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Multi-class confirmatory method for analyzing trace levels of tetracyline and quinolone antibiotics in pig tissues by ultra-performance liquid chromatography coupled with tandem mass spectrometry

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An ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC/MS/MS) method was developed to screen and confirm multi-class veterinary drug residues in pig tissues including pig kidney, liver and meat. Twenty-one drugs of two different classes including seven tetracyclines and four types of quinolones (quinoline, naphthyridine, pyridopyrimidine and cinoline) were determined simultaneously in a single run. The homogenized sample tissues were extracted with EDTA–McIlvaine buffer solution and further purified using a polymer-based Oasis HLB solid-phase extraction (SPE) cartridge. An ACQUITY UPLC^{TM} BEH C_{18} column was used to separate the analytes followed by tandem mass spectrometry using an electrospray ionization source. MS data acquisition was performed in the positive ion multiple reaction monitoring mode, selecting two ion transitions for each target compound. Recovery studies were performed at different fortification levels. The overall average recoveries from pig muscle, kidney, and liver fortified with quinolones and tetracyclines at three levels ranged from 80.2 to 117.8% based on the use of matrix-fortified calibration with the coefficients of variation ranging from 2.1 to 17.8% (n = 6). The limits of quantitation (LOQs) of quinolones and tetracyclines in different tissues ranged from 0.03–4.50 μg/kg and 0.16–10.00 μg/kg, respectively. The effects of the extraction solvent, SPE cartridge, elution solvent and sample matrix on the analyte recovery as well as the effects of the mobile phase composition and column temperature on the chromatographic behavior were also studied. Copyright © 2007 John Wiley & Sons, Ltd.

In modern animal husbandry, animals are reared in restricted accommodations, which inevitably increase the incidence and spread of diseases. Hence, there has been an increasing need for therapeutic agents to safeguard animal welfare and the economic benefits of animal husbandry. Tetracycline and quinolone antibiotics are the two classes of veterinary drugs that are widely used for the prevention or treatment of bacterial infections in various animal species. However, abuse from failure to adhere to prescribed dosages and withdrawal periods may present potential health risks[1–3] and a threat to the eco-environment.[4–6] It is estimated that the veterinary usage of quinolones is about 500 tons per year in China.[7] Although there are no accurate data on the veterinary usage of tetracyclines, it is estimated that one hoggery with 10 000 swine may discharge 300–500 kg chlorotetracycline into the environment per year in China.[8] To protect populations from health risks, food safety regulatory agencies in many countries, including China, have established maximum residue limits (MRLs) for the residues of tetracylines and quinolones[9–11] in foods of animal origin and restricted their use in treating both animals and humans.

Previous analytical methods for screening and confirming tetracyclines and quinolones in foods of animal origin include liquid chromatography coupled with UV[12–14] or fluorescence detection[15-18] and the liquid chromatography/mass spectrometry (LC/MS)[19–22] technique, which were developed only for surveillance at the MRL levels. According to the European Commission Decision 2002/657/EC,[23] the reported method validations were also at 0.5, 1 and 2 times the MRL levels, which were often higher than the true levels. However, the effects of long-term exposure to low levels of residues below the MRLs are still unknown. On the other hand, the European Commission Decision 2002/657/EC[23]

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states that, ''Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods''. Therefore, analytical methods using mass spectrometric technology for screening and confirming trace levels of antibiotic residues in foods of animal origin are necessary for protecting the long-term health of consumers.

After several years of research, especially with the application of ultra-performance liquid chromatography (UPLC), column materials, and the acquisition and transition speed of mass spectrometry, the analytical throughput has been remarkably enhanced, making it possible to monitor more than 50 analytes in a single run.[24] The major benefit from the use of UPLC and the 1.7 μm particles column is the increased column efficiency that results in narrow peaks and an improved separation. Presently, the multi-residue methods have become the prevailing techniques for the analysis of veterinary drug residues in foods of animal origins, such as sulfonamides,[25] tetracyclines,[19,20] quinolones,[21,22] and hormones.[26] To the best of our knowledge, most of these methods aim at one class of drugs for each method. Compared to single-class residue detection methods, the multi-class methods are relatively scarce because of the difficulties in simultaneous extraction and purification of compounds with different physicochemical properties. Nevertheless, a few multi-residue and multi-class methods have been successfully developed for analysis of veterinary drug residues in foods of animal origin.[27–30] Li *et al*. established a multi-class method to monitor veterinary drug residues in shrimp.[27] Heller *et al*. used hydrophilic solid-phase extraction (SPE) cleanup followed by LC/MS/MS to detect a variety of polar drug residues in eggs at levels higher than 10 μg/kg.[28] Granelli *et al*. used LC/MS/MS to screen nineteen analytes from five classes of antibiotics.[29] Yamada *et al*. developed a method to detect 130 analytes of most classes of antibiotics and growth promoters except tetracycline antibiotics.[30]

In this study, a comprehensive sample preparation procedure and UPLC/MS/MS method was developed to simultaneously monitor the wide range polarity of 21 drugs of two different classes including seven tetracyclines and four types of quinolones (quinoline, naphthyridine, pyridopyrimidine, and cinoline) in meat, liver, and kidney tissues. Compounds with different polarities were extracted, concentrated, and purified using the same method. Mobile phase composition and additives were also investigated to achieve the highest sensitivity.

EXPERIMENTAL

Standards and stock solutions

Oxytetracycline, tetracycline, doxycycline, demeclocycline, methacycline, minocycline, chlortetracycline, enrofloxacin, norfloxacin, pefloxacin, ciprofloxacin, oflaxacin, sarafloxacin, enoxacin, lomefloxacin, pipemidic acid, nalidixic acid, oxolinic acid, flumequine, cinoxacin and danofloxacin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

About 10 mg of each reference standard (corrected by purity) was accurately weighed and placed in a 10-mL volumetric flask. Tetracyclines were dissolved in 10 mL methanol and quinolones were first dissolved in 50 μL formic acid and then diluted to 10 mL with methanol. Different volumes of each stock standard solution (200 μL of pipemdilic acid, norfloxacin, pefloxacin, sarafloxacin, 250 μL of ciprofloxacin, 100 μL of other quinolones, 600 μL of chlortetracycline, doxycylines, 900 μL of minocycline and 300 μL of other tertracyclines) were measured precisely and put in the same 10-mL black volumetric flask according to their LC/MS/MS response, diluted with methanol to 10 mL, and mixed to homogeneity as middle stock solution. The six levels of fortification solutions were prepared by serially diluting the middle stock solution 1, 2, 5, 10, 20 and 40 times with methanol. These solutions were stored at −18°C in the dark.

Reagents

All solvents used in sample preparation and chromatographic separations were HPLC grade. Methanol (MeOH) and acetonitrile (ACN) were supplied by Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (HCOOH, 99%) was from Acros Organics (Morris Plains, NJ, USA). Ultra-pure water was obtained using a Milli-Q Ultrapure system (Millipore, Bedford, MA, USA). Ethylenediaminetetraacetic acid disodium salt ($Na_2EDTA{\cdot}H_2O$), disodium hydrogen phosphate ($Na_2HPO_4{\cdot}12H_2O$) and citric acid ($C_6H_8O_7{\cdot}H_2O$) were all from Beijing Chemical Co. (Beijing, China).

Sample preparation

The frozen samples were naturally thawed at room temperature. Fifty pig tissues were minced and mixed. Aliquots of 5 g sample were weighed and transferred into a 50-mL polypropylene centrifuge tube. Then 20 mL EDTA-McIlvaine buffer[31] was added, and the specimens and solution were homogenized for 2 min using a Pro 300A homogenizer (Proscientific, Inc., Monroe, CT, USA). The mixture was centrifuged at 10 000 rpm for 10 min at 0°C. The supernatant was decanted into a conical flask. The residues were vortexed and extracted with 20 mL EDTA-McIlvaine buffer solution for 5 min and then with another 10 mL EDTA-McIlvaine buffer. The supernatants were pooled and subjected to SPE.

An Oasis HLB SPE cartridge (200 mg, 6 mL; Waters Corp., Milford, MA, USA) was sequentially preconditioned with 6 mL methanol, 6 mL water and 6 mL EDTA-McIlvaine buffer solution. The extract was applied to the cartridge at a flow rate of 2–3 mL/min. The solution flask and cartridge were rinsed twice with 3 mL of EDTA-McIlvaine buffer solution and 3 mL water. Then the cartridge was washed with 2 mL water containing 5% methanol and dried with high-purity nitrogen. The analytes were eluted with 6 mL methanol and then evaporated to near dryness under a gentle stream of nitrogen. The residue was reconstituted with 1 mL water containing 0.2% HCOOH.

Liquid chromatography

Chromatographic separation was carried out on a Waters ACQUITY UPLC™ system (Waters Corp.) using an ACQUITY UPLC™ BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm particle size). The column oven temperature was 30°C, the flow rate was 0.2 mL/min, and the injection volume

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was 10 μL. The mobile phase consisted of methanol/acetonitrile (v/v, 40:60) (A) and water containing 0.2% formic acid (B). The initial composition was 10% A and 90% B. A gradient elution was performed where phase A was increased linearly to 30% in the first 6.00 min, then increased to 50% in the next 3.00 min, increased to 100% in 0.50 min, then kept for 2.5 min and finally returned to the initial composition and equilibrated for 3 min before the next injection.

Mass spectrometry

Mass spectrometry was carried out on a Waters Quattro Premier XE mass spectrometer (Waters Corp., Manchester, UK) using the multiple reaction monitoring (MRM) mode and positive electrospray ionization (ESI). The capillary voltage, extractor voltage, RF lens voltage, and the multiplier voltage were set at 3.0 kV, 4.0 V, 0.0 V and 650 V, respectively. The source and desolvation temperatures were held at 110 and 350°C, respectively. The desolvation gas and cone gas were set at flow rates of 500 and 50 L/h, respectively, and the collision gas (ultra-high purity (UHP) argon) was held at 0.06 mL/min. The cone voltages and collision energies used for MRM acquisitions are presented in Table 1. Two transitions were selected for identification but only one was used for quantitation (underlined in Table 1).

Calibration

For quantitation of the 21 drugs in pig tissues, both solvent and matrix-fortified calibration curves using different blank matrices were constructed. Multi-component standard solutions were prepared by spiking 100 μL of serial fortification solutions mentioned above. The specific ranges are listed in Tables 2–4. The areas of the MRM transitions showing the most intense signals were plotted against their respective concentrations.

Table 1. LC/MS/MS acquisition parameters for the 21 compounds

Compound	Tme segment (min)	Ion transitions	Dwell time (s)	Collision energy (eV)	Cone voltage (V)
Pipemidic acid	3.2–4.5	304.3 > 217.1	0.15	21	38
		304.3 > 189.0	0.15	32	38
Minocycline	3.2–4.5	458.5 > 441.4	0.15	18	35
		458.5 > 352.4	0.15	29	35
Pefloxacin	4.5–5.3	334.3 > 290.3	0.03	17	38
		334.3 > 233.2	0.03	25	38
Oflaxacin	4.5–5.3	362.2 > 318.3	0.03	18	38
		362.2 > 261.2	0.03	27	38
Enoxacin	4.5–5.3	321.4 > 303.3	0.03	19	50
		321.4 > 233.9	0.03	22	50
Tetracycline	4.5–5.3	445.4 > 410.4	0.03	19	28
		445.4 > 427.7	0.03	14	28
Norfloxacin	4.5–5.3	320.3 > 302.3	0.03	19	50
		320.3 > 276.3	0.03	17	50
Oxytetracycline	4.5–5.3	461.4 > 426.4	0.03	19	30
		461.4 > 443.6	0.03	13	30
Ciprofloxacin	4.5–5.3	332.2 > 314.3	0.03	19	36
		332.2 > 288.3	0.03	17	36
Enrofloxacin	5.3–7.0	360.3 > 316.4	0.03	19	38
		360.3 > 342.3	0.03	23	38
Danofloxacin	5.3–7.0	358.3 > 340.3	0.03	25	38
		358.3 > 82.0	0.03	42	38
Lomefloxacin	5.3–7.0	352.3 > 265.2	0.03	23	36
		352.3 > 308.3	0.03	17	36
Demeclocycline	5.3–7.0	465.3 > 430.4	0.03	22	36
		465.3 > 448.4	0.03	17	36
Sarafloxacin	5.3–7.0	386.3 > 342.3	0.03	18	40
		386.3 > 299.3	0.03	28	40
Doxycycline	7.4–9.5	445.5 > 428.5	0.05	19	24
		445.5 > 154.0	0.05	32	24
Chlortetracycline	7.4–9.5	479.3 > 444.4	0.05	20	32
		479.3 > 462.3	0.05	18	32
Cinoxacine	7.4–9.5	263.1 > 244.1	0.05	16	35
		263.1 > 188.8	0.05	28	35
Oxolinic acid	7.4–9.5	262.1 > 244.1	0.05	16	50
		262.1 > 155.9	0.05	28	50
Methacycline	7.4–9.5	443.3 > 426.4	0.05	18	28
		443.3 > 201.2	0.05	34	28
Nalidixic acid	10.5–11.7	233.1 > 215.1	0.10	15	26
		233.1 > 187.0	0.10	28	26
Flumequine	10.5–11.7	262.2 > 244.1	0.15	17	50
		262.2 > 202.1	0.15	28	50

[a]The quantitation ion transitions are underlined.

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Table 2. Matrix-fortified standard curves, linear range, LOD, and LOQ of the 21 compounds in the pig muscle

Compound	Calibration curve	Linear range (μg/kg)	Correlation coefficient r^2	LOQ (μg/kg)	LOD (μg/kg)
Pipemidic acid	y = 4511x + 44.6	1–40	0.995	0.06	0.05
Minocycline	y = 2119x−0.986	4.5–120	0.986	1.36	0.79
Pefloxacin	y = 1913x + 233	1–40	0.978	0.26	0.11
Oflaxacin	y = 18204x + 1963	0.5–20	0.978	0.03	0.01
Enoxacin	y = 7416x + 239	1.5–60	0.981	0.63	0.20
Tetracycline	y = 3259x + 1414	1.5–60	0.995	0.45	0.45
Norfloxacin	y = 3509x + 849	1–40	0.975	0.67	0.21
Oxytetracycline	y = 2177x + 4141	3–120	0.980	0.75	0.75
Ciprofloxacin	y = 3186x + 349	1.25–50	0.991	0.29	0.27
Enrofloxacin	y = 8950x + 756	0.5–20	0.982	0.09	0.07
Danofloxacin	y = 8633x + 572	0.5–20	0.968	0.15	0.15
Lomefloxacin	y = 7387x + 600	0.5–20	0.992	0.09	0.04
Demeclocycline	y = 879x + 1072	3–120	0.987	1.30	0.47
Sarafloxacin	y = 2481x + 21	1–40	0.995	0.14	0.07
Doxycycline	y = 1671x + 4048	3–120	0.989	1.15	0.56
Chlortetracycline	y = 568x + 1149	3–120	0.986	0.52	0.47
Cinoxacine	y = 6751x + 2667	0.5–20	0.977	0.12	0.05
Oxolinic acid	y = 4329x + 1148	0.5–20	0.989	0.33	0.14
Methacycline	y = 2116x + 5568	1.5–60	0.996	0.32	0.32
Nalidixic acid	y = 5540x + 1527	0.5–20	0.990	0.07	0.03
Flumequine	y = 7956x + 1291	0.5–20	0.983	0.03	0.01

RESULTS AND DISCUSSION

Optimization of LC/MS/MS

To achieve the highest sensitivity for each analyte, a full-scan mass scan was first applied to identify the precursor ions by direct flow injection. The $[M+H]^+$ ions were found to be the most abundant and were selected as precursor ions. Collision-induced dissociation (CID) spectra of each analyte were then acquired under different collision energies. For the seven tetracyclines, the common fragmentation pathways were found as reported in previous papers,[32,33] which led to the loss of H_2O (18 u) and NH_3 (17 u). However, the $[M+H–H_2O]^+$, $[M+H–H_2O–NH_3]^+$ or $[M+H–NH_3]^+$ ions were abundant in the spectra. Therefore, for tetracyclines, the $[M+H–H_2O]^+$, $[M+H–H_2O–NH_3]^+$ or $[M+H–NH_3]^+$ ions were selected for MRM experiments. Another common ion at *m/z* 154 was found (Fig. 1(a)) with relatively low abundance. Some analytes, such as demeclocycline, oxytetracycline and methacycline, produced $[M+H–H_2O–NH_3–NH(CH_3)_2]^+$ ions with relatively low abundance. As for nalidixic acid, oxolinic acid and cinoxacin, the main fragmentation pathways were similar, which were found to result in the loss of H_2O (18 u) and HCO_2H (46 u), corresponding to the $[M+H–H_2O]^+$ and $[M+H–HCO_2H]^+$ ions in the spectra (Fig. 1(b) for example). For the other analytes involved in this study, except for flumequine and pipemidic acid, common fragmentation modes were found with the loss of H_2O (18 u) and CO_2 (44 u), which corresponded to the $[M+H–H_2O]^+$ and $[M+H–CO_2]^+$ ions in the spectrum. Figures 1(c) and 1(d) show the fragmentation pathways of flumequine and

Table 3. Matrix-fortified standard curves, linear range, LOD, and LOQ of the 21 compounds in the pig liver

Compound	Calibration curve	Linear range (μg/kg)	Correlation coefficient r^2	LOQ (μg/kg)	LOD (μg/kg)
Pipemidic acid	y = 628x + 181	1–40	0.99	0.23	0.17
Minocycline	y = 498x + 6610	4.5–180	0.982	10.00	7.70
Pefloxacin	y = 256x + 292	1–40	0.994	0.51	0.22
Oflaxacin	y = 2375x + 593	0.5–20	0.995	0.11	0.06
Enoxacin	y = 2028x-490	1.5–60	0.995	1.00	1.00
Tetracycline	y = 369x + 2057	1.5–60	0.991	0.50	0.70
Norfloxacin	y = 1164x + 761	1–40	0.995	2.22	1.2
Oxytetracycline	y = 552x + 954	3–120	0.996	0.65	0.65
Ciprofloxacin	y = 707x + 1393	1.25–50	0.987	1.85	1.66
Enrofloxacin	y = 1582x + 1027	0.5–20	0.999	0.21	0.21
Danofloxacin	y = 1902x + 680	0.5–20	0.986	0.60	0.60
Lomefloxacin	y = 935x + 275	0.5–20	0.996	0.29	0.15
Demeclocycline	y = 176x + 505	3–120	0.999	1.20	1.00
Sarafloxacin	y = 446x + 227	1–40	0.997	0.63	0.37
Doxycycline	y = 224x + 1184	3–120	0.995	4.50	4.50
Chlortetracycline	y = 178x + 118	3–120	0.97	0.75	0.75
Cinoxacine	y = 2445x + 165	0.5–20	0.997	0.14	0.103
Oxolinic acid	y = 2445x + 1652	0.5–20	0.997	0.18	0.10
Methacycline	y = 871x + 1664	1.5–60	0.982	1.67	0.90
Nalidixic acid	y = 2846x + 1138	0.5–20	0.996	0.11	0.06
Flumequine	y = 4269x + 659	0.5–20	0.993	0.08	0.07

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Table 4. Matrix-fortified standard curves, linear range, LOD, and LOQ of the 21 compounds in the pig kidney

Compound	Calibration curve	Linear range (μg/kg)	Correlation coefficient r^2	LOQ (μg/kg)	LOD (μg/kg)
Pipemidic acid	y = 549x − 267	1–40	0.988	0.28	0.17
Minocycline	y = 64.8x − 139	4.5–120	0.993	10.00	3.00
Pefloxacin	y = 297x + 268	1–40	0.99	0.68	0.23
Oflaxacin	y = 3193x + 1296	0.5–20	0.995	0.08	0.05
Enoxacin	y = 1483x + 2523	1.5–60	0.978	1.07	0.64
Tetracycline	y = 369x + 2057	1.5–60	0.991	0.69	0.69
Norfloxacin	y = 1123x + 454	1–40	0.988	1.42	0.67
Oxytetracycline	y = 553x + 4079	3–120	0.995	0.82	0.82
Ciprofloxacin	y = 632x + 255	1.25–50	0.99	3.57	2.88
Enrofloxacin	y = 2296x + 1015	0.5–20	0.99	0.20	0.20
Danofloxacin	y = 2295x + 741	0.5–20	0.997	0.60	0.60
Lomefloxacin	y = 931x + 86	0.5–20	0.999	0.47	0.16
Demeclocycline	y = 253x + 888	3–120	0.994	1.05	0.95
Sarafloxacin	y = 1116x + 701	1–40	0.993	0.51	0.24
Doxycycline	y = 788x + 978	3–120	0.995	2.70	2.50
Chlortetracycline	y = 178x + 118	3–120	0.97	0.80	0.80
Cinoxacine	y = 3387x + 1363	0.5–20	0.99	0.22	0.07
Oxolinic acid	y = 3048x + 1176	0.5–20	0.989	0.83	0.30
Methacycline	y = 1358x + 654	1.5–60	0.992	0.88	0.25
Nalidixic acid	y = 3511x + 1024	0.5–20	0.998	0.15	0.06
Flumequine	y = 5923x + 340	0.5–20	0.994	0.06	0.04

pipemidic acid. In addition to the loss of H_2O (18 u), the presence of $[M–CO_2–CH_4+H]^+$ ions and the cleavage of piperazine were observed.

It is well known that tetracyclines have a tendency to form chelation complexes with metal ions and be adsorbed on residual free silanol in sorbents. Therefore, in this study, the column temperature, a mobile phase including formic acid, and an organic mobile phase were optimized to overcome the tailing peaks of tetracyclines (data not shown). The results showed that well-shaped peaks could be obtained

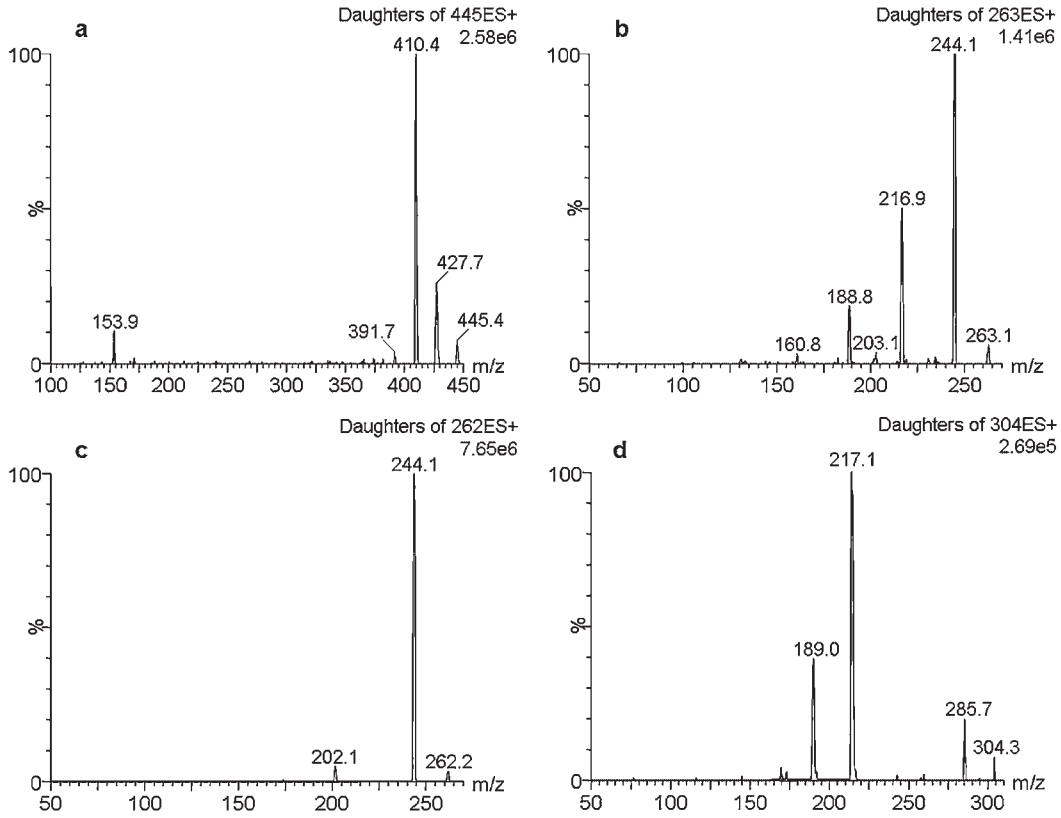

Figure 1. Typical CID spectra for tetracyclines and quinolones: (a) tetracycline; (b) cinoxacin; (c) flumequine; and (d) pipemidic acid.

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when 40:60 methanol/acetonitrile (v/v) and water containing 0.2% formic acid was used as the mobile phase with a column temperature of 30°C. Good resolution was also achieved for most of the analytes during 12 min with peak width no more than 0.2 min.

Optimization of extraction solvent

Acidic methanol, acidic acetonitrile and EDTA-McIlvaine buffer are often used to extract tetracyclines from edible animal products. As for quinolones, organic solvents such as methanol, acetonitrile and acetone are often used, and sometimes acidic organic solvents with pH less than 3 have been suggested. However, under this acidic condition, tetracyclines will form 4-epi-tetracyclines and anhydrotetracyclines reversibly. Therefore, in this study, we compared three extraction solvents with pH = 4, i.e., acidic methanol, acidic acetonitrile and EDTA-McIlvaine buffer. Aliquots of 5 g of sample were weighed, and 1 μg standard mixture was spiked. Three different extraction solvents were used to extract samples three times with 20 mL, 20 mL and 10 mL, respectively. The extracts were pooled and the volume was maintained at 50 mL. Then 100 μL extract was taken and diluted with 0.2% formic acid solution to 0.2 mL. The extract was injected into the UPLC/MS/MS system for analysis. The results indicated that the extraction efficiency was at a desirable level using EDTA-McIlvaine buffer as the extraction solvent.

Selection of SPE cartridge and elution solvent

Reversed-phase octadecylsilyl cartridges (ODS, 500 mg, 6 mL; Waters Corp.) and HLB cartridges were used to compare the concentration and clean-up efficiency. In comparison with the ODS cartridge, the HLB cartridge exhibited significantly higher recoveries for most analytes.

High recoveries (>85%) could be achieved for most of the analytes (except for minocycline) when methanol or acetonitrile was used as the elution solvent. Similar recoveries could be obtained for tetracyclines when using ethyl acetate. However, almost no quinolones could be eluted by ethyl acetate, which might be due to the different solubility.

Selection of reconstitution solvent

Before injection, the reconstitution solvents were optimized using 0%, 10%, 20%, 40%, 50%, and 100% methanol solution containing 0.2% formic acid. Water containing 0.2% formic acid rather the initial composites of the mobile phase was selected as the reconstitution solvent. Considering the conversion into epimers for tetracyclines under acidic conditions, the prepared samples should be kept dry and resuspended just prior to LC/MS/MS analysis.

Method validation

Before method validation, different samples (liver, kidney, and muscle), obtained from a supermarket, were analyzed to screen blank samples as described in the Experimental section. On the basis of the European Commission Decision 2002/657/EC and 96/23/EC, tetracyclines and quinolones belong to group B of Annex I; therefore, a minimum of three identification points are required. This means that two MRM transitions, corresponding to one precursor ion and two fragment ions, are selected when using tandem mass spectrometry. With this choice, four identification points were obtained to ensure the specificity of this method.

Signal suppression is often observed during LC/MS/MS analysis, especially for complicated bio-samples; moreover, the suppression may vary depending on the compound and matrix. To evaluate the matrix effect, the chromatographic responses of multi-component pure standards and matrix-matched standards were compared. Table 5 lists the ratios of ion suppression expressed in percentages and obtained by subtracting the ratio of peak areas between matrix-matched standards and solvent standards. The ion suppression levels ranged between 0 and 51%, 10 and 68%, and 0 and 62% for muscle, kidney, and liver, respectively. In general, the ion suppression for liver and kidney was significantly higher than that for muscle, except for oxolinic acid and cinoxacin, and the suppression for most of the analytes based on kidney was higher than that based on liver samples. This demonstrated the difficulties in quantitative analysis based on the solvent standard curve. The isotopic dilution technique is advantageous to compensate for signal irreproducibility, matrix interference, and recovery loss. However, it is impossible to acquire enough isotopic labeled internal standards for the purpose of multi-component analysis. Therefore, matrix-matched standard curves or matrix-fortified standard curves were applied in the multi-component quantitative analysis. The calibration curves for detection of the target compounds were obtained by performing a linear regression analysis on a series of spiking experiments or by adding a series of standard solutions to the matrix solution and using the area against analyte concentrations. The former can compensate for the matrix effect and the loss of target analytes during sample preparation and the latter can only eliminate the matrix effect. In this study, the

Table 5. Ratio of ion suppression of 21 compounds in pig meat, kidney, liver

	Ion suppression (%)		
Compound	Muscle	Kidney	Liver
Pipemidic acid	7	59	25
Minocycline	0	56	49
Pefloxacin	8.7	67	34
Oflaxacin	42	71	62
Enoxacin	12	39	2
Tetracycline	18	47	32
Norfloxacin	0	46	0
Oxytetracycline	11	33	20
Ciprofloxacin	0	39	5
Enrofloxacin	11	43	38
Danofloxacin	0	33	0
Lomefloxacin	25	74	66
Demeclocycline	18	45	54
Sarafloxacin	18	68	57
Doxycycline	48	51	55
Chlortetracycline	30	20	30
Cinoxacine	35	10	0
Oxolinic acid	51	45	0
Methacycline	18	40	43
Nalidixic acid	27	30	0
Flumequine	0	36	0

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Table 6. Recoveries and RSD of 21 target compounds in pig liver

	Spiking 1.0 μg/kg		Spiking 2.0 μg/kg		Spiking 5 μg/kg	
Compound	Recovery/%	RSD/%	Recovery/%	RSD/%	Recovery/%	RSD/%
Pipemidic acid	116.3	12.5	111.2	8.2	103.8	12.2
Minocycline	113.5	15.6	110.9	16.5	111.2	10.2
Pefloxacin	107.8	11.4	111.5	12.8	110.3	15.2
Oflaxacin	110.6	12.3	115.6	5.4	106.0	6.3
Enoxacin	112.7	14.6	108.7	3.6	104.4	8.4
Tetracycline	115.0	16.7	113.1	13.1	103.2	8.9
Norfloxacin	115.1	11.6	99.9	10.7	106.9	9.4
Oxytetracycline	112.6	15.1	119.1	13.4	112.9	2.9
Ciprofloxacin	113.3	11.2	116.4	5.2	117.5	10.4
Enrofloxacin	117.5	17.8	105.4	6.1	109.6	10.5
Danofloxacin	117.3	10.3	119.7	3.0	105.5	13.4
Lomefloxacin	115.1	12.3	102.1	10.9	100.0	8.7
Demeclocycline	115.5	17.2	116.6	10.8	117.8	12.1
Sarafloxacin	109.8	5.9	108.0	6.0	106.5	11.1
Doxycycline	96.2	12.1	105.1	9.3	111.8	12.8
Chlortetracycline	96.5	15.4	87.9	18.6	85.6	15.4
Cinoxacine	110.8	8.0	119.5	7.4	110.8	5.9
Oxolinic acid	115.3	6.7	117.2	5.0	112.8	9.8
Methacycline	109.5	11.0	117.9	12.3	112.3	12.7
Nalidixic acid	110.4	4.1	111.0	5.6	113.0	13.2
Flumequine	111.8	7.8	113.3	10.7	109.1	13.9

linear dynamic ranges of most target analytes using matrix-fortified mode were verified as being between 0.5 and 120 μg/kg. Good linearity was obtained for all analytes, with correlation coefficients of $r^2 > 0.985$ (Tables 2–4).

The limits of detection (LODs), defined as the concentration that yields a signal-to-noise (S/N) ratio equal to 3 for the transition with the lower response, ranged from 0.01–2.88 μg/kg for quinolones and 0.25–7.70 μg/kg for tetracyclines in different tissues (Tables 2–4). The limits of quantification (LOQs),[34] defined as the concentration that yields an S/N ratio equal to 10 for the transition with the highest chromatographic response, ranged from 0.03–4.50 μg/kg for quinolones and 0.16–10.00 μg/kg for tetracyclines in different tissues (Tables 2–4). The LOQs of this method were well below the MRLs set by the EU and US FDA for residues of tetracyclines and quinolones in animal tissues.

The analyte recovery of this procedure was evaluated by spiking 5, 10 and 25 ng of each standard analyte to 5 g samples at three levels in replicates of six, corresponding to 1, 2 and 5 μg/kg. The results are summarized in Tables 6–8; the

Table 7. Recoveries and RSD of 21 target compounds in pig meat

	Spiking 1.0 μg/kg		Spiking 2.0 μg/kg		Spiking 5.0 μg/kg	
Compound	Recovery/%	RSD/%	Recovery/%	RSD/%	Recovery/%	RSD/%
Pipemidic acid	106.2	3.5	103.8	7.9	88.2	6.9
Minocycline	100.1	16.7	104.4	11.2	96.7	16.0
Pefloxacin	117.8	4.4	101.6	7.4	80.2	11.9
Oflaxacin	115.2	7.9	96.6	6.2	111.4	10.3
Enoxacin	113.9	6.6	105.9	8.8	87.2	9.5
Tetracycline	96.9	16.4	113.7	9.2	108.6	16.0
Norfloxacin	109.8	12.3	94.1	7.2	85.2	7.7
Oxytetracycline	111.5	13.1	119.6	4.2	108.7	8.0
Ciprofloxacin	108.1	5.6	99.6	7.3	87.1	7.6
Enrofloxacin	113.3	3.6	96.1	9.4	83.7	14.5
Danofloxacin	118.3	7.0	95.9	16.9	82.3	7.5
Lomefloxacin	114.6	2.6	117.7	6.6	101.7	9.5
Demeclocycline	104.8	11.0	116.4	5.6	107.8	9.5
Sarafloxacin	119.5	6.4	113.9	8.3	111.4	10.8
Doxycycline	113.3	9.6	103.1	6.8	90.9	9.7
Chlortetracycline	107.6	15.6	110.1	9.8	103.5	10.3
Cinoxacine	112.4	4.1	118.0	9.6	114.7	5.7
Oxolinic acid	112.5	4.5	119.7	7.0	101.9	7.4
Methacycline	111.6	6.1	110.7	12.1	101.9	6.7
Nalidixic acid	116.3	3.4	119.8	7.1	107.0	2.1
Flumequine	117.3	3.4	110.4	7.2	112.0	2.1

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Table 8. Recoveries and RSD of 21 target compounds in pig liver

	Spiking 1.0 μg/kg		Spiking 2.0 μg/kg		Spiking 5.0 μg/kg	
Compound	Recovery/%	RSD/%	Recovery/%	RSD/%	Recovery/%	RSD/%
Pipemidic acid	100.4	18.3	89.3	10.9	87.1	13.1
Minocycline	102.2	15.0	91.0	10.0	88.0	12.2
Pefloxacin	97.7	16.0	114.8	11.1	91.0	8.2
Oflaxacin	108.7	18.9	109.9	10.8	97.8	10.3
Enoxacin	102.3	15.5	110.9	13.3	99.2	15.3
Tetracycline	89.9	15.6	97.8	9.7	104.6	8.9
Norfloxacin	111.3	10.6	104.6	11.3	82.6	10.2
Oxytetracycline	98.1	18.9	104.9	16.9	104.0	10.7
Ciprofloxacin	104.5	18.6	106.3	7.4	96.4	11.3
Enrofloxacin	104.0	10.4	118.2	11.0	98.0	11.7
Danofloxacin	85.3	15.0	105.8	17.8	86.3	10.0
Lomefloxacin	90.2	11.1	104.9	12.3	97.6	9.1
Demeclocycline	90.1	4.2	98.5	17.0	93.7	12.0
Sarafloxacin	87.3	11.1	95.5	7.2	95.7	11.3
Doxycycline	113.2	15.2	110.5	9.0	98.2	8.6
Chlortetracycline	89.7	18.9	96.5	15.5	98.4	12.6
Cinoxacine	89.7	11.4	111.8	5.9	108.6	12.7
Oxolinic acid	115.3	7.9	117.2	5.0	112.8	9.8
Methacycline	104.7	12.1	109.4	15.8	97.6	9.8
Nalidixic acid	90.1	12.6	105.4	6.8	106.7	10.1
Flumequine	89.7	13.5	102.8	6.2	90.1	8.0

average recoveries of each compound ranged from 80.2–117.8%.The positive intercepts of the linear equation for most compounds also documented these results. The reproducibility of this method was represented by the relative standard deviation (RSD) percentage at each fortification level for each compound, and these values are also summarized in Tables 6–8. The results show that the precision of the method was within 20%. The within-day and between-day reproducibilities were evaluated by spiking real samples at the level of 1 μg/kg quinolones and 3 μg/kg tetracyclines, with five replicates each day for five consecutive days. The within-day reproducibility ranged from 4.2–11.0 and the between-day reproducibility ranged from 5.5–14.6 (Table 9).

Real samples commercially available from the local market were detected by this newly developed method. Figure 2 shows the ion chromatograms of a real sample. Two transition ions were monitored, i.e., $461.4 > 426.4$,

Table 9. Within-day and between-day reproducibility of tetracyclines and quinolones in pig muscle, liver, kidney

	Within-day reproducibility/%			Between-day reproducibility/%		
Compound	Muscle	Liver	Kidney	Muscle	Liver	Kidney
Flumequine	8.4	9.8	8.7	7.9	10.2	14.2
Nalidixic acid	5.5	5.6	7.4	6.5	9.8	10.5
Oxolinic acid	4.2	5.0	5.8	8.4	9.5	10.4
Cinoxacine	5.8	8.7	6.6	9.1	10.2	9.7
Sarafloxacin	7.6	8.4	9.2	5.6	8.9	10.7
Enrofloxacin	9.1	8.5	7.9	6.8	7.4	8.4
Danofloxacin	8.4	7.9	10.1	8.5	8.8	10.3
Lomefloxacin	5.5	6.8	8.4	7.7	6.9	8.9
Ciprofloxacin	4.9	5.7	6.0	9.9	10.4	11.5
Norfloxacin	7.0	8.2	9.4	5.5	7.7	6.8
Pefloxacin	10.4	9.9	8.7	8.7	9.4	9.9
Pipemidic acid	6.3	7.4	8.0	10.2	8.8	8.9
Enoxacin	6.9	10.0	9.7	8.0	7.5	9.8
Methacycline	10.1	9.9	10.7	9.4	9.6	8.7
Oflaxacin	8.8	7.9	9.5	5.9	6.8	7.4
Minocycline	9.5	8.8	10.4	10.4	12.4	10.7
Demeclocycline	6.9	7.8	8.4	9.8	10.7	11.7
Oxytetracycline	8.4	6.8	9.7	7.6	8.6	9.7
Tetracycline	5.6	6.0	5.9	8.4	7.9	10.4
Chlortetracyclin	7.9	8.5	10.2	8.9	10.4	12.7
Doxycycline	8.7	9.9	11.0	11.5	14.6	11.8

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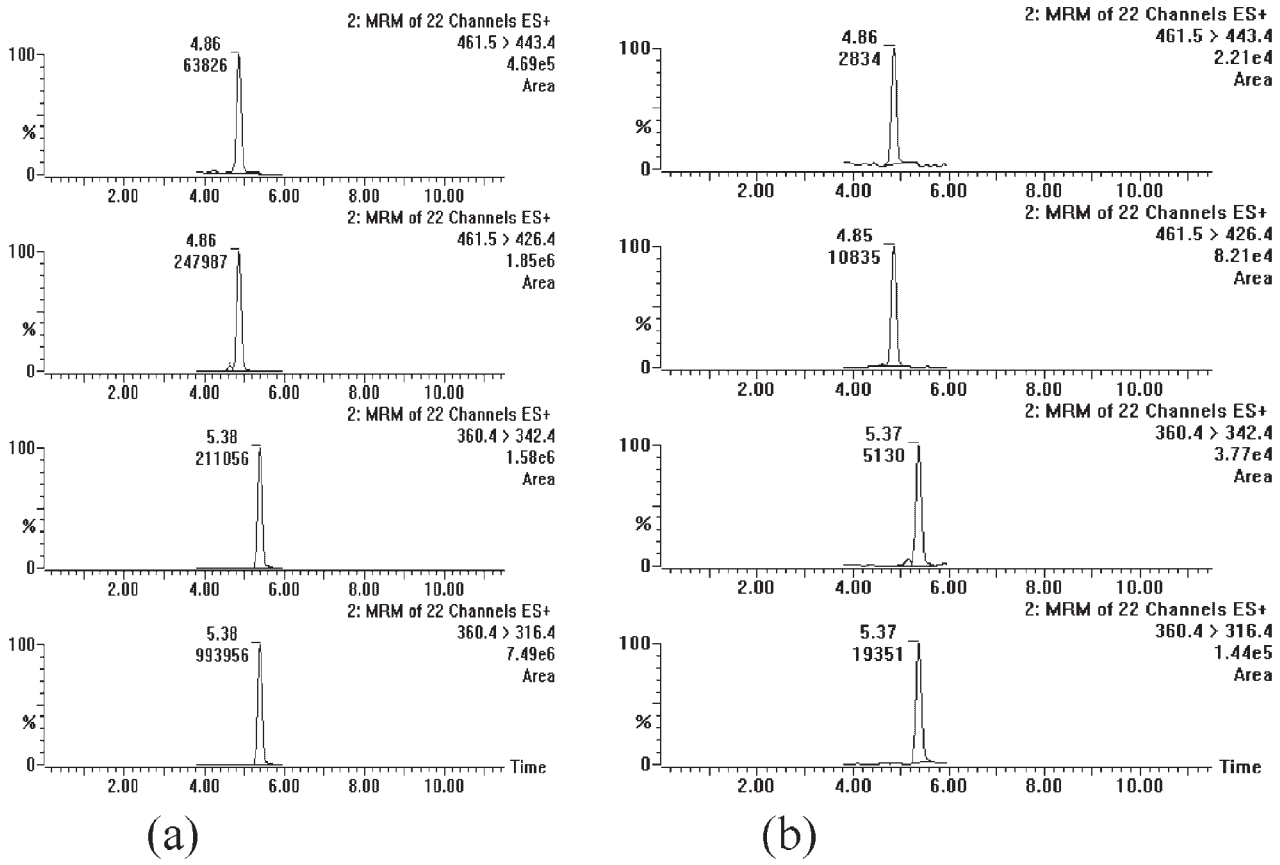

Figure 2. Reconstructed ion chromatograms of real sample (a) and standard (b).

461.4 > 443.6 for oxytetracyclines, and 360.3 > 316.4, 360.3 > 342.3 for enrofloxacin. All ions satisfied the EU analytical criteria; in terms of correspondence of retention times and ion chromatogram area ratio with the standard, enrofloxacin and oxytetracyclines could be confirmed.

CONCLUSIONS

This study comprehensively presented a UPLC/MS/MS multi-class confirmatory method for analyzing traces of 21 antibiotic residues in pig tissues, which involved seven tetracyclines and fourteen quinolones from four categories (quinoline, naphthyridine, pyridopyrimidine and cinoline). The method included extraction of sample tissues with EDTA-McIlvaine buffer and purification using a polymer-based Oasis HLB SPE cartridge. The separation, quantification and confirmation of all of the 21 drugs could be successfully achieved within only 15 min. The LOQs were well below the MRLs of the EU and US FDA, which was useful in regulatory screening programs to monitor antibiotics misuse in animals.

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