Original: Japanese

Provisional translation

Analytical Method for Nitarsone and Roxarsone

1. Instrument

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

2. Reagents

Use the reagents 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 compounds.

Formic acid: Use a reagent not containing any substance that may interfere with the analysis of the target compounds.

Divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge column (500 mg): A polyethylene column of 12-13 mm in inside diameter packed with 500 mg of divinylbenzene-*N*-vinylpyrrolidone copolymer, or a column equivalent to the specified one in separation capability.

Dihexylammonium acetate test solution: Dilute approximately 0.5 mol/L dihexylammonium acetate solution with water by 100 times.

Trimethylammonium salt-modified methacrylate polymer cartridge column (500 mg): A polyethylene column of 12-13 mm in inside diameter packed with 500 mg of trimethylammonium salt-modified methacrylate polymer or a column equivalent to the specified one in separation capability.

Benzenesulfonic-propylsilanized silica gel cartridge column (500 mg): A polyethylene column of 8-9 mm in inside diameter packed with 500 mg of benzenesulfonic-propylsilanized silica gel, or a column equivalent to the specified one in separation capability.

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

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

3. Reference standard

Reference standard of nitarsone: Contains not less than 97% of nitarsone.

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

4. Procedure

1) Extraction

i) Muscle, fat, liver, kidney and milk

Add 50 mL of ammonia solution/water/methanol (1:3:16, v/v/v) to 10.0 g of sample, homogenize, and centrifuge at 3,500 rpm for 10 minutes. Collect the supernatant, add 40 mL of ammonia solution/water/methanol (1:3:16, v/v/v) to the residue, homogenize, and centrifuge as described above. Collect the supernatant, combine with the previously obtained supernatant, and add methanol to make exactly 100 mL. Take a 10 mL aliquot of this solution accurately, concentrate at below 40℃, and remove the solvent. Dissolve the residue in 5 mL of ammonia solution/water (1:19, v/v).

ii) Egg

Add 50 mL of ammonia solution/water/methanol (1:1:18, v/v/v) to 10.0 g of sample, homogenize, and centrifuge at 3,500 rpm for 10 minutes. Collect the supernatant, add 40 mL of ammonia solution/water/methanol (1:1:18, v/v/v) to the residue, homogenize, and centrifuge as described above. Collect the supernatant, combine with the previously obtained supernatant, and add methanol to make exactly 100 mL. Take a 10 mL aliquot of this solution accurately, concentrate at below 40℃, and remove the solvent. Dissolve the residue in 5 mL of ammonia solution/water (1:19, v/v).

2) Clean-up

Add 5 mL each of methanol and ammonia solution/water (1:19, v/v) into a trimethylammonium salt-modified methacrylate polymer cartridge column (500 mg) sequentially, and discard the effluents. Add 5 mL each of methanol and formic acid/water (1:9, v/v) into a benzenesulfonic-propylsilanized silica gel cartridge column (500 mg) sequentially, and discard the effluents. Add 5 mL each of methanol and formic acid/water (1:9, v/v) into a divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge column (500 mg) sequentially, and discard effluents. Transfer the solution obtained in **1) Extraction** to the trimethylammonium salt-modified methacrylate polymer cartridge column, add 5 mL each of methanol and water sequentially, and discard the effluents. Connect the benzenesulfonic-propylsilanized silica gel cartridge column to the lower part of the trimethylammonium salt-modified methacrylate polymer cartridge column, then connect divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge column to the bottom of it, transfer 15 mL of formic acid/water (1:9, v/v), and discard the effluents. Detach the trimethylammonium salt-modified methacrylate polymer cartridge column, add 2 mL of formic acid/water (1:9, v/v) into the benzenesulfonic-propylsilanized silica gel cartridge column, and discard the effluents. Detach the benzenesulfonic-propylsilanized silica gel cartridge column, add 10 mL of water into the divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge column, and

discard the effluents. Then add 10 mL of methanol, take the eluate, concentrate at below 40°C, and remove the solvent. Dissolve the residue in acetonitrile/formic acid/dihexylammonium acetate test solution (1:2:7, v/v/v) to make exactly 1 mL, and use this solution as the test solution.

5. Measurement

1) Calibration curve

Dissolve nitarsone and roxarsone in methanol respectively to prepare standard stock solutions. Mix each stock standard solution appropriately, dilute with acetonitrile/formic acid/dihexylammonium acetate test solution (1:2:7, v/v/v), and prepare standard solutions of several concentrations. Inject each standard solution to LC-MS/MS and make calibration curves by peak-height or peak-area method. When the test solution is prepared following **4. Procedure**, the sample containing 0.002 mg/kg of nitarsone and roxarsone gives the test solution of 0.002 mg/L in concentration.

2) Quantification

Inject the test solution to LC-MS/MS and quantify nitarsone and roxarsone from the calibration curves made in **1) Calibration curve**.

3) Confirmation

Confirm using LC-MS/MS.

4) Measurement conditions

(Example)

Column: Octadecylsilanized silica gel (2.0 mm in inside diameter, 150 mm in length and 3 μm in particle diameter)

Column temperature: Maintaining at 40°C

Mobile phase: Initially 0.02 vol% acetic acid-acetonitrile and dihexylammonium acetate test solution (1:9, v/v) for 5 minutes, followed by a linear gradient from (1:9, v/v) to (1:1, v/v) in 10 minutes.

Ionization mode: Electrospray ionization method (negative ion)

Major monitoring ions (*m/z*): Nitarsone: Precursor ion 246, product ions 138, 108
Roxarsone: Precursor ion 262, product ions 153, 123

Injection volume: 5 μL

Expected retention time: Nitarsone: 11 minutes
Roxarsone: 12 minutes