QUANTITATIVE DETERMINATION OF VETERINARY DRUG RESIDUES IN EGGS BY UPLC-MS/MS USING A SIMPLE, RAPID AND EFFECTIVE CLEANUP APPROACH

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INTRODUCTION

Veterinary drugs are used in chicken farms to control diseases of laying hens. However, these compounds can be transferred to and accumulate in the eggs. The presence of veterinary drug residues in eggs is a potential health risk for the consumer because the residual drugs can provoke allergic reactions or induce pathogen resistance to antibiotics used in human medicine [1]. Sixteen representative veterinary drugs from twelve classes, most of which have MRPLs established in USA, EU and/or China, were chosen for this study [2][3]. Figure 1 presents the structures of a subset of these veterinary drugs.

Sample preparation is a challenging task for the multi-residue determination of veterinary drugs in eggs. The analyst must recover a wide variety of drug classes with different physico-chemical properties. Some of the target compounds may bind to proteins or other matrix components. Also, eggs contain high levels of lecithin (phospholipids) and fats; these co-extracted substances can lead to interference in the LC-MS analysis, contamination of the analytical column and other components of the UPLC system, and contamination of the mass spectrometer itself.

In this work, sample extraction, cleanup and analysis methods were developed for UPLC-MS/MS determination of a wide variety of veterinary drugs in eggs. Samples were treated with an acidified acetonitrile/water solvent to precipitate proteins, release bound residues and to extract the veterinary drugs of interest. Then, to remove fats and phospholipids, a simple pass-through cleanup was performed using a novel SPE device, the Oasis PRiME HLB cartridge.

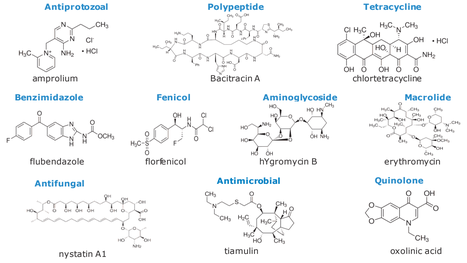

Figure1. *Structures of representative compounds from this study*

METHODS

Standard Compounds

Sixteen veterinary drugs from different classes were chosen for this study. **Table 1** lists their formula, MWs, and MRPLs established in USA, EU or China.

Compounds	Formula	Monoisotopic MW	Market	MRL(ppb)
Amprolium	C14H19ClN4	278.129822	United States	4000
Bacitracin A	C66H103N17O16S	1421.748901	United States/China	Bacitracin:500
Hygromycin B	C20H37N3O13	527.232666	United States/China	No residue allowed
Nystatin A1	C47H75NO17	925.503479	United States	No residue allowed
Colistin B	C52H98N16O13	1154.749878	China	Colistin: 300
Florfenicol	C12H14Cl2FNO4S	357.000458	China	No residue allowed
Flubendazole	C16H12FN3O3	313.086273	China	400
Oxolinic acid	C13H11NO5	261.063721	China	50
Tiamulin	C28H47NO4S	493.322571	China	Tiamulin+8-α-Hydroxymutilin:1000
Chlortetracycline	C22H23ClN2O8	478.114288	United States/China	400/200
Erythromycin	C37H67NO13	733.461243	United States/China	25/150
Lincomycin	C18H34N2O6S	406.213745	China	50
Oxytetracycline	C22H24N2O9	460.148193	China	200
Penicillin G	C16H18N2O4S	334.098724	United States	No residue allowed
Tetracycline	C22H24N2O8	444.153259	China	200
Tylosin	C46H77NO17	915.519165	United States/China	200

Table 1. *Veterinary drugs in this study (bacitracin, colistin and nystatin all contain a mixture of more than two components; one major component was chosen for analysis)*

Sample Preparation

Extraction: Two grams of homogenized whole chicken eggs were weighed into a 50 mL polypropylene centrifuge tube and 8mL of 0.2% formic acid in 80:20 acetonitrile/water were added. Following a 30s vortex and shaking for 30 mins, the mix was centrifuged at 4500rpm for 10min.

Pass-thru SPE Cleanup: An Oasis PRiME HLB cartridge (3cc,60mg) was mounted on a precleaned vacuum manifold. Cartridge conditioning is not required. 0.5 mL of the supernatant was passed-through the cartridge and collected using 1~2 psi vacuum. 0.2 mL of the collected extract was taken and diluted to 0.6 mL with aqueous 10 mM ammonium formate buffer (pH 4.5) prior to UPLC-MS/MS analysis.

LC and MS Conditions

Chromatography Conditions:

System: ACQUITY® UPLC I-Class with Xevo® TQ-S MS
Software: MassLynx® V4.1
Column: ACQUITY UPLC BEH C18,2.1x100mm, 1.7µm
Column Temp.: 30 °C
Injection Volume: 10µL
Flow Rate: 0.4mL/min
Mobile Phase A: 0.1% formic acid in water
Mobile Phase B: 0.1% formic acid in acetonitrile
Gradient: The Initial composition was 85% A and 15% B. Phase B was increased linearly to 40% in the first 2.5min,and then linear ramp to 95%B in 1.4 min, maintained for 2.3 min, then returned to the initial composition and equilibrated for 2 min.

MS Conditions:

Ionization Mode: ES+(ES-for Florfenicol)
Capillary Voltage (kV): 3.00(2.50 for negative ion mode)
Source Temp. (°C): 150
Desolvation Temp. (°C): 600
Cone Gas Flow (L/Hr): 150
Desolvation Gas Flow (L/Hr): 1000
Collision Gas Flow (mL/Min): 0.15
Nebuliser Gas Flow (Bar): 7.00

LC-MS/MS Parameters

MS/MS Transitions are presented in **Table 2**.
Other LC-MS parameters are presented in **Table 3**.
A typical chromatographic separation is presented in **Figure 2**.

	MRM trasition 1				MRM trasition 2		
Compounds	Precursor Ion (m/z)	Product Ion (m/z)	Cone voltage(V)	Collision energy(eV)	Product Ion (m/z)	Cone voltage(V)	Collision energy(eV)
Amprolium	243.26	94.06	20	14	150.17	20	12
Bacitracin A	712.22	199.10	68	40	110.10	68	70
Hygromycin B	528.49	352.20	48	22	177.14	48	32
Nystatin A1	926.82	297.24	22	28	107.13	48	60
Colistin B	578.66	101.07	64	28	86.06	64	40
Florfenicol	356.03	335.96	52	10	184.94	52	22
Flubendazole	314.25	282.19	90	18	123.08	90	36
Oxolinic acid	262.20	244.20	20	12	160.17	50	32
Tiamulin	494.45	119.10	40	42	192.17	40	20
Chlortetracycline	479.27	444.19	12	18	154.06	12	26
Erythromycin	734.72	158.08	48	26	576.52	48	18
Lincomycin	407.20	126.10	40	34	359.30	40	20
Oxytetracycline	461.36	426.22	20	18	201.07	64	36
Penicillin G	335.27	176.05	14	20	159.99	14	16
Tetracycline	445.30	410.20	40	21	154.00	40	26
Tylosin	916.88	174.13	80	36	101.10	45	45

Table 2. *MRM Transition parameters for 16 veterinary drugs*

	Compounds	RT (min)	LOD (ppb)	Linear Range (ppb)	R^2
1	Amprolium	0.61	0.5	80-40000	0.998
2	Bacitracin A	2.52	1	10-5000	0.992
3	Hygromycin B	0.48	4	4-1000	0.990
4	Nystatin A1	3.30	10	40-1000	0.992
5	Colistin B	1.73	30	90-600	0.990
6	Florfenicol	2.69	4	4-1000	0.991
7	Flubendazole	3.29	0.5	8-240	0.993
8	Oxolinic acid	2.83	1	1-500	0.993
9	Tiamulin	3.88	0.5	20-1000	0.990
10	Chlortetracycline	2.49	0.5	4-2000	0.995
11	Erythromycin	2.95	0.5	0.5-250	0.995
12	Lincomycin	1.59	0.5	1-500	0.996
13	Oxytetracycline	1.89	0.5	4-2000	0.995
14	Penicillin G	1.91	1	2-1000	0.991
15	Tetracycline	2.04	0.5	4-2000	0.994
16	Tylosin	3.06	0.5	20-800	0.991

Table 3. *LC-MS Retention times and calibration data*

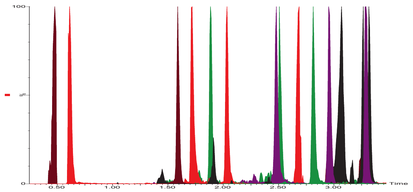

Figure 2. *Overlay of quantitative MRM chromatograms of 16 veterinary drugs separated in 6.20 minutes (at MRL level)*

RESULTS AND DISCUSSION

Oasis PRiME HLB Cartridge Pass-Through Cleanup

The Oasis PRiME HLB cartridge was evaluated with respect to analyte recovery and phospholipids removal from egg matrix. The *total* method recoveries ranged from 50-97%. However, the Oasis PRiME HLB cartridge cleanup contributes little to any method recovery losses. As shown in **Figure 3**, the measured recovery for the SPE cleanup step is better than 80 % for all compounds, with recovery for most compounds greater than 90%.

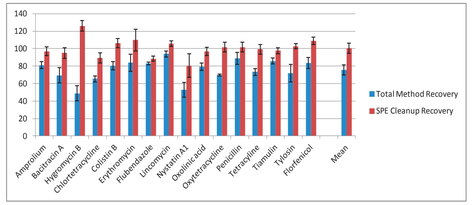

Figure 3. *Recovery data for target veterinary drugs obtained using the Oasis PRiME HLB cartridge clean-up procedure (at 1MRL level)*

Whole eggs contain significant amounts of fat and are among the highest sources of dietary lecithin (phospholipids). The total lipid content of chicken egg is about 11% by weight (excluding the shell) and the phospholipids content is about 0.35% [4]. Significant amounts of these potential interfering substances are extracted along with the target drugs in the initial sample preparation extraction step. Results obtained from gravimetric analysis show that greater than 84% of total lipids were removed from the egg extract after pass-through cleanup with the Oasis PRiME HLB cartridge. LC-MS analysis shows that the cleanup step was even more effective for removal of phospholipids. **Figure 4** shows that the Oasis PRiME HLB cartridge cleanup removed greater than 95% of phospholipids from the egg extract.

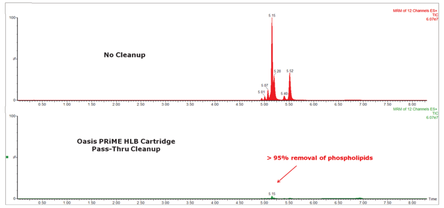

Figure 4. *Effective removal of phospholipids from egg extracts with Oasis PRiME HLB clean-up*

Accuracy and Precision

To evaluate the accuracy and repeatability of the whole method, recovery studies were carried out at three concentration levels (0.4MRL, 1MRL, 2MRL), six replicates per level. Matrix-matched standard calibration curves were used. **Figure 5** shows the results. We concluded that most target compounds show acceptable accuracy results (>70%) except for nystatin A1(<65%). The repeatability results are acceptable (RSD<20%) for all compounds except for hygromycin at 0.4MRL (RSD=34%).

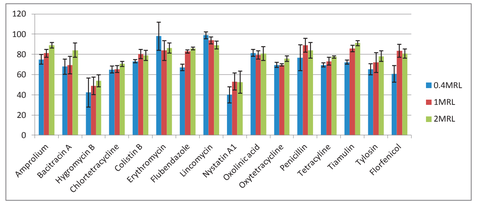

Figure 5. *Summary of recovery data (blank eggs samples spiked at 0.4MRL, 1MRL, 2MRL levels (Hygromycin B, Florfenicol, Penicillin G, Nystatin A1 have no corresponding MRLs, so they were studied at 40, 100, 200ppb levels)*

CONCLUSIONS

- **This analytical method meets the requirement for the simultaneous determination of several classes of veterinary drugs in eggs**
- **A simple pass-through cleanup procedure using Oasis PRiME HLB cartridge can remove more than 95% phospholipids from egg extracts**
- **The Oasis PRiME HLB cartridge cleanup procedure provided effective cleanup and good recoveries for the target veterinary drugs in egg**
- **The ACQUITY® UPLC I-Class coupled with Xevo® TQ-S MS offered good sensitivity for the veterinary drug residues in this study**

References

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