Original: Japanese

Provisional Translation

Analytical Method for Cafenstrole (Animal and Fishery Products)

1. Analyte

Cafenstrole

3-(2,4,6-Trimethylphenylsulfonyl)-1,2,4-triazole (hereafter referred to as the metabolite)

2. Instrument

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

3. Reagents

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

Reference standard of cafenstrole: Contains not less than 95% of cafenstrole.

Reference standard of the metabolite: Contains not less than 95% of the metabolite.

4. Procedure

1) Extraction

i) Muscle, liver, kidney, milk, egg, fish and shellfish

Add 20 mL of 0.1 mol/L hydrochloric acid to 10.0 g of sample. Add 100 mL of acetone, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and filter with suction. Combine the resulting filtrates, and add acetone to make exactly 200 mL. Take a 4 mL aliquot of the extract, and add 16 mL of water.

ii) Honey

Dissolve 10.0 g of sample in 20 mL of 0.1 mol/L hydrochloric acid. Add 100 mL of acetone, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and filter with suction. Combine the resulting filtrates, and add acetone to make exactly 200 mL. Take a 4 mL aliquot of the extract, and add 16 mL of water.

iii) Fat

Add 20 mL of 0.1 mol/L hydrochloric acid to 5.00 g of sample. Add 100 mL of acetone, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and filter with suction. Combine the resulting filtrates, and add acetone to make exactly 200 mL. Take an 8 mL aliquot of the extract, and remove acetone at below 40°C. Add 30 mL of *n*-hexane to the residue, and extract with shaking twice with 30 mL of acetonitrile saturated with *n*-hexane. Combine the extracts, remove solvent at below 40°C. Dissolve the residue in 4 mL of acetone, and add 16 mL of water.

2) Clean-up

Add 5 mL each of acetonitrile and water to an octadecylsilanized silica gel cartridge (1,000 mg) sequentially, and discard the effluents. Transfer the solution obtained in 1) to the cartridge, add 10 mL of acetonitrile/water (1:4, v/v), and discard the effluent. Elute with 10 mL of acetonitrile/water (3:2, v/v), add acetonitrile/water (3:2, v/v) to the eluate to make exactly 10 mL, and use this

solution as the test solution.

5. Calibration curve

Prepare cafenstrole and the metabolite standard solutions (acetonitrile/water (3:2, v/v)) 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 cafenstrole or the metabolite gives the test solution of 0.0002 mg/L in concentration. The concentration of the metabolite is calculated as that of cafenstrole.

6. Quantification

Inject the test solution to LC-MS/MS, and calculate the concentration of cafenstrole and the metabolite from the calibration curve made in **5**. Use the following equation to calculate the concentration of cafenstrole including that of the metabolite.

Concentration (ppm) of cafenstrole (including that of the metabolite) = A + B × 1.395

A: Concentration (ppm) of cafenstrole

B: Concentration (ppm) of the metabolite

7. Confirmation

Confirm using LC-MS/MS.

8. Measurement conditions

Example

Column: Octadecylsilanized silica gel, 2.1 mm in inside diameter, 150 mm in length and 5 μm in particle diameter

Column temperature: 40°C

Mobile phase: Linear gradient from acetonitrile/5 mmol/L ammonium acetate solution (1:4, v/v) to (19:1, v/v) in 15 min and hold for 5 min.

Ionization mode

Cafenstrole: ESI (+)

The metabolite: ESI (-)

Major monitoring ions (*m/z*)

Cafenstrole: precursor ion 351, product ion 100, 72

The metabolite: precursor ion 250, product ion 186, 131

Injection volume: 5 μL

Expected retention time

Cafenstrole: 17 min

The metabolite: 10 min

9. Limit of quantification

0.01 mg/kg for each analyte (The concentration of the metabolite is calculated as cafenstrole.)

10. Explanatory note

1) Outline of analytical method

The method consists of extraction of cafenstrole and the metabolite from sample with acetone under acidic condition (hydrochloric acid), defatting by acetonitrile/hexane partitioning (for fat

only), clean-up with an octadecylsilanized silica gel cartridge, quantification and confirmation using LC-MS/MS.

2) Notes

i) MHLW Director Notice (Syoku-An No.0604002 June 4, 2009) states that cafenstrole should be regarded as a combination of cafenstrole and 3-(2,4,6-trimethylphenylsulfonyl)-1,2,4-triazole for fishery products, and cafenstrole for other foods. Therefore, cafenstrole and the metabolite should be quantified for fishery products, and only cafenstrole for other foods.

ii) Because the metabolite is liable to be lost in the concentration step under reduced pressure, concentrate to about 1 mL under reduced pressure and then remove the solvent under a gentle stream of nitrogen.

iii) Cafenstrole tends to decrease in solvent containing methanol and/or water. To prepare a reference standard solution, use solvents other than those containing methanol and/or water (e.g., acetonitrile). Standard solutions for the calibration curve should be prepared at the time of use and should be refrigerated if stored. The test solutions also should be refrigerated if stored.

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

Cafenstrole

for quantification (*m/z*): precursor ion 351, product ion 100

for confirmation (*m/z*): precursor ion 351, product ion 72

The metabolite

for quantification (*m/z*): precursor ion 250, product ion 186

for confirmation (*m/z*): precursor ion 250, product ion 131

11. References

None

12. Type

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