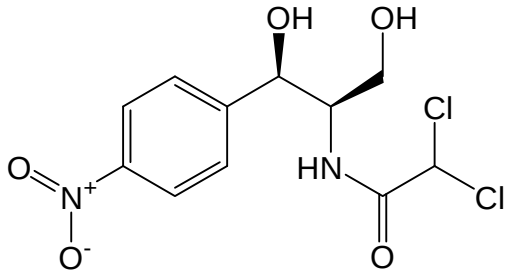
10 Chloramphenicol



2,2-dichloro-*N*-[(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl) ethyl]acetamide

$C_{11}H_{12}Cl_2N_2O_5$ MW: 323.1 CAS No.: 56-75-7

[Summary of Chloramphenicol]

Chloramphenicol (CP) is an antibiotic with a broad antibacterial spectrum and industrially manufactured by a synthetic method. CP can cause side effects, such as aplastic anemia, through toxic effects on the hematopoietic function of the bone marrow.

CP is neither designated nor approved to be used as a feed additive in Japan. However, driven by a report produced by the EU concerning CP contamination of skimmed milk for feeds imported from Russia to Japan in 2001, an analysis method for residual levels of CP was established.

«Standard limits stated in Food Sanitation Act»

Not detected (lower detection limit: 0.0005 ppm)

[Methods listed in the Feed Analysis Standards]

1 Trace quantitative test methods

1.1 Monocomponent analysis by liquid chromatography-tandem mass spectrometry [Feed Analysis Standards Chapter 9, Section 2, 10.1.1]

Scope of application: Fish meal and formula feed (excluding those containing skimmed milk as the main ingredient)

A. Reagent preparation

1) Chloramphenicol standard solution. Weigh accurately 20 mg of chloramphenicol [$C_{11}H_{12}Cl_2N_2O_5$], place in a 200-mL one-mark flask, add acetonitrile to dissolve, and further add acetonitrile up to the marked line to prepare a chloramphenicol standard stock solution (1 mL of this solution contains an amount equivalent to 0.1 mg of chloramphenicol).

Accurately dilute a quantity of the standard stock solution with acetonitrile to prepare a standard solution containing an amount equivalent to 0.5 μg of chloramphenicol in 1 mL.

At the time of use, accurately dilute a quantity of the said standard solution with a mixture of water and acetonitrile (7:3) to prepare a chloramphenicol standard solution that contains an amount equivalent to 0.05 μg of chloramphenicol in 1 mL.

2) Internal standard solution[1]. Place accurately 1 mL of a chloramphenicol standard stock solution labeled with a stable isotope (CP-d_5) (concentration of 100 μg/mL)[Note 1] in a 100-mL one-mark flask, and add acetonitrile up to the marked line to prepare a standard solution that contains an amount equivalent to 1 μg of CP-d_5 in 1 mL.

At the time of use, accurately dilute a quantity of the standard solution with acetonitrile to prepare an internal standard solution that contains an amount equivalent to 50 ng of CP-d_5 in 1 mL.

3) Standard solution for preparing calibration curve. Accurately dilute quantities of the chloramphenicol standard solution and a quantity of the internal standard solution with a mixture of water and acetonitrile (7:3) to prepare several standard solutions for preparing a calibration curve that contain amounts equivalent to 1 to 20 ng of chloramphenicol and 2.5 ng of CP-d_5 in 1 mL.

B. Quantification

Extraction. Weigh 10.0 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, and add accurately 1 mL of the internal standard solution. Add 100 mL of a mixture of methanol and 1 % metaphosphoric acid solution (3:2), and extract with shaking for 30 minutes. Place a 200-mL one-mark flask under a Buchner funnel, and filter the extract under vacuum through glass-fiber filter paper loaded with siliceous earth 2 mm thick. Wash the Erlenmeyer flask and the residue in the funnel with 50 mL of water, filter under vacuum in the same manner, and add water to the one-mark flask up to the marked line. Transfer accurately 20 mL of this liquid to a 300-mL round-bottom flask, condense to approximately 10 mL under reduced pressure in a water bath at 40°C or lower[2], and use as the sample solution subject to column treatment I.

Column treatment I[Note 2]. Wash a divinylbenzene-*N*-vinylpyrrolidone copolymer minicolumn (60 mg)[Note 3] with 5 mL of methanol and 5 mL of water. Place the sample solution in the minicolumn, wash the round-bottom flask that contained the sample solution twice with 10 mL of water, transfer the washing liquid each time to the minicolumn, and allow to flow out until the liquid level reaches the top of the column packing material[3]. Add 4 mL of a mixture of water and methanol (4:1) to wash the minicolumn, and remove the water in the minicolumn under reduced pressure for 10 minutes using a vacuum manifold.

Place a 50-mL round-bottom flask under the minicolumn, add 10 mL of acetonitrile to the minicolumn to elute chloramphenicol, and use the eluate as the sample solution subject to column treatment II.

Column treatment II. Wash a neutral alumina minicolumn (1,710 mg)[Note 4] with 5 mL of acetonitrile. Place the sample solution in the minicolumn, and allow to flow out until the liquid level reaches the top of the column packing material. Then add 5 mL of acetonitrile to the minicolumn and allow to flow out in the same manner.

Place a 100-mL round-bottom flask under the minicolumn and add 40 mL of acetonitrile solution (19:1) to the minicolumn to elute chloramphenicol. Condense the eluate to approximately 1 mL under reduced pressure in a water bath at 40°C or lower, and introduce nitrogen gas to evaporate into dryness. Add accurately 2 mL of a mixture of water and acetonitrile (7:3) to dissolve the residue[4], centrifuge at 5,000×g for 5 minutes, and use the supernatant liquid as the sample solution subject to liquid chromatography-tandem mass spectrometry.

Measurement by liquid chromatography-tandem mass spectrometry. Inject each 10 μL of the sample solution and the standard solutions for preparing a calibration curve into a liquid chromatograph-tandem mass spectrometer to obtain selective reaction monitoring chromatograms.

Operating conditions (example)

(Liquid chromatograph part)

Column: Octadecylsilanized silica gel column (2.0 mm in internal diameter, 150 mm in length, 2.2 μm in particle size)[Note 5]

Eluent: A mixture of 10 mmol/L ammonium formate solution and acetonitrile (7:3) (retained for 1 min) → 9 min → (1:19) (retained for 10 min) → 0.1 min → (7:3) (retained for 10 min)

Flow rate: 0.18 mL/min

Column oven temperature: 40°C

(Tandem mass spectrometer part[Note 6])

Ionization method: Electrospray ionization (ESI) method (negative-ion mode)

Nebulizer gas: N_2 (600 L/h)

Cone gas: N_2 (50 L/h)

Cone voltage: 25 V

Capillary voltage: 1 kV

Collision energy: 20 eV

Ion source temperature: 120°C

Monitored ions: Described below.

Table Monitored ions for each compound

Name of compounds	Precursor ion (m/z)	Product ion (m/z)	Confirmation ion (m/z)
Chloramphenicol	321	152	257
Chloramphenicol d_5	326	157	—

Calculation. Calculate the peak areas of chloramphenicol and CP-d_5 from the selective reaction monitoring chromatograms[5] to prepare a calibration curve by the internal standard method, and estimate the amount of chloramphenicol in the sample.

Note 1. The one manufactured by Wako Pure Chemical Industries, Ltd. or an equivalent

2. Set the flow rate to about 1 mL/min. Use a vacuum manifold as occasion demands.

3. Oasis HLB (Waters, equipped with a reservoir with a capacity of 3 mL) or an equivalent.

4. Sep-Pak Plus Alumina N (Waters) connected with a reservoir with a suitable capacity or an equivalent

5. Shim-pack XR-ODS II (Shimadzu Corporation; the retention time of chloramphenicol under the said operating conditions is approximately 5 minutes) or an equivalent

6. Example operating conditions for Quattro micro API Mass Analyzer (Waters)

«Summary of analysis method»

This method is intended to determine the amount of chloramphenicol in an analysis sample by

liquid chromatography-tandem mass spectrometry using a sample solution prepared by adding a stable isotope-labeled internal standard to the analysis sample, extracting with methanol containing metaphosphoric acid solution, and purifying through a divinylbenzene-N-vinylpyrrolidone copolymer minicolumn and a neutral alumina minicolumn.

The flow sheet of this method is shown in Figure 9.2.10-1.

10.0 g of the sample (200-mL stoppered Erlenmeyer flask)
- Add 0.05 μg of internal standard substance
- Add 100 mL of metahnol-1 % metaphosphoric acid solution (3:2) and shake for 30 min.
- Filter under vacuum through glass-fiber filter paper loaded with siliceous earth (GF/A) (into a 200-mL one-mark flask).
- Wash the Erlenmeyer flask and the residue in the funnel with 50 mL of water.
- Adjust the volume with water.

Filtrate
- Collect 20 mL (into a 300-mL round-bottom flask).
- Condense to about 10 mL under reduced pressure (at 40 °C or lower).

Column treatment I
- Wash a divinylbenzene-*N*-vinylpyrrolidone copolymer minicolumn with 5 mL of methanol and 5 mL of water
- Load the sample solution.
- Wash the flask twice with 10 mL of water and load the washing fluid into the column.
- Wash the column with 4 mL of water-methanol (4:1).
- Remote the water in the column under reduced pressure for 10 min using a manifold.
- Elute with 10 mL of acetonitrile (into a 50-mL round-bottom flask).

Column treatment II
- Wash a neutral alumina minicolumn with 5 mL of acetonitrile.
- Load the sample solution.
- Wash the column with 5 mL of acetonitrile.
- Elute with 40 mL of acetonitrile-water (19:1) (into a 100-mL round bottom flask).
- Condense under reduced pressure (at 40 °C or lower) and evaporate into dryness with nitroger
- Add accurately 2 mL of water-acetonitrile (7:3)
- Centrifuge (at 5,000×g for 5 min).

LC-MS/MS

Figure 9.2.10-1 Trace quantitative test method for chloramphenicol

References: Katsumi Yamamoto, Shinji Oshima: Research Report of Animal Feed, 34, 55 (2009)

History in the Feed Analysis Standards [33] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (μg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Fish meal	2~25	3	98.4~107	9.4
Fattening broiler starter formula feed	5~25	3	94.2~101	12
Sucking piglet grower formula feed	5~25	3	99.0~101	5.5
Silver salmon grower formula feed	2~25	3	89.6~97.7	12

・Collaborative study

Sample type	No. of labs	Spike concentration (μg/kg)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)	HorRat
Fish meal	8	5	104	4.8	5.6	0.25
Fattening broiler starter formula feed	8	5	104	4.5	5.5	0.25

・Lower detection limit: 2 μg/kg in the sample solution (spike recovery and relative standard deviation)

«Notes and precautions»

[1] As it is not acceptable to freeze the standard substance and standard solution, make sure to store them in a refrigerator.

[2] As the sample solution can form bubbles depending on the types of the sample, care should be taken for the reduced-pressure condensing process. Use a round-bottom flask with a larger capacity as occasion demands.

[3] As a large amount of water is loaded, it is recommended to connect a reservoir with a capacity of 20 mL.

[4] Treat with ultrasonic waves for several seconds.

[5] Example selective reaction monitoring (SRM) chromatograms are shown in Figure 9.2.10-2.

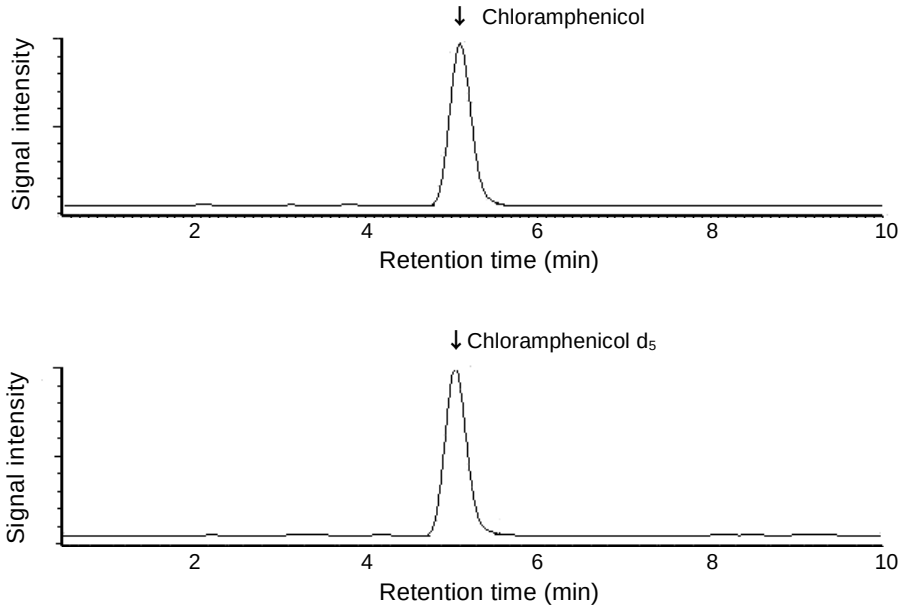


Figure 9.2.10-2 SRM chromatogram for a formula feed spiked with an amount equivalent to 5 μg/kg as chloramphenicol (upper graph: chloramphenicol; lower graph: chloramphenicol d_5)

1.2 Liquid chromatography

[Feed Analysis Standards Chapter 9, Section 2, 10.1.2]

Scope of application: Skimmed milk

A. Reagent preparation

1) Chloramphenicol standard solution. Weigh accurately 20 mg of chloramphenicol [$C_{11}H_{12}Cl_2N_2O_5$][1], place in a brown 200-mL one-mark flask, add acetonitrile to dissolve, and further add acetonitrile up to

the marked line to prepare a chloramphenicol standard stock solution (1 mL of this solution contains an amount equivalent to 0.1 mg of chloramphenicol).

At the time of use, accurately dilute a quantity of standard stock solution with a mixture of acetonitrile and water (1:1) to prepare several chloramphenicol standard solutions that contain amounts equivalent to 0.025 to 0.5 μg of chloramphenicol in 1 mL.

2) Silica gel. Dry silica gel for column chromatography (particle size 63 to 200 μm (230-70 mesh))[Note 1] at 110°C for 2 hours.

B. Quantification

Extraction. Weigh 10.0 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of ethyl acetate, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A). Transfer 50 mL of the filtrate to a 100-mL round-bottom flask, condense almost into dryness under reduced pressure in a water bath at 50°C or lower, and introduce nitrogen gas to evaporate into dryness.

Add 5 mL of chloroform to dissolve the residue, and use as the sample solution subject to column treatment.

Column treatment. Suspend 5 g of silica gel in chloroform, transfer to a column tube (15 mm in internal diameter), and allow to flow out until the liquid level reaches 3 mm above the top of the column packing material to prepare the column.

Place the sample solution in the column, wash the round-bottom flask that contained the sample solution twice with 5 mL of chloroform, and transfer the washing liquid each time to the column. Allow the liquid to flow out at a flow rate of 1 to 2 mL/min until the liquid level reaches 3 mm above the top of the column packing material, add 30 mL of a mixture of chloroform and methanol (97:3) to the column, and allow to flow out in the same manner to wash the column.

Place a 50-mL round-bottom flask under the column, add 30 mL of a mixture of chloroform and methanol (7:3) to the column, and elute chloramphenicol at the said flow rate. Evaporate the eluate almost into dryness under reduced pressure in a water bath at 50°C or lower, and introduce nitrogen gas to evaporate into dryness.

Add accurately 1 mL a mixture of acetonitrile and water (1:1) to dissolve the residue, filter through membrane filter (pore diameter not exceeding 0.5 μm)[2], and use as the sample solution subject to liquid chromatography.

Liquid chromatography. Inject 20 μL each of the sample solution and the chloramphenicol standard solutions into a liquid chromatograph to obtain chromatograms.

Operating conditions (example)

Detector:	Ultraviolet absorption detector (measured wavelength: 278 nm)
Column:	Octadecylsilanized silica gel column (6.0 mm in internal diameter, 150 mm in length, 5 μm in particle size)[Note 2] [3]
Eluent:	A mixture of water and acetonitrile (29:11)
Flow rate:	1.0 mL/min

Column oven temperature: 40°C

Calculation. Calculate the peak areas from the obtained chromatograms[4] to prepare a calibration curve, and estimate the amount of chloramphenicol in the sample.

Note 1. Silica gel 60 (Merck & Co., Inc.) or an equivalent.

2. YMC-Pack ODS-AM (YMC Co., Ltd.) or an equivalent.

«Summary of analysis method»

This method is intended to determine the amount of residual chloramphenicol in skimmed milk by measuring the absorbance of a sample solution prepared by extracting with ethyl acetate and purifying through a silica gel column, using a liquid chromatograph equipped with an ultraviolet absorption detector.

References: Kiyoshi Kanno: Research Report of Animal Feed, 27, 246 (2002)

History in the Feed Analysis Standards [26] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (μg/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Skimmed milk 1	10~50	3	96.0~101.9	3.8
Skimmed milk 2	10~50	3	96.3~96.5	10.0

・Collaborative study

Spike type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)	HorRat
Skimmed milk	9	10	98.1	4.7	6.4	0.29

・Lower quantification limit: 5 μg/kg in the sample (spike recovery and relative standard deviation)

«Notes and precautions»

[1] Commercially available from Wako Pure Chemical Industries, Ltd., Kanto Chemical Co., Inc., etc.

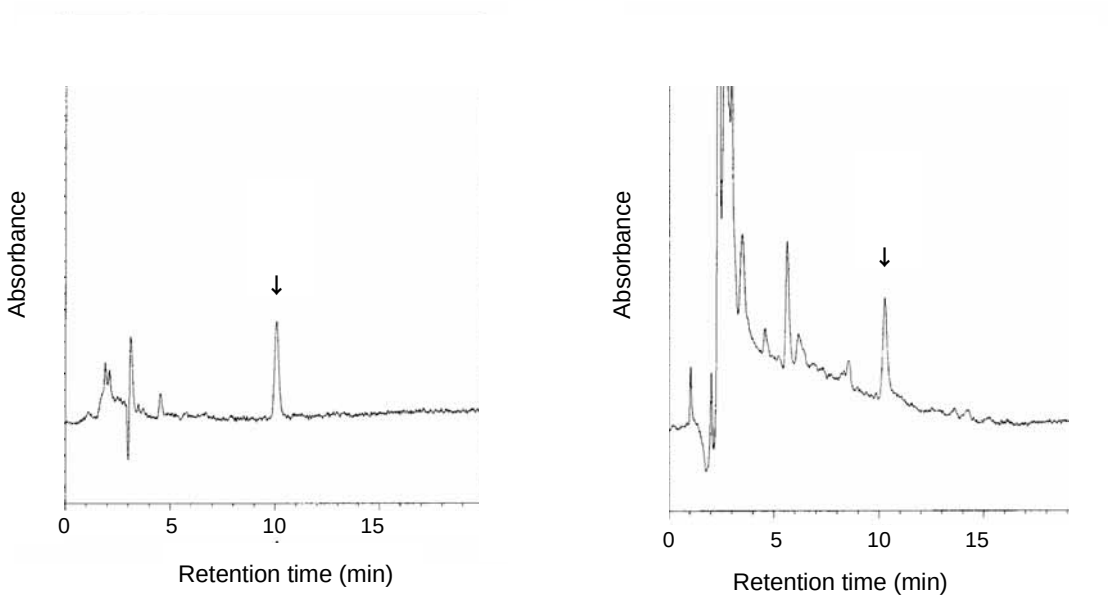
[2] It is recommended to use an acrylic copolymer or polyvinylidene difluoride (PVDF) filter.

When using a hydrophilic polytetrafluoroethylene (PTFE) filter, care should be taken that a peak of an interfering substance can appear at around the same retention time as the peak of CP.

It is permissible to use a high-speed centrifuge to centrifuge the supernatant liquid at 5,000×*g* (10,000 rpm) for 3 minutes to prepare the sample solution.

[3] Any column is applicable as long as its endcapped packing material meets the requirements. The column used in the validation of this method was YMC-Pack ODS-AM-312.

[4] Example chromatograms are shown in Figure 9.2.10-3.



Standard solution (1 ng)

Sample solution of skimmed milk spiked with 10 µg/kg

Figure 9.2.10-3 Chromatogram for chloramphenicol

(The arrow indicates the peak of chloramphenicol.)