Multi-class veterinary drugs analyses of QuEChERS extracts using an automated online µSPE cleanup coupled to LC-MS/MS

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Goal

To demonstrate an automated online sample cleanup solution coupled to LC-MS/MS for rapid and robust screening and quantitation of veterinary drug residues in animal tissues.

Introduction

Veterinary drugs are administered to animals to ensure animal welfare. It is necessary to screen food products for veterinary drug residues at the maximum residue limits (MRL) set by global regulatory agencies. This screening typically involves both identification and quantification of veterinary drugs using LC-MS/MS.

A sample preparation approach often applied to veterinary drug screening in animal tissues is QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction.[1] This process involves a liquid-solid extraction of the sample with acetonitrile and salts. After the extraction, sample cleanup is often preferred. One common cleanup approach is dispersive solid phase extraction (dSPE), which involves adding a fixed amount of a powdered reagent (such as C18 or PSA) to the extract, vortexing for several minutes, then centrifugation and transfer into an autosampler vial. A second approach is solid phase extraction (SPE), in which the extract is passed through a sorbent material contained in a cartridge using a vacuum manifold. The goal of both cleanup approaches is to avoid the loss of target analytes whilst removing as many matrix co-extractives as possible, since they can cause ionization suppression and faster contamination of the LC-MS/MS detection system.

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The dSPE technique requires laboratory technicians to deal with weighing and dispensing reagents into labeled tubes, vortexing, centrifugation, and finally filtration. If using SPE, the extraction flow rate must be carefully controlled by constant monitoring with a manual vacuum manifold. As a result, errors will be made and the overall method extraction precision can be impacted, especially for novice technicians learning the procedure in the lab. An automated, online workflow to address the cleanup procedure required for QuEChERS extract will reduce the potential for errors, save time as well as allow use of lower volumes of solvents and materials compared to manual approaches.[2]

This application note describes a fully automated approach to online cleanup of QuEChERS extracts of animal tissues for LC-amenable veterinary drugs, based on the use of μSPE cartridges. These μSPE cartridges are compatible with the Thermo Scientific™ TriPlus™ RSH μSPE autosampler, which was coupled to a Thermo Scientific™ Vanquish™ Flex binary UHPLC system interfaced with a Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer. Bovine muscle, liver, and kidney extracts were selected as matrices for evaluation of the automated μSPE-LC-MS/MS methods. A total of 103 veterinary drug residues representing a variety of compound classes were spiked into bovine muscle, liver or kidney extracts to evaluate the automated μSPE-LC-MS/MS method.

Experimental

Samples and extraction

Bovine muscle samples were purchased at a local grocery store. Bovine liver and kidney samples were obtained through the Veterinary Diagnostic Laboratory at Iowa State University (ISU VDL). All tissues were homogenized using a laboratory blender. Five grams of tissue 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 at 2500 rpm on a Fisherbrand™ Digital MultiTube Vortexer for 10 minutes. Matrix extracted standards (MES) were prepared by spiking a mix of 103 veterinary drug residues prior to QuEChERS extraction into bovine muscle, kidney, and liver at different concentration levels. Matrix matched standards (MMS) were prepared by spiking the same mixture of veterinary drugs into extracts after the cleanup step. The concentration levels investigated were from 1 to 100 ng/g. All standards were cleaned up using either a manual dSPE and/or two different automated μSPE workflows. Samples were transferred to LC-MS/MS autosampler vials prior to analysis.

Dispersive SPE (dSPE) and Micro SPE (μSPE) Cleanup

Extract cleanup approaches included manual dispersive solid phase extraction (dSPE), as well as two fully automated online μSPE cartridge cleanups utilizing the robotic TriPlus RSH μSPE autosampler. For the dSPE experiments, 500 mg CEC18 was added to the supernatant and shaken on a vortexer for 15 minutes, and then centrifuged at 3000 rpm for 10 minutes. These were then placed into the autosampler for injection.

Automated μSPE cleanup was performed using the robotic TriPlus RSH μSPE autosampler system (P/N C0950-01-00611), based on a PAL3-RTC autosampler from CTC Analytics (CTC Analytics AG, Zwingen, Switzerland). The configuration of the system modules required to perform the automated μSPE cleanup is shown in Figure 1. Thermo Scientific™ TraceFinder™ software was used for system control. Two miniaturized SPE cartridges, each with a different sorbent, were developed and optimized based upon experiments performed earlier at Iowa State University or the PAL System North America Regional Office laboratory located in Lake Elmo, MN. The result were two μSPE cartridges for further evaluation as described in this application note: a) μSPE, QuEChERS blend for LC, P/N 60101-15VDC18 containing 15 mg CEC18 and b) μSPE, QuEChERS blend for LC, P/N 60101-10VDHPR containing 10 mg Thermo Scientific™ HyperSep™ Retain-PEP material (HRP). A new μSPE cartridge design used in these experiments is described in Figure 2. A leak-proof seal is obtained when the needle is compressed by the PAL into the cartridge, which allows the syringe to push sample extracts or solvents through the sorbent bed with a constant force. Essentially, the syringe replaces the vacuum system of the classical SPE methodology working at defined flow rates. Procedurally, uncleaned QuEChERS extracts were transferred into 2 mL autosampler vials and placed into a 54-position tray (sample tray). The corresponding number of collection vials [500 μL, fused insert snap-top (P/N C4011-LV1) sealed with star-slit snap-it caps (P/N C4011-59)] were placed into a second 54-position tray (eluate tray). Uncleaned extracts were loaded onto the cartridges according to the steps shown in Tables 1 and 2 for the CEC18 and HRP μSPE cartridges, respectively, and the extracted samples were injected directly into the LC-MS/MS system.
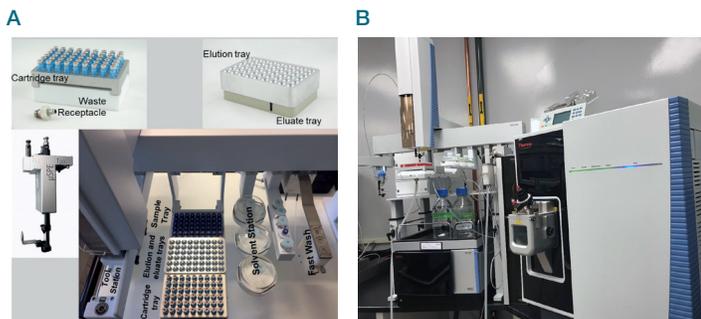
Figure 1. TriPlus RSH with µSPE capabilities system: a) TriPlus RSH modules required to perform the µSPE cleanup procedure and b) coupled to LC-MS/MS for automated on-line injection.
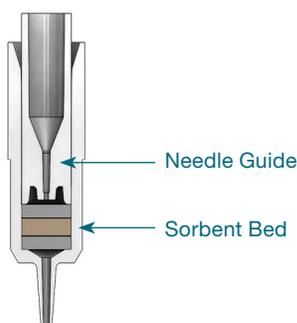

Figure 2. Automated µSPE cartridge. The leak-proof concept shown here requires no rubber seals, minimized dead volume and increases sorbent bed pressure rating.

Table 1. Steps for automated online µSPE cleanup method with LC injection using the CEC18 cartridge

Step	Action
1	Aspirate 300 µL of QuECheRS extract into the syringe
2	Move µSPE cartridge to elution tray
3	Load 300 µL QuECheRS extract onto µSPE cartridge
4	Perform µSPE -push extract through cartridge
5	Move-dispose of cartridge to waste bucket
6	Change to LC/MS injection tool
7	Perform sandwich injection
8	Change to prep syringe for next sample
9	Proceed with prep-ahead for next extract sample upon ready Signal

Table 2. Steps for automated online µSPE cleanup method with LC injection using the HyperSep Retain-PEP cartridge

Step	Action
1	Prep syringe with elution solvent
2	Condition µSPE with 300 µL methanol
3	Condition µSPE with 300 µL water
4	Condition µSPE with 100 µL QuEChERS extract
5	Move µSPE cartridge to elution tray
6	Load 300 µL QuEChERS extract onto µSPE cartridge
7	Perform µSPE-push extract through cartridge
8	Move-dispose of cartridge to waste bucket
9	Change to LC/MS injection tool
10	Perform sandwich injection
11	Change to prep syringe for next sample
12	Proceed with prep-ahead for next extract sample upon ready Signal

LC-MS/MS analysis

The LC-MS/MS system comprised a Vanquish Flex UHPLC binary system interfaced with a TSQ Altis triple quadrupole mass spectrometer equipped with a H-ESI ionization probe. Chromatographic separation was carried out on a Thermo Scientific™ Accucore™ VDX column, 100 mm × 2.1 mm × 2.6 µm (P/N VDX-102130) at a temperature of 40 ˚C, using a gradient elution of 0.05 % formic acid in water (mobile phase A) and 1:1 methanol : acetonitrile with 0.05% formic acid and 5% water (mobile phase B). Table 3 shows HPLC gradient conditions. The mass spectrometer was operated in both positive and negative ionization modes, with the optimized parameters shown in Table 4. A solvent sandwich injection technique (Figure 3a) was used for injection of all samples, which greatly improved the LC peak shapes of the early eluting compounds in the analysis. The sandwich injection contained 20 µL of mobile phase A on each side of a 2 µL volume of sample that is in pure acetonitrile. A rear air gap of 3 µL and a front air gap of 1 µL was used.

Table 3. LC Gradient Conditions

Time (min)	%B	Flow rate (mL/min)
0	2	0.3
2	2	0.3
3	20	0.3
11	100	0.3
13	100	0.4
14.4	100	0.4
14.5	2	0.35
16	2	0.3
20	2	0.3

Table 4. MS Parameter Settings

Parameter	Setting
Spray voltage	3500 V, positive ionization mode 2500 V, negative ionization mode
Sheath gas	50 arb
Auxiliary gas	13 arb
Sweep gas	1 arb
Ion transfer tube temperature	310 °C
Vaporizer temperature	350 °C
Cycle time for scheduled SRM transitions	0.35 s
Q1 resolution	0.7 Da
Q3 resolution	1.2 Da
CID gas	1.5 mTorr

Data Processing

The acquired data were processed using TraceFinder software and Thermo Scientific™ Freestyle™ software was used for qualitative analysis.
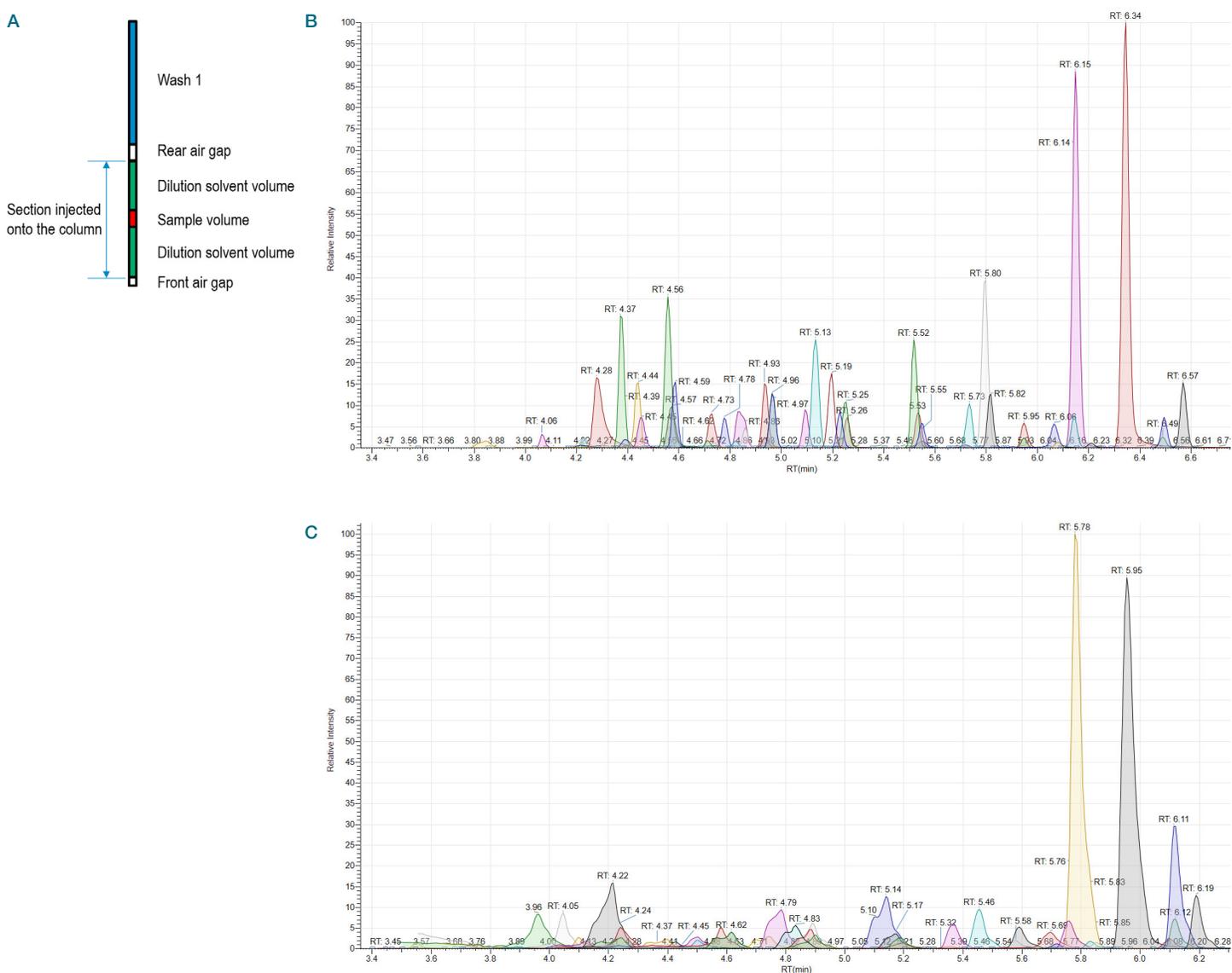

Figure 3. a) Solvent sandwich is an injection procedure which dilutes/mixes the sample in the needle/capillary with two segments of solvent; b) Peak shapes with solvent sandwich injection technique; c) Peak shapes for a 2 µL pure acetonitrile injection.

Results and discussion

The veterinary diagnostic lab at Iowa State University routinely screens for a wide variety of veterinary drugs with QuEChERS extraction followed by manual dSPE or SPE cleanups. Extracts of three tissue types, (bovine muscle, liver, and kidney) were selected to evaluate the automated online µSPE-LC-MS/MS cleanup methods described herein. Method performance criteria, including linearity, absolute recovery, precision, reproducibility, and robustness were evaluated. Absolute recovery was verified at 50 ng/g and reproducibility of the method was verified at 5 and 50 ng/g. Method effectiveness was checked by comparing the conventional manual dSPE with CEC18 QuEChERS cleanup procedure. Also, the effectiveness of the automated µSPE CEC18 cleanup was compared to that of the automated µSPE procedure using the Hypersep Retain-PEP (HRP).

The selected 103 veterinary drugs represent a wide variety of compound classes and are listed in the AOAC Standard Method Performance Requirements document (SMPR 2018.010).[3] An overlay of SRM ion chromatograms for all veterinary drugs analyzed in this method is shown in Figure 4. Good separation and detection of the compounds was achieved using the scheduled-SRM mode.
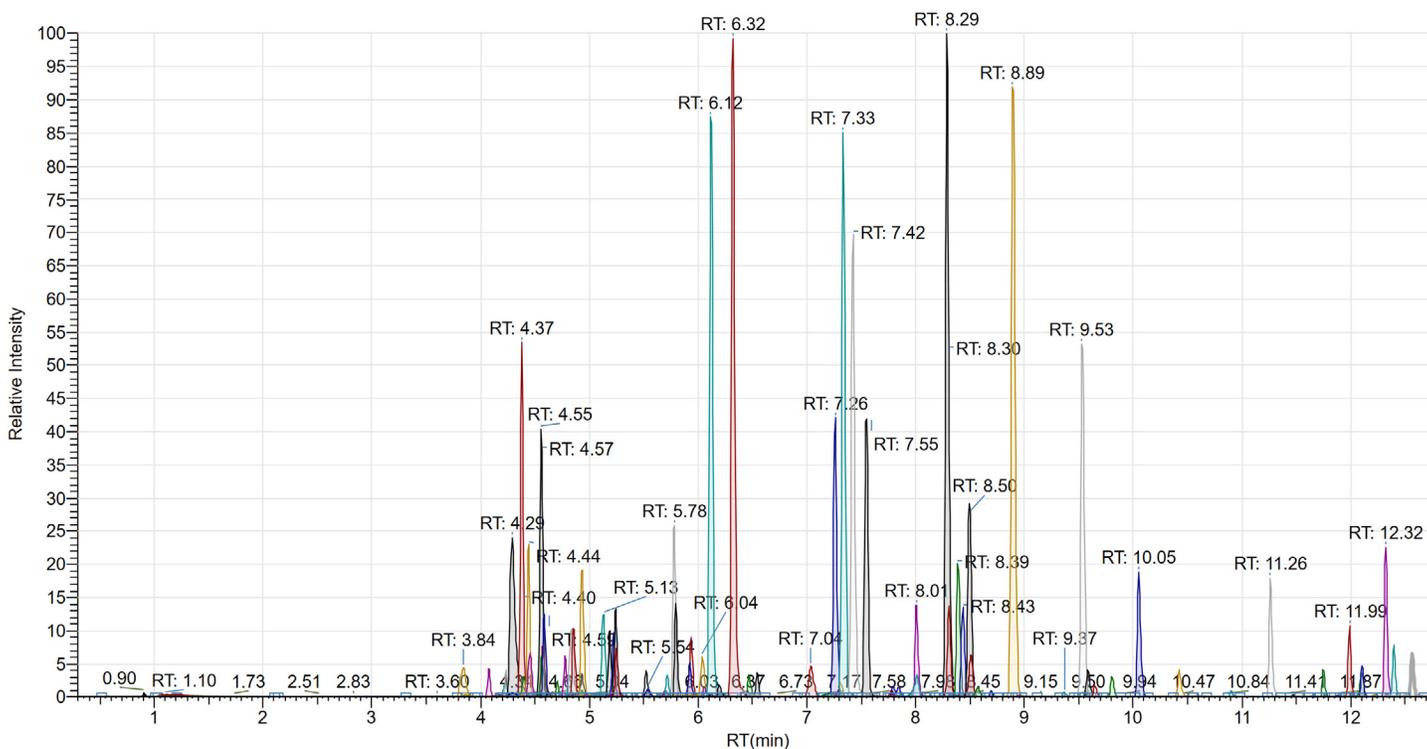

Figure 4. Overlaid chromatograms of all 103 veterinary drugs included in the method, 50 ng/g in bovine kidney extract.

µSPE cleanup effectiveness and matrix effects

Matrix effects in electrospray ionization are typically manifested by a lower signal of the analyte in the matrix than in pure solvent.[4] Signal suppression occurs due to competition for charge between molecules during the ionization process. Therefore, fewer molecules of the analyte are ionized than in a pure solvent. Matrix co-extractives from tissue extracts can also passivate the analytical column, and in some cases enhance the signal of analytes relative to those in pure solvents. Ionized matrix components not monitored in the SRM chromatograms, but present in the background, can affect accuracy and sensitivity not only for the current injection, but also carry over into subsequent injections. As an example, LC-high resolution accurate mass (HRAM) data was acquired in full scan for a bovine muscle extract to compare a non-cleaned up sample total ion chromatogram (TIC) to the same sample cleaned up with the automated CEC18 µSPE procedure. As can be seen in Figure 5, a large amount of matrix is removed between 11 and 13 minutes by the cleanup.

Matrix effects were also evaluated by comparing the responses of veterinary drug standards prepared in solvent (neat solution) and in the different sample matrices. Figure 6 is a comparison of % matrix effect (ME) across the various tissue extract types and cleanup techniques. Percent matrix effect is rated as low (ME<20%), medium (20<ME<50), or high (ME>50). For all combinations, most analytes showed low to moderate ME (70–80% of compounds). For ME>50%, matrix enhancement was observed to be the primary cause (over 95% of compounds in this category).
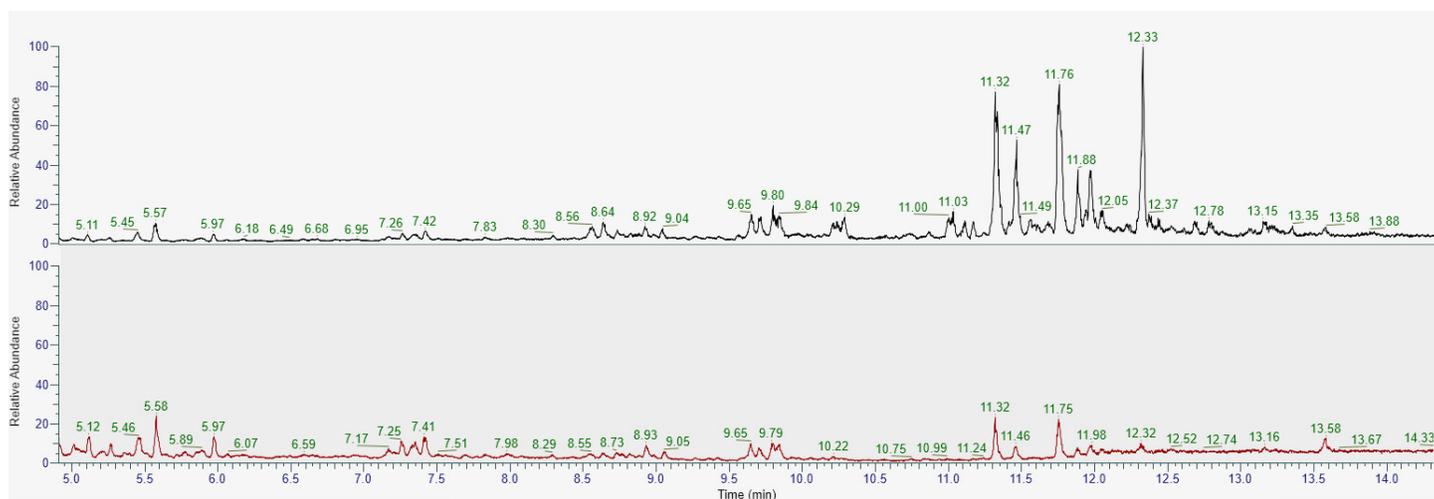

Figure 5. Bovine muscle extract analyzed by LC-HRAM MS in full scan. Top trace is extract injected without cleanup, bottom is extract cleaned up with the automated µSPE procedure containing CEC 18.
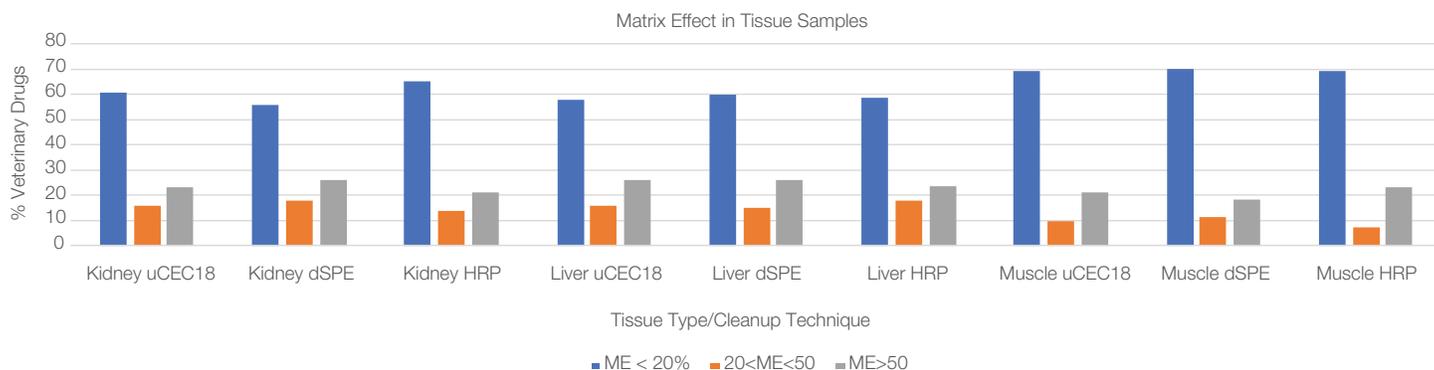

Figure 6: Summary of % matrix effects (ME) for the three tissue types and applied cleanup techniques.

Calibration linearity

For screening and quantitation of the veterinary drugs, calibration levels using matrix extracted standards (MES) and/or matrix matched standards (MMS) were prepared in all three tissue types. Calibration standards ranging from 1 to 100 ng/g were analyzed and excellent linearity was achieved, with coefficient of determination $R^2 > 0.99$ for most compounds in the three different extracts. For screening workflow, the Veterinary Diagnostic Lab at Iowa State University created calibration curves using an automatic calibration script on the TriPlus RSH µSPE system. In this case, the analyst prepared a stock standard in solvent and used the blank QuEChERS matrix as a diluent. The system proceeds to prepare all specified standard levels and mixes them before passing them through the µSPE cartridge and subsequent injection into the LC-MS/MS system. This saves time and reduces potential handling and labeling errors in the lab. Figure 7 shows representative calibration curves for sarafloxacin and sulfamethazine in bovine extract. The MES curves were prepared manually outside of the autosampler (procedural calibration) and the MMS curves were prepared using the automated calibration script.

µSPE method performance

The absolute recoveries of 103 veterinary drugs using the cleanups described above (manual dSPE, CEC18-µSPE, and HRP-µSPE) were evaluated in bovine muscle, liver, and kidney tissues. For each experiment, five biological replicates were prepared as matrix-extracted spikes (MES) containing all the target residues at 50 ng/g. The MES were compared to standards spiked into the cleaned samples (MMS) for each of the different cleanups 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. Absolute recoveries within 30–140% with corresponding %RSDs less than or equal to 20% are required for satisfactory method validation according to the EU SANTE 12682/2019 document for pesticides.[5]
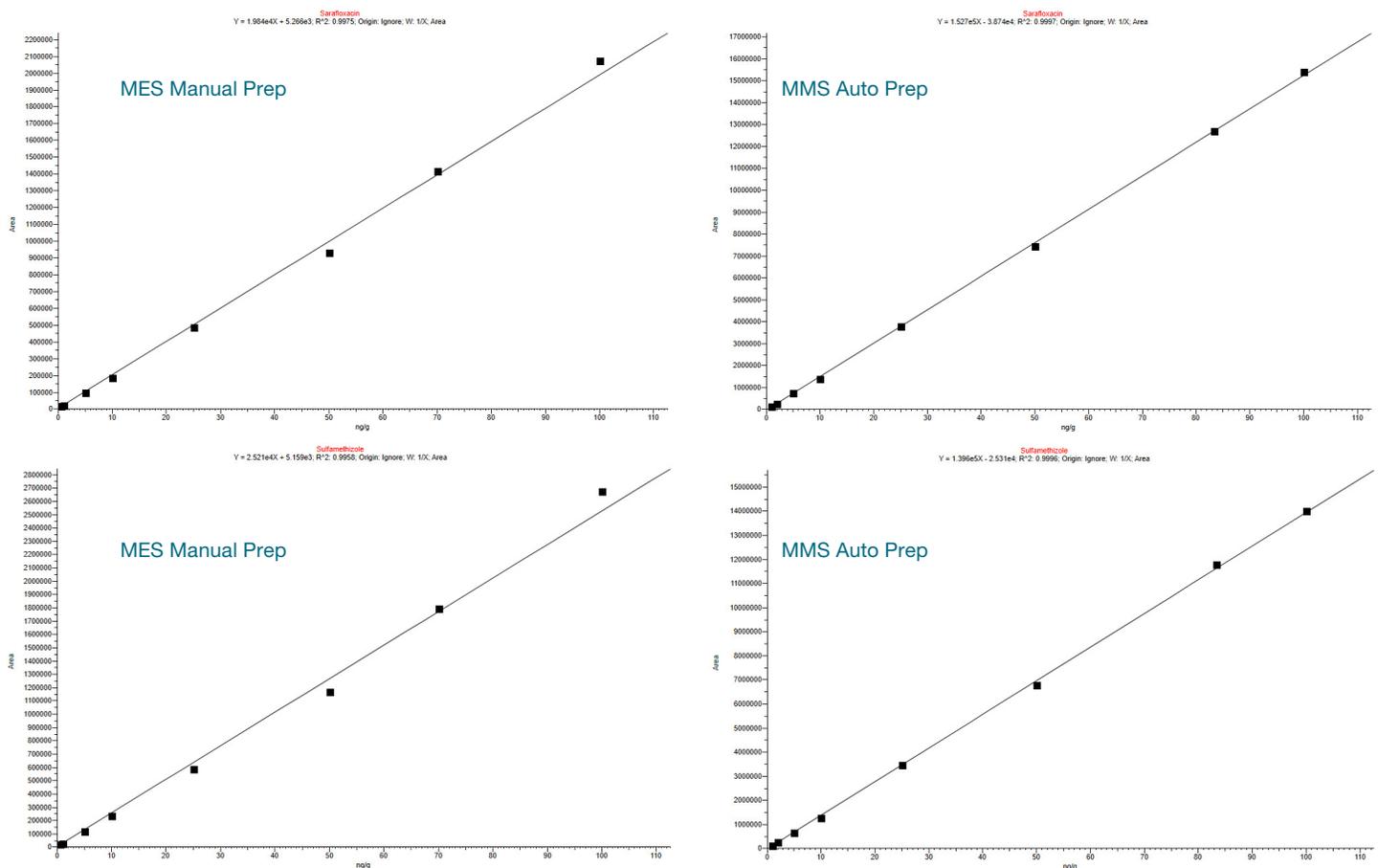
Figure 7. MES and MMS curves in bovine muscle with µSPE CEC18. The MES curves on the left side of the figure were prepared manually by the lab technician. The MMS curves on the right right side of the figure were prepared using the automated calibration script in the TriPlus RHS autosampler.

Recoveries within this range were achieved for over 95% of the target veterinary drugs in the three tissue extract types for both the manual CEC18 dSPE procedure and the automated online cleanup using the miniaturized CEC18 μSPE cartridges. (Figures 8 and 9). Table 5 summarizes the absolute % spike recoveries and %RSD values for all three cleanup techniques in all three matrices. Using the HRP material, it was noted that the recoveries of rafoxanide, closantel, and lasalocid are very poor (typically less than 5%). This is likely due to retention of these analytes on the sorbent during the cleanup, and we are undergoing further study to determine the root cause. Some of the compounds also demonstrated low sporadic recoveries with high %RSD in some matrices. However, these are easily detected at less than 5 ng/g in the bovine extracts.

A precision study was also carried out using the CEC18 μSPE miniaturized cartridge cleanup in all three tissue types. MES calibration curves were prepared at calibration levels of 0.5, 1, 5, 10, 25, 50, 70, and 100 ng/g, along with five biological replicates of each matrix prepared at 5 and 50 ng/g. Excellent precision of less than 20% RSD and recovery between 70 and 120% were obtained for over 90% of the veterinary drugs in each matrix at both concentration levels. An example is shown in Figure 10, with recoveries and %RSD for bovine kidney tissue extracts cleaned up with the CEC18-μSPE miniaturized cartridges.

Figure 8. % Absolute recoveries vs %RSD in bovine muscle, liver, and kidney tissue extracts at 50 ng/g (N=5). The red boxes represent recoveries within 30–140% with %RSD <20%.

Figure 9. % Absolute recoveries vs %RSD in bovine muscle, liver, and kidney tissue extracts at 50 ng/g (N=5). The red boxes represent recoveries within 30–140% with %RSD <20%.

Table 5. LC-MS/MS % absolute spike recoveries in different animal tissues at 50 ng/g using both automated µSPE and manual d-SPE.
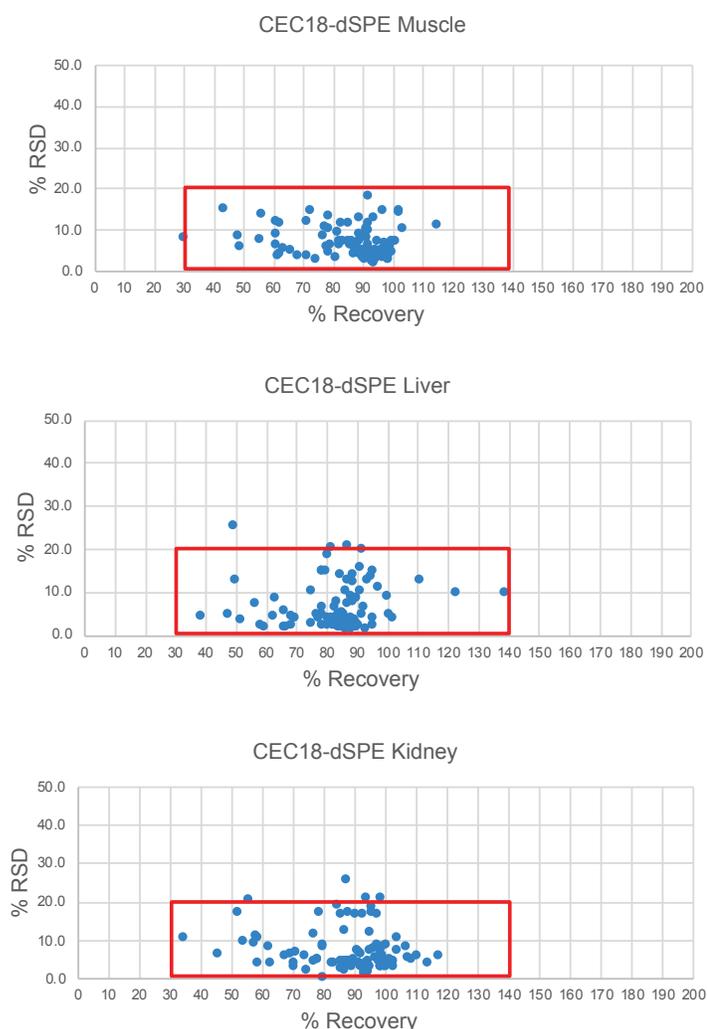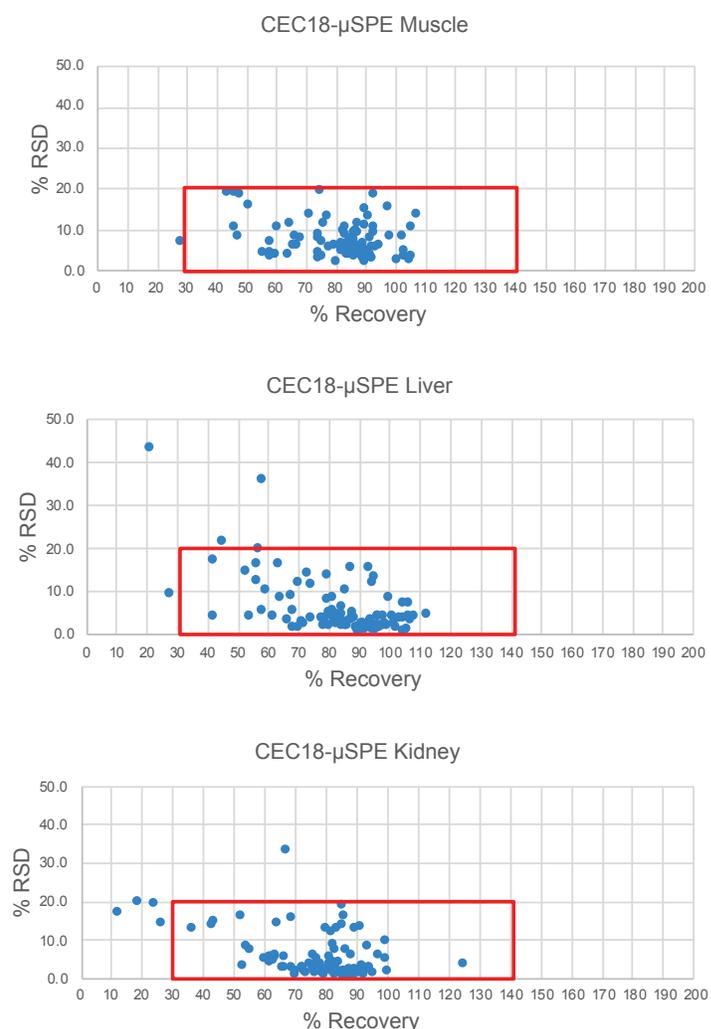Procedural standard spikes (MES) were compared to standards spiked into the cleaned samples (MMS) for each of the different cleanups at 50 ng/g.

Compound	Bovine Muscle						Bovine Liver						Bovine Kidney					
	dSPE-CEC18		µSPE-CEC18		µSPE-HRP		dSPE-CEC18		µSPE-CEC18		µSPE-HRP		dSPE-CEC18		µSPE-CEC18		µSPE-HRP	
	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD
5-hydroxyflunixin	71	3.4	66	8.4	**	23.2	81	3.3	79	3.6	58	1.3	86	2.4	78	3.5	74	8.1
Abamectin B1a	85	11.5	103	8.6	112	9.7	83	7.9	74	11.4	89	4.9	80	8.2	54	8.3	111	13.4
Albendazole	97	5.2	90	4.7	81	4.8	83	6.6	78	3.8	82	4.1	100	2.8	81	2.4	94	11.5
Albendazole 2-aminosulfone	93	3.8	85	4.3	92	3.3	85	2.2	81	8.5	85	2.9	93	2.7	81	5.8	98	7.5
Albendazole sulfone	115	11.2	105	3.6	101	5.2	93	1.4	105	0.7	89	1.9	96	18.3	93	2.1	104	9.0
Albendazole sulfoxide	96	14.5	101	2.5	108	3.5	79	15.0	105	1.0	99	1.1	87	12.4	92	1.1	112	7.6
Amoxicillin	30	7.8	28	7.3	23	8.6	39	4.5	42	3.9	42	6.5	35	10.5	53	3.1	57	5.1
Ampicillin	48	8.3	47	8.5	40	5.2	52	3.4	54	4.2	57	3.2	54	9.6	60	5.0	74	6.5
Betamethazone	100	4.1	87	9.3	97	10.0	89	7.7	84	2.9	84	2.2	92	6.7	84	4.3	100	9.6
Carazolol	97	4.3	90	4.0	105	4.9	85	5.2	96	1.3	96	1.8	94	1.2	92	2.5	109	7.0
Carprofen	88	4.8	91	3.8	107	4.6	79	5.1	83	2.3	98	3.5	87	2.5	79	2.3	107	7.7
Cefalexin	44	14.8	44	19.4	58	13.4	48	4.7	59	10.1	81	8.4	46	6.2	83	12.7	107	4.7
Cefapirin	78	5.6	75	3.7	94	3.5	79	2.3	88	4.9	93	3.3	87	4.2	76	5.8	106	11.5
Cefazolin	82	9.2	83	9.2	98	9.1	87	7.5	81	4.1	109	9.3	91	7.1	67	33.2	127	13.8
Cefoperazone	82	7.2	83	10.7	118	2.9	93	6.4	105	6.9	114	11.2	95	7.0	86	7.2	111	13.5
Cefquinome	92	17.9	80	6.1	161	25.3	75	10.5	67	8.8	132	7.5	95	11.9	86	16.2	136	34.8
Ceftiofur	86	6.8	75	7.2	91	4.1	85	2.4	89	1.5	91	3.6	92	3.6	77	1.2	107	8.3
Chlortetracycline	61	8.6	60	10.6	67	10.3	69	4.6	73	13.9	74	5.1	71	6.6	77	5.3	78	9.2
Ciprofloxacin	74	2.4	68	7.8	78	2.9	70	3.9	80	1.7	79	7.0	77	4.4	81	3.9	94	7.5
Clenbuterol	96	4.6	90	4.6	108	1.4	83	3.0	86	2.8	100	2.1	94	3.3	87	2.5	108	3.9
Clopidol	94	4.9	82	5.0	103	5.7	85	3.4	85	9.9	99	4.2	93	3.4	83	8.7	108	2.7
Clorsulon	92	3.1	86	4.5	108	3.5	90	2.7	102	1.5	96	2.4	99	4.3	91	1.6	108	4.0
Closantel	71	11.9	71	13.7	0	0.0	92	20.1	86	2.0	2	NA	99	20.7	66	2.7	0	NA
Cloxacillin	92	4.6	90	3.2	93	2.4	80	2.5	88	3.7	89	3.3	92	2.9	88	6.2	102	5.0
Danofloxacin	79	6.0	65	11.6	74	7.9	77	3.9	83	2.9	60	2.1	84	3.9	79	0.8	79	20.7
Decoquinate	77	10.6	74	8.1	106	12.4	80	18.9	52	14.5	122	2.6	91	16.7	43	13.9	100	18.9
Dexamethasone	93	2.1	92	5.8	94	2.9	89	2.7	87	3.1	93	3.1	95	2.8	92	3.3	106	8.2
Diclazuril	94	4.2	88	4.3	125	3.9	90	8.5	97	1.3	108	1.6	98	8.8	89	1.5	111	10.4
Diclofenac	90	5.0	82	6.9	71	6.8	78	6.6	58	5.3	97	3.8	88	4.5	62	4.2	89	3.8
Dicloxacillin	94	5.0	89	4.1	97	3.7	83	4.1	93	2.0	92	2.3	94	2.3	92	1.4	103	6.8
Difloxacin	91	4.0	94	5.6	92	3.5	85	3.6	83	4.4	86	3.8	92	3.5	77	3.0	86	8.2
Doramectin	89	8.5	98	15.5	89	10.4	91	15.9	63	16.0	99	8.0	83	3.7	43	14.6	99	12.8
Doxycycline	62	3.6	58	7.1	61	3.5	63	4.5	70	1.4	62	2.9	67	5.5	70	0.9	84	7.6
Emamectin B1a	91	10.2	85	6.7	112	8.5	89	12.3	56	12.1	92	1.5	94	20.6	62	5.7	105	25.4
Enrofloxacin	87	3.8	78	6.0	83	4.5	81	2.8	80	4.9	81	1.9	89	4.1	70	1.9	97	7.1
Eprinomectin B1a	92	11.2	107	13.7	107	7.0	94	12.9	62	3.8	105	2.1	87	25.5	63	4.8	109	16.7
Erythromycin	90	4.8	85	7.7	105	9.0	80	4.2	90	0.5	98	2.4	99	4.4	89	0.8	112	12.7
Febantel	100	7.2	90	5.0	112	9.3	89	2.5	89	1.0	105	3.3	101	3.1	79	1.3	109	11.8
Fenbendazole	94	4.5	86	7.1	90	2.4	88	3.2	78	1.8	91	3.3	100	8.2	78	2.3	97	10.1
Fluazuron	82	11.3	90	15.2	91	9.1	95	13.7	82	3.0	92	4.8	80	8.6	75	3.5	81	22.9
Flubendazole	98	3.0	90	4.9	94	2.5	88	3.8	94	1.8	90	1.9	103	3.1	84	2.2	106	11.5
Flumequine	97	3.2	85	6.7	84	5.2	91	2.3	93	3.0	81	2.1	99	2.8	79	1.8	96	22.9
Flunixin	94	4.5	90	2.2	30	23.0	86	1.9	85	1.7	90	3.0	94	3.6	89	0.8	76	3.3
Gamithromycin	95	6.8	92	8.0	103	11.1	79	15.2	84	4.3	98	3.2	97	7.8	88	2.1	103	8.3
Halofuginone	78	4.6	74	3.2	81	8.1	66	2.1	74	3.7	80	4.4	75	1.9	77	3.1	89	8.2
Ivermectin B1a	72	14.3	75	19.8	106	12.9	86	4.8	56	16.0	102	6.1	85	19.0	91	13.5	107	22.2
Josamycin	102	14.7	93	9.2	105	4.1	97	11.0	92	2.2	107	3.9	104	10.7	69	2.6	109	13.8

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Table 5. Continued

Compound	Bovine Muscle						Bovine Liver						Bovine Kidney					
	dSPE-CEC18		µSPE-CEC18		µSPE-HRP		dSPE-CEC18		µSPE-CEC18		µSPE-HRP		dSPE-CEC18		µSPE-CEC18		µSPE-HRP	
	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD
Ketoprofen	93	3.5	84	4.0	98	2.7	83	3.9	91	2.2	93	2.4	93	1.7	87	1.1	106	6.4
Lasalocid	61	11.7	91	13.6	0	0.0	77	4.7	42	17.0	80	NA	79	16.9	52	16.0	40	NA
Levamisole	93	3.1	85	4.4	106	2.3	84	1.8	80	7.9	104	1.4	93	3.4	83	7.4	104	2.9
Lincomycin	68	3.3	64	4.1	68	3.4	69	2.3	79	13.4	73	1.9	74	5.7	82	11.9	87	4.5
Maduramicin	62	11.4	77	13.6	100	8.8	57	7.2	21	43.1	80	7.5	56	20.3	24	19.4	95	34.0
Marbofloxacin	82	6.2	74	9.0	89	4.4	75	2.9	70	11.8	87	2.9	86	4.2	64	6.1	97	2.6
Mebendazole	98	2.8	89	3.3	95	3.1	87	2.6	94	0.8	90	2.4	100	3.8	86	0.8	106	10.6
Meloxicam	97	3.9	58	3.6	80	9.8	102	4.1	104	3.6	89	3.2	118	5.7	91	1.4	107	4.8
Monensin	79	13.2	93	18.9	125	15.8	86	10.4	58	35.5	172	7.3	59	10.6	69	15.5	107	22.6
Moxidectin	76	8.2	85	7.7	91	9.1	87	21.0	57	19.8	128	5.6	86	16.5	64	14.5	89	12.5
Nafcillin	93	4.5	84	3.9	91	2.0	84	3.3	96	1.7	88	1.9	95	1.4	96	1.6	102	8.9
Nitroxynil	89	7.1	82	5.6	38	18.6	84	1.8	95	1.9	91	2.7	95	3.0	83	1.4	72	8.8
Oxacillin	91	9.8	94	6.4	100	4.3	89	14.3	93	15.5	96	6.7	88	17.1	97	6.1	113	9.2
Oxibendazole	97	4.5	89	5.6	96	6.0	86	4.5	88	3.8	92	1.7	99	4.6	85	0.9	104	2.7
Oxolinic acid	97	5.2	86	7.5	87	9.8	90	3.7	95	1.0	88	3.2	98	4.1	83	1.0	101	10.0
Oxytetracycline	56	7.5	46	10.6	52	7.4	59	1.7	66	3.0	64	6.2	63	3.7	72	2.9	87	9.1
Penicillin G	81	3.1	80	2.2	86	1.8	84	4.8	91	2.3	84	2.4	90	3.0	87	1.8	95	9.6
Penicillin V	88	4.2	87	4.2	83	4.1	82	2.3	94	1.6	82	3.7	90	4.9	88	1.1	98	11.4
Pirlimycin	62	3.9	58	4.6	71	4.9	59	2.5	68	1.5	71	3.1	70	2.9	77	2.1	85	3.8
Ractopamine	87	5.9	86	6.9	102	5.7	85	2.7	98	1.7	96	4.8	103	4.1	91	1.7	106	4.7
Rafoxanide	56	13.5	51	16.1	0	0.0	87	12.9	64	8.3	1	NA	93	16.8	55	7.5	0	NA
Rifaximin	89	12.9	83	9.7	14	31.9	88	9.3	90	0.7	77	13.5	99	7.8	87	1.2	108	14.0
Sarafloxacin	86	6.1	89	6.6	89	4.1	81	2.8	86	1.8	87	2.3	88	4.3	82	3.9	85	7.1
Spiramycin	90	3.2	87	11.6	102	3.5	139	9.9	83	3.3	113	7.1	109	4.8	19	19.7	104	19.0
Sulfachlorpyridazine	97	6.5	103	4.8	96	3.6	86	2.7	106	3.8	85	3.7	80	0.0	93	2.4	100	9.2
Sulfadiazine	94	1.9	89	5.3	32	3.6	92	4.9	94	11.9	27	2.7	98	4.9	85	13.9	30	12.4
Sulfadimethoxine	97	5.3	84	5.7	71	6.0	95	2.5	98	2.3	68	4.3	102	4.6	88	2.0	83	13.1
Sulfadoxine	88	6.1	84	6.9	93	8.0	89	2.0	96	1.9	84	1.5	100	3.8	88	1.8	95	8.8
Sulfaguanidine	91	2.9	74	4.4	89	3.6	87	2.0	91	1.2	75	2.3	95	1.8	73	2.0	84	5.1
Sulfamerazine	93	12.9	105	2.8	88	6.4	82	20.6	108	4.2	85	3.9	77	11.5	100	2.0	107	6.1
Sulfamethazine	95	3.3	86	5.8	96	3.7	88	1.5	102	2.3	91	2.3	100	5.9	93	1.5	131	14.1
Sulfamethizole	88	6.5	103	3.5	90	4.1	83	2.5	101	4.1	79	3.2	107	8.1	94	2.8	80	16.2
Sulfamethoxazole	94	2.5	87	6.1	86	1.0	89	3.6	112	4.3	83	3.7	100	4.1	125	3.6	110	13.1
Sulfamethoxy-pyridazine	96	4.0	86	4.2	96	3.4	87	1.7	98	3.8	88	2.5	99	3.1	94	2.9	116	1.5
Sulfamonomethoxine	96	4.1	86	4.2	96	3.4	87	1.7	107	3.2	88	2.5	99	3.1	99	5.2	116	1.5
Sulfamoxole	91	7.7	92	2.9	85	2.1	111	12.7	96	3.8	92	2.0	78	4.6	86	1.7	108	5.3
Sulfanilamide	88	4.7	83	5.8	100	1.4	88	1.9	95	1.6	98	1.3	95	3.1	82	1.7	100	2.3
Sulfapyridine	90	2.7	83	5.4	111	4.7	90	2.1	103	3.7	102	2.5	99	6.6	94	8.4	111	7.5
Sulfaquinoxaline	90	4.9	85	4.4	86	4.1	87	2.4	97	1.6	81	1.8	87	2.0	88	1.7	98	10.5
Sulfathiazole	99	5.9	86	3.8	93	2.2	87	2.2	87	15.4	87	2.5	94	4.3	85	18.7	103	4.3
Sulfisoxazole	91	7.7	92	2.9	85	2.2	88	2.4	96	3.8	92	2.0	78	4.6	86	1.7	108	5.3
Teflubenzuron	83	6.8	105	10.7	95	11.2	95	15.0	84	5.9	84	6.0	100	8.5	66	2.7	85	17.4
Epitetracycline	79	10.1	66	6.2	40	11.2	63	8.5	71	2.9	52	9.0	62	8.2	72	2.5	78	20.5
Tetracycline	64	5.2	67	6.2	59	3.5	66	5.7	68	5.3	70	3.8	69	6.0	76	3.3	83	5.9
Thiabendazole	99	2.5	85	7.8	111	2.8	88	2.9	95	13.1	104	3.4	97	5.0	80	12.9	111	1.9
Thiamphenicol	99	5.8	90	11.1	119	4.1	96	4.2	106	6.9	116	8.6	104	7.1	90	12.9	114	7.9
Tildipirosin	49	5.6	46	19.1	59	7.6	50	12.7	45	21.5	75	6.1	58	8.9	62	5.7	89	11.1
Tilmicosin	92	9.7	93	10.7	106	11.9	80	14.9	81	5.3	98	4.5	98	8.1	89	2.3	104	11.4
Tolfenamic acid	85	7.2	86	9.4	31	16.6	85	14.1	84	3.3	75	5.2	96	17.1	73	1.4	59	29.2

Table 5. Continued

|	Bovine Muscle						Bovine Liver						Bovine Kidney					
	dSPE-CEC18		µSPE-CEC18		µSPE-HRP		dSPE-CEC18		µSPE-CEC18		µSPE-HRP		dSPE-CEC18		µSPE-CEC18		µSPE-HRP	
Compound	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD	% Rec	%RSD
Triclabendazole	92	6.3	86	8.1	101	8.1	91	10.3	81	4.5	117	5.0	98	16.6	72	2.7	87	16.3
Trimethoprim	88	3.8	76	11.5	95	7.8	82	4.0	100	8.3	91	2.3	92	6.2	99	9.6	111	8.6
Tulathromycin	61	6.3	48	19.0	77	15.7	49	25.6	28	9.2	84	7.5	58	10.7	63	5.7	87	11.9
Tylosin A	103	10.0	87	10.0	101	14.3	123	9.9	92	0.8	103	5.9	108	5.3	37	12.8	97	9.5
Tylvalosin	102	14.0	98	8.4	115	0.5	100	9.2	93	1.8	127	1.2	110	5.6	66	5.8	115	16.3
Virginiamycin M1	101	6.9	56	4.3	82	8.1	101	5.0	99	1.9	83	3.5	114	4.0	89	2.0	87	12.6
Zilpaterol	66	4.9	60	3.9	67	2.6	67	2.1	72	2.1	75	2.5	71	3.9	76	2.2	89	3.9

A

B
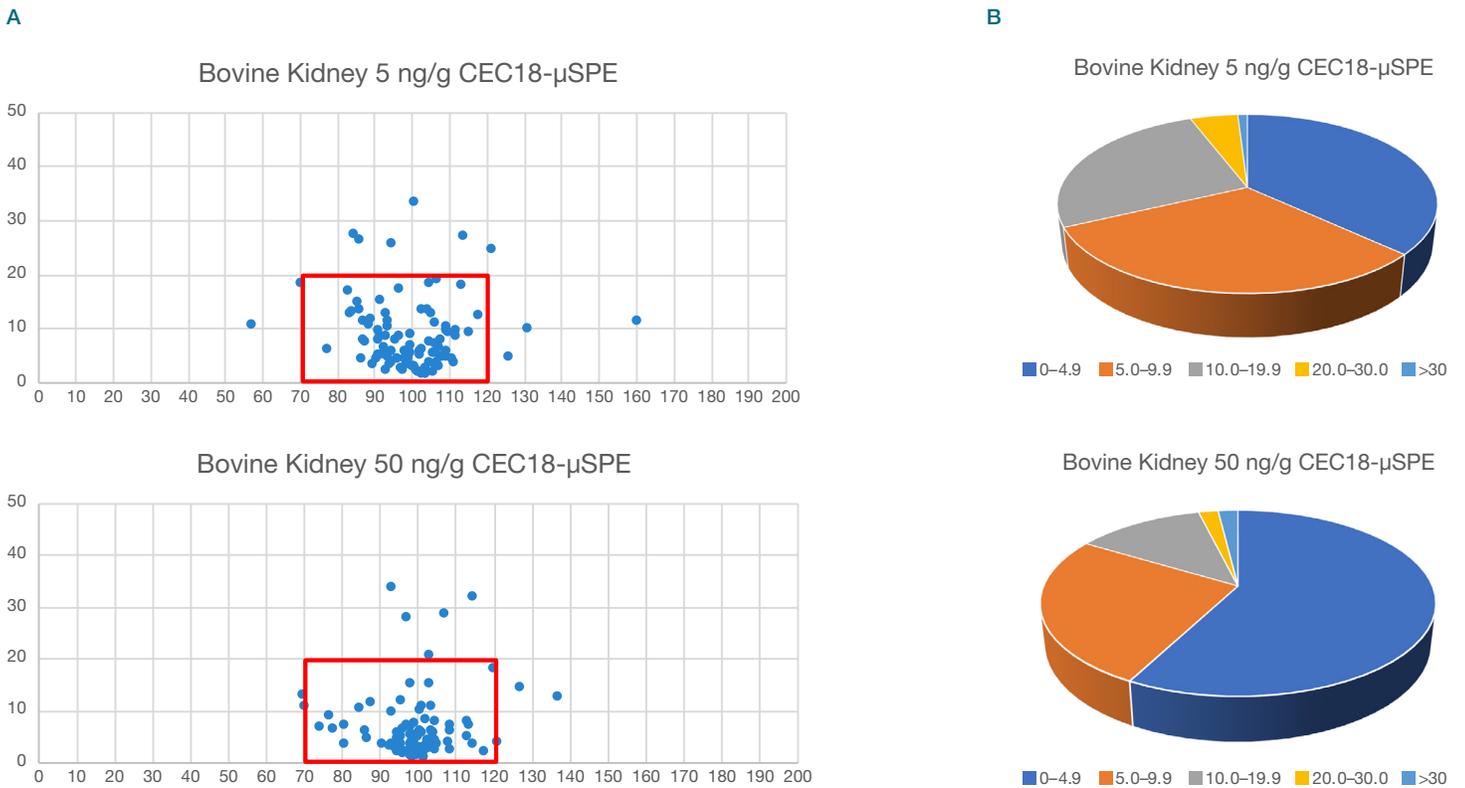
Figure 10. a) Recoveries and %RSD for bovine kidney tissue extracts cleaned up with CEC18-µSPE miniaturized cartridges; b) %RSD for bovine kidney tissue extracts cleaned up with CEC18-µSPE miniaturized cartridges. Data represents 5 biological replicates calculated against procedural calibration curves (MES).

Overall time savings and µSPE method robustness

The online automated approach to cleanup QuEChERS extracts using the TriPlus RSH µSPE autosampler saves a tremendous amount of labor, especially for large batches of samples. At the Iowa State Veterinary Diagnostic lab, measurements were made to determine the operator time saved during a batch preparation of 50 samples. For the µSPE-CEC18 workflow, a time savings of up to 4 hours was achieved as compared to the manual dSPE method. For the HRP method, which normally is an SPE procedure requiring a vacuum manifold, over 5 hours is saved. The operators noticed that labeling and moving vials around the lab costs time and is prone to errors. The time savings noted here can even be improved by the autocalibration function mentioned earlier, as this is clearly another key advantage of the system. Finally, this automation provides an easy way to evaluate different cleanup sorbents packed into the miniaturized cartridges, since control of flow rates is crucial, and these settings are easily adjusted in an automated experiment.

The TSQ Altis triple quadrupole mass spectrometer has been operating routinely in the Iowa State Veterinary Diagnostic laboratory for over three years analyzing veterinary drugs in animal tissues and biological fluids. Method robustness enabling increased uptime is the main reason extract cleanup is so important. Figure 11 is an example for two compounds, levamisole and albendazole-2-aminosulfone in bovine muscle extract, cleaned up using the µSPE CEC18 cartridges. The data shows that the response was well within the expected ±20% range for at least 100 consecutive injections without maintenance. At injection 50, the system was set to stand-by for 24 hours to simulate the start-up of a new batch, then resumed to complete the 100 injections, which demonstrates excellent day-to-day reliability and repeatability.
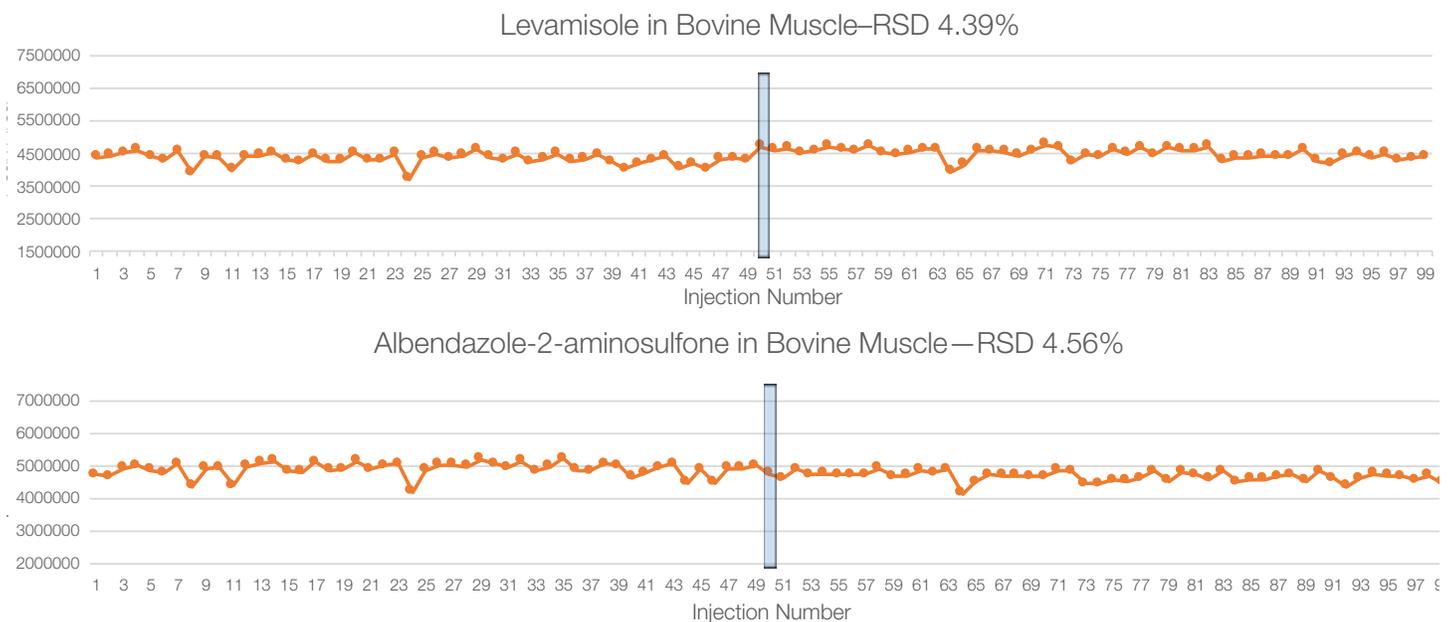

Figure 11. An example for two compounds, levamisole and albendazole 2-aminosulfone in bovine muscle extract, cleaned up using the µSPE CEC18 cartridges. The blue bar represents the point where the system was set into standby for 24 hours, then resuming the analysis of the batch.

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Conclusion

The fully automated and online μSPE cleanup workflow with LC-MS/MS described in this application note is a reliable, accurate, reproducible, and robust solution for multi-class veterinary drug screening and quantitation in a variety of animal tissues (muscle, liver, and kidney).

- On-line automation with the μSPE miniaturized cartridges saves time by reducing labor, saving hours during batch sample preparation over manual methods, and reduces the possibility of technician errors. In addition, lab material costs are reduced in terms of lower solvent usage and elimination the dSPE powdered reagents and/or larger SPE extraction cartridges.

- The sandwich injection technique, incorporated in the workflow will reduce sample handling and enable generation of excellent chromatography for analytes present in pure elution solvents, thus avoiding sample dilution or solvent exchange of extracts.

- The automated μSPE cleanup afforded spike recoveries within 30–140% with corresponding %RSDs below 20% for over 95% of the target veterinary drugs in the tissue extracts, and also demonstrated excellent precision and accuracy when calibrating with procedural standards (MES).

- The autocalibration routine for screening veterinary tissue samples described here is included in the workflow script and saves time, labor, and solvent usage.

- Analyte responses were stable for 100 consecutive injections without maintenance.

References and acknowledgments

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