Original: Japanese
Provisional translation

Analytical Method for Sodium Nifurstyrenate

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.

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

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.

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

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

Quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge column (500 mg): A polyethylene column 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.

3. Reference standard

Reference standard of sodium nifurstyrenate: Contains not less than 95% of sodium nifurstyrenate.

4. Procedure

1) Extraction

Add 50 mL of acetonitrile saturated with *n*-hexane, 50 mL of *n*-hexane and 1 mL of formic acid to 10.0 g of sample, and homogenize. Then add 20 g of anhydrous sodium sulfate, and homogenize again. Centrifuge at 3,000 rpm for 5 minutes, discard the *n*-hexane layer, and take the acetonitrile layer. Add 50 mL of acetonitrile to the residue, homogenize, and centrifuge as described above. Take the acetonitrile layer, combine with the previously obtained acetonitrile layer, and add acetonitrile 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 quaternary ammonium salt-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge column (500 mg), and discard the effluents. Transfer the solution obtained in **1) Extraction** to the column, add 5 mL of water and 5 mL of formic acid/water/methanol (1:2:7, v/v/v) sequentially, and discard the effluents. Then add 10 mL of acetonitrile/formic acid (9:1, v/v), take the eluate, concentrate at below 40℃, and remove the solvent. Dissolve the residue in 0.1 vol% formic acid and 0.1 vol% formic acid/acetonitrile (1:1, v/v) to make exactly 1 mL, and use this solution as the test solution.

5. Measurement

1) Calibration curve

Dissolve sodium nifurstyrenate in methanol to prepare standard stock solutions. Prepare several solutions diluted standard stock solutions with 0.1 vol% formic acid and 0.1 vol% formic acid/acetonitrile (1:1, v/v). 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 0.001 mg/kg of sodium nifurstyrenate gives the test solution of 0.001 mg/L in concentration.

2) Quantification

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

3) Confirmation

Confirm using LC-MS/MS.

4) Measurement conditions

(Example)

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

Column temperature: Maintaining at 40℃

Mobile phase: 0.1 vol% formic acid and 0.1 vol% formic acid/acetonitrile (1:1, v/v)

Ionization mode: Electrospray ionization method (negative ion)

Major monitoring ions (*m/z*): Precursor ion 258, product ions 214, 114

Injection volume: 5 μL

Expected retention time: 5 minutes