# Multiresidue Analysis of Veterinary Drugs in Bovine Liver by LC/MS/MS

## Agilent Bond Elut QuEChERS Enhanced Matrix Removal—Lipid

Application Note

Food Testing and Agriculture


## Authors

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## Abstract

Agilent Bond Elut QuEChERS Enhanced Matrix Removal-Lipid (EMR-Lipid) is the next generation of sample preparation product, and is available for convenient dispersive solid phase extraction (dSPE). The material is highly selective towards coextracted matrix, especially from fatty samples (fat content > 5%) without negatively impacting analyte recovery. This study demonstrates the application of this novel product for the analysis of 30 representative veterinary drugs in bovine liver. The procedure involves a rapid and efficient protein precipitation extraction by acidified acetonitrile, followed by the use of EMR—Lipid dSPE and a polish kit for further cleanup. The amount of matrix removed by the EMR-Lipid protocol was determined by the weight of coextractives and postcolumn infusion experiments. Compared to other matrix cleanup products, EMR—Lipid dSPE provides more effective matrix removal and better analyte recoveries. The optimized EMR—Lipid method delivers superior cleanliness, and excellent accuracy and precision for all 30 veterinary drug compounds at all levels, providing fast, robust, and effective analysis of high-fat samples.


Agilent Technologies

## Introduction

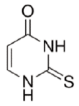
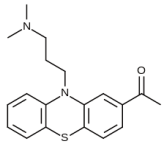
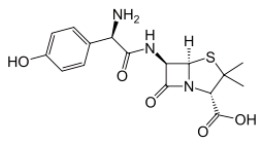
Veterinary drugs are widely used for animals in the food production industry to prevent diseases, or as growth promoters. These drugs accumulate in animal tissue, and improper use can lead to drug residue build-up in edible tissues, which are a known risk to human health. With increased attention on food safety, regulations have been put in place in nearly every country to limit the drugs used in food animal production [1-4].

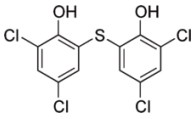
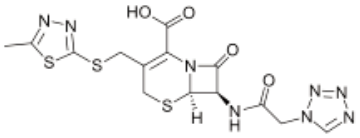
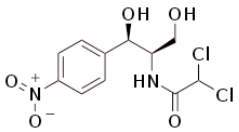
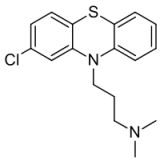
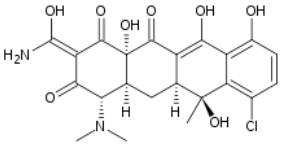
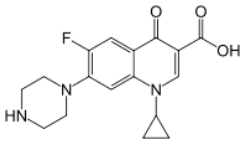
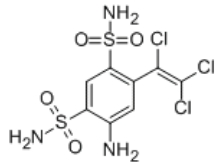
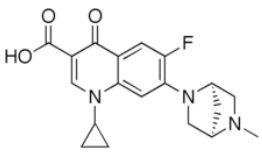
Foods from animal origin such as muscle, liver, and eggs are usually chemically complex and, therefore, it is critical to apply an efficient sample preparation method that includes general extraction and efficient cleanup. The established sample preparation methods include traditional solvent extraction, solid phase extraction (SPE), or a combination of multiple techniques. These methods are usually labor-intensive, time consuming, only suitable for limited classes of compounds, and require additional method development.

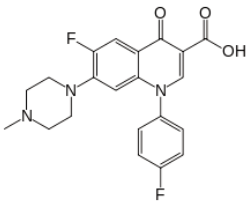
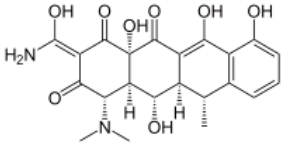
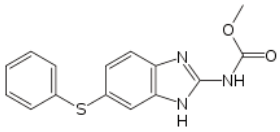
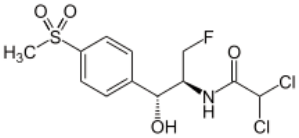
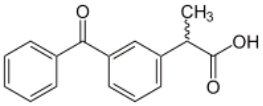
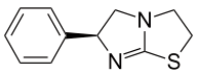
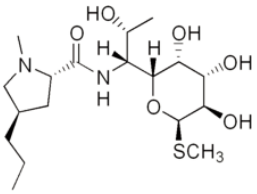
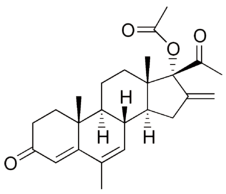
Multiclass, multiresidue methods are becoming increasingly popular in regulatory monitoring programs due to their increased analytical scope and laboratory efficiency. The number of veterinary drugs being monitored has increased in the past few years, and now there are more than 100 reported [5-8]. Sample pretreatment usually involves extraction with a mixture of acetonitrile:water, followed by C18 cleanup, other cleanup techniques, or both. Sorbents such as C18 only provide limited removal of coextracted lipids, which can result in precipitation in the final sample on dilution or reconstitution. The generation of precipitate requires sample filtration before LC/MS/MS injection, and may cause analyte loss. Hexane can be added during the dispersive solid phase extraction (dSPE) to remove coextracted lipids but is nonselective, time-consuming, and removes hydrophobic analytes. The use of zirconia sorbent materials for cleanup provides improved matrix cleanup compared to C18, but also results in more analyte loss, especially for carboxylic acid- and hydroxyl-containing compounds such as fluoroquinolones, tetracyclines, and macrolides [7,8].

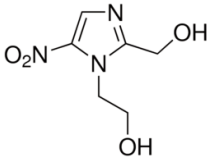
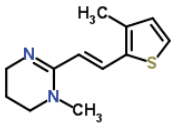
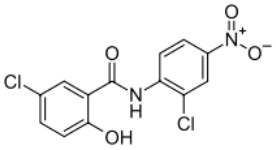
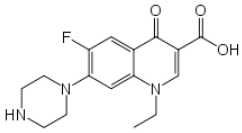
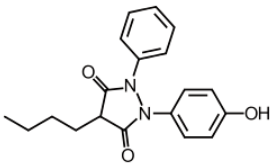
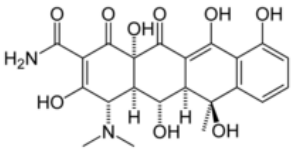
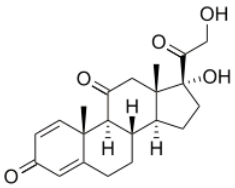
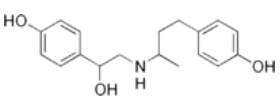
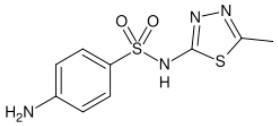
Agilent Bond Elut QuEChERS Enhanced Matrix Removal-Lipid (EMR—Lipid) is a novel sorbent material that selectively removes major lipid classes from the sample without unwanted analyte retention. Removal of lipid interferences from complex matrices is especially important for techniques such as QuEChERS and protein precipitation. Since these simple sample preparation methods cannot remove a large percentage of lipids, the coextractives will remain in the final sample extract with the target analytes. This causes chromatographic anomalies, poor data precision and accuracy, and increased maintenance issues. In this study, we investigate a novel sample preparation approach for the analysis of 30 representative and challenging veterinary drugs in bovine liver using a simple protein precipitation extraction followed by EMR-Lipid cleanup. The selected veterinary drugs represent 17 different classes, including hydrophilic to hydrophobic, acidic, neutral, and basic drugs. Table 1 shows the chemical and regulatory information for these veterinary drugs.

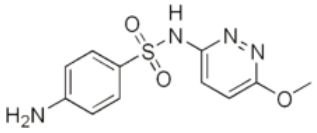
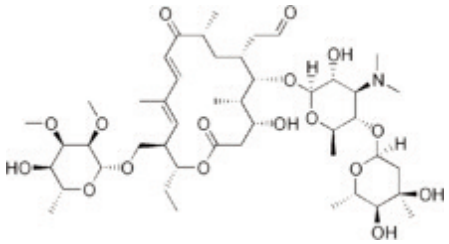
Table 1. Chemical and physical properties of veterinary drugs.

| Name | Drug class | Log P | pKa | Molecular formula | Structure | US tolerance (µg/g) |
|---|---|---|---|---|---|---|
| 2-Thiouracil | Thyreostat | –0.28 | 7.75 | $C_4H_4N_2OS$ |  | N.A |
| Acetopromazine | Tranquilizer | 3.49 | 9.3 | $C_{19}H_{22}N_2OS$ |  | N.A |
| Amoxicillin | $\beta$-Lactam | 0.86 | 2.4 | $C_{16}H_{19}N_3O_5S$ |  | 0.01 |

| Name | Drug class | Log P | pKa | Molecular formula | Structure | US tolerance (µg/g) |
|---|---|---|---|---|---|---|
| Bithionol | Flukicide | 5.51 | 4.82 | $C_{12}H_6Cl_4O_2S$ |  | N.A |
| Cefazolin | Cephalosporin | –1.5 | 2.3 | $C_{14}H_{14}N_8O_4S_3$ |  | N.A. |
| Chloramphenicol | Phenicol | 1.14 | 5.5 | $C_{11}H_{12}C_{12}N_2O_5$ |  | (c) |
| Chlorpromazine | Tranquilizer | 5.20 | 9.3 | $C_{17}H_{19}ClN_2S$ |  | N.A |
| Chlortetracycline | Tetracycline | 0.24 | 3.3, 7.4, 9.3 | $C_{22}H_{23}ClN_2O_8$ |  | 2 (d) |
| Ciprofloxacin | Fluoroquinolone | 0.28 | 6.09, 8.74 | $C_{17}H_{18}FN_3O_3$ |  | (c) |
| Clorsulon | Flukicide | 1.25 | – | $C_8H_8Cl_3N_3O_4S_2$ |  | 0.1 (e) |
| Danofloxacin | Fluoroquinolone | 1.2 | 6.04 | $C_{19}H_{20}FN_3O_3$ |  | 0.02 (a, e) |

| Name | Drug class | Log P | pKa | Molecular formula | Structure | US tolerance (µg/g) |
|---|---|---|---|---|---|---|
| Difloxacin | $\beta$-Lactam | 2.78 | 5.85 | $C_{21}H_{19}F_2N_3O_3$ |  | N.A. |
| Doxycycline | Tetracycline | –0.54 | 3.4 | $C_{22}H_{24}N_2O_8$ |  | N.A |
| Fenbendazole | Anthelmintic | 3.75 | 10.27 | $C_{15}H_{13}N_3O_2S$ |  | 0.4 (e, h), 2 (b) |
| Florfenicol | Phenicol | –0.12 | 10.73 | $C_{12}H_{14}C_{12}FNO_4S$ |  | 0.2 (b) |
| Ketoprofen | Tranquilizer | 2.81 | 3.88 | $C_{16}H_{14}O_3$ |  | N.A |
| Levamisole | Anthelmintic | 1.85 | 8.0 | $C_{11}H_{12}N_2S$ |  | 0.1 (e) |
| Lincomycin | Lincosamide | 0.91 | 7.8 | $C_{18}H_{34}N_2O_6S$ |  | 0.1(b) |
| Melengesterol acetate (MGA) | Other | 4.21 | 11.42 | $C_{25}H_{32}O_4$ |  | 0.025 (g) |

| Name | Drug class | Log P | pKa | Molecular formula | Structure | US tolerance (µg/g) |
|---|---|---|---|---|---|---|
| Methonidazole-OH | Nitroimidazole | –0.81 | 3.09 | $C_6H_9N_3O_4$ |  | (c) |
| Morantel | Anthelmintic | 1.97 | >12 | $C_{12}H_{16}N_2S$ |  | N.A |
| Niclosamide | Flukicide | 5.41 | 5.6 | $C_{13}H_8C_{12}N_2O_4$ |  | N.A |
| Norfloxacin | Fluoroquinolone | 0.82 | 6.32 | $C_{16}H_{18}FN_3O_3$ |  | (c) |
| Oxyphenylbutazone | NSAID | 2.72 | 4.87 | $C_{19}H_{20}N_2O_3$ |  | N.A |
| Oxytertracycline | Tetracycline | –1.5 | 3.27 | $C_{22}H_{24}N_2O_9$ |  | 2 (d) |
| Prednisone | Corticosteroid | 1.57 | 12.58 | $C_{21}H_{26}O_5$ |  | N.A |
| Ractopamine | $\beta$-Agonist | 1.65 | 9.4 | $C_{18}H_{23}NO_3$ |  | 0.03 (e) |
| Sulfamethizole | Sulfonamide | 0.51 | 5.45 | $C_9H_{10}N_4O_2S_2$ |  | N.A |

| Name | Drug class | Log P | pKa | Molecular formula | Structure | US tolerance (µg/g) |
|---|---|---|---|---|---|---|
| Sulfamethoxypyridazine | Sulfonamide | 0.32 | 6.7 | $C_{11}H_{12}N_4O_3S$ |  | N.A |
| Tylosin | Macrolide | 3.27 | 7.7 | $C_{46}H_{77}NO_{17}$ |  | 0.2 (f) |

a Tolerance in cattle liver (for tulathromycin, a marker residue has been established)

b Tolerance in swine muscle

c Banned for extralabel use

d Tolerance is the sum of residues of tetracycline including chlortetracycline, oxytetracycline, and tetracycline in muscle

e Tolerance in cattle muscle

f Tolerance in uncooked cattle fat, muscle, liver, and kidney

g Tolerance in cattle fat

h Tolerance in goat muscle

## Experimental

All regents and solvents were HPLC or analytical grade. Acetonitrile (ACN) was from Honeywell (Muskegon, MI, USA). Dimethyl sulfoxide (DMSO), vet drug standards, and internal standard were from Sigma-Aldrich, Corp. (St Louis, MO, USA). Reagent-grade formic acid (FA) was from Agilent (p/n G2453-86060). Ammonium acetate ($NH_4OAc$) was from Fisher Chemicals (Fair Lawn, NJ, USA).

## Solution and standards

Formic acid (5%) in ACN was freshly prepared by adding 5 mL formic acid to 95 mL ACN. Ammonium acetate stock solution (1 M) was made by dissolving 19.27 g $NH_4OAc$ in 250 mL Milli-Q water. The solution was stored at 4 °C. A 5 mM ammonium acetate in water solution was made by adding 5 mL of 1 M ammonium acetate stock solution to 1 L of Milli-Q water.

Standard and internal standard (IS) stock solutions were made in DMSO at 2.0 mg/mL except for danofloxacin stock solution in DMSO at 1.0 mg/mL and ciprofloxacin stock solution in DMSO at 0.25 mg/mL. Amoxicillin and cefazolin stock solutions were made in water at 2.0 mg/mL. All stock solutions were prepared in amber glass vials, except plastic vials for amoxicillin and cefazolin stock solutions. All stock solutions were stored at –20 °C. The 30 compounds were allocated to two groups, G1 and G2, based on instrument response. A combined 25/5 µg/mL (G1/G2) standard working solution was prepared in 1/1 ACN/water. Flunixin-$d_3$ IS working solution (25 µg/mL) was prepared in 1/1 ACN/water.

## Equipment

Equipment and material used for sample preparation included:

- Geno/Grinder (SPEX, Metuchen, NJ, USA)
- Centra CL3R centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Vortexer and multitube vortexers (VWR, Radnor, PA, USA)
- Bottle top dispenser (VWR, So. Plainfield, NJ, USA)
- Eppendorf pipettes and repeater
- Agilent Bond Elut EMR—Lipid tubes (p/n 5982-1010) Agilent Bond Elut Final Polish for Enhanced Matrix Removal-Lipid (p/n 5982-0101)

## Instrumentation

Analysis was performed on an Agilent 1290 Infinity LC system consisting :

- Agilent 1290 Infinity Quaternary Pump (G4204A)
- Agilent1290 Infinity High Performance Autosampler (G4226A) equipped with an Agilent 1290 Infinity Thermostat (G1330B), and an Agilent1290 Infinity Thermostatted Column Compartment (G1316C)

The UHPLC system was coupled to an Agilent 6490 Triple Quadrupole LC/MS system equipped with an Agilent Jet Stream electrospray ionization source and iFunnel technology. Agilent MassHunter workstation software was used for data acquisition and analysis.

## Instrument conditions

### HPLC conditions

| | |
|---|---|
| Column: | Agilent Poroshell 120 EC-C18, 2.1 × 150 mm, 2.7 µm (p/n 693775-902),<br>Agilent Poroshell 120 EC-C18 UHPLC Guard, 2.1 × 5 mm, 2.7 µm (p/n 821725-911) |
| Mobile phase: | A) 0.1% FA in water<br>B) 0.1% FA in acetonitrile |
| Flow rate: | 0.3 mL/min |
| Column temp: | 40 °C |
| Autosampler temp: | 4 °C |
| Inj vol: | 3 µL |
| Needle wash: | 1:1:1:1 ACN:MeOH:IPA:H2O with 0.2% FA |
| Gradient: | Time (min) %B<br>0 10<br>0.5 10<br>8.0 100 |
| Stop time: | 12 min |
| Posttime: | 3 min |

### Conditions, MS

Positive/negative mode

| | | | |
|---|---|---|---|
| Gas temp: | 120 °C | | |
| Gas flow: | 14 L/min | | |
| Nebulizer: | 40 psi | | |
| Sheath gas heater: | 400 °C | | |
| Sheath gas flow: | 12 L/min | | |
| Capillary: | 3,000 V | | |
| iFunnel parameters: | | Positive | Negative |
| | High-pressure RF | 90 V | 90 V |
| | Low-pressure RF | 70 V | 60 V |

MS DMRM conditions relating to the analytes are listed in Table 2, and a typical chromatogram is shown in Figure 1.

## Sample preparation

The final sample preparation procedure was optimized with the following steps.

1. Weigh 2 g (±0.1 g) homogenized bovine liver into 50 mL centrifuge tube.
2. Add 10 mL acidified acetonitrile (containing 5% FA).
3. Mix samples on a mechanical shaker for 2 min.
4. Centrifuge at 5,000 rpm for 5 min.
5. Add 5 mL ammonium acetate buffer (5 mM) to a 15 mL EMR-Lipid dSPE tube.
6. Transfer 5 mL of supernatant to EMR—Lipid tube.
7. Vortex immediately to disperse sample, followed by 60 s mixing on a multiposition vortexer table.
8. Centrifuge at 5,000 rpm for 3 min.
9. Transfer 5 mL of supernatant into a 15 mL EMR—Lipid polish tube containing 2 g salts (1:4 NaCl:$MgSO_4$) and vortex for 1 min.
10. Centrifuge at 5,000 rpm for 3 min.
11. Combine 200 µL of upper ACN layer and 800 µL water in a 2 mL sample vial, and vortex.

Table 2. LC/MS/MS DMRM parameters and retention times for target analytes.

| Analyte | RT (min) | Delta RT (min) | Polarity | Precursor ion (*m/z*) | Product ion | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Quant ion | CE (V) | Qual ion | CE (V) |
| 2-Thiouracil | 1.41 | 2 | Negative | 127 | 57.9 | 17 | – | – |
| Amoxicillin | 1.84 | 2 | Positive | 366.1 | 349.2 | 5 | 114 | 25 |
| Methonidazole-OH | 2.07 | 2 | Positive | 188.1 | 123.1 | 9 | 126.1 | 13 |
| Levamisole | 3.4 | 2 | Positive | 205.1 | 178.1 | 21 | 91.1 | 41 |
| Lincomycin | 3.35 | 2 | Positive | 407.2 | 126.1 | 37 | 70.1 | 80 |
| Norfloxacin | 4.22 | 2 | Positive | 320.1 | 302.2 | 21 | 276.1 | 17 |
| Oxytetracycline | 4.24 | 2 | Positive | 461.2 | 426.1 | 17 | 443.2 | 9 |
| Ciprofloxacin | 4.31 | 2 | Positive | 332.1 | 231 | 45 | 314.3 | 21 |
| Danofloxacin | 4.42 | 2 | Positive | 358.2 | 340.2 | 21 | 81.9 | 53 |
| Ractopamine | 4.4 | 2 | Positive | 302.2 | 107 | 33 | 77 | 77 |
| Morantel | 4.9 | 2 | Positive | 221.1 | 123.1 | 37 | 76.9 | 80 |
| Cefazolin | 4.65 | 2 | Positive | 455 | 323.1 | 9 | 156 | 13 |
| Sulfamethizole | 4.65 | 2 | Positive | 271 | 156.1 | 13 | 92 | 29 |
| Sulfamethoxypyridazine | 4.69 | 2 | Positive | 281.1 | 92 | 33 | 65.1 | 57 |
| Difloxacin | 4.83 | 2 | Positive | 400.2 | 382 | 25 | 356.3 | 17 |
| Chlortetracycine | 5.11 | 2 | Positive | 479.1 | 444.2 | 21 | 462.1 | 17 |
| Doxycycline | 5.24 | 2 | Positive | 445.2 | 428.1 | 17 | 410.2 | 25 |
| Florfenicol | 5.47 | 2 | Negative | 300.1 | 268.1 | 25 | 159.1 | 41 |
| Chloramphenicol | 5.68 | 2 | Negative | 321 | 152 | 17 | 257.1 | 9 |
| Tylosin | 5.85 | 2 | Positive | 916.5 | 173.9 | 45 | 772.5 | 33 |
| Closulon | 5.86 | 2 | Negative | 377.9 | 341.9 | 9 | – | – |
| Prednisone | 5.88 | 2 | Positive | 359.2 | 147.2 | 33 | 341.2 | 9 |
| Acetopromizine | 5.93 | 2 | Positive | 327.2 | 86 | 21 | 58 | 45 |
| Chlorpromazine | 6.49 | 2 | Positive | 319.1 | 86 | 21 | 58.1 | 45 |
| Fenbendazole | 6.77 | 2 | Positive | 300.1 | 268.1 | 25 | 159.1 | 41 |
| Ketoprofen | 6.8 | 2 | Positive | 255.1 | 208.9 | 13 | 77 | 57 |
| Oxyphenbutazone | 7.27 | 2 | Negative | 323.1 | 133.9 | 25 | 295 | 17 |
| Flunixin-$d_3$ (NEG) | 7.53 | 2 | Negative | 298.1 | 254.2 | 17 | 192 | 37 |
| Flunixin-$d_3$ (POS) | 7.53 | 2 | Positive | 300.1 | 282 | 25 | 264 | 41 |
| Melengestrol acetate | 8.78 | 2 | Positive | 397.2 | 337.4 | 13 | 279.2 | 21 |
| Niclosamide | 8.82 | 2 | Negative | 325 | 170.9 | 25 | 289.1 | 13 |
| Bithionol | 9.49 | 2 | Negative | 352.9 | 161 | 21 | 191.8 | 25 |

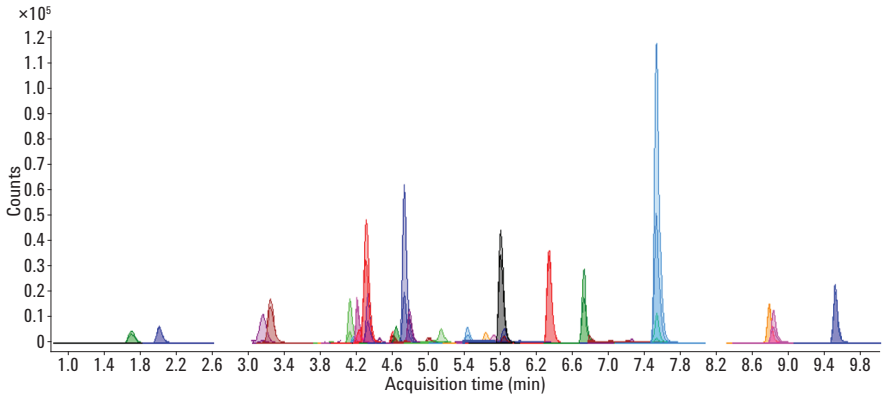



Figure 1. A typical LC/MS/MS chromatogram (DMRM) of a bovine liver sample fortified with a 50 ng/g veterinary drug standard and extracted by protein precipitation followed by cleanup with Agilent Bond Elut EMR—Lipid.

The sample is now ready for LC/MS/MS analysis. The entire sample preparation flow path is shown in Figure 2.

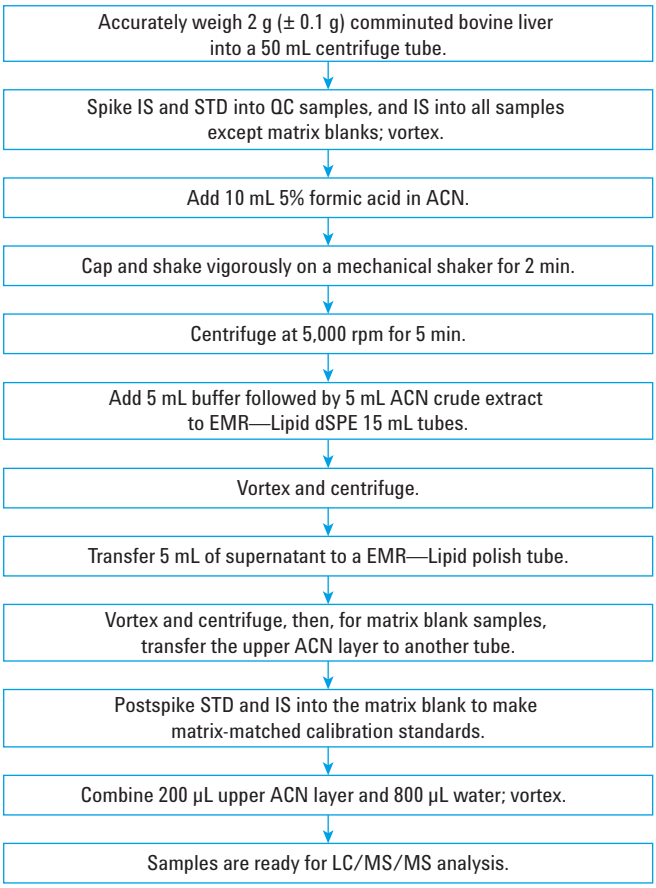



Figure 2, Sample preparation procedure using Agilent Bond Elut EMR—Lipid for the analysis of vet drugs in bovine liver.

## Calibration standards and quality control samples

Prespiked QC samples were fortified with combined standard working solution appropriately, after step 1, for six replicates. For G1 analytes, the QC samples corresponded to 10, 50, 250, and 750 ng/g in liver. For G2 analytes, QC samples corresponded to 2, 10, 50, and 150 ng/g in liver. IS solution was also spiked into all samples except the matrix blank, corresponding to 200 ng/g of flunixin-$d_3$ in liver.

Matrix-matched calibration standards were prepared with standard and IS working solutions. Appropriate concentrations into the matrix blank samples after step 8 corresponded to 5, 25, 50, 250, 750, and 1,000 ng/g in liver (G1), or 1, 5, 10, 50, 150, and 200 ng/g in liver (G2), and 200 ng/g IS in liver.

## Determining amount of coextractives

The amount of coextractives was determined by gravimetric measurement [7] for three different cleanup techniques; C18, zirconia sorbent, and EMR—Lipid. Samples were prepared as follows.

1. Heat glass tubes for ~1 h at 110 °C to remove moisture.
2. Cool tubes to room temperature.
3. Preweigh test tubes.
4. Accurately transfer 1 mL of initial matrix blank extract (no cleanup) and the matrix blanks with various cleanups, each in duplicate.
5. Dry all samples on a CentriVap at 50 °C for 1 h or until dry.
6. Heat the tubes for ~1 h at 110 °C to remove moisture.
7. Cool tubes to room temperature.
8. Reweigh the tubes.

The weight difference between after step 8 and after 3 is the amount of sample coextractive. The amount of coextractives removed by cleanup was the average weight difference of the matrix coextractives before and after cleanup.

## Matrix effect assessment

Chromatographic matrix effect was assessed by a postcolumn infusion experiment. The matrix blank samples were injected with simultaneous postcolumn infusion of 10 ppb neat standard vet drug solution at 60 µL/min. All compound transitions were monitored through the entire LC cycle.

Additionally, the analyte response (peak area) was compared between postspiked liver extracts and the equivalent neat solutions. Postspiked liver extracts were made by postspiking standard solution into the blank liver matrix extract. The difference in response (peak area) is directly correlated to matrix effects.

## Method optimization, validation, and comparison

Different optimization tests were assessed; 5% FA in ACN versus 1% FA in ACN for protein precipitation, ammonium acetate buffer versus water for EMR—Lipid cleanup, and with and without polish salts after EMR—Lipid cleanup. Results were evaluated based on analyte recovery, precision, and other quantitation parameters. The final optimized method was then validated by running a full quantitation batch with duplicated calibration curve standards bracketing all QC samples.

Recovery comparison data were gathered by pre- and postspiking liver samples at 50 ng/g. The extracts were then processed with acceptable cleanup protocols. For the EMR—Lipid protocol with protein precipitation, the cleanup procedure described in Figure 2 was employed. For QuEChERS, a C18 or zirconia sorbent dSPE cleanup was used, as follows.

1. Weigh 2 g of liver in a 50 mL tube.
2. Add 8 mL phosphate buffer (pH 7.0) and 10 mL acidified ACN (5% FA).
3. Vortex sample for 30 s.
4. Add EN salts for partitioning/extraction, and shake vigorously on a mechanical shaker.
5. Centrifuge at 5,000 rpm for 5 min.

The crude ACN liver extract used for further cleanup was prepared as follows.

1. Add 1 mL crude ACN liver extract to a 2 mL vial containing 25 mg C18 and 150 mg $MgSO_4$ (p/n 5982-4921), or into a 2 mL vial containing 100 mg zirconia sorbent.
2. Cap and vortex for 1 min.
3. Centrifuge at 13,000 rpm for 3 min (microcentrifuge).
4. Transfer 200 µL of supernatant into another vial containing 800 µL water.
5. Vortex and filter with a regenerated cellulose 0.45 µm filter.

Samples are then ready for LC/MS/MS analysis. The recovery was calculated by the ratio of analyte peak areas from pre- and postspiked samples.

## Results and Discussion

### Amount of coextractives

Table 3 shows the results from the sample coextractive gravimetric test, clearly demonstrating that EMR—Lipid dSPE provides the best matrix cleanup efficiency by weight than dSPE with C18 or zirconia sorbent.

Table 3. Bovine liver matrix coextractive gravimetric results for Agilent Bond Elut Enhanced Matrix Removal-Lipid, zirconia, and C18 cleanup.

| Cleanup technique | Coextractives per 1 mL of ACN final extract (mg) (n = 2) | Matrix coextractive removal efficiency by cleanup (%) |
|---|---|---|
| No further cleanup | 12.1 | – |
| EMR—Lipid dSPE | 5.3 | 56.2 |
| Zirconia separation with dSPE | 6.0 | 50.4 |
| C18 dSPE | 7.8 | 35.5 |

### Matrix effect assessment

Postcolumn infusion (PCI) of vet drug standards was used for evaluation of matrix effects in the crude ACN extracts and three final ACN extracts cleaned up by EMR—Lipid, C18, and zirconia sorbent. All analytes were monitored through the entire LC cycle. The PCI chromatograms reflect the matrix impact for analytes monitored under positive and negative mode. The final ACN extract was not diluted before injection, and the ACN was injected directly. The PCI profiles are shown in Figure 3.

As shown in the red trace, significant matrix suppression (lower baseline) and matrix enhancement (large peaks) were observed with the injection of crude liver matrix blank without any cleanup. These matrix effects will have dramatic negative impacts on method reliability and data quality. In comparison, as shown in the blue trace, the use of EMR—Lipid cleanup gave significant improvements, as observed by reduced matrix suppression and enhancement.

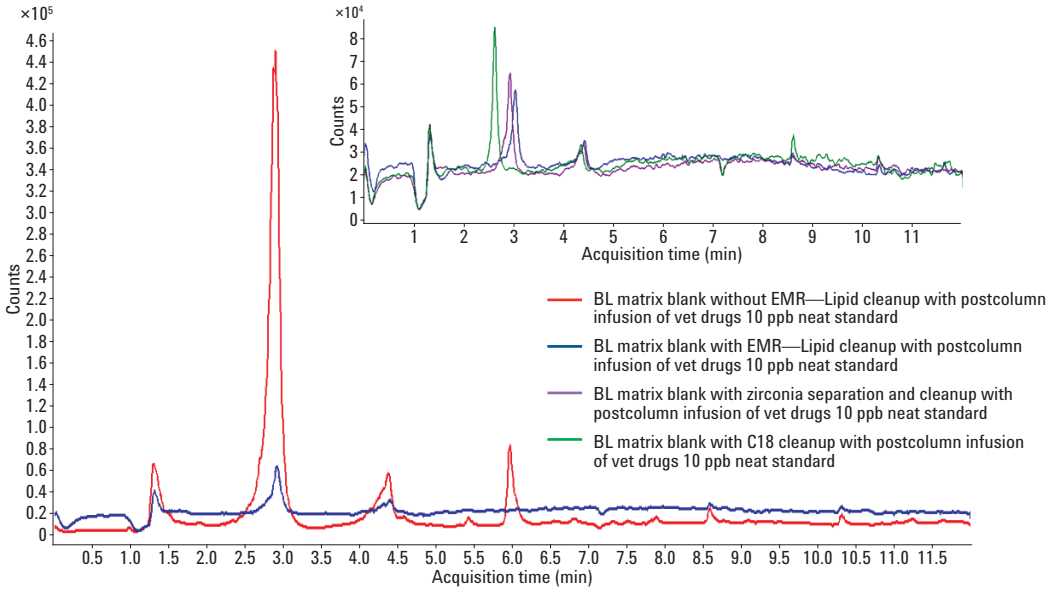



Figure 3. Postcolumn infusion profiles were generated by injecting a bovine liver (BL) matrix blank sample with simultaneous postcolumn infusion of 10 ppb of veterinary drug standard solution. All analytes were monitored for the chromatographic run, and the profile was a combined TIC of all monitored analyte transitions.

The chromatogram insert in Figure 3 shows the PCI profiles with the injection of matrix blank samples using different cleanup. The profiles of EMR—Lipid (blue) and zirconia sorbent (purple) are similar. The C18 cleanup (green) profile also shows similarity with the other two, but with more regions of matrix enhancement and suppression. This comparison corresponds to that of the gravimetric coextractive evaluation, in which EMR—Lipid cleanup gives slightly better cleanup efficiency than zirconia sorbent, and both give better cleanup than C18.

Analyte response comparisons between the postspiked matrix samples and neat standards were also used to evaluate matrix effect. For most analytes, there were no significant differences in analyte responses. However, for the later eluting compounds, which are more hydrophobic, more matrix ion suppression was seen in samples cleaned by C18 and zirconia sorbent. Since most lipid interferences elute late, the reduced matrix ion suppression on hydrophobic analytes confirms that EMR—Lipid efficiently removes coextracted lipids compared to dSPE C18 and zirconia sorbent. Figure 4 shows two examples of how EMR—Lipid cleanup reduced matrix ion suppression effects.

## Method optimization

The solvent used in the protein precipitation step was also investigated. It is known that acetonitrile precipitates proteins efficiently at the sample:organic ratio of 1:3 to 1:5, and acidified acetonitrile offers more protein precipitation. BL is a complex matrix, and the removal of proteins is critical not only for matrix removal, but also to facilitate efficient EMR—Lipid cleanup. In this study, 1% and 5% FA in ACN were evaluated in the protein precipitation step followed by EMR—Lipid cleanup and analysis by LC/MS/MS. Using 5% FA in ACN provided better precision as shown by the calibration curves (Figure 5). The analyte recoveries were also compared, and again 5% FA in ACN provided better precision.

It is important that extra water or buffer is added to activate the EMR-Lipid material. This improves its interaction with unwanted sample matrix, especially lipids, leading to efficient matrix removal. The effect of using an ammonium acetate buffer (5 mM) during EMR—Lipid cleanup was investigated, and results were compared with those using water. Generally, the use of ammonium acetate buffer improved many analyte recoveries by 5 to 10%, except for tetracyclines.

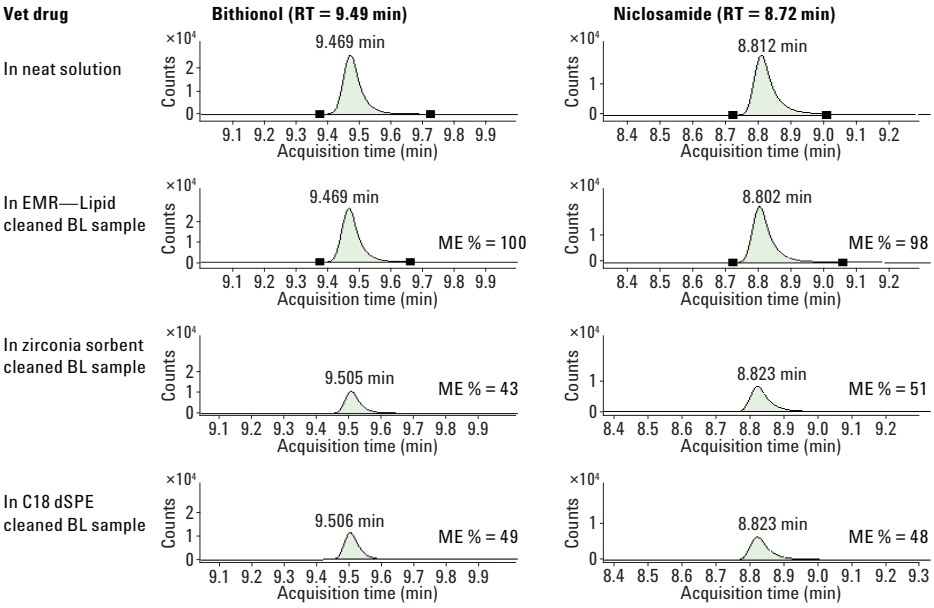



Figure 4. Matrix ion suppression effect comparison for hydrophobic analytes. Matrix effect (ME) was calculated from the ratio of peak area in postspike liver extract and corresponding neat standard.

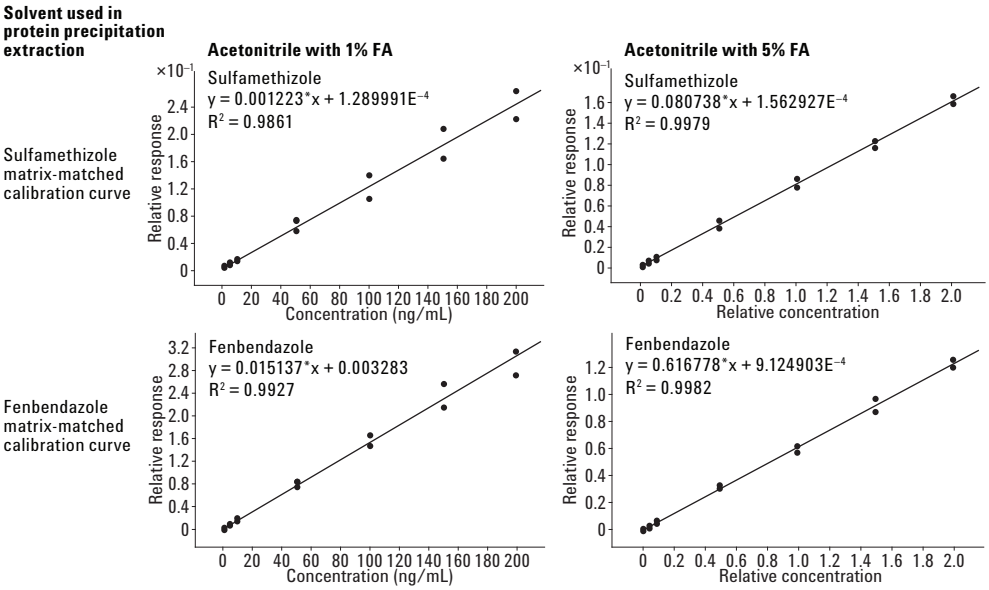



Figure 5. Calibration curve linearity comparison when using 1% FA in ACN versus 5% FA in ACN for protein precipitation.

For these compounds, the use of buffer results in approximately 5% lower recoveries when comparing to the use of unbuffered water. Analytes with obvious recovery differences when buffer versus water was employed were selected for comparison in Figure 6. Since more analyte recoveries improved with the use of buffer, and the tetracycline loss caused by using buffer was minimal, the 5 mM ammonium acetate buffer solution was used for the rest of the study.

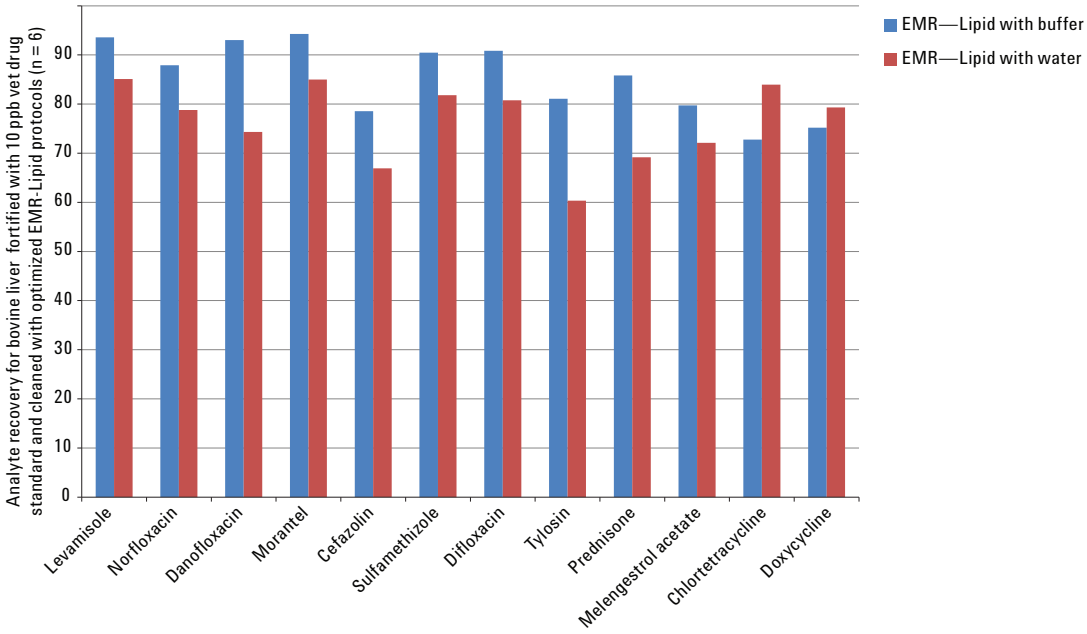



Figure 6. Analyte recovery comparison when using 5 mM ammonium acetate buffer versus water in the Enhanced Matrix Removal dSPE cleanup step.

After EMR-Lipid cleanup, the supernatant is approximately a 1:1 ACN:aqueous mixture. An aliquot of 5 mL supernatant is then transferred into EMR—Lipid polish tube, containing 2 g of salt mix (4:1 $MgSO_4$:NaCl) to separate organic and aqueous phases by salt partition. This step not only partitions but also facilitates further matrix cleanup and removes dissolved extra sorbents and salts. Therefore, this step is highly recommended for both GC and LC analysis. During our method development, good analyte recoveries and precision were achieved for all analytes except tetracyclines. With further investigation, we noticed that the low recoveries of tetracyclines (45 to 68%) were related to the salt partition step. To reduce tetracycline loss, an alternative protocol (Figure 7) was investigated that omits the EMR—Lipid polish salts. The procedure is similar, except that 400 µL of extract (from EMR—Lipid dSPE) and 600 µL of water were mixed in a microcentrifuge vial and vortexed for one minute without using the polish step. Samples were centrifuged on a microcentrifuge at 13,000 rpm for three minutes, and then the supernatant was transferred to a 2 mL sample vial for LC/MS/MS analysis. The standards and IS postspiking in matrix blank was conducted before the mixing/dilution step. This protocol can be considered as nonpartitioning, since the partition salts were not used.

The results in Figure 8 clearly demonstrate that the recoveries for tetracyclines can be substantially improved with this alternative protocol. However, the results from using the polish protocol (as shown in Figure 2) for tetracyclines could be acceptable, since the precision is for quantitation. The low recoveries can be corrected by using an appropriate stable labeled internal standard. In this study, we split extracted samples for the alternative nonpolish protocol after EMR—Lipid cleanup, and reported three results for tetracyclines (oxytetracycline, chlortetracycline, and doxycycline) from the alternative protocol.

## Method comparison

The optimized EMR-Lipid method was then compared with a traditional QuEChERS method with C18 dSPE cleanup and zirconia sorbent cleanup. QuEChERS is often used for analyte or residue extraction, and employs a dSPE cleanup step. Figure 9 shows the statistical recovery comparison results, and Figure 10 shows the comparison for selected, problematic analytes. The optimized EMR—Lipid protocol provides significant improvements for recovery and precision of the problematic analytes, especially with respect to zirconia sorbent, which gives low recoveries for fluoroquinolone and tetracycline classes. Only oxytetracycline and niclosamide gave absolute recoveries of 67% and 68%, respectively. However, the precision for these two compounds for six

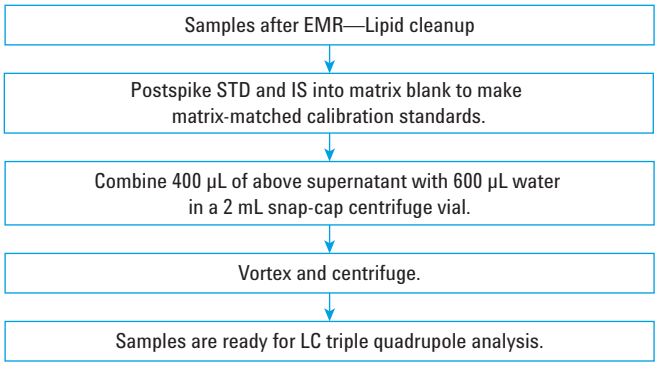



Figure 7. Optional procedure after Agilent Bond Elut EMR—Lipid. cleanup to improve tetracycline recoveries.

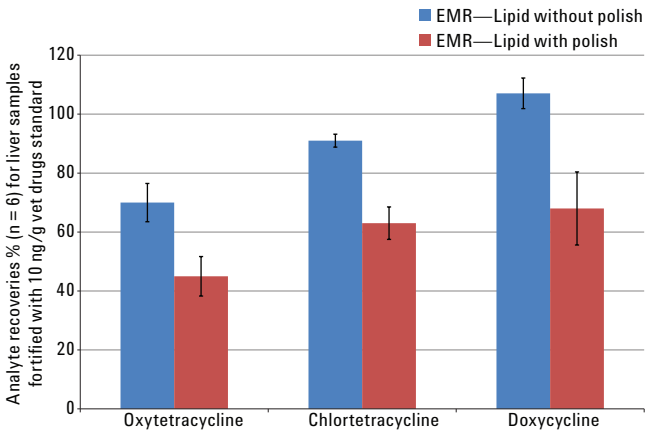



Figure 8. Tetracycline recovery and precision comparison for liver samples prepared with and without a polish step following cleanup with Agilent Bond Elut EMR—Lipid.

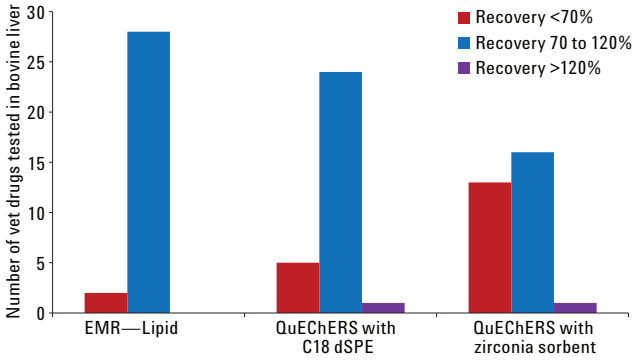



Figure 9. Statistical recovery results for comparison of Agilent Bond Elut EMR—Lipid protocol with traditional QuEChERS protocols.

replicates was acceptable with RSD of 12.8% and 2.0%, respectively, considered as acceptable based on SANCO guidelines [9]. These results are superior to results from the other two protocols employing dSPE with C18 and zirconia sorbents.

## Method validation

The optimized EMR—Lipid method was validated by running a full quantitation batch, using the method described in the sample preparation section. Internal standard (flunixin-$d_3$ for positive and negative mode) was used for quantitation of accuracy and precision. The absolute recovery of flunixin-$d_3$ was from 90 to 100%. Therefore, accuracy closely corresponds to absolute recoveries. Table 4 shows the quantitation results. Summarized accuracy (Figure 11) was generated by determining accuracy and precision for 24 QCs at four different levels (G1 = 10, 50, 250, and 750 ppb and G2 = 2, 10, 50, and 150 ppb; see calibration standard section), with six replicates at each level. Acceptable accuracies (70 to 120%) were achieved for 93% of analytes, except for two outliers, which are slightly below 70% recovery, with good RSDs. The RSD values for six replicates at each level were exceptional, at below 10% for most compounds. It should be noted that ractopamine and ketoprofen were detected at low levels in the BL blank, resulting in the modified calibration range.

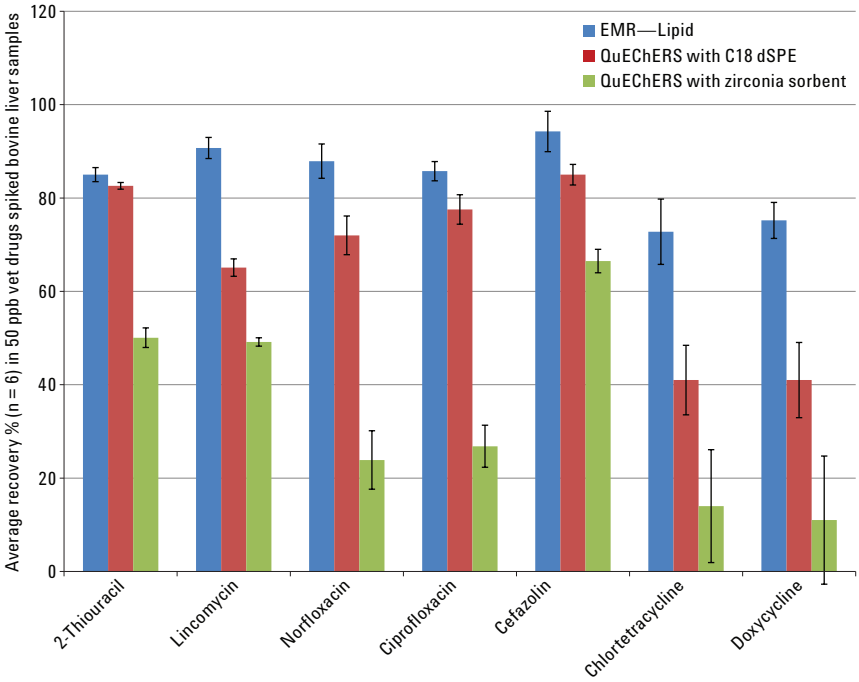



Figure 10. Selected analyte recovery results comparing the Agilent Bond Elut Enhanced Matrix Removal-Lipid protocol with traditional protocols. Chlortetracycline and doxycycline results were generated from an alternative protocol shown in Figure 6. The rest of the compounds used the protocol in Figure 1.

Table 4. Quantitation results for target analytes using Agilent Bond Elut Enhanced Matrix Removal-Lipid. Each analyte was assessed at four concentration levels for six replicates at each level.

| | | Calibration curve | | | Method recovery and precision (ng/g QCs) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | | Regression | | Cal. range | 2 | | 10 | | 50 | | 150 | | 250 | | 750 | |
| no. | Analyte | fit/weight | $R^2$ | (ng/g) | Rec% | RSD | Rec% | RSD | Rec% | RSD | Rec% | RSD | Rec% | RSD | Rec% | RSD |
| 1 | 2-Thiouracil | Linear, 1/x | 0.9976 | 5-1,000 | -- | -- | 106.6 | 4.9 | 93.6 | 5.4 | -- | -- | 87.0 | 3.0 | 85.4 | 8.3 |
| 1 | Amoxicillin | Linear, 1/x | 0.9978 | 5-1,000 | -- | -- | 65.9 | 12.1 | 74.3 | 8.8 | -- | -- | 79.5 | 4.1 | 79.7 | 4.8 |
| 1 | Methonidazole-OH | Linear, 1/x | 0.9981 | 5-1,000 | -- | -- | 92.6 | 15.1 | 89.4 | 6.6 | -- | -- | 89.4 | 3.5 | 89.7 | 3.3 |
| 1 | Oxytetracycline | Linear, 1/x | 0.9963 | 5-1,000 | -- | -- | 73.9 | 19.1 | 71.2 | 14.0 | -- | -- | 67.0 | 12.8 | 63.4 | 9.0 |
| 1 | Cefazolin | Linear, 1/x | 0.9966 | 5-1,000 | -- | -- | 72.1 | 9.4 | 81.2 | 2.7 | -- | -- | 86.7 | 3.8 | 82.9 | 3.0 |
| 1 | Difloxacin | Linear, 1/x | 0.9978 | 5-1,000 | -- | -- | 79.0 | 8.3 | 86.5 | 5.9 | -- | -- | 104.4 | 5.1 | 97.7 | 6.3 |
| 1 | Chlortetracycine | Linear, 1/x | 0.9928 | 5-1,000 | -- | -- | 100.6 | 14.1 | 96.9 | 9.5 | -- | -- | 93.8 | 14.0 | 85.0 | 11.6 |
| 1 | Doxycycline | Linear, 1/x | 0.9972 | 5-1,000 | -- | -- | 118.9 | 12.0 | 102.7 | 10.0 | -- | -- | 110.0 | 7.7 | 104.5 | 8.6 |
| 1 | Florfenicol | Linear, 1/x | 0.9942 | 5-1,000 | -- | -- | 103.4 | 6.2 | 107.9 | 6.0 | -- | -- | 115.2 | 13.5 | 107.0 | 4.0 |
| 1 | Chloramphenicol | Linear, 1/x | 0.9962 | 5-1,000 | -- | -- | 103.6 | 6.6 | 107.1 | 7.9 | -- | -- | 113.7 | 9.8 | 100.9 | 5.2 |
| 1 | Closulon | Linear, 1/x | 0.9954 | 5-1,000 | -- | -- | 77.9 | 10.3 | 104.4 | 6.0 | -- | -- | 102.2 | 7.1 | 94.3 | 3.3 |
| 1 | Prednisone | Linear, 1/x | 0.9984 | 5-1,000 | -- | -- | 105.9 | 9.1 | 92.1 | 11.1 | -- | -- | 103.9 | 10.5 | 94.5 | 2.3 |
| 1 | Oxyphenbutazone | Linear, 1/x | 0.9903 | 5-1,000 | -- | -- | 93.6 | 3.4 | 91.9 | 5.2 | -- | -- | 93.7 | 5.6 | 97.0 | 4.6 |
| 1 | Melengestrol acetate | Linear, 1/x | 0.9994 | 5-1,000 | -- | -- | 70.6 | 1.4 | 77.3 | 3.0 | -- | -- | 82.8 | 2.1 | 77.1 | 2.6 |
| 1 | Bithionol | Quadratic, 1/x | 0.9981 | 5-1,000 | -- | -- | 69.4 | 6.2 | 90.4 | 2.9 | -- | -- | 91.3 | 4.3 | 83.1 | 3.7 |
| 2 | Levamisole | Linear, 1/x | 0.9967 | 1-200 | 84.5 | 11.3 | 95.5 | 5.1 | 103.8 | 5.2 | 89.4 | 9.7 | -- | -- | -- | -- |
| 2 | Lincomycin | Linear, 1/x | 0.9950 | 1-200 | 89.5 | 16.4 | 79.6 | 10.6 | 74.1 | 4.5 | 74.8 | 11.2 | -- | -- | -- | -- |
| 2 | Norfloxacin | Linear, 1/x | 0.9960 | 1-200 | 89.5 | 9.7 | 89.2 | 4.7 | 95.8 | 7.4 | 93.5 | 7.2 | -- | -- | -- | -- |
| 2 | Ciprofloxacin | Linear, 1/x | 0.9980 | 1-200 | 81.0 | 5.6 | 83.6 | 6.9 | 96.9 | 4.1 | 99.5 | 5.9 | -- | -- | -- | -- |
| 2 | Danofloxacin | Linear, 1/x | 0.9985 | 1-200 | 78.2 | 7.8 | 86.2 | 5.8 | 99.5 | 7.8 | 96.9 | 4.8 | -- | -- | -- | -- |
| 2 | Ractopamine | Linear, 1/x | 0.9961 | 10-200[b] | -- | -- | 98.1 | 15.5 | 105.0 | 10.4 | 102.5 | 6.8 | -- | -- | -- | -- |
| 2 | Morantel | Linear, 1/x | 0.9960 | 1-200 | 89.5 | 4.9 | 95.1 | 4.5 | 101.0 | 8.6 | 94.1 | 7.4 | -- | -- | -- | -- |
| 2 | Sulfamethizole | Linear, 1/x | 0.9928 | 1-200 | 85.7 | 14.6 | 89.2 | 8.1 | 93.9 | 4.9 | 88.0 | 10.4 | -- | -- | -- | -- |
| 2 | Sulfamethoxypyridazine | Linear, 1/x | 0.9973 | 1-200 | 84.7 | 8.1 | 84.4 | 2.9 | 89.9 | 5.7 | 84.9 | 6.2 | -- | -- | -- | -- |
| 2 | Tylosin | Linear, 1/x | 0.9967 | 1-200 | 80.6 | 11.2 | 75.6 | 2.7 | 71.0 | 4.5 | 65.3 | 2.3 | -- | -- | -- | -- |
| 2 | Acetopromizine | Linear, 1/x | 0.9973 | 1-200 | 74.1 | 6.3 | 73.5 | 3.0 | 77.2 | 4.9 | 75.2 | 5.3 | -- | -- | -- | -- |
| 2 | Chlorpromazine | Linear, 1/x | 0.9967 | 1-200 | 66.1 | 6.1 | 67.8 | 3.8 | 73.4 | 4.3 | 72.7 | 6.5 | -- | -- | -- | -- |
| 2 | Fenbendazole | Linear, 1/x | 0.9988 | 1-200 | 74.6 | 7.7 | 82.3 | 4.6 | 97.9 | 9.9 | 84.9 | 3.5 | -- | -- | -- | -- |
| 2 | Ketoprofen | Linear, 1/x | 0.9978 | 5-200[c] | -- | -- | 88.3 | 7.2 | 98.1 | 6.9 | 94.5 | 3.8 | -- | -- | -- | -- |
| 2 | Niclosamide | Linear, 1/x | 0.9996 | 1-200 | 60.0 | 15.3 | 66.6 | 4.7 | 71.7 | 2.0 | 67.6 | 3.1 | -- | -- | -- | -- |

[a] Group 1 analytes have a calibration range of 5 to 1,000 ng/g, and QC spiking levels of 10, 50, 250, and 750 ng/g. Group 2 analytes have a calibration range of 1 to 200 ng/g, and QC spiking levels of 2, 10, 50, and 150 ng/g.

[b] Modified calibration range due to ractopamine detected in the BL control blank.

[c] Modified calibration range due to ketoprofen detected in the BL control blank.

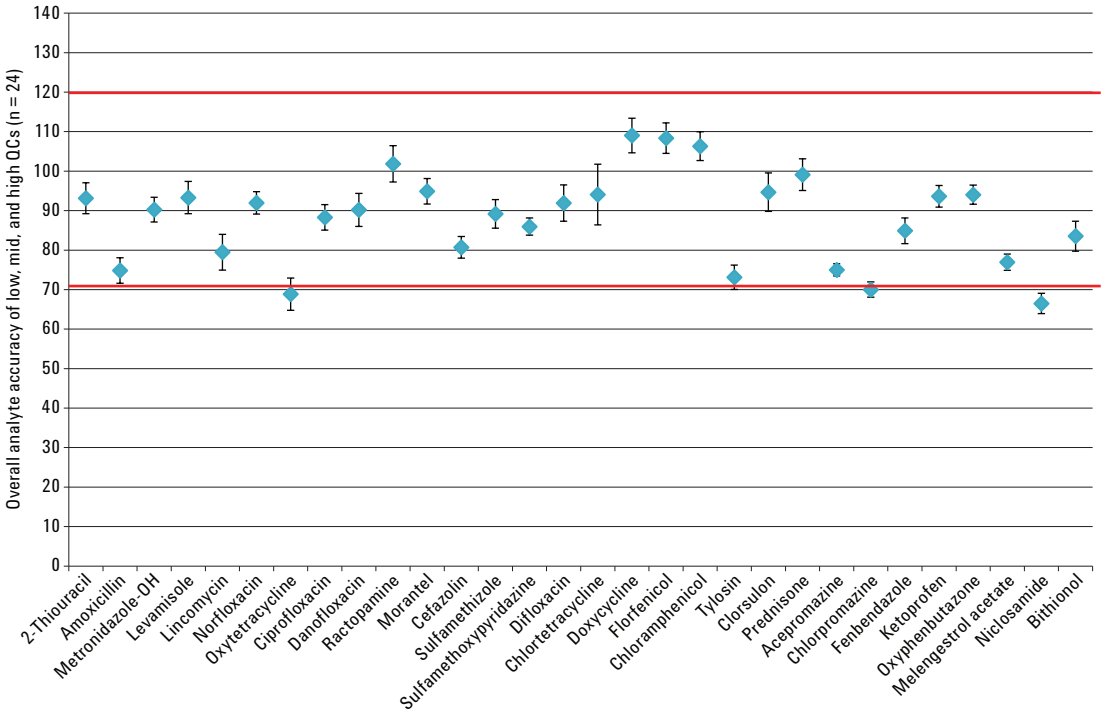



Figure 11. Quantitation of 30 representative vet drugs analyzed in BL using the optimized Agilent Bond Elut Enhanced Matrix Removal-Lipid protocol. The accuracy and precision data were calculated using 24 total replicates at four different spike levels (n = 6 at each level). Error bar = 95% CI. Three tetracycline compound results were generated from an alternative protocol shown in Figure 6. The rest of the compounds used the protocol in Figure 1.

## Conclusions

A rapid, reliable, and robust method using protein precipitation extraction followed by Agilent Bond Elut EMR—Lipid and EMR—Lipid polish cleanup was optimized and validated for the analysis of veterinary drug multiresidues in BL. Matrix effects were carefully assessed and compared with traditional C18 dSPE and zirconia sorbent cleanup. Results demonstrate that the optimized EMR-Lipid method provided superior matrix cleanup, and excellent recovery and precision for this type of application.

It is important to note that direct dilution with water was used before injection to make samples amenable to LC/MS/MS and maintain peak integrity of the early eluting analytes. The LC/MS/MS system we used in this study provided adequate sensitivity for using direct sample dilution while still meeting the detection limit requirement. Compared to common dry-and-reconstitution, this workflow saved significant time and effort, and also prevented potential deviation and analyte loss. If instrument sensitivity cannot meet the desired needs by direct sample dilution, a sample concentration step at the end should still be considered. This is usually achieved by sample evaporation and reconstitution. This application demonstrates that selective matrix removal using EMR-Lipid provides significant advantages for complex samples such as BL, in the form of cleaner samples and higher recoveries and precision for multiresidue veterinary drug analysis.

Future work will investigate other complex, high-lipid matrices and target analytes to demonstrate the advantages of clean samples when using enhanced matrix removal.

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