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 mainly used in chicken farms to control diseases of laying hens. However, these compounds could be transferred and accumulated in eggs. The presence of veterinary drugs in eggs has a potential health risk for the consumers , because they can provoke allergic reactions or induce pathogen resistance to antibiotics used in human medicine[1].

Seventeen representative veterinary drugs from twelve classes, which have their MRPLs established in USA, EU and/or China were chosen in this study[2][3]. Figure 1 presents the structures of a subset of the veterinary drugs studied.

The most difficult task for the determination of veterinary drugs in eggs is the sample treatment, because drugs may bind to the lipoproteins and have different physic-chemical properties, and also egg contains a high level of phospholipids and proteins. Therefore, two main objectives for sample preparation are the removal of phospholipids and proteins and simultaneous extraction of several classes of veterinary drugs. In this work, sample extraction, cleanup and analysis methods were developed for tandem LC/MS determination of a wide variety of veterinary drugs in eggs.

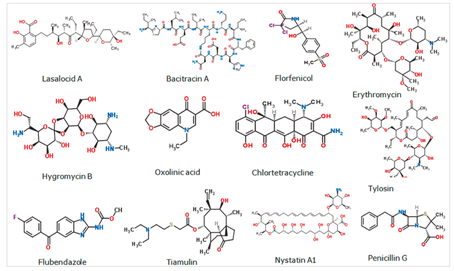

Figure1. *Structures of representative compounds from each class of drug studied*

METHODS

Standards and solutions

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

Individual veterinary drug stock solutions (1mg/mL; lasalocid A 100μg/mL; Flubendazole 50μg/mL) were prepared in methanol or water, except oxolinic acid was dissolved in 0.5M NaOH solution. A mixed stock was prepared by combing a certain amount of each individual stock solution. This mix stock was further diluted with 0.1% formic acid in 50% acetonitrile.

Table 1. *List of veterinary drugs in this study*

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	Not detected
Nystatin A1	C47H75NO17	925.503479	United States	Nystatin :Not detected
Lasalocid A	C34H54O8	590.381897	European Union	Lasalocid:150
Colistin B	C52H98N16O13	1154.749878	China	Colistin: 300
Florfenicol	C12H14Cl2FNO4S	357.000458	China	Not detected
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	Not detected
Tetracycline	C22H24N2O8	444.153259	China	200
Tylosin	C46H77NO17	915.519165	United States/China	200

Note:1. Bacitracin, colistin, lasalocid and nystatin all contain a mixture of more than two components. For each antibiotic we chose one major component to analyze in this study.

Sample preparation

This method was developed based on the previous method[4].

Extraction: 2.0g of homogenized whole 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.

SPE Cleanup: An Oasis PRiME HLB cartridge (3cc,60mg) was mounted on a precleaned vacuum manifold. Cartridge conditioning is not required. 1 mL of the supernatant was passed-through the cartridge and collected using ~1~2 psi vacuum. 0.5 mL elution solvent was taken and diluted two-fold 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 methanol
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 2.3min, 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

Table 2. *MRM Transition parameters for 17 veterinary drugs*

Compounds	MRM trasition 1				MRM trasition 2		
	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
Lasalocid A	613.57	377.40	40	30	577.50	82	32
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

RESULTS AND DISCUSSION

Evaluation of the Oasis PRiME HLB cartridge pass through step

The Oasis PRiME HLB cartridge was evaluated with respect to recovery and phospholipids removal effect using egg samples. To evaluate the cartridge recovery for selected veterinary drugs, two types of solutions were studied: the MRL level standards in pure solvent (0.2% formic acid in 80% acetonitrile) and in the blank egg extracts. Both solutions were divided into two parts, one part was pass-through the cartridge and diluted as the method described before (solution A) and another part was not pass-through the cartridge but also diluted (solution B). The recovery was calculated as follows: the compound response of solution A divided by the response of solution B multiplied by 100. **Figure 2** shows the clean-up procedure using the Oasis PRiME HLB cartridge provided high recoveries (>80%) for most of target compounds in egg extracts except for lasalocid A.

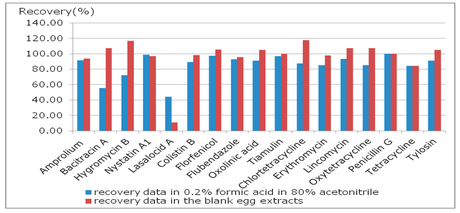

Figure 2. *Recovery data for target veterinary drugs obtained using the Oasis PRiME HLB cartridge clean-up procedure (at MRL level)*

In this study, 12 MRM channels were used for detection of phospholipids[4]. **Figure 3** shows the effectiveness of the Oasis PRiME HLB cartridge for removal of >95% of phospholipids from egg extracts.

Linearity and LOD

The calibration curve linearity was investigated using matrix-matched standard solutions. The LODs were also estimated during the linearity study. The results are presented in **Table 3** and the quantitative MRM chromatograms of 17 compounds are showed in **Figure 4**.

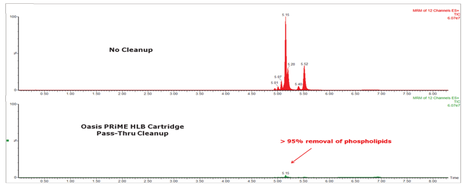

Figure 3. *The effect of removal of phospholipids from egg extracts after a clean-up step*

Table 3. *Linearity of matrix-matched calibration curves and LOD for 17 veterinary drugs in eggs*

Number	Compounds	RT (min)	LOD (ppb)	Linear Range (ppb)	R^2
1	Amprolium	0.57	0.5	80~ 40000	0.998
2	Bacitracin A	3.74	1	10~5000	0.992
3	Hygromycin B	0.46	4	4~1000	0.990
4	Nystatin A1	4.14	10	40~1000	0.992
5	Lasalocid A	5.76	3	3~600	0.992
6	Colistin B	3.86	30	90~600	0.990
7	Florfenicol	2.69	4	4~1000	0.991
8	Flubendazole	4.16	0.5	8~240	0.993
9	Oxolinic acid	3.58	1	1~500	0.993
10	Tiamulin	3.88	0.5	20~1000	0.990
11	Chlortetracycline	3.26	0.5	4~2000	0.995
12	Erythromycin	3.90	0.5	0.5~250	0.995
13	Lincomycin	1.88	0.5	1~500	0.996
14	Oxytetracycline	2.38	0.5	4~2000	0.995
15	Penicillin G	2.97	1	2~1000	0.991
16	Tetracycline	2.30	0.5	4~2000	0.994
17	Tylosin	3.88	0.5	20~800	0.991

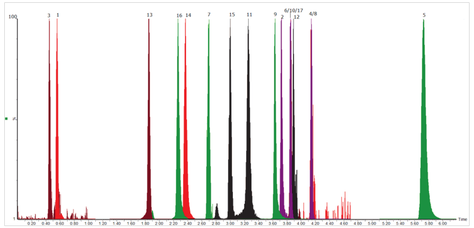

Figure 4. *Overlay of quantitative MRM chromatograms of 17 veterinary drugs separated in 6.20 minutes (at MRL level)*

Accuracy and Precision

To evaluate the accuracy and repeatability of the whole method, recovery studies were carried out at three concentration levels (0.4MRL, MRL, 2MRL) in six replicates. 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%) and lasalocid A (<10%). The repeatability results are acceptable(RSD<20%) for all compounds.

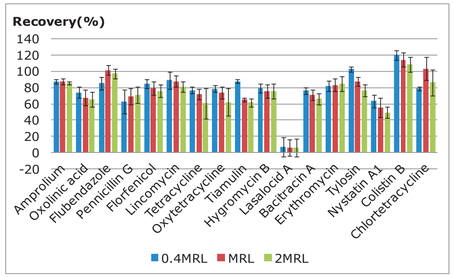

Figure 5. *Summary of recovery data (balnk eggs samples spiked at 0.4MRL, MRL, 2MRL levels)(a.Hygromycin B, Florfenicol, Penicillin G, Nystatin A1 have no corresponding MRLs, so they were studied at 40, 100, 200ppb levels.)*

CONCLUSION

- **This analytical method meets the requirement for the simultaneous determination of several classes of veterinary drugs in eggs.**
- **A simple one-step 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 offered excellent recoveries for the target veterinary drugs in eggs except for lasalocid A.**
- **ACQUITY® UPLC I-Class coupled Xevo® TQ-S MS offered good sensitivity for veterinary drug residues determination in complex matrix like eggs.**

References

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