Original: Japanese
Provisional translation

Analytical Method for Amitraz
(Animal Products)

1. Analyte

Amitraz

N-2,4-Dimethylphenyl-*N'*-methylformamidine (hereinafter referred to as metabolite B)

2. Applicable food

Animal products, milk and honey

3. Instrument

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

4. Reagents

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

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

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

5. Procedure

1) Extraction

For muscle, fat and viscera, weigh the sample accurately. Add a mixture of ethanol and 8 mol/L sodium hydroxide solution (1:1, v/v), which is half the weight of the sample, grind and homogenize, and weigh the sample equivalent to 10.0 g. For milk and honey, add 2.5 mL of 8 mol/L sodium hydroxide solution to 10.0 g of the sample. Add 100 mL of methanol to the sample, homogenize, centrifuge at 3,000 rpm for 5 min, and collect the supernatant. Add 50 mL of methanol to the residue, homogenize, centrifuge as described above. Combine the resulting supernatant, and add methanol to make exactly 200 mL. Take exactly a 2 mL aliquot of the solution, transfer the solution to a porous diatomaceous earth cartridge (for holding 5 mL), let stand for 10 min, add 30 mL of acetonitrile, and collect the eluate. Add 30 mL of *n*-hexane to the eluate, shake for 5 min, and collect the acetonitrile layer. For the *n*-hexane layer, extract with shaking twice using 30 mL each of acetonitrile saturated with *n*-hexane, and combine the resulting extract with the previously collected acetonitrile layer. Concentrate at below 40℃ and remove the solvent. Dissolve the residue in 2 mL of methanol, add 2 mL of acetonitrile, and shake lightly to mix.

2) Clean-up

Inject 10 mL each of acetonitrile into an octadecylsilanized silica gel cartridge (1,000 mg) and an ethylenediamine-*N*-propylsilanized silica gel cartridge (500 mg) and discard each effluent. Connect the ethylenediamine-*N*-propylsilanized silica gel cartridge to the bottom of the octadecylsilanized silica gel cartridge, transfer the solution obtained in 1) to this cartridge, add 10

mL of acetonitrile, and collect the total elute including transferred solutions. Concentrate the eluate at below 40°C and remove the solvent. Dissolve the residue in a mixture of acetonitrile and methanol (1:1, v/v) to make exactly 2 mL, and use this solution as the test solution for metabolite B. Then, take exactly a 1 mL aliquot of the test solution for metabolite B, add a mixture of acetonitrile and methanol (1:1, v/v) to make exactly 10 mL, and use this solution as the test solution for amitraz.

6. Calibration curve

Prepare amitraz standard solution and metabolite B hydrochloride standard solution (acetonitrile and methanol [1:1, v/v]) 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 amitraz and metabolite B in the test solution corresponding to 0.01 mg/kg in the sample results in 0.00005 mg/L for amitraz and 0.0005 mg/L (equivalent to amitraz) for metabolite B.

7. Quantification

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

Concentration (ppm) of amitraz (including metabolite B) = A + B × 1.807

A: Concentration (ppm) of amitraz

B: Concentration (ppm) of metabolite B

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: Initially, 5 mmoL/L ammonium acetate solution and 5 mmoL/L ammonium acetate-methanol solution (9:1, v/v) for 5 min, followed by a linear gradient from (9:1, v/v) to (0:100, v/v) in 10 min, and hold for 10 min.

Ionization mode: ESI (+)

Major monitoring ions (*m/z*)

Amitraz: Precursor ion 294, product ions 163, 122

Metabolite B: Precursor ion 163, product ions 122, 107

Injection volume: 10 μL

Expected retention time:

Amitraz: 18 min

Metabolite B: 14 min

10. Limit of quantification

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

11. Explanatory note

1) Outline of analytical method

The method consists of extraction of amitraz and metabolite B from the sample with methanol under basic conditions, defatting by a porous diatomaceous earth cartridge and acetonitrile/hexane partitioning, clean-up with an octadecylsilanized silica gel cartridge and an ethylenediamine-*N*-propylsilanized silica gel cartridge, and quantification and confirmation using LC-MS/MS. In the method, amitraz and metabolite B are quantified respectively. For the concentration of amitraz including metabolite B, the concentration of metabolite B is converted to the concentration of amitraz by multiplying by the conversion factor, and the sum of the concentrations of amitraz and metabolite B is regarded as the analytical result of amitraz.

2) Notes

i) When the analytical methods for amitraz and metabolite B using LC-MS/MS were developed, the following monitoring ions were used:

Amitraz

for quantitative ions (*m/z*): precursor ion 294, product ion 163

for qualitative ions (*m/z*): precursor ion 294, product ion 122

Metabolite B

for quantitative ions (*m/z*): precursor ion 163, product ion 122

for qualitative ions (*m/z*): precursor ion 163, product ion 107

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

12. References

None

13. Type

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