



# A Multi-Class, Multi-Residue Method for Detection of Veterinary Drugs in Multiple Meat Using a Pass-Through Cleanup SPE Technique and UPLC-MS/MS Analysis

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## Abstract

A quantitative method using ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) was developed for the determination of 60 compounds, belonging to a variety of veterinary drug (VD) classes, in meat. The included analytes belong to the following VD classes:  $\beta$ -agonists, sulfanilamides, quinolones, macrolides, tetracyclines,  $\beta$ -lactams, nitroimidazoles, glucocorticoids, sex hormones, chloromycetins, sedatives, and olaquinox metabolite. The effective PRiME pass-through cleanup procedure was used to ensure high extraction efficiency and good sample cleanup after a simple liquid extraction of the meat samples with acetonitrile/water. The developed method was validated successfully. Mean recoveries for all analytes ranged from 80 to 116%, with the relative standard deviations (RSDs)  $\leq 7.8\%$ . Limits of quantification were in the range 0.05–3.0  $\mu\text{g kg}^{-1}$  and limits of detection were in the range 0.1–10  $\mu\text{g kg}^{-1}$ . The matrix effect was evaluated for the different meat matrices and was found to be markedly different in different matrices. The validated method was used in a pilot study to analyze real samples of pork, beef, mutton, chicken, and pork liver, lambs' liver, and chicken liver. Trace amounts of  $\beta$ -agonists, oxytetracycline, quinolones, chloromycetin sulfadimidine, and 3-methyl-quinoxaline-2-carboxylic acid were detected in these samples. In conclusion, this workflow can provide a simpler and more cost-effective alternative to conventional analytical methods and is compatible with processing large sample numbers in a short time period.

**Keywords** Veterinary drugs · Multi-residue analysis · Meat · Pass-through cleanup · UPLC-MS/MS

## Introduction

Veterinary drugs (VDs), which are widely used in agriculture and animal husbandry to prevent or cure diseases, to increase

production or as growth promoters, may leave residues in food from treated animals, in animal excrement, and even in the environment (Paiga et al. 2017; Teglia et al. 2017; Li et al. 2017; Zhao et al. 2017b). Especially with overdosing and illegal use, VD residues and their metabolites pose a potential risk to both animal and human health since long-term exposure can cause cancer, anaphylactic shock, microbial resistance, and reproductive disorders (Lúcia Santos et al. 2016; Moreno-Gonzalez et al. 2017b; Qin et al. 2017).

To protect public health, pharmacologically active substances are strictly regulated and monitored in food stuffs of animal origin and several countries have established maximum residue limits (MRLs) or tolerances. Commission Regulation (EU) 37/2010 classified VDs in four annexes, on the basis of a scientific assessment of safety (Commission 2010). CAC/MRL 2-2015 recommends MRLs and provides risk management recommendations for residues of VDs, such as chloramphenicol, metronidazole, and dimetridazole, in food (Commission 2015). In China, announcement No. 235 of the Ministry of Agriculture also established MRLs for different substances in food of animal

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origin (China 2002). In all cases, different international regulations require that levels of residues in food should not harm consumers. Because of the high number of VDs that need to be controlled, it is important that regulatory monitoring laboratories have access to multi-class multi-residue analytical methods that permit analysis of a variety of drugs with a single procedure (Mainero Rocca et al. 2017; Wittenberg et al. 2017).

There have been several reports describing analysis of VDs in many products of animal origin, including bovine muscle, porcine muscle (Park et al. 2015; Zhang et al. 2016), chicken (Yoshikawa et al. 2017), eggs (Piatkowska et al. 2017), milk (Wittenberg et al. 2017), and honey (Jin et al. 2017). However, multi-class methods for analysis of VDs in more than two matrices are scarce because of the complexity of animal-origin samples and the large number of analytes (Rizzetti et al. 2017). Although multi-class methods are the most efficient way to maximize the number of analytes that may be simultaneously determined by a single analysis, most of the methodologies proposed in the literature can only be used for compounds from the same class (Matus and Boison 2016; Qin et al. 2017; Lu et al. 2016; Zhao et al. 2017a). There also is a growing trend to develop multi-class multi-residue methods for VD residues in food. Zanella et al. reported a simple extraction procedure, using acetonitrile and trichloroacetic acid for protein precipitation, coupled to liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) for the analysis of 20 VD residues in bovine kidney and liver (Rizzetti et al. 2017). Yoshikawa used a two-step extraction followed by a solid-supported liquid extraction cleanup for LC-MS/MS analysis of 37 VDs in seven different classes in two processed chicken foods (Yoshikawa et al. 2017). A dispersive solid-phase extraction and LC-MS/MS have been used to develop a multi-class method for determination of 30 VDs, belonging to three different classes in milk powder (Li and Wu 2017). The simultaneous detection of a large number of analytes at low concentrations requires the use of a highly sensitive and selective technique. LC–MS/MS has been widely used for this purpose and has enabled the simultaneous analysis of several classes of VDs at low concentrations, even in complex matrices such as bovine muscle (Dasenaki et al. 2016). Taking into account the wide variety of physicochemical properties of VDs and the complexity of meat matrices, an important and complex issue is how to simultaneously analyze multi-class VDs with generic extraction procedures (Yoshikawa et al. 2017). An effective sample extraction technique that can essentially eliminate matrices is necessary to achieve reliable results and maintain instrument performance (Tian et al. 2016). Several extraction approaches, such as the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach (Guo et al. 2016; Jin et al. 2017; Paiga et al. 2017; Piatkowska et al. 2017; Turnipseed et al. 2017), solid phase extraction (SPE) (Jianfeng Wang et al. 2017; Turnipseed et al. 2017; Yoshikawa et al. 2017; Chen et al. 2016), matrix solid phase dispersion (Zhang et al. 2017),

liquid–liquid extraction (Aguilera-Luiz et al. 2008; Pastor-Belda et al. 2017), and molecularly imprinted polymers (Doue et al. 2012; Samanidou et al. 2016) have been described. Because of the complex nature of the matrices, SPE is one of the most widely adopted cleanup steps for determination of VDs in food samples. However, because of the narrow selectivity of SPE, it is difficult to broaden the scope of the analytes and this method is not capable of determining a large number of compounds belonging to different classes (Guo et al. 2016). Waters Corporation has released a new Hydrophilic-Lipophilic Balance (HLB) sorbent called PRiME (process, robustness, improvements, matrix effects, ease of use), which we have found to be highly effective for the removal of both fats and phospholipids from meat extracts (Moreno-Gonzalez et al. 2017a). PRiME HLB sorbent has also been evaluated for the determination of different families of antibiotics in a complex matrix such as milk (Chen et al. 2016) and of multiclass VD residues in fish, shrimp, eel, and swine waste (Turnipseed et al. 2017; Li et al. 2017). This special cartridge does not require activation/equilibration or washing steps, and provides a better cleanup effect than Oasis HLB (Chen et al. 2016). The PRiME pass-through cleanup approach for different mechanisms of action is simple to operate, has high efficiency, and is suitable for high throughput analysis of analytes in complex samples.

In this study, different extraction solvents were investigated and modifications were made to accommodate a variety of target analytes. The efficiency of PRiME HLB was also compared with conventional HLB. The performance of this method was validated by estimating specificity, linearity, trueness, precision, analytical limits, and matrix effects. Finally, the method was successfully used for the analysis of VDs in several meat matrices.

## Experimental

### Chemicals, Reagents, and Solutions

HPLC grade methanol and acetonitrile were obtained from Fisher Scientific, Inc. (Pittsburgh, PA, USA). Formic acid of high-quality grade was purchased from Sigma-Aldrich (St. Louis, MO, USA), (Bedford, MA, USA). Oasis PRiME HLB extraction cartridges (200 mg, 6 mL) were supplied by Waters (Milford, MA, USA). The syringe filter was purchased from Agela (Tianjin, China).

Clenbuterol, salbutamol, ractopamine, mabuterol, cimbuterol, terbutaline, penbutolol, tulobuterol, fenoterol, clorprenaline, sulfamerazine, sulfamethoxazole, sulfadimidine, sulfadimethoxine, sulfamonomethoxine, sulfaquinoxaline, sulphacetamide, sulfapyridine, sulfisoxazole, sulfamethizole, sulfadoxine, sulfathizole, sulfaphenazole, sulfadiazine, sulfamethoxy pyridazine,

sulfamer, sulfachloropyridazine, enrofloxacin, ciprofloxacin, sarafloxacin, danofloxacin, flumequine, ofloxacin, norfloxacin, erythromycin, lincomycin, tetracycline, oxytetracycline, chlorotetracycline, doxycycline, amoxicillin, ampicillin, benzylpenicillin, cloxacillin, dimetridazole, metronidazole, betamethasone, dexamethasone, methyltestosterone, trenbolone, testosterone, mengestrol, chloromycetin, thiamphenicol, florfenicol, chlorpromazine, acetylpromazine, azaperol, phenylethanolamine A, and 3-methyl-quinoxaline-2-carboxylic acid were all purchased from Dr. Ehrenstorfer (Augsburg, Germany) or Sigma-Aldrich.

According to their classification and stability, the aforementioned compounds were divided into 12 different groups:  $\beta$ -agonists (10), sulfanilamides (17), quinolones (7), macrolides (2), tetracyclines (4),  $\beta$ -lactams (4), nitroimidazoles (2), glucocorticoids (2), sex hormones (4), chloromycetins (3), sedatives (4), and olaquinox metabolites (1).

Stock solutions of each compound ( $\sim 1000 \text{ mg L}^{-1}$ ) were prepared in different solvents and stored at  $-20 \text{ }^\circ\text{C}$  in brown glass containers for a maximum period of 6 months. The analytes were dissolved and diluted with an appropriate solvent (acetonitrile, methanol, or water), based on their solubility properties.

Individual stock standard solutions were prepared by weighing approximately 10 mg of all compounds into 10-mL volumetric flasks. Depending on the specific solubility properties, the compounds were dissolved in acetonitrile or methanol. Multi-compound working standard solutions were prepared by diluting an appropriate amount of the standard solutions with methanol. The mixtures were stored at  $4 \text{ }^\circ\text{C}$  in the dark and were stable for 2 weeks.

## Instrumentation

A Waters Acquity™ ultra high-performance liquid chromatography system, equipped with a Waters Acquity autosampler and binary solvent management system, together with a Triple Quad™ 5500 mass spectrometer (AB Sciex, Framingham, USA), with an electrospray ionization source, were used for the analyses. A 3K-30 high speed refrigerated centrifuge (Sigma-Aldrich), MS3 basic vortex mixer (IKA, Germany), and KQ-250-E ultrasonic instrument (Kun Shan, China) were used for sample preparation.

Chromatographic separations were performed using Waters ACQUITY UPLC™ BEH C18 analytical columns ( $100 \times 2.1 \text{ mm i.d.}$ ,  $1.7 \text{ }\mu\text{m}$  particle size) maintained at  $40 \text{ }^\circ\text{C}$ . Nitrogen (purity  $\geq 99\%$ ), generated using a nitrogen generator (Peak Scientific, Billerica, MA, USA), was used in the ESI source. Chromatographic separation was carried out with gradient elution at a flow rate of  $300 \text{ }\mu\text{L min}^{-1}$ , using water (eluent A) and methanol (eluent B), both containing 0.1% formic acid ( $v/v$ ). The gradient elution was performed

as follows, 95–80% A (0–3.0 min); 80–50% A (3.0–5.0 min); 50–0% A (5.0–8.0 min); 0% A (8.0–10.0 min); 0–95% A (10.0–10.1 min); and 95–95% A (10.1–12.0 min).

MS was performed using a Triple Quadrupole Mass Spectrometer, equipped with an ESI source working in positive ion mode (ESI<sup>+</sup>) as follows: ion spray (IS) voltage, 5500 V; curtain gas, 30 psi; nebulizer gas (GS1), 55 psi; auxiliary gas (GS2), 55 psi; and source temperature,  $400 \text{ }^\circ\text{C}$ . When working in negative ion mode (ESI<sup>-</sup>), the same parameters were used, except the IS voltage, which was set at  $-4500 \text{ V}$ . Nitrogen was used for nebulization and collision. To optimize the mass spectrometer parameters for each compound, the compounds were infused separately using selective reaction monitoring (SRM) mode, with a continuous flow of  $10 \text{ }\mu\text{L min}^{-1}$  and a concentration of  $1 \text{ mg L}^{-1}$ . The Analyst 1.6.2 software (AB Sciex) was used for data acquisition and processing. Optimal values of declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP), and SRM for each analyte used for identification of VD residues are summarized in Table 1.

## Meat Samples

Samples of pork, beef, mutton, chicken, pork liver, lambs' liver, and chicken liver were purchased from a local supermarket. The samples were minced using a meat grinder and stored at  $-18 \text{ }^\circ\text{C}$  until analysis.

## Sample Preparation

A meat sample ( $2.50 \pm 0.01 \text{ g}$ ) was weighed into a 50-mL conical centrifuge tube. Ten milliliters acetonitrile/water (80/20,  $v/v$ ) was added and the tube was vortexed for 10 s. The sample was sonicated for about 30 min then centrifuged at 7000 rpm at  $4 \text{ }^\circ\text{C}$  for 5 min. The resulting supernatant solution was slowly transferred into a 15-mL centrifuge tube. A Waters Oasis PRiME HLB cartridge (200 mg, 6 mL) was set up for pass-through filtration. The extraction mixture (5 mL) was passed through the cartridge under gravity and collected in a 15-mL centrifuge tube. The extract was concentrated to near dryness under nitrogen at  $45 \text{ }^\circ\text{C}$ . The extract was then reconstituted with 20% acetonitrile-water (1 mL) and vortexed for 30 s. Finally, the extract was pressed through a  $0.22\text{-}\mu\text{m}$  syringe filter into a vial and analyzed by UPLC-MS/MS.

## Validation Procedure

The method was validated by evaluating linearity, the limits of detection (LODs), limits of quantification (LOQs), accuracy, repeatability and reproducibility, and matrix effect using UPLC-MS/MS. The linearity of the method was demonstrated using blank matrix-matched calibration standard for each analyte. A calibration curve was prepared for blank meat matrix

**Table 1** Mass spectrometer parameters and performance characteristics of linearity equation,  $R^2$ , linearity ranges, limits of detection (LODs), and limits of quantification (LOQs) of the 60 VDs

Compounds	SRM transition (m/z)	DP/V	EP/V	CXP/V	CE/V	Linear equation	$R^2$	Linear <sup>a</sup> range ( $\mu\text{g kg}^{-1}$ )	LODs ( $\mu\text{g kg}^{-1}$ )	LOQs ( $\mu\text{g kg}^{-1}$ )
<b><math>\beta</math>-agonist (10)</b>										
Clenbuterol	277.1/203.1, 277.1/259.0	80	10	12	22, 15	$y = 1.12 \times 10^5 x + 3.46 \times 10^4$	0.9999	0.5–500	0.2	0.5
Salbutamol	240.2/148.1, 240.2/22.3	68	10	12	24, 14	$y = 4.27 \times 10^5 x + 3.13 \times 10^5$	0.9968	0.5–500	0.2	0.5
Ractopamine	302.3/107.0, 302.3/164.0	90	10	12	50, 20	$y = 4.74 \times 10^5 x + 3.20 \times 10^4$	0.9979	0.5–500	0.2	0.5
Mabuterol	311.2/237.1, 311.2/293.2	100	10	17	20, 14	$y = 3.21 \times 10^4 x + 1.54 \times 10^4$	0.9989	0.5–500	0.2	0.5
Cimbuterol	220.1/160.1, 220.1/143.0	70	10	17	23, 30	$y = 1.81 \times 10^4 x + 1.13 \times 10^5$	0.9996	0.5–500	0.2	0.5
Terbutaline	226.0/152.1, 226.0/170.1	80	10	12	22, 16	$y = 1.94 \times 10^5 x + 1.70 \times 10^4$	0.9994	0.5–500	0.2	0.5
Penbutolol	292.2/236.2, 228.0/201.0	110	10	12	23, 28	$y = 3.25 \times 10^5 x + 2.39 \times 10^4$	0.9996	0.5–500	0.2	0.5
Tulobuterol	228.0/154.0, 228.0/118.1	85	10	12	21, 36	$y = 1.95 \times 10^4 x + 1.49 \times 10^4$	0.9974	0.5–500	0.2	0.5
Fenoterol	304.1/135.1, 304.1/107.2	100	10	12	23, 36	$y = 1.14 \times 10^4 x + 1.60 \times 10^4$	0.9997	0.5–500	0.2	0.5
Clorprenaline	214.0/154.1, 214.0/118.0	85	10	12	23, 34	$y = 1.19 \times 10^4 x + 7.5 \times 10^3$	0.9998	1.0–500	0.3	0.8
<b>Sulfamillamide (17)</b>										
Sulfamerazine	265.2/156.1, 265.2/172.1	82	10	10	25, 25	$y = 1.42 \times 10^5 x + 3.48 \times 10^4$	0.9991	2.0–500	0.5	2.0
Sulfamethoxazole	254.2/156.1, 254.2/108.1	85	10	10	24, 37	$y = 2.27 \times 10^5 x + 3.05 \times 10^4$	0.9998	2.0–500	0.5	2.0
Sulfadimidine	279.2/186.1, 279.2/156.1	90	10	4	27, 27	$y = 1.74 \times 10^4 x - 4.28 \times 10^3$	0.9994	2.0–500	0.5	2.0
Sulfadimethoxine	311.3/156.1, 311.3/218.0	100	10	4	28, 28	$y = 1.21 \times 10^5 x + 1.34 \times 10^5$	0.9992	2.0–500	0.8	2.0
Sulfamonomethoxine	281.2/156.1, 281.2/108.0	87	10	10	26, 38	$y = 2.85 \times 10^4 x + 3.16 \times 10^4$	0.9987	3.0–500	1.2	3.0
Sulfaquinoxaline	301.1/155.8, 301.1/107.8	90	10	10	27, 23	$y = 2.90 \times 10^5 x + 1.90 \times 10^4$	0.9999	5.0–500	1.5	5.0
Sulphacetamide	215.0/156.1, 215.0/108.1	65	13	10	15, 27	$y = 1.78 \times 10^4 x + 8.11 \times 10^3$	0.9992	2.0–500	0.5	2.0
Sulfapyridine	250.2/156.1, 250.2/184.1	90	10	4	25, 25	$y = 9.88 \times 10^4 x + 5.01 \times 10^4$	0.9994	2.0–500	0.5	2.0
Sulfisoxazole	268.0/156.0, 268.0/113.0	90	10	4	20, 23	$y = 1.20 \times 10^4 x + 4.53 \times 10^4$	0.9987	2.0–500	0.5	2.0
Sulfamethizole	271.0/155.9, 271.0/107.8	100	13	10	28, 32	$y = 1.42 \times 10^5 x + 1.14 \times 10^5$	0.9994	2.0–500	0.5	2.0
Sulfadoxine	311.3/108.1, 311.3/156.0	100	10	10	28, 28	$y = 1.73 \times 10^5 x + 1.08 \times 10^5$	0.9998	2.0–500	0.5	2.0
Sulfathizole	256.1/156.1, 256.1/108.1	65	13	10	22, 32	$y = 1.17 \times 10^5 x + 7.77 \times 10^4$	0.9996	2.0–500	0.5	2.0
Sulfaphenazole	315.0/156.0, 315.0/160.0	100	10	4	32, 35	$y = 1.58 \times 10^5 x + 6.70 \times 10^4$	0.9988	2.0–500	0.5	2.0
Sulfadiazine	251.2/156.1, 251.2/92.2	80	13	8	23, 40	$y = 1.03 \times 10^5 x + 6.97 \times 10^4$	0.9991	2.0–500	0.5	2.0
Sulfamethoxyipyridazine	281.2/156.1, 281.2/92.2	95	10	10	25, 45	$y = 1.04 \times 10^5 x + 5.92 \times 10^4$	0.9978	2.0–500	0.5	2.0
Sulfamer	281.2/156.1, 281.2/108.1	90	10	10	27, 42	$y = 2.65 \times 10^5 x + 2.01 \times 10^4$	0.9995	2.0–500	0.5	2.0
Sulfachloropyridazine	285.1/156.1, 285.1/108.0	60	10	10	22, 34	$y = 1.66 \times 10^5 x - 2.01 \times 10^5$	0.9991	2.0–500	0.5	2.0
<b>Quinolone (7)</b>										
Enrofloxacin	360.6/316.4, 360.6/245.3	45	10	12	30, 40	$y = 1.25 \times 10^5 x - 2.04 \times 10^4$	0.9968	2.0–250	0.8	2.0
Ciprofloxacin	332.4/288.3, 332.4/245.3	45	10	12	25, 33	$y = 2.15 \times 10^4 x - 1.49 \times 10^4$	0.9979	5.0–250	2.0	5.0
Sarafloxacin	240.2/148.1, 240.2/222.3	68	10	17	24, 14	$y = 2.87 \times 10^4 x - 1.28 \times 10^4$	0.9989	5.0–250	2.0	5.0
Danofloxacin	358.2/255.0, 358.2/340.0	45	10	17	18, 10	$y = 8.29 \times 10^4 x + 8.88 \times 10^5$	0.9996	5.0–250	2.0	5.0

Table 1 (continued)

Compounds	SRM transition (m/z)	DP/V	EP/V	CXP/V	CE/V	Linear equation	R <sup>2</sup>	Linear <sup>a</sup> range (µg kg <sup>-1</sup> )	LODs (µg kg <sup>-1</sup> )	LOQs (µg kg <sup>-1</sup> )
Flumequine	262.1/202.1, 262.1/244.4	45	10	12	32, 20	$y = 3.21 \times 10^5 x + 1.14 \times 10^5$	0.9994	2.0–500	0.8	2.0
Ofloxacin	362.4/318.4, 362.4/261.3	45	10	12	30, 40	$y = 1.18 \times 10^4 x + 1.05 \times 10^3$	0.9989	5.0–250	2.0	5.0
Norfloxacin	320.4/276.6, 320.4/233.2	45	10	12	30, 25	$y = 6.70 \times 10^4 x + 2.79 \times 10^3$	0.9996	5.0–250	2.0	5.0
Macrolides (2)										
Erythromycin	734.7/576.5, 734.7/158.1	100	10	15	20, 32	$y = 4.44 \times 10^4 x + 2.05 \times 10^3$	0.9991	1.0–200	0.2	1.0
Lincomycin	407.3/126.2, 407.3/359.3	100	10	15	30, 24	$y = 5.11 \times 10^5 x + 3.15 \times 10^4$	0.9968	1.0–200	0.2	1.0
Tetracycline (4)										
Tetracycline	445.2/1410.0, 445.2/427.2	35	10	12	31, 22	$y = 6.09 \times 10^5 x + 2.77 \times 10^4$	0.9979	10–1000	2.0	10
Oxytetracycline	461.0/426.2, 461.0/443.1	35	10	12	26, 20	$y = 5.14 \times 10^5 x + 5.33 \times 10^4$	0.9992	10–1000	3.0	10
Chlorotetracycline	479.1/444.2, 479.1/462.2	35	10	12	31, 28	$y = 7.15 \times 10^4 x + 6.15 \times 10^3$	0.9968	10–1000	3.0	10
Doxycycline	445.2/154.1, 445.2/428.2	35	10	12	40, 27	$y = 4.32 \times 10^4 x + 1.55 \times 10^3$	0.9979	10–1000	3.0	10
β-lactam (4)										
Amoxicillin	366.0/208.0, 366.0/349.0	130	10	17	16, 47	$y = 5.22 \times 10^5 x + 3.77 \times 10^4$	0.9989	5–500	1.8	5
Ampicillin	350.3/106.0, 350.3/192.0	120	10	13	22, 16	$y = 7.13 \times 10^5 x + 5.24 \times 10^4$	0.9997	2–500	0.5	2
Benzylpenicillin	335.4/160.3, 335.4/176.1	120	10	13	17, 18	$y = 4.77 \times 10^5 x + 1.09 \times 10^4$	0.9995	5–500	1.2	5
Cloxacillin	436.2/160.3, 436.2/277.3	120	10	13	18, 20	$y = 2.42 \times 10^5 x + 5.48 \times 10^4$	0.9994	5–500	1.5	5
Nitimidazole (2)										
Dimetridazole	142.1/96.1, 142.1/81.1	100	10	20	22, 26	$y = 2.66 \times 10^4 x + 4.05 \times 10^4$	0.9974	1.0–500	0.3	1.0
Metronidazole	172.1/128.5, 172.1/82.1	100	10	20	22, 26	$y = 3.74 \times 10^4 x + 4.28 \times 10^4$	0.9997	1.0–500	0.3	1.0
Glucocorticoid (2)										
Betamethasone	393.2/355.2, 393.2/337.3	70	10	15	17, 19	$y = 1.29 \times 10^4 x + 1.36 \times 10^4$	0.9988	1.0–500	0.3	1.0
Dexamethasone	393.2/373.4, 393.2/355.2	70	10	15	13, 18	$y = 1.85 \times 10^4 x + 2.16 \times 10^4$	0.9969	1.0–500	0.3	1.0
Sex hormones (4)										
Methyltestosterone	303.1/109.1, 303.1/97.1	106	10	18	37, 19	$y = 2.90 \times 10^5 x + 1.90 \times 10^4$	0.9992	1.0–500	0.3	1.0
Trenbolone	271.0/253.1, 271.0/199.0	100	10	17	16, 20	$y = 4.42 \times 10^4 x + 3.48 \times 10^5$	0.9958	1.0–500	0.3	1.0
Testosterone	345.7/97.2, 345.7/109.0	130	10	13	25, 30	$y = 2.27 \times 10^4 x + 3.05 \times 10^4$	0.9971	1.0–500	0.3	1.0
Mengestrol	386.9/327.3, 386.9/285.4	130	10	12	20, 25	$y = 1.79 \times 10^4 x + 4.28 \times 10^4$	0.9978	1.0–500	0.3	1.0
Chloromycetins (3)										
Chloromycetin	320.9/152.0, 320.9/257.0	–110	–10	–16	–22, –15	$y = 1.34 \times 10^4 x + 1.34 \times 10^4$	0.9996	0.1–500	0.05	0.1
Thiamphenicol	354.0/185.0, 354.0/290.0	–130	–10	–16	–27, –16	$y = 4.85 \times 10^4 x + 3.16 \times 10^5$	0.9994	0.5–500	0.2	0.5
Florfenicol	356.0/336.0, 356.0/185.0	–120	–10	–16	–12, –25	$y = 2.90 \times 10^4 x + 1.90 \times 10^4$	0.9959	0.5–500	0.2	0.5
Sedative (4)										
Chlorpromazine	319.3/58.2, 319.3/86.2	80	10	17	30, 25	$y = 1.24 \times 10^5 x + 5.04 \times 10^5$	0.9988	0.5–500	0.2	0.5
Acetylpromazine	327.2/58.2, 327.2/86.0	80	10	17	32, 22	$y = 3.27 \times 10^4 x + 2.60 \times 10^4$	0.9979	0.5–500	0.2	0.5

**Table 1** (continued)

Compounds	SRM transition (m/z)	DP/V	EP/V	CXP/V	CE/V	Linear equation	R <sup>2</sup>	Linear <sup>a</sup> range (µg kg <sup>-1</sup> )	LODs (µg kg <sup>-1</sup> )	LOQs (µg kg <sup>-1</sup> )
Azaperol	330.2/121.1, 330.2/312.3	80	10	17	32, 20	$y = 1.57 \times 10^4 x + 1.72 \times 10^4$	0.9991	0.5–500	0.2	0.5
Phenylethanolamine A	345.1/117.8, 345.1/150.0	100	10	12	40, 30	$y = 1.74 \times 10^4 x + 1.56 \times 10^4$	0.9996	0.5–500	0.2	0.5
Olaquinox metabolite (1)										
3-Methyl-quinoxaline-2-carboxylic acid	189.0/143.1, 189.0/145.1	100	10	16	17, 18	$y = 1.25 \times 10^5 x + 2.19 \times 10^4$	0.9999	0.5–500	0.2	0.5

<sup>a</sup>Matrix-matched calibration curves for each compound with different linearity ranges at six different concentration levels, as follows, 0.5–500; 5, 10, 50, 100, 500 µg kg<sup>-1</sup>; 1.0–500; 1, 5, 10, 50, 100, 500 µg kg<sup>-1</sup>; 2.0–500; 2, 5, 10, 50, 100, 500 µg kg<sup>-1</sup>; 3.0–500; 3, 5, 10, 50, 100, 500 µg kg<sup>-1</sup>; 5.0–500; 5, 10, 50, 100, 200, 500 µg kg<sup>-1</sup>; 5.0–250; 2, 5, 10, 50, 100, 250 µg kg<sup>-1</sup>; 2.0–250; 2, 5, 10, 50, 100, 250 µg kg<sup>-1</sup>; 5.0–250; 5, 10, 20, 50, 100, 250 µg kg<sup>-1</sup>; 1.0–200; 1, 5, 10, 50, 100, 200 µg kg<sup>-1</sup>; and 10–1000; 10, 50, 100, 200, 500, 1000 µg kg<sup>-1</sup>

with six different concentration ranges at levels of 0.1–1000 µg kg<sup>-1</sup> for analytes, shown in Table 1. For each compound, the matrix-matched calibration curve was obtained by plotting the ratio of each analyte quantitative ion peak area (y) versus the corresponding mass concentrations (x). LODs and LOQs were estimated by fortifying blank samples with the 60 analytes and applying the extraction procedure before chromatographic determination. For each analyte, LODs and LOQs in meat samples were defined as the concentration giving a signal to noise ratio of 3 and 10, respectively.

The accuracy of the method was evaluated based on the recoveries of VD residues. The blank meat samples spiked with all analytes were prepared at three levels ((LOQ) (low), 20 × LOQ (medium), and 50 × LOQ (high)), then processed and measured under the experimental conditions. Repeatability and reproducibility was determined in six replicates at each level by measuring corresponding relative standard deviations (RSDs). Matrix effect (ME) was evaluated by comparing the response of each VD residue obtained from a standard solution in solvent and that from a matrix at the same concentration.

## Results and Discussion

### Optimization of Analytical Conditions

Various LC conditions, including different chromatography columns, mobile phases, modes of elution, flow rates, and column temperatures, were evaluated to obtain good response, peak shape, and separation. A comparison of acetonitrile and methanol as the organic solvent showed that higher intensities and better peak shapes were obtained for most analytes using methanol. Addition of 0.1% formic acid to both the aqueous phase and the organic phase enhanced the response of most analytes in positive ion mode and gave an outstanding response for chloromycetins (negative ion mode), with negligible suppression of the response. As a result of these preliminary studies, 0.1% formic acid in methanol and water using a BEH C18 column were chosen as the best LC conditions for further investigations.

The MS ESI parameters were optimized for both positive and negative ion modes by direct infusion of standard solutions of each target compound. For most compounds, the protonated ion [M + H]<sup>+</sup> was present as the base peak and was selected as the precursor ion for quantification. Chloromycetins, however, gave the deprotonated ion [M - H]<sup>-</sup>. Cone voltage and collision energy were optimized to acquire two product ions and two transitions for each compound in SRM mode. The higher abundance transition with less interference was used for quantification and the other transition was used for identification. The MS parameters,

cone voltage, collision energy, and SRM transition selected for each analyte are shown in Table 1.

## Optimization of Sample Preparation

### Sample Extraction

One of the greatest challenges faced was to develop extraction conditions suitable for a wide range of VD analytes in a single extraction procedure and to eliminate the complex interferences in meat tissue matrices. Acetonitrile, methanol, and water mixtures are frequently used in the extraction step in the analysis of VD residues. Acetonitrile gave less co-extraction of matrix and facilitated protein precipitation.

Various extraction solvent combinations were investigated in terms of the absolute recoveries obtained for each individual analyte. In the present study, we tested different acetonitrile/water ratios (50:50, 80:20, and 100:0, *v/v*) for the extraction step in the analysis of VD residues. Acetonitrile/water (80/20, *v/v*) extracted the most drug from the spiked samples, providing acceptable absolute recoveries of 80–110%. The recoveries obtained under the different test conditions are presented in Fig. 1. Recoveries were very low (< 10%) for tetracyclines and too high (> 120%) for  $\beta$ -lactams and glucocorticoids when 100% acetonitrile was used as the extraction solvent for our list of analytes. Increasing the amount of water in the extraction solvent gave more co-extraction of matrix. Acetonitrile/water (50/50, *v/v*) was not compatible with all the compound classes, and absolute

recoveries were outside the acceptable range for certain  $\beta$ -lactams, nitroimidazoles, glucocorticoids, sex hormones, chloromycetins, and for olaquinox metabolite.

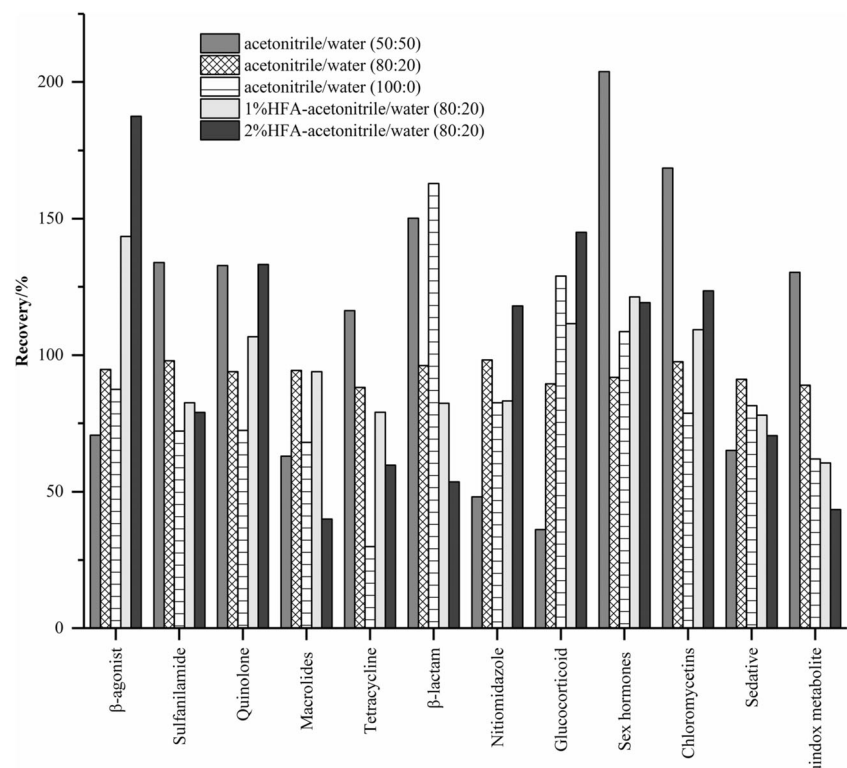
Addition of 1 or 2% formic acid to the extraction solvent gave acceptable recoveries for most analytes, but low recoveries for  $\beta$ -agonists, macrolides, glucocorticoids, and olaquinox metabolite. Taking into account the different chemical properties of VD analytes, we decided not to use acid in the extractions.

The volume of the extraction solvent was then optimized by evaluating the difference between 5, 10, and 15 mL of 80% acetonitrile–water. When 5 mL of solvent was used, the recoveries of most analytes were very low since extraction was inadequate and the protein may not have precipitated completely. Although a slight improvement in recoveries was observed when the volume of the extraction solvent was increased, the improvement did not justify the higher amount of solvents needed and the increased time needed for the evaporation step. To avoid increasing the cost and decreasing the rapidity of the developed method, 10 mL was chosen as the best volume for the extraction solvent.

### Selection of Procedures for Cleanup

The suitability of this pass-through SPE for analyzing multi-residue VDs was evaluated in various meat matrices. Several cleanup procedures, including no clean-up, MCX, HLB, and PRiME HLB, have previously been used in the determination of VDs. The performance of different purification technologies

**Fig. 1** Recoveries for different classes of veterinary drugs with different extracting solution



was evaluated by spiking meat samples with  $50 \mu\text{g kg}^{-1}$  of VDs to choose the best eluting mixture. The recovery (%RE) was determined by comparing the response area by the spiked sample (Ab), the blank matrix (As), and theoretical addition (M) using Eq. 1. The PRiME HLB SPE cartridge was the most efficient sorbent for purifying the extracts for most VDs (Fig. 2). With the Oasis PRiME HLB, most compounds had recoveries between 80 and 116%, with all the relative standard deviations (RSDs)  $< 7.8\%$ , shown in Table 2. Higher variability was seen with other cleanup methods. Without cleanup, the recovery of sex hormones was  $> 150\%$ , which is unsatisfactory. With the Oasis MCX, low recoveries were obtained for almost all glucocorticoids, sex hormones, sedatives, and for olaquinox metabolite, although the  $\beta$ -agonists had better recoveries (85–116%). The common RP C18 SPE, Oasis HLB gave good recovery for most analytes, with the exception of glucocorticoids and sex hormones.

$$\%RE = \frac{(Ab-As)}{M} \times 100 \quad (1)$$

## Method Validation

### Matrix Effect

The ME is widely used to evaluate the performance of sample pretreatment methods. ME was investigated by

comparing the peak areas of drugs in blank matrices (Am) to those of aqueous standards (Ae), using Eq. 2 (Yoshikawa et al. 2017):

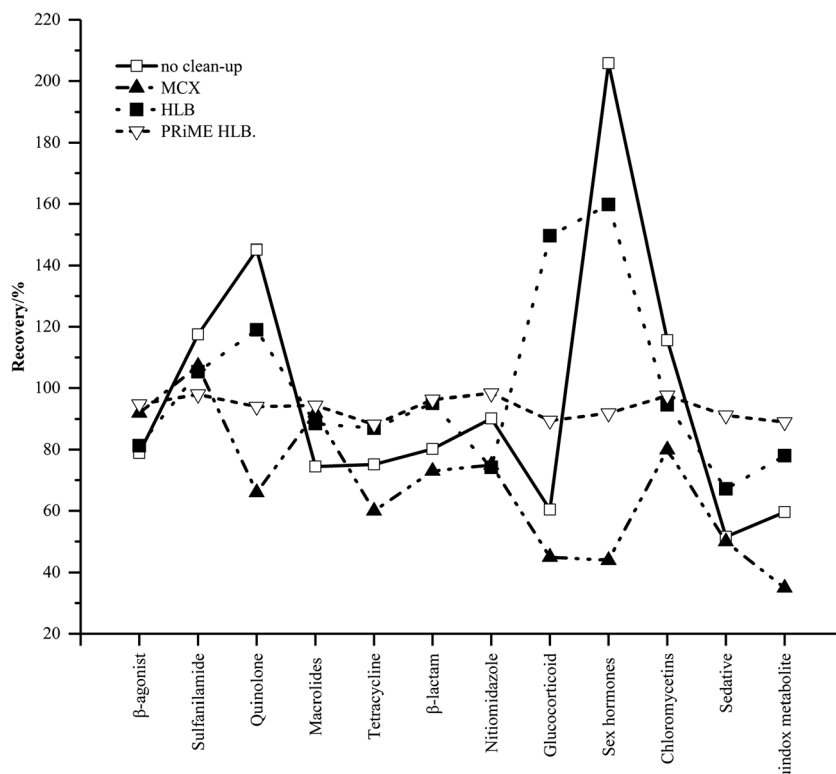
$$ME(\%) = \left( \frac{A_m}{A_e} - 1 \right) \times 100 \quad (2)$$

An ME(%) value between  $-20$  and  $20\%$  indicates an acceptable matrix effect, a value  $< -20\%$  indicates ion suppression, and a value  $> 20\%$  indicates an enhancement effect (Valese et al. 2017). MEs were evaluated for the different meat matrices (Fig. 3). It is noteworthy that ME is different in different matrices. All 60 compounds had an acceptable ME in pork, beef, mutton, and chicken whereas, in the liver samples, most compounds had a large ME because of the complex matrix. Some analytes that showed  $> 20\%$  false negatives, especially sex hormones, exhibited apparent signal suppression, with ME values  $> 40\%$ . Quinolones and chloromycetins showed matrix enhancement, ranging from 10 to 50%, and quinolones gave significant matrix enhancement ( $> 40\%$ ), which is in agreement with a report that all quinolones are subject to signal enhancement (Ortelli et al. 2009).

### Removal of Phospholipids

Because of their special physical and chemical properties, phospholipids co-extract with analytes, caused ionization suppression in the LC-ESI-MS analysis and an obvious

**Fig. 2** Comparison of extraction efficiencies of different cleanup strategies for all analytes





**Table 2** Validation results of 60 VDs in seven matrices ( $n=6$ )

Compound	Spiked ( $\mu\text{g kg}^{-1}$ )	%Recovery (%RSD)						
		Beef	Mutton	Pork	Chicken	Pork liver	Chicken liver	Lambs' liver
$\beta$ -agonist (10)								
Clenbuterol	0.5, 2.0, 5.0	85 (4.3), 91 (3.3), 96 (0.6)	90 (3.5), 95 (3.6), 97 (1.9)	90 (3.7), 92 (3.1), 95 (2.2)	81 (5.1), 88 (3.3), 93 (3.0)	90 (4.0), 91 (3.1), 94 (1.6)	84 (5.2), 85 (3.7), 94 (2.8)	82 (7.1), 87 (3.7), 90 (4.4)
Salbutamol	0.5, 2.0, 5.0	106 (3.6), 107 (3.1), 94 (1.6)	109 (3.9), 105 (4.1), 95 (2.4)	109 (4.6), 89 (3.5), 97 (2.1)	83 (5.1), 85 (4.4), 90 (3.6)	89 (3.9), 91 (3.6), 103 (2.8)	113 (4.1), 84 (4.5), 91 (3.2)	82 (6.9), 91 (4.3), 115 (4.6)
Ractopamine	0.5, 2.0, 5.0	88 (4.1), 92 (3.2), 98 (1.3)	82 (3.8), 94 (3.3), 91 (1.1)	85 (5.2), 92 (3.1), 94 (2.7)	115 (4.2), 88 (3.6), 91 (2.8)	88 (4.5), 89 (2.9), 95 (2.1)	112 (6.2), 108 (2.1), 102 (2.8)	111 (3.8), 84 (3.6), 89 (3.6)
Mabuterol	0.5, 2.0, 5.0	91 (3.3), 95 (2.3), 95 (1.9)	95 (4.5), 92 (3.1), 94 (2.7)	104 (3.9), 96 (3.1), 92 (3.3)	83 (6.3), 104 (5.5), 93 (3.9)	106 (5.6), 87 (3.1), 95 (3.2)	88 (5.3), 106 (4.9), 93 (3.1)	83 (5.3), 84 (5.5), 91 (4.9)
Cimbuterol	0.5, 2.0, 5.0	105 (5.3), 88 (3.8), 91 (3.1)	86 (4.9), 86 (2.9), 95 (1.6)	80 (4.7), 82 (4.4), 95 (3.9)	82 (7.3), 82 (5.6), 89 (4.5)	85 (4.8), 88 (3.7), 91 (2.5)	81 (4.9), 80 (4.5), 91 (2.9)	116 (7.1), 114 (2.6), 89 (3.5)
Terbutaline	0.5, 2.0, 5.0	105 (4.6), 107 (4.1), 97 (3.9)	112 (4.8), 104 (3.6), 105 (2.9)	101 (4.8), 89 (4.2), 95 (3.8)	80 (7.1), 88 (5.6), 93 (3.4)	114 (3.5), 88 (4.3), 95 (3.3)	108 (5.1), 84 (4.8), 90 (3.4)	81 (6.5), 83 (5.4), 90 (4.4)
Penbutolol	0.5, 2.0, 5.0	88 (4.5), 89 (3.7), 94 (1.9)	83 (2.9), 89 (2.3), 92 (1.2)	85 (4.2), 94 (3.9), 94 (1.7)	115 (6.4), 102 (3.8), 106 (2.0)	89 (4.6), 90 (3.9), 97 (2.9)	112 (5.7), 103 (3.5), 95 (2.6)	116 (6.1), 90 (3.5), 90 (3.1)
Tulobuterol	0.5, 2.0, 5.0	89 (3.7), 87 (2.9), 94 (3.1)	82 (4.4), 90 (3.7), 94 (2.7)	90 (4.9), 90 (3.1), 96 (1.9)	85 (6.5), 89 (4.5), 93 (4.6)	85 (4.1), 91 (3.8), 94 (0.5)	88 (5.4), 106 (3.9), 93 (3.3)	81 (4.3), 88 (4.4), 101 (3.2)
Fenoterol	0.5, 2.0, 5.0	83 (3.7), 84 (3.1), 98 (1.2)	89 (4.5), 88 (3.1), 97 (1.9)	90 (3.7), 92 (3.1), 95 (2.2)	112 (5.7), 86 (2.9), 94 (3.1)	82 (4.4), 87 (2.3), 92 (0.8)	83 (5.2), 87 (3.7), 96 (3.8)	110 (5.6), 86 (3.5), 88 (3.4)
Cloproprenaline	0.5, 2.0, 5.0	83 (4.9), 87 (3.6), 96 (2.8)	107 (5.6), 108 (3.5), 102 (3.0)	88 (5.6), 89 (3.0), 97 (2.9)	88 (5.5), 85 (4.1), 90 (3.9)	86 (3.8), 105 (2.6), 98 (2.1)	85 (4.8), 89 (4.1), 89 (2.7)	85 (5.5), 82 (4.1), 95 (3.9)
Sulfamiamide (17)								
Sulfamerazine	2.0, 10, 20	92 (5.3), 93 (2.3), 94 (0.7)	93 (2.7), 92 (3.3), 94 (1.1)	90 (2.7), 95 (3.1), 94 (0.2)	85 (4.4), 86 (3.3), 95 (3.7)	89 (4.4), 90 (3.3), 94 (0.8)	88 (3.5), 91 (3.7), 91 (2.1)	84 (4.4), 84 (2.3), 95 (3.7)
Sulfamethoxazole	2.0, 10, 20	105 (4.2), 107 (3.7), 96 (2.8)	104 (5.7), 105 (3.1), 101 (2.2)	94 (4.6), 93 (3.5), 93 (0.5)	83 (5.9), 90 (4.1), 90 (3.3)	93 (4.9), 92 (3.6), 98 (1.8)	88 (4.1), 89 (4.1), 94 (2.6)	88 (4.9), 90 (4.3), 97 (1.3)
Sulfadimidine	2.0, 10, 20	103 (2.2), 105 (3.12), 94 (0.6)	89 (4.2), 95 (2.9)	86 (4.7), 92 (3.6)	84 (5.0), 89 (2.9)	93 (5.4), 97 (2.1), 97 (2.7)	82 (5.1), 85 (3.7), 95 (1.8)	85 (5.2), 89 (3.9)

Table 2 (continued)

Compound	Spiked ( $\mu\text{g kg}^{-1}$ )	%Recovery (%RSD)						
		Beef	Mutton	Pork	Chicken	Pork liver	Chicken liver	Lambs' liver
Sulfadimethoxine	2.0, 10, 20	110 (4.3), 103 (2.3), 95 (0.7)	94 (1.9)	94 (1.3)	90 (1.4)	92 (5.3), 87 (3.3), 96 (0.6)	82 (5.5), 88 (4.7), 91 (3.8)	96 (2.4)
		108 (4.4), 92 (2.3), 96 (0.9)	88 (4.1), 94 (2.7), 95 (0.7)	85 (5.2), 88 (3.1), 94 (2.7)	116 (6.7), 102 (3.8), 90 (3.4)	88 (4.2), 89 (2.9), 95 (2.1)	114 (5.2), 108 (3.7), 101 (2.8)	114 (4.1), 105 (2.8), 94 (1.4)
Sulfaquinoxaline	2.0, 10, 20	85 (4.8), 89 (3.2), 96 (2.6)	87 (3.7), 91 (3.1), 97 (2.2)	88 (4.7), 93 (3.5), 95 (2.4)	89 (3.8), 107 (3.9), 103 (3.7)	90 (3.2), 91 (3.1), 94 (4.2)	90 (3.4), 95 (3.3), 98 (2.1)	89 (4.8), 91 (3.6), 101 (2.7)
		82 (5.3), 90 (2.3), 96 (0.7)	93 (4.7), 92 (3.3), 97 (1.9)	82 (3.7), 90 (3.1), 94 (2.2)	111 (4.3), 86 (3.3), 91 (2.8)	89 (4.6), 86 (3.3), 94 (2.2)	82 (5.2), 85 (3.7), 91 (2.1)	115 (5.3), 87 (2.3), 96 (2.7)
Sulfapyridine	2.0, 10, 20	105 (5.2), 107 (3.7), 97 (2.8)	104 (5.7), 105 (3.1), 106 (2.2)	91 (4.6), 90 (3.2), 95 (0.9)	108 (4.9), 101 (3.4), 90 (3.7)	105 (4.8), 91 (3.6), 96 (1.7)	106 (3.7), 84 (4.5), 89 (0.9)	106 (3.9), 111 (4.4), 90 (3.4)
		103 (2.8), 96 (3.8), 94 (2.5)	89 (4.2), 93 (2.5), 96 (0.7)	86 (2.7), 97 (3.6), 99 (2.4)	109 (6.2), 107 (3.8), 90 (3.9)	82 (3.4), 91 (3.1), 94 (1.1)	107 (5.4), 85 (3.5), 96 (2.1)	112 (4.2), 106 (1.8), 98 (3.4)
Sulfamethizole	2.0, 10, 20	110 (2.2), 103 (4.2), 96 (1.6)	92 (2.6), 91 (3.0), 98 (3.2)	83 (5.7), 92 (3.3), 95 (1.7)	80 (4.1), 91 (4.3), 94 (2.6)	83 (4.3), 94 (1.3), 96 (0.9)	82 (5.5), 85 (4.7), 91 (3.8)	88 (3.1), 91 (4.7), 92 (1.6)
		108 (2.1), 92 (5.1), 96 (2.6)	88 (3.5), 94 (1.9), 95 (2.3)	85 (6.2), 88 (3.4), 94 (3.1)	113 (2.7), 106 (3.3), 93 (2.4)	88 (3.2), 89 (3.4), 95 (2.3)	107 (4.2), 108 (3.6), 92 (2.8)	112 (4.7), 92 (3.0), 94 (1.8)
Sulfathiazole	2.0, 10, 20	89 (0.8), 94 (4.2), 98 (2.7)	86 (3.7), 94 (2.8), 101 (1.7)	111 (6.4), 104 (3.5), 94 (2.9)	88 (4.9), 93 (3.4), 94 (2.1)	82 (6.1), 91 (3.4), 104 (1.6)	90 (4.7), 95 (4.4), 92 (1.6)	89 (5.9), 91 (3.2), 101 (1.1)
		91 (2.5), 107 (3.8), 97 (2.6)	89 (4.6), 103 (3.6), 96 (2.8)	88 (2.6), 91 (3.5), 97 (1.6)	83 (4.7), 85 (4.7), 90 (4.6)	92 (4.9), 91 (3.6), 97 (2.8)	89 (4.9), 91 (4.3), 89 (2.7)	112 (3.9), 88 (4.2), 91 (3.6)
Sulfadiazine	2.0, 10, 20	103 (2.8), 105 (3.7), 94 (3.1)	89 (4.2), 86 (2.9), 94 (2.2)	86 (4.7), 87 (3.6), 94 (2.5)	84 (5.2), 103 (3.1), 90 (2.9)	88 (5.4), 87 (3.1), 95 (2.3)	82 (5.4), 85 (3.7), 95 (2.8)	86 (5.0), 86 (4.1), 94 (1.9)
		110 (2.2), 96 (3.1), 102 (1.9)	92 (3.6), 91 (3.5), 98 (1.3)	83 (2.7), 92 (2.3), 95 (1.2)	80 (6.3), 84 (4.3), 101 (3.2)	83 (5.1), 87 (2.3), 96 (1.6)	83 (5.1), 89 (3.3), 91 (3.6)	80 (5.3), 86 (5.2), 89 (3.3)
Sulfameter	2.0, 10, 20	108 (2.2), 92 (3.8), 96 (3.3)	88 (1.9), 94 (2.1), 95 (0.3)	90 (4.2), 91 (2.2), 97 (2.6)	109 (4.8), 112 (3.1), 101 (2.8)	105 (4.2), 89 (3.1), 97 (0.9)	86 (5.1), 91 (3.9), 95 (2.2)	107 (3.8), 92 (3.0), 93 (2.2)
			86 (3.7),	88 (4.7),	81 (3.6),	82 (4.1), 87 (2.7),	106 (4.4), 91 (2.3),	83 (3.7),

**Table 2** (continued)

Compound	Spiked ( $\mu\text{g kg}^{-1}$ )	%Recovery (%RSD)						
		Beef	Mutton	Pork	Chicken	Pork liver	Chicken liver	Lambs' liver
<b>Quinolone (7)</b>								
Enrofloxacin	5.0, 10, 50	87 (2.1), 103 (4.8), 97 (0.7)	88 (3.1), 95 (2.2)	92 (3.5), 93 (3.2)	84 (3.3), 88 (0.9)	98 (2.1)	96 (0.7)	88 (3.2), 99 (1.1)
Ciprofloxacin	5.0, 10, 50	115 (3.6), 107 (3.1), 92 (1.6)	109 (4.7), 105 (4.1), 106 (2.3)	108 (4.6), 89 (3.5), 97 (3.0)	114 (6.5), 88 (3.3), 95 (2.7)	86 (3.9), 105 (3.6), 96 (2.8)	83 (4.2), 84 (4.5), 89 (1.2)	84 (3.5), 87 (3.3), 101 (2.1)
Sarafloxacin	5.0, 10, 50	112 (2.1), 108 (0.9), 104 (0.4)	89 (4.5), 108 (3.3), 97 (1.9)	90 (3.7), 92 (3.1), 95 (2.2)	115 (4.9), 109 (2.9), 93 (3.6)	82 (3.4), 92 (3.1), 98 (0.6)	80 (6.1), 85 (3.7), 89 (3.1)	90 (4.4), 92 (1.9), 99 (1.6)
Danofloxacin	5.0, 10, 50	115 (4.5), 104 (3.3), 97 (1.9)	116 (4.2), 102 (2.9), 106 (2.2)	92 (4.1), 87 (3.6), 104 (1.8)	82 (6.7), 94 (3.6), 92 (2.9)	88 (6.3), 87 (2.3), 96 (2.1)	82 (6.3), 90 (5.4), 91 (3.7)	82 (6.7), 94 (3.6), 92 (2.9)
Flumequine	5.0, 10, 50	94 (3.8), 89 (2.3), 96 (1.1)	107 (5.2), 88 (3.7), 94 (2.8)	87 (3.7), 92 (3.1), 95 (2.2)	108 (6.5), 84 (3.9), 91 (4.1)	85 (5.8), 88 (3.1), 94 (2.9)	83 (5.7), 85 (3.7), 90 (2.9)	107 (3.5), 90 (1.9), 91 (1.1)
Ofloxacin	5.0, 10, 50	88 (5.8), 94 (3.3), 95 (1.7)	90 (4.4), 92 (3.3), 95 (2.6)	88 (4.6), 89 (3.5), 97 (3.0)	83 (5.4), 85 (4.1), 90 (3.6)	86 (3.9), 91 (3.6), 102 (2.8)	85 (5.5), 88 (4.5), 93 (2.7)	85 (4.3), 87 (4.2), 98 (3.0)
Norfloxacin	5.0, 10, 50	94 (2.9), 89 (3.3), 96 (0.5)	107 (5.2), 106 (3.7), 94 (2.8)	87 (3.7), 92 (3.1), 95 (2.2)	81 (4.8), 90 (3.3), 95 (3.6)	85 (5.4), 88 (3.7), 94 (0.6)	83 (5.2), 85 (3.8), 90 (2.9)	88 (2.8), 90 (3.3), 94 (1.6)
<b>Macrolides (2)</b>								
Erythromycin	1.0, 5.0, 10	88 (3.6), 94 (3.1), 95 (1.6)	90 (4.4), 92 (3.3), 95 (2.6)	88 (4.6), 89 (3.5), 97 (3.0)	85 (4.7), 85 (4.4), 94 (2.4)	90 (3.9), 91 (3.6), 96 (2.8)	85 (6.1), 87 (4.5), 93 (1.5)	85 (3.7), 85 (1.4), 94 (3.4)
Lincomycin	1.0, 5.0, 10	86 (4.2), 91 (3.2), 99 (0.6)	82 (2.1), 94 (1.8), 91 (0.4)	85 (5.2), 88 (3.1), 94 (2.7)	115 (5.1), 112 (3.3), 106 (2.8)	86 (4.2), 89 (2.9), 95 (2.2)	83 (5.2), 84 (4.2), 89 (2.8)	115 (5.0), 112 (4.3), 106 (1.8)
<b>Tetracycline (4)</b>								
Tetracycline	10, 50, 100	84 (3.3), 89 (2.3), 95 (1.9)	82 (4.5), 89 (3.3), 89 (2.7)	84 (4.9), 89 (3.1), 94 (3.3)	83 (6.3), 84 (5.5), 88 (3.9)	88 (5.6), 89 (3.1), 97 (3.2)	81 (5.1), 106 (4.9), 93 (3.1)	83 (6.4), 88 (2.5), 91 (1.6)
Oxytetracycline	10, 50, 100	92 (4.2), 91 (4.7), 98 (2.2)	90 (4.5), 92 (3.3), 95 (1.9)	91 (4.7), 92 (3.2), 96 (0.9)	88 (5.2), 85 (3.9), 99 (3.1)	85 (5.1), 88 (3.1), 91 (2.9)	82 (6.2), 85 (3.3), 91 (2.7)	83 (5.7), 88 (4.8), 99 (3.1)
Chlortetracycline	10, 50, 100	86 (4.6), 87 (3.1), 94 (0.9)	89 (4.5), 88 (3.7), 97 (0.7)	86 (3.7), 92 (3.1), 95 (2.2)	81 (5.6), 86 (3.3), 93 (3.6)	84 (3.4), 89 (2.2), 94 (0.7)	81 (5.9), 82 (4.4), 95 (3.6)	82 (3.6), 82 (3.7), 97 (3.2)

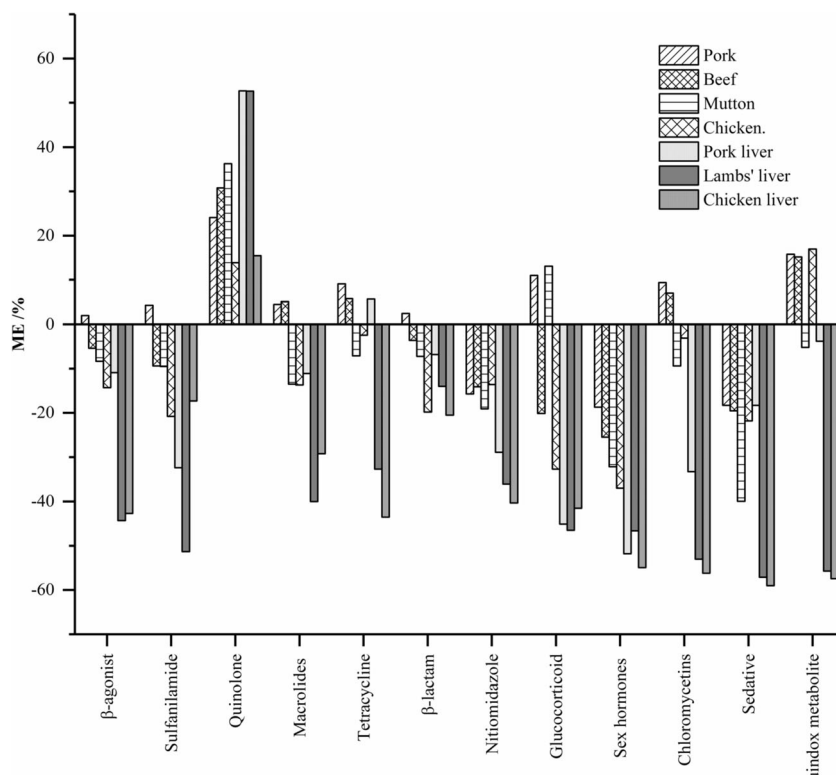
Table 2 (continued)

Compound	Spiked ( $\mu\text{g kg}^{-1}$ )	%Recovery (%RSD)							
		Beef	Mutton	Pork	Chicken	Pork liver	Chicken liver	Lambs' liver	
Doxycycline	10, 50, 100	113 (3.6), 97 (3.1), 101 (1.6)	109 (4.1), 105 (4.1), 106 (2.3)	89 (3.5), 97 (2.7)	85 (4.4), 90 (3.7)	97 (2.8)	84 (4.5), 89 (3.2)	85 (5.4), 87 (2.6)	
$\beta$ -lactam (4)									
Amoxicillin	5.0, 10, 20	88 (3.3), 89 (2.3), 92 (1.9)	88 (4.9), 89 (2.9)	84 (4.9), 89 (3.1), 94 (3.3)	84 (6.6), 84 (5.5), 90 (3.9)	83 (5.6), 87 (2.8), 95 (3.2)	85 (5.3), 106 (4.1), 94 (2.2)	89 (4.4), 86 (2.5), 97 (3.2)	
Ampicillin	5.0, 10, 20	85 (2.7), 87 (2.8), 94 (0.6)	89 (4.5), 92 (3.3), 97 (1.9)	90 (3.7), 92 (3.1), 95 (2.2)	81 (6.4), 88 (2.2), 91 (2.9)	82 (4.5), 86 (3.1), 94 (2.1)	82 (5.4), 85 (2.6), 91 (1.1)	81 (4.4), 86 (4.2), 94 (1.6)	
Benzylpenicillin	5.0, 10, 20	104 (3.6), 92 (3.6), 94 (0.7)	82 (4.7), 97 (1.5), 94 (0.6)	88 (4.1), 92 (3.7), 97 (3.0)	82 (3.9), 85 (3.6), 91 (2.8)	112 (3.9), 91 (3.6), 97 (2.8)	83 (6.1), 84 (4.5), 92 (1.6)	81 (5.9), 88 (3.4), 95 (2.1)	
Cloxacillin	5.0, 10, 20	91 (4.7), 92 (1.1), 97 (0.4)	89 (4.0), 88 (2.9), 97 (1.9)	88 (4.6), 92 (3.3), 95 (2.2)	82 (4.2), 94 (2.9), 91 (2.2)	85 (4.6), 86 (3.1), 93 (2.9)	85 (5.7), 85 (3.3), 96 (3.3)	83 (5.2), 94 (3.6), 98 (4.2)	
Nitimidazole (2)									
Dimetridazole	1.0, 5.0, 10	86 (4.4), 87 (3.6), 96 (1.8)	83 (4.5), 90 (3.3), 95 (1.9)	86 (3.9), 93 (3.6), 97 (2.8)	81 (4.4), 89 (2.9), 89 (3.6)	84 (4.4), 85 (3.1), 90 (2.4)	105 (5.1), 95 (4.6), 103 (3.6)	107 (3.4), 86 (2.8), 85 (4.1)	
Metronidazole	1.0, 5.0, 10	85 (3.7), 89 (3.1), 96 (2.2)	86 (4.5), 92 (3.3), 102 (1.9)	91 (3.9), 89 (3.6), 95 (2.8)	85 (5.7), 88 (4.1), 89 (3.4)	82 (3.3), 89 (2.3), 91 (3.9)	84 (4.4), 87 (3.3), 89 (3.3)	84 (5.2), 91 (3.4), 91 (1.4)	
Glucocorticoid (2)									
Betamethasone	1.0, 5.0, 10	82 (3.3), 87 (1.3), 96 (1.5)	89 (4.5), 88 (3.3), 97 (1.9)	86 (4.2), 85 (2.9), 96 (2.2)	84 (4.5), 89 (2.7), 94 (3.1)	82 (5.7), 94 (3.0), 91 (2.2)	83 (3.2), 87 (1.6), 95 (1.7)	84 (3.5), 86 (2.6), 96 (3.0)	
Dexamethasone	1.0, 5.0, 10	83 (5.1), 87 (3.1), 96 (2.1)	109 (4.7), 105 (4.1), 106 (3.9)	86 (4.6), 89 (3.5), 96 (3.0)	107 (5.9), 85 (4.2), 90 (3.6)	84 (6.1), 91 (3.6), 93 (3.5)	83 (6.1), 84 (4.7), 89 (2.7)	80 (4.9), 85 (2.2), 90 (1.6)	
Sex hormones (4)									
Methyltestosterone	1.0, 5.0, 10	88 (4.8), 92 (2.9), 98 (2.1)	82 (4.5), 94 (2.8), 91 (2.1)	85 (5.2), 88 (3.1), 94 (2.7)	85 (5.1), 82 (4.3), 86 (2.8)	88 (4.2), 89 (2.9), 95 (2.2)	82 (5.7), 88 (3.8), 92 (2.8)	87 (3.1), 111 (4.4), 94 (0.8)	
Trenbolone	1.0, 5.0, 10	85 (5.6), 84 (3.1), 90 (1.2)	85 (4.5), 89 (3.3), 94 (2.7)	81 (4.6), 89 (3.6), 90 (0.8)	82 (6.3), 90 (5.5), 88 (3.9)	83 (5.5), 87 (3.1), 89 (4.1)	83 (5.3), 86 (4.4), 95 (3.1)	112 (6.3), 90 (2.1), 102 (0.9)	

**Table 2** (continued)

Compound	Spiked ( $\mu\text{g kg}^{-1}$ )	%Recovery (%RSD)						
		Beef	Mutton	Pork	Chicken	Pork liver	Chicken liver	Lambs' liver
Testosterone	1.0, 5.0, 10	88 (3.9),	89 (4.5),	90 (3.7),	91 (4.9),	81 (3.7), 87 (2.6),	86 (5.8),	92 (4.1),
		89 (3.6),	94 (2.3),	92 (3.1),	89 (2.5),	91 (1.2)	84 (3.7),	91 (1.8), 107 (2.6)
Mestrol	1.0, 5.0, 10	96 (2.8)	93 (1.9)	95 (3.7)	107 (2.6)	86 (3.9), 91 (3.6),	88 (2.3)	83 (3.9),
		85 (3.6),	113 (4.6), 105 (4.5),	115 (4.6), 89 (4.1),	81 (5.9),	92 (2.8)	86 (6.1),	85 (2.0),
		92 (3.8),	104 (4.6)	95 (3.0)	85 (4.4),		84 (4.7),	98 (3.3)
		95 (3.2)			90 (3.6)		90 (5.1)	
Chloromycetins (3)								
Chloromycetin	0.1, 0.5, 1.0	88 (2.7),	89 (5.1),	90 (5.6),	83 (4.7),	84 (2.7), 89 (1.9),	85 (5.1),	114 (4.7), 97 (2.6),
		93 (1.3),	88 (3.3),	92 (3.6),	87 (2.6),	96 (0.5)	85 (3.7),	95 (0.8)
Thiamphenicol	0.5, 2.0, 5.0	96 (0.7)	97 (1.8)	95 (3.9)	95 (0.8)	86 (3.9), 91 (3.6),	91 (2.5)	82 (4.1),
		103 (3.6), 90 (3.1),	109 (4.3), 105 (4.1),	107 (4.2), 89 (3.5),	88 (5.7),	94 (2.8)	83 (5.7),	95 (5.1),
		96 (1.9)	106 (1.7)	97 (4.3)	85 (5.1),		84 (4.5),	
					90 (4.2)		89 (3.1)	
Florfenicol	0.5, 2.0, 5.0	86 (4.1),	82 (3.1),	85 (5.2),	85 (4.6),	87 (4.2), 92 (5.0),	82 (5.5),	88 (6.6),
		90 (3.9),	94 (2.5),	88 (3.1),	92 (3.5),	93 (4.2)	88 (3.7), 102 (1.9)	90 (5.5), 101 (2.0)
		99 (1.3)	91 (0.5)	94 (2.7)	106 (3.0)			
Sedative (4)								
Chlorpromazine	0.5, 2.0, 5.0	86 (4.7),	108 (4.4), 89 (3.3),	84 (4.1),	83 (5.2),	83 (5.6), 87 (3.1),	80 (5.9), 106 (4.9), 93	113 (6.2), 84 (3.1),
		92 (3.5),	96 (2.7)	90 (4.2),	84 (3.1),	95 (3.2)	(3.1)	85 (3.7)
Acetylpromazine	0.5, 2.0, 5.0	93 (1.2)		94 (1.1)	85 (3.7)	90 (3.5), 87 (3.1),	88 (5.2),	82 (3.9),
		85 (4.6),		91 (3.7),	87 (4.9),	102 (0.7)	87 (4.7),	84 (3.7),
		91 (3.5),		92 (3.1),	84 (3.1),		93 (4.6)	
		96 (3.0)		91 (2.2)	94 (4.3)			
Azaperol	0.5, 2.0, 5.0	90 (3.7), 107 (2.9),	109 (4.7), 105 (4.1),	88 (4.6),	81 (5.4),	88 (3.9), 91 (3.6),	81 (4.9),	84 (7.8),
		94 (2.2)	106 (1.1)	89 (2.5), 102 (3.0)	91 (4.4),	95 (2.8)	84 (4.6),	97 (4.2),
					90 (1.7)	90 (4.2)	90 (1.4)	
Phenylethanolamine A	0.5, 2.0, 5.0	88 (3.7),	82 (2.5),	85 (5.3),	114 (5.5), 102	81 (4.6), 89 (3.9),	87 (5.5),	114 (5.1), 102 (1.2),
		92 (3.2),	94 (2.7),	90 (3.1),	(2.2),	95 (1.2)	92 (3.8),	90 (0.8)
		98 (1.8)	91 (1.3)	97 (1.9)	90 (2.8)		95 (4.1)	
Olaquinox metabolite (O) (1)								
3-Methyl-quinoxaline-2-carboxylic acid	0.5, 2.0, 5.0	90 (3.3),	88 (4.7),	82 (5.7),	85 (5.8),	83 (5.8), 87 (3.1),	82 (5.7),	109 (5.4), 81 (4.7),
		89 (2.3),	89 (3.1),	95 (2.8),	84 (4.9),	95 (3.1)	89 (4.9),	94 (3.2)
		95 (1.9)	97 (2.7)	93 (2.1)	95 (3.9)		93 (4.2)	

**Fig. 3** Matrix effects in HPLC-MS analysis of VDs in different matrix samples



matrix effect of analytes (José Ángel Salatti-Dorado et al. 2017). Since they are strongly retained on the chromatographic column, phospholipids can also produce shifts in retention time, increased baseline noise, and poor precision.

Both phosphatidylcholine (PC) and sphingomyelin (SM) have a phosphocholine head group and would be fragmented to generate a characteristic ion at  $m/z$  184. The total amount of PC and SM in the sample can be determined by measuring the total precursor ion signals that generate product ion with  $m/z$  184. This value can then be used in lipidomic studies (Chao et al. 2017). The removal of phospholipids was evaluated in a single injection by measuring the positive precursor ion scan at  $m/z$  184 after sample extraction with no cleanup or cleanup using PRiME HLB. Large amounts of phospholipids were detected in positive ion mode in both muscle and yolk extracts, when no cleanup was carried out. A PRiME HLB cleanup step completely removed phospholipids (Fig. 4). PRiME HLB cartridges have a packed bed of a novel water-wettable reversed-phase SPE sorbent, designed for the gross level removal of phospholipids from biological tissue prior to UPLC-MS/MS analysis.

#### Accuracy, Repeatability, and Reproducibility

To evaluate the trueness of the proposed method, three concentrations, limits of quantification (LOQ) (low),

$20 \times \text{LOQ}$  (medium) and  $50 \times \text{LOQ}$  (high) for each analyte, were analyzed in seven meat samples. Each analysis was repeated six times in parallel. Repeatability was evaluated at three levels as previously described, with six replicates at each level. The percentage recovery and precision results expressed as RSDs are given in Table 2. All of the compounds had recoveries between 80 and 116% in various meat and tissue samples. Although recoveries were slightly lower in the liver samples, these also met the quantitative requirements. The RSDs of all compounds, in all investigated matrices, was  $< 7.8\%$ , indicating that the method can be carried out with a satisfactory level of precision. The results also indicate that good recoveries can be obtained using the developed method. Total ion current (TIC) chromatograms of blank and spiked pork samples were showed in Fig. 5.

#### Linearity and Limit of Detection

Because of the difference in sensitivity of the analytes in the LC/MS, linearity of the analytical method was evaluated using matrix- matched calibration curves for each compound in blank meat samples at six different concentration levels, and for different compounds over different concentration ranges. All 60 compounds showed good linear regression, with  $R^2$  values  $> 0.995$  (Table 1).

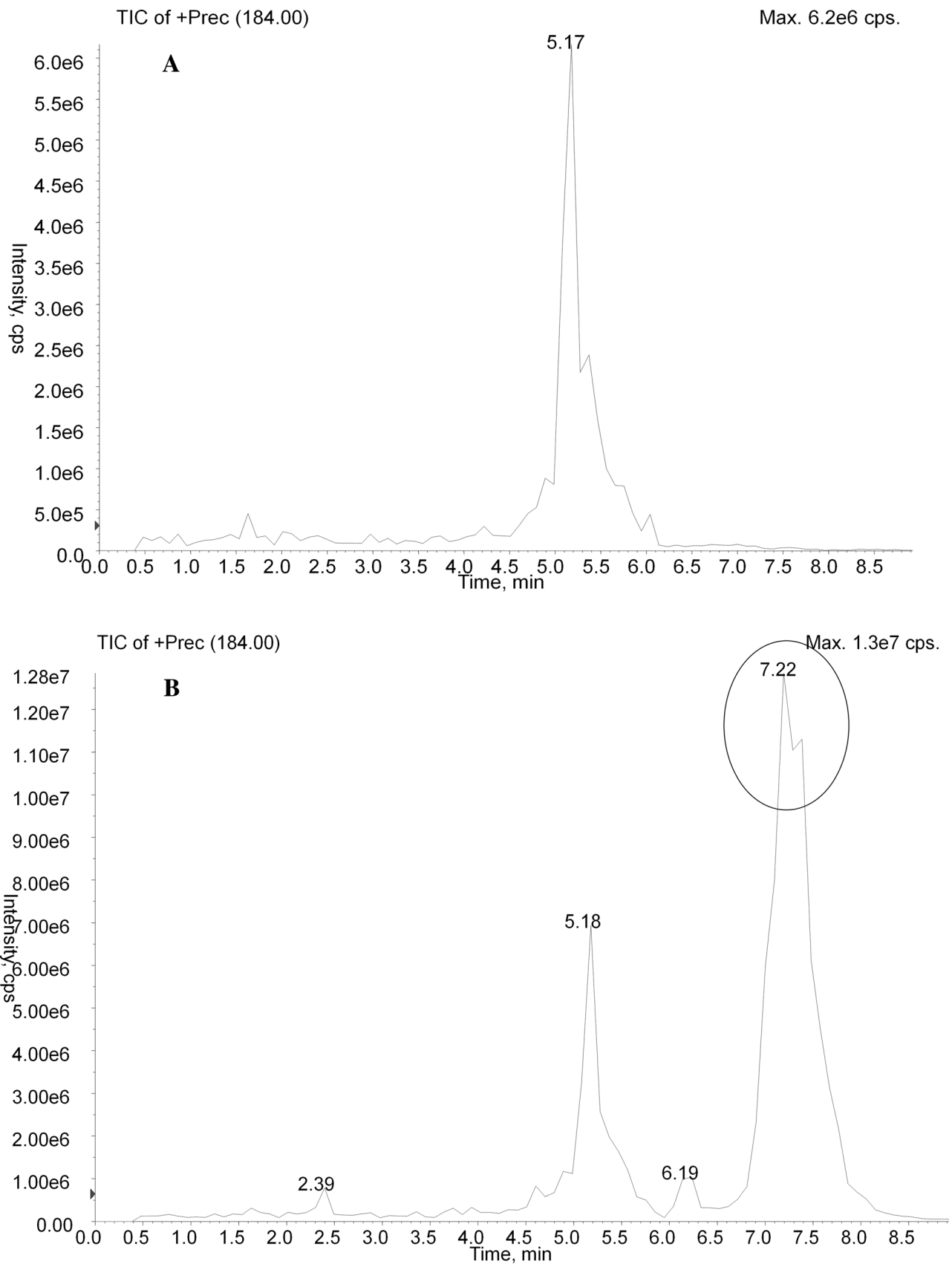


Fig. 4 Parent scan mass spectrum at m/z 184 of pork blank with PRiME HLB (A) and no purification (B)

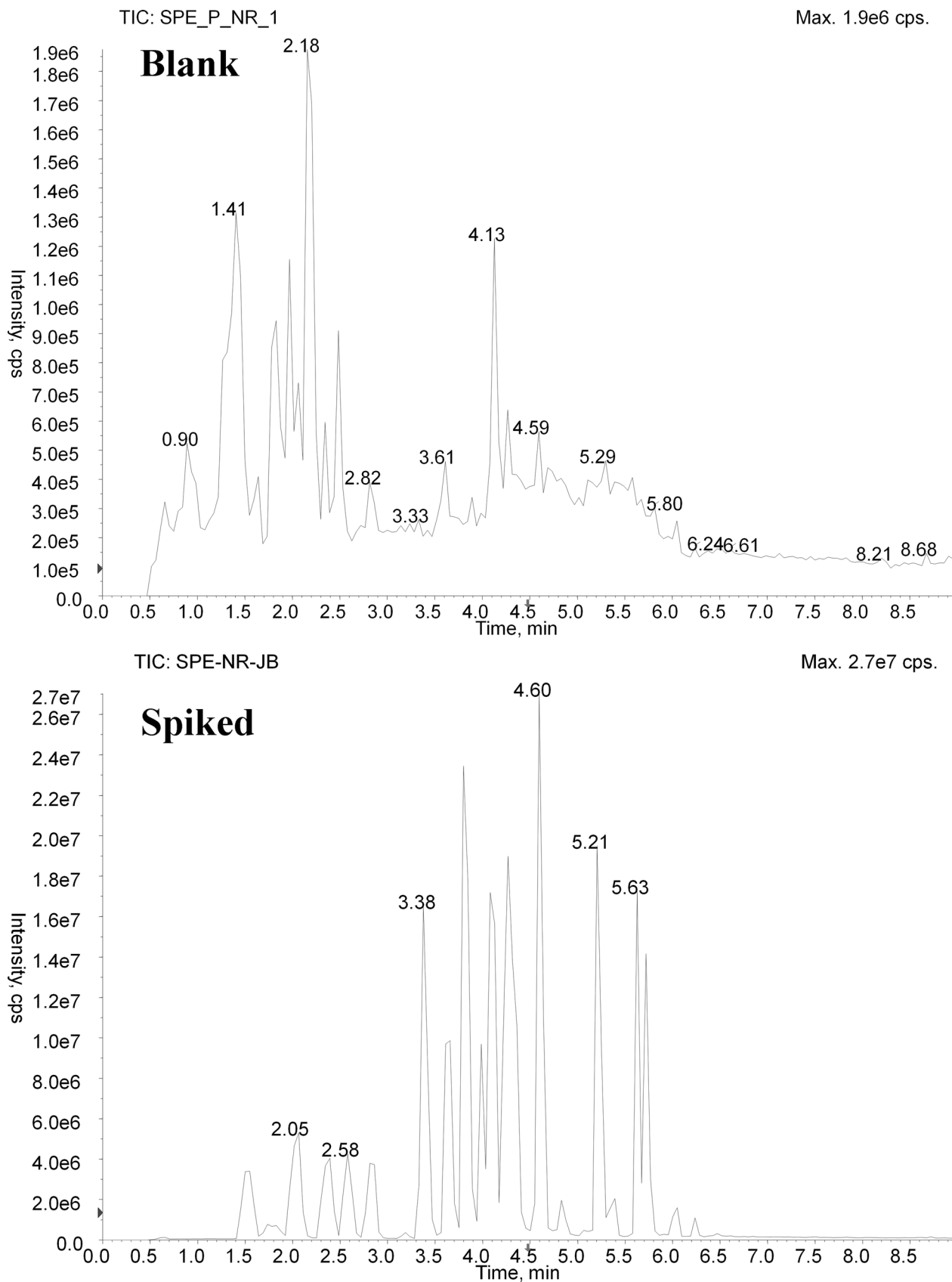
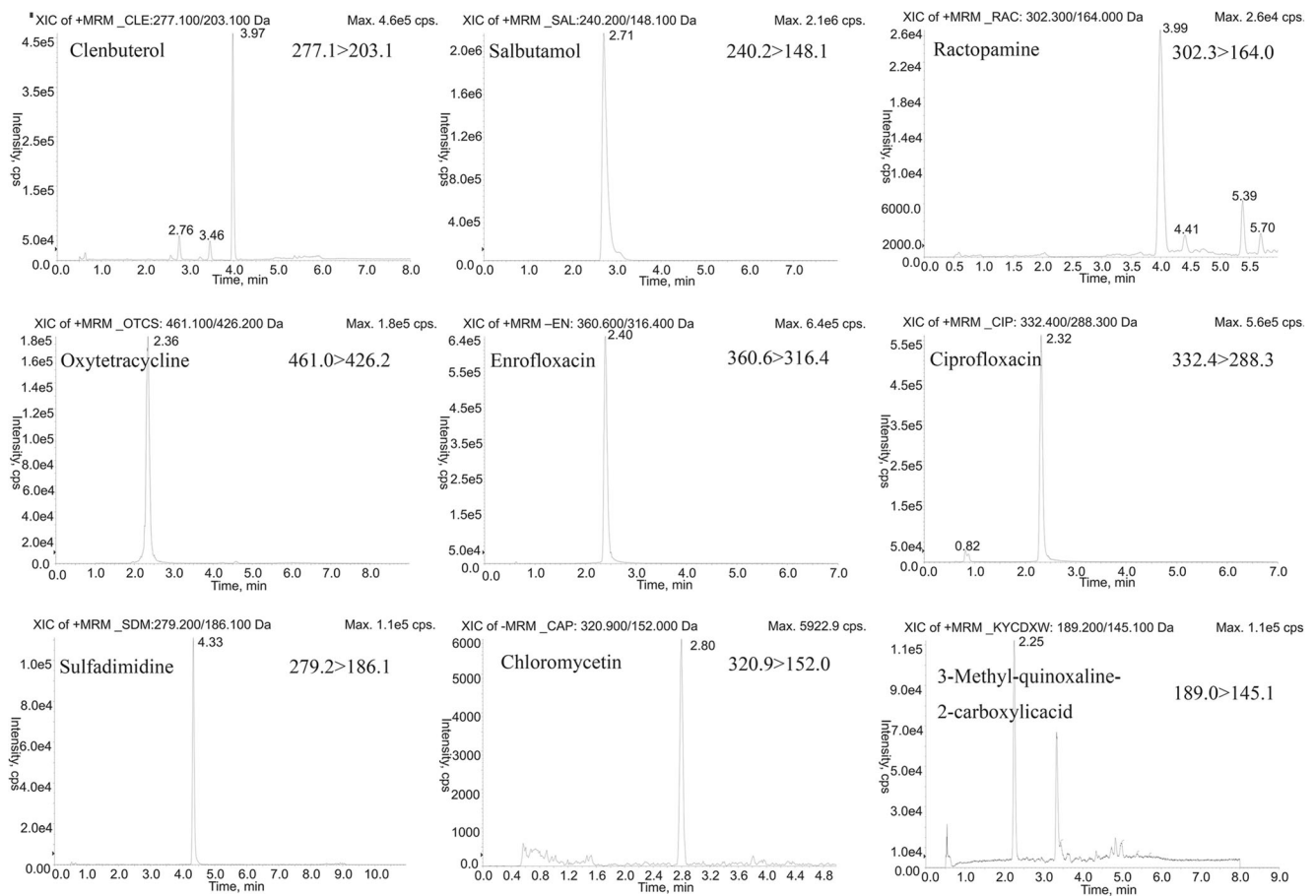


Fig. 5 Total ion current (TIC) chromatograms of blank and spiked pork samples





**Fig. 6** SRM chromatograms of nine VDs in real samples (clenbuterol, salbutamol, ractopamine, oxytetracycline, enrofloxacin, ciprofloxacin, chloromycetin, sulfadimidine, and 3-methyl-quinoxaline-2-carboxylic acid)

The LODs and LOQs were determined as explained in the “Validation Procedure” section. Concentration levels with signal to noise  $\geq 3$  were defined as LODs and concentration levels with signal to noise  $\geq 10$  were defined as LOQs. As shown in Table 1, the LODs and LOQs for 60 analytes ranged from 0.2 to 3  $\mu\text{g kg}^{-1}$  and 0.5 to 10  $\mu\text{g kg}^{-1}$ , respectively, indicating the satisfactory sensitivity for the proposed analytical protocol.

### Application to Real Samples

The developed method was used for the analysis of VDs in 280 meat samples, including pork, beef, mutton, pork liver, lambs’ liver, and chicken liver. Three  $\beta$ -agonists (clenbuterol, salbutamol, ractopamine) were identified in samples of mutton, beef, lambs’ liver, pork, and chicken with concentrations ranging from 0.90 to 410  $\mu\text{g kg}^{-1}$ . Oxytetracycline, enrofloxacin, ciprofloxacin, and chloromycetin were also found in chicken and chicken liver samples, with the concentrations  $< 222 \mu\text{g kg}^{-1}$ . Sulfadimidine and 3-methyl-quinoxaline-2-

carboxylic acid were detected in a pork and pork liver sample, at concentration levels of 607 and 2.73  $\mu\text{g kg}^{-1}$ , respectively. The typical SRM chromatograms of positive samples were presented in Fig. 6. These results are consistent with previous studies. Several analytical methods for the determination of different groups of VDs in meat samples have been developed by LC-MS/MS and applied in real meat samples. A method was developed for detecting sulfonamides, tilmicosin, and avermectins residues in animal samples, and sulfamethazine (sulfadimidine) was detected at concentration levels of 15 and 72  $\mu\text{g kg}^{-1}$  in 2 bovine meat samples (Qin et al. 2017). LC-MS/MS had also been applied in 137 pilot meat samples and ractopamine had been identified with the concentration ranging from 1.8 to 6.3  $\mu\text{g kg}^{-1}$  and 0.6 to 64  $\mu\text{g kg}^{-1}$  in 12 bovine muscle and 15 bovine liver samples, respectively (Matus and Boison 2016). For all positive samples, the differences between values obtained using the new method and those obtained using the National Standard Method of China were  $< 7.78\%$ , which are presented in Table 3.

**Table 3** Detection data and relative deviations (RDs) of several drugs in positive samples

Sample number	Sample	Compound	M-1 (µg/kg)	M-2 <sup>a,b,c</sup> (µg/kg)	RDs%
S-81	Mutton	Clenbuterol	1.66	1.63 <sup>a</sup>	+ 1.8
S-82	Mutton	Clenbuterol	4.73	5.99 <sup>a</sup>	− 5.88
S-89	Mutton	Clenbuterol	14.6	13.9 <sup>a</sup>	+ 1.23
S-98	Mutton	Clenbuterol	12.7	13.4 <sup>a</sup>	− 1.34
S-102	Mutton	Clenbuterol	45.0	44.5 <sup>a</sup>	+ 0.28
S-103	Mutton	Clenbuterol	2.90	3.12 <sup>a</sup>	− 1.83
S-111	Mutton	Clenbuterol	0.90	0.89 <sup>a</sup>	+ 0.27
S-112	Mutton	Clenbuterol	53.0	56.7 <sup>a</sup>	− 1.69
S-45	Beef	Clenbuterol	10.0	10.2 <sup>a</sup>	− 0.50
S-51	Beef	Clenbuterol	15.9	15.2 <sup>a</sup>	+ 1.13
S-52	Beef	Clenbuterol	11.8	12.0 <sup>a</sup>	− 0.42
S-241	Lambs' liver	Clenbuterol	134	106 <sup>a</sup>	+ 5.83
S-249	Lambs' liver	Clenbuterol	5.65	5.83 <sup>a</sup>	− 0.78
S-266	Lambs' liver	Clenbuterol	410	428 <sup>a</sup>	− 1.11
S-278	Lambs' liver	Clenbuterol	1.66	1.59 <sup>a</sup>	+ 1.08
S-15	Pork	Salbutamol	0.99	1.02 <sup>a</sup>	− 1.5%
S-146	Chicken	Salbutamol	148	159 <sup>a</sup>	− 1.81
S-62	Beef	Ractopamine	1.60	1.62 <sup>a</sup>	− 0.31
S-122	Chicken	Oxytetracycline	35.4	34.2 <sup>b</sup>	+ 3.5%
S-134	Chicken	Oxytetracycline	130	139 <sup>b</sup>	− 1.76
S-158	Chicken	Oxytetracycline	222	164 <sup>b</sup>	+ 7.51
S-138	Chicken	Enrofloxacin	113.5	110.1 <sup>c</sup>	+ 3.0%
S-138	Chicken	Ciprofloxacin	27.1	19.8 <sup>c</sup>	+ 7.78
S-32	Pork	Sulfadimidine	607	528 <sup>d</sup>	+ 3.47
S-211	Chicken liver	Chloromycetin	0.651	0.567 <sup>e</sup>	+ 3.45
S-186	Pork liver	3-methyl-quinoxaline-2-carboxylicacid	2.73	2.32 <sup>f</sup>	+ 4.06

M-1 is the new method, M-2<sup>a</sup> is GB/T 22286-2008, M-2<sup>b</sup> is GB/T 21317-2007, M-2<sup>c</sup> is GB/T 21312-2007, M-2<sup>d</sup> is GB/T 21316-2007, M-2<sup>e</sup> is GB/T 22338-2008 and M-2<sup>f</sup> is GB/T 20746-2006

## Conclusion

The Oasis PRiME HLB UPLC-MS/MS method described here is a reliable procedure for the simultaneous determination of 60 VD residues in different meat samples. The new method was satisfactorily validated in terms of linearity, sensitivity, precision, repeatability, and MEs. The use of acetonitrile/water (80/20, *v/v*) and Oasis PRiME HLB for extraction and cleanup gave excellent recovery and purification. Oasis PRiME HLB, when used as a pass-through cleanup SPE, can remove most of the phospholipids from biological tissues prior to LC-MS analysis. Notably, the properties of the pass-through SPE technique mean that more compounds in more food matrices could easily be included in the future.

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## Compliance with Ethical Standards

**Conflict of Interest** Yanxia Zhang declares no conflict of interest. Xia Xue declares no conflict of interest. Shufang Su declares no conflict of interest. Zhimou Guo declares no conflict of interest. Jun Wang declares no conflict of interest. Lisheng Ding declares no conflict of interest. Yanming Liu declares no conflict of interest. Jianhua Zhu declares no conflict of interest.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed Consent** Informed consent is not applicable for this study.

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