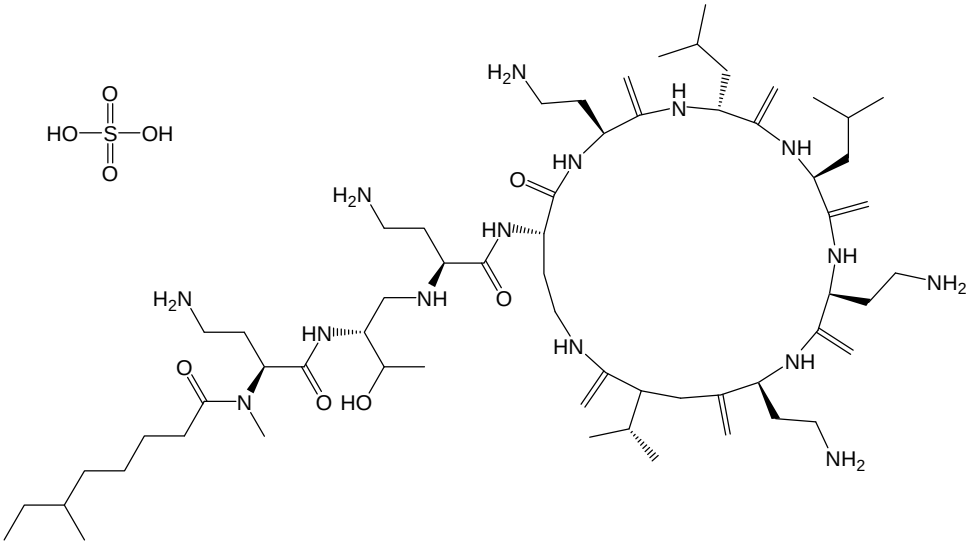
30 Colistin sulfate

(Polymyxin E)



Colistin A (Polymyxin E1)

Colistin	$C_{53}H_{100}N_{16}O_{13}$	MW: 1170	CAS No.: 1066-17-7
Colistin sulfate	$2(C_{52}H_{98}N_{16}O_{13})\cdot 5(H_2SO_4)$	MW: 2801	CAS No.: 1264-72-8

[Summary of colistin sulfate]

Colistin is a basic polypeptide antibiotic obtained by the incubation of *Bacillus polymyxa* and a mixture composed of colistinA and colistinB as main ingredients.

For physicochemical properties, pure-grade colistin sulfate (CL) technical occurs as a light grayish white to brown powder. It is freely soluble in water, and practically insoluble in acetone and in ether. CL has a strong antibacterial effect mainly on Gram-negative bacteria (especially bacilli) and a growth promoting effect on chickens (including broilers) and pigs.

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

CL was designated as a feed additive as of July 24, 1976, and the specifications are specified for pure grade and feed grade separately depending on the difference in the method of manufacture. 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 pigs	
	Starting chicks Growing chicks	Starting period broilers Finishing period broilers	Suckling piglets	Piglents
Added amount	2~20	2~20	2~40	2~20

The amount of CL added to a commercial premix is roughly 0.5 to 50 g (potency)/kg.

[Methods listed in the Feed Analysis Standards]

1 Quantitative test method - Plate method

1.1 Permix

[Feed Analysis Standards, Chapter 9, Section 2, 30.1.1]

A. Reagent preparation

1) Buffer solution: Buffer No.5

2) Colistin standard solution. Dry a suitable amout of colistin 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 Buffer No.5 and dissolve to prepare a colistin standard stock solution with a concentration of 1 mg(potency)/mL[2].

At the time of use, accurately dilute a quantity of the standard stock solution with Buffer No.5 to prepare high- and low-concentration standard solutions with concentrations of 2 and 0.5 μg (potency)/mL, respectively[3].

3) Culture medium: Medium F-9

4) Suspension of test organism and amount of addition. Use *Bordetella bronchiseptica* ATCC 4617[4] as the test organism. Add about 0.1 mL of a suspension of the test organism per 100 mL of the culture medium.

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

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 hydrochloric acid (1 mol/L), extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A). Transfer exactly 20 mL of the filtrate to a 50-mL beaker, and adjust the pH to 5.9 to 6.1 with ammonia solution [5]. Transfer the whole amount of this liquid with Buffer No.5 to a 100-mL volumetric flask, further add Buffer No.5 up to the marked line, and filter through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with Buffer No.5 to prepare high- and low-concentration sample solutions with concentrations of 2 and 0.5 μg (potency)/mL, respectively[6].

C. Quantification[7]

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

«Summary of analysis method»

This method is intended to determine the amount of CL in a premix by microbiological assay using a sample solution prepared by extracting with hydrochloric acid (1 mol/L), adjusting the pH to 5.9 to 6.1, and diluting with Buffer No.5.

The flow sheet of this method is shown in Figure 9.2.30-1.

Sample (3.0-5.0 g)
↓
Extract with 100 mL of hydrochloric acid (1 mol/L)
(with a magnetic stirrer for 20 min).
↓
Filter (through filter paper No.5A).
↓
Collect 20 mL of the filtrate (into a 50-mL beaker).
↓
Adjust the pH to 5.9-6.1 (with ammonia solution).
↓
Add Buffer No.5 to make 100 mL in a volumetric flask.
↓
Filter (through filter paper No.5A).
↓
Dilute a quantity of the filtrate with Buffer No.5 to prepare high- and low-concentration sample solutions (2 and 0.5 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 9.2.30-1 Quantitative test method for colistin sulfate (premix)

References: Shoichi Yamatani: Research Report of Animal Feed, 11, 174 (1985)

History in the Feed Analysis Standards [3] New [8] Revision

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Premix 1	0.5~10	3	96.4~101.9	3.3
Premix 2	0.5~10	3	96.7~104.5	4.7
Premix 3	0.5~10	3	95.6~99.9	5.1

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Premix	6	2	95.1	3.6	4.3

«Notes and precautions»

[1] For the definition etc. of colistin 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.

Method of preparation: Example (when the weighed amount is 50 mg)

When the labeled potency of the working standard is 662 μg (potency)/mg, 50 mg of the working standard contains 33,100 μg (potency) (i.e., 50 mg × 662 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 33.1 mL (i.e., 33,100 μg (potency) / 1,000 μg (potency)/mL. Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 33.1 mL of Buffer No.5 and dissolve to prepare the 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 colistin standard solution is shown in Table 9.2.30-1.

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

[5] It is recommended to add 5 to 10 mL of Buffer No.5 to the filtrate before adjusting the pH.

[6] 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 9.2.30-1.

Table 9.2.30-1 Method of preparation for colistin standard solution and sample solution

1) Method of preparation for colistinstandard solution (premix, example)

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	4	5
Amount (mL) of Buffer No.5	18	18	16	15
Concentration (μg(potency)/mL)	100	10	2	0.5

Note: "②mL" 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 4,000 μg (potency) of CL, the concentration of colistin in the filtrate is calculated to be 8 μg (potency)/mL.

Test tube No.	1	2
Amount (mL) of sample solution	⑤	5
Amount (mL) of Buffer No.5	15	15
Concentration (μg(potency)/mL)	2	0.5

Note: "⑤mL" means "5 mL of filtrate (8 μg (potency)/mL)".

[7] An example standard response line for CL is shown in Figure 9.2.30-2.

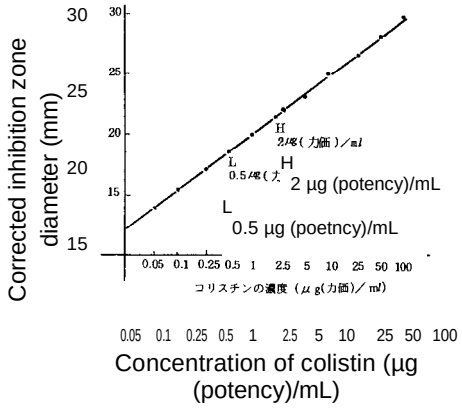


Figure 9.2.30-2 Standard response line for colistin (premix, example)

(*Bordetella bronchiseptica* ATCC 4617, Medium F-9, Agar well method)

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

1.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 30.2.1]

A. Reagent preparation

1) Buffer solution: Buffer No.5

2) Dilution solvent

i) When the CL content is not less than 10 g(potency)/t, use a mixture of Buffer No.5, acetone and pyridine (91:8:1)

ii) When the CL content is less than 10 g(potency)/t, use a mixture of Buffer No.5, acetone and pyridine (83:15:2)

3) Colistin standard solution. Dry a suitable amout of colistin 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 Buffer No.5 and dissolve to prepare a colistin 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 dilution solvent to prepare standard solutions with concentrations of 0.8, 0.4, 0.2, 0.1 and 0.05 µg (potency)/mL[1].

4) Culture medium: Medium F-9

5) Suspension of test organism and amount of addition. Use *Bordetella bronchiseptica* ATCC 4617[2] as the test organism. Add about 0.5 mL of a 100-fold diluted suspension of the test organism per 100 mL of the culture medium.

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

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

B. Preparation of sample solution

1) When the CL content is not less than 10 g(potency)/t

Weigh accurately a quantity of the analysis sample (equivalent to 0.1 mg (potency) as CL), place in a 200-mL stoppered Erlenmeyer flask, add 5 mL of pyridine and 5 mL of hexane[3], and stir for 2 to 3 minutes. Further add 95 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 water-acetone phase (lower phase) through filter paper (No.5A).

Transfer exactly 20 mL of the filtrate to a 50-mL beaker and adjust the pH to 5.9 to 6.1 with ammonia solution[4]. Transfer the whole amount of this liquid with Buffer No.5 to a 100-mL volumetric flask, further add Buffer No.5 to the marked line, and filter through filter paper (No.5A) to prepare a sample solution with a concentration of 0.2 µg (potency)/mL.

2) When the CL content is less than 10 g(potency)/t

Weigh accurately a quantity of the analysis sample (equivalent to 50 µg (potency) as CL), place in a 200-mL stoppered Erlenmeyer flask, add 5 mL of pyridine and 5 mL of hexane[3], and stir for 2 to 3 minutes. Further add 95 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 1,500×*g* for 5 minutes, and filter the

water-acetone phase (lower phase) through filter paper (No.5A).

Transfer exactly 20 mL of the filtrate to a 50-mL beaker and adjust the pH to 5.9 to 6.1 with ammonia solution[4]. Transfer the whole amount of this liquid with Buffer No.5 to a 50-mL volumetric flask, further add Buffer No.5 to the marked line, and filter through filter paper (No.5A) to prepare a sample solution with a concentration of 0.2 μg (potency)/mL.

C. Quantification[5]

Proceed by the standard response line method[6].

«Summary of analysis method»

This method is intended to determine the amount of CL in a feed by microbiological assay using a sample solution prepared by adding pyridine and hexane, mixing, extracting with a mixture of water, acetone and hydrochloric acid (51:40:9), and adjusting the pH to 5.9 to 6.1. None of the antibacterial substances approved for combined use with CL interfere with the quantification of CL.

The flow sheet of this method is shown in Figure 9.2.30-3.

Sample (equivalent to 0.1 mg or 50 μg (potency) as CL)
↓
Add 5 mL of pyridine and 5 mL of hexane and stire for 2-3 min).
↓
Extract with 95 mL of water-acetone-hydrochloric acid（51:40:9) (with a magnetic stirrer for 20 min).
↓
Centrifuge (at 1,500×*g* for 5 min).
↓
Filter (through filter paper No. 5A).
↓
Collect 20 mL of the filtrate (into a 50-mL beaker).
↓
Adjust the pH to 5.9-6.1 (with ammonia solution).
↓
Add Buffer No.5 to make 100 mL (for the sample amount of 0.1 mg (potency)) or 50 mL (for the sample amount of 50 μg (potency)) in a respective one-mark
↓
Filter (through 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 9.2.30-3　Quantitative test method for colistin sulfate (feed)

References: Noriyuki Koyama, Toshitake Kono: Research Report of Animal Feed, 6, 95 (1980)

History in the Feed Analysis Standards [3] New

«Validation of analysis method»

・Spike recovery and repeatability

Added ingredient	Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Colistin sulfate (pure grade)	Starting chick grower formula feed	2~40	3	98.2~99.2	3.5
	Growing chick grower formula feed	2~40	3	100.2~100.5	2.5
	Finishing chick grower formula feed	2~40	3	97.8~101.8	2.8
	Piglet grower formula feed	2~40	3	98.5~100.4	2.3
	Suckling piglet grower formula feed	2~40	3	98.1~99.6	2.0
Colistin sulfate (feed grade)	Suckling piglet grower formula feed	2~40	4	98.3~100.2	2.2
	Starting chick grower formula feed	2~40	3	98.1~99.2	3.5
	Growing chick grower formula feed	2~40	3	98.1~99.7	3.5
	Finishing chick grower formula feed	2~40	3	97.2~99.4	2.4
	Piglet grower formula feed	2~40	3	98.0~99.1	2.4

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Starting chick formula feed	4	10	105.2	6.9	6.3

«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 colistinstandard solution is shown in Table 9.2.30-2.

Table 9.2.30-2 Method of preparation for colistinstandard solution (feed, example)

Test tube No.	1	2	3	4	5	6	7
Amount (mL) of standard solutic	②	2	2	10	10	10	5
Amount (mL) of Buffer No.5-acetone-pyridine (91:8:1)	23	18	18	10	10	10	5
Concentration (μg(potency)/mL)	80	8	0.8	0.4	<0.2>	0.1	0.05

Note: ②mL" 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] The addition of hexane is intended to remove fat and oil. The hexane phase can be separated from the sample solution by centrifugation.

[4] It is recommended to add 5 to 10 mL of Buffer No.5 to the filtrate before adjusting the pH.

[5] An example standard response line for CL is shown in Figure 9.2.30-4.

Linearity is observed in the quantification range for CL (CL concentrations between 0.05 and 1 μg (potency)/mL).

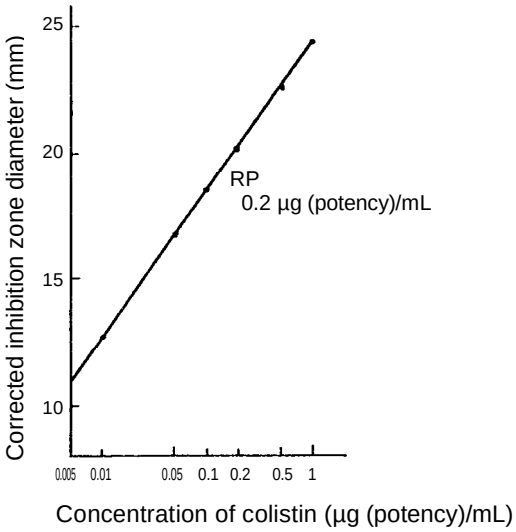


Figure 9.2.30-4 Standard response line for colistin (feed, example)
(*Bordetella bronchiseptica* ATCC 4617, Medium F-9, Agar well method)

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