Original: Japanese
Provisional translation

Analytical Method for Tylosin
(Animal Products)

1. Analytes

Tylosin A

Tylosin B

2. Applicable food

Tylosin A: Animal products and honey

Tylosin B: 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 tylosin A: Contains not less than 97% of tylosin A.

Reference standard of tylosin B: Use a reference standard that clearly shows its purity.

5. Procedure

1) Extraction

i) Muscle, fat, liver and kidney

Freeze the sample, cut it into small pieces about 5 mm square or less, weigh it accurately, and add the same amount of ethanol and 20 vol% acetic acid (1:1, v/v) in an equal weight ratio. After grind and homogenize, weigh the sample equivalent to 10.0 g. Add 50 mL of acetone to the sample, homogenize, centrifuge at 3,000 rpm for 5 min, and collect the supernatant. Add 25 mL of acetone to the residue, homogenize, centrifuge as described above, combine the resulting supernatants, and add acetone to make exactly 100 mL. Take exactly a 2 mL aliquot of the solution, concentrate to approximately 1 mL under a nitrogen stream, and add 10 mL of water. After adding 10 mL of *n*-hexane and shaking, repeat the operation of centrifuging at 3,000 rpm for 5 min and discarding the *n*-hexane layer twice.

ii) Milk and eggs

Weigh the ice-cooled sample accurately, add the same amount of ethanol and 20 vol% acetic acid (1:1, v/v) in an equal weight ratio, homogenize, and weigh the sample equivalent to 10.0 g. Add 50 mL of acetone to the sample, homogenize, centrifuge at 3,000 rpm for 5 min, and collect the supernatant. Add 25 mL of acetone to the residue, homogenize, centrifuge as described above, combine the resulting supernatants, and add acetone to make exactly 100 mL. Take exactly a 2 mL aliquot of the solution, concentrate it to approximately 1 mL under a

nitrogen stream, and add 10 mL of water. After adding 10 mL of *n*-hexane and shaking, repeat the operation of centrifuging at 3,000 rpm for 5 min and discarding the *n*-hexane layer twice.

iii) Honey

Weigh the ice-cooled sample accurately, add the same amount of ethanol and 20 vol% acetic acid (1:1, v/v) in an equal weight ratio, homogenize, and weigh the sample equivalent to 10.0 g. Add 50 mL of acetone to the sample, homogenize, centrifuge at 3,000 rpm for 5 min, and collect the supernatant. Dissolve the residue in 5 mL of water, add 25 mL of acetone, homogenize, and centrifuge as described above. Combine the resulting supernatants, and add acetone to make exactly 100 mL. Take exactly a 2 mL aliquot of the solution, concentrate to approximately 1 mL under a nitrogen stream, and add 10 mL of water.

2) Clean-up

Inject 5 mL each of methanol and water into an octadecylsilanized silica gel cartridge (500 mg) sequentially and discard each effluent. After transferring the solution obtained in 1) to the cartridge, add 5 mL each of water and a mixture of water and methanol (1:1, v/v) sequentially, and discard each effluent. Then, add a 10 mL mixture of acetic acid, water and methanol (1:29:70, v/v/v), take the eluate, add a mixture of acetic acid, water and methanol (1:29:70, v/v/v) to make exactly 10 mL, and use this solution as the test solution.

6. Calibration curve

Prepare stock standard solutions using tylosin A reference standard and tylosin B reference standard, respectively. Dilute each stock standard solution with a mixture of acetic acid, water and methanol (1:29:70, v/v/v) 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 tylosin A and tylosin B in the test solution corresponding to 0.005 mg/kg (equivalent to tylosin A) in the sample results in 0.0001 mg/L (equivalent to tylosin A) for each analyte.

7. Quantification

1) Except for honey

Inject the test solution into LC-MS/MS, and calculate the concentration of tylosin A from the calibration curve made in 6.

2) Honey

Inject the test solution into LC-MS/MS, calculate the concentration of tylosin A and tylosin B from the calibration curve made in 6, and calculate the concentration of tylosin A including tylosin B using the following equation.

Concentration of tylosin A (including tylosin B) (ppm) = $A + B \times 1.187$

A: Concentration of tylosin A (ppm)

B: Concentration of tylosin B (ppm)

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, 0.1 vol% formic acid-acetonitrile solution and 5 mmol/L ammonium formate solution containing 0.1 vol% formic acid (1:4, v/v) for 5 min, followed by a linear gradient from (1:4, v/v) to (19:1, v/v) in 10 min.

Ionization mode: ESI (+)

Major monitoring ion (*m/z*)

Tylosin A: Precursor ion 916, product ions 772, 174

Tylosin B: Precursor ion 772, product ions 174, 88

Injection volume: 5 μL

Expected retention time

Tylosin A: 12 min

Tylosin B: 12 min

10. Limit of quantification

0.005 mg/kg for each analyte (equivalent to tylosin A)

11. Explanatory note

1) Outline of analytical method

The method consists of extraction of tylosin A and tylosin B with acetone from the ground and homogenized sample using a mixture of ethanol and 20 vol% acetic acid (1:1, v/v), defatting by *n*-hexane (omit for honey), clean-up with an octadecylsilanized silica gel cartridge, and quantification and confirmation using LC-MS/MS. In the method, tylosin A and tylosin B are quantified respectively. To calculate the concentration of tylosin A including tylosin B, the concentration of tylosin B is converted to the concentration of tylosin A by multiplying by a conversion factor, and the sum of the concentrations of tylosin A and tylosin B is regarded as the analytical result of tylosin.

2) Notes

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

Tylosin A:

for quantitative ions *(m/z)*: precursor ion 917, product ion 174

for qualitative ions (*m/z*): precursor ion 917, product ion 772

Tylosin B:

for quantitative ions *(m/z)*: precursor ion 772, product ion 174

for qualitative ions (*m/z*): precursor ion 772, product ion 88

ii) Tylosin A may decompose in samples (especially liver) at room temperature. Therefore, solid samples (such as muscle, fat, and viscera) should be frozen, thawed on ice until they can be shredded using a cooking knife, etc. (semi-thawed state), and then be shredded. To the shredded samples, add the same amount of ethanol and 20 vol% acetic acid (1:1, v/v) in a weight ratio immediately and homogenize by grinding.

iii) For liquid samples such as milk, eggs, and honey, chill on ice, add the same amount of ethanol and 20 vol% acetic acid (1:1, v/v) in a weight ratio immediately, and homogenize.

iv) The mixtures of ethanol and 20 vol% acetic acid (1:1, v/v) are chilled on ice prior to addition.

v) Homogenized samples should be kept below 0°C until extraction begins.

vi) If *n*-hexane remains when the operation of discarding the *n*-hexane layer repeated twice after centrifugation, remove it completely under a nitrogen stream.

vii) In 4.-Reagents, it is stated that "Reference standard of tylosin B: Use the reference standard that clearly shows its purity", because highly pure reference standards of tylosin B were not available when the analytical method was developed. However, it is desirable to use reference standards with a purity of 95% or higher for analysis if available.

viii) Tylosin A can be converted to tylosin B even in standard solutions. Therefore, the standard solutions of tylosin A and tylosin B should be prepared separately.

ix) Food items used to develop the analytical method

Tylosin A: cattle muscle, cattle fat, cattle liver, milk, chicken egg, and honey

Tylosin B: honey

12. References

None

13. Type

C