Chlortetracycline	57-62-5
	Doxycycline	564-25-0
	Oxytetracycline	6153-64-6
	Tetracycline	60-54-8
β-lactams	Amoxicillin	26787-78-0
	Ampicillin	69-53-4
	Benzylpenicillin/Procaine Benzylpenicillin/Penicillin G	61-33-6
	Oxacillin	66-79-5
	Sulbactam	68373-14-8
Triphenylmethanes	Crystal Violet/Basic violet 3	548-62-9
	Leucomalachite green	129-73-7
	Malachite green	569-64-2
Diterpene	Valnemulin	133868-46-9
Phenothiazines	Chlorpromazine	50-53-3
	Xylazine	7361-61-7
Furans	Nifurstyrenate	54992-23-3
Quinoxalines	Olaquindox	23696-28-8
Trematocides	Closantel	57808-65-8
	Nitroxynil	1689-89-0
	Rafoxanide	22662-39-1
Anticoccidiosis	Clopidol	2971-90-6
	Decoquinate	18507-89-6
	Diclazuril	101831-37-2
	Ethopabate	59-06-3
	Halofuginone	55837-20-2
	Nequinate	13997-19-8
	Nicarbazine	330-95-0
	Robenidine	25875-50-7
	Toltrazuril	69004-03-1
	Toltrazuril sulfone	69004-04-2
	Toltrazuril sulfoxide	69004-15-5
	Zoalene	148-01-6

Table 1. Veterinary Drugs Analyzed (continued)

Classification	English name	CAS
Antivirus	Amantadine	768-94-5
Pesticides	Carbofuran	1563-66-2
	Chlordimeform	6164-98-3
	Coumaphos	56-72-4
	Deltamethrin	52918-63-5
	Diazinon	333-41-5
	Dichlorvos	62-73-7
	Fenthion sulfoxide	3761-41-9
	Malathion	121-75-5
	Pentachlorophenol (PCP)	131-52-2
	Propetamphos	31218-83-4
	Trichlorfon	52-68-6
Lincosamide	Lincomycin	7179-49-9
Dapsones	Dapson	80-08-0
	N-Acetyl dapsone	565-20-8
Organic acids	Heptadecafluorooctanesulfonic acid (PFOS)	1763-23-1
	Perfluorooctanoic acid (PFOA)	335-67-1
Nitros	3-Amino-5-nitro-*o*-toluamide (ANOT)	3572-44-9
	4,6-Dinitro-*o*-cresol (DNOC)	534-52-1
	Nitrovin	2315-20-0
	Sodium nitrophenolate/ 4-nitrophenol	100-02-7/ 63317-67-9
Tranquilizer	Azaperol	2804-05-9
	Azaperone	1649-18-9
Non-steroid anti-inflammatory drugs (NSAIDs)	4-Acetylamino antipyrine	83-15-8
	4-Formylaminoantipyrine	1672-58-8
	Carprofen	53716-49-7
	Diclofenac/Diclofenac acid	15307-86-5
	Flufenamic acid	530-78-9
	Flunixin	42461-84-7
	Indomethacin	53-86-1
	Indoprofen	31842-01-0
	Ketoprofen	22071-15-4
	Mefenamic acid	61-68-7
	Meloxicam	71125-38-7
	Piroxicam	36322-90-4
	Sulindac	38194-50-2
	Tenoxicam	59804-37-4
	Tolfenamic acid	13710-19-5
	Tolmetin	26171-23-3
Parasiticide	Levamisole	14769-73-4

A set of nine matrix spiked calibration standards (0.1 ng/g, 0.2 ng/g, 0.5 ng/g, 1.0 ng/g, 2.0 ng/g, 5.0 ng/g, 10 ng/g, 20 ng/g, and 40 ng/g) were analyzed consecutively, and linear fittings were generated with coefficient of correlation values (R^2). Figures 1A and 1B show calibration curves obtained in pork on a 6495 Triple Quadrupole LC/MS for 12 compounds that belong to different chemical classes: ractopamine, testosterone, albendazole, cefapirin, chloramphenicol, prednisolone, oleandomycin, metronidazole, ciprofloxacin, sulfabenzamide, amoxicillin, and carprofen.

The LODs and LOQs were calculated by Agilent MassHunter Quantitative Analysis software (Ver. B.06.00 or above) under *Replicate Injection MDL-LOQ-LOD Calculation* using data files from seven replicates of each level (1 ng/g, 5 ng/g, and 20 ng/g). The level chosen for LOD and LOQ calculation should meet the following criteria:

- The concentration level chosen was within the calibration range.
- The %RSD of replicates was less than or equal to 20.
- The concentration level should be as low as possible.

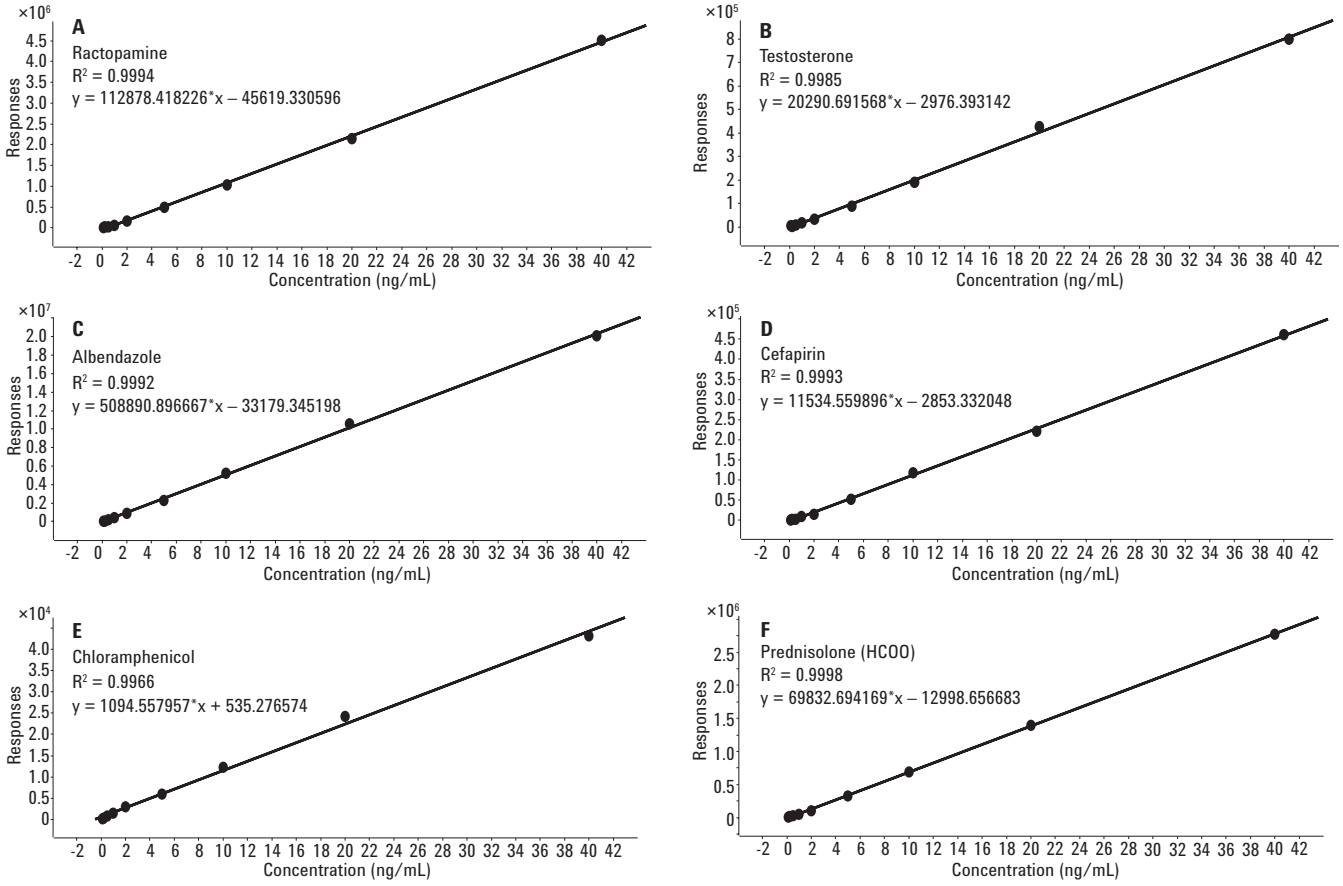

Figure 1A. Calibration curves of ractopamine (A), testosterone (B), albendazole (C), cefapirin (D), prednisolone (E), and oleandomycin (F) from 0.1 ng/g to 40 ng/g in pork on an Agilent 6495 Triple Quadrupole LC/MS.

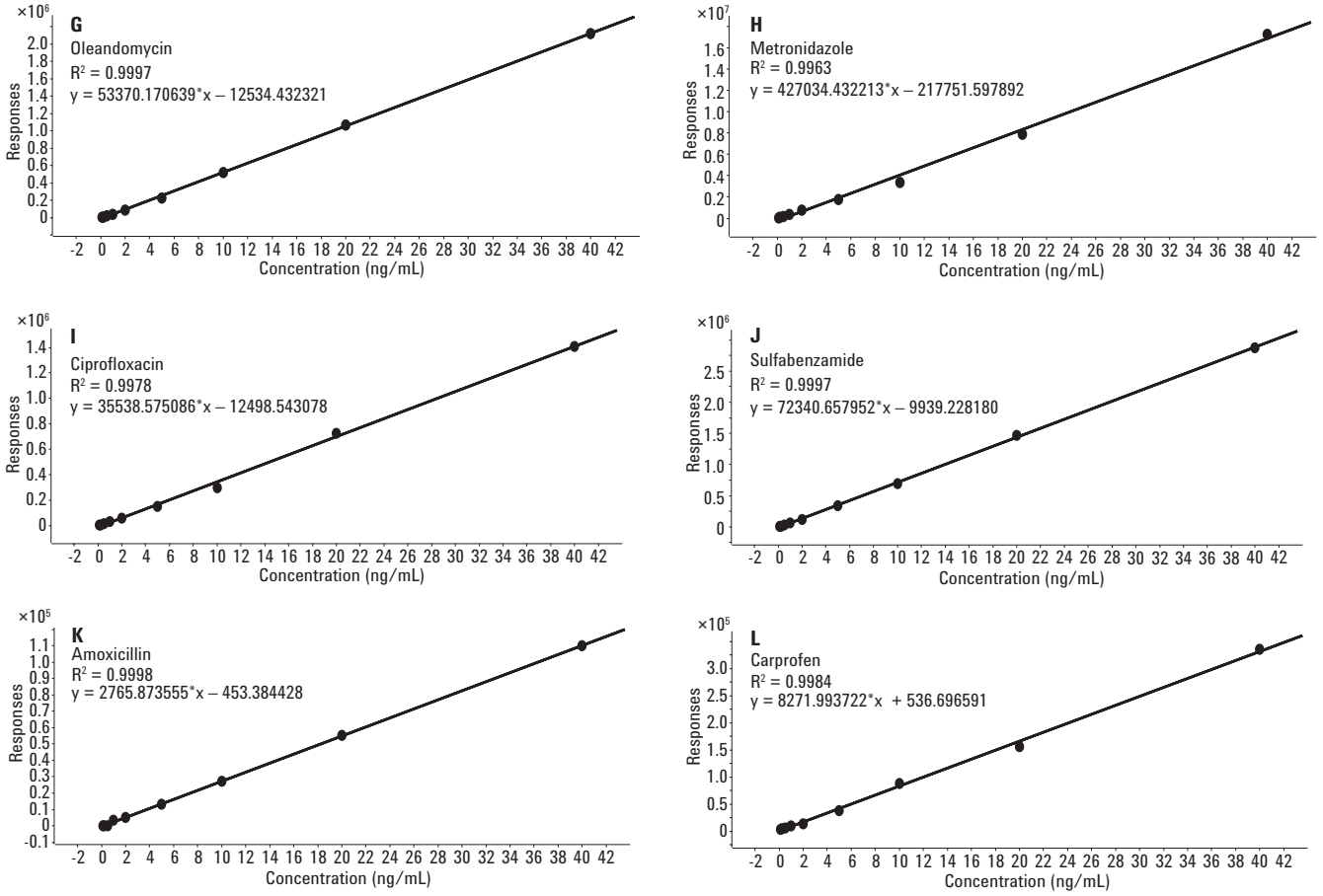

Figure 1B. Calibration curves of metronidazole (G), ciprofloxacin (H), sulfabenzamide (I), amoxicillin (J), carprofen (K), and chloramphenicol (L) from 0.1 ng/g to 40 ng/g in pork on an Agilent 6495 Triple Quadrupole LC/MS.

The validation results in pork on a 6495 Triple Quadrupole LC/MS are summarized to demonstrate the performance of our workflow solution:

- Good linearity results were achieved, with 98 % of compounds having R^2 ≥0.99.
- The recovery data at three different levels were grouped into four categories: % recovery between 50–79, % recovery between 80–120, % recovery between 121–150, and % recovery greater than 150. The results shown in Figure 2 demonstrate good recoveries achieved through matrix spiked calibration. For example, at 5 ng/g spiking level, the recoveries of 92 % compounds were within 80–120.
- The repeatability at three different levels were also grouped in four categories:
  - % RSD between 0–10
  - %RSD between 11–15
  - %RSD between 16–20
  - %RSD greater than 20

  Figure 3 shows the results for all veterinary drugs tested in this method. Only a few compounds had %RSD greater than 20 %. For example, at 5 ng/g spiking level, 11 compounds (sulfanitran, cephradine, diclazuril, rafoxanide, closantel, tylvalosin, oxacillin, toltrazuril-sulfoxide, sodium nifurstyrenate, amoxicillin, and doramectin) had RSDs greater than 20 %, that could be caused either by weak signal response or greater variance in signal response for unstable analytes.

In this application note, an additional nitrogen concentration step was applied for the 6460 Triple Quadrupole LC/MS and the 6470 Triple Quadrupole LC/MS to match the detection sensitivity of the 6495 Triple Quadrupole LC/MS. The validation results showed that similar performance results could be achieved through this additional step. Table 2 lists validation data for some representative compounds on different tandem mass spectrometers. Note that some unstable compounds, such as penicillins, undergo decomposition during the nitrogen concentration step. The following nine compounds failed to be detected on the 6460 Triple Quadrupole LC/MS and the 6470 Triple Quadrupole LC/MS due to decomposition under the nitrogen concentration process: carprofen, closantel, deltamethrin, diazinon, sodium nifurstyrenate, oxacillin, rafoxanide, sulbactam, and β-trenbolone. In the final reconstitution step, using membrane filtration (not centrifugation) also caused great losses of the following compounds: avermectin B1a, cephradine, decoquinate, dichlorvos, diclazuril, doramectin, eprinomectin, flufenamic acid, heptadecafluorooctanesulfonic acid (PFOS), ivermectin, lasalocid, maduramicin, mefenamic acid, monensin, nequinate, pentachlorophenol (PCP), perfluorooctanoic acid (PFOA), robenidine, and tolfenamic acid.

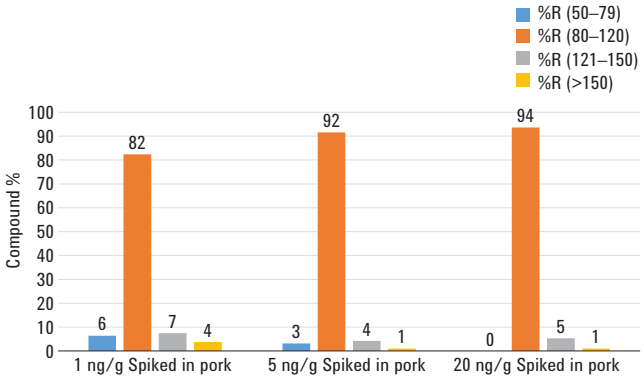

Figure 2. Analyte recoveries at different spiking levels in pork on an Agilent 6495 Triple Quadrupole LC/MS.

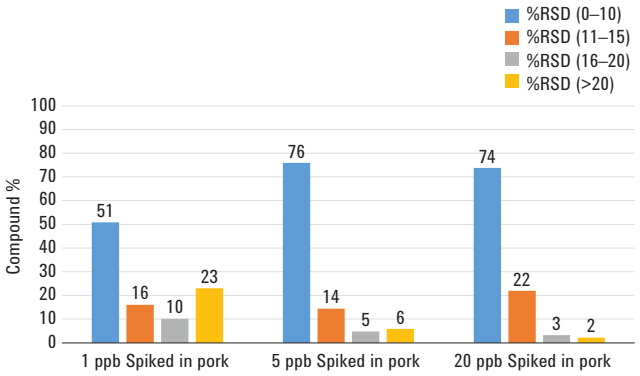

Figure 3. Analysis repeatability at different spiking levels in pork on an Agilent 6495 Triple Quadrupole LC/MS.

Table 2. Representative Validation Data in Pork on Different Tandem Mass Spectrometers (Agilent 6460 Triple Quadrupole LC/MS, Agilent 6470 Triple Quadrupole LC/MS, and Agilent 6495 Triple Quadrupole LC/MS)

				Spiking level							
				1 ng/g		5 ng/g		20 ng/g			
Name	**Model**	**R^2**	**Dynamic range (ng/g)**	**% Recovery***	**%RSD**	**% Recovery***	**%RSD**	**% Recovery***	**%RSD**	**LOQ (ng/g)**	**LOD (ng/g)**
Albendazole	G6460CA	0.998	0.1–40	97	3	99	3	109	4	0.3	0.1
	G6470AA	0.999	0.2–40	101	7	102	3	102	5	0.7	0.2
	G6495A	0.999	0.1–40	98	6	105	5	108	4	0.6	0.2
Amoxicillin	G6460CA	1.000	5–40	103	21	87	24	108	10	22.0	7.3
	G6470AA	0.999	2–40	78	33	103	11	107	5	5.9	1.8
	G6495A	1.000	1–40	154	73	80	21	117	8	8.5	2.6
Cefapirin	G6460CA	0.999	0.5–40	121	44	107	16	123	7	8.6	2.9
	G6470AA	0.995	2–40	148	39	130	10	101	7	6.7	2.0
	G6495A	0.999	1–40	101	43	150	10	110	19	7.3	2.2
Chloramphenicol	G6460CA	0.999	2–40	211	36	111	23	85	13	12.9	4.3
	G6470AA	0.998	1–40	92	16	90	13	106	9	1.4	0.4
	G6495A	0.997	0.2–40	84	15	96	11	95	5	1.3	0.4
Ciprofloxacin	G6460CA	0.999	1–40	191	27	93	19	101	9	8.9	3.0
	G6470AA	0.989	1–40	189	66	94	9	96	8	1.4	0.4
	G6495A	0.998	0.5–40	91	15	133	8	103	7	1.4	0.4
Metronidazole	G6460CA	0.999	0.1–40	92	9	104	6	102	3	0.8	0.3
	G6470AA	0.999	0.5–40	101	11	104	7	103	6	1.1	0.3
	G6495A	0.996	0.5–40	87	7	105	4	113	12	0.6	0.2
Oleandomycin	G6460CA	0.998	0.1–40	105	5	100	2	104	4	0.6	0.2
	G6470AA	0.995	0.2–40	119	7	111	8	97	6	0.9	0.3
	G6495A	1.000	0.1–40	120	8	100	8	95	4	0.9	0.3
Prednisolone	G6460CA	1.000	0.5–40	113	8	91	3	101	2	0.9	0.3
	G6470AA	1.000	0.2–40	88	12	112	3	104	7	1.0	0.3
	G6495A	1.000	0.2–40	101	3	100	5	91	6	0.3	0.1
Ractopamine	G6460CA	0.997	0.1–40	102	6	103	7	100	5	0.7	0.2
	G6470AA	0.999	0.2–40	78	16	116	8	99	5	1.3	0.4
	G6495A	0.999	0.1–40	94	11	93	4	95	11	1.0	0.3
Sulfabenzamide	G6460CA	0.998	0.5–40	100	6	87	3	105	4	0.6	0.2
	G6470AA	0.999	1–40	139	23	107	8	98	5	4.4	1.3
	G6495A	1.000	0.1–40	104	4	96	3	115	10	0.4	0.1
Testosterone	G6460CA	0.999	1–40	91	11	110	13	111	6	1.0	0.3
	G6470AA	0.999	1–40	62	13	105	13	101	5	0.8	0.2
	G6495A	0.999	0.5–40	112	28	98	5	102	10	2.5	0.8

* Average recovery of seven replicates.

Conclusions

The China multiclass residue veterinary drug workflow solution is developed to screen and quantify multiclass veterinary drugs in different animal-derived food samples. It supports all Agilent liquid chromatography tandem mass spectrometers coupled with an Agilent Jet Stream Ionization Source. We have demonstrated that our total workflow solution, from sample preparation to results, can be applied to the analyses of most types of animal-derived food samples with high sensitivity, and good accuracy and precision. With >180 veterinary drugs spanning 32 different classes in one method, our workflow solution would significantly help increase sample throughput without sacrificing data quality, robustness, or sensitivity.

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