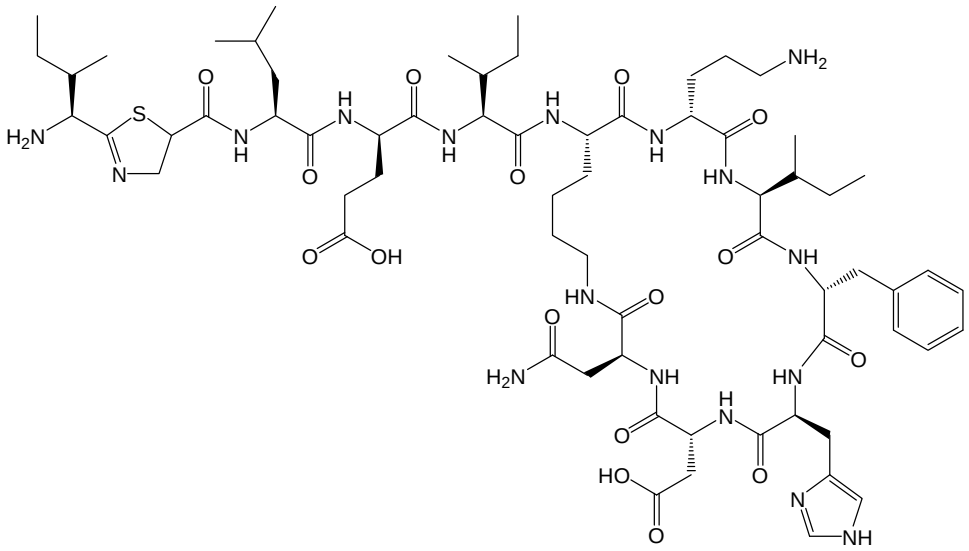
1 Zinc bacitracin or manganese bacitracin

Zinc bacitracin, Manganese bacitracin



Bacitracin A

$C_{66}H_{103}N_{17}O_{16}S$ MW: 1423 CAS No.: 1405-87-4 (bacitracin A), 1405-89-6 (zinc bacitracin)

[Summary of zinc bacitracin or manganese bacitracin]

Bacitracin (BC) is a polypeptide antibiotic obtained by the incubation of *Bacillus licheniformis*. The chemical structure shown above is of bacitracin A, the main ingredient.

For physicochemical properties, zinc bacitracin technical occurs as a yellowish gray-brown to brown powder, and has a characteristic odor. It is freely soluble in dilute hydrochloric acid, in water, in pyridine and in methanol, and slightly soluble in chloroform and in benzene. Although stable in the dry state, zinc bacitracin becomes inactivated in an alkaline solution with a pH of 9 or higher, and in the presence of high-concentration cupper or iron and of hydrogen peroxide.

Bacitracin has a strong antibacterial effect mainly on Gram-positive bacteria and an antibacterial effect on Gram-negative bacteria, leptospirae, and actinomycete, and has a growth promoting effect on chickens (including broilers), pigs, and cattle.

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

Zinc bacitracin and manganese bacitracin were designated as feed additives as of July 24, 1976, but manganese bacitracin was deleted from the list as of October 15, 1985.

Zinc bacitracin is a feed-grade antibiotic, and 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 (Ordinance No. 35, issued by the Ministry of Agriculture and Forestry).

(in 1,000,000 units/t)

Feed of interest	For chickens (except for broilers)	For broilers	For pigs		For cattle	
	Starting Growing chicks	Starting broilers Finishing broilers	Sucking piglets	Piglets	Sucking calves	Calves
Added amount	16.8~168	16.8~168	42~420	16.8~168	42~420	16.8~168

The amount of zinc bacitracin added to a commercial premix is roughly 10,000 to 4,000,000 units/kg.

[Methods listed in the Feed Analysis Standards]

1 Quantitative test methods - Plate method

1.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 1.1.1]

A. Reagent preparation

1) Buffer solution: Buffer No.3

2) Bacitracin standard solution. Dry a suitable amount of bacitracin 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.3 and dissolve to prepare a bacitracin standard stock solution with a concentration of 100 units/mL[2].

At the time of use, accurately dilute a quantity of standard stock solution with Buffer No.3 to pepare high- and low-concentration standard solutions with concentrations of 0.2 and 0.05 unit/mL, respectively[3].

3) Culture medium: Medium F-1.

4) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 10240[4] as the test organism, and add about 0.1 mL of a 10-fold diluted suspension of the test organism to 100 mL of the culture medium.

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

6) Extracting solvent: A mixture of water, pyridine, and hydrochloric acid (1 mol/L) (62:18:9)[5]

B. Preparation of sample solution

1) When the analysis sample does not contain SL or MN

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) [6].

Accurately dilute a quantity of the filtrate with Buffer No.3 to prepare high- and low-concentration sample solutions with concentrations of 0.2 and 0.05 unit/mL, respectively[7].

2) When the analysis sample contains SL or MN[8]

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) [6].

Transfer accurately 25 mL of the filtrate to a 50 mL beaker, adjust the pH to 1.0 or lower with hydrochloric acid, allow to stand for 1 hour, and again adjust the pH to 5.9 to 6.1 with ammonia solution. Transfer the whole amount of this liquid to a 50-mL one-mark flask with Buffer No.3, add Buffer No.3 to the marked line, and filter through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with Buffer No.3 to prepare high- and low-concentration sample solutions with concentrations of 0.2 and 0.05 unit/mL, respectively[7].

C. Quantification[9]

Proceed by the 2-2 dose method[10].

«Summary of analysis method»

This method is intended to determine the amount of BC in a premix by microbiological assay using a sample solution prepared by extracting with a mixture of water, pyridine, and hydrochloric acid (1 mol/L) (62:18:9) and diluting with Buffer No.3. To remove the effects of SL or MN, a premix containing these ingredients shall be treated with hydrochloric acid.

The flow sheet of the analysis method is shown in Figure 9.2.1-1.

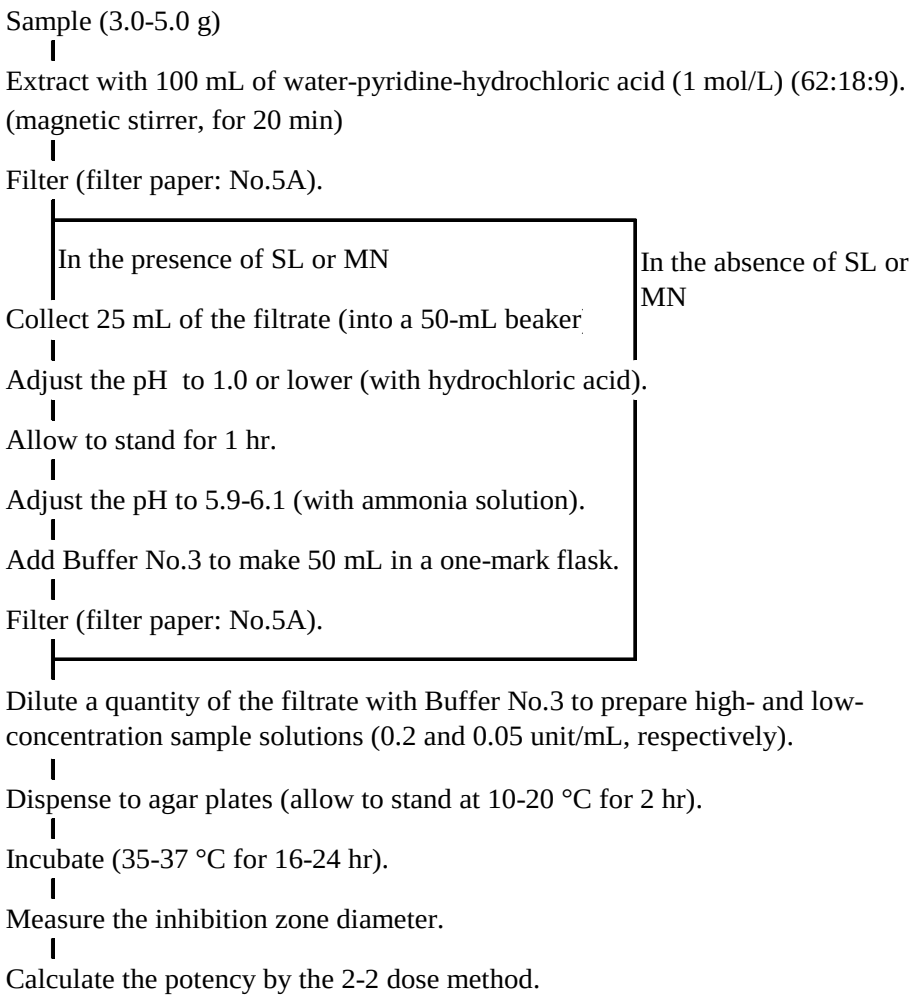


Figure 9.2.1-1 Quantitative test method for zinc bacitracin and manganese bacitracin (premix)

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

Name of spiked component	Sample type	Spike concentration (10,000)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Zinc bacitracin	Vitamin premix	24-168	3	99.6-100.8	2.8
	Vitamin/mineral premix	24-168	3	99.3-100.6	3.4

«Notes and precautions»

[1] For the definition etc. of bacitracin working standard, refer to «Notes and precautions» [9] in Secton 1, 1 of this Chapter.

[2] For the method of preparation for the standard stock solution, refer to «Notes and precautions» [10] in Secton 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 62.5 units/mg, 50 mg of the working standard contains 3,125 units (i.e., 50 mg × 62.5 units/mg). To prepare a standard stock solution with a concentration of 100 units/mL, the required amount of solvent is thus calculated to be 31.25 mL (i.e., 3,125 units / 100 units/mL). Therefore, completely transfer 50 mg of working standard to an Erlenmeyer flask containing 31.25 mL of Buffer No.3 and dissolve to prepare a standard stock solution with a concentration of 100 units/mL.

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

An example method of preparation for bacitracin standard solution is shown in Table 9.2.1-1.

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

[5] Corresponds to a mixture of water, pyridine, and hydrochloric acid (355:95:4).

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

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

An example method of preparation is shown in Table 9.2.1-1.

Table 9.2.1-1 Method of preparation for bacitracin standard solution and sample solution

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

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	2	5
Amount (mL) of Buffer No.3	8	18	18	15
Concentration (unit/mL)	20	2	0.2	0.05

Note: ②mL" means "2 mL of standard stock solution (100 unit/mL).

2) Method of preparation for the sample solution (premix not containing SL or MN, example)

When the analysis sample is collected in an amount equivalent to 2,000 units of BC, the concentration of bacitracin in the filtrate is calculated to be 20 units/mL.

Test tube No.	1	2	3
Amount (mL) of sample solution	②	2	5
Amount (mL) of Buffer No.3	18	18	15
Concentration (unit/mL)	2	0.2	0.05

Note: "②mL" means "2 mL of filtrate (20 units/mL).

[8] Test organism *Micrococcus luteus* ATCC 10240 becomes sensitive to the sample solution if it contains not less than 10 μg (potency)/mL of SL or MN, resulting in a larger zone of growth inhibition (Figure 9.2.1-2). To remove this effect, treat the sample solution with hydrochloric acid if the analysis sample contains SL or MN in combination.

As SL and MN gradually lose their antibacterial activities in an acid solution, inactivate the SL or MN contained together with the antibiotic of interest in the sample solution, to a level that would not affect the quantified results, by adjusting the pH of the sample solution to 1.0 or lower and allowing to stand for 1 hour (see Figure 9.2.1-3). Although this method of quantifiction is applicable to many antibiotics that are used in combination with SL or MN, it is not applicable if the antibiotic of intrest is unstable in an acid solution.

For LS, a substance related to SL and MN, it is difficult to reduce its antibacterial activity by treating with hydrochloric acid as directed above.

[9] An example standard response line for BC is shown in Figure 9.2.1-4.

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

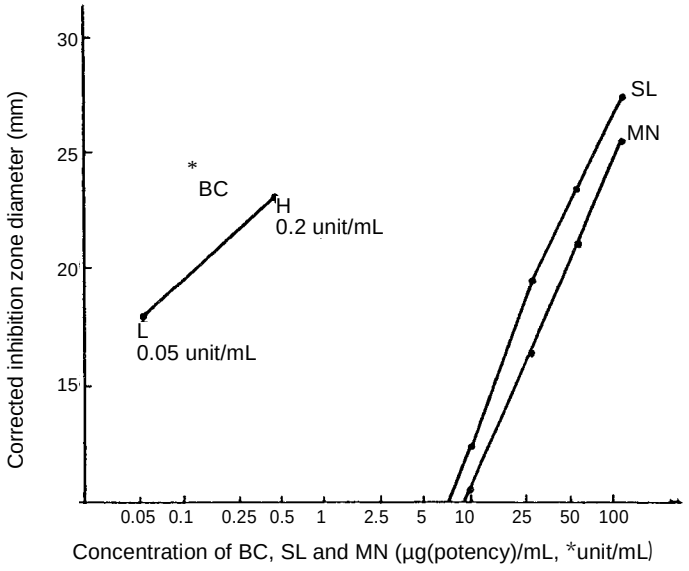


Figure 9.2.1-2 Sensitivity curves for bacitracin, salinomycin, and monesin

(*Micrococcus luteus* ATCC 10240, Medium F-1, Agar well method)

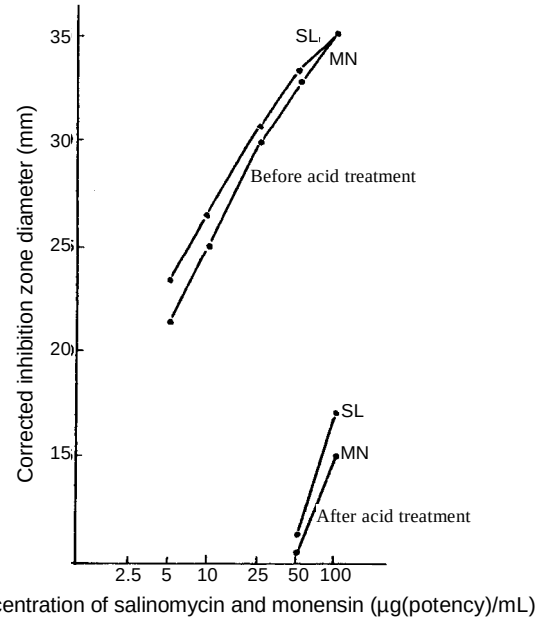


Figure 9.2.1-3 Effect of hydrochloric acid treatment on salinomycin and monensin
(*Micrococcus luteus* ATCC 10240, Medium F-1, Agar well method)

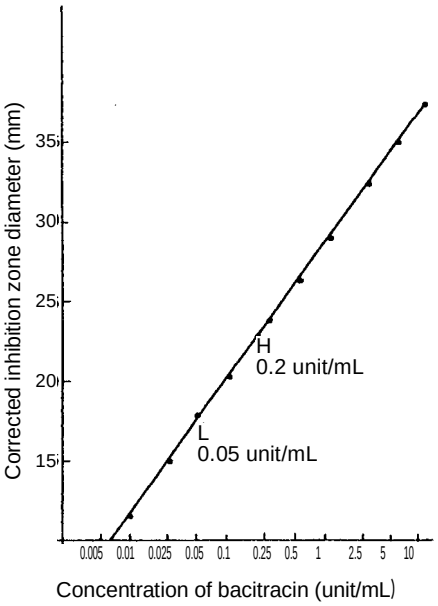


Figure 9.2.1-4 Standard response line for bacitracin (premix, example)
(*Micrococcus luteus* ATCC 10240, Medium F-1, Agar well method)

2.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 1.2.1]

A. Reagent preparation

1) Buffer solution: Buffer No.3

2) Bacitracin standard solution. Dry a suitable amount of bacitracin 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.3 and dissolve to prepare a bacitracin standard stock solution with a concentration of 100 units/mL.

At the time of use, accurately dilute a quantity of standard stock solution with a mixture of Buffer No.3 and methanol (3:1) to prepare standard solutions with concentrations of 0.4, 0.2, 0.1, 0.05, and 0.025 unit/mL[1].

3) Culture medium: Medium F-1.

4) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 10240[2] as the test organism, and add about 0.1 mL of a 10-fold diluted suspension of the test organism to 100 mL of the culture medium.

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

6) Extracting solvent: A mixture of methanol and hydrochloric acid (0.3 mol/L) (1:1)[3].

B. Preparation of sample solution

Weigh a quantity of the analysis sample[4] (equivalent to 20 units as BC), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, extract with stirring for 20 minutes. Place 50 mL of the extract in a stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, filter the

supernatant liquid through filter paper (No.5A).

Transfer accurately 25 mL of the filtrate into a 50-mL beaker, and adjust the pH to 5.9 to 6.1 with ammonia solution (6 mol/L) [5]. Transfer the total amount of this liquid with Buffer No.3 to a 50-mL one-mark flask, add Buffer No.3 to the marked line, and filter through filter paper (No.5A) to prepare a sample solution with a concentration of 0.1 unit/mL.

C. Quantification[6]

Proceed by the standard response line method[7].

«Summary of analysis method»

This method is intended to determine the amount of BC in a feed by microbiological assay using a sample solution prepared by extracting with a mixture of methanol and hydrochloric acid (0.3 mol/L) (1:1) and adjusting the pH to 5.9 to 6.1.

The flow sheet of the analysis method is shown in Figure 9.2.1-5.

Sample (equivalent to 20 units as BC)
↓
Extract with 100 mL of methanol-hydrochloride acid (0.3 mol/L) (1:1).
(magnetic stirrer, 20 min)
↓
Centrifuge (at 3,000 rpm for 5 min).
↓
Filter (filter paper: No.5A).
↓
Collect 25 mL of the filtrate (into a 50-mL beaker).
↓
Adjust the pH to 5.9-6.1 (with ammonia solution).
↓
Add Buffer No. 3 to make 50 mL in a one-mark flask.
↓
Filter (filter paper: No.5A).
↓
Dispense to agar plates (allow to stand at 10-20 °C at 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 9.2.1-5 Quantitative test method for zinc bacitracin and manganese bacitracin (feed)

References: Tetsuo Chihara: Livestock Research, 37, 371 (1983)

History in the Feed Analysis Standards [4] New

«Validation of analysis method»

・Spike recovery and repeatability

Name of spiked component	Sample type	Spike concentration (10,000 units/t)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Zinc bacitracin	Starting chick formula feed	84~420	6	96.0~99.6	6.1
	Sucking piglet formula feed	84~420	6	96.6~98.0	5.9
	Sucking calf formula feed	84~420	6	94.6~96.2	5.0

«Notes and precautions»

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

An example method of preparation for bacitracin standard solution is shown in Table 9.2.1-2.

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

Table 9.2.1-2 Method of preparation for bacitracin standard solution (feed, example)

Test tube No.	1	2	3	4	5	6	7
Amount (mL) of standard solution	②	2	10	10	10	10	5
Amount (mL) of Buffer No.3-methanol (3:1)	23	18	10	10	10	10	5
Concentration (unit/mL)	8	0.8	0.4	0.2	<0.1>	0.05	0.025

Note: "②mL" means "2 mL of standard stock solution (100 units/mL)".

[3] Corresponds to a mixture of methanol, water, and hydrochloric acid (77:75:2).

[4] An example amount of sample collection is shown in Table 9.2.1-3.

Table 9.2.1-3 Labeled potency and collected amount of bacitracin (example)

Labeled potency (unit/t)	Amount of sample collection
4200000	4.8
1680000	11.9
840000	23.8

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

[6] An example standard response line for BC is shown in Figure 9.2.1-6.

The standard response line shows linearity in the quantification range for BC (BC concentrations between 0.025 and 0.4 unit/mL).

Of the antibacterial substances that are approved to be used in combination with BC, DM-A, CL and sulfaquinoxaline have weak antibacterial effects on the test organism; however, they do not interfare with the quantification of BC within the usual range of spiked amount. Although SL and MN have strong antibacterial effects on the test organism, they do not interfare with the quantification of BC because they lose their antibacterial activities in the acidic extracting solvent.

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

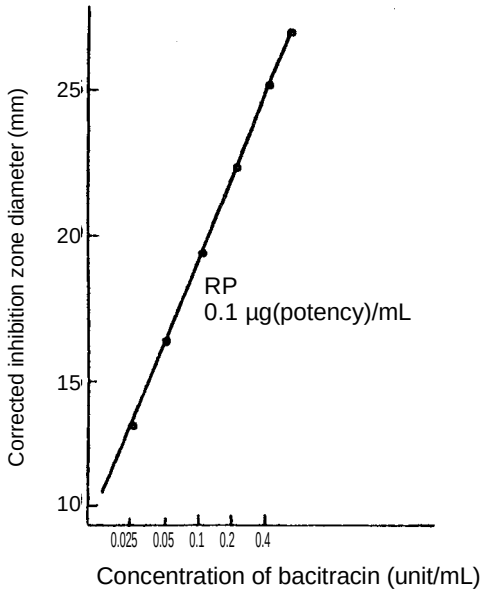


Figure 9.2.1-6 Standard response line for bacitracin (feed, example)
(*Micrococcus luteus* ATCC 10240, Medium F-1, Agar well method)