

## An Executive Summary

# Multi-class Veterinary Drug Screening and Quantitation with a Comprehensive Workflow



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Analysis of more than 170 veterinary drugs using a single high-throughput method.

### Overview

The screening and routine quantitation of veterinary drugs in food products is one of the most important and demanding applications in food safety. Despite the recent technological advancements in LC–MS, it is still challenging to obtain excellent chromatographic peak shapes, adequate sensitivity, and accurate quantitation of more than 170 veterinary drugs from different chemical classes within a single method.

To address this, a comprehensive, multi-class veterinary drug LC–MS/MS method has been developed, using a generic QuEChERS sample preparation. The method was validated in bovine muscle, salmon fillet, and milk to demonstrate applicability to a wide range of matrices. The detection limits were compliant with the lowest global maximum residue limits for each analyte/matrix combination. This method provides researchers fast, reliable, cost-effective solutions for the analysis of veterinary drug residues in food.

### Introduction

Veterinary medicines are pharmacologically active compounds that are used to treat and prevent animal diseases. They restore, correct, or modify physiological functions by exerting a pharmacological, immunological, or metabolic action. Although it happens infrequently, residues of the drugs or their transformation products can remain in foods after the treatment of animals. As a result, the approvals and usage of veterinary drugs are highly regulated and monitored.

Regulators have established the maximum residue limits (MRLs) for these compounds and some of their metabolites to allow the practice of using veterinary medicines in animals at safe doses. There are numerous approved compounds and the range of acceptable concentrations covers from sub-microgram per kilogram quantities all the way up to a thousand micrograms per kilogram. The EU has categorized the drugs into Annex I, II, III, and IV, which classify drugs with fixed MRLs, those requiring no MRL, drugs with provisional MRLs, and those that are banned from use. The EU also sorts the drugs into two main groups:

- Group A, which includes substances with anabolic effects as well as unauthorized substances; and
- Group B, which includes antibiotics, common veterinary drugs, and environmental contaminants

Understandably, the US FDA and China also have specific regulations regarding veterinary medicines used in food production.

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## Analytical Method Development

To comply with regulations that ensure consumer safety, veterinary medicines must be identified and quantified in food products. The diversity of compound classes and wide range of concentrations make efficient analysis challenging. Historically, multiple time-consuming, class-specific analytical methods were required to quantify the multiple classes of medicines in a sample. More recently, there has been a move to the use of generic extraction approaches, such as the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) extraction-based methodology, where screening for as many compounds as possible is done in a single analysis. This technique has a broad scope, but less clean up and lower recoveries for some compounds.

A mass spectrometry (MS) screening approach is also accepted for the analysis of veterinary medicines. It involves a validation that is based on detectability, such as method detection limit (MDL). The use of internal standards or matrix-extracted calibrations is often employed by laboratories to ascertain that the proper recovery is recognized and reported. Many labs use a combination of MS screening techniques and class-specific methods to reliably cover the range of veterinary analytes.

Recently, a single workflow that incorporates a wide variety of veterinary compound classes was developed and tested. This method, using LC–MS/MS and QuEChERS sample preparation with simple clean-up, is generic enough to apply to several different matrices, including meat, fish, and dairy. The simple, cost-effective, and broadly applicable sample preparation is combined with an MS instrument, which has appropriate sensitivity for all analytes. The Thermo Scientific™ Accucore™ VDX liquid chromatography (LC) column is rugged and handles a wide range of polarities, while the Thermo

Scientific™ TSQ Altis™ triple-quadrupole MS with the Thermo Scientific™ Vanquish™ Flex Binary pump handles polarity switching with ease.

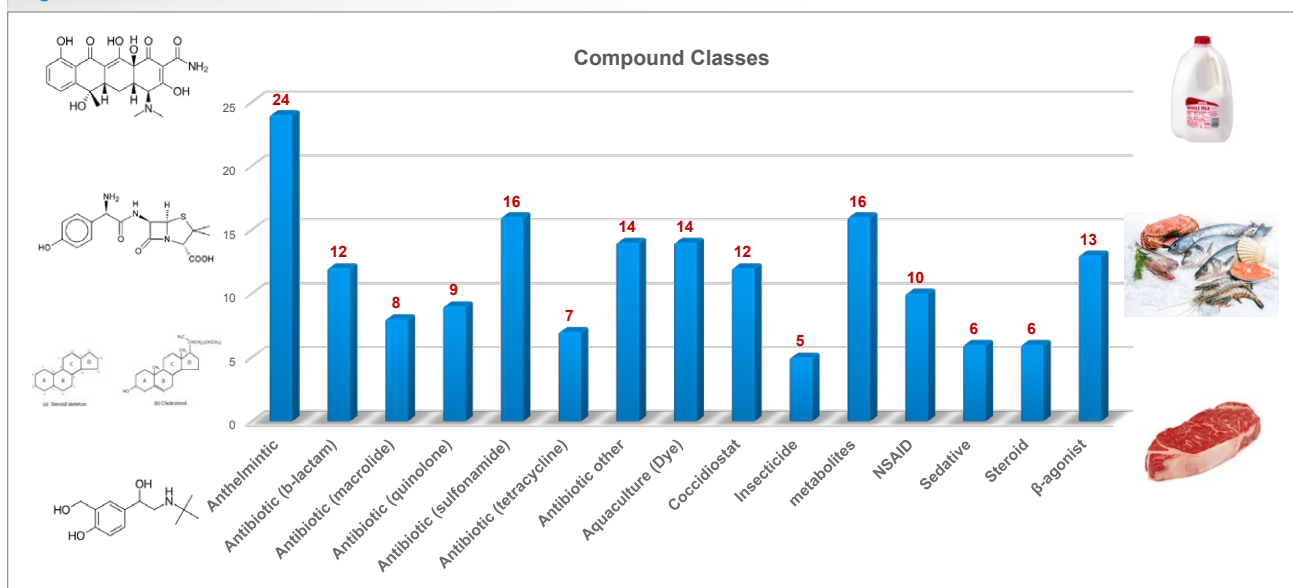
QC and analytical standards are included in the workflow. The QC samples are used to evaluate the status of the instrument, and the standards can be used for matrix spikes, instrument checks, or selective reaction monitoring (SRM). These system performance checks verify the quality of data. Thermo Scientific™ TraceFinder™ software unifies all aspects of data handling, from acquisition to reporting.

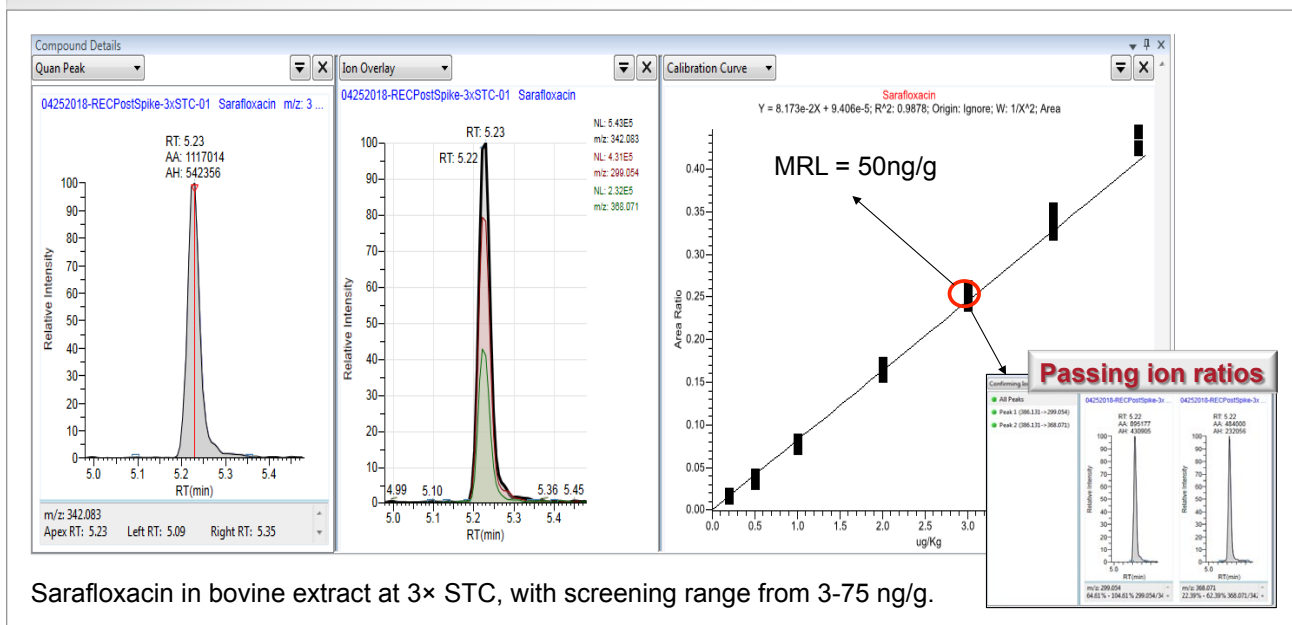
A comprehensive User Guide, analytical standards, and information on preparation of samples, QC checks, spiking cocktails and standards can be obtained from Thermo Scientific. The User Guide also includes advice on installation and operating conditions for the LC and MS, as well as detailed descriptions of sample preparation for meat, fish, and dairy matrices. In addition, the kit contains an Excel spreadsheet with compound information, exact mass, formula, polarities, adducts, and retention times for the analytical standards. When the workflow is used, 170+ veterinary drugs can be reliably analyzed, and false positives and negatives are avoided. **Figure 1** shows the widespread applicability of the workflow. Fifteen different compound classes covering 172 analytes have proven to be amenable to the new procedure. Additionally, the results are in compliance with regulations and accreditation requirements. The kit from Thermo Scientific enables easy set up and proper use of the workflow's procedures and method.

## Experimental

The QuEChERS extraction uses an ethylenediaminetetraacetic acid (EDTA)/NH<sub>4</sub> oxalate solution and acetonitrile. The sample is homogenized until it is fully dispersed and then sodium sulphate

**Figure 1:** Chemical classes within the method.



**Figure 2:** Quantitative results- 0.2 to 5 x STC-bovine Matrix Extracted Spike (MES).

Sarafloxacin in bovine extract at 3× STC, with screening range from 3-75 ng/g.

is added before centrifugation to dry the sample. Dispersive solid-phase extraction (SPE) is used as the final clean-up step. At the very end of the extraction, 1 mL of water is added to 3 mL of the extract and then it is filtered and injected into the LC instrument. This generic method is designed to capture as many compounds as possible and get the best recoveries.

A critical piece of the workflow is the Accucore VDX LC column. The solid-core particle, designed for high-resolution separations, has column chemistry selectivity similar to a C18 column. It separates the complex sample constituents, including tetracycline epimers, as required by regulatory agencies. Optimized for MS detection and low tailing, it exhibits low column bleed. Most importantly, the column is very robust against matrix extracts. The column can be stable for over 500 injections, which is critical when working with heavy matrix samples.

The method's mobile phase A is 0.05% formic acid and B is 0.05% formic acid with 1:1 methanol and acetonitrile. Only 2 µL are injected, which is important for maintaining robustness. The TSQ Altis triple-quadrupole mass spectrometer uses positive-negative switching along with a comprehensive compound database that includes all of the optimized compound SRMs.

The TSQ Altis instrument also offers the advantage of highly selective reaction monitoring (H-SRM). This is a very powerful technique for improving sensitivity and specificity of compounds, particularly in heavy matrix samples. H-SRM essentially increases the resolution of the first quadrupole and can lead to improved signal-to-noise and lower MDLs.

## Applications

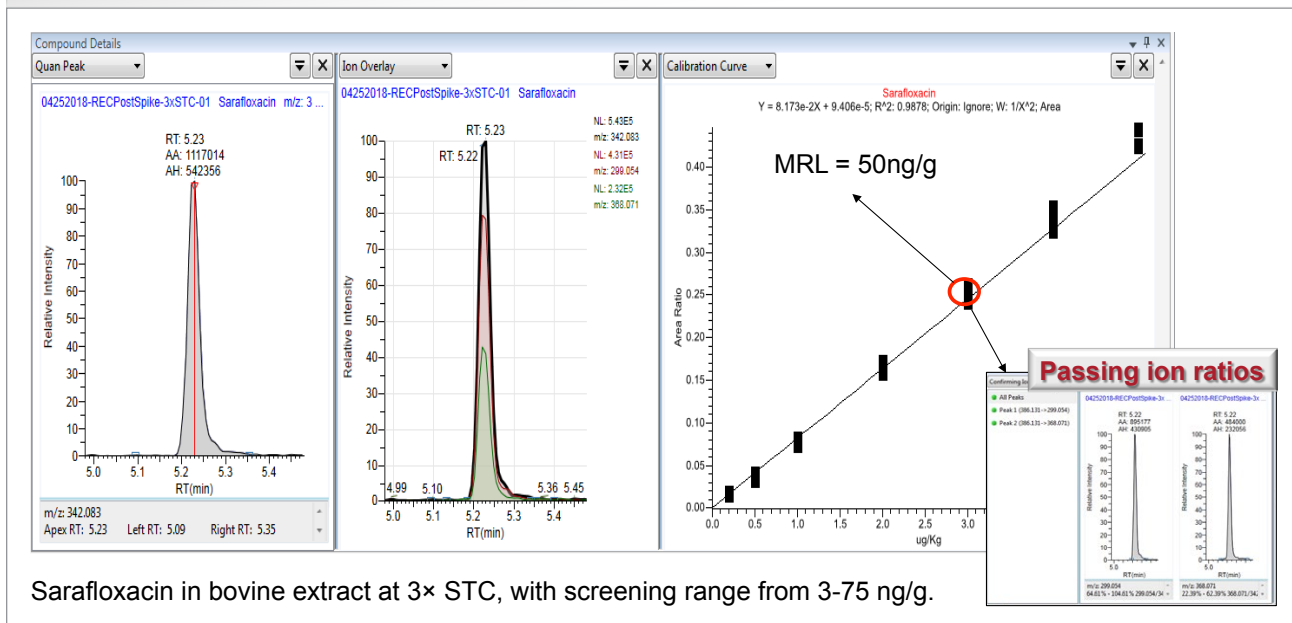
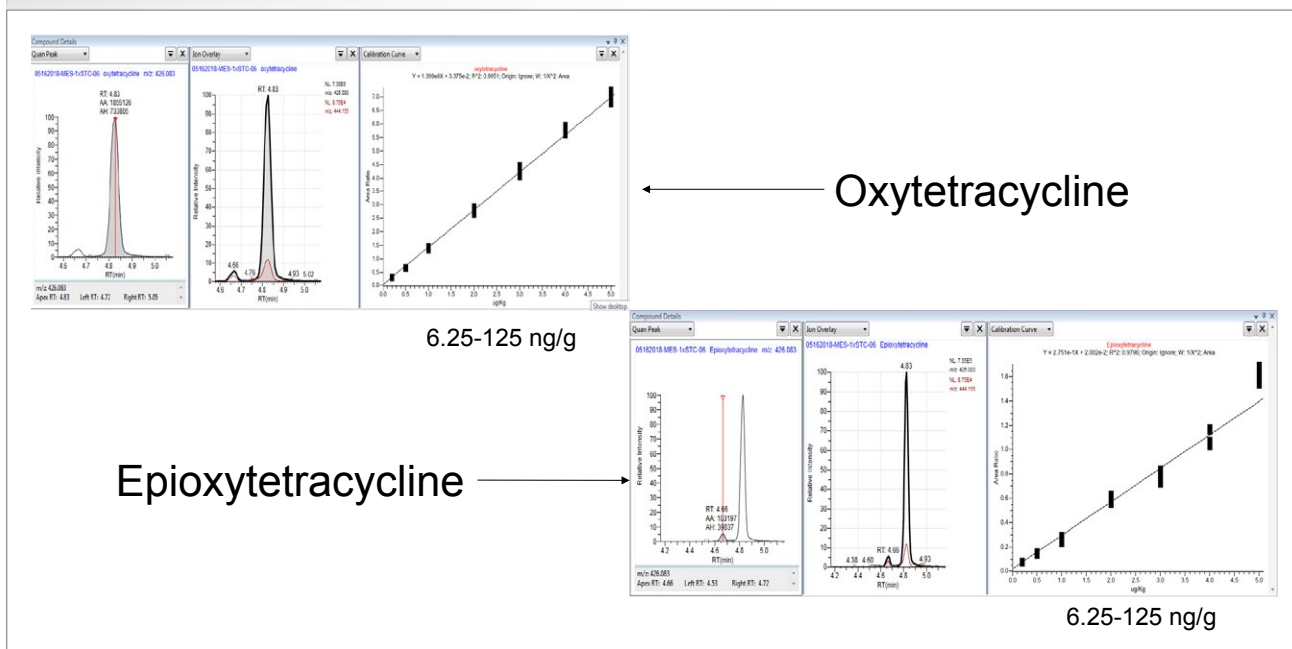
**Figure 2** shows a Bovine Matrix Extracted Spike (MES) of Sarafloxacin. An MES is prepared by spiking the matrix at

the front end of the extraction and taking it through the entire extraction process. A semblance of a calibration curve is created across a concentration range that is determined by the Maximum Residue Limit (MRL) of the compound. The screening target concentration (STC) level is typically between one-fourth and one-third the MRL. A concentration range made from factors of the STC is created around the MRL to confirm that the compound can be observed and quantified confidently at that level. In this case, the screening range was 3-75 ng/g, which encompassed the 50 ng/g MRL.

The Emamectin in salmon extract in **Figure 3** demonstrates that even at the lowest point, 2 ng/g, good detectability and passing ion ratios can be obtained at very low concentrations with the new workflow. Tetracycline epimers in milk are shown in **Figure 4**, illustrating that each of these stereoisomers can be separated and quantified at meaningful concentrations.

## Validation

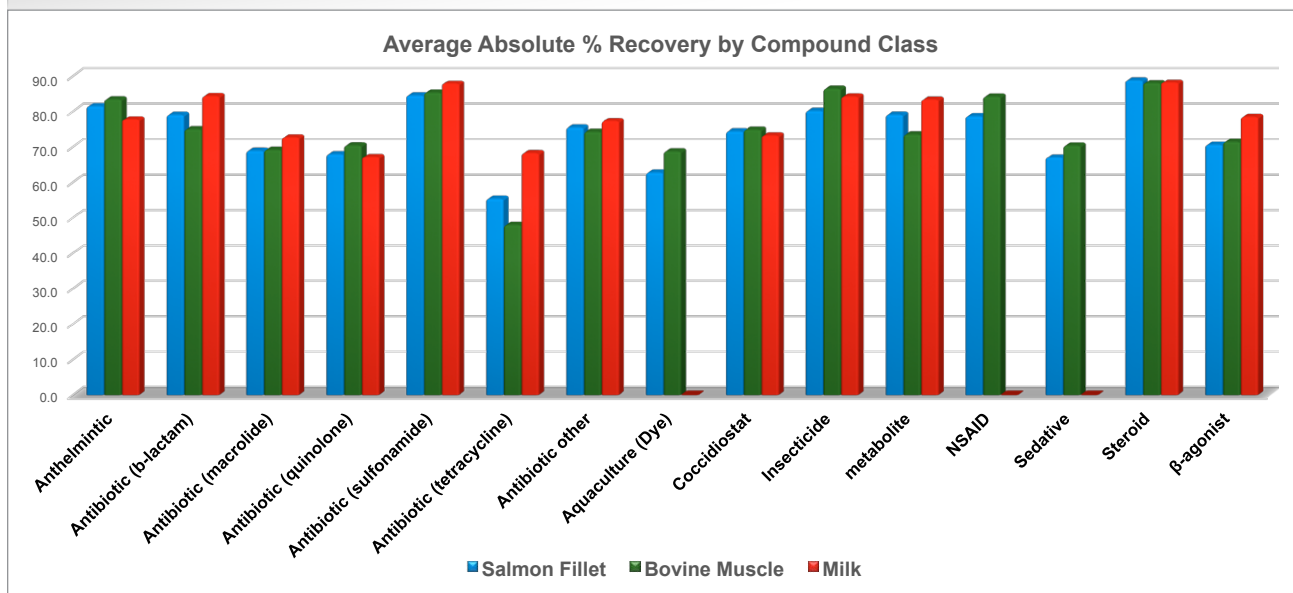
In addition to determining the STC and appropriate range of MES concentrations to bracket the MRL with the pseudo-calibration curve, there are further considerations for securing dependable method performance. The next step is to calculate the relative standard deviations (RSDs) and check the precision at each level to determine how well the method is working. With those statistics, the MDLs can be calculated from the lowest STC factor that is at or below 15% RSD. Absolute percent recovery is then based on a post-spike at three times the STC. Whereas a matrix-extracted spike is a pre-spike, a post-spike is prepared on a blank matrix after the extraction process. Comparison of pre-spike to post-spike concentration provides the percent recovery to determine the efficiency of the extraction. **Figure 5** shows the percent

**Figure 3:** Quantitative results- 0.2 to 5 x STC - salmon fillet.**Figure 4:** Quantitative results - 0.2 to 5 x STC-milk.

recoveries resulting from the new workflow for many common veterinary drug classes. Note that for the milk matrix, NSAIDs, sedatives, and dyes were not spiked into the MES.

Finally, the method should be tested on multiple LC-MS/MS systems to verify that it is reproducible and transferable. Retention time (RT) stability was assessed on four different systems: three were located at Thermo Fisher Scientific's San Jose facility, and one was at Iowa State University. Compounds throughout the gradient range showed excellent RT stability

with agreement between the different systems. In addition, the four instruments were used to compare quantitation capabilities with regulated MRL values, as shown in the salmon matrix data in **Figure 6**. Note that the method easily achieved the necessary sensitivity to quantify drug residues well below the MRLs, and data from the different instruments matched. This sensitivity and reproducibility were also attained in milk and bovine matrices. The excellent precision across the four independent systems provides confidence in the multi-class method.

**Figure 5:** Compound class–average % recovery (absolute-uncorrected).**Figure 6:** Example data of salmon matrix-precision at STC Levels across four LC–MS/MS systems.

Compound	Lowest Global MRL	STC	System 1	System 2	System 3	System 4	Average	SDEV	%RSD
Amoxicillin	50	25	24.4	24.8	25.0	27.3	25.4	1.31	5.2
Chlortetracycline	200	100	87.3	97.1	86.4	104.0	93.7	8.39	9.0
Danofloxacin	100	50	50.9	49.8	47.3	52.3	50.1	2.09	4.2
Dicloxacin	300	150	144.5	148.2	147.7	152.2	148.2	3.15	2.1
Difloxacin	300	150	150.0	149.2	145.6	159.3	151.0	5.80	3.8
Doxycycline	10	10	9.2	9.1	9.2	10.1	9.4	0.47	5.0
Enrofloxacin	100	50	50.2	49.4	55.0	52.3	51.7	2.51	4.9
Flumequine	500	200	200.8	201.9	201.5	199.3	200.9	1.17	0.6
Oxolinic Acid	50	25	28.2	29.3	27.5	25.8	27.7	1.48	5.3
Oxytetracycline	200	100	102.2	96.4	94.6	101.1	98.6	3.65	3.7
Penicillin G	50	25	23.6	24.0	21.6	25.4	23.6	1.57	6.7
Sarafloxacin	10	3	3.0	3.0	2.9	3.1	3.0	0.06	2.0
Sulfadoxine	SUM 100	10	9.1	8.7	9.4	10.1	9.3	0.59	6.3
Tetracycline	200	100	98.8	98.3	91.7	100.4	97.3	3.81	3.9
Thiamphenicol	50	25	23.6	24.0	22.2	26.5	24.1	1.80	7.5
Tilmicosin	50	25	27.5	28.0	25.4	23.2	26.0	2.21	8.5
Trimethoprim	50	25	21.8	18.7	24.0	22.0	21.6	2.19	10.1
Tylosin	100	50	46.8	47.5	45.9	47.7	47.0	0.80	1.7

## Conclusion

The new workflow addresses the complexity of setting up a multi-class LC–MS/MS method for routine screening and quantitation. It allows for the consolidation of several single-class methods into a universal screening method for a diverse group of analytes in multiple matrices. Some 172 veterinary medicines within 15 classes of compounds were quantified in a single analysis, which achieved MDLs that were compliant with the lowest global MRLs. The method

has proven to be robust, providing stable response and retention times with good detection limits over 500 injections on multiple instruments. The generic sample preparation method demonstrated good recovery of multi-class compounds in the meat, fish, and milk matrices that were included in the study. H-SRM capability of the MS resulted in increased specificity and lower MDLs. Laboratories involved in the analysis of veterinary drug residues in food will benefit from this streamlined, validated workflow.