Validation and development of a multi-residue method for quantitation and confirmation of 30 veterinary drug residues in milk by high-resolution mass spectrometry (HRMS)

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Abstract

Veterinary drugs are used on dairy farms to treat animal diseases. FDA needs to monitor for drug residues in raw milk against the established tolerances or safe levels, to ensure judicious use of approved animal drugs. Therefore, there is a need for a rapid and reliable analytical method to monitor milk for illegal veterinary drug residues, to help keep the nation's milk supply safe for human consumption.

We developed and validated a new high-resolution mass spectrometric method for the quantitation and confirmation of selected 30-veterinary drug residues in raw milk. It has a simple sample preparation procedure, including a quick protein precipitation step followed by solvent dilution, which allows many samples to be processed within a short timeframe.

Chromatographic separation and the mass spectrometric detection was accomplished by Full Scan-All Ion Fragmentation (FS-AIF) using a Q-Exactive mass spectrometer coupled with an UPLC. This approach allowed quantitation of both polar and non-polar veterinary drugs representing different classes including macrolides, b-lactams, penicillins, fluoroquinolones, sulfonamides, tetracyclines, and amphenicols in a single analysis. Raw milk samples were spiked with 30-veterinary drugs at different levels depending on their tolerances or target testing levels. This method has been successfully validated to meet performance criteria by pertinent FDA guidelines.

Introduction

- Veterinary drugs are used in dairy farms. FDA needs to determine their residue concentrations in raw milk to see whether their concentration exceeds the established tolerances.
- We are interested in the development of a rapid and reliable multi-residue HRMS method for this purpose.
- Liquid chromatography- High Resolution Mass Spectrometry (LC-HRMS) using Orbitrap Q-Exactive instruments allows both targeted quantitation and non-targeted screening, with the advantage of retrospective data analysis for degradants and other metabolites. Additionally, the Full-scan data-independent MS/MS All-Ion Fragment (FS-AIF) option allows MS2 information for compound confirmation and reliable quantitation.
- There was no validated method using HRMS to quantify all 30-drugs in our list.

Materials and Methods

Raw Milk

Protein precipitation & dilution

LC-HRMS

- To 1 mL raw milk
- Add 1ml water
- Spike with 30 stds, wait 30 min
- Add 4 ml ACN 1% Formic acid (protein precipitation)
- Vortex for 60 min ("end-to-end" shaker)
- Dilute with water (1 to 1.5) **(Final dilution 15-fold)**
- Analyze with "LC-QE/HF HRMS"

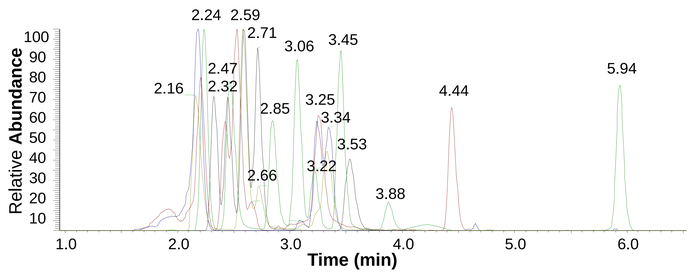
MS: Q-Exactive HF

Full-Scan data-independent All-Ion Fragment (FS-AIF). Positive mode Full Scan Resolution setting - 60,000 at m/z 200; AGC 3E6; Max IT 200 ms; AIF Resolution setting - 30,000 at m/z 200; AGC 5E6; Max IT 200 ms. Negative mode Full Scan Resolution setting - 120,000 at *m/z* 200; AGC 3E6; Max IT =350 ms; AIF Resolution setting 15,000 at m/z 200; AGC 1E6; Max IT 150 ms.

FS Mass range 150–1500 m/z (positive mode); 300-360 *m/z* (negative mode) NCE 10,35,70; Spray voltage 4.5 kV; Sheath gas 40 arb; Aux gas 12 arb; Sweep gas 1 arb; Capillary temperature 310°; Heater temperature 350°C; RF-lens level 60. MS2 mass rage for positive mode 100-1500 *m/z* and negative mode 80-360 m/z.

Improved Performance with Low-Flow (150 mL/min) LC Method

Phenomenex Kinetex biphenyl,100A 2.1x 50 mm, 1.7 µm column



Time (min)	Flow rate mL/min	B %
0	0.15	15
0.2	0.15	15
1.0	0.15	55
6.0	0.15	90
6.5	0.40	100
8.0	0.40	100

Mobile Phase A
5 mM Ammonium Formate, 0.1% formic acid in water
Mobile Phase B
5 mM Ammonium Formate, 0.1% formic acid in Methanol

Validation Criteria: (guideline;2019)

Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition

	Matrix 1, cow 78 12-28-2019	Matrix 2 cow 79 12-28-2019	Matrix 3 cow 78 01-07-2019	Matrix4 cow79 01-07-2019
Day 1	Blank 1	QC1-1 (0.5X)	QC2-1 (1X)	QC3-1 (2X)
	Blank 2	QC1-2 (0.5X)	QC2-2 (1X)	QC3-2 (2X)
Day 2	QC3-1 (2X)	Blank 1	QC1-1 (0.5X)	QC2-1 (1X)
	QC3-2 (2X)	Blank 2	QC1-2 (0.5X)	QC2-2 (1X)
Day 3	QC2-1 (1X)	QC3-1 (2X)	Blank 1	QC1-1 (0.5X)
	QC2-2 (1X)	QC3-2 (2X)	Blank 2	QC1-2 (0.5X)
Day 4	QC1-1 (0.5X)	QC2-1 (1X)	QC3-1 (2X)	Blank 1
	QC1-2 (0.5X)	QC2-2 (1X)	QC3-2 (2X)	Blank 2

- Four different milk matrices were used during 4-day validation
- Total of 8-replicates for each fortification level
- X is the Tolerance or Target Testing level for each drug in milk (ppb)

Veterinary Drugs	Tolerance or Target Testing Level in milk (ppb = 1X)	Fortification Levels (ppb) 0.5X; 1X, 2X
Ampicillin	10	5, 10, 20
Bacitracin A	500	250, 500, 1000
Cefapirin	20	10, 20, 40
Chlortetracycline	100	50, 100, 200
Ciprofloxacin	5	2.5, 5, 10
Cloxacillin	10	5, 10, 20
Doxycycline	100	50, 100, 200
Enrofloxacin	5	2.5, 5, 10
Erythromycin	50	25, 50, 100
Oxytetracycline	100	50, 100, 200
Penicillin G	5	2.5, 5, 10
Sarafloxacin	5	2.5, 5, 10
Sulfachlorpyridazine	10	5, 10, 20
Sulfadiazine	10	5, 10, 20
Sulfadimethoxine	10	5, 10, 20
Sulfamerazine	10	5, 10, 20
Sulfamethazine	10	5, 10, 20
Sulfapyridine	10	5, 10, 20
Sulfaquinoxaline	10	5, 10, 20
Sulfathiazole	10	5, 10, 20
Tetracycline	100	50, 100, 200
Thiabendazole	50	25, 50, 100
Tilmicosin	100	50, 100, 200
Tripelennamine	20	10, 20, 40
Tulathromycin A	100	50, 100, 200
Tylosin	50	25, 50, 100
Virginiamycin M1	100	50, 100, 200

List of 30 compounds and tested levels in milk

Negative mode

Veterinary Drugs	Tolerance or Target Testing Level in milk (ppb = 1X)	Fortification Levels (ppb) 0.5X; 1X, 2X
Chloramphenicol	0.3	0.15, 0.3, 0.6
Florfenicol	1	0.5, 1, 2
5-hydroxyflunixin	2	1, 2, 4

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Disclaimer

The views expressed in this poster are those of the authors and do not necessarily reflects the official policy if the department of Health and Human Services, the US Food and Drug Administration, or the US Government

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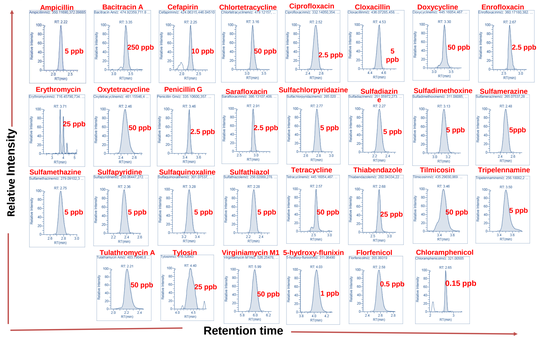
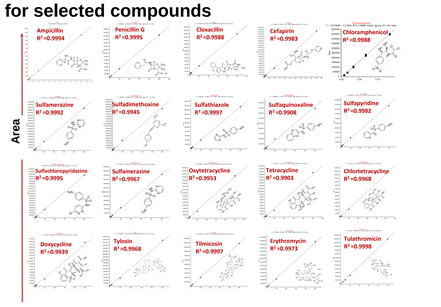
Results and Discussion

Extracted ion chromatograms (5 ppm mass window) for low-QC levels (0.5X) for all 30 compounds



Representative matrix-matched calibration curves for selected compounds



Recoveries for the selected compounds by TraceFinder

Compound	Sample ID	Day1 Recov%	Day2 Recov%	Day3 Recov%	Day4-Recov%	TTL/ppb	Acceptance %	Average Recovery %	STDEV
	Negative mode								
5-hydroxy-flunixin	Matrix Blank								
	Matrix Blank								
	QC1-1 (0.5X)	107.46	107.46	105.97	107.46	1.00		105.41	6.67
	QC1-2 (0.5X)	89.55	107.46	111.94	105.97	1.00	40 - 120		
	QC2-1 (1X)	106.77	128.57	102.26	112.03	2.00		114.00	10.90
	QC2-2 (1X)	112.03	108.27	109.02	133.08	2.00			
	QC3-1 (2X)	88.39	100.37	105.24	105.24	4.00		104.35	7.55
	QC3-2 (2X)	112.36	103.75	108.24	111.24	4.00			
Chloramphenicol	Matrix Blank								
	Matrix Blank								
	QC1-1 (0.5X)	80.00	120.00	80.00	110.00	0.15		98.75	21.00
	QC1-2 (0.5X)	70.00	120.00	90.00	120.00	0.15			
	QC2-1 (1X)	95.00	135.00	95.00	110.00	0.30	40-120	109.38	15.45
	QC2-2 (1X)	100.00	110.00	100.00	130.00	0.30			
	QC3-1 (2X)	77.50	110.00	87.50	120.00	0.60		103.13	16.78
	QC3-2 (2X)	102.50	112.50	90.00	125.00	0.60			
Florfenicol	Matrix Blank								
	Matrix Blank								
	QC1-1 (0.5X)	109.09	118.18	96.97	118.18	0.5		108.71	10.68
	QC1-2 (0.5X)	90.91	118.18	103.03	115.15	0.5			
	QC2-1 (1X)	105.97	131.34	95.52	114.93	1	40-120	114.37	13.86
	QC2-2 (1X)	113.43	119.40	100.00	134.33	1			
	QC3-1 (2X)	84.96	104.51	90.98	118.05	2		104.51	13.35
	QC3-2 (2X)	109.02	112.78	93.98	121.80	2			
	Positive mode								
Sulfathiazole	Matrix Blank								
	Matrix Blank								
	QC1-1 (0.5X)	99.70	102.40	98.50	97.90	5	40 - 120	96.47	6.48
	QC1-2 (0.5X)	81.68	96.10	94.59	100.90	5	40 - 120		
	QC2-1 (1X)	102.25	123.99	93.10	97.90	10	60 - 115	106.09	11.23
	QC2-2 (1X)	103.60	105.40	100.00	122.49	10	60 - 115		
	QC3-1 (2X)	79.77	96.49	96.62	99.40	20	60 - 115	98.43	9.02
	QC3-2 (2X)	106.32	106.09	92.33	101.43	20	60 - 115		
Tetracycline	Matrix Blank								
	Matrix Blank								
	QC1-1 (0.5X)	99.58	97.12	103.12	100.51	50	40 - 120 %	97.84	7.26
	QC1-2 (0.5X)	86.40	90.96	109.88	95.14	50	40 - 120 %		
	QC2-1 (1X)	111.05	117.89	89.76	101.95	100	60 - 115 %	107.41	12.32
	QC2-2 (1X)	108.46	96.42	104.71	129.07	100	60 - 115 %		
	QC3-1 (2X)	82.54	106.08	99.32	99.35	200	60 - 115 %	100.17	7.75
	QC3-2 (2X)	106.93	104.91	99.83	102.35	200	60 - 115 %		

Validation summary (quantitation & confirmation)

		TOL/TTL (ppb)	Quantitation QC1-0.5X	Quantitation QC2-1X	Quantitation QC3-2X	Number confirmed/total replicates QC1-0.5X	Number confirmed/total replicates QC2-1X	Number confirmed/total replicates QC3-2X
1	5-hydroxy-flunixin	2	Pass	Pass	Pass	8/8	8/8	8/8
2	Chloramphenicol	0.3	Pass	Pass	Pass	8/8	8/8	8/8
3	Florfenicol	1	Pass	Pass	Pass	8/8	8/8	8/8
4	Ampicillin	10	Pass	Pass	Pass	3/8	5/8	7/8
5	Bacitracin A	500	Pass	Pass	Pass	8/8	8/8	8/8
6	Cefapirin	20	Pass	Pass	Pass	6/8	8/8	8/8
7	Chlortetracycline	100	Pass	Pass	Pass	8/8	8/8	8/8
8	Ciprofloxacin	5	Pass	Pass	Pass	8/8	8/8	8/8
9	Cloxacillin	10	Pass	Pass	Pass	0/8	4/8	8/8
10	Doxycycline	100	Pass	Pass	Pass	8/8	8/8	8/8
11	Enrofloxacin	5	Pass	Pass	Pass	7/8	8/8	8/8
12	Erythromycin	50	Pass	Pass	Pass	8/8	8/8	8/8
13	Oxytetracycline	100	Pass	Pass	Pass	8/8	8/8	8/8
14	Penicillin G	5	Not Pass	Not Pass	Pass	2/8	5/8	8/8
15	Sarafloxacin	5	Pass	Pass	Pass	8/8	8/8	8/8
16	Sulfachlorpyridazine	10	Pass	Pass	Pass	8/8	8/8	8/8
17	Sulfadiazine	10	Pass	Pass	Pass	8/8	8/8	8/8
18	Sulfadimethoxine	10	Pass	Pass	Pass	8/8	8/8	8/8
19	Sulfamerazine	10	Pass	Pass	Pass	8/8	8/8	8/8
20	Sulfamethazine	10	Pass	Pass	Pass	8/8	8/8	8/8
21	Sulfapyridine	10	Pass	Pass	Pass	8/8	8/8	8/8
22	Sulfaquinoxaline	10	Pass	Pass	Pass	8/8	8/8	8/8
23	Sulfathiazole	10	Pass	Pass	Pass	8/8	8/8	8/8
24	Tetracycline	100	Pass	Pass	Pass	8/8	8/8	8/8
25	Thiabendazole	50	Pass	Pass	Pass	8/8	8/8	8/8
26	Tilmicosin	100	Pass	Pass	Pass	8/8	8/8	8/8
27	Tripelennamine	20	Pass	Pass	Pass	8/8	8/8	8/8
28	Tulathamycin A	100	Pass	Pass	Pass	8/8	8/8	8/8
29	Tylosin	50	Pass	Pass	Pass	8/8	8/8	8/8
30	Virginiamycin M1	100	Pass	Pass	Pass	8/8	8/8	8/8

Quantitation: Pass/Not pass acceptance criteria for average recovery and RSD. Guideline for validation of chemical methods for FDA food program-3rd Ed.2019
Confirmation: Number confirmed/total replicates: Pass 8/8

Conclusions

- A simple, efficient and fast protein precipitation-dilution procedure for veterinary drug analysis in milk was developed, with good recoveries for both polar and non-polar compounds in a single UPLC/HRMS method
- Method yielded acceptable recoveries (84-120%) and %RSD for all veterinary drugs in the study, bacitracin A, recovery improved after use of plastic volumetric flasks and vials instead of glass
- All the quantitation ions [MS1] and fragment ions [MS2] were found within 5 ppm mass window
- Chromatographic Retention Time (Rt) for all QCs are acceptable, within +/- 0.2 min
- Erythromycin was quantified by adding the peak area of dehydro erythromycin to the parent erythromycin
- Some replicates of lower QCs (0.5X and 1X) for beta-lactams could not be confirmed lacking a fragment ion
- 25 vet-drugs were successfully quantitated and confirmed, except for the four beta-lactam drugs at low levels (0.5X and 1X)