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Application News

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Liquid Chromatograph Mass Spectrometry

Multi-Residue Veterinary Drug Analysis of >200 Compounds using MRM Spectrum Mode by LC-MS/MS

Veterinary drugs are used for therapeutic, metaphylactic, prophylactic and growth promotion purposes. To provide an assurance that food from animals is safe with regards to residues of veterinary medicines, regulatory authorities have established Maximum Residue Limits (MRL's) for certain drugs in target tissues and animal species. Some pharmacologically active compounds identified by regulatory authorities have been prohibited and their hazardousness at all levels are being considered (EU regulation EC 37/2010; Commission Decision 2003/181/EC; 21CFR Part 556 Tolerances for Residues of New Animal Drugs in Food). In this article, we describe how a triple quadrupole mass spectrometer, which is both highly sensitive and selective, contributes to reducing false positive and false negative reporting when using a measurement mode called MRM Spectrum mode. MRM Spectrum mode acquires a high number of fragment ion transitions for each target compound and generates fragmentation spectra that can be used in routine library searching and compound verification using reference library match scores.

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Samples and Analysis Conditions

Samples of beef, egg, honey, milk and salmon were extracted and spiked with veterinary drugs in the calibration range of 0.001 to 0.1 mg/kg. Repeatability was assessed at low and high concentrations. Samples were measured using Shimadzu's Nexera X2 UHPLC and LCMS-8060 triple quadrupole mass spectrometer (Table 1 and 2). Over 200 veterinary drugs were targeted and over 2,000 MRM transitions in both ESI +/- were monitored during a gradient elution time of 12 minutes.

Table 1 UHPLC Conditions

Liquid chromatography				
UHPLC	Nexera LC system			
Analytical column	Restek Biphenyl (100 × 2.1, 2.7 µm)			
Column temperature	40 °C			
Flow rate	0.4 mL/minute			
Solvent A	0.1 % formic acid 0.5 mM ammonium formate solution			
Solvent B	0.1 % formic acid in methanol			
Binary Gradient	Time (mins)	%B	Time (mins)	%B
	0.00	2	14.60	2
	12.50	100	17.50	Stop
	14.50	100		

Table 2 MS/MS Acquisition Parameters

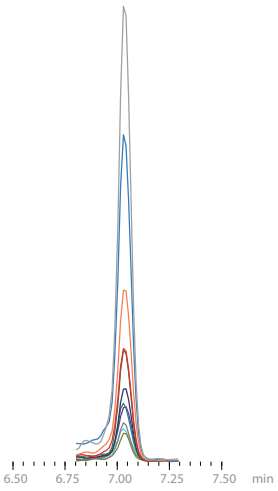
Mass spectrometry	
Mass spectrometer	Shimadzu LCMS-8060
Pause time/dwell time	1 msec/3 msec
Polarity switching time	Pos/neg switching time set to 5 msec
Scope	218 drugs in positive ion mode (including internal standards)
	11 drugs in negative ion mode
	Structure Analytics (in house development tool)
Source temperatures (interface; heat block; DL)	350 °C; 300 °C; 150 °C
Gas flows (nebulising; heating; drying)	3 L/min; 10 L/min; 10 L/min

Advantages of MRM Spectrum Mode

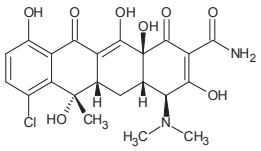
The measurement method can be easily set using the MRM optimization tool and measurement window (MRM Synchronization) settings of LabSolutions LCMS. The method achieves high data densities and a high data sampling rate across each elution peak. This approach generates a consistent loop time and sampling rate producing reliable quantitation and peak integration. It also provides great operator-friendliness in routine simultaneous analysis of veterinary drugs by enhancing flexibility in qualifier and quantifier ion selection. The number of fragment ion transitions generated from a single precursor ion is limited only by the chemical structure of the veterinary drug.

Results

MRM Spectrum mode was used to acquire a high number of fragment ion transitions for each veterinary drug target. For chlortetracycline, 11 precursor-fragment ion transitions were acquired using optimized collision energies (Fig. 1). Acquiring a high number of fragment ion transitions enables generation of fragmentation spectra which can be used in library searching and compound verification for each veterinary drug. (Chlortetratcycline is a tetracycline class of antimicrobials. According to the Sixth ESVAC report published in 2016, of the overall sales of antimicrobials in the 29 EU countries in 2014, the largest amount, expressed as a proportion of mg/PCU, was accounted for by tetracyclines (33.4 %). This is followed by penicillins (25.5 %) and sulfonamides (11.0 %). Chlortetracycline was selected as a representative target).



Compound name Chlortetracycline
Accurate mass 479.1216 [M+H]+
Formula C22H23ClN2O8
CAS 57-62-5



MRM Spectrum Mode
11 MRM's acquired for chlortetracycline at 10pg/uL in egg.

1:479.10>444.00 (+)	CE: -23V	7:479.10>300.80(+)	CE: -45V
2:479.10>461.95 (+)	CE: -35V	8:479.10>287.90(+)	CE: -53V
3:479.10>154.00 (+)	CE: -34V	9:479.10>274.95(+)	CE: -44V
4:479.10>98.05(+)	CE: -45V	10:479.10>370.95(+)	CE: -31V
5:479.10>260.05(+)	CE: -60V	11:479.10>285.85(+)	CE: -56V
6:479.10>303.05(+)	CE: -37V		

MRM Spectrum mode
Higher specificity
Higher reporting confidence
Library searchable fragment data.
The number of precursor-fragment ion transitions monitored is limited only by the structural chemistry of the molecule. Typically more than 10 precursor-fragment ion transitions were monitored for each veterinary drug.

Fig. 1 Utilization of MRM Spectrum Mode (Chlortetracycline)

Fig. 2 shows the MRM reference spectrum for chlortetracycline with assigned fragment structures. The MRM Spectrum mode is a measurement mode which combines MRM with the generation of a product ion spectrum. The product ion spectrum can be used for compound identification by searching a library.

As the collision energy was optimized for each fragment ion to generate a product ion spectrum, the library spectrum is highly specific and selective.

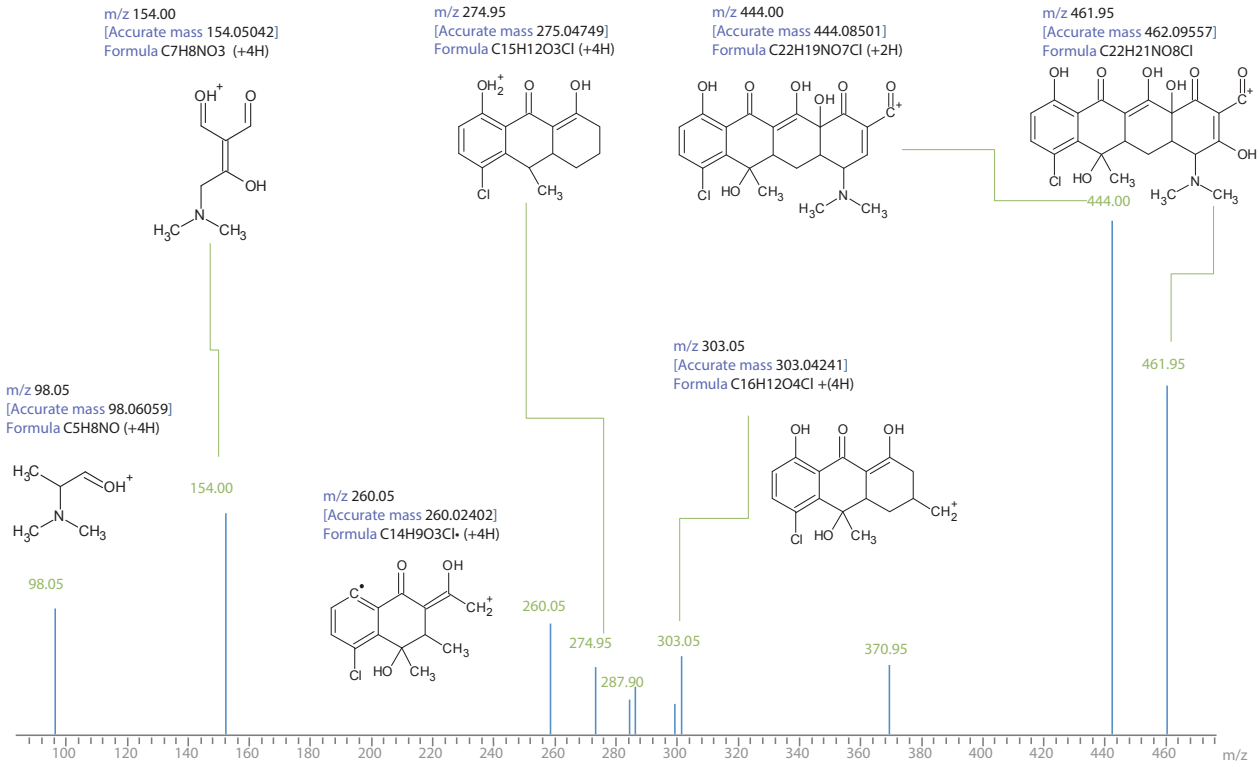


Fig. 2 MRM Reference Spectrum with Assigned Fragment Structures (Chlortetracycline)

Library Identification using MRM Spectrum Mode

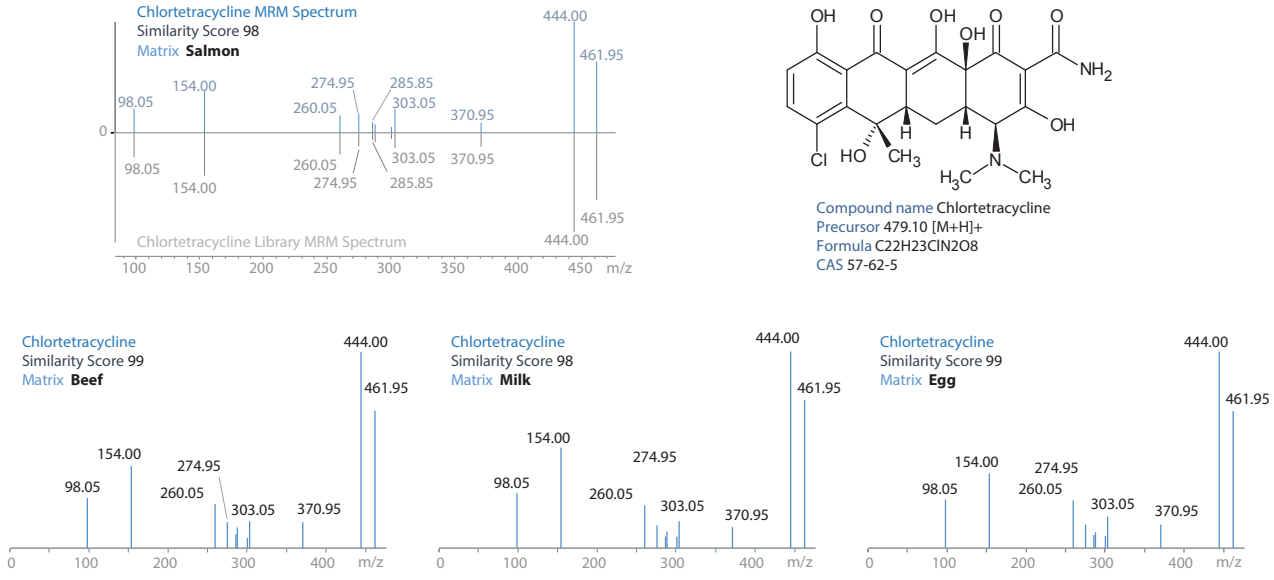
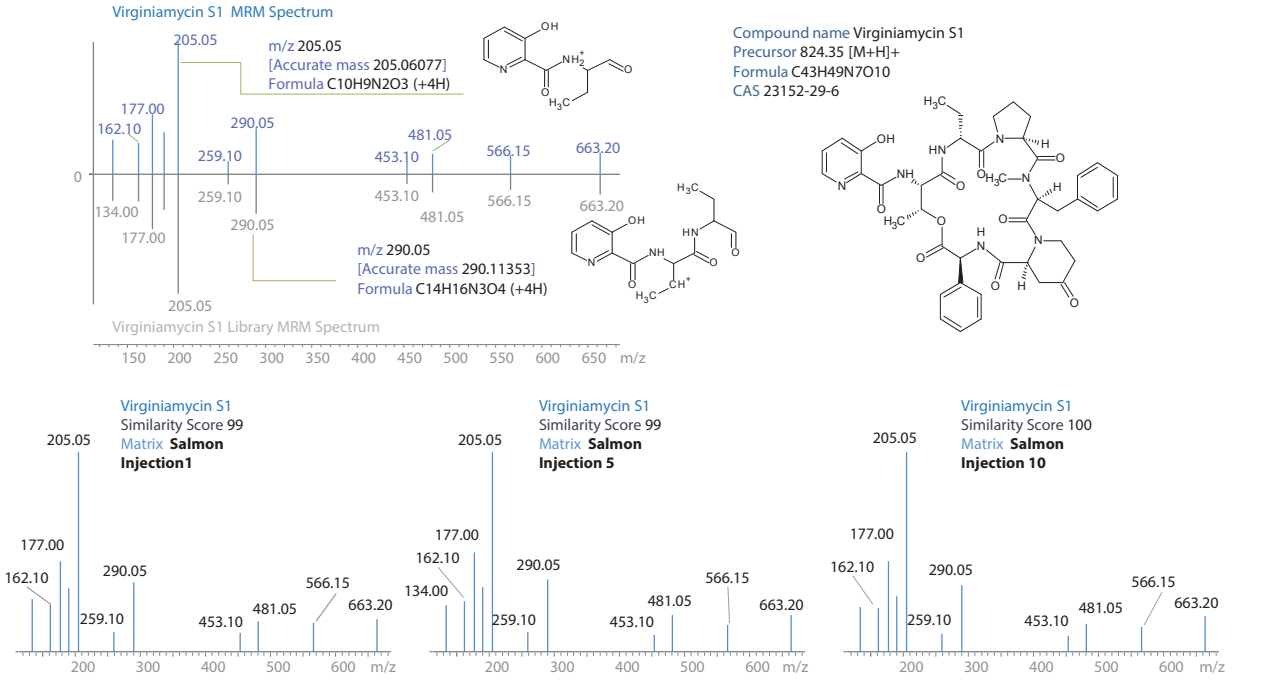


Fig. 3 Library Searchable MRM Spectra in Different Matrices Spiked at 10 pg/µL (Chlortetracycline)

Fig. 4 shows the MRM spectra and the n=10 measurement results of four compounds for salmon extract spiked with virginiamycin S1 at a concentration of 10 pg/µL. The library match score was above 99 in all injections (MRM spectra of injections 1, 5 and 10 are indicated). Also, the %RSD for oxytetracycline, sulfadimethoxine, ormetoprim, and virginiamycin spiked into salmon extract (n=10; 10 pg/uL) acquired using a conventional 2-MRM method was compared with that of the MRM spectrum method.



Compound name	Oxytetracycline		Sulfadimethoxine		Ormetoprim		Virginiamycin	
Number of MRM's	2MRM's	8MRMs	2MRM's	11MRMs	2MRM's	11MRMs	2MRM's	11MRMs
Mean peak area								
Quantitation ion	1890170	1729171	7809989	7227748	8291171	8160952	2232967	1956045
%RSD	**3.74**	**3.04**	**1.49**	**1.46**	**1.54**	**1.18**	**0.91**	**1.65**

Fig. 4 MRM Spectra and n=10 Results of Salmon Extract Spiked with Virginiamycin S1 at 10 pg/µL

Quantitation Results using MRM Spectrum Mode

To assess the robustness of the MRM Spectrum mode, the same sample was repeatedly injected. The method used complies with the identification criteria set out in the EU guidelines SANTE/11945/2015 that require the retention time and the ion ratio from at least 2 MRM ion ratios to be within acceptable tolerance limits. The absolute response and signal variability were compared to those of the MRM Spectrum mode (Fig. 4). Both methods resulted in a variance of less than 4 %RSD (n=10 for each method; 10 pg/uL spiked into salmon matrix). Fig. 5 indicates MRM spectra and the calibration curve obtained for sulfamerazine as an example of quantitation results.

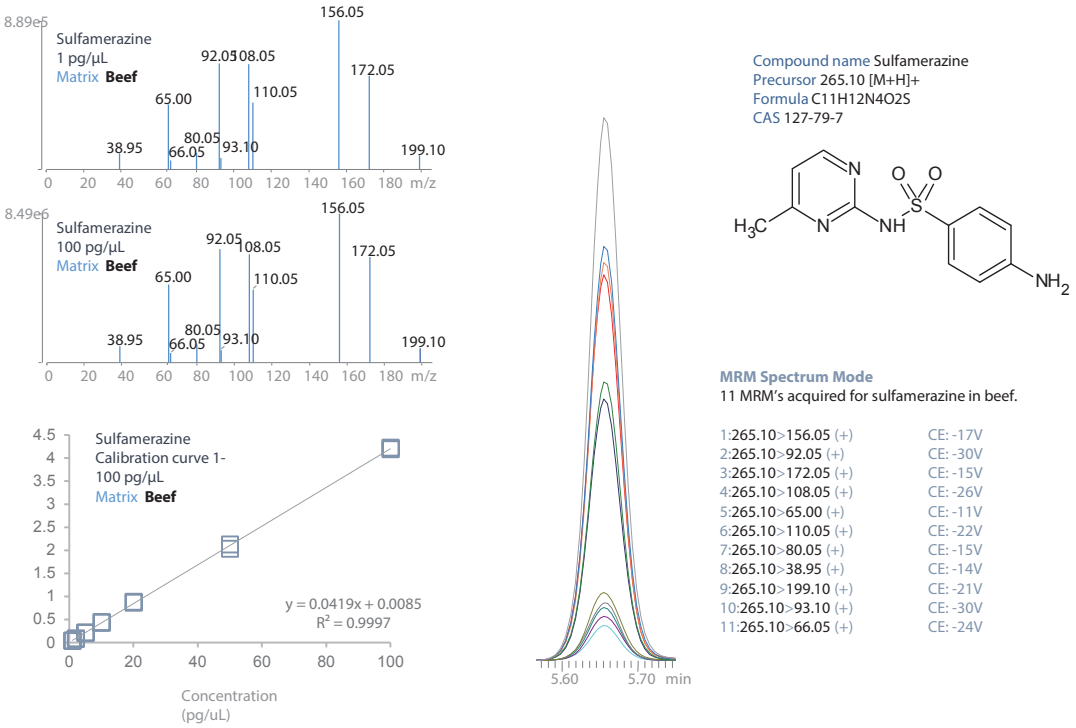


Fig. 5 MRM Spectra and Calibration Curve of Sulfamerazine (1 pg/µL to 100 pg/µL)

Conclusion

The level of confidence in compound identification and verification was increased by using a higher number of MRM transitions for each veterinary drug target and thereby reducing false negative and false positive reporting. Although the number of transitions for each target is dependent upon the chemical structure of the target, typically more than 10 transitions can be monitored for each compound. MRM Spectrum mode combines conventional quantitation with the generation of a high quality product ion spectrum which can be used to achieve highly reliable compound identification and verification by library searching. In this research, use of the MRM Spectrum mode was examined by quantifying and identifying 212 veterinary drugs (the method included 2,009 MRM transitions). Limits of detection, linearity or repeatability were not compromised compared to a conventional 2-MRM method.

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