Rapid screening 73 antibiotic drugs in animal feeds using ultra-performance liquid chromatography coupled to FT-Orbitrap high resolution mass spectrometry

Yu-an Sun, Qiuxia Zhang, Wei Ke, Zhenxing Li, Ke Wang, and Guoqing Wang

***Abstract*—**In this paper, we proposed a rapid screening method for the determination and confirmation of 73 antibiotic drugs in multiclass animal feeds, including 7 β—lactams, 3 lincosamides, 11 macrolides, 23 sulfonamides, 18 quinolones, 3 quinoxalines, 5 tetracyclines, and 3 amphenicols, using ultra-high performance liquid chromatography coupled to FT-Orbitrap high resolution mass spectrometry (UHPLC—Orbitrap HRMS). Feed samples were extracted with 6 mL water-acetonitrile (1:1, v/v), and then extracted with 4 mL acetonitrile. The extracts was purified by an -NH_2 cartridge, concentrated by N_2, and filtrated prior to analysis by C18 column. The method was validated in fortified samples, including pre-mixture, concentrates and compound feeds. The results show that the recoveries are in the range of 34.51%-129.64% with relative standard deviations (RSD) 0.1%-24.4%, and all of the drugs analyzed can be detected at 0.3-156.3 ng/g.

***Keywords*—**Antibiotics drugs, animal feeds, ultra-performance liquid chromatography, high resolution mass spectrometry.

I. Introduction

As feed additives, antibiotics are used mainly for three purposes, *i.e.*, therapeutic use to treat sick animals, prophylactic use to prevent infection in animals, and as growth promoters to improve feed utilization and production [1]. The frequent but regulated use of antibiotics may leave residues in edible tissues that can lead to an allergic reaction, disorder of intestinal flora or emergence of resistant strains of bacteria [2]. The majority of the recently developed methods for the analysis of antibiotics in feed are simultaneously analyses within a single class of compounds [3]-[10]. There are a few multiclass/multiresidue liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for the determination of antibiotics in various food matrices, such as meat, egg, milk, and honey, etc. [11]-[14]. It is obvious need for multiclass analytical methods for monitor of antibiotics in animal feeds.

Multiclass residue rapid screening methods require low selectivity and simple preparation procedure, no sample discrimination, high flux and high precision. However, there are problems lead by the strong ion suppression and the matrix interference. The high resolution mass spectrometry (HRMS) instruments, such as Orbitrap MS, which can operate in the full scan mode, theoretically, there is no limitations in number of monitored compounds [15], can provide accurate mass measurements. Since Orbitrap MS was first introduced in 2000 [16] and commercially used in 2005, it was incorporated in hybrid-MS systems and typically used in proteomics [17],[18], metabolomics [19],[20], drug monitor [21],[22], environmental monitor [23],[24], and food security [25],[26]. With the introduction of a single stage mass spectrometer, Orbitrap technology has become an attractive option for full scan screening in the field of food safety [27]. Gómez-Pérez *et al*. [28] created a database for the simultaneous analysis of more than 350 pesticides and veterinary drugs in honey using UHPLC–Orbitrap HRMS. Kaufmann *et al*. [29] validated the multi-residue methods for veterinary drugs or pesticides in food using TOF and Orbitrap MS, the results showed that because of the higher resolution and the superior mass stability, the method using Orbitrap instruments had significantly better performance parameters, e.g., linearity, reproducibility, and detection limits.

This study reported the development of an exact mass database for determination of 73 antibiotics drugs by UHPLC–Orbitrap HRMS, and it was applied to multiclass analysis of animal feeds using generic extraction and chromatographic conditions.

This work was supported by the National Natural Science Foundation of China (21075113).

Yu-an Sun is with School of Materials and Chemical Engineering, Zhengzhou University of Light Industry, Zhengzhou 450000, China (e-mail: sya@zzuli.edu.cn).

Qiuxia Zhang is with School of Food and Biological Engineering, Zhengzhou University of Light Industry, Zhengzhou 450000, China; School of Chemistry and Chemical Engineering, Pingdingshan University, Pingdingshan 467000, China (e-mail: zqx@pdsu.edu.cn).

Wei Ke is with China National Tobacco Quality Supervision and Test Center, Zhengzhou 450000, China (e-mail: yamei0926@163.com).

Zhenxing Li is with School of Materials and Chemical Engineering, Zhengzhou University of Light Industry, Zhengzhou 450000, China (e-mail: 279170556@qq.com).

Guoqing Wang is with School of Materials and Chemical Engineering, Zhengzhou University of Light Industry, Zhengzhou 450000, China (corresponding author to provide phone: +86-371-63556063; fax: +86-371-63556320; e-mail: gqwang@ zzuli.edu.cn).

II. Materials and methods

A. Reagents and chemicals

The following sources of antibiotics were used in this study:

β—lactams, lincosamides, macrolides, quinoxalines, tetracyclines, amphenicols (National institutes for food and drug control, China); sulfonamides and quinolones (Dr.Ehrenstorfer, Augsburg, Germany). HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from J.T. Baker (Deventer, Holland). Formic acid (ACS grade) was purchased from Amethyst Chemicals (J&K Scientific, Beijing). LC-MS water was generated in-house from a Milli-Q Advantage water system. For accurate mass calibration, a mixture of caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and Ultramark 1600 (ProteoMass LTQ/FT-Hybrid ESI positive mode calibration mix) from Sigma–Aldrich was used in the Orbitrap analyzer.

Oasis HLB, MCX and MAX (60 mg, 3 mL), Sep-Pak Alumina (500 mg, 3 mL) solid phase extraction (SPE) columns were obtained from Waters (Manchester, UK); Supeldean SCX (500 mg, 3 mL) and Supelclean ENVI-Carb (500 mg, 6 mL) SPE columns were obtained from Supelco (Bellefonte, PA); MEGA BE-NH2 cartridges (500 mg, 6 mL) were purchased from Aglient cooperation, USA.

B. Apparatus

A refrigerated centrifuge (Sigma 30K, Osterede am Harz, Germany), a vortexer (IKA MS 3 basic; IKA Werke GmbH& Co. KG, Staufen, Germany), a multitube vortexer (VX-III; Beijing Targin Technology Co. Ltd, China), a nitrogen evaporator (N-EVAP-112; Organomation Associates Inc., Berlin, MA), and the adjustable pipettes, range: 10~100 μL, 20~200 μL, 100~1000 μL, 500~5000 μL (Eppendorf Research, Hamburg, Germany) were also used in this study.

C. Preparation of Standard Stock Solutions

Between 9.5 and 10.5 mg of antibiotics in the same class were weighed into 10 mL volumetric flask. Macrolides, sulfonamides, quinolones and tetracyclines were filled up to the mark with MeOH, *β*—lactams, lincosamides and quinoxalines fill up to the mark with 50% MeOH (MeOH:H_2O,v/v), amphenicols were filled up to the mark with ACN. And then, approximately 1.5 mL aliquots were transferred into amber autosampler vials and stored below 0°C.

Working standard solutions were prepared on the day of analysis by successive dilutions of the mixed-standard stock solution using 50% ACN (ACN:H_2O, v/v).

D. Sample preparaion

2 g homogenized feed was weighed into a 35 mL centrifuge tube, added 6 mL 50% ACN (ACN: H2O=1:1,v/v), and vortexed 30 s. The mixture was shaken by a multitube vortexer for 10 min and centrifuged at 8000 r/min for 5 min. After centrifugation, the remaining supernatant was removed to a 10 mL centrifuge tube, and the residue was extracted by 4 mL ACN in the same steps. Then the supernatant were combined and loaded onto a MEGA BE-NH2 cartridge previously conditional with 6 mL ACN. The extraction passed through the cartridge was evaporated under a stream of nitrogen and redissolved with 1 mL of a mixture of ACN/ H_2O (50:50 v/v). Finally, 5 μL of the sample extract that filtered through a 0.2 μm Pall GHP Acrodisc filter (Pall Corporation, East Hill, NY, USA) were injected into the UPLC system.

E. Feed samples

Three different feed matrices (including pre-mixture, concentrates and compound feed) and 15 different commodities used in this work were supported by Henan Institute of Veterinary Drug Control. In these feed matrices used as blank samples during the optimization and validation of the method, the target compounds can not be detected.

F. UPLC-Qrbitrap MS analysis

For detection of multiclass veterinary drugs , an method developed by Gómez-Pérez *et al*. [28] was modified. The separation of the analytes was carried out using an Accela UPLC (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Hypersil GOLD Q C18 column (100 mm ×2.1 mm, 1.9 μm particle size) from Thermo (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase consisted of 0.1% (v/v) formic acid in water (eluent A) and ACN (eluent B). The analysis started with 98% of eluent A. After 0.5 min, this percentage was linearly decreased up to 5% in 8.5 min. This composition was held during 1.5 min and increased again up to 98% in 1.5 min, followed by a re-equilibration time of 3 min (total running time = 15.0 min). The flow rate was 0.45 mL/min and the column temperature was set at 20 ^{o}C.

The UHPLC system was coupled to a single stage Orbitrap mass spectrometer (ExactiveTM, Thermo Fisher Scientific, Bremen, Germany) operating with a heated electrospray interface (HESIII, Thermo Fisher Scientific, San Jose, CA, USA), in positive (ESI+) and negative ionization mode (ESI−) using the following operational parameters: spray voltage, 3.0 kV(−2.5 kV in ESI−); skimmer voltage, 20 V (−20 V in ESI−); capillary voltage, 60 V (−60 V in ESI−); tube lens voltage, 120 V (−120 V in ESI−); sheath gas (N2, >95%), 35 (a dimensional); auxiliary gas (N2, >95%), 10 (a dimensional); heater temperature, 300 ^{o}C; and capillary temperature, 350 ^{o}C.

The mass spectra were acquired using two independent acquisition functions: (1) full MS, ESI+, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power is 25,000 FWHM; scan time is 0.25 s; (2) full MS, ESI− using the aforementioned settings. All the analyses were performed without lock mass. Mass accuracy was carefully monitored as follows: checked everyday with multi-compound standards; evaluated (once a week) and calibrated when necessary (every two weeks at least) with mass accuracy standards. External calibration mode was adopted.

All data were processed using XcaliburTM version 2.2.1 (Thermo Fisher Scientific, Les Ulis, France) with Qual and Quanbrowser. Genesis peak detection was applied. ToxIDTM 2.1.1 (automated compound screening software, Thermo Scientific) was used for screening and LCQuanTM 2.6 software (Thermo Scientific) was used for quantification during method validation and sample analysis.

III. RESULTS AND DISCUSSION

A. Database development

A good UPLC-MS method can separate the isomer drugs and less affected by matrix effects. To verify this, a comparative study was provided. The standard solutions were mixed by ACN/H_2O (50:50 v/v) to 1 μg/mL, and then injected in the system with the extracted ion chromatogram of m/z at 311.07990 shown as Fig.1a. It shows that sulfadimoxine and sulfadimethoxine can be separated completely. Every individual class standard solution was mixed by blank matrix extraction to 1 μg/mL and then injected in the system. Compared to the extracted ion chromatogram in Fig.1, it can be seen that the response value and retention time were affected less by matrix effects. Therefore, retention time obtained by detected by the standard solutions in this method can be used to recognized target drugs.

The isotopic pattern was used to investigate the matrix effects on mass spectrometry analysis. Clindamycin that has characteristic atoms Cl and S was selected. Comparation of the blank matrix sample added with mixed standards, mixed standard solution and theoretical isotopic mass was shown in Table 1. It can be seen that the single Orbitrap mass analyzer

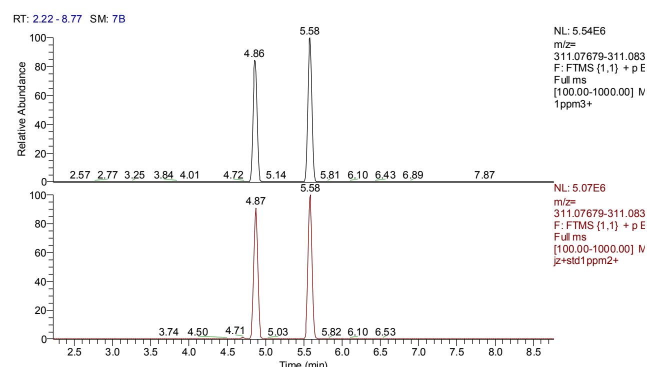

Fig. 1 Extracted ion chromatogram of m/z at 311.07990: (a) standard solution; (b) standard solution was mixed by blank matrix extraction. RT 4.86 min is sulfadimoxine and RT 5.58 min is sulfadimethoxine.

can identify the target compounds.

The information used to build the database such as retention time (RT), ionization mode (including polarity), characteristic ions and possible adducts with Na^+ or H_3O^+ can be obtained by the UHPLC-MS system. Furthermore, the automatic screening method was developed by ToxID software. The information of the database was shown in Table 2.

Table 1 Comparing of isotopic pattern

Sample type	Isotopic patterns（m/z）			Relative abundance		
Theoretical	425.18715	426.19050	427.18420	100.00	19.47	31.96
Standard	425.18706	426.19055	427.18403	100.00	19.57	36.52
Matrix added standard	425.18701	426.19050	427.18395	100.00	19.59	36.18

Table 2 The information of the database

Compound Name	Formula	$[M+H]^+$ Theoretical	Detected (m/z)	Delta (ppm)	RT
Benzylpenicillin G	$C_{16}H_{18}N_2O_4S$	335.10600	335.10516	-2.51	5.48
Ampicillin	$C_{16}H_{19}N_3O_4S$	350.11690	350.11658	-0.93	3.67
Oxacillin	$C_{19}H_{19}N_3O_5S$	402.11182	402.11148	-0.84	6.02
Flucloxacillin	$C_{19}H_{17}ClFN_3O_5S$	454.06342	454.06302	-0.89	6.48
Cephalexin	$C_{16}H_{17}N_3O_4S$	348.10125	348.10089	-1.04	3.72
Cefradine	$C_{16}H_{19}N_3O_4S$	350.11690	350.11658	-0.93	3.90
Ceftiofur	$C_{19}H_{17}N_5O_7S_3$	524.03629	524.03564	-1.22	4.93
Lincomycin	$C_{18}H_{34}N_2O_6S$	407.22103	407.22028	-1.86	3.45
Clindamycin	$C_{18}H_{33}ClN_2O_5S$	425.18715	425.18665	-1.18	4.56
Pirlimycin	$C_{17}H_{31}ClN_2O_5S$	411.17150	411.17102	-1.16	4.16
Tiamulin	$C_{28}H_{47}NO_4S$	494.32985	494.32830	-1.55	5.76
Tylosin	$C_{46}H_{77}NO_{17}$	916.52643	916.52478	-1.80	5.22
Tilmicosin	$C_{46}H_{80}N_2O_{13}$	869.57332	869.57208	-1.42	4.68
Azithromycin	$C_{38}H_{72}N_2O_{12}$	749.51580	749.51447	-1.78	4.31
Clarithromycin	$C_{38}H_{69}NO_{13}$	748.48417	748.48230	-2.50	5.68
Erythromycin	$C_{37}H_{67}NO_{13}$	734.46852	734.46692	-2.18	5.08
Roxithromycin	$C_{41}H_{76}N_2O_{15}$	837.53185	837.52966	-2.61	5.76
Josamycin	$C_{42}H_{69}NO_{15}$	828.47400	828.47357	-0.51	6.12
Natamycin	$C_{33}H_{47}NO_{13}$	666.31202	666.31158	-0.65	5.25
Spiramycin I	$C_{43}H_{74}N_2O_{14}$	843.52128	843.51770	-4.24	5.76

ISBN: 978-1-61804-284-2

Table 2 *Continued.*

Compound Name	Formula	$[M+H]^+$ Theoretical	Detected (m/z)	Delta (ppm)	RT
Kitasamycin A5	$C_{39}H_{65}NO_{14}$	772.44778	772.44666	-1.46	5.64
Sulfamonomethoxine	$C_{11}H_{12}N_4O_3S$	281.07029	281.06979	-1.76	4.54
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	254.05939	254.05907	-1.27	4.84
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	311.08085	311.08038	-1.51	5.29
Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	301.07537	301.07501	-1.20	5.25
Sulfadimidine	$C_{12}H_{14}N_4O_2S$	279.09102	279.09079	-0.84	4.09
Sulfachlorpyridazine	$C_{10}H_9ClN_4O_2S$	285.02075	285.02039	-1.28	4.69
Sulfapyridine	$C_{11}H_{11}N_3O_2S$	250.06447	250.06425	-0.88	3.69
Sulfaguanidine	$C_7H_{10}N_4O_2S$	215.05972	215.05949	-1.06	1.70
Sulfamoxol	$C_{11}H_{13}N_3O_3S$	268.07504	268.07468	-1.35	4.05
Sulfamethizole	$C_9H_{10}N_4O_2S_2$	271.03179	271.03140	-1.44	4.18
Sulfisomidine	$C_{12}H_{14}N_4O_2S$	279.09102	279.09079	-0.84	3.4
Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$	281.07029	281.06979	-1.76	4.33
Sulfachloropyrazine	$C_{10}H_9ClN_4O_2S$	285.02075	285.02039	-1.28	5.30
Sulfadimoxine	$C_{12}H_{14}N_4O_4S$	311.07990	311.08038	-1.51	4.75
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	251.05972	251.05940	-1.28	3.49
Sulfacetamide	$C_8H_{10}N_2O_3S$	215.04849	215.04825	-1.12	3.31
Sulfamerazine	$C_{11}H_{12}N_4O_2S$	265.07537	265.07465	-2.72	4.15
Sulfafurazole	$C_{11}H_{13}N_3O_3S$	268.07504	268.07468	-1.35	5.04
Sulfanitran	$C_{14}H_{13}N_3O_5S$	336.06487	336.06451	-1.05	5.93
Sulfametoxydiazine	$C_{11}H_{12}N_4O_3S$	281.07029	281.06979	-1.76	4.27
Sulfathiazole	$C_9H_9N_3O_2S_2$	256.02089	256.02057	-1.27	3.70
Trimethoprim	$C_{14}H_{18}N_4O_3$	291.14517	291.14468	-1.66	3.77
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	360.17180	360.17133	-1.31	4.01
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	332.14050	332.14005	-1.36	3.86
Sarafloxacin	$C_{20}H_{17}F_2N_3O_3$	386.13107	386.13031	-1.98	4.29
Danofloxacin	$C_{19}H_{20}FN_3O_3$	358.15615	358.15509	-2.95	5.16
Difloxacin	$C_{21}H_{19}F_2N_3O_3$	400.14672	400.14594	-1.97	4.34
Nadifloxacin	$C_{19}H_{21}FN_2O_4$	361.15581	361.15527	-1.49	5.48
Sparfloxacin	$C_{19}H_{22}F_2N_4O_3$	393.17327	393.17233	-2.39	4.28
Norfloxacin	$C_{16}H_{18}FN_3O_3$	320.14050	320.14001	-1.50	3.81
Oxilinic acid	$C_{13}H_{11}NO_5$	262.07100	262.07074	-0.99	4.98
Pefloxacin	$C_{17}H_{20}FN_3O_3$	334.15615	334.15570	-1.33	3.84
Marbofloxacin	$C_{17}H_{19}FN_4O_4$	363.14631	363.14518	-1.14	3.68
Orbifloxacin	$C_{19}H_{20}F_3N_3O_3$	396.15295	396.15157	-1.40	4.03
Flumequine	$C_{14}H_{12}FNO_3$	262.08740	262.07021	1.27	5.04
Nalidixic acid	$C_{12}H_{12}N_2O_3$	233.09207	233.09175	-1.36	5.66
Cinoxacin	$C_{12}H_{10}N_2O_5$	263.06625	263.06552	-2.77	4.69
Lomefloxacin	$C_{17}H_{19}F_2N_3O_3$	352.14672	352.14621	-1.46	3.93
Ofloxacin	$C_{18}H_{20}FN_3O_4$	362.15106	362.15045	-1.68	3.82
Fleroxacin	$C_{17}H_{18}F_3N_3O_3$	370.13730	370.13618	-3.03	3.78
Mequindox	$C_{11}H_{10}N_2O_3$	219.07642	219.07617	-1.13	4.00
Olaquindox	$C_{12}H_{13}N_3O_4$	264.09788	264.09750	-1.43	3.01
Quinocetone	$C_{18}H_{14}N_2O_3$	307.10772	307.10690	-2.73	6.01
Oxytetracycline	$C_{22}H_{24}N_2O_9$	461.15546	461.15485	-1.32	3.83

ISBN: 978-1-61804-284-2

Table 2 *Continued.*

Compound Name	Formula	$[M+H]^+$ Theoretical	Detected (m/z)	Delta (ppm)	RT
Tetracycline	$C_{22}H_{24}N_2O_8$	445.16054	445.16003	-1.14	4.02
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	479.12157	479.12106	-1.06	4.18
Doxycycline	$C_{22}H_{24}N_2O_8$	445.16054	445.16003	-1.14	4.78
Metacycline	$C_{22}H_{22}N_2O_8$	443.14489	443.14417	-1.64	4.65
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	321.00395$[M-H]^-$	321.00534	0.90	4.90
Thiamphenicol	$C_{12}H_{15}Cl_2NO_5S$	353.99643$[M-H]^-$	353.99786	0.97	3.87
Florfenicol	$C_{12}H_{14}Cl_2FNO_4S$	355.99209$[M-H]^-$	355.99472	3.45	4.75

B. Extraction procedure optimization

For a screening method, the generic extraction method that allowed the determination of multiclass compounds needed to establish at first. Depending on the nature of the feed sample and the target compounds, the extraction solution was selected according to the "Like Dissolves Like" principle. Hydrophilic solvents such as MeOH, ACN and H_2O were tested for the extraction procedure, and the hydrophilic solvents had three advantages:1) feed samples mixed easily and extracted acceleratedly; 2) can effectively reduce the loss of adsorption of polar analytes; 3) can deproteinized and defatted. Method A: 6 mL MeOH/H_2O (50:50, v/v) extracted and 4 mL MeOH secondary extracted. Method B: ACN was used to extract like method A. The recoveries of method A and B were compared as shown as Fig. 2. It can be seen that program B shows batter recoveries of 73 drugs.

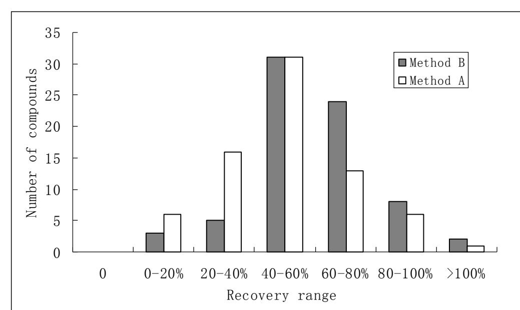

Fig. 2 Recovery results at different extraction method

C. Purification procedure optimization

In order to establish a generic purification method, two different program based on SPE were used to study with the workflows shown as Fig. 3. The blank matrix sample added concentration 1 μg/g was used in the two different purification programs to compare the recoveries.

In program A, four type cartridges were used to compare, such as SCX, MAX, MCX, and HLB. The recovery profile was shown in Fig. 4. The results of recoveries show that different cartridges have different selectivity. For example, after purified by SCX cartridge, the recoveries of macrolides are in range of 64% to 92%, but sulfonamides are not retained. Therefore, this purification method can't meet the requirement for multiclass drugs analysis.

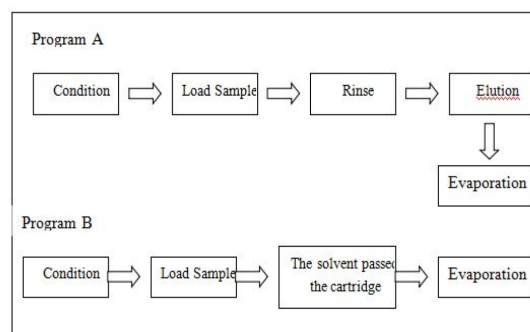

Fig. 3 Workflow of purification SPE programs

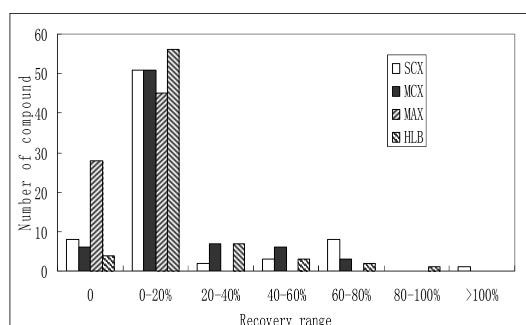

Fig. 4 Recovery results of program A

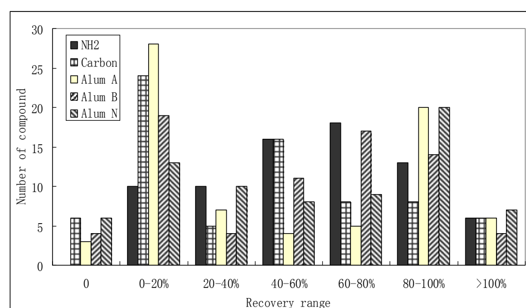

Fig. 5 Recovery results of program B

In program B, five type cartridges were used to compare, such as Alumina cartridges (Neutral, Acidic and Alkaline), NH_2 and Carbon cartridges. The recovery profile was shown in Fig. 5. The experimental results show that, for Alumina cartridges, the recoveries of tetracyclines and quinolones are extremely low; and for Carbon cartridges, the recoveries of quinolones are poor. However, for NH_2 cartridges, the recoveries of 73 drugs are

satisfying. Therefore NH_2 cartridges can be used to establish multiclass antibiotics analysis.

D. Method validation

The validation procedure for the developed method was carried out in order to ensure the adequate identification and quantification of the target compounds. Several parameters such as linearity, intra-day precision and limits of detection (LODs) were studied.

Three different blank feed matrices, including pre-mixture, concentrates, and compound feed, were added equal volumes of different concentrations of mixed standard solution with added in an amount of 100, 250, 500 ng/g feed, and then carried out according to the preparation method. The concentration of inject into the UPLC-MS were 40, 100, 200 ng/mL. LODs for the 73 antibacterials were calculated as ten times signal to noise ratio, respectively. The results were shown in Table 3. It can be seen that this proved method can meet the requirement of multiclass screening of animal feed.

Intra-day precision, or within-day reproducibility, is expressed as the average of the relative standard deviation (RSD%) of the areas obtained for each analyte after the replicate (n=6) analysis, and three different blank feed matrices fortified at the level (2.5 μg/g) with all 73 analytes during the same day. The results were shown in Table 3. The recoveries of different class feed prove that the combined capability of different component feeds and different drugs is obvious distinction.

For internal standard based quantification, six point calibration curves, were constructed using least-squares linear regression analysis based on peak areas, from application of the overall method to blank matrix extraction containing the standard antibiotic mixture at concentrations ranging from 10 to 1000 ng/mL. The data proved that validation linearity range of macrolides, tetracyclines, β—lactams, quinoxalines is 50-1000 ng/mL, and sulfonamides, quinolones, lincosamides, amphenicols are 10-1000 ng/mL. The correlation coefficients (r^2) were shown in Table 3.

Table 3 Summary of validation results (part of drugs)

Compound Name	Correlation Coefficient (r^2)	Recovery/%(n=6)			RSD/%(n=3)			LOD (ng/g)		
		A	B	C	A	B	C	A	B	C
Cephalexin	0.9946	14.07	38.20	30.62	5.6	16.6	22.0	6.0	9.8	17.5
Oxacillin	0.9981	45.68	71.10	24.28	1.7	12.3	5.5	10.9	18.5	26.3
Flucloxacillin	0.9980	48.04	52.90	19.36	5.5	11.0	10.4	131.6	156.3	119.0
Cefradine	0.9992	46.93	59.17	50.30	3.2	7.9	7.6	8.4	6.4	8.7
Lincomycin	0.9966	75.64	127.69	108.52	7.4	4.2	7.2	2.3	1.5	9.2
Clindamycin	0.9980	58.83	75.46	75.76	8.8	2.6	8.1	2.1	1.2	1.3
Tylosin	0.9999	50.50	50.84	35.77	4.5	5.1	5.6	6.1	7.9	11.5
Tiamulin	0.9989	69.64	65.31	59.29	6.1	5.4	34.4	0.5	0.8	0.9
Tilmicosin	0.9993	111.48	125.81	94.05	4.5	12.0	12.7	5.5	5.8	3.8
Azithromycin	0.9857	109.09	114.17	98.44	6.2	4.0	8.6	2.4	1.8	3.6
Erythromycin	0.9996	65.78	66.82	50.61	0.3	5.1	10.4	0.4	1.3	0.7
Natamycin	0.9995	20.54	27.18	29.30	4.1	10.0	21.7	23.8	55.6	13.9
Sulfamonomethoxine	0.9994	54.47	74.90	68.33	6.7	3.5	12.4	0.7	0.7	0.8
Sulfamethoxazole	0.9996	49.34	70.14	74.62	5.3	8.0	15.7	1.5	1.2	0.9
Sulfadimethoxine	0.9980	73.76	76.06	67.62	4.5	9.4	10.2	1.4	1.2	1.5
Sulfaquinoxaline	0.9992	56.66	72.84	70.24	5.9	4.9	10.5	1.0	0.7	1.0
Sulfachlorpyridazine	0.9991	49.13	63.42	72.82	10.2	12.5	15.5	5.0	4.2	4.7
Sulfapyridine	0.9980	48.03	61.05	62.96	7.2	7.6	2.3	0.4	0.3	1.5
Sulfamoxol	0.9997	70.12	75.81	24.06	1.8	3.8	10.0	0.9	1.0	5.0
Sulfamethizole	0.9996	48.30	69.34	65.28	5.0	6.0	10.6	2.0	3.0	2.2
Sulfadimoxine	0.9968	81.63	70.59	57.55	3.3	6.7	12.9	1.5	1.0	1.5
Enrofloxacin	0.9962	24.48	36.14	19.15	3.2	5.9	13.2	14.7	3.0	3.8
Ciprofloxacin	0.9986	8.84	25.44	11.22	5.3	14.0	24.4	14.3	4.3	90.9

ISBN: 978-1-61804-284-2

Table 3 *Continued.*

Compound Name	Correlation Coefficient (r^2)	Recovery/%(n=6)			RSD/%(n=3)			LOD (ng/g)		
		A[a]	B[b]	C[c]	A	B	C	A	B	C
Sarafloxacin	0.9983	29.53	34.51	14.27	4.2	5.7	19.7	3.1	5.1	5.5
Difloxacin	0.9993	42.56	51.62	18.60	2.9	1.7	15.7	90.9	8.5	10.4
Nadifloxacin	0.9993	61.54	0.85	57.10	16.1	7.3	3.5	2.5	78.1	2.2
Pefloxacin	0.9998	13.80	23.20	23.47	4.9	3.0	23.7	6.0	8.5	76.9
Mequindox	0.9960	68.96	82.29	78.44	5.8	3.5	5.7	3.0	11.0	32.3
Olaquindox	0.9982	73.66	117.40	73.46	11.5	10.2	17.5	4.7	2.5	2.7
Quinocetone	0.9880	57.38	71.60	51.89	0.1	4.6	9.2	2.0	1.8	2.1
Oxytetracycline	0.9894	2.68	10.03	0.34	14.5	7.4	/[d]	11.6	35.7	27.0
Tetracycline	0.9978	10.66	29.32	0.97	14.7	12.4	/	14.1	2.4	45.5
Chlortetracycline	0.9822	6.01	99.22	0.04	15.0	19.1	/	58.5	1.3	/
Doxycycline	0.9964	11.97	27.22	2.23	5.9	4.9	17.3	29.4	8.6	47.2
Chloramphenicol	0.9864	87.55	96.82	88.89	9.9	11.5	19.1	0.7	5.2	0.8
Thiamphenicol	0.9929	100.76	99.04	129.64	11.0	10.5	9.5	2.5	3.3	1.2
Florfenicol	0.9971	98.99	99.23	129.08	6.2	11.1	8.1	2.8	8.3	2.1

[a] Concentrate feed; [b] Compound feed; [c] Pre-mixture feed; [d] not detected.

E. Sample analysis

The method applicability was assessed by analyzing 15 feed commodities, including 5 pre-mixtures, 5 concentrates, and 5 compound feeds. Chlortetracycline was detected in one pig compound feed at 8.5 µg/g feed.

The extracted ion chromatogram was shown in Fig. 6. It can be seen the peak width of the standard is less than that the real feed sample, and the real sample can be identified by the retention time.

The isotopic abundance was shown in Table 4. It is noteworthy that the results obtained by using ToxID automated screening have some false positives. Therefore, the manual confirmation is necessary.

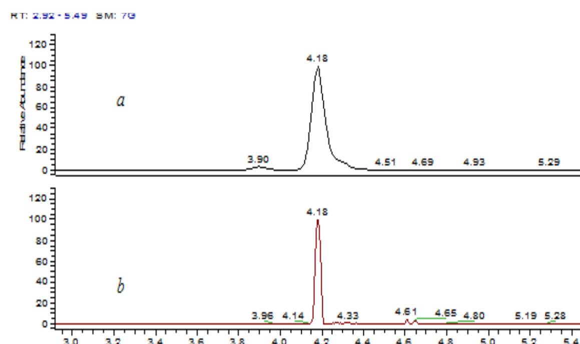

Fig. 6 Extraction ion chromatograms of real feed sample (a) and standard solution (b)

Table 4 Comparing of isotopic pattern about chlortetracycline in a real feed sample

Sample type	Isotopic patterns（m/z）			Relative abundance		
Theoretical	479.12157	480.12492	481.11862	100.00	23.79	31.96
Standard	479.12096	480.12450	481.11767	100.00	24.78	30.60
Matrix added standard	479.12071	480.12432	481.11771	100.00	25.81	29.86

The proposed method was compared to that of reference [12] and [15]. It shows that the recovery, RSD, LOD of the proposed method are comparable to the reported results with automated screening based on the developed database.

IV. Conclusion

A multiclass analytical method was developed for determination of 73 antibacterials with 8 families in feed by UPLC–Orbitrap-MS. The generic methods of instrumental analysis and pretreatment can facilitate the determination of a wide range of compounds. And the database including retention time, characteristic ions and accurate mass information of the target compounds can be used to automate screening of the analytes through ToxID. The proposed method is practical with the recoveries 34.51%-129.64%, RSD 0.1%-24.4% and LODs 0.3-156.3 ng/g.

ISBN: 978-1-61804-284-2

ACKNOWLEDGMENT

The authors are grateful to the experts from Henan Institute of Veterinary Drug Control of China for their assistance of providing some antibiotics drugs standards.

REFERENCES

[1] M.D. Barton MD, "Antibiotic use in animal feed and its impact on human health," *Nutr. Res. Rev.,* vol. 13, no. 2, pp. 279-300, Feb. 2000.

[2] M. Gaugain-Juhel, B. Delépine, S. Gautier, M-P Fourmond, V. Gaudin, D. Hurtaud-Pessel, E. Verdon, and P. Sanders, "Validation of a liquid chromatography-tandem mass spectrometry screening method to monitor 58 antibiotics in milk: a qualitative approach," *Food Add. Contam.,* vol. 26, no. 11, pp. 1459-1471, Nov. 2009.

[3] L. Kantiani, M. Farré, and D. Barceló, "Development and validation of a pressurised liquid extraction liquid chromatography–electrospray–tandem mass spectrometry method for β-lactams and sulfonamides in animal feed," *J. Chromat. A,* vol. 1217, no. 26, pp. 4247-4254, Mar. 2010.

[4] I. Pecorelli, R. Galarini, R. Bibi, A. I. Floridi, E. Casciarri, and A. Floridi, "Simultaneous determination of 13 quinolones from feeds using accelerated solvent extraction and liquid chromatography," *Anal. Chim. Acta,* vol. 483, no. 1, pp. 81-89, Apr. 2003.

[5] S. Croubels, P. Wassink, and P. DeBacker, "Simultaneous determination of sulfadiazine and trimethoprim in animal feed by liquid chromatography with UV and tandem mass spectrometric detection," *Anal. Chim. Acta,* vol. 473, no. 1, pp. 183-194, Aug. 2002.

[6] C. VanPoucke, K. DeKeyser, A. Baltusnikiene, J. McEvoy, and C. VanPeteghem, "Liquid chromatographic–tandem mass spectrometric detection of banned antibacterial growth promoters in animal feed," *Anal. Chim. Acta,* vol. 483, no. 1, pp. 99-109, May 2003.

[7] V. Jimenez, J. Adrian, J. Guiteras, M-P Marco, and R. Companyo, "Validation of an enzyme-linked immunosorbent assay for detecting sulfonamides in feed resources," *J. Agr. Food Chem.,* vol. 58, no. 13, pp. 7526-7531, Jun. 2010.

[8] L. Kantiani, M. Farré, J. M. G. Freixiedas, and D. Barceló, "Determination of antibacterials in animal feed by pressurized liquid extraction followed by online purification and liquid chromatography-electrospray tandem mass spectrometry," *Anal. Bioanal. Chem.*, vol.398, no. 3, pp. 1195-1205, Mar. 2010.

[9] R. Liu, P. He, Z. Li, and R. Li R, "Simultaneous Determination of 16 Sulfonamides in Animal Feeds by UHPLC-MS-MS," *J. Chromat. Sci.*, vol.49, no. 8, pp. 640-646, Aug. 2011.

[10] T. MingChih, and S. BorLing , "Determination of tetracyclines in feed, chicken blood, muscle and liver by liquid chromatography tandem mass spectrometry," *J. Taiwan Livestock Res.*, vol. 42, no. 4, pp. 351-360, Apr. 2009.

[11] R. Peters, Y. Bolck, P. Rutgers, A. Stolker, and M. Nielen, "Multi-residue screening of veterinary drugs in egg, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry," *J Chromat. A,* vol. 1216, no. 46, pp. 8206-8216, Oct. 2009.

[12] M. I. Lopez, J. S. Pettis, I. B. Smith, and P-S Chu, "Multiclass determination and confirmation of antibiotic residues in honey using LC-MS/MS," *J. Agr. Food Chem.*, vol. 56, no. 5, pp. 1553-1559, May 2009.

[13] C. Chiaochan, U. Koesukwiwat, S. Yudthavorasit, and N. Leepipatpiboon, "Efficient hydrophilic interaction liquid chromatography–tandem mass spectrometry for the multiclass analysis of veterinary drugs in chicken muscle," *Anal. Chim. Acta*, vol. 682, no. 1, pp. 117-129, Aug. 2010.

[14] A. Stolker, P. Rutgers, E. Oosterink, J. Lasaroms, R. Peters, J. Van Rhijn, and M. Nielen, "Comprehensive screening and quantification of veterinary drugs in milk using UPLC-ToF-MS," *Anal. Bioanal. Chem.*, vol. 391, no. 6, pp. 2309-2322, Mar. 2008.

[15] E. Van der Heeft, Y. Bolck, B. Beumer, A. Nijrolder, A. Stolker, and M. Nielen M, "Full-scan accurate mass selectivity of ultra-performance liquid chromatography combined with time-of-flight and orbitrap mass spectrometry in hormone and veterinary drug residue analysis," *J. Am. Soc. Mass Spectr.*, vol. 20, no. 3, pp. 451-463, Feb. 2009.

[16] A. Makarov, "Electrostatic axially harmonic orbital trapping: a high-performance technique of mass analysis," *Anal. Chem.*, vol. 72, no. 6, pp. 1156-1162, Mar. 2000.

[17] Y. Luo, T. Li, F. Yu, T. Kramer, and I. M. Cristea, "Resolving the composition of protein complexes using a MALDI LTQ Orbitrap," *J. Am. Soc. Mass Spectr.*, vol. 21, no. 1, pp. 34-46, Jan. 2010.

[18] S. A. Müller, T. Kohajda, S. Findeiß, P. F. Stadler, S. Washietl, M. Kellis, M. vonBergen, and S. Kalkhof S, "Optimization of parameters for coverage of low molecular weight proteins," *Anal. Bioanal. Chemistry*, vol. 398, no. 7-8, pp. 2867-2881, Apr. 2010.

[19] L. Van Meulebroek, J. V. Bussche, K. Steppe, and L. Vanhaecke, "Ultra-high performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry for metabolomic profiling of the endogenous phytohormonal status of the tomato plant," *J. Chromat. A*, vol. 1260, no. 19, pp. 67-80, Oct. 2012.

[20] L. MacIntyre, L. Zheng, P. Scullion, P. Keating, and D. G. Watson, "Metabolomic profiling of biomarkers of liver X receptor-induced toxicity in mouse liver tissue," *Metabolomics*, vol. 7, no. 1, pp. 54-70, Apr. 2011.

[21] E. Viryus, T. Sobolevskii, and G. Rodchenkov, "Detection of oxandrolone and its metabolite in urine by high-performance liquid chromatography-high-resolution mass spectrometry with atmospheric pressure chemical ionization and orbitrap detection after ceasing drug administration," *J. Anal. Chem.*, vol. 64, no. 1pp. 31-35, Jan. 2009.

[22] A. Thomas, H. Geyer, W. Schänzer, C. Crone, M. Kellmann, T. Moehring, and M. Thevis, "Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer," *Anal. Bioanal. Chem.*, vol. 403, no. 5, pp. 1279-1289, Mar. 2012.

[23] A. Hogenboom, J. Van Leerdam, and P. de Voogt, "Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography–hybrid linear ion trap Orbitrap mass spectrometry," *J. Chromat. A*, vol. 1216, no. 3, pp. 510-519, Jan. 2009.

[24] J. A. van Leerdam, A. C. Hogenboom, M. M. van der Kooi, and P. de Voogt, "Determination of polar 1H-benzotriazoles and benzothiazoles in water by solid-phase extraction and liquid chromatography LTQ FT Orbitrap mass spectrometry," *Int. J. Mass Spectr.*, vol. 282, no. 3, pp. 99-107, Mar. 2009.

[25] M. Nielen, M. Van Engelen, R. Zuiderent, and R. Ramaker, "Screening and confirmation criteria for hormone residue analysis using liquid chromatography accurate mass time-of-flight, Fourier transform ion cyclotron resonance and orbitrap mass spectrometry techniques," *Anal. Chim. Acta*, vol. 586, no. 1, Nov. 122-129, Spt. 2007.

[26] S. E. Edison, A. Lin, and L. Parrales, "Practical considerations for the rapid screening for pesticides using ambient pressure desorption ionisation with high-resolution mass spectrometry," *Food Add. Contam. A*, vol. 28, no. 10, pp.1393-1404, Oct. 2011.

[27] M. Kellmann, H. Muenster, P. Zomer, and H. Mol, "Full scan MS in comprehensive qualitative and quantitative residue analysis in food and feed matrices: how much resolving power is required?" *J. Am. Soc. Mass Spectr.*, vol. 20, no. 8, pp. 1464-1476, Aug. 2009.

[28] M. L. Gómez-Pérez, P. Plaza-Bolaños, R. Romero-González, J. L. Martínez-Vidal, and A. Garrido-Frenich, "Comprehensive qualitative and quantitative determination of pesticides and veterinary drugs in honey using liquid chromatography-Orbitrap high resolution mass spectrometry," *J. Chromat. A*, vol. 1248, no. 27, pp. 130-138, Jul. 2012.

[29] A. Kaufmann, P. Butcher, K. Maden, S. Walker, and M. Widmer, "Development of an improved high resolution mass spectrometry based multi-residue method for veterinary drugs in various food matrices," *Anal. Chim. Acta*, vol. 700, no. 1-2, pp. 86-94, Aug. 2011.