Original: Japanese

Provisional Translation

Analytical Method for Brodifacoum and Warfarin (Animal and Fishery Products)

1. Analytes

Brodifacoum

Warfarin

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 brodifacoum: Contains not less than 96% of brodifacoum.

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

4. Procedure

1) Extraction

For muscle, liver, kidney, fish, shellfish, milk and egg, weigh 10.0 g of sample. For fat, weigh 5.00 g of sample. For honey, weigh 10.0 g of sample and dissolve in 20 mL of water.

Add 1 mL of acetic acid and 100 mL of acetone, homogenize, and filter with suction. Add 50 mL of acetone to the residue on the filter paper, homogenize, and filter as described above. Combine the resulting filtrates, and concentrate to about 10 mL (30 mL for honey) at below 40°C. Add 100 mL of 10% sodium chloride solution, and extract with shaking twice with 100 mL and 50 mL of ethyl acetate/*n*-hexane (1:1, v/v). Dehydrate the extract with anhydrous sodium sulfate, filter out the anhydrous sodium sulfate, and add ethyl acetate/*n*-hexane (1:1, v/v) to make exactly 200 mL.

Take a 20 mL (40 mL for fat) aliquot of the extract, concentrate at below 40°C and remove the solvent. Add 30 mL of *n*-hexane to the residue, and extract with shaking three times with 30 mL of acetonitrile saturated with *n*-hexane. Combine the resulting extracts, concentrate at below 40°C, and remove the solvent. Dissolve the residue in 2 mL of acetone/*n*-hexane (1:1, v/v).

2) Clean-up

Add 10 mL of acetone/*n*-hexane (1:1, v/v) to an ethylenediamine-*N*-propylsilanized silica gel cartridge (500 mg) and discard the effluent. Transfer the extract obtained in 1) to the cartridge, add 8 mL of acetone/*n*-hexane (1:1, v/v), and discard effluent. Elute with 20 mL of acetone/formic acid/*n*-hexane (25:1:25, v/v/v), concentrate the eluate at below 40°C, and remove the solvent. Dissolve the residue in methanol to make exactly 1 mL, and use this solution as the test solution.

5. Calibration curve

Prepare 1 mg/L brodifacoum stock standard solution (acetonitrile) and 1 mg/mL warfarin stock standard solution (acetonitrile). After mixing each stock standard solution, dilute with methanol to prepare standard solutions 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 0.0005 mg/kg of brodifacoum gives the test solution of 0.0005 mg/L in concentration, and the sample containing 0.001 mg/kg of warfarin gives the test solution of 0.001 mg/L in concentration.

6. Quantification

Inject 5 μL of the test solution to LC-MS/MS, and calculate the concentration of brodifacoum and warfarin from the calibration curves made in **5**.

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 3 μm in particle diameter

Column temperature: 40°C

Mobile phase: Linear gradient from 10 mmol/L ammonium acetate solution/10 mmol/L ammonium acetate-methanol solution (4:1, v/v) to (1:19, v/v) in 15 min and hold for 10 min.

Ionization mode: ESI (−)

Major monitoring ions (*m/z*)

Brodifacoum: precursor ion 523, product ion 135, 81

Warfarin: precursor ion 307, product ion 250, 161

Expected retention time

Brodifacoum: 20 min

Warfarin: 14 min

9. Limit of quantification

Brodifacoum: 0.0005 mg/kg

Warfarin: 0.001 mg/kg

10. Explanatory note

1) Outline of analytical method

The method consists of extraction of brodifacoum and warfarin from sample with acetone acidified with acetic acid, transferring of the extract into ethyl acetate/*n*-hexane (1:1, v/v), defatting by acetonitrile/hexane partitioning, clean-up with an ethylenediamine-*N*-propyl-silanized silica gel cartridge, and quantification and confirmation using LC-MS/MS.

2) Notes

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

for quantification (*m/z*): precursor ion 523, product ion 135

for confirmation (*m/z*): precursor ion 523, product ion 81

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

for quantification (*m/z*): precursor ion 307, product ion 161

for confirmation (*m/z*): precursor ion 307, product ion 250

iii) It is recommended that the LC column is washed with a mobile phase containing a high percentage of methanol after brodifacoum has been eluted to decrease the effect of carry-over of interfering components.

iv) Defatting by acetonitrile/hexane partitioning can be omitted for extracts of foods that contain only small amounts of fat.

v) If emulsification occurs during the extraction procedure, the sample should be centrifuged at 3,000 rpm for 5 minutes.

vi) Interfering components, such as fatty acids, are eluted from the ethylenediamine-*N*-propyl-silanized silica gel cartridge under the conditions described in the method; therefore, it is recommended that the test solution is diluted with methanol before the LC-MS/MS analysis if the sensitivity of instrument is sufficiently high.

11. References

None

12. Type

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