

**Analytical Method for Enrofloxacin, Oxolinic Acid, Ofloxacin, Orbifloxacin,
Sarafloxacin, Difloxacin, Danofloxacin, Nalidixic Acid, Norfloxacin, Flumequine and
Marbofloxacin
(Honey)**

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
Marbofloxacin	Marbofloxacin

2. Applicable food

Honey

3. Instrument

Liquid chromatograph-tandem mass spectrometer (LC-MS/MS)

4. Reagents

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

Quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge (500 mg): A polyethylene tube of 12–13 mm in inside diameter packed with 500 mg of quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer, or a cartridge equivalent to the specified one in separation capability.

Reference standard of enrofloxacin: Contains not less than 98% of enrofloxacin.

Reference standard of ciprofloxacin: Contains not less than 95% of ciprofloxacin, ciprofloxacin hydrochloride, or ciprofloxacin hydrochloride monohydrate.

Reference standard of oxolinic acid: Contains not less than 98% of oxolinic acid.

Reference standard of ofloxacin: Contains not less than 98% of ofloxacin.

Reference standard of orbifloxacin: Contains not less than 98% of orbifloxacin.

Reference standard of sarafloxacin hydrochloride: Contains not less than 95% of sarafloxacin hydrochloride.

Reference standard of difloxacin: Contains not less than 98% of difloxacin, or difloxacin hydrochloride.

Reference standard of danofloxacin: Contains not less than 98% of danofloxacin, or danofloxacin mesilate.

Reference standard of nalidixic acid: Contains not less than 98% of nalidixic acid.

Reference standard of norfloxacin: Contains not less than 98% of norfloxacin.

Reference standard of flumequine: Contains not less than 98% of flumequine.

Reference standard of marbofloxacin: Contains not less than 98% of marbofloxacin.

25 mmol/L ammonium formate solution containing 25mmol/L formic acid: Add water to 1.15 g of formic acid and 1.58 g of ammonium formate to make exactly 1,000 mL

25 mmol/L ammonium formate-methanol solution containing 25 mmol/L formic acid: Add methanol to 1.15 g of formic acid and 1.58 g of ammonium formate to make exactly 1,000 mL.

5. Procedure

1) Extraction

Weigh 5.00 g of sample, add 20 mL of 1 mmol/L ammonia solution, shake for 10 minutes, centrifuge at 3,000 rpm for 10 minutes, and collect the supernatant.

2) Clean-up

i) Quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer column chromatography

Add 5 mL each of methanol and 1 mol/L ammonia solution to a Quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge sequentially, and discard the effluents. Transfer the solution obtained in 1) to the cartridge, add 10 mL of water, and discard the effluent. Elute with 5 mL of 250 mmol/L formic acid-methanol solution, collect the total eluate.

ii) Ethylenediamine-*N*-propylsilanized silica gel column chromatography

Add 5mL of 250 mmol/L formic acid-methanol solution to ethylenediamine-*N*-propylsilanized silica gel cartridge (500 mg), and discard the effluents. Transfer the solution obtained in i) to the cartridge, elute with 1 mL of 250 mmol/L formic acid-methanol solution, collect the total eluate. Add 50 mmol/L ammonium formate solution to the resulting eluate to make exactly 10 mL, and use this solution as the test solution.

6. Calibration curve

Dissolve reference standard of each analyte in methanol, prepare 100 mg/L stock standard solutions. Mix these stock standard solutions appropriately, dilute with 50 mmol/L ammonium formate solution and 250 mmol/L formic acid/ methanol (2:3, v/v), prepare standard solutions of several concentrations, inject each standard solution to LC-MS/MS, and make a calibration

curve by peak-height or peak-area method. When the test solution is prepared following the above procedure, the sample containing 0.01 mg/kg of each analyte gives the test solution of 0.005 mg/L in concentration.

7. Quantification

Inject the test solution to LC-MS/MS and calculate the concentration of each analytes from the calibration curve made in 6). For analytical result of enrofloxacin, use the sum of enrofloxacin and its metabolite, ciprofloxacin.

8. Confirmation

Confirm using LC-MS/MS.

9. Measurement conditions

Example

Column: Octadecylsilanized silica gel, 3.0 mm in inside diameter, 150 mm in length and 3 μm inparticle diameter

Column temperature: 40°C

Mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

Mobile phase A: 25 mmol/L ammonium formate solution containing 25mmol/L formic acid

Mobile phase B: 25 mmol/L ammonium formate-methanol solution containing 25 mmol/L formic acid

Time (min)	Mobile phase A (%)	Mobile phase B: (%)
0.0	80	20
5.0	80	20
20.0	65	35
25.0	20	80
30.0	0	100
35.0	0	100

Ionization mode: ESI (+)

Major monitoring ions (m/z):

Enrofloxacin ;Precursor ion 360, product ion 342, 316

Ciprofloxacin ; Precursor ion 332, product ion 314, 231

Oxolinic Acid ; Precursor ion 262, product ion 244, 216

Ofloxacin ; Precursor ion 362, product ion 318, 261

Orbifloxacin ; Precursor ion 396, product ion 378, 352

Sarafloxacin ; Precursor ion 386, product ion 368, 299

Difloxacin ; Precursor ion 400, product ion 382, 299
Danofloxacin ; Precursor ion 358, product ion 340, 255
Nalidixic Acid ; Precursor ion 233, product ion 215, 187
Norfloxacin ; Precursor ion 320, product ion 302, 276
Flumequine ; Precursor ion 262, product ion 242, 202
Marbofloxacin ; Precursor ion 363, product ion 345, 72

Injection volume : 10 μ L

Expected retention time:

Enrofloxacin 17 min
Ciprofloxacin 16 min
Oxolinic Acid 25 min
Ofloxacin 14 min
Orbifloxacin 18 min
Sarafloxacin 20 min
Difloxacin 19 min
Danofloxacin 17 min
Nalidixic Acid 26 min
Norfloxacin 15 min
Flumequine 26 min
Marbofloxacin 12 min

10. Limit of quantification

0.01 mg/kg for each analyte

11. Outline of analytical method

The method consists of extraction of enrofloxacin, ciprofloxacin, oxolinic acid, ofloxacin, orbifloxacin, sarafloxacin, difloxacin, danofloxacin nalidixic acid, norfloxacin, flumequine and marbofloxacin from sample of honey with 1 mmol/L ammonia solution, clean-up with a quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge and ethylenediamine-*N*-propylsilanized silica gel cartridge, quantification, and confirmation using LC-MS/MS.

12. Notes

i) When the analytical method for each analyte using LC-MS/MS was developed, the following monitoring ions were used:

Enrofloxacin

for quantification (m/z): precursor ion 360, product ion 342

for confirmation (m/z): precursor ion 360, product ion 316

Ciprofloxacin

for quantification (m/z): precursor ion 332, product ion 314

for confirmation (m/z): precursor ion 332, product ion 231

Oxolinic Acid

for quantification (m/z): precursor ion 262, product ion 244

for confirmation (m/z): precursor ion 262, product ion 216

Ofloxacin

for quantification (m/z): precursor ion 362, product ion 318

for confirmation (m/z): precursor ion 362, product ion 261

Orbifloxacin

for quantification (m/z): precursor ion 396, product ion 352

for confirmation (m/z): precursor ion 396, product ion 378

Sarafloxacin

for quantification (m/z): precursor ion 386, product ion 368

for confirmation (m/z): precursor ion 386, product ion 299

Difloxacin

for quantification (m/z): precursor ion 400, product ion 382

for confirmation (m/z): precursor ion 400, product ion 299

Danofloxacin

for quantification (m/z): precursor ion 358, product ion 340

for confirmation (m/z): precursor ion 358, product ion 255

Nalidixic Acid

for quantification (m/z): precursor ion 233, product ion 215

for confirmation (m/z): precursor ion 233, product ion 187

Norfloxacin

for quantification (m/z): precursor ion 320, product ion 302

for confirmation (m/z): precursor ion 320, product ion 276

Flumequine

for quantification (m/z): precursor ion 262, product ion 202

for confirmation (m/z): precursor ion 262, product ion 244

Marbofloxacin

for quantification (m/z): precursor ion 363, product ion 72

for confirmation (m/z): precursor ion 363, product ion 345

- ii) The linearity of calibration curve for the analytes of this analytical method may deteriorate at low temperature. In such a case, use a cartridge which has few metal impurities on the filler, silicagel surface.
- iii) The analytes of this analytical method may be adsorbed to glass. In the case, use the appliance made by polypropylene.
- iv) Because some analytes determined on this analytical method easily cause the photolysis, procedures in the preparation of test solution should be performed as far as possible promptly.
- v) The foods examined in the development of the analytical method: Honey

13. Reference

None.

14. Type

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