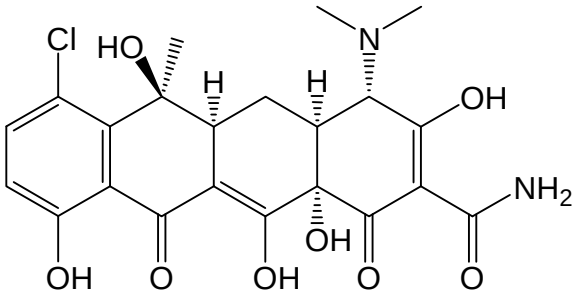
11 Chlorotetracycline



(2*Z*,4*S*,4a*S*,5a*S*,6*S*,12a*S*)-2-[amino(hydroxy)methylidene]-7-chloro-4-(dimethylamino)-6,10,11,12a-tetrahydroxy-6-methyl-4,4a,5,5a-tetrahydrotetracene-1,3,12-trione

$C_{22}H_{23}ClN_2O_8$ MW: 478.9 CAS No.: 57-62-5

[Summary of chlorotetracycline]

Chlorotetracycline (CTC) is a tetracycline antibiotic obtained by the incubation of *Streptomyces aureofaciens* and has the chemical structure shown above. The one used as a feed additive (feed grade) is prepared by filtering the incubated culture and drying the solid residue.

For physicochemical properties, CTC occurs as yellow crystals or crystalline powder and has no odor. It is sparingly soluble in water, slightly soluble in ethanol, and likely to lose the potency in an alkaline solution.

CTC has a broad antibacterial spectrum against Gram-positive bacteria, Gram-negative bacteria, rickettsia, large viruses, etc., and has a growth promoting effect on pigs and cattle.

«**Standards and specifications in the Act on Safety Assurance and Quality Improvement of Feeds**»

Chlorotetracycline is a feed-grade antibiotic that was designated as a feed additive on July 24, 1976. The specifications for feeds containing this ingredient are specified in Appended Table 1, 1-(1)-C of the Ministerial Ordinance Concerning the Ingredient Specifications for Feeds and Feed Additives.

(in g(potency)/t)

Feed of interest	For chickens (except for broilers)	For broilers	For cattle	
	Starting chicks Growing chicks	Starting broilers	Suckling calves	Calves
Added amount	10~55	10~55	10~50	10~50

The amount of CTC added to a commercial premix is roughly 4 to 40 g (potency)/kg.

[Methods listed in the Feed Analysis Standards]

1 Quantitative test method - Plate method

1.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 11.1.1]

A. Reagent preparation

1) Buffer solution: Buffer No.1

2) Chlorotetracycline standard solution. Dry a suitable amount of chlorotetracycline working standard[1] under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, accurately add water and dissolve to prepare a chlorotetracycline standard stock solution with a concentration of 1 mg (potency)/mL[2].

At the time of use, accurately dilute a quantity of standard stock solution with Buffer No.1 to prepare high- and low-concentration standard solutions with concentrations of 4 and 1 μg (potency)/mL, respectively[3].

3) Culture medium: Medium F-4

4) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 9341[4] as the test organism. Add about 0.1 mL of the suspension of the test organism per 100 mL of the culture medium.

5) Agar plate. Proceed by the agar well method.

6) Extracting solvent: A mixture of water, acetone, and hydrochloric acid (51:40:9)

B. Preparation of sample solution

Weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A)[5].

Accurately dilute a quantity of the filtrate with Buffer No.1 to prepare high- and low-concentration sample solutions with concentrations of 4 and 1 μg (potency)/mL, respectively[6].

C. Quantification[7]

Proceed by the 2-2 dose method [8].

«Summary of analysis method»

This method is intended to determine the amount of CTC in a premix by microbiological assay using a sample solution prepared by extracting with a mixture of water, acetone, and hydrochloric acid (51:40:9) and diluting with Buffer No.1.

The flow sheet of this method is shown in Figure 9.2.11-1.

Sample (3.0-5.0 g)
↓
Extract with 100 mL of water-acetone-hydrochloric acid (51:40:9).
(magnetic stirrer, 20 min)
↓
Filter (filter paper: No.5A).
↓
Dilute a quantity of the filtrate with Buffer No.1 to prepare high- and low-concentration sample solutions (4 and 1 μg(potency)/mL, respectively).
↓
Dispense to agar plates (allow to stand at 10-20°C for 2 hr).
↓
Incubate (at 35-37°C for 16-24 hr).
↓
Measure the inhibition zone diameter.
↓
Calculate the potency by the 2-2 dose method.

Figure 9.2.11-1 Quantitative test method for chlorotetracycline (premix)

References: Noriyuki Koyama: Research Report of Animal Feed, 6, 163 (1980)

History in the Feed Analysis Standards [3] New

«Validation of analysis method»

・Spike recovery and repeatability

Sampletype	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Vitamin premix	10~40	3	99.3~100.2	1.7
Vitamin/mineral premix	10~40	3	99.1~101.1	1.0

«Notes and precautions»

[1] For the difinition etc. of chlorotetracycline working standard, refer to «Notes and precautions» [9] in Section 1, 1 of this Chapter.

[2] For the method of preparation for the standard stock solution, refer to «Notes and precautions» [10] in Section 1, 1 of this Chapter.

Mmethod of preparation: example (when the weighed amount is 50 mg)

When the labeled potency of the working standard is 967 μg (potency)/mg, 50 mg of the working standard contains 48,350 μg (potency) (i.e., 50 mg × 967 μg (potency)/mg). To prepare a standard stock solution with a concentration of 1,000 μg (potency)/mL, the required amount of solvent is thus calculated to be 48.35 mL (i.e., 48,350 μg (potency) / 1,000 μg (potency)/mL). Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 48.35 mL of sterilized water and dissolve to prepare a standard stock solution with a concentration of 1,000 μg (potency)/mL.

[3] For the method of preparation for the standard solution, Refer to «Notes and precautions» [8] in Section 1, 1 of this Chapter.

An example method of preparation for the chlorotetracycline standard solution is shown in Table 9.2.11-1.

[4] For the number of bacteria, refer to «Notes and precautions» [33] in Section 1, 1 of this Chapter.

[5] When the filtration is difficult, it is permissible to transfer the extract to a stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and use the supernatant liquid.

[6] For the method of preparation for sample solution, refer to «Notes and precautions» [8] in Section 1, 1 of this Chapter.

An example method of preparation is shown in Table 9.2.11-1.

Table 9.2.11-1 Methods of preparation for chlorotetracycline standard solution and sample solution

1) Method of preparation for chlorotetracycline standard solution (premix, example)

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	4	5	5
Amount (mL) of Buffer No.1	23	16	15	15
Concentration (μg(potency)/mL)	80	16	4	1

Note: "② mL" means "2 mL of standard stock solution (1 mg(potency)/mL)".

2) Method of preparation for sample solution (premix, example)

When the analysis sample is collected in an amount equivalent to 200,000 μg (potency) of CTC, the concentration of chlorotetracycline in the filtrate is calculated to be 2,000 μg (potency)/mL.

Test tube No.	1	2	3	4
Amount (mL) of sample solution	②	2	4	5
Amount (mL) of Buffer No.1	18	18	16	15
Concentration (μg(potency)/mL)	200	20	4	1

Note: "② mL" means "2 mL" of the filtrate (2,000 μg(potency)/mL)".

[7] An example standard response line for CTC is shown in Figure 9.2.11-2.

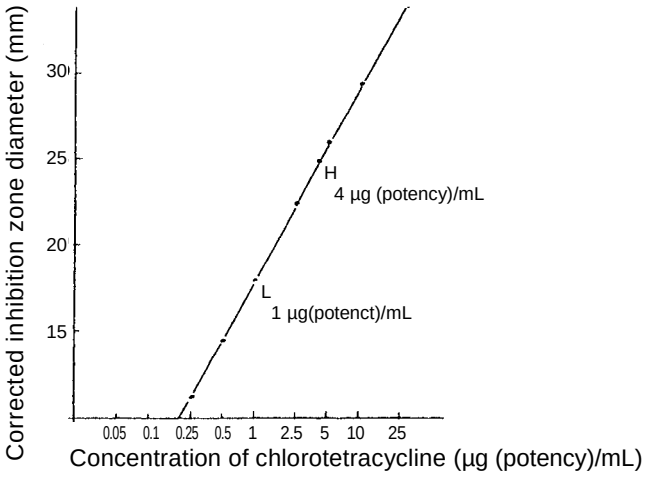


Figure 9.2.11-2 Standard response line for chlorotetracycline (premix, example)

(*Micrococcus luteus* ATCC 9341, Medium F-4, Agar well method)

[8] Refer to «Notes and precautions» [53] to [60] in Section 1, 1 of this Chapter.

1.2 Mineral-rich premix [Feed Analysis Standards, Chapter 9, Section 2, 11.1.2]

Scope of application: Mineral-rich premix

A. Reagent preparation

1) Buffer solution: Buffer No.11

2) Chlorotetracycline standard solution: Dry a suitable amount of chlorotetracycline working standard under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, accurately add water and dissolve to prepare a chlorotetracycline standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with Buffer No.11 to prepare high- and low-concentration standard solutions with concentrations of 4 and 1 μg (potency)/mL, respectively[1].

3) Culture medium: Medium F-4

4) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 9341[2] as the test organism. Add about 0.1 mL of the suspension of the test organism per 100 mL of the culture medium.

5) Agar plate. Proceed by the agar well method.

6) Extracting solvent: A mixture of water, acetone, and hydrochloric acid (51:40:9)

B. Preparation of sample solution

Weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 3 g of 2,4,6-tri-2-pyridyl-1,3,5-triazine[3] and 100 mL of the extracting solvent, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A)[4].

Transfer accurately 5 mL of the filtrate to a 50-mL beaker, add 30 mL of Buffer No.11, and adjust the pH to 3.9 to 4.1 with sodium hydroxide solution (2 mol/L). Transfer the whole amount of this liquid with Buffer No.11 to a 50-mL one-mark flask, add Buffer No.11 up to the marked line, accurately dilute a quantity of this liquid with Buffer No.11 to prepare high- and low-concentration sample solutions with concentrations of 4 and 1 μg (potency)/mL, respectively[5].

C. Quantification[6]

Proceed by the 2-2 dose method[7].

«Summary of analysis method»

This method is intended to determine the amount of CTC in a premix that is rich in minerals, such as iron and cupper. When determined as described in 1, 1.1 Plate method (Part 1), CTC recoveries from some mineral-rich premixes were found to be low. This is considered to be because the antibacterial acitivity of CTC had been reduced by chelation with minerals, such as iron and cupper. The method described here prevents this effect by masking such minerals in sample solution with a chelating agent before determined by the microbiological assay.

The flow sheet of this method is shown in Figure 9.2.11-3.

Sample (3.0-5.0 g)
↓
Add 3 g of TPTZ (2,4,6-tri-2-pyridyl-1,3,5-triazine).
↓
Extract with 100 mL of water-acetone-hydrochloric acid (51:40:9).
(magnetic stirrer, 20 min)
↓
Filter (filter paper: No.5A).
↓
Collect 5 mL of the filtrate (into a 50-mL beaker) and add 30 mL of Buffer No.1
↓
Adjust the pH to 3.9-4.1 (with sodium hydroxide solution (2 mol/L)).
↓
Add Buffer No.11 up to 50 mL in a one-mark flask.
↓
Dilute a quantity with Buffer No.11 to prepare high- and low-concentration sample solutions with concentrations of 4 and 1 µg(potency)/mL, respectively.
↓
Dispense to agar plates (allow to stand at 10-20°C for 2 hr).
↓
Incubate (35-37°C for 16-24 hr).
↓
Measure the inhibition zone diameter.
↓
Calculate the potency by the 2-2 dose method.

Figure 9.2.11-3 Quantitative test method for chlorotetracycline (mineral-rich premix)

References: Toyoko Kusama: Research Report of Animal Feed, 14, 35 (1989)

History in the Feed Analysis Standards [11] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Chicken premix	10~50	3	100.0~102.3	3.6
Pig permix	10~50	3	95.7~100.3	4.8
Cattle premic	10~50	3	96.3~101.0	4.3

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/kg	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)
Cattle premix	8	20	100.2	2.3	3.0

«Notes and precautions»

[1] For the method of preparation for the standard solution, refer to «Notes and precautions» [8] in Section 1, 1 of this Chapter.

An example method of preparation for chlorotetracycline standard solution is shown in Table 9.2.11-2.

[2] For the number of bacteria, refer to «Notes and precautions» [33] in Section 1, 1 of this Chapter.

[3] 2,4,6-Tri-2-pyridyl-1,3,5-triazine, commonly called TPTZ, is commercially available from Tokyo Chemical Industry Co., Ltd.

[4] When the filtration is difficult, it is permissible to transfer the extract to a stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and use the supernatant liquid.

[5] For the method of preparation for the sample solution, refer to «Notes and precautions» [8] in Section 1, 1 of this Chapter.

An example method of preparation is shown in Table 9.2.11-2.

Table 9.2.11-2 Method of preparation for chlorotetracycline standard solution and sample solution

1) Method of preparation for chlorotetracycline standard solution (mineral-rich premix, example)

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	4	5	5
Amount (mL) of Buffer No.11	23	16	15	15
Concentration (μg(potency)/mL)	80	16	4	1

Note: "② mL" means "2 mL of standard stock solution (1 mg(potency)/mL)".

2) Method of preparation for sample solution (mineral-rich premix, example)

When the analysis sample is collected in an amount equivalent to 200,000 μg (potency) of CTC, the concentration of chlorotetracycline in the filtrate is calculated to be 2,000 μg (potency)/mL.

Test tube No.	1	2	3	4
Amount (mL) of sample solution	②	2	4	5
Amount (mL) of Buffer No.11	18	18	16	15
Concentration (μg(potency)/mL)	200	20	4	1

Note: "② mL" means "2 mL of the filtrate (2,000 μg(potency)/mL)".

[6] An example standard response line for CTC is shown in Figure 9.2.11-4.

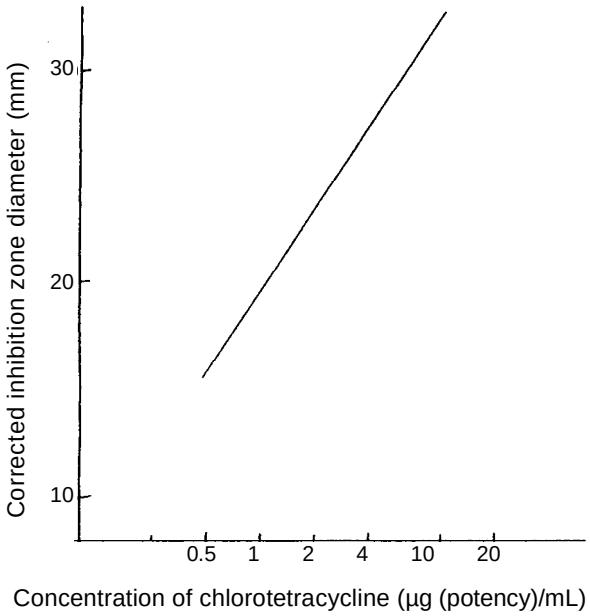


Figure 9.2.11-4 Standard response line for chlorotetracycline (mineral-rich premix, example)
(*Micrococcus luteus* ATCC 9341, Medium F-4, Agar well method)

[7] Refer to «Notes and precautions» [53] to [60] in Section 1, 1 of this Chapter.

1.3 Feeds with CTC (over 20 g (potency)/t)

[Feed Analysis Standards, Chapter 9, Section 2, 11.2.1]

Scope of application: Feeds with CTC content not less than 20 g (potency)/t

A. Reagent preparation

1) Buffer solution: Buffer No.1

2) Chlorotetracycline standard solution. Dry a suitable amount of chlorotetracycline working standard under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, accurately add water and dissolve to prepare a chlorotetracycline standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with a mixture of Buffer No.1 and acetone (4:1) to prepare standard solutions with concentrations of 8, 4, 2, 1 and 0.5 µg (potency)/mL[1].

3) Culture medium: Medium F-4

4) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 9341[2] as the test organism. Add about 0.5 mL of a 10-fold diluted suspension of the test organism to 100 mL of Medium F-4.

5) Agar plate. Proceed by the agar well method.

6) Extracting solvent: A mixture of water, acetone, and hydrochloric acid (51:40:9)

B. Preparation of sample solution

Weigh accurately a quantity of the analysis sample (equivalent to 0.4 mg (potency) as CTC), place 200 mL in a stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer the extract to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and filter the supernatant liquid through filter paper (No.5A).

Transfer accurately 25 mL of the filtrate to a 50-mL beaker, and adjust the pH to 4.4 to 4.6 with ammonia solution[3]. Transfer the whole amount of this liquid with Buffer No.1 to a 50-mL one-mark flask, add Buffer No.1 up to the marked line, and filter through filter paper (No.5A) to prepare a sample solution with a concentration of 2 µg (potency)/mL.

C. Quantification[4]

Proceed by the standard response line method[5].

«Summary of analysis method»

This method is intended to determine the amount of CTC in a feed by microbiological assay using a sample solution prepared by extracting with a mixture of water, acetone, and hydrochloric acid (51:40:9) and adjusting the pH to 4.4 to 4.6.

Of the antibacterial substances approved for combined use with CTC, SL and MN have strong antibacterial effects on *Micrococcus luteus* ATCC 9341. When spiked with SL (50 g (potency)/t) or MN (80 g (potency)/t) in combination with CTC and determined with this method, however, neither of the spiked ingredients affected the quantified results of CTC. This is considered to be because the

antibacterial activity of SL or MN had been reduced by hydrochloric acid in the extract.

The flow sheet of this method is shown in Figure 9.2.11-5.

Sample (in an amount equivalent to 0.4 mg (potency) of CTC)
↓
Extract with 100 mL of water-acetone-hydrochloric acid (40:9).
(magnetic stirrer, 20 min)
↓
Centrifuge (at 1,500×g for 5 min).
↓
Filter (filter paper: No.5A).
↓
Collect 25 mL of the filtrate (into a 50-mL beaker).
↓
Adjust the pH to 4.4-4.6 (with ammonia solution).
↓
Add Buffer No.1 up to 50 mL in a one-mark flask.
↓
Filter (filter paper: No.5A).
↓
Dispense to agar plates (allow to stand at 10-20°C for 2 hr)
↓
Incubate (at 35-37°C for 16-24 hr).
↓
Measure the inhibition zone diameter.
↓
Calculate the potency by the standard response line method.

Figure 9.2.11-5 Quantitative test method for chlorotetracycline (feed with CTC content not less than 20 g (potency)/t)

References: Kiyoshi Kanno: Research Report of Animal Feed, 6, 106 (1980)

History in the Feed Analysis Standards [3] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Starting chick formula feed	50~100	6	94.3~99.8	3.7
Suckling piglet formula feed	50~100	6	96.3~96.8	3.2
Suckling calf formula feed	50~100	6	97.7~98.2	3.4

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)
Calf formula feed	3	50	101.6	3.1	6.9

«Notes and precautions»

[1] For the method of preparation for the standard solution, refer to «Notes and precautions» [8] in Section 1, 1 of this Chapter.

An example method of preparation for chlorotetracycline standard solution is shown in Table 9.2.11-3.

Table 9.2.11-3 Method of preparation for chlorotetracycline standard solution (feed with CTC content not less than 20 g (potency)/t, example)

Test tube No.	1	2	3	4	5	6
Amount (mL) of standard solution	②	2	10	10	10	5
Amount (mL) of Buffer No.1-acetone (4:1)	23	18	10	10	10	5
Concentration (μg(potency)/mL)	80	8	4	<2>	1	0.5

Note: "② mL" means "2 mL of standard stock solution (1 mg(potency)/mL)".

[2] For the number of bacteria, refer to «Notes and precautions» [33] in Section 1, 1 of this Chapter.

[3] To facilitate the pH adjustment, it is advisable to add 5 to 10 mL of Buffer No.1 to 25mL of the filtrate in advance.

[4] An example standard response line for CTC (feed with CTC content not less than 20 g (potency)/t) is shown in Figure 9.2.11-6.

Linearity is observed in the quantification range for CTC (CTC concentrations between 0.5 and 8 μg (potency)/mL).

Of the antibacterial substances approved for combined use with CTC, SL and MN have strong antibacterial effects on *Micrococcus luteus* ATCC 9341. When spiked with SL (50 g (potency)/t) or MN (80 g (potency)/t) in combination with CTC and determined with this method, however, neither of the spiked ingredients affected the quantified results of CTC. This is considered to be because the antibacterial activity of SL or MN had been reduced by hydrochloric acid in the extract.

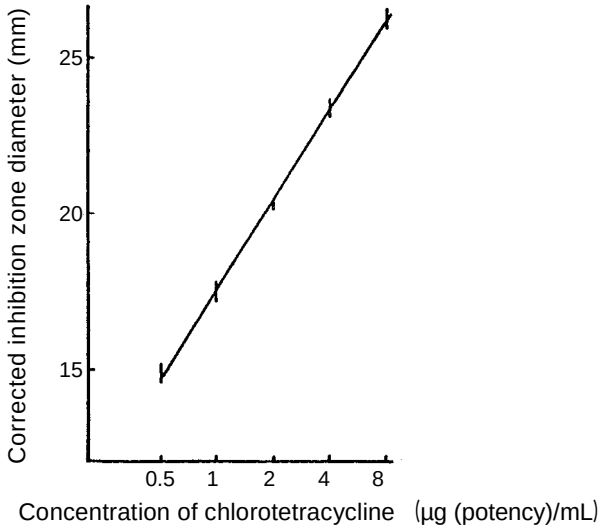


Figure 9.2.11-6 Standard response line for chlorotetracycline (feed with CTC content not less than 20 g (potency)/t, example)

(*Micrococcus luteus* ATCC 9341, Medium F-4, Agar well method)

[5] Refer to «Notes and precautions» [53] to [57] and [61] in Section 1, 1 of this Chapter.

1.4 Feed with CTC (less than 20g(potency)/t) [Feed Analysis Standards, Chapter 9, Section 2, 11.2.2]

Scope of application: Feeds with CTC content less than 20 g (potency)/t

A. Reagent preparation

1) Buffer solution: Buffer No.1

2) Chlorotetracycline standard solution. Dry a suitable amount of chlorotetracycline working standard under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, accurately add water and dissolve to prepare a chlorotetracycline standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of standard stock solution with a mixture of Buffer No.1 and acetone (4:1) to prepare standard solutions with concentrations of 1.28, 0.64, 0.32, 0.16 and 0.08 μg (potency)/mL[1].

3) Culture medium: Medium F-17

4) Spore suspension and amount of addition. Use *Bacillus cereus* ATCC 11778 as the test organism. Add about 0.5 mL of the spore suspension at a concentration of 1×10^6 spores/mL per 100 mL of the culture medium.

5) Agar plate. Add the spore suspension to the culture medium that has been melted and maintained at 49 to 51°C, stir thoroughly, dispense 15 mL into each Petri dish to form a uniform layer, and allow to stand horizontally to solidify. On this layer again dispense 5 mL of the culture medium to form a uniform layer, and allow to stand horizontally to solidify [2]. Then proceed by the agar well method.

6) Extracting solvent: A mixture of water, acetone, and hydrochloric acid (51:40:9)

B. Preparation of sample solution

Weigh accurately a quantity of the analysis sample (equivalent to 64 μg (potency) as CTC), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer the extract to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and filter the supernatant liquid through filter paper (No.5A).

Transfer accurately 25 mL of the filtrate to a 50-mL beaker, and adjust the pH to 4.4 to 4.6 with ammonia solution[3]. Transfer the whole amount of this liquid with Buffer No.1 to a 50-mL one-mark flask, add Buffer No.1 up to the marked line, and filter through filter paper (No.5A) to prepare a sample solution with a concentration of 0.32 μg (potency)/mL.

C. Quantification[4]

Proceed by the standard response line method [5].

«Summary of analysis method»

This method is intended to determine the amount of CTC in a feed by microbiological assay using a sample solution prepared by extracting with a mixture of water, acetone, and hydrochloric acid (51:40:9) and adjusting the pH to 4.4 to 4.6. The major difference from the quantitative test method for CTC content not less than 20 g (potency)/t is the use of a sensitive test organism and culture medium that are more sensitive to CTC.

Of the antibacterial substances approved for combined use with CTC, SL and MN have strong

antibacterial effects on *Bacillus cereus* ATCC 11778. When spiked with SL (50 g (potency)/t) or MN (80 g (potency)/t) and determined by this method, however, neither of the spiked ingredients affected the quantified results of CTC. This is considerd to be because the antibacterial activity of SL or MN had been reduced by hydrochloric acid in the extract.

The flow sheet of this method is shown in Figure 9.2.11-7.

Sample (in an amount equivalent to 64 μg(potency) as CTC)
↓
Extract with 100 mL of water-acetone-hydrochloric acid (51:40:9).
(magnetic stirrer, 20 min)
↓
Centrifuge (at 1,500×g for 5 min).
↓
Filter (filter paper: No.5A).
↓
Collect 25 mL of the filtrate (into a 50-mL beaker).
↓
Adjust the pH to 4.4-4.6 (with ammonia solution).
↓
Add Buffer No.1 up to 50 mL in a one-mark flask.
↓
Filter (filter paper: No.5A).
↓
Dispense to agar plates (10-20°C for 2 hr).
↓
Incubate (at 35-37°C for 16-24 hr).
↓
Measure the inhibition zone diameter.
↓
Calculate the potency by the standard resonse line method.

Figure 9.2.11-7 Quantitative test method for chlorotetracycline (feed with CTC content of less than 20 g (potency)/t)

References: Kiyoshi Kanno: Research Report of Animal Feed, 6, 106 (1980)

History in the Feed Analysis Standards [3] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD(%)
Starting chick formula feed	5	6	93.2	4.5
Suckling piglet formula feed	5	6	98.5	3.9
Suckling calf formula feed	5	6	96.5	5.4

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)
Calf formula feed	3	10	104.2	4.7	9.2

«Notes and precautions»

[1] For the method of preparation for the standard solution, Refer to «Notes and precautions» [8] Section

1, 1 of this Chapter.

An example method of preparation for chlorotetracycline standard solution is shown in Table 9.2.11-4.

Table 9.2.11-4 Method of preparation for chlorotetracycline standard solution

(feed with CTC content not less than 20 g (potency)/t, example)

Test tube No.	1	2	3	4	5	6	7
Amount (mL) of standard solution	②	2	4	10	10	10	5
Amount (mL) of Buffer No.-acetone (4:1)	23	18	21	10	10	10	5
ɔncentration (μg(potency)/m	80	8	1.28	0.64	<0.32>	0.16	0.08

Note: "② 2 mL" means "2 mL of standard stock solution (1 mg(potency)/mL)".

[2] *Bacillus cereus* ATCC 11778 grows strongly on the surface of the culture medium and therefore produces an ill-defined edge of the inhibition zone. The procedure described here inhibits the superficial growth of the test organism and thus helps it produce a clear-cut zone of inhibition.

Alternatively, it is permissible to incubate the test organism in 20 mL of Medium F-17 by the single layer agar well method to inhibit the superficial growth. In this case, the amount of the spore suspension to be added to the culture medium should be about 20 times the specified amount.

[3] To facilitate the pH adjustment, it is advisable to add 5 to 10 mL of Buffer No.1 to 25 mL of the filtrate in advance.

[4] An example standard response line for CTC (feed with CTC contant less than 20 g (potency)/t) is shown in Figure 9.11-8.

Linearity is observed in the quantification range for CTC (CTC concentrations between 0.08 and 1.28 μg (potency)/mL).

Of the antibacterial substances approved for combined use with CTC, SL and MN have strong antibacterial effects on *Bacillus cereus* ATCC 11778. When spiked with SL (50 g (potency)/t) or MN (80 g (potency)/t) in combination with CTC and determined by this method, however, neither of the spiked ingredients affected the quantified results of CTC. This was considered to be because the antibacterial activity of SL or MN had been rduced by hydrochloric acid present in the extract.

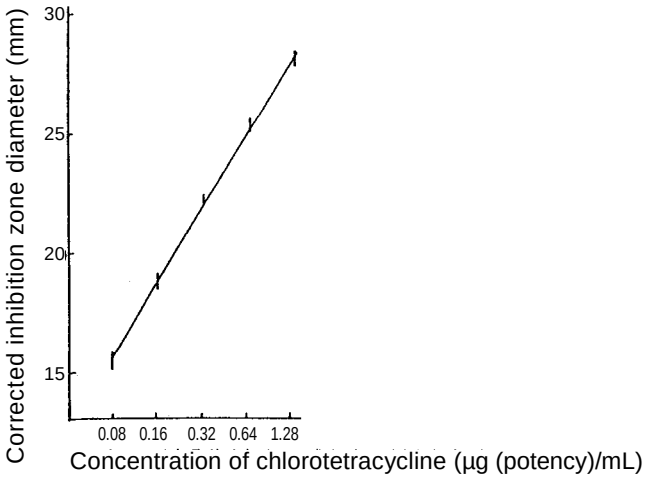


Figure 9.2.11-8 Standard response line for chlorotetracycline

(feed with CTC contnt less than 20 g (potency)/t, example)
(*Bacillus cereus* ATCC 11778, Medium F-17, Agar well method)

[5] Refer to «Notes and precautions» [53] to [57] and [61] in Section 1, 1 of this Chapter.

2. Liquid chromatography [Feed Analysis Standards, Chapter 9, Section 2, 11.2.3]

Scope of application: Feed

A. Reagent preparation

1) Buffer solution: Buffer No.1

2) Extracting solvent: A mixture of Buffer No.1, methanol and hydrochloric acid (4 mol/L) (50:49:1)

3) Chlorotetracycline standard solution: Dry a suitable amount of chlorotetracycline working standard under reduced pressure (0.67 kPa not exceeding) at 60 °C for 3 hours, weigh accurately not less than 40 mg, accurately add water and dissolve to prepare a chlorotetracycline standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of standard stock solution with the extracting solvent to prepare several chlorotetracycline standard solutions containing amounts equivalent to 0.25 to 5.0 µg (potency) as chlorotetracycline in 1 mL.

B. Quantification

Extraction. Weigh accurately 2 to 4 g of the analysis sample (equivalent to 0.2 mg (potency) as CTC), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer 50 mL of the extract to a stoppered centrifuge tube, centrifuge at 1,000×*g* for 5 minutes, filter the supernatant liquid through membrane filter (pore size not exceeding 0.5 µm), and use the filtrate as the sample solution subject to liquid chromatography.

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

Operating conditions (example)

Detecotor: Fluorescence detector (excitation wavelength: 380 nm, emission wavelength: 520 nm)

Column: Octadecylsilanized silica gel column (6 mm in internal diameter, 150 mm in length, 5 µm in particle size)[Note 1]

Eluent: A mixture of imidazole buffer solution[Note 2] and methanol (7:3)

Flow rate: 0.8 mL/min

Column oven temperature: 40 °C

Calculation. Calculate the peak height or peak area from the obtained chromatogram[1] to prepare a calibration curve and estimate the amount of chlorotetracycline in the sapmle.

Note 1. YMC-Pack ODS-AM (YMC Co., Ltd.) or an equivalent[2]

2. Weigh 68.08 g of imidazole, 10.72 g of magnesium acetate tetrahydrate and 0.37 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate, dissolve in 750 mL of water, add about 25 mL of acetic acid to adjust the pH to 7.1 to 7.3, and add water to make 1,000 mL.

«Summary of analysis method»

This method is intended to determine the amount of CTC in a feed by extracting the analysis sample with a mixture of Buffer No.1, methanol, and hydrochloric acid (4 mol/L) (50:49:1), chelating CTC with magnesium ions in the eluent to produce a fluorescent derivative, separing through an octadecylsilanized silica gel (ODS) colum, and measuring with a fluorescence detector.

The flow sheet of this method is shown in Figure 9.2.11-9.

2-4 g of the sample (equivalent to not more than 0.2 mg(potency) as CTC)
↓
Add 100 mL of extracting solvent and extract with stirring for 20
↓
Centrifuge (at 1,000×g for 5 min).
↓
Filter through a membrane filter (pore size not exceeding 0.5 μm)
↓
LC-FL (Ex: 380 nm, Em: 520 nm)

Figure 9.2.11-9 Liquid chromatography for chlorotetracycline (feed)

References: Daisaku Makino, Tomotaro Yoshida, Tetsuo Chihara: Research Report of Animal Feed, 30, 34 (2005)

History in the Feed Analysis Standards [28] New

«Validation of analysis method»

・Spike recovery and repeatability

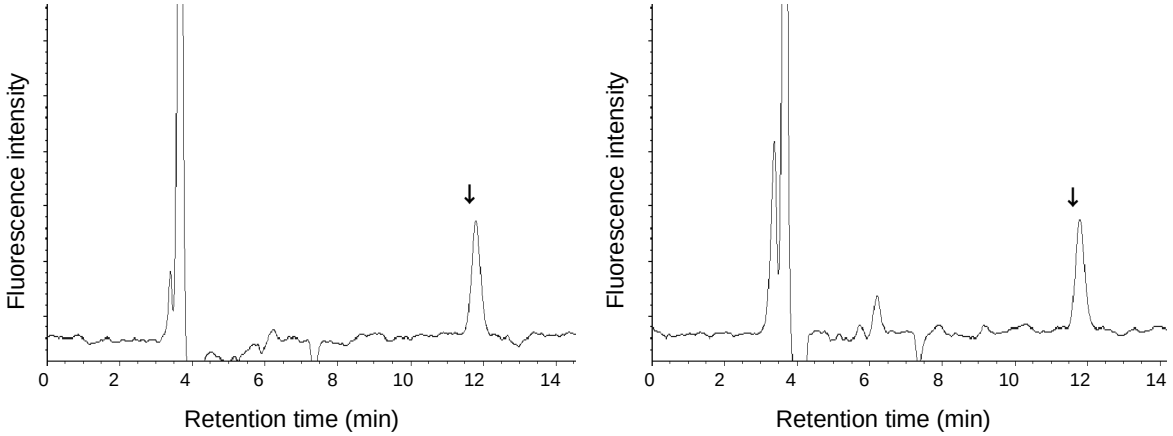
Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Starting chick formula feed	10~50	3	93.4~104.1	7.5
Suckling calf grower formula feed	10~50	3	95.3~99.6	6.2
Calf grower formula feed	10~50	3	91.6~98.4	5.2

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/kg	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)	HorRat
Suckling calf formula feed	8	50	96.8	2.1	4.3	0.48

«Notes and precautions»

[1] An example chromatogram is shown in Figure 9.2.11-10.



Chlorotetracycline standard solution
(equivalent to 25 ng (potency))

Suchking calf grower formula feed
(50 g (potency)/t)
(equivalent to 25 ng (potency))

Figure 9.2.11-10 Chromatogram for chlorotetracycline
(The arrow indicates the peak of a chlorotetracycline derivative.)

[2] CTC has a strong tendency to bind to metals. Therefore, depending on the type of the ODS column, tailing of the peak is likely to occur due to residual silanol groups in the packing material remaining after end-capping and traces of metals contained in the silica gel. It is therefore necessaty to perform a trial run using the ODS column to be used to confirm the absence of such effect. The column used in the validation of this method was YMC-Pack ODS-AM.