Simultaneous determination of 88 veterinary drug residues in pork liver using LC-MS/MS

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In recent years, the use of illicit drugs in animal breeding and husbandry has become increasingly widespread. Many veterinary drugs can effectively prevent and treat diseases in poultry and animals and improve breeding efficiency and product quality. However, due to the lack of scientific knowledge and economic benefits, some farmers overuse veterinary drugs, use illegal drugs or use drugs during the breeding withdrawal period, resulting in drug residues that are present in animal-derived foods. Some of these drugs can cause allergic reactions or carcinogenic or teratogenic effects in humans. Alternatively, they can cause bacterial resistance or environmental toxicity. For these reasons, illegal drug residues in animal-derived foods are problematic and have resulted in widespread public concern.

To protect public safety, the Chinese Ministry of Agriculture Announcement No. 2292 in 2015 prohibited the use of lomefloxacin, pefloxacin, ofloxacin and norfloxacin and various salts, esters and preparations of these 4 raw materials. In 2020, a bulletin was issued, listing 21 categories of drugs and other compounds that are prohibited in animal food. In addition, the published GB 31650-2019 stipulates 9 veterinary drugs that are allowed for therapeutic use but must not be detected in animal-derived foods. These actions indicate that China's supervision of veterinary drugs and illegally added drugs is increasing.[1,2]

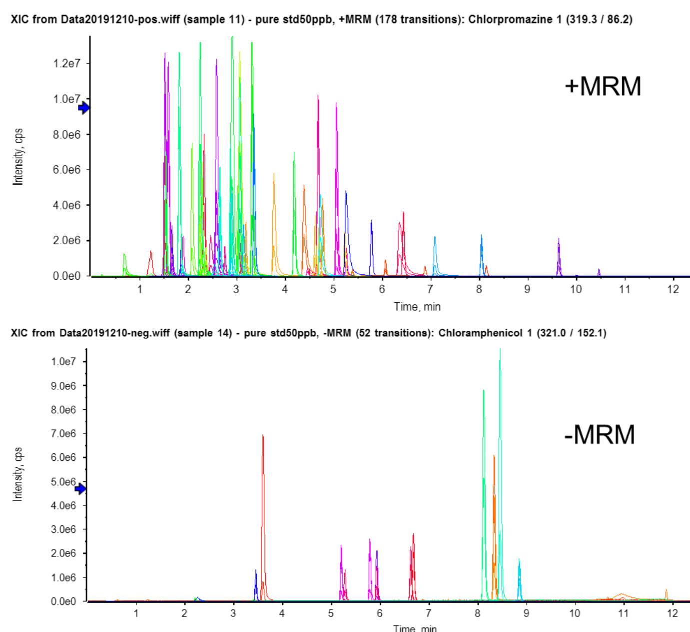

Figure 1. LC-MS/MS analysis of 88 drugs. Ion chromatogram of drug compounds measured in positive mode (top) and negative ion mode (bottom).

Due to the wide variety of animal-derived foods, complex sample matrices, multiple types of veterinary drugs and large differences in physical and chemical properties, food safety testing is challenging. Usually, multiple sample preparation steps and multiple instruments are needed, both of which are costly. To improve efficiency and reduce testing costs, this technical note uses pig liver as a matrix to establish a quantitative analytical method to quantify 88 veterinary drugs prohibited in China. The method aims to provide a complete solution for simple and efficient testing for veterinary drugs and illegally added drugs in animal-derived foods.

Key features of the method for veterinary drug residue analysis using the QTRAP 4500 system

- A panel of 88 chemically diverse veterinary drugs was effectively monitored in both positive and negative ion modes
- Calibration curves tested concentrations ranging from 0.2 to 200 ng/mL, revealing good linear correlation and excellent method %CV values for triplicate injections
- Sample preparation and sample cleanup protocols were optimized
- Sensitivity and analyte recovery metrics were sufficient to meet Chinese regulations
- Method can be implemented for routine analysis

Methods

Sample preparation: Two variations of the sample preparation method were tested. This technical note describes both methods, however, final sample analyses were performing using method 2.

Method 1:

The pig liver sample was homogenized and a 2.5 g sample was mixed with 10 mL of 80% acetonitrile aqueous solution. The samples were vortexed for 1 min, sonicated for 30 min and centrifuged at 10,000 rpm for 10 min. A cleanup procedure was then performed on the supernatant. An 4.0 mL aliquot of supernatant was loaded directly into the PEP-PLUS (P/N: PE0603X) solid-phase extraction (SPE) column without activation. An approximate flow rate of 1 drop/s was maintained and all effluent collected. Nitrogen gas was used to evaporate the sample to a volume of less than 1.0 mL at 45°C before the sample was reconstituted to 1.0 mL with 10% acetonitrile aqueous solution. Finally, the sample was vortexed, filtered through a 0.22 µm microporous membrane and analyzed on the liquid chromatography-tandem mass spectrometer (LC-MS/MS).

Method 2:

A 2.5 g sample of homogenized pig liver was mixed with 2 mL of water and vortexed for 1 min. The resulting sample was then mixed with 10 mL of 80% acetonitrile aqueous solution, vortexed for 1 min, sonicated for 30 min and centrifuged at 10,000 rpm for 10 min. The entire volume of supernatant was dried under nitrogen gas to 2 mL at 40° C. This volume was added to the activated PEP-2 SPE column, rinsed with 5 mL of water, drained and eluted with 5 mL of methanol and 5 mL of 5% formic acid in methanol. The eluent was then blown to dryness with nitrogen gas and 1 mL of 10% acetonitrile aqueous solution was added. The sample was sonicated for 5 min, vortexed briefly and then passed through a 0.22 µm microporous membrane for LC-MS/MS analysis.

Table 1. LC gradient for chromatographic separation.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
1.0	*97*	*3*
1.1	*80*	*20*
9.5	*25*	*75*
9.6	*1*	*99*
11.0	*1*	*99*
11.1	*97*	*3*
13.5	*97*	*3*

Method 2 was a more useful procedure because the SPE column in method 1 adsorbed carbachol, QCA, diethylstilbestrol, diethylstilbestrol, pentachlorophenol and estradiol benzoate.

Chromatography: The LC system used was an ExionLC AD system. The analytical column used was a Phenomenex Kinetex C18 column (100 mm X 2.1 mm, 1.7 µm). Gradient conditions used are outlined in Table 1. In positive ion mode, mobile phase A was an aqueous solution containing 0.1% formic acid and mobile phase B was an acetonitrile solution containing 0.1% formic acid. In negative ion mode, mobile phase A was an aqueous solution containing 0.2 mM ammonium fluoride and mobile phase B was pure acetonitrile.

Mass spectrometry: The mass spectrometer used was a QTRAP 4500 system. The multiple reaction monitoring (MRM) scan mode was used and operated in both positive and negative ion modes. Refer to SCIEX How for the full list of analytes monitored and MRM parameters used. Ionization was conducted in electrospray (ESI) mode. Ion source parameters included: spray voltage (IS) of 5500 V in positive ion mode or -4500 V in negative ion mode, ion source temperature of 550°C, CUR gas at 35 psi, CAD gas at 7 psi, GS1 gas at 50 psi and GS2 gas at 60 psi.

Results

First, concentration curves were generated to investigate the sensitivity and reproducibility of the method. Triplicate calibration standards were injected continuously to determine method reproducibility. These analyses revealed that the relative standard deviation (RSD) of the calculated concentration was less than 5% for each concentration level (Table 2). An example of the observed chromatographic data for ofloxacin across 6 replicate injections is shown in Figure 3.

To investigate the extraction recovery rate, a blank sample was selected and the standard addition experiment was carried out at low and high concentration levels. Three parallel experiments were carried out for the addition of different spiking concentrations. The extraction recovery rate for 85% of the compounds ranged from 60% to 120% and the relative standard deviation RSD (n=3) of all compounds was less than 20% (Figure 2).

In the context of regulatory compliance, it is important that a developed analytical method can reach a lower limit of quantification that meets Chinese regulatory requirements. The limit of quantification determined for all compounds was between 0.1 µg/kg and 2 µg/kg in the original sample. This quantification ability meets the requirements of government regulations.

Table 2. Reproducibility and accuracy for the example analyte – levonorgestrel. Very good reproducibility was observed with RSD of calculated concentration <5% across concentrations ranging from 0.2 ng/mL to 100 ng/mL (n=3).

Std. Conc. (ng/mL)	CV (%)	Accuracy (%)
0.2	*1.2*	*108.3*
0.5	*4.8*	*99.4*
1.0	*2.6*	*99.0*
2.0	*0.93*	*98.6*
5.0	*1.2*	*94.4*
10	*2.2*	*100.9*
50	*1.5*	*98.3*
100	*1.4*	*101.1*

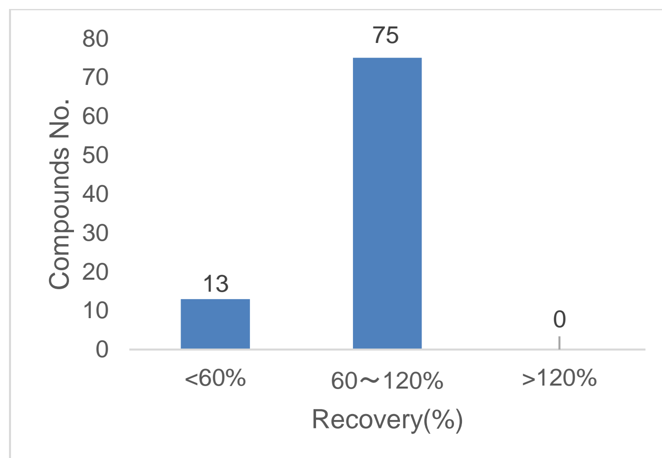

Figure 2 Analyte extraction recovery rate (%) frequency binned for <60%, 60-120%, >120%.

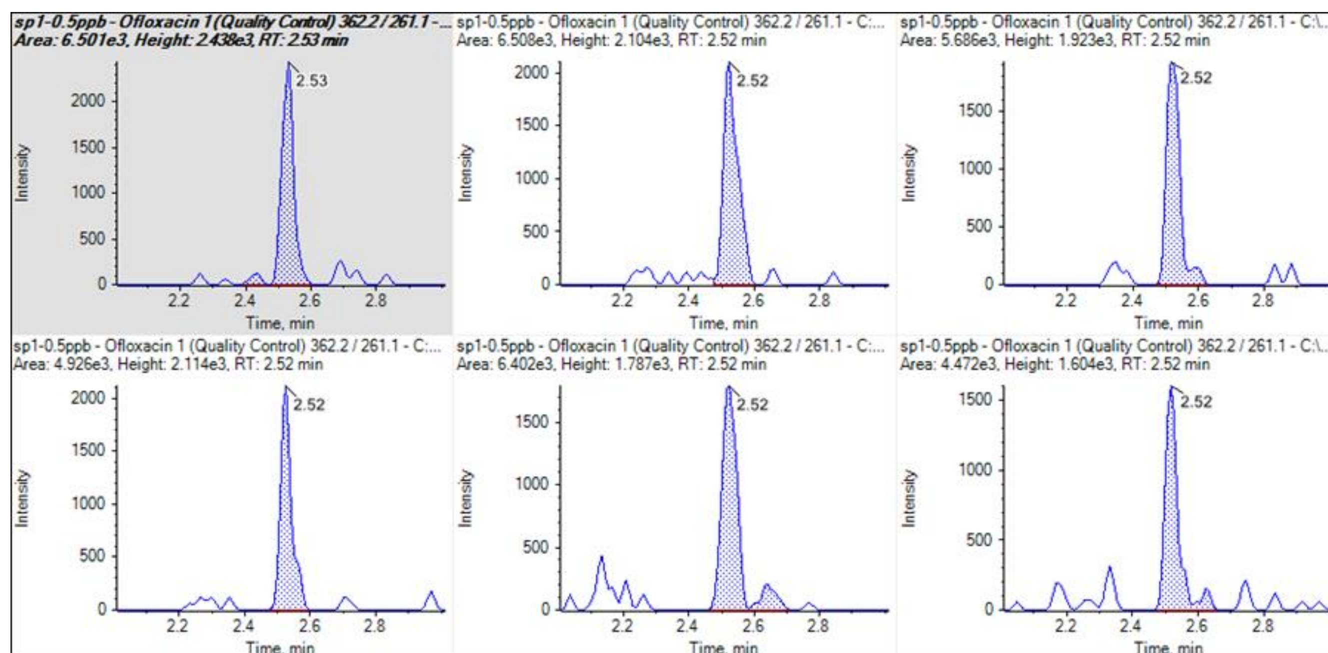

Figure 3. Chromatographic peaks of the example veterinary drug, ofloxacin. The target analyte, ofloxacin, was spiked into blank matrix extract at the lowest concentration standard, 0.5 µg/kg. Individual panels represent each replicate injection. Analysis of replicates (n=6) showed excellent reproducibility in sample matrix with RSD <10%.

Conclusions

In this technical note, a method was established using the QTRAP 4500 system for the analysis of 88 veterinary drug residues in animal-derived foods. Sample extraction was optimized using pork as an example matrix. The sample cleanup step was performed using a special cleanup column for animal residues, without activation and equilibration to simplify the pre-processing steps and save sample processing time and labor.

After method development and verification, the recovery rate was high and the reproducibility was good. The limit of quantification for all compounds was between 0.1 µg/kg and 2 µg/kg, which meets regulatory requirements.

References

1. The Ministry of Agriculture Announcement No. 250 List of drugs and other compounds prohibited to be used in food animals.
2. GB 31650-2019 National Food Safety Standard Maximum Residue Limits of Veterinary Drugs in Foods.
3. SCIEX How Method.

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