Analytical Method for Chloramphenicol
(Targeted to animal and fishery products)

The target compound to be determined is Chloramphenicol and Chloramphenicol Glucuronic Acid Conjugate.

1. Instrument

Liquid Chromatograph/Tandem Mass Spectrometer (LC-MS/MS)

2. Reagents and test solutions

Use the reagent listed in Section C Reagent/Test Solution,Etc.,Part II Food additives, except the following.

Acetonitrile: Use a reagent not containing any substance that may interfere with the analysis of the target compositional substances.

Ethyl acetate: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

Divinylbenzene-*N*-vinylpyrrolidone Co-polymer Cartridge Column (500 mg): A polyethylene column of 12–13 mm in inner diameter packed with 500 mg of divinylbenzene-*N*-vinylpyrrolidone co-polymer or a column equivalent to the specified one in separation capability.

Methanol: Use a reagent not containing any substance that may interfere with analysis of the target compositional substances.

Water: Use water suitable for chemical analysis, including distilled water, purified water, or pure water. If it contains any substance that may interfere with analysis of the target compositional substances, wash with an appropriate solvent, such as *n*-hexane, before use.

β-Glucuronidase (type IX-A): Use β-glucuronidase type IX-A derived from *Escherichia coli.* One unit of this substance is the amount of enzyme that produces 1.0 μg of phenolphthalein at pH 6.8 at 37°C in one hour when phenolphthalein β-D-glucuronido is used as the substrate.

β-Glucuronidase Solution: Dissolve an appropriate amount of β-glucuronidase (type IX-A) in 0.1 mol/L phosphate buffer (pH 6.8) to make a solution of 1500 units/mL. Prepare before use.

0.1 mol/L Phosphate Buffer (pH 6.8): Dissolve 1.36 g of potassium dihydrogen phosphate in water to make 100 mL (Solution 1). Dissolve 1.42 g of disodium hydrogenphosphate in water to make 100 mL (Solution 2). Mix the two solutions and adjust the pH to 6.8.

3. Reference standard

Reference standard of Chloramphenicol : Contains not less than 98% of Chloramphenicol

4. Procedure

a. Extraction

In the case of muscle, fat, liver, kidney, and fish/shellfish, weigh 10.0 g of the test sample, previously chopped and homogenized. In the case of milk, egg, honey, and royal jelly, weigh 10.0 g of the test sample, previously mixed well uniformly. In the case of dried royal jelly, weigh 5.00 g of the test sample, previously mixed well uniformly, add 10 mL of water, and allow to stand for 30 minutes.

To the weighed sample, add 50 mL of methanol, homogenize the mixture, and centrifuge at 3,000 rpm for 5 minutes to collect the supernatant. To the residue, add 30 mL of methanol, homogenize the mixture, and centrifuge under the same conditions to collect the supernatant. Combine the two supernatants to make exactly 100 mL with methanol. Transfer exactly 4 mL and remove the solvent at a temperature not exceeding 40ºC. To the residue, add 9 mL of 0.1 mol/L phosphate buffer (pH 6.8) and mix well using an ultrasonic treatment.

b. Hydrolysis

To the solution prepared in section 4-a, add 1 mL of β-glucuronidase solution and warm for 60 minutes at 37ºC to hydrolyze. Add 10 mL of ethyl acetate and extract by shaking. Centrifuge the mixture at 3000 rpm for 5 minute and collect the ethyl acetate layer. To the water layer, add 10 mL of ethyl acetate, extract by shaking, and centrifuge under the same conditions to collect the ethyl acetate layer. Combine the ethyl acetate layers, evaporate at a temperature not exceeding 40ºC to remove the solvent. To the residue, add 5 mL of a 1:1 mixture of water and methanol and mix well by an ultrasonic treatment.

c. Clean-up

Pour 5 mL of methanol and 5 mL of a 1:1 mixture of water and methanol sequentially into a divinylbenzene-*N*-vinylpyrrolidone co-polymer cartridge column (500 mg) and discard the effluent. Charge the column with 5 mL of the solution obtained in section 4-b, pour 5 mL of a 1:1 mixture of water and methanol, and discard the effluent. Then pour 10 mL of a 1:4 mixture of water and methanol, and evaporate the eluate at a temperature not exceeding 40ºC to remove the solvent. Dissolve the residue in a 3:7 mixture of acetonitrile and water to make exactly 2 mL. For dried royal jelly, make exactly 1 mL.

5. Measurement

a. Calibration curve

Prepare several solutions of Chloramphenicol Reference Standard in a 3:7 mixture of acetonitrile and water with different concentrations. Inject them into LC-MS/MS to prepare a calibration curve using the peak-height or peak-area method. When the test solution is prepared as directed in this method, the concentration of chloramphenicol in the test solution that is equivalent to 0.0005 mg/kg in the test sample is 0.0001 mg/L.

b. Quantification

Inject the test solution in LC-MS/MS and determine the content of chloramphenicol from the calibration curve prepared in section 5-a.

c. Confirmation tests

Conduct confirmation tests using LC-MS/MS.

d. Measurement conditions

Column: Octadecylsilanized silica gel (2.1 mm in inner diameter, 150 mm in length, 3 μm in particle size)

Column temperature: 40°C

Mobile phase: A 3:7 mixture of acetonitrile and 10 mmol/L ammonium acetate

Ionization mode: ESI (–)

Main ions (m/z): Precursor ion 321; product ions 152

Precursor ion 323; product ions 152

Injection volume: 5 μL

Retention time: About 4 minutes

6. Limit of Quantification

0.0005mg/kg (royal jelly:0.005mg/kg)