Multi-residue veterinary drug analysis of >200 compounds using MRM Spectrum mode by LC-MS/MS

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1. Introduction

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 and has also identified pharmacologically active compounds that are prohibited and considered a hazard at any level (EU regulation EC 37/2010; Commission Decision 2003/181/EC; 21CFR Part 556 Tolerances for Residues of New Animal Drugs in Food). In this work, we describe a method that delivers highly sensitive and selective triple quadrupole detection together with MRM Spectrum mode to reduce false positive and false negative reporting. MRM Spectrum mode acquires a high number of fragment ion transitions for each target compound generating a fragmentation spectra that could be used in routine library searching and compound verification using reference library match scores.

2. Materials and Methods

Samples of beef, egg, honey, milk and salmon were extracted and spiked in the calibration range 0.001 – 0.1 mg/kg. Repeatability was assessed at low and high concentrations. Samples were measured using a Nexera UHPLC and the LCMS-8060 triple quadrupole detector (Table 1). Over 200 veterinary drugs were targeted, with more than 2,000 MRM transitions in both ESI +/- during the 12minute gradient.

Liquid chromatography				
UHPLC	Nexera LC system			
Analytical column	Restek Biphenyl (100 x 2.1, 2.7µm)			
Column temperature / Flow rate	40ºC ; 0.4mL/minute			
Solvent A	0.1% formic acid 0.5mM 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		

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)	350ºC; 300ºC; 150ºC
Gas flows (nebulising; heating; drying)	3L/min; 10 L/min; 10L/min

Table 1. LC and MS/MS acquisition parameters used to create the LC-MS/MS method.

2.1 Advantages of MRM Spectrum mode

The method is straightforward to set-up using conventional MRM optimization procedures and acquisition windows (scheduled MRM) resulting in high data densities and a high data sampling rate across a peak. This approach generates a consistent loop time and sampling rate producing reliable quantitation and peak integration. It also enables greater flexibility in routine veterinary drug monitoring programs by supporting a change in the qualifier and quantifier ion selection. The number of precursor-fragment ion transitions used to generate a product ion spectrum is only limited by the chemical structure of the veterinary drug.

3. Results

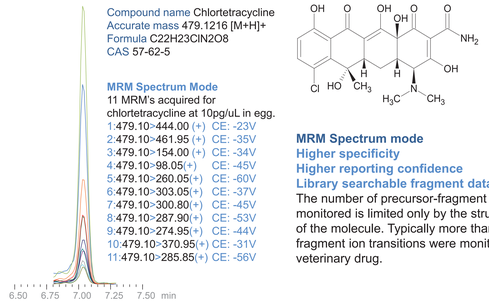


Figure 1. MRM Spectrum mode was used to acquire a high number of fragment ions for each veterinary drug target. For chlortetracycline, 11 precursor-fragment ion transitions were acquired using optimized collision energies. By acquiring a high number of fragment ion transitions, each target veterinary drug had a corresponding fragmentation spectra which could be used in routine library searching and compound verification using reference library match scores. (Chlortetratcycline is a tetracycline class of antimicrobial. According to the Sixth ESVAC report published in 2016, of the overall sales of antimicrobials in the 29 EU countries in 2014, the largest amounts, expressed as a proportion of mg/PCU, were accounted for by tetracyclines (33.4 %), penicillins (25.5%) and sulfonamides (11.0 %). Chlortetracycline was selected as a representative target).

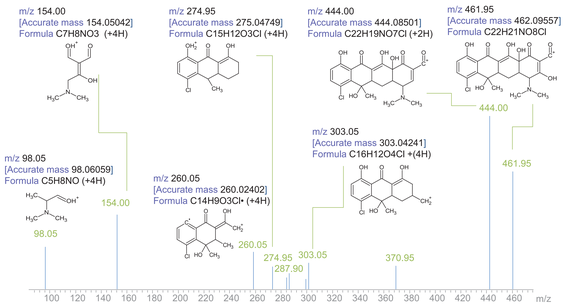


Figure 2. MRM reference spectrum for chlortetracycline with assigned fragment structures. MRM Spectrum mode combines MRM with the generation of a product ion spectrum. The product ion spectrum can be used for compound identification by searching a library. As the collision energy was optimized for each fragment ion to generate a product ion spectrum, the library spectrum is highly specific and selective. (Each precursor-fragment ion transitions structure may be verified by using Structure Analytics to show commonly described losses and charge migration; the hydrogen deficit is shown in brackets).

3.1 Library identification using MRM Spectrum mode

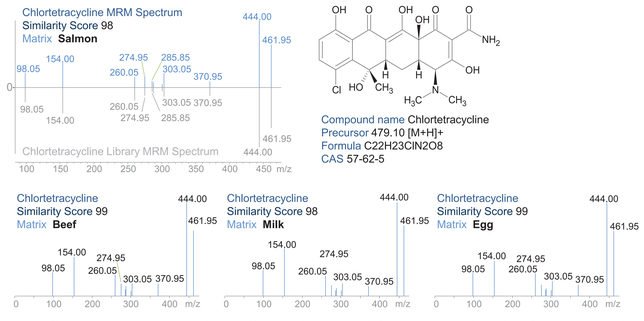
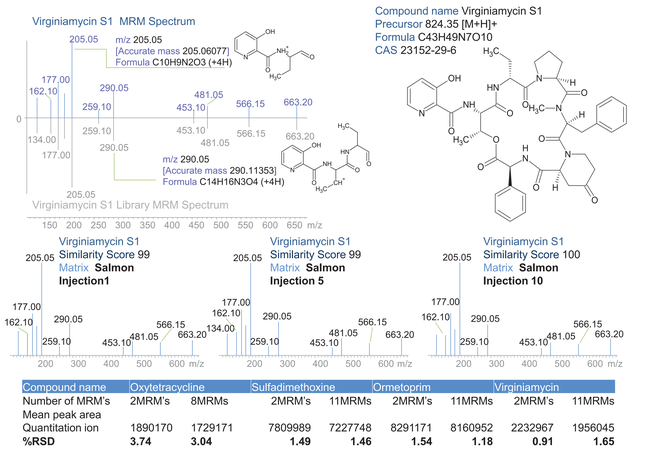


Figure 3. Library searchable MRM spectrum data for chlortetracycline in different matrices spiked at 10pg/uL.



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

Figure 4. Virginiamycin S1 was spiked into an extract of salmon at a concentration of 10pg/uL and repeatedly injected (n=10). The library score was above 99 in all injections (injection 1, 5 and 10 shown above) using 11 MRM's to generate a product ion spectrum (Structure Analytics was used to propose fragment structures; the hydrogen deficit is shown in brackets). %RSD for oxytetracycline, sulfadimethoxine, ormetoprim, and virginiamycin spiked into salmon (n=10; 10pg/uL) acquired using a conventional 2 MRM method compared to a MRM spectrum method with a higher number of fragment ions. [Compound selection based on antibiotics used in aquaculture; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4254636/].

3-2. Quantitation using MRM Spectrum mode

To assess the robustness of this approach the same sample was repeatedly injected using a method that compiled with the identification criteria set out in the EU guidelines SANTE/11945/2015 that requires the retention time and the ion ratio from at least 2 MRM transitions to be within acceptable tolerance limits. The absolute response and signal variability was compared to a method using a higher number of fragment ions (Table 2). Both methods resulted in a variance of less than 4%RSD (n=10 for each method; 10pg/uL spiked into salmon matrix).

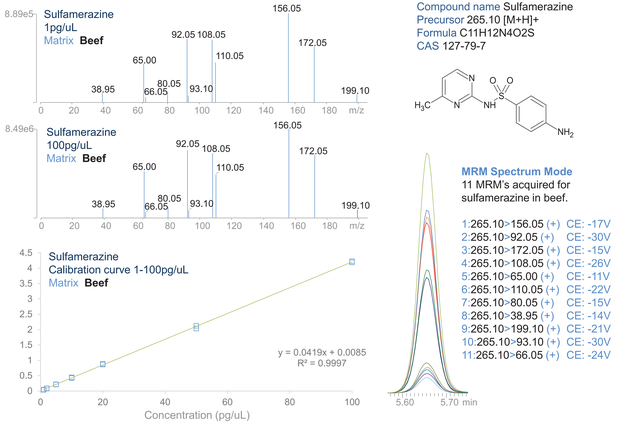


Figure 5. Library searchable MRM spectrum data for sulfamerazine over three orders of magnitude (1pfg/uL to 100pg/uL). Linear calibration curves were generated for all compounds with r2 typically greater than 0.9996. Sulfamerazine was selected as a representative target.

4. Conclusions

- To reduce false negative and false positive reporting a higher number of MRM transitions were used for each veterinary drug target to increase the level of confidence in compound identification and verification. The number of fragment ion transitions monitored for each target pesticide was dependent upon the chemical structure with typically more than 10 fragment ions for each compound. MRM Spectrum mode combines conventional MRM quantitation with the generation of a high quality MRM product ion spectrum which can be used in routine library searching and compound verification and identification.
- MRM Spectrum mode was used to quantify and identify 212 drugs (the method included 2,009 MRM transitions) without compromising limits of detection, linearity or repeatability compared to a conventional 2MRM method.