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Method of Test for Veterinary Drug Residues in Foods- Multiresidue Analysis (2)

1. Scope

This method is applicable to the determination of 48 veterinary drug residues (ciprofloxacin etc. listed in the attached **Table 1** and **Table 2**) in poultry, livestock and aquatic products.

2. Method

After extraction, analytes are determined by liquid chromatography/ tandem mass spectrometry (LC-MS/MS).

2.1. Equipments

2.1.1. Liquid chromatograph/tandem mass spectrometer.

2.1.1.1. Ion source: electrospray ionization, ESI.

2.1.1.2. Column: ACQUITY UPLC® HSS T3, 1.8 μm, 2.1 mm × 10 cm, or an equivalent product.

2.1.2. Homogenizer.

2.1.3. Vortex mixer.

2.1.4. Ultrasonicator.

2.1.5. Centrifuge: centrifugal force > 3500 ×g, temperature control < 4°C.

2.1.6. Shaker.

2.1.7. Rotary evaporator.

2.2. Chemicals

Acetonitrile, HPLC grade;
Methanol, HPLC grade;
Formic acid, reagent grade;
Dimethylformamide, reagent grade;
Sodium sulfate anhydrous, reagent grade;
n-Hexane, reagent grade;
Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);
Ciprofloxacin and other veterinary drugs listed in the attached tables, reference standards.

2.3. Apparatus

2.3.1. Centrifuge tube: 50 mL, PP.

2.3.2. Membrane filter: 0.22 μm, Nylon.

2.3.3. Volumetric flask: 100 mL.

2.4. Reagents

2.4.1. 50% Methanol

Dilute 50 mL of methanol with deionized water to 100 mL.

2.4.2. 5% Methanol in acetonitrile

Dilute 50 mL of methanol with acetonitrile to 1000 mL.

2.4.3. *n*-Hexane saturated with acetonitrile

Add 50 mL of acetonitrile to 500 mL of *n*-hexane, shake and then stand until complete layering. Take the *n*-hexane layer.

2.5. Mobile phase

2.5.1. Solvent A

Mix deionized water and formic acid at the ratio of 99.9: 0.1 (v/v), and filter with a membrane filter.

2.5.2. Solvent B

Mix methanol and formic acid at the ratio of 99.9: 0.1 (v/v), and filter with a membrane filter.

2.6. Standard solution preparation

Transfer about 10 mg of reference standards accurately weighed into each 100-mL volumetric flask, dissolve and dilute with methanol to volume as standard stock solutions. For piromidic acid reference standard, dissolve and dilute with dimethylformamide to volume. When to use, mix appropriate volume of each standard stock solution, and dilute with 50% methanol to 0.005-1.0 μg/mL as the standard solutions.

2.7. Sample solution preparation

Transfer about 5 g of the fine-cut and homogenized sample accurately weighed into a 50-mL centrifuge tube. Add 25 mL of 5% methanol in acetonitrile, and homogenize for 3 min. Add 10 g of sodium sulfate anhydrous, shake for 10 min, centrifuge at 3500 ×g for 10 min at 4°C, and collect the supernatant. Add 25 mL of 5% methanol in acetonitrile to the residue, shake for 10 min, and centrifuge at 3500 ×g for 10 min at 4°C. Combine the supernatants in a separately funnel, add 30 mL of *n*-hexane saturated with acetonitrile, and shake for 3 min for liquid-

liquid partition. Collect the acetonitrile layer, and evaporate to dryness under vacuum at 40°C. Dissolve and dilute the residue with 50% methanol to 1 mL, and filter with a membrane filter. Take the filtrate as the sample solution.

2.8. Matrix-matched calibration curve preparation

Take a blank sample, and follow the procedure described in section 2.7 to obtain the acetonitrile solution after liquid-liquid partition. Take the solution, add 1 mL of the standard solutions at different concentrations separately, and evaporate to near dryness. Dissolve and dilute the residues with 50% methanol to 1 mL, and filter with a membrane filter. Take the filtrates as the matrix-matched standard solutions. Operate LC-MS/MS according to the following conditions. Establish the matrix-matched calibration curve of each veterinary drug by the peak areas of each veterinary drug vs. the added concentrations.

LC-MS/MS operating conditions(note)

Column: ACQUITY UPLC® HSS T3 1.8 μm, 2.1 mm i.d.× 10 cm.

Column temperature: 35°C.

Mobile phase: a gradient program of solvent A and solvent B is as follows.

Time (min)	A (%)	B (%)
0.0 → 2.0	96 → 95	4 → 5
2.0 → 3.0	95 → 80	5 → 20
3.0 → 6.0	80 → 75	20 → 25
6.0 → 8.6	75 → 73	25 → 27
8.6 → 14.5	73 → 63	27 → 37
14.5 → 14.7	63 → 0	37 → 100
14.7 → 18.7	0 → 96	100 → 4
18.7 → 20.0	96 → 96	4 → 4

Flow rate: 0.3 mL/min.

Injection volume: 10 μL.

Capillary voltage: 3.3 kV, ESI^+/ESI^-.

Ion source temperature: 120°C.

Desolvation temperature: 450°C.

Cone gas flow rate: 100 L/hr.

Desolvation flow rate: 800 L/hr.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, cone voltage and collision energy are shown in **Table 1** and **Table 2**.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.9. Identification and quantification

Accurately inject 10 μL of the sample solution and the matrix-matched standard solutions into LC-MS/MS separately. Operate according to the conditions in section 2.8. Identify each veterinary drug based on the retention time and the relative ion intensities(note). Calculate the amount of each veterinary drug in the sample by the following formula:

$$\text{The amount of each veterinary drug in the sample (ppm)} = \frac{C \times V}{M}$$

Where,

C: the concentration of each veterinary drug in the sample solution calculated by the matrix-matched calibration curve (μg/mL)

V: the final make-up volume of the sample (mL)

M: the weight of the sample (g)

Note: Relative ion intensities are calculated by peak areas of quantitative ions divided by peak areas of qualitative ions (≤100%). Maximum permitted tolerances of relative ion intensities are as the follows:

Relative ion intersity (%)	Tolerance (%)
> 50	± 20
> 20 ~ 50	± 25
> 10 ~ 20	± 30
≤ 10	± 50

Remark

1. Limits of quantification (LOQs) for 48 veterinary drugs are listed in **Table 3**.
2. Further validation shall be done when interfering compounds appear in

the samples.

Reference

1. Yamada, R., Kozono, M., Ohmori, T., Morimatsu, F. and Kitayama, M. 2006. Simultaneous determination of residual veterinary drugs in bovine, porcine, and chicken muscle using liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Biosci. Biotechnol. Biochem. 70: 54-65.
2. Sheridan, R. and Desjardins, L. 2006. Determination of abamectin, doramectin, emamectin, eprinomectin, ivermectin, and moxidectin in milk by liquid chromatography electrospray tandm mass spectrometry. J. AOAC Int. 89: 1088-1094.

Table 1. MRM parameters of 46 veterinary drugs (positive ion mode)

No.	Analyte	Quantitative ion pair			Qualitative ion pair		
		Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
1	Azaperol	330 > 121	30	30	330 > 149	30	25
2	Azaperone	328 > 165	30	20	328 > 121	30	20
3	Carazolol	299 > 116	30	20	299 > 222	30	20
4	Ciprofloxacin	332 > 314	30	25	322 > 231	30	45
5	Clopidol	192 > 101	45	25	192 > 87	40	30
6	Danofloxacin	358 > 340	35	30	358 > 283	40	25
7	Dicyclanil	191 > 150	30	25	191 > 175	30	20
8	Difloxacin	400 > 356	35	20	400 > 299	35	30
9	Enrofloxacin	360 > 316	35	20	360 > 245	35	25
10	Eprinomectin	936.5 > 490	15	10	936.4 > 352	15	10
11	Fleroxacin	370 > 326	30	20	370 > 269	35	25
12	Flumequine	262 > 244	25	20	262 > 202	25	30

Table 1. MRM parameters of 46 veterinary drugs (positive ion mode) (continued)

No.	Analyte	Quantitative ion pair			Qualitative ion pair		
		Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
13	Lomefloxacin	352 > 265	30	25	352 > 308	30	15
14	Marbofloxacin	363 > 345	35	20	363 > 72	30	25
15	Morantel	221 > 164	35	25	221 > 149	35	35
16	Nalidixic acid	233 > 215	20	15	233 > 187	20	25
17	Norfloxacin	320 > 302	30	20	320 > 276	30	15
18	Ormetoprim	275 > 259	35	25	275 > 123	35	25
19	Oxolinic acid	262 > 244	25	20	262 > 216	25	35
20	Pefloxacin	334 > 316	30	20	334 > 233	35	25
21	Pipemidic acid	304 > 217	30	20	304 > 189	30	30
22	Piromidic acid	289 > 243	25	30	289 > 271	25	20
23	Sarafloxacin	386 > 368	40	20	386 > 342	35	20
24	Succinylsulfathiazole	356 > 256	35	15	356 > 192	30	25

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Table 1. MRM parameters of 46 veterinary drugs (positive ion mode) (continued)

No.	Analyte	Quantitative ion pair			Qualitative ion pair		
		Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
25	Sulfabenzamide	277 > 156	20	15	277 > 92	20	30
26	Sulfacetamide	215 > 156	15	10	215 > 92	15	25
27	Sulfachlorpyridazine	285 > 156	25	15	285 > 92	20	30
28	Sulfadiazine	251 > 156	25	15	251 > 92	25	25
29	Sulfadimethoxine	311 > 156	35	20	311 > 92	30	35
30	Sulfadoxine	311 > 156	25	20	311 > 92	30	30
31	Sulfaethoxypyridazine	295 > 156	30	20	295 > 92	30	30
32	Sulfaguanidine	215 > 156	20	15	215 > 92	25	25
33	Sulfamerazine	265 > 156	25	15	265 > 92	25	30
34	Sulfameter	281 > 156	25	20	281 > 92	30	30
35	Sulfamethazine	279 > 156	30	20	279 > 186	30	15
36	Sulfamethizole	271 > 156	25	25	271 > 92	25	25

Table 1. MRM parameters of 46 veterinary drugs (positive ion mode) (continued)

No.	Analyte	Quantitative ion pair			Qualitative ion pair		
		Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
37	Sulfamethoxazole	254 > 156	25	15	254 > 92	25	25
38	Sulfamethoxypyridazine	281 > 156	25	15	281 > 92	30	30
39	Sulfamonomethoxine	281 > 156	25	10	281 > 92	30	30
40	Sulfapyridine	250 > 156	25	15	250 > 92	30	30
41	Sulfaquinoxaline	301 > 156	25	15	301 > 92	25	30
42	Sulfathiazole	256 > 156	25	15	256 > 92	25	25
43	Sulfatroxazole	268 > 156	25	15	268 > 92	25	30
44	Tetramisole	205 > 178	35	20	205 > 123	25	30
45	Trichlorfon	259 > 109	20	20	257 > 109	20	20
46	Trimethoprim	291 > 230	35	25	291 > 123	35	25

Table 2. MRM parameters of ethopabate and fluazuron (negative ion mode)

No.	Analyte	Quantitative ion pair			Qualitative ion pair		
		Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
1	Ethopabate	236 > 192	30	25	236 > 132	30	35
2	Fluazuron	504 > 305	30	15	506 > 307	30	15

Table 3. Limits of quantification (LOQs) for 48 veterinary drugs

No.	Analyte	Muscle (ppm)	Visceral (ppm)	Acquatic products (ppm)	Milk (ppm)
1	Azaperol	0.01	0.02	0.01	0.01
2	Azaperone	0.01	0.02	0.01	0.01
3	Carazolol	0.002	0.01	0.002	0.002
4	Ciprofloxacin	0.01	0.02	0.01	0.01
5	Clopidol	0.05	0.10	0.01	0.01
6	Danofloxacin	0.01	0.02	0.01	0.01
7	Dicyclanil	0.01	0.02	0.01	0.01
8	Difloxacin	0.01	0.02	0.01	0.01
9	Enrofloxacin	0.01	0.02	0.01	0.01
10	Eprinomectin	0.01	0.05	0.05	0.01
11	Ethopabate	0.01	0.02	0.01	0.01
12	Fleroxacin	0.01	0.02	0.01	0.01
13	Fluazuron	0.05	0.1	0.05	0.05
14	Flumequine	0.01	0.02	0.01	0.01
15	Lomefloxacin	0.01	0.02	0.01	0.01
16	Marbofloxacin	0.01	0.02	0.01	0.01
17	Morantel	0.01	0.02	0.01	0.01
18	Nalidixic acid	0.01	0.02	0.01	0.01
19	Norfloxacin	0.01	0.02	0.01	0.01
20	Ormetoprim	0.05	0.05	0.05	0.05
21	Oxolinic acid	0.01	0.02	0.01	0.01
22	Pefloxacin	0.01	0.02	0.01	0.01
23	Pipemidic acid	0.01	0.02	0.01	0.01
24	Piromidic acid	0.01	0.02	0.01	0.01
25	Sarafloxacin	0.005	0.02	0.01	0.01
26	Succinylsulfathiazole	0.01	0.02	0.01	0.01
27	Sulfabenzamide	0.01	0.02	0.01	0.01
28	Sulfacetamide	0.01	0.02	0.01	0.01
29	Sulfachlorpyridazine	0.02	0.02	0.01	0.01
30	Sulfadiazine	0.01	0.02	0.01	0.01

Table 3. Limits of quantification (LOQs) for 48 veterinary drugs (continued)

No.	Analyte	Muscle (ppm)	Visceral (ppm)	Acquatic products (ppm)	Milk (ppm)
31	Sulfadimethoxine	0.01	0.02	0.01	0.01
32	Sulfadoxine	0.01	0.02	0.01	0.01
33	Sulfaethoxypyridazine	0.01	0.02	0.01	0.01
34	Sulfaguanidine	0.01	0.02	0.01	0.01
35	Sulfamerazine	0.01	0.02	0.01	0.01
36	Sulfameter	0.01	0.02	0.01	0.01
37	Sulfamethazine	0.01	0.02	0.01	0.01
38	Sulfamethizole	0.01	0.02	0.01	0.01
39	Sulfamethoxazole	0.01	0.02	0.01	0.01
40	Sulfamethoxypyridazine	0.01	0.02	0.01	0.01
41	Sulfamonomethoxine	0.01	0.02	0.01	0.01
42	Sulfapyridine	0.01	0.02	0.01	0.01
43	Sulfaquinoxaline	0.01	0.02	0.01	0.01
44	Sulfathiazole	0.01	0.02	0.01	0.01
45	Sulfatroxazole	0.01	0.02	0.01	0.01
46	Tetramisole	0.01	0.02	0.01	0.01
47	Trichlorfon	0.01	0.02	0.005	0.01
48	Trimethoprim	0.01	0.02	0.01	0.01