[APPLICATION NOTE]

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Optimized Extraction and Cleanup Protocols for LC/MS/MS Multi-Residue Determination of Veterinary Drugs in Milk

Michael S. Young and Kim Van Tran

APPLICATION BENEFITS

- Efficient, timesaving multi-class/multi-residue methodology
- Straightforward sample preparation for diverse range of analytes
- Fast, sensitive UPLC/MS analysis

WATERS SOLUTIONS

- ACQUITY UPLC®
- ACQUITY® TQD
- Sep-Pak® C_{18} Cartridge

KEY WORDS

Milk, UPLC/MS/MS, Sep-Pak Cartridges, Veterinary Drugs

OVERVIEW

In order to insure public health and safety, a reliable screening analysis is necessary to determine veterinary drug residue levels in milk samples. The compounds of interest range from highly polar water-soluble compounds to very non-polar fat-soluble compounds. There exists very effective extraction and cleanup procedures for individual compounds or compound classes, but these methods are not well suited for a multi-class, multi-residue screening analysis.

- Solvent extraction (with excess acetonitrile or methanol) can be effective for many veterinary drug residues in milk, but highly water-soluble drugs, such as salbutamol, are not well recovered using this approach.
- Aqueous buffer extraction can also be effective for many compounds, but fat-soluble compounds, such as dexamethasone, are not well recovered using this approach.
- Traditional solid-phase extraction (SPE) enrichment and cleanup (retention/wash/elution) has limited utility for multi-residue analysis. Because the range of acidity/polarity/solubility among the compounds is so broad, dispersive or pass-through SPE is preferred for multi-residue methods.

INTRODUCTION

Optimized sample preparation and analysis protocols were developed for tandem LC/MS/MS determination of a wide variety of veterinary drug residues in milk samples. Samples are initially precipitated and extracted with an equal volume of acetonitrile. After the resulting extract is treated with acidified acetonitrile to precipitate remaining proteins, a simple SPE cleanup is performed using a Sep-Pak C_{18} cartridge. After evaporation and reconstitution, the sample is analyzed using tandem LC/MS. Representative compounds were chosen from major classes of veterinary drugs including tetracyclines, fluoroquinolones, sulfonamides, macrolides, beta-lactams, NSAIDS, steroids, and beta-andrenergids.

EXPERIMENTAL

LC Conditions

LC system:	ACQUITY UPLC system
Column:	ACQUITY UPLC CSH™ C_{18}, 1.7 µm, 100 mm x 2.1 mm (i.d.)
Mobile phase A:	0.1% formic in water
Mobile phase B:	0.1% formic acid acetonitrile
Injection volume:	7 µL
Injection mode:	Partial loop injection
Column temperature:	30 °C
Weak needle wash:	10:90 acetonitrile:water (600 µL)
Strong needle wash:	50:30:20 water:acetonitrile:IPA (200 µL)
Seal wash:	10:90 acetonitrile:water

Gradient:

Time (min)	Flow (mL/min)	Profile %A	%B	Curve
Initial	0.4	85	15	6
2.5	0.4	60	40	6
3.9	0.4	5	95	6
4.9	0.4	5	95	6
5.0	0.4	85	15	6
7.0	0.4	85	15	6

MS Conditions

Mass spectrometer:	Waters ACQUITY TQD
Source temperature:	150 °C
Desolvation temperature:	500 °C
Desolvation gas flow:	1000 L/Hr
Cone gas flow:	30 L/Hr
Collision gas flow:	0.15 mL/min
Data management:	MassLynx™ v4.1

Table 1 summarizes the MRM transitions and instrument parameters used for this study. Also presented in Table 1 are matrix-matched calibration data for each compound (calculated using the primary transition).

Compound	MRM	Cone (V)	Collision (eV)	Calibration Range (ng/g)	Correlation (r^2)	LOQ (ng/g)
Carbadox	263>231 263>145	15 15	17 25	25-200	0.9901	50
Ciprofloxacin	332>288 332>314	20 20	20 20	25-200	0.9911	25
Chloramphenicol	321>152 321>257	10 20	15 12	25-200	0.9903	25
Chlortetracyline	479>444 479>462	25 25	25 15	25-200	0.9945	25
Dexamethasone	393>355 393>373	18 18	13 10	25-200	0.9955	25
Enrofloxacin	360>316 360>342	15 15	15 15	50-400	0.9917	50
Erythromycin	734>158 734>576	25 25	25 20	2.5-20	0.9979	2.5
Lincomycin	407>126 407>359	15 15	35 15	12.5-100	0.9903	12.5
Oxacillin	402>160 402>243	30 30	15 10	25-200	0.9948	25
Oxytetracycline	461>426 461>381	22 22	20 20	25-200	0.9914	25
Penicillin-G	335>160 335>176	15 15	15 15	12.5-100	0.9961	12.5
Phenylbutazone	309>160 309>146	15 15	25 27	25-200	0.9908	25
Ractopamine	302>107 302>284	25 25	20 10	75-600	0.9935	75
Salbutamol	240>148 240>222	25 25	10 20	25-200	0.9902	25
Sulfamerazine	265>92 265>156	25 25	33 15	25-200	0.9951	25
Sulfamethazine	279>92 279>186	15 15	33 15	25-200	0.9915	25
Sulfanilamide	173>156 173>92	20 20	12 20	25-200	0.9910	50
Tetracycline	445>154 445>410	25 25	25 20	25-200	0.9929	25

Table 1. MRM transitions and calibration data.

SAMPLE PREPARATION PROTOCOL

1. Initial Extraction/Precipitation:

Transfer
2 mL sample into a 15 mL centrifuge tube

Add
2 mL acetonitrile (ACN) and vortex for 30 seconds

Centrifuge
8000 x g for 4 minutes[1]

[1] This step gives good extraction of most compounds of interest but also extracts significant amounts of protein and some fats that may interfere with the LC/MS analysis.

2. Residual Protein Precipitation:

Transfer
2 mL of supernatant (from step 1)
to a second centrifuge tube

Add
3 mL of acidified acetonitrile (0.2 % formic acid)

Centrifuge
30 seconds[2]

[2] This step effectively precipitates the residual protein.

3. SPE Cleanup:

Take 1 mL of the supernatant (from step 2)
for SPE cleanup using a Sep-Pak C_{18} cartridge
(see SPE details in Figure 1).[3]

[3] This step removes fats and non-polar interferences.

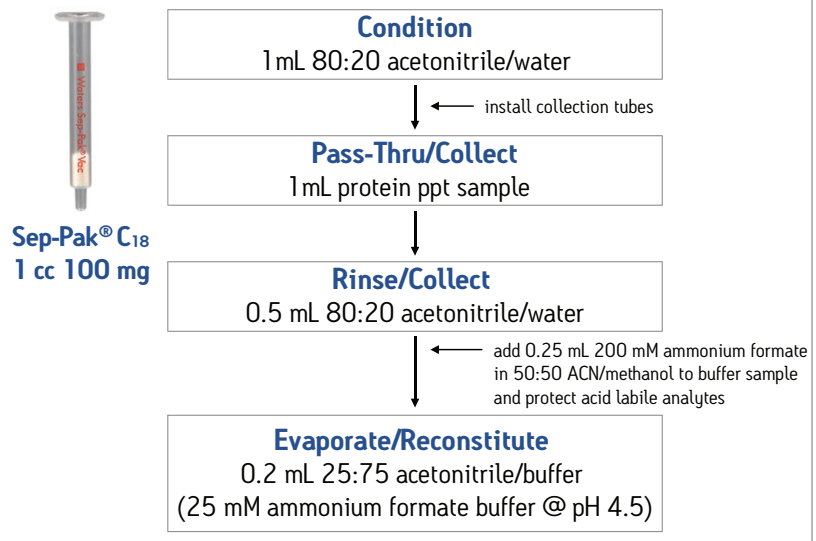

Figure 1. SPE cleanup protocol.

RESULTS

Figure 2 shows a typical LC/MS chromatogram obtained from analysis of a matrix matched standard of erythromycin at 6.7 ng/g. Performance of the other compounds was similar. Table 2 shows the recovery and matrix effects observed for multi-residue milk analysis.

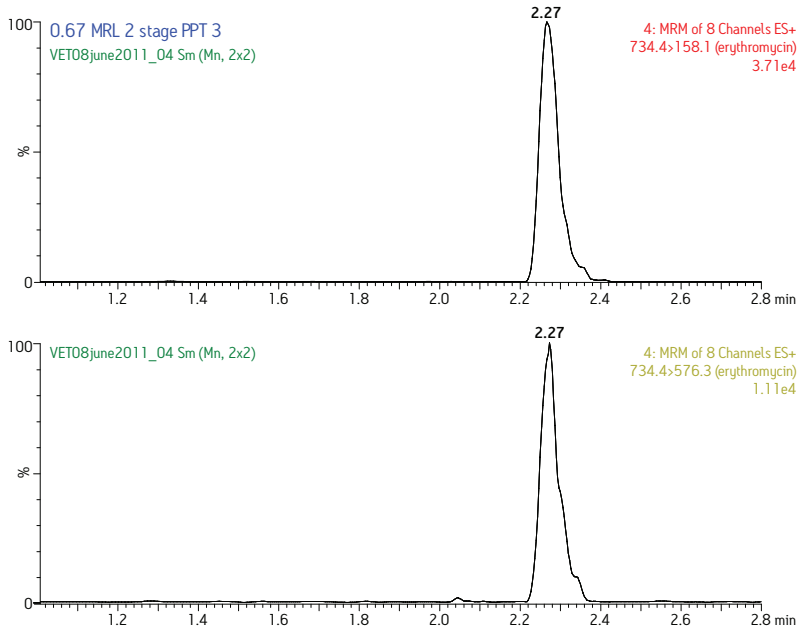

Figure 2. Typical LC/MS/MS Chromatogram obtained from milk spiked with erythromycin at 6.7 ng/g (primary MRM transition on top).

Compound	Spike Level (ng/g)	%REC (%RSD) (n=3)	%Suppression*
Carbadox	67.0	27 (27)	-43
Ciprofloxacin	67.0	67 (20)	32
Chloramphenicol	67.0	94 (16)	10
Chlortetracyline	67.0	22 (20)	7
Dexamethasone	67.0	87 (6)	-8
Enrofloxacin	134.0	76 (11)	26
Erythromycin	6.7	59 (10)	5
Lincomycin	33.0	102 (9)	25
Oxacillin	67.0	79 (12)	-9
Oxytetracycline	67.0	24 (16)	-9
Penicillin-G	33.0	73 (8)	-8
Phenylbutazone	67.0	67 (18)	20
Ractopamine	200.0	65 (14)	0
Salbutamol	67.0	80.4 (3)	96
Sulfamerazine	67.0	71 (4)	-16
Sulfamethazine	67.0	71 (6)	-74
Sulfanilamide	67.0	110 (30)	60
Tetracycline	67.0	31 (18)	-21

Table 2. Recovery and matrix effects.
** Negative number signifies matrix enhancement*

DISCUSSION

The procedure chosen for the milk analysis was to initially extract and precipitate a milk sample with an equivalent amount of acetonitrile and then to precipitate the remaining protein from the supernatant with acidified acetonitrile. A single step procedure was also considered by which the milk sample was directly precipitated with 0.2% formic acid in 80% acetontrile. This procedure was more straightforward and produced a final extract of similar cleanliness compared with the chosen protocol. However, there was significantly lower recovery for the most polar compounds, such as sulfanilamide, and virtually no recovery of chlorotetracycline. Another approach was considered, by which two separate extractions were performed. The first extraction, for the water soluble compounds, was accomplished using aqueous succinic buffer. The second, performed on the re-suspended pellet, was with acetonitrile. This approach requires that each fraction be worked up independently before ultimately combining fractions for a single injection. Performance was marginally better than the chosen procedure but at a much greater cost of time and materials. Although the chosen procedure requires two precipitation steps, it provided the best balance of preparative time with good method performance.

CONCLUSIONS

- A two step extraction/protein precipitation procedure was developed and demonstrated for milk analysis.
  - The procedure was suitable for screening for a wide range of veterinary drug residues.
  - Recoveries averaged 67% (22-110) with the lowest values for tetracyclines.
- A pass-thru SPE cleanup protocol using Sep-Pak C_{18} was utilized for effective removal of residual fats.
- The sample preparation methodology for milk produced an extract for LC/MS that was free of particulates and required no subsequent filtration prior to LC/MS analysis.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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