APPLICATION NOTE 65889

Multiclass veterinary drug screening and quantitation by high-resolution mass spectrometry (HRMS) using the Orbitrap Exploris 120 mass spectrometer

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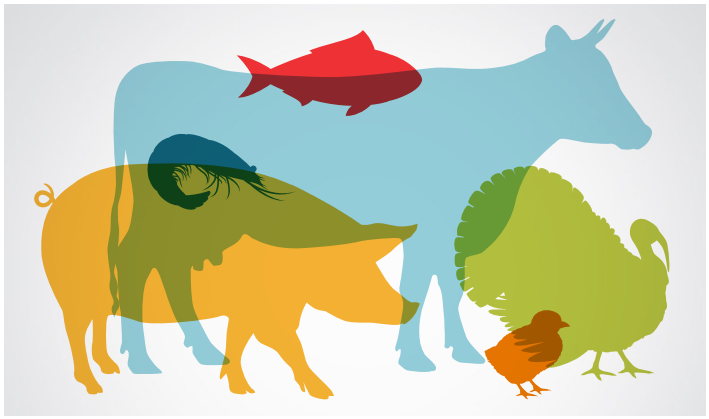


Goal

Demonstrate a HRMS method for screening and quantitation of a multi-class veterinary drug panel in bovine muscle matrix based upon AOAC Standard Method Performance Requirements (SMPR 2018.010).[3]

Introduction

Veterinary drugs are frequently administered to production animals to ensure their health and well-being throughout the lifetime of the animal. Inappropriate administration of these drugs however, can adversely affect the health of both animals and humans. Analysis of drug residues in animal tissue matrices is challenging because of the complexity and diversity of chemical structures represented among the various classes of veterinary drugs. Global agencies provide regulatory information regarding acceptable veterinary drug residue levels in various animal tissues destined for human consumption.[1,2] LC-MS/MS methods are frequently used to screen animal tissues for veterinary drugs because they provide the sensitivity needed to identify and quantify these compounds over the wide range of maximum residue limits (MRLs) set by regulatory agencies.

The Association of Analytical Chemists (AOAC) has released a call for multi-class veterinary drug residue methods capable of identifying over 200 residues in several animal matrices at one-half the lowest global MRL as published in their Standard Methods Performance Requirements document (SMPR 2018.010).[3] Multi-residue screening methods are difficult to validate because they are semi-quantitative in nature and often contain large panels of compounds of varying chemical classes in the applicable matrices. The SMPR document references a statistical model known as Probability of Detection (POD) that is used to validate semi-quantitative screening methods. The POD model is based on the probability of a drug residue being

correctly detected and identified in a given matrix at one-half the MRL. The POD calculation combines false positive, false negative, sensitivity, and specificity parameters into a single calculation.[4]

Here, an HRMS UHPLC-MS/MS method for analysis of veterinary drugs in bovine muscle matrix was developed using a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer. Drug residues were screened using both data-independent acquisition (DIA) and data-dependent acquisition (DDA) workflows, with confirmation against a highly curated veterinary drug spectral library and fragment compound database. The method was evaluated using a limited number of biological replicates in a POD experiment based upon the AOAC SPMR for 109 vetdrugs. Recovery, precision, and quantitative results obtained from the analysis of incurred residues in a certified reference material (CRM) known as BOTS-1 provided by the National Research Council of Canada (NRCC) are presented.

Experimental

Reagents and standards

Standards for 109 veterinary drugs were obtained as neat from a variety of manufacturers, including Sigma Aldrich (St. Louis, MO), Crescent Chemical Co. (Islandia, NY), Ultra Scientific (North Kingstown, RI). The Vet Drugs Check Standard Mixture (P/N 00590-01-00519) was from Thermo Fisher Scientific.

Mobile phase reagents used for the analysis

- Formic acid, >99% (P/N 28905)
- Water, ultra grade (P/N W8-1)
- Acetonitrile, ultra grade (P/N A956-1)
- Methanol, ultra grade (P/N A458-1)

Sample preparation supplies

- 0.2 M ammonium oxalate monohydrate/0.1 M disodium EDTA dihydrate buffer
- 5 g Sodium sulfate, slim pouch—50 pk (P/N 60105-368-SP)
- 500 mg CEC18, Slim Line Pouch—50 pk (P/N 60105-367-SP)
- Falcon tubes (50 mL)—50 pk (P/N 60106-425)
- 0.45 µm PTFE filters, 17 mm—100 pk (P/N F2513-3)
- 10 mL luer-lock syringe—100 pk (P/N S7515-10)
- Thermo Scientific™ HyperSep™ Retain-PEP 60 mg/3 mL SPE column—50 pk (P/N 60107-203)

Development of the multi-class spiking mixtures

Due to the wide variety of chemical classes and MRLs of the veterinary drugs used in the method, a set of ten family spiking mixtures was created for preparation of matrix-extracted spikes (MES) used in the POD procedure. The mixtures were chosen to ensure their stability when the components were mixed together in the appropriate solvents and then were stored at -20 °C prior to use. The β-lactam antibiotics were stored at -80 °C. The stock concentration of each analyte was adjusted such that the final spike level provided the appropriate MRL in the bovine muscle matrix. The chemical classes included in the mixtures were avermectins, benzimidazoles, β-lactams, coccidiostats/ionophores/growth promoters, antibiotics, anthelmintics, NSAIDS/misc. class compounds, quinolones, sulfonamides, and tetracyclines. Figure 1 shows the overall distribution of the MRL concentrations for the analytes studied, which were based upon the information provided in SMPR 2018.010. The MRLs ranged from 0.1–1000 ng/g.

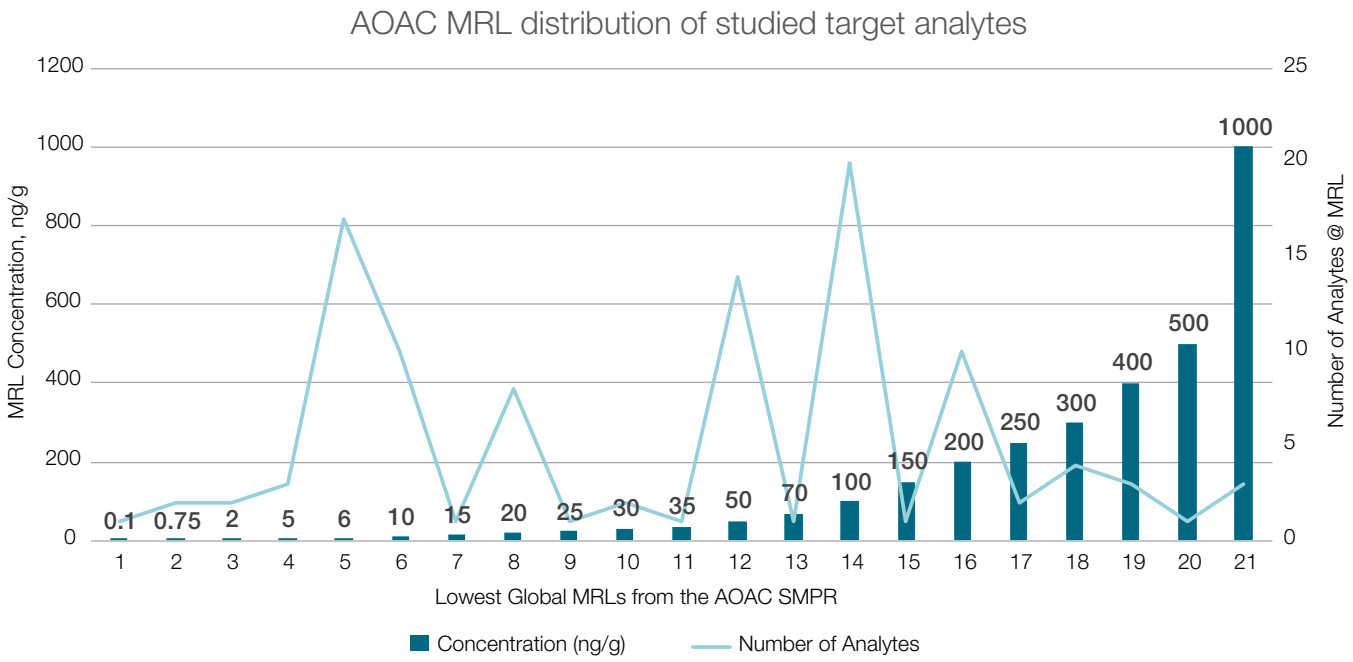


Figure 1. Maximum residue limits (MRLs) for the compounds studied in a bovine muscle matrix taken from the AOAC SMPR. The light blue line indicates the number of analytes at the specified MRL (left-hand scale), and the teal bars indicate the concentration of the MRLs (right-hand scale).

Sample preparation—extraction step

Bovine muscle samples were purchased at a local grocery store and homogenized using a blender. The control bovine muscle was screened and determined to be free of veterinary drug residues. Sample extraction used a modified QuEChERS preparation protocol. Five grams of bovine muscle was added to a 50 mL Falcon tube. Next, 0.5 mL of 0.2 M ammonium oxalate/EDTA solution was added to the tube followed by acetonitrile to bring the total volume to 15 mL. The tubes were shaken vigorously on a Fisherbrand™ Digital MultiTube Vortexer for 10 minutes. Five grams of sodium sulfate were added and the tubes were vortexed by hand for 30 seconds. All tubes were allowed to stand for 30 minutes at room temperature before centrifugation at 3000 rpm for 10 minutes.

Sample preparation—cleanup steps

Extract cleanup approaches included dispersive solid phase extraction (dSPE), extract freezing at -20 °C for 1 hour, and solid phase extraction (SPE). For the dSPE experiments, 500 mg CEC18 was added to the supernatant and shaken on the vortexer for 30 minutes, and then centrifuged at 3000 rpm for 10 minutes. One mL was evaporated to near dryness at 30 °C (Biotage® TurboVap® LV) and reconstituted to a final volume of 0.5 mL in 75:25 mobile phase A:B. The SPE cleanup was performed by conditioning a HyperSep Retain-PEP 60 mg × 3 mL SPE column with 3 mL methanol followed by 3 mL water. One mL of the sample extract was passed though the column and discarded to waste. Two mL of the extract was then added to the cartridge and collected. One mL of either frozen or SPE cleaned up extract was then evaporated and reconstituted in the same manner as described above.

Separation	
LC	Thermo Scientific™ Vanquish™ Flex UHPLC system, consisting of a binary pump, autosampler, and column heater set at 40 °C
Column	Thermo Scientific™ Accucore™ VDX column, 100 × 2.1 mm, 2.6 µm
Mobile phase	A: Water with 0.05% formic acid B: 1:1 methanol: acetonitrile with 0.05% formic acid and 5% water
Gradient	Start at 2% B, hold for 2 minutes and then use a linear gradient to 30% B for 1 minute, hold for 0 minutes, then apply a linear gradient to 100% B for 8 minutes, hold 3.4 minutes, then reduce to 2% B in 0.5 minutes, hold for 2.6 minutes for a total run time of 17 minutes.
Flow rate	0.300 mL/min
Injection volume	5.0 µL

Orbitrap Exploris 120 mass spectrometer scan modes and settings

The Orbitrap Exploris 120 mass spectrometer was evaluated using two scan modes:

1. Data-independent acquisition (DIA) using full-scan analysis at mass resolution 60,000 (FWHM) with a resolution setting of 15,000 for MS^2 (with four precursor isolation windows across the scan range)
2. Data-dependent acquisition ($ddMS^2$) using full-scan analysis at a mass resolution setting of 60,000 (FWHM) and a target mass list for MS^2 with a resolution setting of 15,000

Mass spectrometry settings	
Instrument	Orbitrap Exploris 120 mass spectrometer
Spray voltage	3.5 kV POS/2.5 kV NEG
Sheath gas	50
Aux gas	13
Sweep gas	1
Capillary temperature	280 °C
Vaporizer temperature	350 °C
Ion polarity	One positive and one negative analysis

Data acquisition and processing

Data was acquired and processed using Thermo Scientific™ TraceFinder™ software which provides full automation from instrument setup to raw data collection, processing, and reporting. Data acquired from the two scan modes were analyzed with an extraction mass tolerance of ≤ 5 ppm for both precursor and product ions. Analytes were quantified based on full-scan information. In addition, confirmation of target compounds was performed using MS^2 fragment matching along with searches against a highly curated mass spectral fragmentation library in the Thermo Scientific™ mzVault™ application.

Results and discussion

Several combinations of sample preparation cleanup steps were evaluated at the Iowa State University Veterinary Diagnostic laboratory. These included the following: dSPE cleanup using CEC18; dSPE cleanup with CEC18 with a 1-hour freeze step at -20 °C; freeze step alone at -20 °C for 1 hour; SPE Hypersep cleanup only; CEC18 dSPE plus Hypersep SPE cleanups, and finally CEC18 dSPE and Hypersep SPE cleanups plus a 1-hour freeze step at -20 °C. For each experiment, five biological replicates were prepared as matrix-extracted spikes (MES) containing all the target residues at their MRL concentration. The MES were compared to post-spiked standards after each of the different cleanups (MMS or matrix-matched standards) at the same concentrations. Recovery was calculated as the ratio of the average peak area response of the MES to the average peak area response of the MMS.

The authors quickly determined that the -20 °C freeze step did not provide reproducible results when used by itself or in combination with the other techniques. This may have been due to sample handling after the freeze step. For example, after removing a large batch of samples from the freezer, a portion of samples might have experienced warming before all were transferred to the final evaporation and concentration step. Also, from a logistical point of view, the authors speculated that the freezing step would be difficult to perform on a large scale and would add significantly more time to the overall extraction process.

The best results were obtained using either the dSPE cleanup step or HyperSep SPE cleanup alone. A comparison of the recoveries and the %RSDs obtained are shown in Figure 2. Both techniques provided a good basis for performing the method with bovine muscle matrix. The authors decided to use the dSPE cleanup for the HRMS UHPLC-MS/MS screening experiments, noting that although both methods provided good results, it was easier to implement dSPE for the analysis of large numbers of samples.

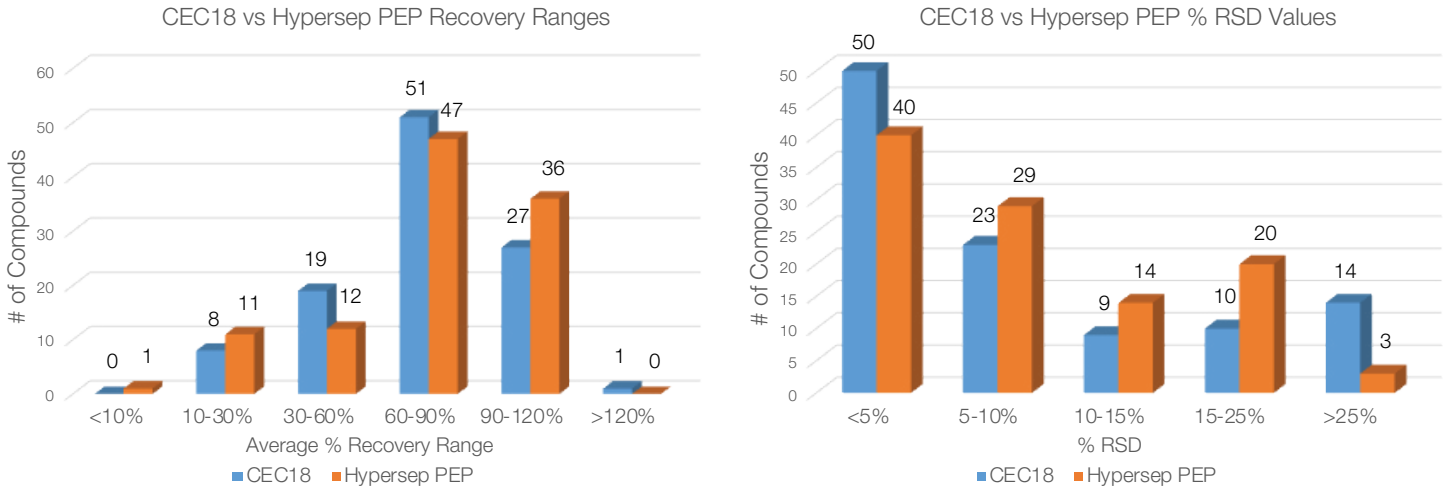


Figure 2. Recovery and %RSD comparison of results for 5 biological MES replicates at the MRL concentration in bovine muscle matrix. The blue bar represents cleanup of the QuEChERS extracts using the CEC18 dSPE procedure, and the orange bar represents cleanup with the Hypersep PEP solid phase extraction material. The number on top of each bar represents the number of analytes in the described ranges.

The AOAC SMPR and the mini-POD experiment

The AOAC SMPR 2018.010 document for veterinary drugs focuses on the screening of a large panel of veterinary drug residues in a variety of matrices, preferably using the fewest possible number of methods. It is intended for use in developing LC-MS/MS methods for routine surveillance and GMP compliance with respect to multi-class regulated residues, marker residues, and metabolites. The screening procedure is based upon the lowest global MRL for each analyte/matrix combination published in the SMPR.

The probability of detection (POD)[4] procedure was used to evaluate the method's performance for a given matrix/analyte combination. POD is defined as the proportion of positive analytical outcomes for a qualitative method in a given matrix at a given analyte level or concentration. It is calculated as the ratio of the number positive outcomes (×) to the total number of samples tested (N):

$$POD = \times/N$$

To evaluate the HRMS screening method, a 'mini-POD' experiment was designed for the bovine muscle matrix. Ten MES samples at one-half the MRL level for each analyte, along with ten MES at one times the MRL and ten blanks, were prepared and cleaned up using the dSPE procedure as described above. The POD was then calculated for each analyte. A POD score of 0.8 or greater was considered a positive result. In addition, confirmations of analytes were obtained according to the procedure described in Guidance Document SANTE/12682/2019.[1] Confirmation was described as an analyte with a precursor ion in full scan data and at least one fragment ion in MS^2 data, both with mass accuracy ≤ 5 ppm and signal-to-noise (S/N) ≥ 3.

The TraceFinder software compound database used contains the precursor and corresponding MS^2 fragments required for compound confirmation. An important aspect of this database is that it was derived from the highly curated Thermo Scientific™ mzCloud™ mass spectral fragmentation library which ensures the mass accuracy of all compounds. To increase confidence in the results, searches were performed against a veterinary drug offline mzCloud mass spectral library.

Figure 3 shows the typical results for the DIA and DDA analysis of 5-Hydroxyflunixin at a concentration of 10 ng/g displayed in TraceFinder software. The DIA experiment consisted of four precursor isolation windows over a mass range of *m/z* 140–1100 Da, with stepped normalized collision energies of 30 and 80 V. The DDA experiment used an inclusion list with an isolation precursor width of 1.5 Da over the specified 0.5 minute retention time window, and a single optimized normalized collision energy of 25 V. The fragments were detected with a mass tolerance of < 5 ppm mass accuracy. In both cases, excellent library match scores of >90% were obtained, with all fragments detected.

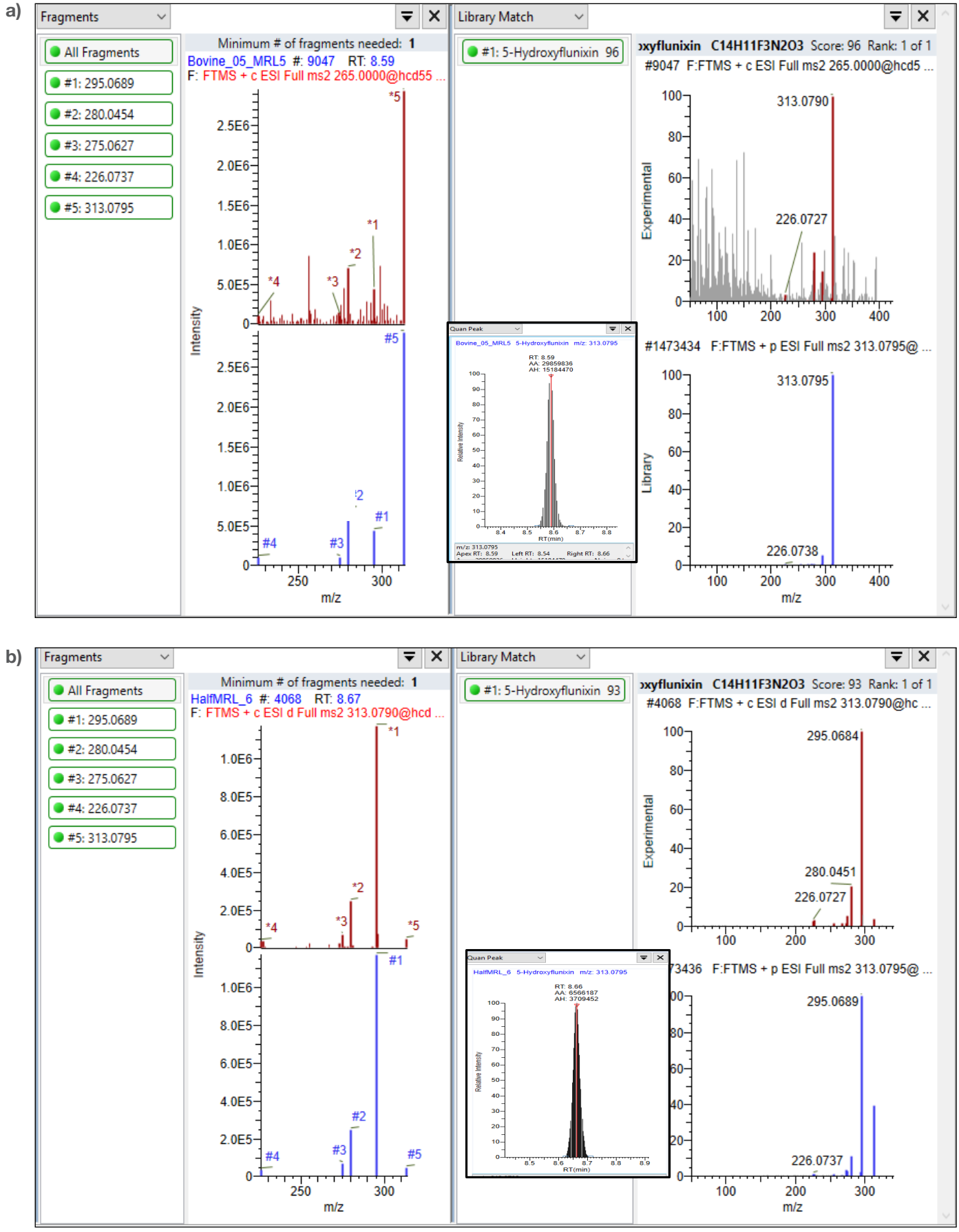


Figure 3. 5-Hydroxyflunixin detected and confirmed at one-half MRL (10 ng/g) in bovine muscle MES. a) DIA acquisition method with four precursor isolation windows and b) Data dependent MS^2 acquisition method. All full scan precursor and all MS^2 fragments detected at < 5 ppm mass accuracy with excellent library match scores, and POD = 1.0.

Table 1 provides a summary of select compounds representing several chemical classes of the veterinary drugs studied. Both the DIA and DDA workflows provided similar results for detection and confirmation using the POD procedure.

Table 1. Probability of Detection (POD), fragment matches, and library scores for a selection of compounds representing several drug classes at 1/2 the MRL in bovine muscle. The two different acquisition methods (DIA and DDA) are shown for comparison.

Compound	Class	1/2 MRL (ng/g)	DIA POD score	Library match score (%)	# fragments match	ddMS^2 POD Score	Library match score (%)	# fragments match
Albendazole	Benzimidazoles	50	1	99	5/5	1	93	5/5
Cefapirin	Cephalosporin antibiotics	25	1	88	4/5	1	86	5/5
Ciprofloxacin	Floxacins	50	1	100	3/5	1	100	3/5
Imidocarb	Coccidiostats	150	1	95	2/5	1	97	4/5
Flunixin	NSAIDS	10	1	98	5/5	1	98	5/5
Levamisole	Anthelmintic	5	1	100	4/5	1	100	5/5
Lincomycin	Antibiotics	50	1	97	5/5	1	97	5/5
Oxytetracycline	Tetracyclines	100	1	96	5/5	1	90	5/5
Penicillin V	Penicillin antibiotics	25	1	96	5/5	1	94	5/5
Ractopamine	β-agonists	5	1	89	2/2	1	no score	1/2
Sulfamethazine	Sulfanilamides	3	1	100	5/5	1	100	5/5

Table 2 summarizes the results for all compounds in the study that were either a) not detected, b) detected but not confirmed, and c) detected and confirmed at one-half the MRL for both workflows. A detected analyte was defined as the precursor ion detected at < 5 ppm mass accuracy with S/N ≥ 3 and retention time ≤ 0.1 minutes in at least eight of the ten biological replicates (POD ≥ 0.8/1.0). A detected and confirmed analyte was defined as the precursor ion detected at <5 ppm mass accuracy with S/N ≥ 3 and POD ≥ 0.8/1.0, AND at least one MS^2 fragment detected at < 5 ppm. A compound was not detected if the POD is < 0.8/1.0 and/or the precursor has S/N ≤ 3. Some of the compounds that were not detected included a few that were not stable in the spiking mixture (amoxicillin, cloxacillin, and sulfanilamide) and others with a very low MRL value or poor response (clenbuterol, zilpaterol, ivermectin). The DDA had more analytes that were not confirmed with MS^2 fragment ions, likely due to the need for further optimization of the MS^2 trigger thresholds in the acquisition method inclusion list.

Table 2. Summary of all compounds analyzed in bovine muscle matrix on the Orbitrap Exploris 120 MS using the AOAC POD and SANTE confirmation guidelines.

Compound	Class	RT	Polarity	MRL ng/g	Screening level ng/g	DIA	DDA
5-Hydroxyflunixin	Metabolite	8.67	POS	20	10	●	●
Abamectin B1a	Antihelmintic	11.78	POS	10	5	●	✕
Albendazole	Antihelmintic; antiparasitic	7.47	POS	100	50	●	●
Albendazole 2-aminosulfone	Metabolite	4.43	POS	100	50	●	●
Albendazole sulfone	Metabolite	6.23	POS	100	50	●	●
Albendazole sulfoxide	Metabolite	5.58	POS	100	50	●	●
Amoxicillin	Antibiotic (β-lactam)	5.10	POS	10	5	✕	✕
Ampicillin	Antibiotic (β-lactam)	5.33	POS	10	5	●	●
Betamethasone	Steroid anti-inflammatory	7.95	POS	0.75	0.375	●	●
Carazolol	Beta blocker	5.89	POS	5	2.5	●	●
Carprofen	NSAID	9.46	NEG	500	250	●	●
Cefalexin	Antibiotic (β-lactam)	5.33	POS	200	100	●	●
Cefapirin	Antibiotic (β-lactam)	4.20	POS	50	25	●	●
Cefazolin	Antibiotic (β-lactam)	5.29	POS	100	50	●	●
Cefoperazone	Antibiotic (β-lactam)	5.79	POS	100	50	●	●
Cefquinome	Antibiotic (β-lactam)	4.68	POS	50	25	●	●
Ceftiofur	Antibiotic (β-lactam)	6.58	POS	1000	500	●	●
Chlortetracycline	Antibiotic (tetracycline)	5.25	POS	200	100	●	●
Ciprofloxacin	Antibiotic (quinolone)	4.76	POS	100	50	●	●
Clenbuterol	β-agonist	5.37	POS	0.1	0.05	●	✕
Clopidol	Coccidiostat (antiprotozoal)	4.39	POS	200	100	●	●
Clorsulon	Antihelmintic	6.32	NEG	35	17.5	●	●
Closantel	Antihelmintic	11.84	POS	1000	500	●	●
Cloxacillin	Antibiotic (β-lactam)	9.40	POS	10	5	●	●
Cyromazine	Insecticide; ectoparasiticide	0.83	POS	200	100	●	●
Danofloxacin	Antibiotic (quinolone)	4.92	POS	70	35	●	●
Decoquinate	Coccidiostat (antiprotozoal)	11.42	POS	1000	500	●	●
Dexamethasone	Steroid anti-inflammatory	7.98	POS	0.75	0.375	●	●
Diclazuril	Coccidiostat (antiprotozoal)	9.81	NEG	10	5	●	●
Diclofenac	NSAID	9.68	POS	5	2.5	●	●
Dicloxacillin	Antibiotic (β-lactam)	8.85	POS	300	150	●	●
Difloxacin	Antibiotic (quinolone)	5.32	POS	400	200	●	●
Doramectin	Antihelmintic	11.94	POS	10	5	●	●
Doxycycline	Antibiotic (tetracycline)	6.17	POS	100	50	●	●
Emamectin B1a	Antihelmintic	9.95	POS	20	10	●	●
Enrofloxacin	Antibiotic (quinolone)	5.00	POS	100	50	●	●
Epichlorotetracycline	Antibiotic (tetracycline)	5.03	POS	200	100	●	●
Epitetracycline	Antibiotic (tetracycline)	4.65	POS	100	50	●	●
Eprinomectin B1a	Antihelmintic	11.61	POS	50	25	●	●
Erythromycin	Antibiotic (macrolide)	7.17	POS	100	50	●	●
Febantel	Antihelmintic	9.68	POS	50	25	●	●

● Detected and confirmed; POD = 8 or greater, Mass accuracy precursor and at least on MS^2 fragment < 5 ppm

● Detected, not confirmed; POD = 8 or greater, Mass accuracy of precursor < 5 ppm

✕ Not detected; POS < 0.8/1.0 and/or S/N ≤ 3.

Table 2. Continued.

Compound	Class	RT	Polarity	MRL ng/g	Screening level ng/g	DIA	DDA
Fenbendazole	Antihelmintic	8.43	POS	50	25	●	●
Fluazuron	Insecticide	11.07	NEG	200	100	●	●
Flubendazole	Antihelmintic	7.67	POS	20	10	●	●
Flumequine	Antibiotic (quinolone)	7.56	POS	200	100	●	●
Flunixin	NSAID	9.02	POS	20	10	●	●
Gamithromycin	Antibiotic (macrolide)	6.00	POS	20	10	●	●
Halofuginone	Coccidiostat (antiprotozoal)	6.20	POS	10	5	●	●
Imidocarb	Antiparasitic; antiprotozoal	4.13	POS	300	150	●	●
Ivermectin B1a	Antihelmintic	12.36	POS	10	5	●	✕
Josamycin	Antibiotic (macrolide)	8.14	POS	200	100	●	●
Ketoprofen	NSAID	8.61	POS	250	125	●	●
Lasalocid	Coccidiostat (antiprotozoal)	12.13	POS	50	25	●	●
Levamisole	Antihelmintic	4.31	POS	10	5	●	●
Lincomycin	Antibiotic (macrolide)	4.30	POS	100	50	●	●
Maduramicin	Coccidiostat (antiprotozoal)	12.55	POS	250	125	●	●
Marbofloxacin	Antibiotic (quinolone)	4.62	POS	150	75	●	●
Mebendazole	Antihelmintic	7.37	POS	20	10	●	●
Meloxicam	NSAID	8.49	POS	20	10	●	●
Monensin	Antibiotic (ionophore)	12.16	POS	2	1	●	●
Moxidectin	Antihelmintic	12.15	POS	20	10	●	●
Nafcillin	Antibiotic (β-lactam)	8.67	POS	300	150	●	●
Narasin	Coccidiostat (antiprotozoal)	12.73	POS	15	7.5	●	●
Nitroxinil	Antihelmintic/antiparasitic	7.40	NEG	400	200	●	●
Oxacillin	Antibiotic (β-lactam)	8.10	POS	300	150	●	●
Oxibendazole	Antihelmintic	6.24	POS	100	50	●	●
Oxolinic acid	Antibiotic (quinolone)	6.41	POS	100	50	●	●
Oxytetracycline	Antibiotic (tetracycline)	4.70	POS	200	100	●	●
Penicillin G	Antibiotic (β-lactam)	7.46	POS	50	25	●	●
Penicillin V	Antibiotic (β-lactam)	7.94	POS	50	25	●	●
Pirlimicyn	Antibiotic	6.06	POS	100	50	●	●
Ractopamine	β-agonist	4.96	POS	10	5	●	●
Rafoxanide	Antihelmintic	12.24	NEG	30	15	●	●
Rifaximin	Antibiotic	9.52	POS	50	25	●	●
Robenidine	Coccidiostat	8.14	POS	5	2.5	●	●
Salinomycin	Coccidiostat (ionophore)	12.48	POS	50	25	●	●
Sarafloxacin	Antibiotic (quinolone)	5.33	POS	30	15	●	●
Spiramycin	Antibiotic (macrolide)	5.68	POS	200	100	●	●
Sulfachloropyridazine	Antibiotic (sulfonamide)	5.64	POS	6	3	●	●
Sulfadiazine	Antibiotic (sulfonamide)	4.20	POS	6	3	●	●
Sulfadimethoxine	Antibiotic (sulfonamide)	6.60	POS	6	3	●	●
Sulfadoxine	Antibiotic (sulfonamide)	5.89	POS	6	3	●	●

● Detected and confirmed; POD = 8 or greater, Mass accuracy precursor and at least on MS^2 fragment < 5 ppm

● Detected, not confirmed; POD = 8 or greater, Mass accuracy of precursor < 5 ppm

✕ Not detected; POS < 0.8/1.0 and/or S/N ≤ 3.

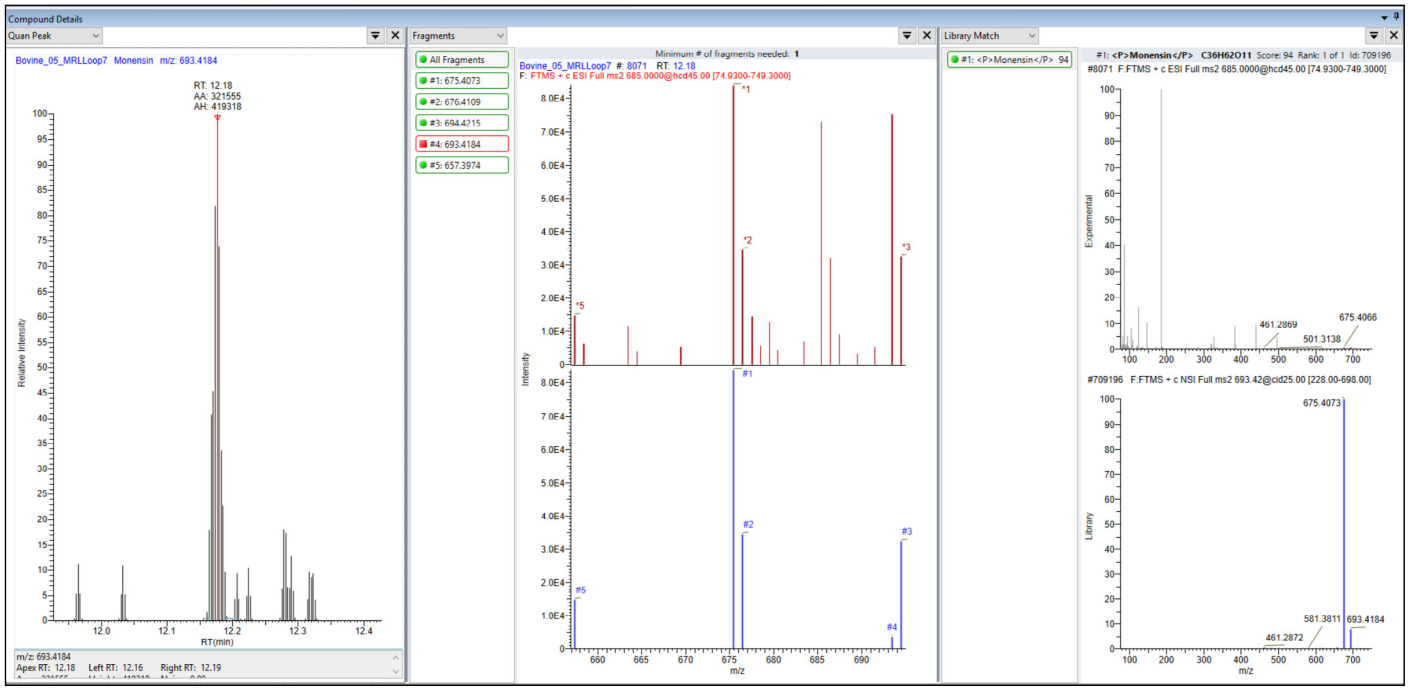
Table 2. Continued.

Compound	Class	RT	Polarity	MRL ng/g	Screening level ng/g	DIA	DDA
Sulfaguanidine	Antibiotic (sulfonamide)	1.00	POS	6	3	●	●
Sulfamerazine	Antibiotic (sulfonamide)	4.76	POS	6	3	●	●
Sulfamethazine	Antibiotic (sulfonamide)	5.19	POS	6	3	●	●
Sulfamethizole	Antibiotic (sulfonamide)	5.17	POS	6	3	●	●
Sulfamethoxazole	Antibiotic (sulfonamide)	5.79	POS	6	3	●	●
Sulfamethoxypyridazine	Antibiotic (sulfonamide)	5.26	POS	6	3	●	●
Sulfamonomethoxine	Antibiotic (sulfonamide)	5.62	POS	6	3	●	●
Sulfamoxole	Antibiotic (sulfonamide)	5.03	POS	6	3	●	●
Sulfanilamide	Antibiotic (sulfonamide)	1.15	POS	6	3	✕	✕
Sulfapyridine	Antibiotic (sulfonamide)	4.60	POS	6	3	●	●
Sulfaquinoxaline	Antibiotic (sulfonamide)	6.67	POS	6	3	●	●
Sulfathiazole	Antibiotic (sulfonamide)	4.47	POS	6	3	●	●
Sulfisoxazole	Antibiotic (sulfonamide)	6.06	POS	6	3	●	●
Teflubenzuron	Insecticide	10.77	NEG	100	50	●	●
Tetracycline	Antibiotic (tetracycline)	5.00	POS	100	50	●	●
Thiabendazole	Antiparasitic; antiroundworm	4.58	POS	100	50	●	●
Thiamphenicol	Antibiotic	5.00	POS	50	25	●	●
Tildipirosine	Antibiotic (macrolide)	4.30	POS	400	200	●	●
Tilmicosin	Antibiotic (macrolide)	6.33	POS	50	25	●	●
Tolfenamic acid	NSAID	10.58	POS	50	25	●	●
Triclabendazole	Antihelmintic	10.14	POS	200	100	●	●
Trimethoprim	Antibiotic	4.65	POS	50	25	●	●
Tulathromycin A	Antibiotic (macrolide)	5.02	POS	100	50	●	●
Tylosin	Antibiotic	7.33	POS	100	50	●	●
Tylvalosin	Antibiotic (macrolide)	8.70	POS	25	12.5	●	●
Virginiamycin M1	Antibiotic	8.41	POS	100	50	●	●
Zilpaterol	β-agonist	3.67	POS	2	1	✕	✕

● Detected and confirmed; POD = 8 or greater, Mass accuracy precursor and at least on MS^2 fragment < 5 ppm
● Detected, not confirmed; POD = 8 or greater, Mass accuracy of precursor < 5 ppm
✕ Not detected; POS < 0.8/1.0 and/or S/N ≤ 3.

The Orbitrap Exploris 120 mass spectrometer has a high-field Thermo Scientific™ Orbitrap™ mass analyzer, which provides fast scanning for accurate quantitation and more flexibility in the DIA experiment setup (shown in Figure 4a and b). Further experiments were performed using more precursor isolation windows, which can improve specificity for compound confirmation, especially at lower concentrations in heavily co-eluting matrices. Monensin formed a sodium adduct during ionization and was detected as a narrow chromatographic peak at 12 minutes. Using only four precursor mass isolation windows in the DIA experiment, the compound can be detected at 1 ng/g, but is not able to be confirmed, presumably due to more matrix background taken during the MS^2 experiment. By breaking up the mass range into 17 precursor isolation windows, the compound was detected and confirmed in all ten biological replicates with fragment matches and library searches.

a)



b)

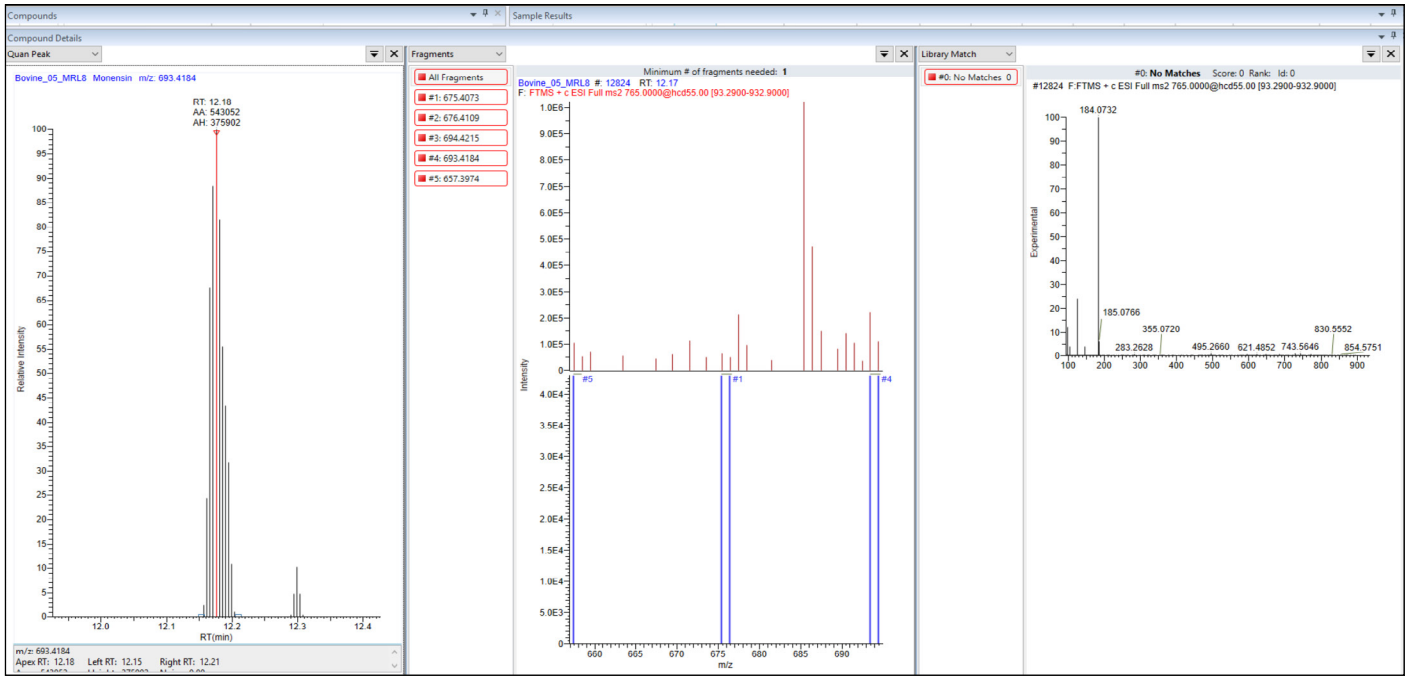


Figure 4. Monensin Na adduct detected at 1 ng/g in bovine muscle matrix. a) DIA acquisition method with 17 precursor isolation windows and b) DIA acquisition method with 4 precursor isolation windows. Note that the precursor and fragments were detected in all 10 biological replicates (POD = 1.0) with library match scores >90% when more isolation windows were used (a). Fragments were not detected when 4 precursor isolation windows were used (b).

Certified reference material (CRM)

Quantitation with the HRMS DIA method was evaluated using the certified reference material known as BOTS-1, which contains incurred veterinary drug residues in bovine muscle. The certified values are based on results from the National Research Council Canada (NRC), the Canadian Food Inspection Agency (CFIA), the USDA, and the German Federal Office of Consumer Protection and Food Safety (BVL) using tandem LC-MS/MS. Sample preparation was performed on three biological replicates of the BOTS-1 sample, using the QuEChERS extraction method with dSPE cleanup (CEC18). A calibration range of 0.5 to 1000 ng/g for all the analytes was prepared by spiking blank bovine muscle with all of the target analytes. All calibration standards were treated as matrix-extracted spikes (MES) and then used to quantitate the target analytes in the BOTS-1 sample. Table 3 and Figure 5 provide a summary of the results obtained.

Table 3. Average experimental results for three biological replicates versus the true value of incurred residues in BOTS-1. All results were confirmed with MS^2 fragment matches and precursor ions with mass accuracies < 5 ppm.

Compound	Experimental (ng/g)	True value (ng/g)
Chlorpromazine	164	147
Ciprofloxacin	15.4	15.7
Clenbuterol	2.4	1.1
Dexamethasone	3.3	3.2
Enrofloxacin	21	19
Meloxicam	0.9	1
Ractopamine	5.1	4.1
Sulfadiazine	699	763

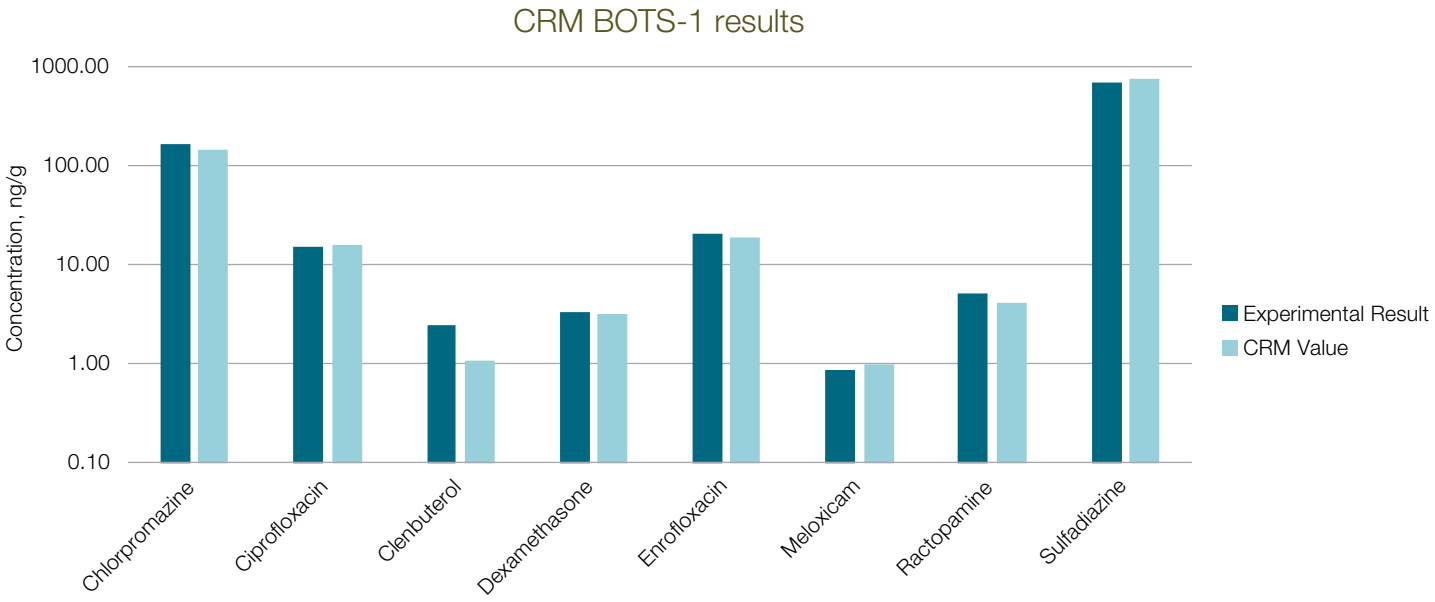


Figure 5. Average experimental results for three biological replicates versus the true value of incurred residues in BOTS-1. All results were confirmed with MS^2 fragment matches and precursor ions with mass accuracies < 5 ppm.

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Conclusion

The Orbitrap Exploris 120 mass spectrometer provided high-quality data in both DIA and ddMS^2 scan modes for both screening and quantitation assays applied to veterinary drugs. This preliminary method development for analysis of bovine muscle matrix using the AOAC SMPR and POD guidelines clearly demonstrates the instrument's ability to confidently screen samples over a very wide range of MRLs with confirmation per Guidance Document SANTE/12682/2019. The high-field Orbitrap mass analyzer provides added scan speed that is useful when increasing the number of precursor mass isolation windows used in a DIA experiment to improve specificity, while maintaining enough scans for quantitative analysis. The analysis of incurred residues in the BOTS-1 CRM sample demonstrated that the method is also quantitative and fit-for-purpose.

References and Acknowledgements

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