Analytical Method for Ipronidazole, Dimetridazole, Metronidazole, Ronidazole (Targeted to animal and fishery products)

Target compounds to be determined

Ipronidazole: Ipronidazole, 1-Methyl-2-(2′-hydroxyisopropyl)-5-nitroimidazole (Ipronidazole metabolite B)

Dimetridazole: Dimetridazole, 2-Hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI)

Metronidazole: Metronidazole, 1-(2-Hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (Metronidazole metabolite A)

Ronidazole: Ronidazole, HMMNI

1. Instrument

Liquid Chromatograph/Tandem Mass Spectrometer (LC-MS/MS)

2. Reagents and test solutions

Use the reagent listed in Section C Reagent/Test Solution, Etc., Part II Food additives, except the following.

Acetonitrile: Use a reagent not containing any substance that may interfere with the analysis of the target compositional substances.

Acetone: Use a reagent not containing any substance that may interfere with the analysis of the target compositional substances.

Formic Acid: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

Strongly Acidic Cation Exchange Resin Cartridge Column (500 mg): A polyethylene column of 12–13 mm in inner diameter packed with 500 mg of strongly acidic cation exchange resin or a column equivalent to the specified one in separation capability.

n-Hexane: Use a reagent not containing any substance that may interfere with the analysis of the target compositional substances.

Water: Use water suitable for chemical analysis, including distilled water, purified water, or pure water. If it contains any substance that may interfere with analysis of the target compositional substances, wash with an appropriate solvent, such as *n*-hexane, before use.

Anhydrous Sodium Sulfate: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

Methanol: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

3. Reference standard

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

Reference standard of Ipronidazole Metabolite B: Contains not less than 98% of Ipronidazole Metabolite B.

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

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

Reference standard of Metronidazole Metabolite A: Contains not less than 98% of Metronidazole Metabolite A.

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

4. Procedure

a. Extraction

Homogenize 10.0 g of the test sample (for honey, 10.0 g of the test sample dissolved in 10 mL of water) with 1 mL of acetic acid and 50 mL of acetone. Centrifuge the mixture at 3,000 rpm for 5 minutes and collect the supernatant. Homogenize the residue (for honey, the residue dissolved in 5 mL of water) again with 30 mL of acetone, centrifuge under the same conditions, and collect the supernatant. Combine the supernatants and add acetone to make exactly 100 mL.

Transfer exactly 10 mL of the resulting solution and evaporate to about 2 mL at a temperature not exceeding 40ºC. Shake the concentrate with 10 mL of acetonitrile saturated by *n*-hexane and 10 mL of *n*-hexane and centrifuge at 3,000 rpm for 5 minutes. Evaporate the acetonitrile layer at a temperature not exceeding 40ºC to remove the solvent. Dissolve the residue by adding 5 mL of 2% (vol) formic acid.

b. Clean-up

Pour 5 mL of acetonitrile and 5 mL of 2% (vol) formic acid into a strongly acidic cation exchange resin cartridge column (500 mg) and discard the effluent. Pour the solution obtained in section 4-a, then pour 5 mL of 2% (vol) formic acid, 5 mL of methanol, and 5 mL of 0.5% ammonia solution sequentially, and discard the effluent. Pour 10 mL of a 1:3 mixture of acetonitrile and water and collect the eluate.

Dissolve 2 g of ammonium sulfate in the resulting eluate and extract twice by shaking with 10 mL of ethyl acetate each time. Combine the ethyl acetate extracts, add anhydrous sodium sulfate to dehydrate, and remove the anhydrous sodium sulfate by filtration. Evaporate the filtrate at a temperature not exceeding 40ºC to remove the solvent. Dissolve the residue by adding 0.1% (vol) formic acid to make exactly 1 mL. Use this solution as the test solution.

5. Measurement

a. Calibration curve

Prepare several solutions of each Reference Standard in 0.1% (vol) formic acid with different concentrations. Inject them into LC-MS/MS to prepare a calibration curve for each Reference Standard using the peak-height or peak-area method. When the test solution is prepared as directed in this method, the concentration of each target compound in the test solution that is equivalent to 0.0001 mg/kg (0.0002 mg/kg) in the test sample is 0.0001 mg/L (0.0002 mg/L).

b. Quantification

Inject the test solution in LC-MS/MS and determine the content of each compound from the corresponding calibration curve prepared in section 5-a.

c. Confirmation tests

Conduct confirmation tests using LC-MS/MS.

d. Measurement conditions

Column: Octadecylsilanized silica gel (3 mm in inner diameter, 150 mm in length, 3 µm in particle size)

Column temperature: 40°C

Mobile phase: Run 0.1% (vol) formic acid (solution A) and 0.1% formic acid solution in acetonitrile (solution B) at the following concentration gradients:

Time (minute)	Solution A (%)	Solution B (%)
0	98	2
5	90	10
15	5	95
20	5	95

Ionization mode: Electrospray ionization (positive ion mode)

Main ions (m/z)

Ipronidazole: Precursor ion 170; Product ions 124, 109

Ipronidazole Metabolite B: Precursor ion 186; Product ions 168, 122

Dimetridazole: Precursor ion 142; Product ions 96, 95

Metronidazole: Precursor ion 172; Product ions 128, 82

Metronidazole Metabolite A: Precursor ion 188; Product ions 126, 123

Ronidazole: Precursor ion 201; Product ions 140, 55

HMMNI: Precursor ion 158; Product ions 94, 55

Injection volume: 5 µL

Retention time

Ipronidazole: about 15 minutes
Ipronidazole Metabolite B: about 13 minutes
Dimetridazole: about 12 minutes
Metronidazole: about 11 minutes
Metronidazole Metabolite A: about 11 minutes
Ronidazole: about 12 minutes
HMMNI: about 11 minutes

6. Limit of Quantification
Ipronidazole: 0.0001mg/kg
Ipronidazole Metabolite B: 0.0001mg/kg
Dimetridazole: 0.0002mg/kg
Metronidazole: 0.0001mg/kg
Metronidazole Metabolite A: 0.0001mg/kg
Ronidazole: 0.0002mg/kg
HMMNI: 0.0002mg/kg