Original: Japanese
Provisional translation

Analytical Method for Hexazinone (Animal Products)

1. Analytes

Hexazinone

3-Cyclohexyl-6-(methylamino)-1-methyl-1,3,5-triazine-2,4-(1*H*,3*H*)-dione (hereinafter referred to as metabolite B)

3-(4-Hydroxycyclohexyl)-6-(methylamino)-1-methyl-1,3,5-triazine-2,4-(1*H*,3*H*)-dione (hereinafter referred to as metabolite C)

3-Cyclohexyl-6-amino-1-methyl-1,3,5-triazine-2,4-(1*H*,3*H*)-dione (hereinafter referred to as metabolite F)

2. Application

Animal products

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.

Trimethylaminopropylsilanized silica gel/ethylenediamine-*N*-propylsilanized silica gel layered cartridge (500 mg/500 mg): A polyethylene tube of 12−13 mm inside diameter packed with 500 mg each of trimethylaminopropylsilanized silica gel in the upper layer and ethylenediamine-*N*-propylsilanized silica gel in the lower layer, or a cartridge equivalent to the specified one in separation capability.

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

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

Reference standard of metabolite C: Contains not less than 98% of metabolite C.

Reference standard of metabolite F: Contains not less than 98% of metabolite F.

5. Procedure

1) Extraction

Add 50 mL of *n*-hexane and 50 mL of acetonitrile saturated with *n*-hexane to 10.0 g (5.00 g for fat) of sample, homogenize, centrifuge at 3,000 rpm for 10 min, and collect the acetonitrile layer. Add 25 mL of acetonitrile saturated with *n*-hexane to the residue and *n*-hexane layer, homogenize, and centrifuge as described above. Combine the acetonitrile layer with the previous acetonitrile layer and add acetonitrile to make exactly 100 mL.

2) Clean-up

Inject 10 mL of acetonitrile into a trimethylaminopropylsilanized silica gel/ethylenediamine-*N*-propylsilanized silica gel layered cartridge (500 mg/500 mg) and discard the effluent.

Transfer exactly 4 mL of the solution obtained in 1) to the cartridge, add 20 mL of acetonitrile, concentrate the total eluate at below 40°C, and remove the solvent. Dissolve the residue in methanol to make exactly 4 mL (2 mL for fat) and use these solutions as the test solution.

6. Calibration curve

Dissolve each reference standard of hexazinone, metabolite B, metabolite C and metabolite F in methanol respectively to make stock standard solutions. Mix each stock standard solution appropriately, dilute with methanol, and prepare standard solutions of several concentrations. Inject each solution into LC-MS/MS, and make calibration curves by peak-height or peak-area method. When the test solution is prepared following the above procedure, the concentration of each analyte in the test solution corresponding to 0.0025 mg/kg (equivalent to hexazinone) in the sample is 0.00025 mg/L (equivalent to hexazinone).

7. Quantification

1) Animal products, except milk

Inject the test solution into LC-MS/MS, and calculate each concentration of hexazinone, metabolite B and metabolite F from the calibration curve prepared in **6**. Use the following equation to calculate the concentration of hexazinone including metabolite B and metabolite F.

Concentration (ppm) of hexazinone (including metabolite B and metabolite F) = A + B × 1.059 + C × 1.125

A: Concentration (ppm) of hexazinone

B: Concentration (ppm) of metabolite B

C: Concentration (ppm) of metabolite F

2) Milk

Inject the test solution into LC-MS/MS, and calculate each concentration of hexazinone, metabolite B, metabolite C and metabolite F from the calibration curve prepared in **6**. Use the following equation to calculate the concentration of hexazinone including metabolite B, metabolite C and metabolite F.

Concentration (ppm) of hexazinone (including metabolite B, metabolite C and metabolite F) = A + B × 1.059+ C × 0.9922 + D × 1.125

A: Concentration (ppm) of hexazinone

B: Concentration (ppm) of metabolite B

C: Concentration (ppm) of metabolite C

D: Concentration (ppm) of metabolite F

8. Confirmation

Confirm using LC-MS/MS.

9. Measurement conditions

(Example)

Column: Octadecylsilanized silica gel, 2.1 mm inside diameter, 150 mm in length and 3 μm in particle 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: 5 mmol/L ammonium acetate solution

Mobile phase B: 5 mmol/L ammonium acetate-methanol solution

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	85	15
1	60	40
3.5	60	40
6	50	50
8	45	55
17.5	5	95

Ionization mode: ESI (+)

Major monitoring ions (*m/z*)

Hexazinone: Precursor ion 253, product ions 171, 71

Metabolite B: Precursor ion 239, product ions 157, 71

Metabolite C: Precursor ion 255, product ions 157, 71

Metabolite F: Precursor ion 225, product ions 143, 101

Injection volume: 5 μL

Expected retention time

Hexazinone: 15 min

Metabolite B: 14 min

Metabolite C: 6 min and 7 min

Metabolite F: 13 min

10. Limit of quantification

0.0025 mg/kg for each analyte (equivalent to hexazinone)

11. Explanatory note

1) Outline of analytical method

The method consists of extracting hexazinone, metabolite B, metabolite C and metabolite F from sample with acetonitrile in the presence of *n*-hexane, cleaning up with a trimethylaminopropylsilanized silica gel/ethylenediamine-*N*-propylsilanized silica gel layered cartridge, and quantifying and confirming using LC-MS/MS. In the method, for animal products (except milk), hexazinone, metabolite B and metabolite F are quantified respectively and the concentration of hexazinone, metabolite B and metabolite F is converted to the concentration of hexazinone, and the sum of these concentrations is regarded as the

analytical result of hexazinone. For milk, hexazinone, metabolite B, metabolite C and metabolite F are quantified respectively and the concentration of hexazinone, metabolite B, metabolite C and metabolite F is converted to the concentration of hexazinone and the sum of these concentrations is regarded as the analytical result of hexazinone.

2) Notes

i) Metabolite C is a mixture of isomers and is quantified for the sum of each isomer.

ii) When the analytical method for hexazinone, metabolite B, metabolite C and metabolite F using LC-MS/MS was developed, the following monitoring ions were used:

Hexazinone

for quantitative ions (*m/z*): precursor ion 253, product ion 171

for qualitative ions (*m/z*): precursor ion 253, product ion 71

Metabolite B

for quantitative ions (*m/z*): precursor ion 239, product ion 157

for qualitative ions (*m/z*): precursor ion 239, product ion 71

Metabolite C

for quantitative ions (*m/z*): precursor ion 255, product ion 157

for qualitative ions (*m/z*): precursor ion 255, product ion 71

Metabolite F

for quantitative ions (*m/z*): precursor ion 225, product ion 143

for qualitative ions (*m/z*): precursor ion 225, product ion 101

iii) In the extraction operation, it is recommended to filter the acetonitrile layer through filter paper if residues from the sample are observed in the acetonitrile layer after centrifugation.

iv) Food items used to develop the analytical method: cattle muscle, cattle fat, cattle liver and cattle milk.

12. Reference

None

13. Type

C