# Recent Advancements in LC-MS/MS Technology for Enabling Improvements in the Multi-residue Analysis of Veterinary Drugs in Food

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## ABSTRACT

**Purpose:** To demonstrate recent advances in LC-MS/MS technology to enable laboratories to more efficiently create, screen, quantify, and modify methods of multi-class veterinary drug residues.

**Methods:** A modified QuEChERS sample preparation with no cleanup was used to prepare pork samples spiked with multi-class veterinary drugs for LC-MS/MS analysis. A solvent sandwich injection technique using aqueous mobile phase was optimized to allow for larger injection volumes with minimal or no peak shape distortions. A novel tandem MS/MS instrument containing advanced ion optics with a power supply enabling fast polarity switching was employed, along with instrument control software that allows direct import of the precursor ion and the most abundant product ions with corresponding optimal collision energies (CEs) directly into a triple quadrupole acquisition method from an on-line, curated mass spectral data base.

**Results:** Calibration standards were spiked into final pork extracts ranging from 10 ppt to 5 ppb and analyzed, with correlation coefficients greater than 0.99 obtained for most compounds. Most LOQs were below 0.5 ppb. The fast polarity switching, along with advanced ion optics for fast SRM scan rates, provided excellent performance at very short dwell times with high quality quantitative and qualitative results in densely populated areas of the chromatogram. The solvent sandwich injection technique greatly improved peak shapes for analytes eluting under 5 minutes. Excellent correlation in peak response was observed between SRM methods derived from experimentally optimizing via a classic infusion experiment and SRMs imported from the online mzCloud™ mass spectral database.


## INTRODUCTION

Veterinary drugs are administered to animals to ensure animal welfare. It is necessary to screen food products for veterinary drug residues at the maximum residue limits (MRLs) set by global regulatory agencies. This screening typically involves identification and quantification of veterinary drugs using LC-MS/MS. Many publications and newer methodologies cite screening and quantification of large panels of multi-class veterinary drugs in a variety of matrices using a single or limited number of LC-MS/MS methods[1].

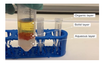
Here we describe an LC-MS/MS method for the quantitation of a diverse group of veterinary compounds spiked into pork muscle extract at concentrations equivalent to or below MRLs set in the China GB standard[2]. Mobile phase composition, column, and liquid chromatography injection techniques were optimized to provide the best separation, peak shape, and response for the analytes studied. Importantly, the liquid chromatography flow path was evaluated prior to analysis with a system suitability mixture containing multiple classes of veterinary drug residues that elute over the retention time range of the method. The method takes full advantage of the Thermo Scientific™ TSQ Quantis™ Plus triple quadrupole mass spectrometer's advanced ion optics and fast polarity switching capability, which are critical for accurate quantification in high density SRM acquisition areas within the analytical run.

## MATERIALS AND METHODS

**Sample Preparation**

1. Weigh 2 g homogeneous ground pork in 50 mL plastic tube
2. Add 10 mL acetonitrile, shake for 10 minutes
3. Centrifuge @ 4000 rpm for 10 min at 5 C
4. Freeze Step: -20 C for 10 minutes
5. Transfer all supernatant to 15 mL glass tube
6. Evaporate to dryness @ 40C, reconstitute to 1mL 1:1 MeOH:H2O
7. Pass through 0.2 um filter, ready for injection





## MATERIALS AND METHODS- cont.

**Liquid Chromatography and Mass Spectrometry Conditions**

The LC-MS/MS system comprised a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system interfaced with a TSQ Quantis Plus triple quadrupole mass spectrometer equipped with a heated electrospray ionization (H-ESI) probe. Thermo Scientific™ TraceFinder™ software was used for instrument control, analysis, data review, and reporting.

| | |
|---|---|
| Inj. Volume | 10 µL (Sandwich Injection) |
| Col Temp. | 40 C |
| Analytical Column | Thermo Scientific™ Accucore™ VDX, 100 x 2.1 mm, 2.6 µm |
| Run Time | 15 min |
| Mobile Phase A | 0.1% Formic Acid + 2mM Ammonium formate in water |
| Mobile Phase B | 0.1% Formic Acid in 1:1 Acetonitrile:Methanol + 2% Water+ 2mM ammonium formate |

| Time | Flow (ml/min) | % B |
|---|---|---|
| 0.0 | 0.400 | 2.0 |
| 2.0 | 0.400 | 2.0 |
| 3.0 | 0.400 | 35 |
| 9.0 | 0.400 | 100 |
| 12.0 | 0.400 | 100 |
| 12.5 | 0.400 | 100 |
| 12.6 | 0.450 | 2.0 |
| 15.0 | 0.400 | 2.0 |

**Tables 1 and 2: Liquid chromatography column, gradient and mobile phase conditions.**

Replace normal injection
Normal injection with liquid handling

| No | Command | Parameters |
|---|---|---|
| 1 | UDP_PrepareLiquidHa... | Volume=99.00 [µl] |
| 2 | UDP_NeedleWash | Duration=10.000 [s] |
| 3 | UDP_Draw | Position=SB:1, Volume=38.00 [µl] |
| 4 | UDP_NeedleWash | Duration=10.000 [s] |
| 5 | UDP_Draw | |
| 6 | UDP_NeedleWash | Duration=10.000 [s] |
| 7 | UDP_Draw | Position=SB:1, Volume=38.00 [µl] |
| 8 | UDP_NeedleWash | Duration=10.000 [s] |
| 9 | UDP_InNeedleMix | Volume=10.00 [µl], DrawSpeed=20 [µl/s], DispenseSpeed=20 [µl/s], Cycles=20 |
| 10 | UDP_Wait | 10 [s] |
| 11 | UDP_PrepareInject | |

**Table 3: LC autosampler custom program for solvent sandwich injection. Total sample loop volume is 100 µL.**

| | |
|---|---|
| Neg. Voltage | 2500 V |
| Pos. Voltage | 3500 V |
| Sheath Gas | 50 units |
| Auxiliary Gas | 13 units |
| Sweep Gas | 1 unit |
| Ion Trans Tube | 310 C |
| Vaporizer | 350 C |

**Table 4: TSQ Quantis Plus mass spectrometer API settings**

**Standards and system suitability test (SST) solution**

Forty-two neat veterinary drug standards characterizing several compound classes and representative of low quantitation levels required in the China GB standard were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in appropriate solvents to create individual stock solutions at a concentration of 1.0 mg/mL. It is important to note that some beta lactam antibiotics, for example penicillin G, were prepared in water and stored frozen to prevent rapid degradation. Storage at -20 C or colder for all compound stock solutions is recommended.

A system suitability check standard containing 34 veterinary drugs in acetonitrile at a stock concentration of 1.0 mg/mL was developed by the Veterinary Diagnostic Laboratory at Iowa State University. A 50 ppb substock was prepared in 80:20 mobile phase A/mobile phase B and used to check peak shape, retention, and general inertness of the liquid chromatography system.

## RESULTS

**Sandwich Solvent Injection Technique**

Use of a sandwich injection improves peak shapes when injecting larger volumes of extract, since stronger solvents like methanol and acetonitrile are required to keep analytes stable and in solution. Modern LC systems are designed to reduce dead volume through the sampling valve and syringe, resulting in a sharp solvent plug arriving at the head of the column. A sandwich injection works by bracketing the injected sample volume (8 µL) between mobile phase A plugs (38 µL for each solvent plug) that allows solvent mixing prior to the column head. Figure 1 is an example of peak shape improvements gained by the technique.

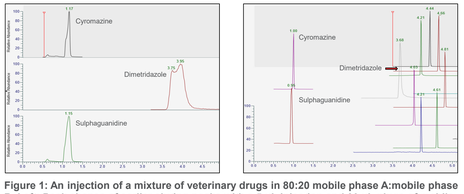



**Figure 1: An injection of a mixture of veterinary drugs in 80:20 mobile phase A:mobile phase B. Left: Peak shapes of earlier eluting peaks with a 5 µL injection and 20 µL plugs of mobile phase A surrounding the sample volume. Right: Peak shape improvement of earlier eluting peaks with an 8 µL injection and 38 µL plugs of mobile phase A surrounding the sample.**

**System Suitability**

The system suitability check helps ensure analyte response, peak shape, and LC analytical flow path inertness (minimal reactivity with target compounds) is acceptable prior to starting the run. Tables 5a and 5b list the components in the mix covering several challenging compounds and wide RT range.

| | |
|---|---|
| Amprolium | 0.89 |
| Cyromazine | 0.96 |
| Amoxicillin | 1.73 |
| Cefapirin | 3.81 |
| Levamisole | 3.86 |
| Lincomycin | 4.00 |
| Tulathromycin | 4.16 |
| Ciprofloxacin | 4.17 |
| oxytetracycline +epimer | 4.18 |
| Ractopamine | 4.2 |
| Carbadox | 4.21 |
| Chlortetracycline +epimer | 4.22 |
| Penicillin G | 4.25 |
| Sulfamethazine | 4.3 |
| Clenbuterol | 4.41 |
| Florfenicol | 4.67 |
| Carazolol | 4.73 |
| Halofuginone | 4.93 |

| | |
|---|---|
| Chloramphenicol | 5.00 |
| Tilmicosin | 5.04 |
| Tiamulin | 5.83 |
| Tylosin A | 5.84 |
| Albendazole | 5.96 |
| Betamethazone | 6.07 |
| Fenbendazole | 6.71 |
| Flunixin_pos | 6.99 |
| Flunixin_neg | 6.99 |
| Carprofen | 7.41 |
| Melengesterol Acetate | 8.04 |
| Decoquinate | 9.09 |
| Closantel | 9.39 |
| Lasalocid_NH4 | 9.67 |
| Rafoxanide | 9.83 |
| Monensin_Na | 9.90 |
| Ivermectin B1a_NH4 | 9.99 |

**Tables 5a and 5b: System suitability check compounds and retention times**

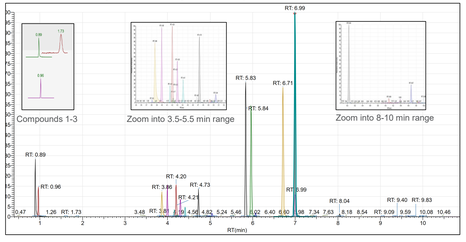



**Figure 2: System suitability test (SST) mixture at 50 ppb for veterinary drug residues. Shown insets are expanded views of the above extracted TIC.**

## RESULTS- cont.

**Calibration, Quantitation, LOD/LOQs**

The TSQ Quantis Plus mass spectrometer provides key features that enable accurate detection and quantification of the target veterinary drug residues in the pork matrix. Fast polarity switching at 5 ms, combined with its advanced ion optics, enable excellent quantitation and ion ratio stability in densely SRM-populated regions of the chromatogram. See Figures 3a, 3b, and 3c below.

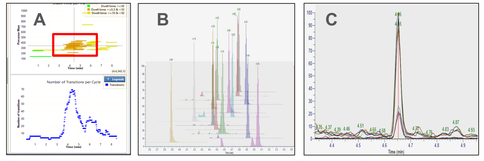



**Figure 3: A- View in the instrument control software of the TSQ Quantis Plus showing high density SRMs between 4-5 minutes. B- Stick scan view of SRMs shown in Thermo Scientific FreeStyle™ software to help visualize number of scans across a chromatographic peak to ensure accurate quantification. C- Ion ratio stability overlay of 5 consecutive injections, m/z 284.2/107.04 for 50 ppt Isoxsuprine in pork tissue extract.**

To assess the instrument response, calibration standards were spiked into the final pork extract ranging from 10 ppt to 5 ppb and analyzed with excellent linearity achieved, with correlation coefficients greater than 0.99 for over 95% of the compounds.

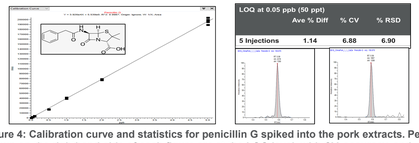



**Figure 4: Calibration curve and statistics for penicillin G spiked into the pork extracts. Peaks shown on the righthand side of each figure are at the LOQ level, with CV, accuracy, and percent relative standard deviation (RSD) statistics listed above them.**

A summary of instrument LODs and LOQs for select compounds in the China GB Standard is presented in Table 6. LOD is defined as the concentration with RSD < 25%, and for the LOQ a concentration with RSD <15%. In both cases, the percent difference between the calculated value from the calibration curve and true value are ≤ 20%. Based on 8 replicate injections in pork extract.

| Compound name | GB Standard requirement LOD ng/g | GB Standard requirement LOQng/g | TSQ Quantis Plus LOD ng/g in Pork | TSQ Quantis Plus LOQ ng/g in Pork | AOAC Lowest Global MRL ng/g |
|---|---|---|---|---|---|
| Ritodrine | 0.5 | -- | 0.025 | 0.025 | ** |
| Isoxsuprine | 0.5 | -- | 0.005 | 0.005 | ** |
| Penicillin G | 0.5 | -- | 0.005 | 0.025 | 50 |
| Oxacillin | 1.0 | -- | 0.25 | 0.25 | 30 |
| Danofloxacin | 1.0/0.5 | 3.0/2.0 | 0.005 | 0.025 | 30 |
| Sarafloxacin | 2.0/1.0 | 6.0/3.0 | 0.05 | 0.25 | 10 |
| Pipemidic Acid | 2.0/1.0 | 6.0/3.0 | 0.25 | 0.25 | ** |
| Pefloxacin | 2.0/1.0 | 6.0/3.0 | 0.025 | 0.05 | ** |
| Norfloxacin | 2.0/1.0 | 6.0/3.0 | 0.05 | 0.05 | ** |
| Ciprofloxacin | 2.5/1.2 | 8.0/4.0 | 0.025 | 0.025 | ** |
| Thiamphenicol | 0.3 | 1.0 | 0.25 | 0.25 | 50 |
| Florfenicol | 1.0 | -- | 0.05 | 0.25 | 100 |
| Sodium Cyclamate | 20.0 | 100.0 | 0.05 | 0.05 | ** |
| Prednisone | 0.2 | -- | 0.5 | 1 | 0.7 |
| Cortisone | 0.2 | -- | 0.5 | 0.5 | ** |
| Methylprednisolone | 0.2 | -- | 0.05 | 0.05 | 2 |
| Betamethasone | 0.2 | -- | 0.025 | 0.05 | 0.3 |
| Beclomethasone | 1 | -- | 0.25 | 0.5 | ** |
| Fluorohydrocortisone acetate | 1 | -- | 0.5 | 1 | ** |
| Methyl testosterone | 0.5 | 0.4 | 0.05 | 0.05 | ** |
| Stanozolol | 0.5 | 0.4 | 0.005 | 0.005 | ** |
| Testosterone | 0.5 | 0.4 | 0.05 | 0.25 | ** |
| Trenbolone | 0.5 | 1 | 0.025 | 0.05 | 2 |
| Gamithromycin | * | 0.1 | 0.005 | 0.005 | 20 |
| Tilmicosin | 2.0 | -- | 0.025 | 0.025 | 50 |
| Chlormadinone | * | 0.5 | 0.05 | 0.25 | 2.5 |

**Table 6: Calculated instrument LODs and LOQs obtained from the final pork spiked extracts as compared to the expected levels in the China GB standard and AOAC SMPR. ** Indicate that the analyte is not listed in AOAC SPMR 2018.010 document.**

## RESULTS- cont.

**On-line Mass Spectral Database for Method Development**

Utilizing an advanced high-resolution mass spectral database, known as mzCloud (www.mzcloud.org), one can greatly reduce the time required to build SRM tables. This is accomplished by a software feature that directly imports the precursor ion and the most abundant product ions with corresponding optimal collision energies (CEs) directly into a triple quadrupole acquisition method.

Figure 5 compares the peak area response between the SRM method derived from experimentally optimizing via a classic infusion experiment (QC) and SRMs imported from mzCloud. A very high correlation is observed between the two methods, in terms of product ions selected, CEs, and the intensity of SRMs. Importing from mzCloud can dramatically decrease method development times, and methods can even be developed when analytical standards are not available or prohibitively costly. Compounds are continually being added to the database to address emerging research requests. The mzCloud database currently contains over 20,000 entries covering 16 different compound classes.

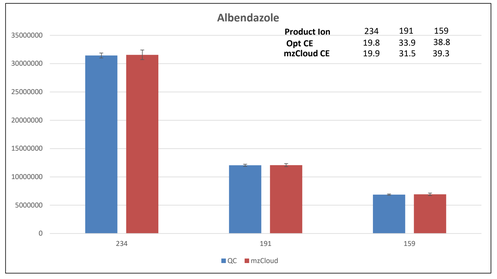



**Figure 5: Comparison of mean peak areas (n=7) between Optimized (blue) and mzCloud (red) methods, for three SRMs of Albendazole.**

## CONCLUSIONS

- An optimized mobile phase and an inert LC system checked using an SST solution with solvent sandwich injection capability improves peak shape and sensitivity.
- The TSQ Quantis Plus triple quadrupole mass spectrometer with fast polarity switching (5 ms), advanced ion optics for fast SRM scan rates, and excellent performance at very short dwell times provides high quality quantitative and qualitative results in densely populated areas of the chromatogram.
- An on-line, highly curated advanced mass spectral database (mzCloud) enables easy method development, allowing direct import of optimized CEs with SRM precursor and product ions into acquisition methods using a simple software tool.

## REFERENCES


1. Screening of 154 Veterinary Drug Residues in Foods of Animal Origin Using LC–MS/MS: First Action 2020.04. https://academic.oup.com/jaoac/article-abstract/104/3/650/6044156
2. National Food Safety Standard on Maximum Residue Limits for Veterinary Drugs in Foods (GB 31650-2019). https://food.chemlinked.com/database/view/1661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## TRADEMARKS/LICENSING



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