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Development and validation of a streamlined method designed to detect residues of 62 veterinary drugs in bovine kidney using ultra-high performance liquid chromatography – tandem mass spectrometry

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In the USA, the US Department of Agriculture's Food Safety and Inspection Service (FSIS) conducts the National Residue Program designed to monitor veterinary drug and other chemical residues in beef and other slaughtered food animals. Currently, FSIS uses a 7-plate bioassay in the laboratory to screen for antimicrobial drugs in bovine kidneys from those animals tested positive by inspectors in the slaughter establishments. The microbial inhibition bioassay has several limitations in terms of monitoring scope, sensitivity, selectivity, and analysis time. Ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) has many advantages over the bioassay for this application, and this study was designed to develop, evaluate, and validate a fast UHPLC-MS/MS method for antibiotics and other high-priority veterinary drugs in bovine kidney. Five existing multi-class, multi-residue methods from the literature were tested and compared, and each performed similarly. Experiments with incurred samples demonstrated that a 5-min shake of 2 g homogenized kidney with 10 ml of 4/1 (*v/v*) acetonitrile/water followed by simultaneous clean-up of the initial extract with 0.5 g C18 and 10 ml hexane gave a fast, simple, and effective sample preparation method for the <10 min UHPLC-MS/MS analysis. An extensive 5-day validation process demonstrated that the final method could be used to acceptably screen for 54 of the 62 drugs tested, and 50 of those met qualitative MS identification criteria. Quantification was not needed in the application, but the method gave ≥70% recoveries and ≤25% reproducibilities for 30 of the drugs. Published 2012. This article is a U.S. Government work and is in the public domain of the USA.

Keywords: veterinary drugs residues analysis; UHPLC-MS/MS; bovine kidney; method validation; qualitative screening

Introduction

Nearly all countries regulate the use of veterinary drugs for food animal production to ensure that drug residues in the food (or target tissue) do not exceed maximum residue levels (MRLs) or 'tolerances' in the USA.[1–5] Internationally, the Codex Committee on Residues of Veterinary Drugs in Foods makes MRL and other recommendations designed to provide international standards.[2] Monitoring of veterinary drug residues is conducted by many government and contract laboratories around the world to enforce regulations domestically and in international food trade. In the USA, the Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) is responsible for the scheduled surveillance and inspector-generated enforcement monitoring of veterinary drugs and other adulterants in meat and poultry products.[3] The FSIS National Residue Program (NRP) has been in place since 1967 to conduct monitoring of imported and domestic meat, poultry, and egg products collected from ports and processing establishments across the USA.[6]

In the USA, all slaughter establishments that meet USDA-certification criteria require online FSIS inspection to legally produce food. To check for antibiotic residues in suspect carcasses, the inspectors assay the kidney (typical target tissue for antimicrobial drugs) using simple microbial inhibition screening tests, such as the fast antibiotic screening test (FAST) or kidney inhibition

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swab (KIS™) kit.[7,8] In 2009, ≈137,000 of 33.7 million cattle slaughtered (0.41%) were screened for antibiotic residues in inspector-generated sampling.[6] When the 3–6 h test of the kidney swab yields a positive result (3–5% of tested samples), the carcass is detained, and kidney, liver, and muscle samples are sent by overnight delivery to the FSIS Midwestern Laboratory. Speed of analysis by the laboratory is important for the establishment to recover the value of the carcass if it is not found to be violative.

Currently, personnel at the FSIS laboratory perform a simple stomaching extraction of the kidneys followed by a 7-plate microbial bioassay.[9] This test uses a group of 7 agar plates with media and microorganisms designed to assess which class of antibiotic has triggered the positive response in the field. A team of microbiologists and technicians perform the 7-plate microbial inhibition bioassay in batch fashion, and the growth patterns on the plates are interpreted after an overnight incubation period. Due to insensitivity to sulfonamides in the 7-plate bioassay, that antimicrobial drug class is screened separately using thin-layer chromatography.[10] Furthermore, all bovine kidneys are currently analyzed in the laboratory using a commercial enzyme-linked immunosorbent assay (ELISA) for flunixin, a non-steroidal anti-inflammatory drug (NSAID) of heightened concern.

In practice, ≈40% of the 7-plate bioassay sample results do not indicate an antibiotic residue violation, and those carcasses are cleared with respect to antibiotics soon after the results are known. For the indicated positives, the samples are then analyzed depending on the microbial inhibition of growth patterns on the plates by one or more single-class chemical-based identification methods internally validated by FSIS. These include different liquid chromatography (LC) or LC-tandem mass spectrometry (MS/MS) methods for aminoglycosides,[11] β-lactams,[12] sulfonamides,[13] tetracyclines,[14] macrolides,[15] and fluoroquinolones.[16] Confirmatory results by these chemical methods are sufficient to take an enforcement action if the drug is not permitted for use in the given slaughter class. However, if the confirmed residues have US tolerances, then quantitative analyses are needed using the official enforcement method dictated by the US Food and Drug Administration (FDA) Center for Veterinary Medicine (CVM). These methods were approved by the FDA-CVM during the drug registration process,[3] and they are typically very old, single-analyte methods using microbial inhibition or complicated chemical assays. On average since 1996, the violation rate for tested veterinary drug residues in inspector-generated cattle has been ≈0.9% with up to 25% of those animals being violative for multiple residues.[6]

Considering the adage, 'You can only find what you're looking for', there are several problems with the 7-plate bioassay screen with respect to the true need of the analysis: (1) it only works for microbial growth inhibiting residues (certain antibiotics within and among classes); (2) it is not sensitive enough for sulfonamides and fluoroquinolones in relation to their tolerances, but it is much too sensitive for tetracyclines and certain aminoglycosides with high tolerances; (3) it does not distinguish one drug from another in the same class; (4) the results can be difficult to interpret, especially when multiple drugs are present even from more than one class; (5) it is not quantitative by current standards; (6) it is prone to unknown microbial inhibition responses; and (7) it takes a team of personnel to set up the assay and >16 h to obtain the results.

For these reasons, FSIS wishes to replace the 7-plate bioassay with another approach, and we believe that a multi-class, multi-residue method (MMM) using streamlined sample preparation and ultra-high performance liquid chromatography UHPLC-MS/MS,[17] is the best option at this time. FSIS is already using LC-MS/MS for individual classes, so combining these methods into a single, faster MMM and eliminating the 7-plate bioassay will give a much more efficient laboratory analysis scheme. UHPLC-MS/MS has several advantages including the potential for fast (<15 min runtime) screening, identification, and/or quantification of hundreds of targeted drugs in the extracts. Furthermore, it can distinguish one drug from another independent of class, and it can be used for more than just antibiotics. Drawbacks include that the instrumentation is expensive and requires expertise to operate and interpret results. Also, although the UHPLC-MS/MS analyses are short, which gives a quick turnaround time for high-priority samples; sample injections are made sequentially, which limits sample throughput in large batches. Other concerns relate to the potential for time-consuming and complicated sample preparation needs and ion suppression due to matrix effects.[18]

In this respect, the key to success for the MMM depends mostly on sample preparation due to its repercussions in chromatography, ionization, and mass spectrometric analysis. For example, the aminoglycosides usually require different sample preparation and chromatographic conditions than other antibiotics.[17] We developed, validated, and implemented in FSIS a separate streamlined sample preparation method using disposable pipette extraction (DPX)[19] followed by UHPLC-MS/MS analysis of 10 common aminoglycosides.[11] Thus, our MMM does not need to include aminoglycosides (although it would be an advantage to include them).

Among the MMMs described in the scientific literature for veterinary drugs in animal tissues,[17] there were six that we considered for this application.[20–25] Each method was described to give more or less equivalent quality of results overall, but certain methods were better than others for some drug classes. None of the MMMs was ideal for all drug classes evaluated, and each MMM had advantages and disadvantages in terms of time, labour, and cost. Our evaluation of the different MMMs will be described in Results and Discussion.

The aim of this study was to develop and validate a streamlined and reliable sample preparation and UHPLC-MS/MS method for screening, identifying, and/or quantifying 60 priority veterinary drugs in bovine kidney. The expectation was that not all analytes would meet validation criteria for quantification, but the main purpose was to improve upon the 7-plate bioassay screen. For method development, we sought to compare different existing MMMs for veterinary drug residues, and analyzed incurred tissues to determine the conditions most conducive to extraction in the fastest time and minimal effort. We also sought to define identification criteria and evaluate matrix effects in the UHPLC-MS/MS analysis.

Materials and methods

Materials

For method development, beef kidneys from organic-certified cattle were obtained from Blackwing (Antioch, IL, USA) and Natural Acres (Millersburg, PA, USA), and several kidneys were mixed together during homogenization. For method validation, >20 different bovine kidneys from many different slaughtered animals were obtained. For analysis of real samples, FSIS Midwestern Laboratory provided 10 pre-homogenized kidney samples previously found to contain drug residues. All kidneys were shipped in dry ice and stored in a freezer at −18°C (organic) or −80°C (FSIS samples).

Table 1 lists the prioritized drugs chosen for analysis. Standards were stated to be 90–99.9% purity from Sigma (St Louis, MO, USA), US Pharmacopeia (Rockville, MD, USA) and Dr Ehrenstorfer (Augsburg, Germany) except for DCCD, which was unknown purity provided by Pfizer (Groton, CT, USA) and sulfabromomethazine, also of unknown purity synthesized previously in the laboratory. All reagents for extraction were ACS-grade or higher quality, including 88% formic acid from Spectrum (Gardena, CA, USA). The hexanes were saturated in HPLC grade acetonitrile (MeCN) by adding MeCN to 1 L until distinct layers were observed after mixing. Distilled and deionized water of 18.2 Ω was prepared with a Barnstead E-Pure system (Dubuque, IA, USA). Trichloroacetic acid was from Sigma and anhydrous sodium sulfate was from Mallinckrodt (Paris, KY, USA). Stock and mixed drug solutions for spiking and calibration were prepared in MeCN and water for β-lactams (glass vials were used for storage except for the β-lactams in water, which were stored at 4°C in plastic vessels due to concerns with their retention or degradation on glass surfaces). Sorbents were obtained from multiple sources: bulk C18 (40 μm particles) was from J.T. Baker (Phillipsburg, NJ, USA), Supelco (Bellefonte, PA, USA), and Applied Separations (Allentown, PA, USA). Graphitized carbon black was from Supelco, ENV+ designed to sink in MeCN was from Biotage (Hengoed, UK), and aminopropyl was from Varian (Harbor City, CA, USA). Reagents for UHPLC mobile phase were Optima quality from Fisher (Suwanee, GA, USA), and 98% purity formic acid from Sigma.

Instrumentation

A Waters (Milford, MA,USA) Acquity TQD UHPLC-MS/MS was used for analysis, and MassLynx software was used for instrument control and data integrations. Intellistart software was used to optimize MS/MS parameters for choice of ions, cone voltages, and collision energies for all targeted compounds as they were being infused into the source. The dwell times were 5 ms in all cases in order to maximize the number of data collection points per peak. Fixed MS parameters were 3 kV capillary voltage, 2 V extractor voltage, and 150°C and 450°C source and desolvation temperatures, respectively. The spray cones were exchanged and cleaned after each sequence.

The autosampler kept the vials at 4°C during sequences to reduce chances of degradation. The column was a Waters Acquity HSS T3, 10 cm long, 2.1 mm i.d., 1.8 μm particle size. The mobile phase consisted of 0.1% formic acid each in (A) 95/5 (v/v) water/MeCN and (B) MeCN. The gradient was 100% A for 0.1 min, then ramped to 100% B until 8 min, where it was held for 1.5 min. The column was re-requilibrated to 100% A for 3.3 min until the next injection. The column temperature was 40°C, flow rate was 0.5 ml/min, and injection volume was 20 μl. Table 1 lists the retention times (t_R) and MS/MS ion transitions for analysis.

Shaking of extracts was done by multiplex vortexers or a Glas-Col (Terre Haute, IN, USA) platform pulse mixer. Solvent evaporation was done on a Zymark (Hopkinton, MA, USA) TurboVap LV system, and centrifugation was conducted with a Thermo Fisher (Waltham, MA, USA) Sorvall Legend RT.

Sample processing

The incurred samples from FSIS were previously homogenized before receipt. In the case of the organic kidneys, 100 g portions from each of 20 kidneys were diced while frozen by hand with a knife and individually homogenized into slurry using a kitchen food processor with plastic bowls. Equal portions of the homogenized kidneys were mixed together for method development experiments, matrix-matched calibration, and 40% of the replicates in the validation study. In validation experiments, a quality control standard (400 ng/g of the pesticide, imidacloprid) was added to each kidney to check the homogenization process and assess the appropriateness of the sample size used for analysis.

Sample preparation

Several different MMMs were compared in experiments prior to final method development and validation. Sample size was 2.0 g weighed into polypropylene (PP) centrifuge tubes, and spiking was done for 6 replicates each at ½X, 1X, and 2X the spiking levels listed in Table 1. In each method, the last step was to filter a 0.5-ml portion of the final extract using a Whatman (Florham Park, NJ, USA) Mini-Uniprep PVDF 0.2 μm filter vial followed by analysis using UHPLC-MS/MS.

MMM 1 was from Mol *et al.*[20] with minor modifications. The samples in 50-ml PP tubes were extracted with 15 ml of MeCN/water (75/25 *v/v*) with 1% formic acid by vortex shaking for 30 min. The extracts were then centrifuged at 25°C for 5 min at 3716 rcf (the maximum using the swinging bucket rotor on our centrifuge). A 7.5-ml portion of the extract was transferred to a 15-ml graduated PP tube containing 7.5 ml hexane pre-saturated with MeCN, which was shook for 30 s and centrifuged as before. The hexane was aspirated to waste and the extract was taken to 2.0 ml final volume on the TurboVap at 40°C to yield 0.5 g/ml sample equivalent.

MMM 2 was from Martos *et al.*[21] with minor modifications. The samples in 15-ml PP tubes were vortexed for 30 s with 4 ml of MeCN/water (86/14 *v/v*) and then placed in a water bath at 60°C for 60 min. The extracts were then put on ice for 10 min, and 100 μL of 88% formic acid was added to each tube, which was vortexed for 30 s followed by centrifugation at 5°C for 10 min at 3716 rcf. The extract was transferred to a 15-ml graduated PP centrifuge tube containing 4 ml hexane pre-saturated with MeCN, which was shook for 30 s and centrifuged as before. The hexane was aspirated to waste and 2 ml of extract was taken to <0.5 ml on the TurboVap at 40°C, and water was added to reach the 1-ml mark (1 g/ml sample equivalent).

MMM 3 was modified from Mastovska and Lightfield,[22] which became the final validated method. The 2 g samples in 50-ml PP tubes were vortexed for 5 min with 10 ml of MeCN/water (4/1 *v/v*) and centrifuged at 25°C for 5 min at 3716 rcf. The extract was transferred to a 50-ml graduated PP centrifuge tube containing 500 mg C-18, and 10 ml hexane pre-saturated with MeCN was added; the tube was shook for 30 s and centrifuged as before. The hexane was aspirated to waste and 5 ml of extract was taken to <0.7 ml on the TurboVap at 40°C (45°C in the final method), and water was added to reach the 1-ml mark (1 g/ml sample equivalent).

MMM 4 was from Stubbings *et al.*[23] with slight modifications. The 2 g samples in 50-ml PP tubes were homogenized with 6 ml of MeCN containing 1% acetic acid. Then, 2 g anhydrous sodium sulfate was added, followed by vortexing for 30 s. To help remove water, the samples were allowed to stand for 15 min, and then they were centrifuged at 15°C for 10 min at 3716 rcf. The extract was transferred to a 50-ml graduated PP centrifuge tube containing 200 mg aminopropyl sorbent, which was vortexed intermittently over a 15 min timeframe and centrifuged at 20°C for 5 min at 3716 rcf. In this case, 3 ml of extract was taken to <0.5 ml on the TurboVap at 40°C, and water was added to reach the 1-ml mark (1 g/ml sample equivalent).

Table 1. List of priority veterinary drugs targeted in the study, their US tolerances in bovine kidney and lowest spiking levels, retention times (t_R), and MS/MS quantifier and qualifier ions

#	Analyte	Drug Class	US Tolerance (µg/g)	½X Spike Level (µg/g)	t_R (min)	Data Collection (min)	Precursor Ion (m/z)	Product Ions (m/z)		
								Quant. Ion 1	Qual. Ion 2	Qual. Ion 3
1	Desacetyl Cephapirin	β-Lactam	0.1[a]	0.05	0.65	0.40-1.10	382.1	152.0	124.2	111.3
2	Florfenicol Amine	Phenicol	0.3[b]	0.15	0.65	0.40-1.10	248.1	230.2	130.1	131.1
3	Sulfanilamide	Sulfonamide		0.05	1.15	0.80-1.40	172.9	92.9	75.9	65.9
4	Amoxicillin	β-Lactam	0.01	0.005	1.46	1.27-1.67	366.1	114.0	349.3	208.1
5	Salbutamol	β-Agonist		0.003	1.46	1.27-1.67	240.2	148.2	222.3	166.2
6	Zilpaterol	β-Agonist	0.012[c]	0.003	1.46	1.27-1.67	262.3	244.3	185.2	202.2
7	Cimaterol	β-Agonist		0.003	1.50	1.32-1.72	220.0	143.0	115.9	88.9
8	DCCD*	β-Lactam	0.4	0.05	1.70	1.55-1.95	549.1	183.0	241.1	125.9
9	Lincomycin	Lincosamide	0.1[d]	0.05	1.86	1.69-2.09	407.3	126.1	359.2	389.2
10	Sulfadiazine	Sulfonamide		0.05	1.95	1.79-2.19	251.1	156.1	108.0	158.1
11	Ampicillin	β-Lactam	0.01	0.005	2.00	1.82-2.22	350.1	106.1	114.0	160.1
12	Desethylene Ciprofloxacin	Fluoroquinolone	0.1[c]	0.025	2.05	1.90-2.30	306.2	288.2	245.2	289.3
13	Sulfathiazole	Sulfonamide	0.1[e]	0.05	2.08	1.90-2.30	256.1	156.1	108.0	101.0
14	Sulfapyridine	Sulfonamide		0.05	2.15	1.97-2.37	250.1	92.0	156.1	108.1
15	Norfloxacin	Fluoroquinolone	[f]	0.025	2.15	2.03-2.43	320.2	276.2	233.1	219.0
16	Tulathromycin	Macrolide	5.5[c]	1.0	2.17	1.98-2.38	806.8	72.0	577.5	116.0
17	Oxytetracline	Tetracycline	2[g]	0.5	2.20	2.00-2.40	461.2	426.4	443.4	201.2
18	Ciprofloxacin	Fluoroquinolone	[f]	0.025	2.20	2.07-2.47	332.2	288.4	245.2	203.2
19	Ractopamine	β-Agonist	0.03[h]	0.003	2.27	2.08-2.48	302.2	164.0	107.0	121.0
20	Methomyl (QC)			0.4	2.27	2.09-2.49	163.0	105.9	121.9	87.8
21	Sulfamerazine	Sulfonamide		0.05	2.27	2.09-2.49	265.0	91.9	155.9	107.9
22	Carbadox	Other	0.03[i]	0.015	2.30	2.05-2.55	263.1	231.1	129.1	102.1
23	Danofloxacin	Fluoroquinolone	0.2[c]	0.025	2.31	2.12-2.52	358.1	96.0	314.2	283.1
24	Tetracyline	Tetracycline	2[g]	0.5	2.35	2.16-2.56	445.2	154.1	410.2	427.3
25	Enrofloxacin	Fluoroquinolone	0.1[j]	0.025	2.37	2.20-2.60	360.2	316.4	245.2	203.2
26	2-Quinoxalinecarboxylic Acid	Other		0.015	2.38	2.30-2.60	175.0	129.0	131.0	102.0
27	Sulfamethizole	Sulfonamide		0.05	2.48	2.36-2.76	271.1	156.1	92.0	108.0
28	Sulfamethazine	Sulfonamide	0.1[a]	0.05	2.51	2.33-2.73	279.1	186.1	156.1	108.1
29	Sulfamethazine-^{13}C6 (IS)			0.4	2.51	2.33-2.73	285.2	186.1	98.0	124.0
30	Cefazolin	Cephalosporin		0.05	2.52	2.36-2.76	455.1	323.2	156.0	112.1
31	Sulfamethoxypyridazine	Sulfonamide		0.05	2.56	2.38-2.78	281.1	156.1	126.2	108.1
32	Difloxacin	β-Lactam		0.025	2.59	2.38-2.78	400.2	356.4	299.2	285.3
33	Sarafloxacin	Fluoroquinolone	[f]	0.025	2.59	2.38-2.78	386.1	342.2	299.2	270.2
34	Clenbuterol	β-Agonist	[f]	0.003	2.61	2.36-2.76	277.2	259.2	132.1	203.1
35	Pirlimycin	Lincosamide	0.3[h]	0.25	2.69	2.54-2.94	411.3	112.2	363.3	110.3
36	Chlortetracycline	Tetracycline	2[g]	1.0	2.79	2.63-3.03	479.2	154.1	444.3	462.2
7	Clindamycin	Lincosamide		0.05	2.84	2.68-3.08	425.3	126.2	377.4	124.3
38	Gamithromycin	Macrolide		0.05	2.87	2.70-3.10	777.8	83.0	116.0	158.1
39	Sulfachloropyridazine	Sulfonamide	0.1[a]	0.05	2.88	2.73-3.13	285.0	156.1	108.0	92.0
40	Imidacloprid (QC)			0.4	2.91	2.77-3.17	256.0	174.9	209.0	83.9

41	Tilmicosin	Macrolide	0.1[h]	0.06	3.02	2.85-3.25	869.8	174.2	132.2	696.6
42	Sulfadoxine	Sulfonamide		0.05	3.03	2.88-3.28	311.2	156.1	108.1	140.1
43	Sulfamethoxazole	Sulfonamide		0.05	3.04	2.89-3.29	254.0	92.1	156.0	107.9
44	Sulfaethoxypyridazine	Sulfonamide	0.1[a]	0.05	3.07	2.91-3.31	295.1	156.1	140.2	108.1
45	Florfenicol	Phenicol	0.2[d]	0.1	3.08	2.93-3.33	358.1	241.0	206.0	130.4
46	Chloramphenicol	Phenicol	[f]	0.003	3.30	3.12-3.52	323.1	275.0	165.0	118.8
47	Erythromycin	Macrolide	0.1[a]	0.05	3.46	3.28-3.68	734.8	158.2	115.9	576.5
48	Sulfadimethoxine	Sulfonamide	0.1[a]	0.05	3.51	3.35-3.75	311.1	156.1	108.0	245.2
49	Sulfaquinoxaline	Sulfonamide	0.1[a]	0.05	3.53	3.35-3.75	301.1	156.1	108.0	92.0
50	Prednisone	Corticosteroid		0.05	3.62	3.35-3.85	359.2	341.1	146.9	267.3
51	Tylosin	Macrolide	0.2[k]	0.1	3.64	3.46-3.86	916.8	174.2	101.1	145.2
52	Penicillin G-d7 (IS)			0.4	3.81	3.63-4.03	342.1	160.1	183.1	98.1
53	Penicillin G	β-Lactam	0.05[a]	0.025	3.81	3.63-4.03	335.1	176.0	160.1	114.0
54	Beta/Dexa-methasone	Corticosteroid		0.05	4.06	3.89-4.29	393.2	373.2	147.1	355.3
55	Sulfanitran	Sulfonamide		0.05	4.10	3.93-4.33	336.2	156.1	134.1	92.7
56	Sulfabromomethazine	Sulfonamide	0.1[a]	0.05	4.16	3.98-4.38	357.1	92.0	156.1	108.1
57	Zeranol (α-Zearalanol)	Other	[l]	0.003	4.31	4.13-4.53	323.2	305.2	189.1	149.0
58	Oxacillin	β-Lactam		0.05	4.35	4.17-4.57	402.1	160.0	243.1	144.1
59	Atrazine (QC)			0.4	4.43	4.26-4.66	216.1	174.0	103.9	96.0
60	Cloxacillin	β-Lactam	0.01[a]	0.005	4.61	4.43-4.83	436.2	160.1	277.1	114.1
61	Nafcillin	β-Lactam		0.05	4.73	4.55-4.95	415.2	199.2	171.1	115.1
62	Oxyphenylbutazone	NSAID		0.05	4.73	4.59-4.99	325.2	120.1	148.2	204.1
63	Flunixin	NSAID	0.025[h]	0.0125	4.82	4.63-5.03	297.0	279.0	109.0	364.0
64	Flunixin-d3 (IS)			0.4	4.82	4.63-5.03	300.0	282.1	112.0	264.0
65	Dicloxacillin	β-Lactam		0.05	4.97	4.80-5.20	470.2	160.1	311.1	114.1
66	Phenylbutazone	NSAID		0.05	5.87	5.69-6.09	309.1	120.0	76.9	91.8
67	Melengesterol Acetate	Other	0.025[m]	0.01	6.23	6.04-6.44	397.4	279.3	337.5	221.3

* Desfuroylceftiofur cysteine disulfide (DCCD) is a marker residue metabolite of ceftiofur

[a] - tolerance in uncooked edible tissue of cattle

[b] - Marker residue for florfenicol in cattle muscle

[c] - Tolerance in cattle liver, (for tulathromycin, a marker residue has been established)

[d] - Tolerance in swine muscle

[e] - Tolerance in uncooked edible tissue of swine

[f] - Banned for extralabel use

[g] - Tolerance is for the sum of residues of tetracycline including chlortetracycline, oxytetracycline, and tetracycline in muscle

[h] - Tolerance in cattle muscle

[i] - Tolerance in swine liver

[j] - Tolerance is for desethylene ciprofloxacin (marker residue) in cattle liver

[k] - Tolerance in uncooked cattle fat, muscle, liver, and kidney

[l] - A tolerance is not needed

[m] - Tolerance in cattle fat.

MMM 5 was from Chiaochan *et al.*[24] with slight modifications. The 2 g samples in 15-ml PP tubes were vortexed for 10 min with 4 ml of 1/1 (*v/v*) MeCN/water containing 2% trichloroacetic acid. The tubes were centrifuged at 25°C for 5 min at 3716 rcf, and 2 ml was transferred to a 15-ml graduated PP centrifuge tube containing 2 ml hexane pre-saturated with MeCN; the tube was shook for 30 s and centrifuged as before. The hexane was aspirated to waste and 2 ml of extract was taken to <0.5 ml on the TurboVap at 40°C, and water was added to reach the 1-ml mark (1 g/ml sample equivalent). An observable difference in this method from the others was that a large amount of precipitation occurred.

Analysis of incurred samples

To help finalize the method prior to validation, 10 samples containing drug residues from the field (incurred samples) were extracted with 4/1 MeCN/water (10 ml for 2 g sample) to evaluate different factors: use of 1% formic acid or not, use of 60°C heating for 60 min, or shaking for 5 or 30 min. These factors involved the main differences during extraction in the five given methods tested. The clean-up step with 500 mg C18 and 10 ml hexane pre-saturated with MeCN was the same as in the third method above, and final extracts were 1 g/ml sample equivalents.

The results of the previous analyses of the kidney samples by FSIS were unknown to the chemists when conducting the experiment. Analysis of known drug analytes fortified in samples at known levels in the laboratory is not as realistic of an assessment as analyzing unknown residues in real samples. In addition to investigate extraction conditions, the experiment was designed to test the final method in the real-world application.

Method validation

The final method given above for MMM 3 was validated according to FSIS Laboratory Quality Assurance Division requirements in a streamlined, multi-faceted approach. The requirements dictate that several variables in the method be evaluated to assess its performance and ruggedness, and typically one factor is varied per experiment. We had done many method development experiments prior to validation, including analyses of incurred samples, and we were confident enough in the method validation to vary multiple factors each day. In all, 20 different bovine kidney samples (plus a mixture of them all) were analyzed over the course of 5 days by 5 different chemists using different sets of pipettes, in different laboratory temperature and humidity environments, using 3 different sources of C18, and comparing glass and PP centrifuge tubes during the evaporation step. On each day, 10 different blank kidneys were analyzed along with 10 replicate spikes at 3 levels each (½X, 1X, and 2X with ½X levels listed in Table 1). On one of the days, the β-lactams/cephalosporins were accidentally spiked at 4-fold higher concentrations.

Quality control spikes at 400 ng/g were added during the method to determine the limiting steps that required greater care on the part of the analyst. Atrazine (a triazine herbicide) was added just prior to the PVDF filtration and UHPLC-MS/MS analysis to isolate the final step in the method from the sample preparation. Methomyl (a carbamate insecticide) was added to the extracts during the clean-up step with C18 and hexane, and 3 isotopically labelled internal standards (sulfamethazine-^{13}C6, flunixin-d3, and penicillin G-d7) were added along with the drug analytes prior to extraction. As already noted, imidacloprid was added during the sample processing step of the kidneys.

Matrix effect assessment

Calibration was done in both matrix-matched and solvent-only standards at 0, ¼X, ½X, 1X, 2X, and 3X equivalent sample concentrations with duplicate injections of each standard dispersed throughout the UHPLC-MS/MS sequence. Carry-over was assessed by injecting the reagent blank after a 3X standard (no carry-over was observed in any of the experiments). The matrix effect (ME) for each analyte was calculated as the difference between the linear best-fit slopes of the solvent-only and matrix-matched calibration curves divided by the slope of the reagent-only calibration curve. $\mathrm{ME} \leq |20|\%$ indicated that quantification should be reasonably correct when using the solvent-only calibration standards for that analyte.

Results and discussion

Choice of drug analytes and target levels in bovine kidney

The first issue in the study concerned what drugs should be included in the method and at what desired detection levels in bovine kidney. Originally, we had in mind to target as many veterinary drugs as possible at levels as low as possible. We obtained standards for 219 drugs in all and optimized the MS/MS conditions for the analysis of each. We also used a generic UHPLC column and mobile phase gradient and determined the retention times (t_R) for each drug in a <10 min chromatographic separation. However, the sheer number of analytes and details involved to follow the data trends for each one became overwhelming and caused excessive delays.

We decided to reduce the number of analytes to a more manageable level, and add other drugs to the list in the future. We chose to include the highest priority drugs first, which consisted of 44 targeted antibiotics (plus aminoglycosides, which are analyzed separately[11]) screened by the 7-plate bioassay[9] and sulfonamide TLC test.[10] As listed in Table 1, these consist of 16 sulfonamides, 10 β-lactams/cephalosporins, 8 macrolides/lincosamides, 7 fluoroquinolones, and 3 tetracyclines. We also included other analytes currently covered separately in methods used in FSIS, including 6 NSAIDs/corticosteroids, 5 β-agonists, 3 phenicols, and 4 others. The NSAID, flunixin, is a priority drug already routinely monitored by the FSIS, and we also included the corticosteroids, betamethasone/dexamethasone (only one was added, but both co-eluted with same MS/MS ion transitions) and prednisone because they had also been found previously in a study designed to screen for 121 veterinary drugs in 235 culled dairy cows collected from a slaughter establishment in the USA.[26]

In terms of concentrations, regulatory needs dictate that the detection threshold ('yes/no' screening level) should be at ½X tolerances. Those drugs with tolerances in bovine kidney are noted in Table 1. If the drug did not have a US tolerance, we checked for the tolerances of the drug in other food animals/tissues and considered the maximum residue levels (MRLs) in other countries and Codex. For tetracyclines, the 12 μg/g tolerance represents the sum of the drugs in that class, and we lowered the individual tetracyclines spiking levels to reflect that (and because preparing solutions in the mix at higher concentrations posed problems). In the case of DCCD, we had a limited supply of reference standard material, and we lowered the spiking level to get through the experiments. We attempted to match the detection limit of the current FSIS method for β-agonists of 3 ng/g, and otherwise, we set a default ½X target level at our estimated limit of quantification.

The final chosen concentrations are listed as the lowest spiking levels (½X tolerances/targets) in Table 1.

Filtration

Experiments were conducted to compare different clean-up options and use of different types of Mini-Uniprep filter vials. For greater long-term column performance and lower back-pressure, UHPLC benefits from a high degree of mobile phase and sample extract filtration even moreso than traditional HPLC. The Mini-Uniprep filter vials provide a convenient filtration of the extracts when capping the autosampler vials. However, some of the drug analytes may be 'filtered' along with some of the fine particulates during this step. To assess this effect, we compared Nylon (polyamide), polytetrafluoroethylene (PTFE), polyvinylidene fluoride (PVDF), and polyethersulfone (PES) filter types for our 60 drugs spiked in kidney extracts at 100 ng/g. The peak areas were integrated and normalized to the greatest peak area for each analyte among the 4 filter types. The normalized signals were categorized into 5 'recovery' regions as listed in Figure 1 with 100% being the largest signal among the 4 filter materials. As Figure 1 shows, PVDF and PTFE performed similarly and better than the other two filter types, and 0.2 μm PVDF was chosen as the overall best in all experiments to follow.

Clean-up

Sample clean-up is often an unavoidable step in any analytical method to reduce chemical interferences (direct and indirect) due to co-extracted matrix components. Assuming desired detection limits are still met, greater (UHP)LC-MS/MS instrument sensitivity may allow dilution of extracts without (or with) clean-up to reduce matrix suppression effects (indirect chemical interferences), but we could not afford that option. Proper clean-up tends to improve performance and reduce instrument maintenance needs, but at the cost of more labour, time, and expense in the analysis. In MMMs, clean-up is more challenging due to greater diversity of analyte properties, which tends to cause analyte losses when more extensive clean-up is performed. The original method of Mastovska and Lightfield used only dispersive solid-phase extraction (d-SPE) with 500 mg C18 per g equivalent of sample.[22] We experimented with different sorbents and options in an attempt to improve removal of matrix co-extractives without reduced drug recoveries in a fast, easy, and inexpensive approach. We evaluated graphitized carbon black and the polymeric sorbent, ENV+, as clean-up sorbents in replacement of C18, and even though they gave greater clean-up, they also removed many of the analytes in the method.

The easiest option was to add a liquid-liquid partitioning clean-up step at the same time as the d-SPE step using 1 ml hexane (saturated with MeCN) per ml kidney extract. The drug analytes are all too polar to partition into the hexane phase, but more of the remaining lipids in the extracts are removed. Figure 2 compares the clean-up effects by weight of co-extractives before and after the d-SPE and/or hexane partitioning clean-up procedures. The simultaneous combination of the two gave slighter greater removal of co-extractives, and we eventually incorporated it into the final method. No significant differences were observed in comparing total ion chromatograms or matrix effects differences between the two clean-up approaches, but less material seemed to appear on the spray shield after analytical sequences using the d-SPE+hexane clean-up. Prior to filtration, the final method led to ≈0.62 mg of bovine kidney co-extractives in the 20 μl injection of 1 g/ml equivalent extracts.

Comparison of different methods

Out of the six MMMs we considered[20–25] for implementation by FSIS, we evaluated 5 of them.[20–24] The MMM of Kaufmann *et al.*[25] was also performed, but it was deemed to be too laborious and time-consuming for our purposes. Each method had advantages in some respect over the others. For example, Mol *et al.* devised their approach to cover mycotoxins, pesticides, environmental contaminants, and other chemical residues in addition to veterinary drugs.[20] The methods of Martos *et al.*[20] (60°C extraction) and Chiaochan *et al.*[24] (use of 2% trichloroacetic acid) were also reported to be applicable to aminoglycosides, but we did not evaluate that aspect as explained in Introduction. The method of Stubbings *et al.*[23] potentially gave the most clean-up due to the use of aminopropyl sorbent in d-SPE, and Mastovska and Lightfield[22] devised the fastest and easiest approach among the MMMs considered.

The comparison experiment involved spiking 6 bovine kidney replicates each at ½X, 1X, and 2X tolerance/target levels. A complete evaluation of the 62 drug analyte recoveries, repeatabilities, qualitative factors, detectabilities, linearity, matrix effects, and practical matters among the different MMMs was made using

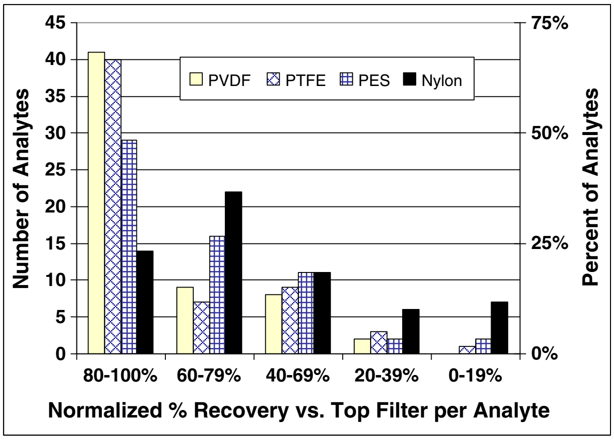

Figure 1. Relative performances of different types of Mini-UniPrep filter vials in bovine kidney extracts spiked at 100 ng/g for the drug analytes.

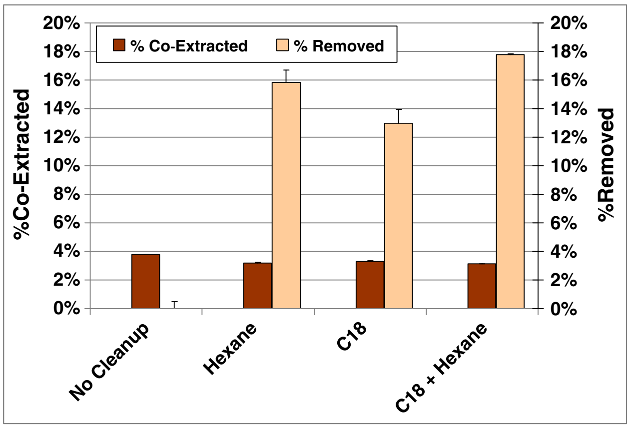

Figure 2. Extraction and clean-up of bovine kidneys by weight of co-extractives using clean-up variations in the final method. '%Co-extracted' is kidney weight equivalent remaining in the extract, and '%Removed' is the difference in weights before and after the given clean-up technique.

the same UHPLC-MS/MS method. The results were compiled, and Tables 2a and 2b and Figure 3 show a comparison of the different MMMs.

Qualitatively, the fewest false negatives occurred for the method of Martos *et al*., with only 4 of the 62 analytes giving >10% false negatives (identification criteria not met) for the 18 spikes and 4 matrix-matched calibration standards. For the other MMMs 7–8 analytes were not sufficiently able to be identified in the kidney matrix. These drugs were pretty much the same 'bad actors' among the methods as shown in Table 2. The analytes with t_R at the front of the chromatogram (e.g. florfenicol amine, sulfanilamide, and desacetyl cephapirin) were very difficult to measure due to their high polarity leading to broader peak shapes and co-elution with the most polar matrix co-extractives.

Quantitatively, the Martos *et al*. MMM gave the fewest number of drugs (20) that did not achieve 70–120% recovery and ≤20% RSD among the 18 replicates at 3 spiking levels. Table 2b lists all of the drugs for which these criteria were not met depending upon the MMM tested, and the 'All' row indicates the 7 analytes that did not give acceptably quantitative results with any of the MMMs at the spiking levels in bovine kidneys. As in Table 2a, many of the same drugs appear in Table 2b for more than one of the different MMMs, and these are generally considered problematic drugs for analysis. There are reasons for the worse results for each of the listed drugs, which include: lower than ideal spiking levels (*e.g*. amoxicillin); LC-MS matrix effects and calibration problems (e.g. DCCD); poor chromatography (e.g. desacetyl cephapirin); analyte degradation (e.g. penicillin G); MS/MS interferants (e.g. cimaterol), and/or particular chemistry of the problematic analytes (e.g. tulathromycin, tetracyclines, phenylbutazone).

Approximately 60–70% of the 62 drugs could meet quantification criteria by any of the different methods, and several borderline drug analytes were affected more so by day-to-day differences in instrument performance aspects than method-to-method differences. Figure 3 gives an overview of the different methods by drug class, without normalization to an internal standard in this case. A different picture emerges when assessing the different methods according to drug class, and ultimately, we believe that all of the MMMs essentially perform much the same with only small differences qualitatively and quantitatively for some drug analytes over others. Practical considerations of sample throughput, ease of use, and costs become more prominent factors, and the MMM of Mastovska and Lightfield is the most advantageous in those regards.

Analysis of incurred samples

Analysis of real samples (field-incurred) is a more important test of method performance than spiked samples. Fortified analytes do not interact via animal metabolism with the matrix, and the true extractabilities of the analytes from the matrix are not determined. We were concerned that the 5 min shake at room temperature without the presence of acid would not be sufficient to fully extract incurred drugs in bovine kidney samples. Table 3 shows that our concerns were misplaced, at least for the majority of

Table 2a. Summary of qualitative results in the comparison of 5 MMMs for the 62 veterinary drugs in bovine kidney: drugs that gave >2 non-identifications (false negatives) among 22 sample extracts

Mol *et al.*[20]	Martos *et al.*[21]	Mastovska & Lightfield[22]	Stubbings *et al.*[23]	Chiaochan *et al.*[24]
Erythromycin	Erythromycin	Amoxicillin	Amoxicillin	Amoxicillin
Florfenicol Amine	Florfenicol Amine	Florfenicol	Danofloxacin	Cimaterol
Penicillin G	Sulfamethazine	Florfenicol Amine	Florfenicol	Danofloxacin
Sulfamethoxazole	Sulfanilamide	Gamithromycin	Oxyphenylbutazone	Desacetyl Cephaprin
Sulfanilamide		Lincomycin	Phenylbutazone	Florfenicol Amine
Sulfanitran		Oxyphenylbutazone	Sulfanilamide	Salbutamol
Sulfapyridine		Sulfanilamide	Tulathromycin	Sulfanilamide
Tylosin		Tulathromycin		Zilpaterol

Table 2b. Summary of quantitative results in the comparison of 5 MMMs for the 62 veterinary drugs in bovine kidney: drugs that did not give 70-120% recovery and ≤20%RSD *vs* sulfamethazine-^{13}C6 in the comparison experiment

MMM	#	Drug Analytes
All	7	Cimaterol; DCCD; Desacetyl Cephapirin; Melengesterol Acetate; Nafcillin; Penicillin G; Phenylbutazone
Mol *et al.*[20]	23	Amoxicillin; Ampicillin; Carbadox; Cefazolin; Chlortetracycline; Cloxacillin; Dicloxacillin; Erythromycin; Florfenicol Amine; Oxacillin; Ractopamine; Salbutamol; Sulfanilamide; Sulfapyridine; Tetracyline; Tylosin
Martos *et al.*[21]	20	Ampicillin; Carbadox; Chlortetracycline; Cloxacillin; Difloxacin; Enrofloxacin; Erythromycin; Florfenicol Amine; Oxacillin; Oxyphenylbutazone; Oxytetracycline; Sulfanilamide; Tetracyline
Mastovska & Lightfield[22]	25	Amoxicillin; Ampicillin; Cefazolin; Chlortetracycline; Ciprofloxacin; Clindamycin; Cloxacillin; Dicloxacillin; Gamithromycin; Oxacillin; Oxyphenylbutazone; Oxytetracycline; Pirlimycin; Sulfanilamide; Tetracyline; Tilmicosin; Tulathromycin; Tylosin
Stubbings *et al.*[23]	21	Amoxicillin; Ampicillin; Beta/Dexamethasone; Carbadox; Cefazolin; Chloramphenicol; Chlortetracycline; Erythromycin; Oxyphenylbutazone; Oxytetracycline; Pirlimycin; Tetracyline; Tylosin
Chiaochan *et al.*[24]	26	Amoxicillin; Cefazolin; Clindamycin; Cloxacillin; Dicloxacillin; Erythromycin; Florfenicol Amine; Flunixin; Gamithromycin; Oxacillin; Oxyphenylbutazone; Salbutamol; Sulfabromomethazine; Sulfanilamide; Tilmicosin; Tulathromycin; Tylosin; Zeranol; Zilpaterol

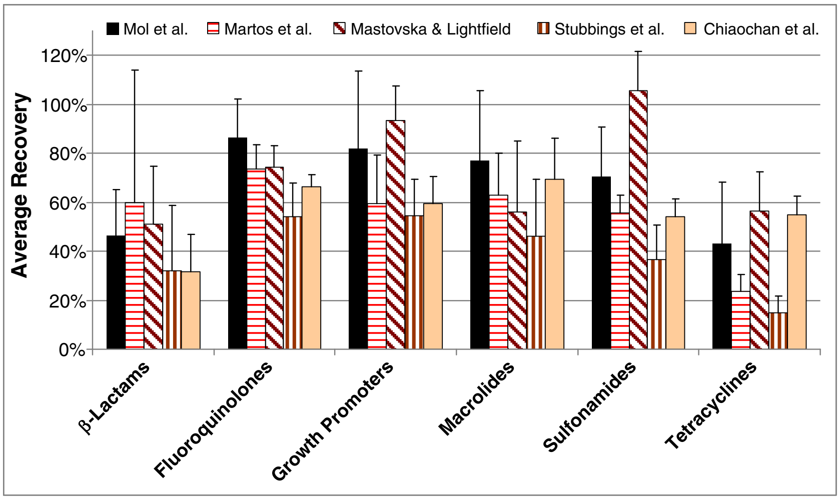

Figure 3. Comparison of average recoveries of the analytes from different drug classes using different MMMs[20–24] (no internal standard used). The error bars represent the standard deviations of the average recoveries of the drugs within the given class.

drug analytes determined in the 10 real samples provided. As shown in the table, the use of a 5 min shake gave the highest concentrations 17 out of the 26 instances when a drug was determined in the samples. With respect to inclusion of 1% formic acid or not in the 4/1 MeCN/water extraction solvent, the highest drug concentrations were found to occur in only 5 out of the 26 instances when acid was present.

The sulfonamides in the samples (sulfamethazine and sulfadimethoxine) gave rather similar results independent of extraction time and temperature, and the absence of acid during extraction led to nearly double the determined concentrations for sulfadimethoxine than when acid was present. Significantly lower concentrations also occurred for flunixin and beta/dexa-methasone when acid was added. In the case of tetracyclines, no clear trend

Table 3. Average drug concentrations (ng/g), n = 2, w/o normalization to an internal standard, of 10 incurred bovine kidney samples extracted by different conditions with and without 1% formic acid in the development of the final method

#	Detected Drugs	60 min at 60°C		30 min shake		5 min shake	
		w/ acid	w/o acid	w/ acid	w/o acid	w/ acid	w/o acid
1	DCCD		108 ± 3		73 ± 1		194 ± 0
	Pirlimycin	123 ± 15	156 ± 12	153 ± 4	162 ± 5	126 ± 5	189 ± 4
	Penicillin G		13 ± 0				10 ± 2
	Flunixin	99 ± 9	127 ± 2	101 ± 5	126 ± 6	102 ± 6	125 ± 4
2	DCCD		136 ± 11		91 ± 3		250 ± 5
	Penicillin G		10 ± 0				17 ± 3
3	Sulfadimethoxine	104 ± 10	184 ± 5	70 ± 3	172 ± 47	79 ± 8	181 ± 6
	Penicillin G		18 ± 0		12 ± 1		36 ± 2
4	Tetracyclines (sum)	944 ± 241	539 ± 11	1,190 ± 287	1,610 ± 792	1,292 ± 20	2,400 ± 44
	Tulathromycin	969 ± 179	650 ± 36	1,380 ± 90	1,140 ± 0	1,142 ± 68	1,050 ± 58
	Sulfamethazine	99 ± 24	128 ± 6	130 ± 6	112 ± 1	102 ± 1	153 ± 2
5	Sulfamethazine	117 ± 17	132 ± 2	132 ± 1	145 ± 11	117 ± 5	141 ± 1
	Penicillin G						10 ± 0
6	DCCD		35 ± 0	13 ± 13	14 ± 0		40 ± 2
	Flunixin	303 ± 52	456 ± 15	341 ± 22	392 ± 6	276 ± 10	456 ± 3
7	Tetracyclines (sum)		19 ± 1	32 ±1			
	Tilmicosin	1,850 ± 60	814 ± 3	1,760 ± 226	556 ± 37	1,900 ± 66	609 ± 12
8	Tilmicosin	1,210 ± 47	358 ± 7	998 ± 17	265 ± 11	1,200 ± 158	259 ± 1
9	Tetracyclines (sum)	2,350 ± 212	1,020 ± 92	3,320 ± 754	3,310 ± 1,760	2,830 ± 33	3,660 ± 99
	Tilmicosin	9,240 ± 492	8,250 ± 75	45,200 ± 4,700	12,100 ± 567	12,600 ± 334	4,550 ± 403
	Beta/Dexa-methasone		10 ± 0	11 ± 1			
	Flunixin	373 ± 42	504 ± 1	434 ± 9	488 ± 12	355 ± 21	546 ± 3
10	Tetracyclines (sum)		30 ± 2				
	Penicillin G		38 ± 2		24 ± 2		51 ± 3
	Beta/Dexa-methasone	281 ± 12	376 ± 8	268 ± 1	333 ± 40	253 ± 1	396 ± 5
	Flunixin	241 ± 9	325 ± 14	248 ± 5	345 ± 2	268 ± 27	351 ± 26

was observed, and results were rather variable as would be expected considering their lower recoveries in all of the MMMs tested (Figure 3). However, in the case of macrolides, extracted amount of tulathromycin and tilmicosin increased significantly in the presence of 1% formic acid in all cases, but this was not the case of pirlimycin, a lincosamide, for which the amount extracted was higher and more constant without acid present. Most prominently and importantly, the β-lactams, penicillin G and DCCD, disappeared (degraded) when acid was present during extraction.

Penicillin and ceftiofur (the parent drug of the DCCD metabolite) are two of the most widely used antibiotics in veterinary medicine, and the analytical screening method must be able to detect them. Therefore, the final analytical method must not use strong acidic conditions during extraction, and even though results were reduced without acid for the incurred macrolides determined, they were still readily detected at ½X tolerance levels in the samples. Quantification of those drugs with tolerances would still need to be done using the official regulatory method, thus quantitative accuracy is not as important as qualitative performance in this application.

Qualitative analysis

In this article, we use the terminology of screening, identification, confirmation, and determination described in Lehotay *et al*.[27] In brief, 'screening' involves simple yes/no decisions from moderately selective tests, such as the bioassay or the UHPLC-MS screen using t_R and signal from one ion transition (as described in the next section); 'identification' entails yes/no decisions from highly selective methods, such as the UHPLC-MS/MS approach using 3 ion transitions (as described in the section to follow); 'confirmation' involves at least two analyses in agreement, one or both of which meet identification criteria and preferably entail different aspects of chemistry for analysis (orthogonal selectivity); and 'determination' is quantification using an appropriately validated method (the official regulatory method in this case).

In our application, UHPLC-MS/MS will serve as the screening/identification method, and re-analysis of presumptive positives by the official regulatory method will serve as the determinative/confirmatory method. Regulatory action will only be taken if the tolerance is exceeded in the determinative method and the FSIS identification criteria are met in at least one of the analyses. For those drugs without a tolerance, quantification is not required, and identification and confirmation must be achieved to take regulatory action. Presumptive positives from the UHPLC/MS-MS qualitative analysis would be confirmed by another method validated by FSIS.[12–17]

In the screening approach, no false positives or false negatives above the ½X tolerance levels occur ideally, but the reality is that analytical certainty cannot be 100% simultaneously for yes and no outcomes in qualitative analyses at the threshold level[28] (½X tolerance, or limit of detection for those drugs without tolerances). In our application, we strived for 0% false negatives and a false positive rate of <5% was deemed acceptable for our screening and identification purposes. However, determinative/confirmatory methods can tolerate a low degree of false negatives, but should not yield any false positives. This is the context of our decisions when setting the UHPLC-MS/MS qualitative analysis criteria discussed in the next two sections.

Screening analysis

Our main intent in this project was to replace the 7-plate bioassay screen in the FSIS laboratory. According to FSIS screening criteria, an LC-MS screening test is a presumptive positive if one of the ion transitions gives a chromatographic peak with t_R within 5% of the reference standard's t_R, the signal/noise (S/N) must be >3 for drugs that have no tolerance and if the blank shows a chromatographic peak it must be lower than 10% of the ½X tolerance level of the reference standard. In the case of drugs with a tolerance value (Table 1), the detected peak in the sample must equal or exceed the signal threshold setting of a contemporaneously analyzed fortified sample at ½X tolerance level and the fortified sample must exceed 10% of the ½X tolerance level of the reference standard to be considered a presumptive positive for further analysis.

The first consideration is t_R. In the UHPLC method, t_R was extremely consistent for nearly all analytes with average standard deviation (SD) of merely ±0.8 s ($n = 250$ over the course of 5 days per analyte), excluding desacetyl cephapirin, florfenicol amine, tulathromycin, and clenbuterol. In those cases, there was a consistent discrepancy between the t_R of the calibration standard in reagent-only solution vs. matrix-matched standards and sample spikes. The discrepancy indicated matrix interferences (direct or indirect) for those analytes, whereas the exceptional consistency in t_R for the other 64 compounds (including IS and QC standards) shows that t_R becomes an even more important qualitative tool in UHPLC than in typical HPLC applications.

The arbitrary criterion of ±5% of t_R gives a very tight range of ±2 s for the first-to-elute analytes and very loose ±19 s for the last-to-elute drug. In reality, the variation in t_R is consistent along the entire chromatogram, and it is not proper to use RSD rather than SD. Thus, our more scientific approach empirically measures the average and SD of the t_R and sets the acceptable range at ±4 SD from the average t_R. This led to ±3.2 s on average and no more than ±4.8 s for any analyte other than the 4 mentioned previously. An alternate simpler approach for the method in practice is to set the criterion to be ±0.1 min (±6 s) for all drugs, which corresponds to an exceptionally low random chance of a false negative, and also gives a lower chance of false positives for most of the analytes compared to the ±5% RSD criterion.

The 5-day validation study involved 50 blanks and 50 spikes each at 3 levels from 20 different kidneys altogether. In the study, the screening criteria were met for 53 of the 62 analytes at all spiking levels, which yielded ≤5% false positives and no false negatives. Additionally, the criteria were met for chloramphenicol at the 12 ng/g level ($n = 50$ blanks and 50 spikes). The 8 drugs that did not meet these screening criteria included the 4 listed above with t_R consistency problems (desacetyl cephapirin, florfenicol amine, clenbuterol, and tulathromycin) and 4 others: amoxicillin, cimaterol, phenylbutazone, and oxyphenylbutazone. Amoxicillin yields acceptable qualitative and quantitative results at spiking levels >20 ng/g, but its 10 ng/g tolerance is lower than the method's capabilities at this time. In the case of cimaterol, an interferant was present in the kidneys (see Figures 4 and 5). Some of the kidneys also contained an interferant for clenbuterol, which led to the aforementioned different t_R between reagent-only and matrix-matched standards, but this was not observed for cimaterol. The method was not suitable for the NSAIDs, phenylbutazone and oxyphenylbutazone, and we intend to follow up with further investigations in the future for all of the drugs that did not meet screening criteria by this method.

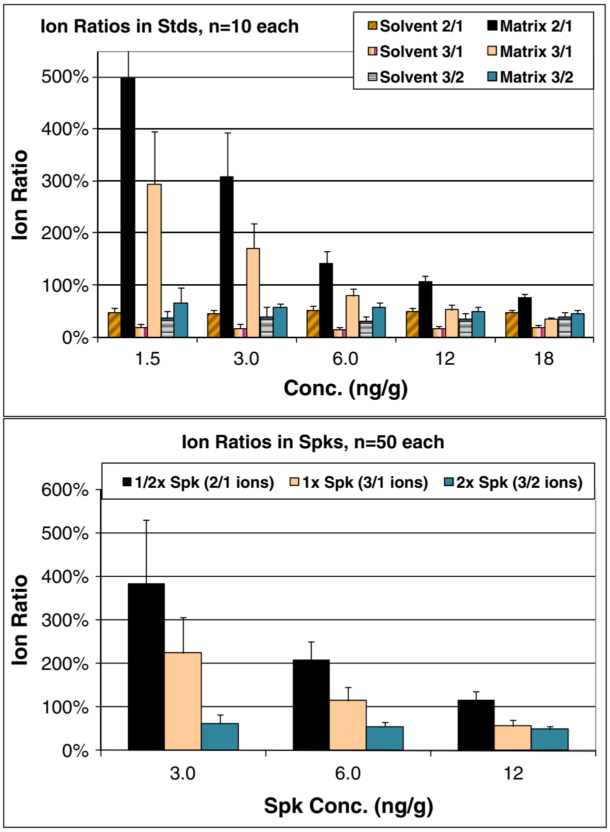

Figure 4. Plots of 2/1, 3/1 and 3/2 ion ratios for cimaterol solvent-only and matrix-matched calibration standards and spikes in the bovine kidney samples. A lower level matrix interferant occurred for ions 2 and 3 in a consistent ratio, but not for ion 1 (the quantification ion).

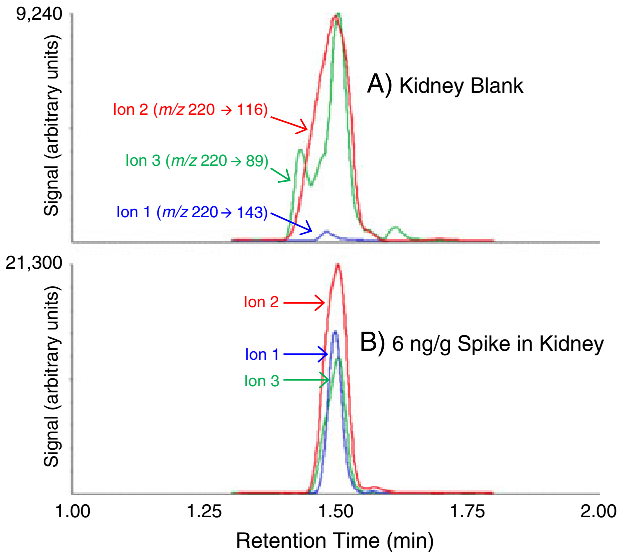

Figure 5. Chromatograms of the 3 ion transitions for cimaterol in (A) blank kidney extract and (B) kidney spiked with 6 ng/g cimaterol (1X spiking level), which shows the matrix interferences for ions 2 and 3, but not for the quantification ion (ion 1). In addition to the ion ratios shown in Figure 4, the peak shapes and intensity levels provide helpful qualitative information.

UHPLC-MS/MS identification

There are numerous MS identification criteria used for different purposes, or similar purposes with different subjective choices.[4,27–31] The FSIS criteria are based on those suggested by the FDA-CVM for guidance to industry when developing methods for veterinary drug registrations.[29] We chose to monitor 3 ion transitions for each compound due to the greater analytical selectivity of the method. For this reason, FSIS qualitative identification/confirmation criteria allow up to ±20% absolute differences from the reference standard ratio when two ion ratios are used and ±10% maximum differences if only one ion transition is used. When monitoring 2 ions, only one ion ratio is produced (ion 2 *vs* ion 1), whereas collecting 3 ions yields 3 ratios (2/1, 3/1, and 3/2). As in typical MS identification systems, the less intense ion should be divided by the more intense ion to keep the ion ratio $<100\%$. In keeping with our goal to negate false negatives provided that false positives are ≤5%, we took advantage of having 3 ion ratios with which to meet the criteria.

In the 5-day validation study, we analyzed 10 blank kidneys per day and 10 spikes each at 3 levels plus 6 calibration standards each (including zero concentrations) in reagent solution and blank matrix extracts (matrix-matching). The calibration standards were injected twice each interspersed among the spiked samples. For each sample in each sequence, we entered the t_R and integrated peak areas for each ion transition into an Excel spreadsheet and set up a macro that could be used to evaluate identification criteria and their subsequent rates of false positives and negatives.

As in screening, the first consideration was t_R, and of course, the same findings as described in the section on screening applies to UHPLC-MS/MS identification. It must be recognized that very few false positives occurred even when only t_R was taken into consideration. Few false positives or negatives occurred if a distinguishable chromatographic peak was formed for any of the 3 monitored ion data channels having t_R within ±5 s of the average t_R from the calibration standards. In this situation, the ion ratio identification criteria could be eliminated and still not increase the number of false positives among the 20 kidney samples repetitively tested. Therefore, we employed relatively liberal identification criteria to reduce false negatives and rely on confirmation to eliminate the chance of false positives.[27] The final identification criteria for our purposes were defined as follows:

1. t_R falls within ±0.1 min of the average t_R of the calibration standards analyzed in the same sequence.
2. t_R and peak shape of each monitored ion for the given analyte coincide.
3. The quantification ion signal (peak area) exceeds that of the ½X tolerance matrix-matched calibration standard(s) measured in the same analytical sequence, or has S/N >3 for those drugs that have no tolerance.
4. Two ion ratios are $\leq|20\%|$ or one ion ratio is $\leq|10\%|$ of the average ion ratios from the calibration standards analyzed in the same sequence.
5. All sample and reagent blanks do not contain a positive identification for the analyte (*e.g.* no lab contamination or carry-over).

A wealth of information was collected in the validation study and evaluated using Excel spreadsheets. Detailed analysis of the data revealed discrepancies, such as the t_R differences mentioned earlier, and the matrix interference in the case of cimaterol. Figure 4 shows how cimaterol's 2/1, 3/1, and 3/2 ion ratios in reagent-only standards are rather consistent from 3–18 ng/g, but in matrix, the 2/1 and 3/1 ion ratios separate greatly from those without matrix present. Ion chromatograms of each ion transition for cimaterol in blank and spiked kidneys are shown in Figure 5. This figure highlights how peak shapes (criterion #2 listed above) are very helpful in practice for identification purposes, and it is unfortunate that this aspect involving common sense is considered to be too nuanced in other identification schemes.[27] Figures 4 and 5 demonstrate that a consistent low-level interferant occurs for ions 2 and 3, but not ion 1 (the quantification ion). Indeed, response linearity and quantification results based on ion 1 for cimaterol were very good, even though the identification results based on ions 2 and 3 were poor. Interestingly, follow-up choices of different ion transitions for cimaterol did not improve the situation. In any case, the quality of the identification capability of the method for cimaterol is expected to be acceptable at concentrations >18 ng/g as surmised from Figure 4.

In current quality assurance programs, qualitative methods must be validated[4,27–31] just as in the case of quantitative methods, and it is most efficient to conduct both aspects of the validation process simultaneously. Table 4 gives the rates of false positives and false negatives for the drugs in the validation study using the 5-factor identification criteria list above. As already discussed, false positives were not found to be a concern, and even for cimaterol, only a single case occurred when the interfering peak in a blank kidney happened to yield ion ratios within the correct range. In the case of penicillin G, 30% of the samples met the t_R and ion ratio identification criteria, but none exceeded the ½X tolerance peak area threshold.

In practice, false negatives in the identification results are a secondary consideration given that the less stringent screening results would already trigger re-analysis at a higher rate of the true positive samples by the single-class confirmatory method and/or official regulatory method. In any case, meeting identification criteria provides even greater confidence in the finding for justifications of potential regulatory actions. With this in mind, the identification acceptability threshold was set at ≤10% false negatives, as indicated in bold text in Table 4. In that case, 43 out of the 62 drugs met the criterion at all spiking levels, and 18 gave no false negatives at all. Another 4 drugs met the criterion at the 1X spiking concentrations, and sarafloxacin and melengestrol acetate met the criterion at the 2X level (100 and 40 ng/g, respectively). As can be inferred from the table, a substantial number of true positives would still be identified at the lower levels, even for all but a few of the 12 drugs that did not meet the arbitrary ≤10% cut-off rate that we set.

As stated before, the method was not trustworthy for desacetyl cephapirin, florfenicol amine, phenylbutazone, and oxyphenylbutazone. The worse results for 7 other drugs was a matter of low levels, and improvements vs. concentration can be observed for salbutamol, amoxicillin, cimaterol, ractopamine, zeranol, chloramphenicol, and carbadox (note: 2-quinoxalinecarboxylic acid is a metabolite of carbadox). We also believe that the worse results for clenbuterol is related to concentration, but the interference and t_R discrepancy remain to be explained.

Most notable is tulathromycin, which is unique among the analytes. This is an issue related to its chemical nature, and it gave a higher variability in t_R and ion ratios than nearly all of the other drugs. No chemical interference was observed, and its better results under acidic conditions were the probable cause. The official regulatory method requires acid hydrolysis and determination of CP-60,300 (a marker residue of tulathromycin), and even so, our method was demonstrated in the 150 spiked samples to yield the correct identification 90% of the time overall.

Quantitative analysis

At this time, possible quantification is a bonus in this application, but FSIS may bridge the quantitative results of this method with the official regulatory method by conducting side-by-side comparisons of incurred samples. In this way, this method may become the official regulatory method if acceptably consistent results are achieved between the two methods. This would not preclude the need for confirmatory analysis for enforcement actions, but determination would not have to be performed in that case and a simple test such as ELISA could suffice.

The first aspect of the quantitative validation study is to check the performance of the IS and QC standards. We added atrazine as the QC standard to the final extracts prior to filtration and analysis; we added methomyl prior to the clean-up step; we spiked the IS compounds to the kidney samples along with the drug analytes prior to extraction; and we added imidacloprid to the kidneys during homogenization with the food processor (each compound was added at 400 ng/g). The contribution of each step to the overall uncertainty in the analysis can be deduced from the sum of squares of the RSD for the different QC standards.

Table 5 shows the recovery results from this analysis for the 200 spiked samples (including blanks) vs. the 50 matrix-matched calibration standards in the 5-day validation study involving 20 different kidneys. The table also gives the average RSD calculated from each day of $n = 40$ replicate spikes (average repeatability). A few main conclusions can be made from this assessment: (1) average %bias was -10 ± 10 in the preparation of the blank and spiked samples vs. the matrix-matched standards because atrazine had 90% 'recovery' even though there was no difference in how it was added to the final extracts; (2) the filtration/analytical step contributed 100% to the overall method uncertainty (U) of ≈10% RSD in the method because no real difference occurred in the repeatability of atrazine results from the other QC standards; and (3) homogeneity of the samples was excellent, but ≈11–15% of imidacloprid was lost during the sample processing, transfer, and −18°C storage steps. Essentially, the QC results demonstrate that method performance by the 5 analysts was sound, and the quantitative performance depended on the chemistry of the method, quality of the instrumentation, and variability of matrix effects.

As in the case of the qualitative validation, a large amount of data was compiled and processed using Excel spreadsheets. The details for the 68 compounds in the study are too numerous to show in multiple tables and figures, but the overall quantitative results for the spiked samples are shown in Table 5 and conclusions discussed in the paragraphs to follow. Recoveries <70%, average repeatabilities (rep) >20% and reproducibilities (Rep) >25% are given in bold text in the table. Since we knew that we added the analytes to the samples, qualitative results were not taken account, which is how florfenicol amine and cimaterol were able to be calculated in the assessment. As mentioned before, the quantification MS/MS transition for cimaterol gave acceptable results, but the chemical interference affected the two qualifier ions. Florfenicol amine in matrix could only be determined on 3 of the 5 days, and its t_R was different but consistent between reagent-only standards and kidney extracts.

In all, 44 drugs gave >70% recoveries and another 13 drugs averaged 50-69% (these are not corrected for the recoveries of

Table 4. Summary of the qualitative identification and quantitative validation results with the final method in bovine kidneys. Values in bold did not achieve false negatives ≤10%, average recovery (Rec.) >70%, average repeatability (rep) <20%, and reproducibility (Rep) <25%. No IS was used for quantification

Analyte	1X Spk (ng/g)	%False Positives (n = 50)	%False Negatives				Avg %Rec. (n = 150)	Avg %rep (n = 5)	%Rep (n = 150)
			½X Spks (n = 50)	1X Spks (n = 50)	2X Spks (n = 50)	Overall (n = 150)			
Desacetyl Cephapirin*	100	0	**100**	**100**	**100**	**100**	**0**	N/A	N/A
Florfenicol Amine	300	0	**100**	**100**	**100**	**100**	$86^{\ddagger}$	**21**‡	$25^{\ddagger}$
Sulfanilamide	100	0	**20**	2	0	7.3	84	18	23
Salbutamol	6	0	**42**	**28**	**30**	**33**	75	**26**	**29**
Zilpaterol	6	0	4	2	0	2.0	75	14	16
Amoxicillin*	10	0	**68**	**74**	**78**	**73**	74	**45**	**47**
Cimaterol	6	2	**92**	**54**	**18**	**55**	78	17	18
DCCD*	100	0	5	2.5	0	2.3	**54**	16	18
Lincomycin	100	0	2	0	0	0.7	76	10	12
Sulfadiazine	100	0	0	0	0	0.0	79	10	14
Ampicillin*	10	0	5	0	0	1.5	73	17	18
Carbadox	30	0	**90**	**80**	**66**	**79**	88	**28**	**31**
Desethylene Ciprofloxacin	50	0	2	2	4	2.7	**64**	14	16
Sulfathiazole	100	0	0	0	0	0.0	82	9	11
Norfloxacin	50	0	10	2	0	4.0	**68**	**21**	24
Tulathromycin	2000	0	**14**	8	8	10	**39**	**71**	**87**
Sulfapyridine	100	0	0	0	4	1.3	86	13	15
Oxytetracycline	1000	0	4	0	0	1.3	**55**	16	18
Ciprofloxacin	50	0	6	2	0	2.7	**67**	**24**	25
Ractopamine	6	0	**42**	**60**	**66**	**56**	81	**24**	22
Sulfamerazine	100	0	2	2	0	1.3	84	9	11
Danofloxacin	50	0	4	0	0	1.3	**61**	**24**	25
Enrofloxacin	50	0	6	0	0	2.0	**59**	**28**	**30**
2-Quinoxalinecarboxylic Acid	30	0	10	4	0	4.7	80	17	20
Tetracyline	1000	0	0	0	0	0	**59**	19	21
Sulfamethazine	100	0	2	0	0	0.7	97	**22**	**26**
Difloxacin*	50	0	6	2	0	2.7	**69**	14	16
Sarafloxacin	50	0	**30**	**18**	6	**18**	73	**24**	**28**
Cefazolin*	100	0	10	2	0	4.0	87	**24**	**28**
Sulfamethoxypyridazine	100	0	2	4	2	2.7	83	16	19
Clenbuterol	6	0	**100**	**100**	**100**	**100**	**0**	N/A	N/A
Sulfamethizole	100	0	2	2	0	1.3	92	**22**	**26**
Pirlimycin	500	0	0	0	0	0.0	71	10	10
Chlortetracycline	2000	0	0	0	0	0.0	**54**	18	21
Gamithromycin	100	0	4	2	2	2.7	**66**	15	22
Clindamycin	100	0	4	0	0	1.3	81	13	15
Sulfachloropyridazine	100	0	0	0	4	1.3	82	10	14
Sulfamethoxazole	100	0	0	0	0	0.0	88	9	15

(continues)

Table 4. *(Continued)*

Analyte	1X Spk (ng/g)	%False Positives (n = 50)	%False Negatives				Avg %Rec. (n = 150)	Avg %rep (n = 5)	%Rep (n = 150)
			½X Spks (n = 50)	1X Spks (n = 50)	2X Spks (n = 50)	Overall (n = 150)			
Sulfadoxine	100	0	0	0	0	0.0	83	9	10
Tilmicosin	120	0	2	0	0	0.7	74	**22**	**28**
Sulfaethoxypyridazine	100	0	0	0	0	0.0	84	10	16
Florfenicol	200	0	10	0	0	3.3	93	19	**32**
Chloramphenicol	6	0	**94**	**82**	**58**	**78**	84	**66**	**75**
Erythromycin	100	0	0	0	0	0.0	75	14	17
Sulfaquinoxaline	100	0	0	0	0	0.0	81	11	15
Sulfadimethoxine	100	0	0	0	0	0.0	82	9	11
Prednisone	100	0	4	2	0	2.0	82	13	17
Tylosin	200	0	0	0	0	0.0	**61**	12	21
Penicillin G*	50	0†	2.5	2.5	0	1.5	75	12	17
Beta/Dexa-methasone	100	0	0	0	0	0.0	80	10	12
Sulfanitran	100	0	**24**	2	0	8.7	72	17	22
Sulfabromomethazine	100	0	2	0	0	0.7	76	11	13
Zeranol	6	0	**96**	**80**	**38**	**71**	95	**36**	**60**
Oxacillin*	100	0	0	0	0	0.0	75	9	10
Cloxacillin*	10	0	**12.5**	2.5	0	4.6	74	**23**	**30**
Nafcillin*	100	0	0	0	0	0.0	80	8	10
Oxyphenylbutazone	100	0	**100**	**98**	**96**	**98**	**0**	N/A	N/A
Flunixin	25	0	0	0	0	0.0	80	10	12
Dicloxacillin*	100	0	0	0	0	0.0	76	13	14
Phenylbutazone	100	0	**56**	**70**	**74**	**67**	**0**	N/A	N/A
Melengesterol Acetate	20	0	**14**	**12**	0	8.7	**59**	**47**	**62**

* n = 40 at ½X and 1X and n = 130 overall; N/A = not applicable.

† 30% of blanks met t_R and ion ratio criteria, but all concentrations <½X tolerance.

‡ Florfenicol amine was detected on 3 of the 5 days, and at t_R in matrix different from the reagent-only calibration stds.

Table 5. Performance of the quality control (QC) standards in the 5-day validation study with the final method in spiked bovine kidney samples (n = 200). Total uncertainty (U) of the method is limited by the filtration and analysis step, which is isolated by the addition of the atrazine QC std added just prior to the filtration of the final extracts for injection in UHPLC-MS/MS

Method Step	QC Std	Avg. % Recovery	Avg. % RSD	% of U
UHPLC-MS/MS	Atrazine	90	11	100
Clean-up	Methomyl	92	9	0
Extraction	Flunixin-d3	94	10	0
Homogenization	Imidacloprid	79	10	0

the IS nor compensated for the −10% bias observed for atrazine). As discerned from Table 5, 30 drugs in all met the common quantitative method acceptability criteria of 70-120% recovery and ≤25% reproducibility (depending on concentration). Four drugs already listed could not be reliably detected (desacetyl cephapirin, clenbuterol, oxyphenylbutazone, and phenylbutazone), and tulathromycin averaged 39% recovery with high variability.

In a previous method for macrolides using similar extraction as this method, tulathromycin gave excellent quantitative results.[32] The main differences between the methods are the use of C18 in the d-SPE step of this method and different conditions for UHPLC-MS/MS analysis. Retrospective analysis of the tulathromycin results in the MMM comparison study showed that MMMs 3 and 4 yielded 51 ± 22 and 68 ± 24 %recoveries, respectively, for tulathromycin, whereas MMMs 1, 2, and 5 had %recoveries of 103 ± 20, 72 ± 10, and 81 ± 9, respectively. The use of acid during sample preparation is one similarity among the MMMs that achieved higher and more consistent recoveries, but also, MMMs 3 and 4 both used d-SPE for clean-up. We intend to use this information to improve the tulathromycin results as this MMM approach is extended to more veterinary drug residues and animal tissue matrices.

In the interest of brevity, additional quantitative aspects of note are briefly summarized as follows: (1) Isotopically-labelled IS solutions for sulfamethazine and flunixin led to highly trustworthy and excellent results for those two analytes, but not others in general, and the penicillin G-d7 IS degraded readily in the spiking solution, which worsened the quality of penicillin G results (precautions to avoid that problem can be made during method implementation if it is deemed important enough). (2) Matrix effects tended to be rather variable even for the same analytes on different days. Less than ideal calibration plots explains part of the reason for the variability (average R^2 in matrix-matched calibration curves ranged from 0.96-0.99 on the different days), and highly complex kidney extracts was probably the main underlying reason. (3) Interestingly, the matrix typically caused an enhancement in the signal, not suppression as typically found in electrospray MS techniques. This was very likely caused by the filtration step rather than UHPLC-MS/MS because the matrix can serve to reduce retention of analytes on active surfaces in the vial or slow degradation of susceptible drugs. (4) Matrix-matching using a mixture of the 20 blank kidneys minimized the variable matrix effects in quantification. However, the results for the 12 spikes each day in the mixed matrix kidneys were not noticeably different than the other 18 spikes in 6 different kidney samples. (5) No differences resulted between the extracts evaporated in glass tubes vs. those in PP tubes. The glass tubes transferred heat better during the evaporation step, which shaved 15–30 min from the time to perform the method.

Conclusions

In summary, this study demonstrated that a rapid sample preparation and UHPLC-MS/MS MMM for qualitative (and quantitative) analysis of veterinary drug residues in bovine kidney could be used to replace the current 7-plate bioassay used by FSIS. The new MMM was able to acceptably screen for 54 out of the 62 drugs studied, qualitatively identify 50, and quantify 30. The scope of drugs screened affords not only elimination of the 7-plate bioassay, but also the TLC and ELISA screening methods for sulfonamides and flunixin, respectively. In addition, the new method adds to the FSIS screen several fluoroquinolones and β-agonists, beta/dexa-methasone, prednisone, florfenicol, melengestrol acetate, and carbadox and it 2-quinoxalinecarboxylic acid metabolite. The sensitivity and selectivity of the method was also superior over the 7-plate bioassay, with the added ability to distinguish drug mixtures and non-violative positives below the regulatory tolerance threshold. For the drugs meeting validation criteria, not a single false positive occurred among the 20 certified-organic kidneys analyzed in 50 replicates over 5 days by 5 different analysts. In this respect among the others mentioned, the new approach should greatly reduce the number of follow-up analyses needed by single-class LC-MS/MS methods.

Sample turnaround time from receipt to results for a small batch of a few samples was ≈2 h, and within an 8-h day, a single analyst was able to prepare a large batch of 60 pre-homogenized samples and start the analytical sequence for overnight UHPLC-MS/MS analysis. The material costs per sample were 1/5 less than the 7-plate bioassay costs, and overall operating costs particularly for personnel and space needs, are also expected to be reduced.

Despite these advantages, a major limitation of MS/MS is that it is capable of only detecting targeted drugs. Furthermore, it is prone to matrix effects that can increase detection limits and reduce quantitative performance. In the future, we intend to resolve problems, including choice of ion transitions, to yield better results for the drugs that failed in the method, and expand the approach to cover more drugs and animal tissue matrices.

Also, comparisons can be made between quantification results from the final method and the official regulatory method. If acceptable consistency is observed, this new method, which uses up-to-date technology, may be used as the quantitative analysis for enforcement actions.

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Conflicts of interest

This project was directly funded by the US Department of Agriculture. There are no other conflicts of interest.

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