Original: Japanese

Provisional Translation

# Analytical Method for Enrofloxacin, Oxolinic Acid, Ofloxacin, Orbifloxacin, Sarafloxacin, Difloxacin, Danofloxacin, Nalidixic Acid, Norfloxacin and Flumequine (Animal and Fishery Products)

## 1. Analytes

| Compositional substances of agricultural chemicals | Analytes |
|---|---|
| Enrofloxacin | Enrofloxacin, Ciprofloxacin |
| Oxolinic Acid | Oxolinic Acid |
| Ofloxacin | Ofloxacin |
| Orbifloxacin | Orbifloxacin |
| Sarafloxacin | Sarafloxacin |
| Difloxacin | Difloxacin |
| Danofloxacin | Danofloxacin |
| Nalidixic Acid | Nalidixic Acid |
| Norfloxacin | Norfloxacin |
| Flumequine | Flumequine |

## 2. Instruments

High performance liquid chromatograph-fluorometric detector (HPLC-FL)

Liquid chromatograph-mass spectrometer (LC-MS)

## 3. Reagents

Use the reagents listed in Section 3 of the General Rules, except the following.

Acetonitrile: Prepared for liquid chromatography.

Water: Prepared for liquid chromatography.

Methanol: Prepared for liquid chromatography.

Divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge (60 mg): Polyethylene tube of 12-13 mm in inside diameter packed with 60 mg of divinylbenzene-*N*-vinylpyrrolidone copolymer, or other cartridge with equal separation characteristics.

Reference standard of enrofloxacin: Contains not less than 98% of enrofloxacin. Melting point of the standard is 219-225°C.

Reference standard of ciprofloxacin: Contains not less than 98% of ciprofloxacin. Melting point of the standard is 255-257°C.

Reference standard of oxolinic acid: Contains not less than 99% of oxolinic acid. Melting point of the standard is 314-316°C.

Reference standard of ofloxacin: Contains not less than 98% of ofloxacin. Melting point of the standard is 250-257°C.

Reference standard of orbifloxacin: Contains not less than 95% of orbifloxacin. Decomposition point of the standard is 263°C.

Reference standard of sarafloxacin hydrochloride: Contains not less than 95% of sarafloxacin hydrochloride. Melting point of the standard is 200°C.

Reference standard of difloxacin hydrochloride: Contains not less than 95% of difloxacin hydrochloride. Melting point of the standard is not less than 275°C.

Reference standard of danofloxacin mesilate: Contains not less than 95% of danofloxacin mesilate. Melting point of the standard is 328°C.

Reference standard of nalidixic acid: Contains not less than 99% of nalidixic acid. Melting point of the standard is 225-230°C.

Reference standard of norfloxacin: Contains not less than 98% of norfloxacin. Melting point of the standard is 220-221°C.

Reference standard of flumequine: Contains not less than 99% of flumequine. Melting point of the standard is 253-255°C.

**4. Procedure**

1) Extraction

Weigh 5.00 g of sample, add 100 mL of acetonitrile/0.2% metaphosphoric acid (2:3, v/v), homogenize, and filter with suction. Add 20 mL of acetonitrile/0.2% metaphosphoric acid (2:3, v/v) to the residue on the filter paper, mix, treat as described above, combine the resulting filtrates, and concentrate to about 30 mL at below 40°C.

2) Clean-up

Add 5 mL each of methanol and water to a divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge (60 mg) sequentially, and discard the effluents. Transfer the solution obtained in 1) to the cartridge, add 5 mL of water, and discard the effluent. Elute with 5 mL of methanol to the cartridge, transfer the eluate to a vacuum rotary evaporator flask, and remove methanol at below 40°C. Dissolve the residue in 1.0 mL of water/methanol (7:3, v/v), and use this solution as the test solution.

**5. Calibration curve**

Prepare 10 mg/100 mL solutions (methanol) of each reference standard. Dilute with water/methanol (7:3, v/v), and prepare 0.05-5 mg/L standard solutions of several concentrations. Inject each standard solution to HPLC, and make calibration curves by peak-height or peak-area method.

**6.** Quantification

Inject the test solution to HPLC, and calculate the concentrations of each analyte from the calibration curves made in **5**.

Quantify enrofloxacin and its metabolite, ciprofloxacin, individually, and regard the sum of the

results as the analytical result of enrofloxacin.

**7. Confirmation**

Confirm using LC-MS.

**8. Measurement conditions**

HPLC

Detector: FL

Enrofloxacin, ciprofloxacin, ofloxacin, orbifloxacin, sarafloxacin, difloxacin, danofloxacin and norfloxacin: Excitation wavelength 290 nm, emission wavelength 450 nm

Oxolinic acid, nalidixic acid and flumequine: Excitation wavelength 325 nm, emission wavelength 365 nm

Column: Octadecylsilanized silica gel, 2.0-6.0 mm in inside diameter, 100-250 mm in length and 2-5 μm in particle diameter

Column temperature: 40°C

Mobile phase: Linear gradient from acetonitrile/0.1% formic acid (1:99, v/v) to (1:0, v/v) in 35 min and hold for 5 min.

Expected retention time: 20 min (enrofloxacin)

**9. Limits of quantification**

0.01 mg/kg for each analyte

**10. Explanatory notes**

1) Outline of analytical method

The method consists of extraction of enrofloxacin, ciprofloxacin, oxolinic acid, ofloxacin, orbifloxacin, sarafloxacin, difloxacin, danofloxacin nalidixic acid, norfloxacin and flumequine from sample with acetonitrile/0.2% metaphosphoric acid, clean-up with a divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge, quantification using HPLC-FL, and confirmation using LC-MS.

2) Notes

i) Typical injection volume of the standard solutions and the test solutions in HPLC-FL and LC-MS for a column with 3.0 mm in inside diameter is 10 μL. The optimum injection volume may vary with the column and instrument used. Consider the optimum injection volume for each column/instrument.

ii) The optimum ionization method and monitoring ions in LC-MS may vary with the instrument used. Consider optimum conditions for each instrument.

**11. References**

Horie, et al., Shokuhin Eiseigaku Zasshi, **36**, 62 (1995)

**12. Type**

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