APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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A Multiclass Multiresidue Method for Analysis of Veterinary Drugs in Chicken by UHPLC/MS/MS

Introduction

Veterinary drugs are used in animal production to treat diseases, prevent infection and protect growth of animals, which helps provide quality food products at a profit in the marketplace. However, improper use of drugs in animal production can lead to residue violations in food products and possible health risks, especially the potential of developing antibiotics resistance effects in animals and human beings. Regulatory agencies around the world have established maximum residue levels (MRLs) or tolerances of veterinary drugs in foods (for example, the European Union, Canada, China and many other countries set "maximum residue levels of drug residues in foods", while in the US, these are called "tolerances").[1-5] Veterinary drug residues are now monitored worldwide by many government and private contract laboratories to enforce regulations domestically and in international food trade. A common goal in drug analysis is to get acceptable results for many analytes by a cost-effective method in a single run. However, it is challenging to develop such a method for veterinary drugs in animal tissues due to the complexity of sample matrices and diversity of analytes from various classes of chemical properties. In the past, veterinary drugs methods were developed for specific analytes or groups of closely related analytes with less selective instruments and extensive sample clean up steps, many single class methods had to be used for monitoring all the targeted drugs.[6] Recently, with the advance of ultra-high-performance separation and high sensitive and selective mass detection techniques, the single-class methods have been gradually replaced by multiclass, multiresidue methods (MMMs).[7-19] In this study, such a method was developed for analysis of over 70 drugs in chicken by UHPLC/MS/MS.

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Although QuEChERS extraction method has been applied for analysis of multiresidue veterinary drugs in food samples,[7-8,15] most of the recent studies used simple solvent extractions, such as a mixture of acetonitrile and water (4/1 in v/v),[9-14] followed by different sample clean up steps to reduce sample matrix effects (mainly fat, proteins and lipids), such as protein precipitation at low temperature,[16] defatting with hexane,[17] and/or clean-up by dispersive solid phase extraction (d-SPE) with C18 or other sorbents,[8-13] or by cartridge SPE with HLB.[18] Proper sample clean-up can improve method performance and reduce instrument maintenance needs, but at the cost of more labour, time and expense for the analysis. In addition, in MMMs, due to the nature of the multiclass analytes (containing different chemical properties) in the samples, sample cleanup steps could lead to low recoveries for some analytes.[8-14] To meet the required detection limits with less sensitive instrumentation in the previous studies, it was also necessary to evaporate solvents and concentrate analytes prior to LC/MS/MS analysis.[7-12] In this study, a fast, sensitive and selective method has been developed for analysis of 73 veterinary drugs (covering 13 different chemical classes) in chicken samples by coupling solvent extraction method with LC/MS/MS. It was found that better recoveries were obtained for all the analytes without sample clean-up when comparing with different sample cleanup methods. Due to the high sensitivity of QSight mass spectrometer and its unique StayClean™ technology (hot-surface induced desolvation (HSID™) and Laminar Flow Ion Guide™),[20] sample extracts can be analyzed directly without time-consuming solvent evaporation and analyte concentration steps.

Experimental

Hardware/Software

Chromatographic separation of veterinary drugs was conducted by a PerkinElmer UHPLC system and analyte determination was achieved using a PerkinElmer QSight™ 220 triple quadrupole mass detector with a dual ionization source. Since the mass detector is capable of fast polarity switching, both positive and negative ionization modes were used. All instrument control, data acquisition and data processing was performed using Simplicity 3Q™ software.

Method

Solvents, Standards and Sample Preparation

LC/MS grade methanol (MeOH), acetonitrile (ACN), formic acid, and water were obtained from Fisher Scientific. Veterinary drugs desethylene ciprofloxacin HCl, hydroxy dimetridazole and pirlimycin HCl were obtained from Toronto Research Chemicals (Toronto, ON, Canada). Haloxon was supplied by Cedarlane Labs (Burlington, ON, Canada), all other veterinary drugs, internal standards and other reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada). Chicken samples were purchased from local grocery stores in Waterloo,ON, Canada. For d-SPE clean up adsorbent tubes, AOAC 2007.01 clean-up kit ($MgSO_4$ 1200 mg, PSA 400 mg and C18 400 mg) was obtained from PerkinElmer with part number N9306911, end-capped C18 d-SPE (500 mg C18) was obtained from UCT (Bristol, PA, USA), and Z-Sep+ (500 mg in 12 mL) tube was obtained from Sigma-Aldrich (Oakville, ON, Canada).

Stock solutions of each veterinary drug standard and deuterated internal standard were prepared separately by accurately weighing about 5 mg of each individual standard and dissolving it in a 5-mL of either ACN, MeOH, water or dimethyl sulfoxide, depending on the solubility of each compound. An internal standard (IS) mix solution containing three internal standards, flunixin-d3, $^{13}C_6$-sulfamethazine, and acepromazine-d3, each at 10 µg/mL, was prepared by appropriate dilution of IS stock solutions in ACN. A composite solution containing β-Lactams/cephalosporins was prepared in water at a concentration of 200X (X are the MRLs). All other analytes were mixed in a composite solution and diluted to 100X with ACN. All stock and composite solutions were stored at –20 °C, and spiking solutions were prepared and used within a week of preparation in the validation experiments. Calibration standards were prepared by diluting the spiking solutions each day during validation and analysis. All standard solutions were stored in amber glass vials and closed with fitted PTFE except for Lactams/cephalosporins solutions which were stored in plastic vials.

Chicken samples were homogenized and ground with dry ice to obtain uniformed powder and kept at -20 °C overnight to allow sublimation of residual dry ice and then stored at -20 °C until analysis. Certified organic chicken samples were used as blank matrix for method validation. 2 g of sample was weighed into a centrifuge tube, spiked with internal standards and fortified with targeted analytes, and extracted with 10-mL of extraction solution (acetonitrile/water: 4/1 in v/v). After centrifugation, the extract was analyzed by LC/MS/MS directly or after clean-up procedures, that was done by dispersive solid phase extraction (d-SPE) with different sorbents. The extract was transferred to the cleanup tubes, after agitating for two min and centrifugation (five min at 4 °C), the top clear extract solution was analyzed without filtration.

LC Method and MS Source Conditions

The LC method and MS source parameters are shown in Table 1. The multiple reaction monitoring mode (MRM) transitions of the studied veterinary drugs are shown in Table 2 in order of retention time. At least two MRM transitions were monitored for each analyte to reduce the number of false positive and negative in the method.[21] Optimization of MS/MS parameters, including choice of parent ions and product ions, collision energies (CE), entrance voltages (EV), the voltages on the flat lens prior to collision cell (CCL2) and so on, was done by infusion of standards and use of the software. Source conditions were optimized by flow injection (FIA) method. Based on the optimized conditions, the acquisition MS method is generated automatically by selecting the veterinary drugs of interest from the built-in compound library in the time-managed-MRM module of the Simplicity software, including both positive and negative MRM transitions of the targeted analytes.

Table 1. LC Method and MS source conditions.

LC Conditions	
LC Column	Brownlee, SPP Phenyl-Hexyl, 100 x 2.1 mm, 2.7 µm (Cat#N9308485)
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid and 10 % methanol in acetonitrile
Mobile Phase Gradient (Flow Rate: 0.4 mL/min)	Start at 3% mobile phase B and hold it for one min, then increase B to 100% in 11 min and keep at 100% B for two mins. Finally equilibrate the column at initial condition for three min.
Column Oven Temperature	40 °C
Auto Sampler Temperature	4 °C
Injection Volume	3.0 µL

MS Source Conditions	
ESI Voltage (Positive)	5000 V
ESI Voltage (Negative)	-4000V
Drying Gas	120
Nebulizer Gas	200
Source Temperature	400 °C
HSID Temperature	320 °C
Detection Mode	Time-managed MRM™

Table 2. MRM Transitions.

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Florfenicol amine	Positive	248.1	230.1	-17	16	-50
Florfenicol amine-2	Positive	248.1	130.2	-29	16	-50
Hydroxy-dimetridazole	Positive	158	140.1	-17	10	-56
Hydroxy-dimetridazole-2	Positive	158	112	-25	10	-88
5-Hydroxy-thiabendazole	Positive	217.7	191.1	-33	46	-36
5-Hydroxy-thiabendazole-2	Positive	217.7	147.1	-43	46	-36
Levamisole	Positive	205	178	-27	31	-30
Levamisole-2	Positive	205	91	-49	31	-42
Lincomycin	Positive	407.2	126.2	-36	18	-56
Lincomycin-2	Positive	407.2	359.1	-24	18	-52
ALBZ 2-aminosulfone	Positive	240	133.2	-38	25	-63
ALBZ 2-aminosulfone-2	Positive	240	198	-27	25	-53
Sulfathiazole	Positive	256.2	156	-19	10	-34
Sulfathiazole-2	Positive	256.2	108.2	-36	10	-50
Desethylene ciprofloxacin	Positive	306.1	288	-25	10	-90
Desethylene ciprofloxacin-2	Positive	306.1	268	-35	10	-90
Oxytetracycline	Positive	461.1	426	-24	10	-100
Oxytetracycline-2	Positive	461.1	201	-50	10	-128
Sulfamerazine	Positive	265.2	108.2	-38	28	-53
Sulfamerazine-2	Positive	265.2	172.1	-22	28	-37
Tetracycline	Positive	445.4	154	-35	19	-96
Tetracycline-2	Positive	445.4	410	-23	19	-44
Enrofloxacin	Positive	360.4	316.1	-27	38	-52
Enrofloxacin-2	Positive	360.4	245.1	-35	38	-47
Sulfamethizole	Positive	271.2	156	-19	30	-102
Sulfamethizole-2	Positive	271.2	92.1	-41	30	-69
Orbifloxacin	Positive	396.2	295.3	-32	10	-75
Orbifloxacin-2	Positive	396.2	352.1	-25	10	-40
Sulfamethazine	Positive	279.2	186	-23	10	-42
Sulfamethazine-2	Positive	279.2	124.1	-36	10	-44
Sulfamethazine-C13	Positive	285	124.1	-32	25	-100

Table 2. MRM Transitions *continued.*

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Sulfamethazine-C13-2	Positive	285	186.2	-23	26	-100
Sulfamethoxypyridazine	Positive	281.2	92.2	-47	10	-68
Sulfamethoxypyridazine-2	Positive	281.2	108.2	-35	10	-72
ALBZ sulfoxide	Positive	282.3	240.1	-19	22	-89
ALBZ sulfoxide-2	Positive	282.3	208.2	-31	22	-89
Sarafloxacin	Positive	386.1	342.2	-26	36	-76
Sarafloxacin-2	Positive	386.1	299.1	-38	36	-98
Xylazine	Positive	221	164	-35	25	-46
Xylazine-2	Positive	221	90	-29	25	-44
Hydroxy-ipronidazole	Positive	186	168	-16	26	-24
Hydroxy-ipronidazole-2	Positive	186	121.2	-36	26	-20
Clenbuterol	Positive	276.8	203.2	-20	11	-144
Clenbuterol-2	Positive	276.8	132.2	-45	11	-61
Difloxacin	Positive	400.2	356.2	-26	36	-60
Difloxacin-2	Positive	400.2	299.2	-38	36	-92
Morantel	Positive	221	123.1	-46	28	-74
Morantel-2	Positive	221	150.1	-38	28	-68
Morantel-3	Positive	221	111	-46	28	-74
6-Phenyl-2-thiouracil	Positive	205	188	-23	30	-36
6-Phenyl-2-thiouracil-2	Positive	205	146.1	-25	30	-42
Pirlimycin	Positive	411.1	112.2	-35	28	-92
Pirlimycin-2	Positive	411.1	363.1	-23	28	-36
2-Amino-flubendazole	Positive	256.1	123.1	-35	48	-60
2-Amino-flubendazole-2	Positive	256.1	133.2	-50	48	-88
Sulfachloropyridazine	Positive	285.2	156.1	-21	25	-95
Sulfachloropyridazine-2	Positive	285.2	92	-45	25	-62
Clindamycin	Positive	425.4	126.2	-38	12	-80
Clindamycin-2	Positive	425.4	377.1	-30	12	-40
Sulfamethoxazole	Positive	254.1	92.1	-38	30	-60
Sulfamethoxazole-2	Positive	254.1	108.3	-39	30	-70
Carazolol	Positive	299.3	116.2	-25	31	-72
Carazolol-2	Positive	299.3	222.2	-25	31	-37
Doxycycline	Positive	445.5	428	-24	16	-68
Doxycycline-2	Positive	445.5	267	-47	16	-112
Sulfadoxine	Positive	311.2	92.2	-49	21	-60
Sulfadoxine-2	Positive	311.2	108.2	-37	21	-60
Sulfaethoxypyridazine	Positive	295.2	156	-25	25	-50
Sulfaethoxypyridazine-2	Positive	295.2	92.1	-47	25	-66
ALBZ sulfone	Positive	297.9	159.1	-50	35	-70
ALBZ sulfone-2	Positive	297.9	266.2	-27	35	-39
Oxfendazole	Positive	315.9	191	-29	36	-90
Oxfendazole-2	Positive	315.9	284.1	-25	36	-33
Tilmicosin	Positive	435.4	174	-31	35	-94
Tilmicosin-2	Positive	435.4	99.1	-30	35	-54
Tilmicosin-3	Positive	435.4	696	-23	35	-94

Table 2. MRM Transitions *continued.*

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Ipronidazole	Positive	170	109.1	-33	25	-55
Ipronidazole-2	Positive	170	123.1	-33	25	-55
Sulfaquinoxaline	Positive	301.3	155.9	-22	30	-48
Sulfaquinoxaline-2	Positive	301.3	108.1	-38	30	-48
Sulfadimethoxine	Positive	311.3	156	-27	28	-76
Sulfadimethoxine-2	Positive	311.3	92	-48	28	-64
Prednisone	Positive	359.1	147.1	-40	12	-62
Prednisone-2	Positive	359.1	171.1	-45	12	-68
Fenbendazole sulfone	Positive	332.2	300.1	-29	35	-54
Fenbendazole sulfone-2	Positive	332.2	159.1	-52	35	-94
Haloperidol	Positive	376.1	165.2	-32	25	-68
Haloperidol-2	Positive	376.1	123.1	-50	25	-86
Acetopromazine	Positive	327.1	86.1	-25	30	-65
Acetopromazine-2	Positive	327.1	222.2	-50	30	-115
Acetopromazine-3	Positive	327.1	254	-35	30	-115
Acepromazine-dimethyl-d6	Positive	333	92.2	-27	20	-70
Promethazine	Positive	285	86.1	-17	25	-94
Promethazine-2	Positive	285	198	-33	25	-94
Albendazole (ALBZ)	Positive	266	234.1	-26	33	-45
Albendazole (ALBZ)-2	Positive	266	191	-44	33	-75
Mebendazole	Positive	296.5	264.3	-29	30	-110
Mebendazole-2	Positive	296.5	105.1	-52	30	-106
Flubendazole	Positive	314	282.1	-30	15	-44
Flubendazole-2	Positive	314	123.1	-49	15	-94
Betamethasone	Positive	393.3	373.3	-12	15	-48
Betamethasone-2	Positive	393.3	355.2	-18	15	-58
Propionylpromazine	Positive	341.1	86.2	-25	25	-52
Propionylpromazine-2	Positive	341.1	236.2	-50	25	-102
Chlorpromazine	Positive	319	86.1	-26	25	-60
Chlorpromazine-2	Positive	319	246.1	-31	25	-135
Oxacillin	Positive	402.3	160	-27	19	-78
Oxacillin-2	Positive	402.3	243.2	-20	19	-108
Nitroxynil	Negative	288.8	126.7	32	-24	70
Nitroxynil-2	Negative	288.8	162	25	-24	60
Fenbendazole	Positive	299.9	268.2	-28	34	-64
Fenbendazole-2	Positive	299.9	159.1	-47	34	-86
Triflupromazine	Positive	353	86.2	-27	10	-52
Triflupromazine-2	Positive	353	248.1	-55	10	-122
Virginiamycin	Positive	526.2	355.2	-23	21	-78
Virginiamycin-2	Positive	526.2	337.1	-29	21	-76
Cloxacillin	Positive	437.3	278.1	-19	17	-116
Cloxacillin-2	Positive	437.3	160.1	-25	17	-76
Ketoprofen	Positive	255	177.1	-26	25	-104
Ketoprofen-2	Positive	255	194.1	-33	25	-104

Table 2. MRM Transitions *continued.*

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Oxyphenylbutazone	Positive	325.1	204.1	-22	12	-48
Oxyphenylbutazone-2	Positive	325.1	120.1	-29	12	-44
Nafcillin	Positive	415.1	199.1	-26	25	-54
Nafcillin-2	Positive	415.1	171.1	-49	25	-78
Dicloxacillin	Positive	470.3	160	-28	17	-72
Dicloxacillin-3	Positive	470.3	311.1	-20	17	-126
Flunixin	Positive	297	279.2	-29	30	-50
Flunixin-2	Positive	297	236.2	-55	30	-150
Flunixin-d3	Positive	300	282	-29	35	-35
Haloxon	Positive	415.2	211.1	-46	42	-98
Haloxon-2	Positive	415.2	353	-29	42	-64
Triclabendazole Sulfoxide	Positive	375	360	-30	25	-72
Triclabendazole Sulfoxide-2	Positive	375	313	-35	25	-156
Diclofenac	Positive	296	215	-25	28	-122
Diclofenac-2	Positive	296	250	-15	28	-134
Phenylbutazone	Positive	309.1	120.1	-25	10	-42
Phenylbutazone-2	Positive	309.1	188.1	-20	10	-40
Emamectin	Positive	886.5	158.2	-48	30	-140
Emamectin-2	Positive	886.5	302.1	-40	30	-144
Triclabendazole	Positive	359	344	-34	25	-92
Triclabendazole-2	Positive	359	274	-48	25	-96
Oxyclozanide	Negative	399.8	363.8	24	-10	90
Oxyclozanide-2	Negative	399.8	201.8	35	-10	70
Niclosamide	Negative	324.9	170.8	31	-25	60
Niclosamide-2	Negative	324.9	288.9	24	-25	60
Melengestrol acetate	Positive	397.4	337.3	-18	25	-54
Melengestrol acetate-2	Positive	397.4	279.2	-26	25	-62
Tolfenamic acid	Positive	262	244	-22	12	-44
Tolfenamic acid-2	Positive	262	209.1	-37	12	-70
Bithionol	Negative	354.9	160.8	27	-10	60
Bithionol-2	Negative	354.9	162.8	33	-10	60
Closantel	Negative	661.1	315	41	-30	160
Closantel-2	Negative	661.1	344.9	47	-30	160

Results and Discussion

Selection of Drug Analytes and Target Levels in Chicken Tissue Samples

In this study, 73 veterinary drugs were selected from 13 important classes with various physical and chemical properties. The maximum residue levels (MRLs) established for veterinary drugs in foods by the Health Canada or tolerances by US FDA were used as the reference target levels (X in Table 3) and the detection threshold ('yes/no' screening level) should be at or below 0.5X.

UHPLC/MS/MS Method Optimization

In this study, different columns and mobile phase conditions were evaluated to get better separation and sensitivity for the target compounds. It was found that three columns can be used for the separation of the compounds with slightly different retention time profiles. The columns are: Waters Acquity UPLC HSS T3 (1.8 µm, 2.1 x 100 mm), PerkinElmer Brownlee SPP Phenyl-Hexyl (2.7 µm, 2.1 x 100 mm) and Restek Raptor Biphenyl (2.7 µm, 2.1 x 100 mm). It was also found that 10% methanol in acetonitrile as organic mobile phase provided the overall best performance in terms of analyte peak shape, retention and mass signal intensity. In particular, mass signal intensity was improved for the late-eluting compounds when 10% methanol present in the organic mobile phase.

Because the advanced time-managed-MRM acquisition method can intelligently utilize the retention time of a chromatographic peak and automatically optimize the residence time of the MRM to achieve the best quantitative data, a time-managed-MRM is applied for method development in this study to improve efficiency, data quality and method performance. The fast polarity switching capability of the QSight mass spectrometer provided high sensitive detection for both positive and negative ions in a single analytical run.

Sample Matrix Effects

Sample matrix effects (MEs) are the main concerns for LC/MS/MS method development, especially for food analysis due to the diversity and complexity of food sample matrices. ESI is notoriously susceptible to ionization suppression of analytes in the presence of charge-competing matrix components.[22] Matrix-induced enhancement effects are also known to occur in ESI, which can also introduce a large bias in quantification. To overcome sample MEs, several approaches have been used, such as sample dilution, use of stable isotope internal standards, matrix-matched (MM) calibration, standard addition, sample clean-up, use of high efficiency columns for improved separation, and the use of alternative ionization sources.[22-23] Using stable isotopically labeled internal standard (IS) in the method for each analyte would be very useful to compensate for MEs, but this is not practical for so many analytes due to aspects of availability, cost, and convenience. Thus, MM calibration is commonly used to reduce MEs in practice.

In this study, sample MEs were evaluated by comparing the slopes of calibration curves obtained from chicken sample matrix to slopes obtained from reagent-only (RO). Sample ME (%) for each analyte was calculated by the percentage difference between the slopes. When the percentage difference is positive, there is a signal enhancement effect, whereas a negative value indicates signal suppression effect. As shown in Figure 1 and Table 3, the MEs for about 83% of the studied analytes were less than 20%. However, significant MEs were observed for the rest 17% of the analytes studied, some showed enhancement such as hydroxyl-dimetridazole and nafcillin, but most of them showed suppression effects at retention times between three and 10 min when most of the analytes were eluted from column to detector, possibly because MEs result not only from matrix–analyte competitive interactions but also from the many co-eluting analytes in the mixture.[22]
To overcome matrix effects and reduce variations in analytical results, matrix-matched calibrations were used in this study for quantification of all analytes.

Sample Preparation and Recovery

Sample preparation has always been the major bottleneck in any analytical procedure for the determination of chemical residues in food products. For all multiclass, multiresidue methods (MMMs), there are always trade-offs between reduced matrix effects by cleaning up samples and loss of some of the analytes (low recoveries) during clean up steps. The more clean up steps to use, the more time/expense will need and more potential analytes will lose in the processes. However, less or no sample cleanup will lead to more sample matrix effects and maintenance needs. Lehotay's group from US Department of Agriculture (USDA) have published many papers on MMMs for veterinary drugs in animal

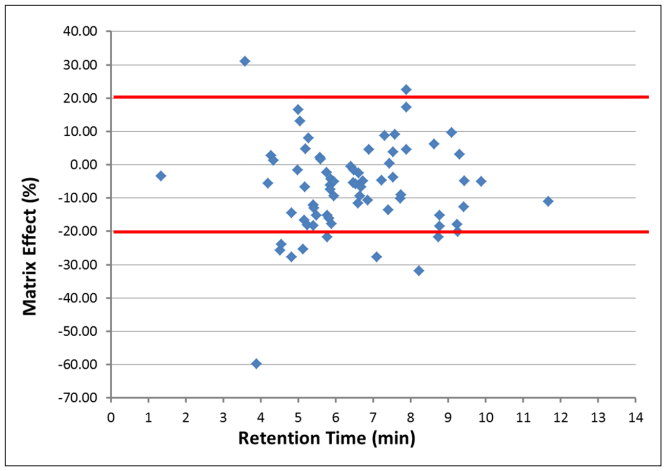

Figure 1. Sample matrix effects of chicken samples on the drug analytes vs. retention time.

foods.[9-14] In their work on bovine kidney,[9] they tested and compared six MMMs available from literature and found that all methods performed similarly, but some methods were better than others for some drug classes and none of the methods was ideal for all drug classes evaluated. From the experiments with incurred samples, they found that the method of five min shake of 2 g homogenized kidney with 10 mL of 4/1 (v/v) acetonitrile/water followed by simultaneous clean-up of the extract with 0.5 g C18 and 10 mL hexane gave a fast, simple and effective sample preparation method. In this study, they also evaluated different sorbents for dispersive solid phase extraction (d-SPE) clean-up, such as carbon black, polymeric sorbent ENV+, and aminopropyl, and found that although these sorbent gave better clean-up for matrix components, they also removed many of the analytes in the samples in comparison with C18. In another study on the ruggedness of a practical method for more than 100 drugs in bovine muscle,[11] they tested relatively new d-SPE sorbents Z-Sep and Z-Sep+ together with C18 and end-capped C18, studied nine different clean-up conditions with combinations of different d-SPE and/or partitioning with hexane. Although the use of Z-Sep+ and Z-Sep+hexane could remove matrix components effectively but they also significantly lower the recoveries for many drugs studied. The best compromise in terms of matrix effects and analyte recoveries was obtained using end-capped C18+hexane. In their recent studies,[12-14] they did not use hexane for clean-up, because oxyphenylbutazone, phenylbutazone and other less hydrophilic drugs partially partitioned into the hexane, resulting in low and variable recoveries. More recently, they tested the so-called "enhanced matrix removal for lipids" (EMR-L) new product designed to selectively remove lipids and proteins from fatty food samples such as animal-derived foods.[13] The results showed that the EMR-L method gave cleaner extracts and improved results for some less polar compounds such as anthelmintics and tranquilizers compared to C18 d-SPE method, but the EMR-L method showed much lower recoveries for β-lactam antibiotics and some polar drugs. In addition, EMR-L method involved extra steps compared to C18 d-SPE. For tetracyclines, low recoveries were obtained by both EMR-L and C18 d-SPE sample preparation methods. It was recommended to use stable isotope labeled IS for tetracyclines in the future to compensate for the losses in sample preparation.[8,12] Thus, practically, the C18 d-SPE method was faster, easier and

less expensive than the EMR-L method. However, even using this end-capped C18 d-SPE clean up, only 75 % of the drugs showed recoveries within 70-120% and some less polar drugs can still be partially retained on the C18 sorbent during d-SPE in the aqueous ACN extract, leading to lower recoveries.[12-14] In addition, all the polar matrix components and interferences could not be removed by any of the clean-up methods studied so far. Therefore, for MMMs, the best approach for reducing matrix effects and minimizing maintenance needs would be diluting the final sample extracts or injecting small volume of samples on column,[11,12,14,22,23] which can also improve chromatography for the early eluting polar analytes. Recently, the commercial availability of modern high sensitive mass spectrometers with fast polarity switching has made this approach possible for analyzing over 100 veterinary drugs without sample clean-up.[14] The enhanced sensitivity of the new LC/MS/MS systems also helped to eliminate the time-consuming solvent evaporation, concentration and reconstitution steps that were necessary to meet the detection limits by the previous MMMs using less sensitive instruments.[7-11] Although it is common practice to filter sample extracts before LC analysis, especially for UHPLC applications, different filter materials need be evaluated carefully to avoid contaminations from filters or analyte losses due to adsorption onto the filters. In their previous studies,[9-13] researchers from Lehotay's group assessed four different types of filters and found that polyvinylidenefluoride (PVDF) filter gave the overall best performance. However, they also found that filtration not only filtered out some of the drug analytes but also introduced potential interfering components and signal enhancement matrix effects in sample matrices.[9,12,14] Thus, they decided to abandon the clean-up and filtration steps altogether in their latest method using modern instrumentation and injecting small volume of samples, which showed increased analytical scope without affecting method performance and maintenance needs.[14]

In this study, different sample preparation methods were further evaluated. The recovery study was carried out by spiking the analytes to the samples at two concentration levels of 0.5X and 1X, respectively. Recoveries were calculated by comparison of peak areas of fortified samples with the matrix-matched calibration curve. As shown from Table 3, the average recoveries of analytes ranged from 70% to 120 % with RSD < 20% for most of the analytes studied when the extract was analyzed directly without clean-up. However, the average recoveries for several classes of analytes are lower after d-SPE clean-up using AOAC 2007.01 clean-up kit ($MgSO_4$ 1200 mg, PSA 400 mg and C18 400 mg), such as for fluoroquinolones, tetracyclines and some tranquilizers. Similar results were also obtained using end-capped C18 as d-SPE sorbent in this study, which are in good agreement with the published results.[8-13] The results of using Z-Sep+ sorbent during d-SPE clean-up steps are the same as the published results[11] and therefore, are not included in this report.

Linearity, Precision and Limit of Quantification

Calibration was performed in both matrix-matched (MM) and reagents-only (RO) standards at 0, 0.1X, 0.2X, 0.5X, 1X, and 2X (X are the regulatory limits or tolerances in Table 3) equivalent sample concentrations with duplicate injections of each standard dispersed throughout the UHPLC/MS/MS sequence. All calibration curves built from both RO and chicken sample matrix showed good linearity with correlation coefficient (R^2) larger than 0.99 (see Figures 2 and 3 for typical examples of calibration curves). Carry-over was assessed by injecting the reagent blank after a 2X standard and no carry-over was observed in any of the experiments. The method demonstrated good precision with RSD less than 20% for most of the drugs studied. The estimated limits of quantification

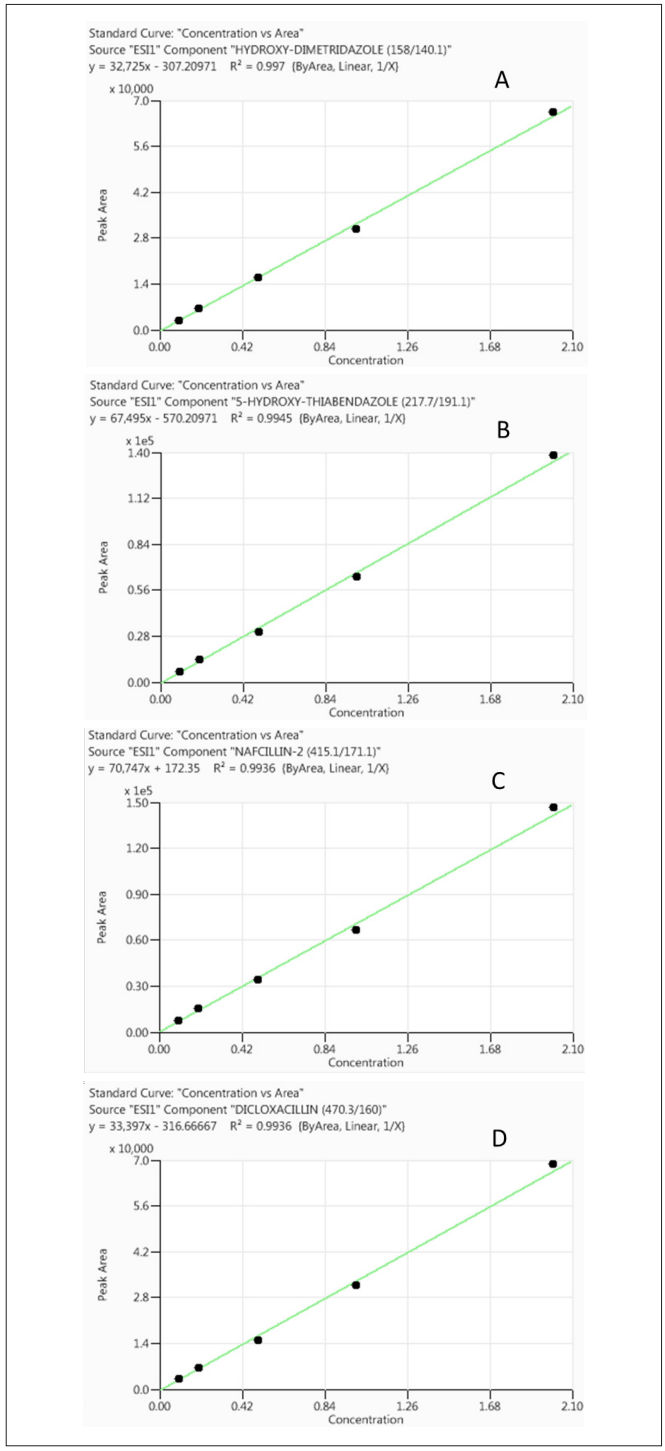

Figure 2. Calibration curves for hydroxyl-dimetridazole (A), 5-hydroxy-thiabendazole (B), nafcillin (C) and dicloxacillin (D) obtained from standards prepared in reagents only (analyte concentrations range from 0.1X to 2X).

(LOQs) for the method were the concentrations with signal/noise (S/N) ratio of 10 and all the LOQs for the drugs studied are below the 0.5 X tolerance limits or the maximum residue levels (MRLs). The results demonstrated that the developed method can be applied for the fast screening and quantification of multiclass veterinary drug residues in chicken samples.

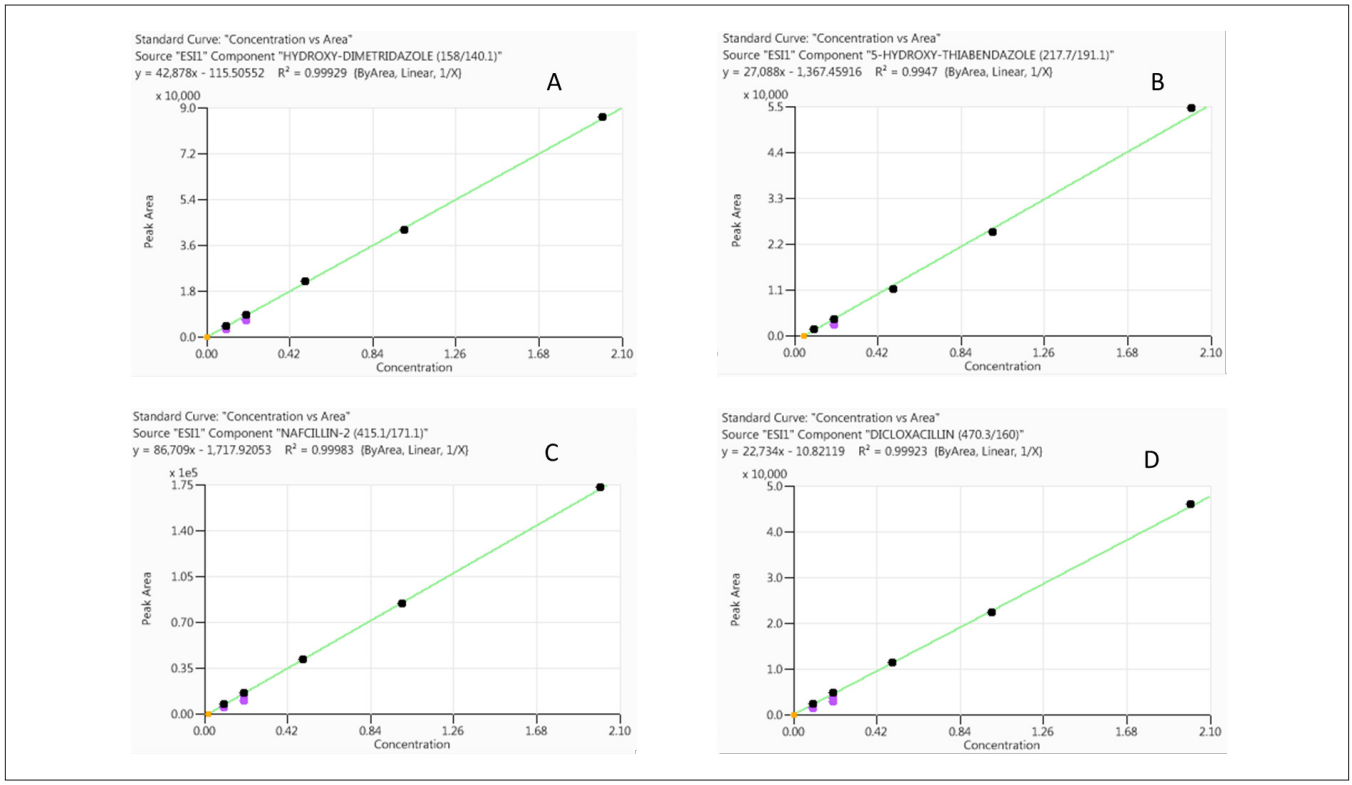

Figure 3. Calibration curves for hydroxyl-dimetridazole (A), 5-hydroxy-thiabendazole (B), nafcillin (C) and dicloxacillin (D) obtained from standards prepared in chicken sample matrix (analyte concentrations range from 0.1X to 2X).

Table 3. Results of retention time, matrix effect (ME), linearity, recovery, and reproducibility (%RSD)*.

Compound Name	Drug Class	X Level (ng/g)	RT (min)	ME (%)	Linearity (R^2)	Recovery (%) (Non d-SPE)	Recovery (%) (d-SPE)
Florfenicol amine	Phenicols	100	1.34	-3.4	0.9999	88 (16)*	92 (18)
Hydroxy-dimetridazole	Coccidiostats	50	3.58	31.0	0.9993	90 (10)	103 (11)
5-Hydroxy-thiabendazole	Anthelmintics	100	3.89	-59.9	0.9947	111 (13)	92 (17)
Lincomycin	Macrolides/lincosamides	100	4.19	-5.6	0.9969	85 (11)	102 (7)
ALBZ 2-aminosulfone	Anthelmintics	100	4.28	2.7	0.9998	108 (8)	87 (11)
Levamisole	Anthelmintics	100	4.34	1.3	0.9989	89 (10)	87 (6)
Desethylene ciprofloxacin	Fluoroquinolones	100	4.51	-25.7	0.9988	113 (13)	4 (18)
Sulfathiazole	Sulfonamides	100	4.55	-23.8	0.9997	104 (6)	95 (9)
Sulfamerazine	Sulfonamides	100	4.82	-27.8	0.9999	110 (4)	98 (8)
Oxytetracycline	Tetracyclines	200	4.82	-14.5	0.9994	99 (12)	11 (15)
ALBZ sulfoxide	Anthelmintics	50	4.99	-1.7	0.9995	71 (4)	69 (9)
Hydroxy-ipronidazole	Coccidiostats	10	5.00	16.6	0.9955	107 (5)	107 (7)
Tetracycline	Tetracyclines	200	5.05	13.0	0.9963	85 (2)	11 (17)
Enrofloxacin	Fluoroquinolones	20	5.13	-25.5	0.9997	78 (11)	40 (14)
Xylazine	Tranquilizers	10	5.14	-13.6	0.9987	121 (9)	92 (11)
Sulfamethazine	Sulfonamides	100	5.16	-16.6	0.9990	103 (10)	89 (8)
Sulfamethizole	Sulfonamides	100	5.18	-6.6	0.9973	115 (7)	87 (5)
Clenbuterol	β-Agonists	10	5.19	4.7	0.9994	80 (12)	72 (10)

Table 3. Results of retention time, matrix effect (ME), linearity, recovery, and reproducibility (%RSD)* *continued.*

Compound Name	Drug Class	X Level (ng/g)	RT (min)	ME (%)	Linearity (R^2)	Recovery (%) (Non d-SPE)	Recovery (%) (d-SPE)
Orbifloxacin	Fluoroquinolones	50	5.24	-18.1	0.9933	111 (14)	44 (9)
Sulfamethoxypyridazine	Sulfonamides	100	5.27	8.0	0.9988	120 (2)	103 (3)
Sarafloxacin	Fluoroquinolones	50	5.40	-12.1	0.9977	117 (4)	45 (6)
Pirlimycin	Macrolides/lincosamides	300	5.41	-18.2	0.9994	102 (9)	88 (7)
6-Phenyl-2-thiouracil	Thyreostats	400	5.41	-12.1	0.9999	117 (4)	93 (10)
Morantel	Anthelmintics	150	5.42	-13.1	1.0000	112 (11)	89 (5)
Difloxacin	Fluoroquinolones	50	5.48	-15.2	0.9998	106 (8)	90 (12)
2-Amino-flubendazole	Anthelmintics	10	5.59	2.2	0.9977	106 (3)	89 (6)
Clindamycin	Macrolides/lincosamides	100	5.60	1.7	0.9997	99 (8)	87 (11)
Sulfachloropyridazine	Sulfonamides	100	5.76	-2.3	0.9992	109 (7)	103 (3)
ALBZ sulfone	Anthelmintics	50	5.78	-15.3	0.9999	100 (12)	98 (8)
Oxfendazole	Anthelmintics	800	5.78	-21.7	0.9997	95 (14)	83 (9)
Carazolol	Tranquilizers	10	5.83	-16.1	0.9975	117 (7)	122 (13)
Doxycycline	Tetracyclines	100	5.85	-6.1	0.9931	114 (11)	26 (15)
Sulfamethoxazole	Sulfonamides	100	5.86	-7.4	0.9998	110 (2)	92 (4)
Sulfadoxine	Sulfonamides	100	5.87	-4.3	1.0000	92 (6)	101 (4)
Tilmicosin	Macrolides/lincosamides	100	5.89	-17.7	0.9991	119 (1)	95 (10)
Sulfaethoxypyridazine	Sulfonamides	100	5.94	-9.5	0.9999	104 (5)	87 (7)
Ipronidazole	Coccidiostats	10	5.96	-5.0	0.9965	115 (13)	101 (8)
Sulfadimethoxine	Sulfonamides	100	6.41	-0.6	0.9989	94 (4)	89 (10)
Prednisone	Anti-inflammatories	100	6.46	-5.4	0.9979	105 (3)	66 (9)
Sulfaquinoxaline	Sulfonamides	100	6.48	-1.7	0.9997	111 (6)	100 (10)
Albendazole (ALBZ)	Anthelmintics	50	6.53	-5.8	1.0000	100 (3)	93 (7)
Mebendazole	Anthelmintics	10	6.59	-11.5	0.9997	117 (2)	77 (5)
Fenbendazole sulfone	Anthelmintics	400	6.62	-2.6	1.0000	104 (6)	92 (11)
Acetopromazine	Tranquilizers	10	6.64	-9.4	0.9999	118 (2)	88 (9)
Promethazine	Tranquilizers	10	6.68	-6.8	0.9890	95 (10)	33 (8)
Haloperidol	Tranquilizers	10	6.72	-4.8	0.9993	109 (5)	98 (13)
Flubendazole	Anthelmintics	10	6.85	-10.6	0.9989	106 (4)	103 (11)
Betamethasone	Anti-inflammatories	100	6.89	4.5	0.9982	110 (7)	126 (2)
Propionylpromazine	Tranquilizers	10	7.10	-27.8	0.9994	121 (5)	85 (8)
Chlorpromazine	Tranquilizers	10	7.31	8.6	0.9993	92 (3)	68 (7)
Fenbendazole	Anthelmintics	100	7.40	-13.6	0.9999	104 (7)	87 (5)
Oxacillin	β-Lactams/cephalosporins	100	7.43	0.3	0.9998	87 (6)	89 (11)
Triflupromazine	Tranquilizers	10	7.53	3.8	0.9993	116 (4)	74 (11)
Flunixin	Anti-inflammatories	20	7.54	-3.7	0.9997	114 (3)	90 (10)
Virginiamycin	Miscellaneous	100	7.59	9.1	0.9903	109 (8)	118 (3)
Nitroxynil	Anthelmintics	50	7.72	-10.2	0.9990	84 (10)	71 (7)
Ketoprofen	Anti-inflammatories	10	7.75	-9.0	0.9964	105 (13)	119 (9)
Nafcillin	β-Lactams/cephalosporins	100	7.88	22.6	0.9998	104 (7)	85 (4)
Cloxacillin	β-Lactams/cephalosporins	100	7.89	4.6	0.9999	103 (2)	96 (12)
Oxyphenylbutazone	Anti-inflammatories	100	7.89	17.3	0.9990	115 (5)	103 (8)

Table 3. Results of retention time, matrix effect (ME), linearity, recovery, and reproducibility (%RSD)* *continued.*

Compound Name	Drug Class	X Level (ng/g)	RT (min)	ME (%)	Linearity (R^2)	Recovery (%) (Non d-SPE)	Recovery (%) (d-SPE)
Dicloxacillin	β-Lactams/cephalosporins	100	8.23	-31.9	0.9992	88 (7)	79 (6)
Triclabendazole Sulfoxide	Anthelmintics	50	8.63	6.1	0.9989	96 (4)	88 (10)
Haloxon	Anthelmintics	100	8.75	-21.8	0.9998	82 (3)	79 (10)
Emamectin	Anthelmintics	10	8.77	-15.1	0.9998	105 (12)	76 (19)
Diclofenac	Anti-inflammatories	200	8.77	-18.5	0.9998	89 (9)	80 (5)
Phenylbutazone	Anti-inflammatories	100	9.10	9.7	0.9999	117 (3)	90 (5)
Triclabendazole	Anthelmintics	50	9.24	-17.9	0.9988	103 (5)	80 (14)
Melengestrol acetate	Miscellaneous	25	9.25	-20.0	0.9990	103 (4)	87 (7)
Oxyclozanide	Anthelmintics	10	9.30	3.1	0.9997	111 (6)	78 (11)
Tolfenamic acid	Anti-inflammatories	200	9.42	-12.6	0.9991	88 (9)	67 (10)
Niclosamide	Anthelmintics	10	9.43	-4.8	0.9991	107 (2)	85 (9)
Bithionol	Anthelmintics	10	9.90	-5.0	0.9973	87 (4)	59 (8)
Closantel	Anthelmintics	50	11.68	-11.0	0.9995	93 (6)	86 (5)

Sample Analysis

The developed method was applied for the analysis of veterinary drugs in five chicken samples fortified with internal standards. None of the studied drugs were detected based on the retention time and mass spectra information (two MRM transitions) in comparison with the corresponding reference standards.

Conclusions

Analytical method development for veterinary drugs in animal tissues is highly challenging because the matrices are complex with high amounts of fat, proteins and lipids, and the residue analytes of interest are highly diverse from different classes with varying physical and chemical properties. Although sample preparation is the key to many successful methods due to its effects on chromatography, ionization and mass spectrometric analysis, sample clean-up is more challenging in MMMs because it can cause analyte losses when more extensive clean-up is carried out, as demonstrated by the results with d-SPE cleanup in this study and in the previous publications. A compromise has to be made between maintaining enough analyte recovery and reducing sample matrix effects to analyze as many analytes as possible. Perhaps, with the commercial availability and further advancement in high sensitive instrumentation, the dilute-and- shoot method could be the best approach to reduce MEs, minimize instrument maintenance needs, and improve chromatography for all analytes in the MMMs.

In this study, a cost-effective, MMM for veterinary drugs analysis in chicken was developed by coupling a UHPLC system to a QSight 220 triple-quad mass spectrometer. The method can be applied for the fast screening and analysis of over 70 veterinary drugs in chickens with LOQs well below the limits set by regulatory agencies. The results from different sample preparation methods demonstrated that the simple direct injection method without sample clean up gave the overall best recovery for all the compounds studied although sample matrices could affect the life time of the analytical column. With the stay-clean ion source and the commercial availability of more advanced and more sensitive mass spectrometer such as QSight 300 series in the near future, it is possible to further reduce matrix effects and improve the method robustness by more sample dilutions or less sample injection without affecting the method's detectability.

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