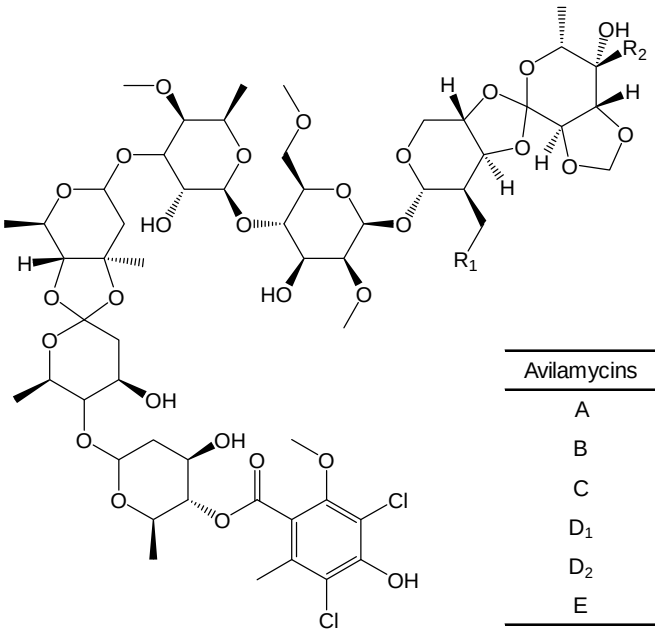
2 Avilamycin



Avilamycins	R_1	R_2
A	$COCH(CH_3)_2$	$COCH_3$
B	$COCH_3$	$COCH_3$
C	$COCH(CH_3)_2$	$CH(OH)CH_3$
D_1	H	$COCH_3$
D_2	$COCH_3$	$CH(OH)CH_3$
E	H	$CH(OH)CH_3$

$C_{61}H_{88}Cl_2O_{32}$ MW: 1403 CAS No.: 69787-79-7

[Summary of avilamycin]

Avilamycin (AVM) is a orthosomycin antibiotic obtained by the incubation of *Streptomyces viridochromogenes*, and is a mixture composed of multiple components.

For physicochemical properties, AVM occurs as a gray to black-brown powder or particles, and has a characteristic odor. Avilamycin A is freely soluble in chloroform, freely soluble in acetone, slightly soluble in ethyl acetate, inmethanol and in ethanol, and very slightly soluble in water.

AVM has an antibacterial effect mainly on Gram-positive bacteria 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»

AVM is a feed-grade antibiotic that was designated as a feed additive as of May 14, 1992. The specifications for feeds containing AVM are specified in Appended Table 1, 1-(1)-C in 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 broilers Finishing broilers	Sucking piglets	Piglets
Added amount	2.5~10	2.5~10	10~40	5~40

The amount of AVM added to a commercial premix is roughly 2.5 to 25 g (potency)/kg.

[Methods listed in the Feed Analysis Standards]

1 Quantitative test methods - Plate maethod (premix)

1.1 Premix not containing SL, MN or LS [Feed Analysis Standards Chapter 9, Section 2, 2.1.1.]

Scope of application: Premix not containing SL, MN or LS

A. Reagent preparation

1) Buffer solution: Buffer No.7

2) Dilution solvent: A mixture of Buffer No.7 and acetone (4:1)

3) Avilamycin standard solution. Dry a suitable amount of avilamycin working standard[1] under reduced pressure (not exceeding 2.67 to 3.33 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, add accurately acetone and dissolve to prepare an avilamycin 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 the dilution solvent to prepare a high- and low-concentration standard solutions with concentrations of 2 µg and 0.5 µg (potency)/mL[3].

4) Culture medium: Medium F-8

5) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 10240[4] as the test organism. Add about 0.2 mL of the suspension of the test organism to 100 mL of the culture medium.

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

B. Preparation of the sample solution

Weigh accurately 3 to 5 g of the analysis sample[Note 1], place in a 200-mL stoppered Erlenmeyer flask, add 20 mL of Buffer No.7, and stir for 5 minutes. Add 80 mL of acetone, extract with stiring for 20 minutes[5], and filter the extract through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with Buffer No.7 or dilution solvent so that the concentration of acetone in the sample solution is 20 v/v% to prepare high- and low-concentration sample solutions with concentrations of 2 µg and 0.5 µg (potency)/mL, respectively[6].

C. Quantification[7]

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

Note 1 Pulverize the analysis sample so that the particles pass through a 0.5-mm sieve[9].

(Additional notes) For a premix whose extract has a pH of 4.5 or lower, prepare the extract as directed below.

Weigh accurately 3 to 5 g of the analysis sample, place in a 50-mL beaker, add 20 mL of Buffer No.7, and stir for 5 minutes. Add sodium hydroxide solution (10 mol/L) until the pH of this solution becomes 4.5 to 5.0, and confirm the amount required.

Separately, weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, and add 20 mL of Buffer No.7. Further, add the previously confirmed required amount of sodium hydroxide solution (10 mol/L), and stir for 5 minutes. Then, add an amount of acetone calculated by subtracting the already added amount of sodium hydroxide solution (10 mol/L) from 80 mL, and extract with stirring for 20 minutes.

«Summary of analysis method»

This method is intended to determine the amount of AVM in a premix not containing SL, MN or LS by microbiological assay using a sample solution prepared by extracting with Buffer No.7 and acetone and diluting with Buffer No.7 or a mixture of Buffer No.7 and acetone (4:1).

The flow sheet of the analysis method is shown in Figure 9.2.2-1.

Sample (3.0-5.0 g)
↓
Add 20 mL of Buffer No.7 and stir (using a magnetic stirrer for 5 min).
↓
Add 80 mL of acetone and stir (using a magnetic stirrer for 20 min).
↓
Filter (filter paper: No.5A).
↓
Dilute a quantity of the filtrate with Buffer No.7 or Buffer No.7-acetone (4:1) so that the acetone concentration is 20 v/v% 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 ℃ for 2 hr).
↓
Incubate (at 35-37 ℃ for 16-24 hr).
↓
Measure the inhibition zone diameter.
↓
Calculate the potency by the 2-2 dose method.

Figure 9.2.2-1 Quantitative test method for avilamycin (premix not containing SL, MN or LS)

References: Seishi Araki: Research Report of Animal Feed, 22, 87 (1997)

History in the Feed Analysis Standards [19] 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	0.2~5	3	95.3~99.7	9.9
Chicken premix	0.2~5	3	99.7~105.0	12.1
Pig premix	0.2~5	3	94.7~103.3	8.0

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/kg	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Chicken premix	8	1	102.4	3.4	6.6

«Notes and precautions»

[1] For the definition etc. of avilamycin 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 1,206 µg (potency)/mg, 50 mg of the working standard contains 60,300 µg (potency) (i.e., 50 mg × 1,206 µg (potency)/mg). To prepare a standard stock solution with a concentration of 1,000 µg (potency)/mL, the required amount of the solvent is thus calculated to be 60.3 mL (i.e., 60,300 µg (potency) / 1,000 µg (potency)/mL). Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 60.3 mL of acetone 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 Secton 1, 1 of this Chapter.

An example method of preparation for avilamycin standard solution is shown in Table 9.2.2-1.

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

[5] When the pH of the extract is significantly low, the quantified amount of AVM can be low. In this case, proceed as directed in Additional notes.

[6] 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 9.2.2-1.

Table 9.2.2-1 Method of preparation for avilamycin standard solution and sample solution

1) Method of preparation for avilamycin standard solution (premix not containing SL, MN or LS, example)

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	4	5
Amount (mL) of Buffer No.7-acetone (4:1)	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 of sample solution (premix not containing SL, MN or LS, example)

When the analysis sample was collected in an amount equivalent to 1,000 µg (potency) of AVM, the concentration of avilamycin in the filtrate is calculated to be 10 µg (potency)/mL.

Test tube No.	1	2	3
Amount (mL) of sample solution	⑤	16	5
Amount (mL) of Buffer No.7	15		
Amount (mL) of Buffer No. 7-acetone (4:1)		4	15
Concentration (µg(potency)/mL)	2.5	2	0.5

Note: ⑤ mL" means "5 mL of the filtrate (10 µg(potency)/mL)".

When the amount of the analysis sample is collected in an amount equivalent to 8,000 µg (potency) of AVM, the concentration of avilamycin in the filtrate is calculated to be 80 µg (potency)/mL.

Test tube No.	1	2	3
Amount (mL) of sample solution	⑤	2	5
Amount (mL) of Buffer No.7	15		
Amount (mL) of Buffer No.7-acetone (4:1)		18	15
Concentration (μg(potency)/mL)	20	2	0.5

Note: "⑤ mL" means "5 mL of the filtrate (80 μg(potency)/mL)".

[7] An example standard response line for AVM is shown in Figure 9.2.2-2.

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

[9] As the particle size of AVMformulation is coarse, pulvelize the analysis sample to particles not exceeding 0.5 mm to remove the variability of quantified values.

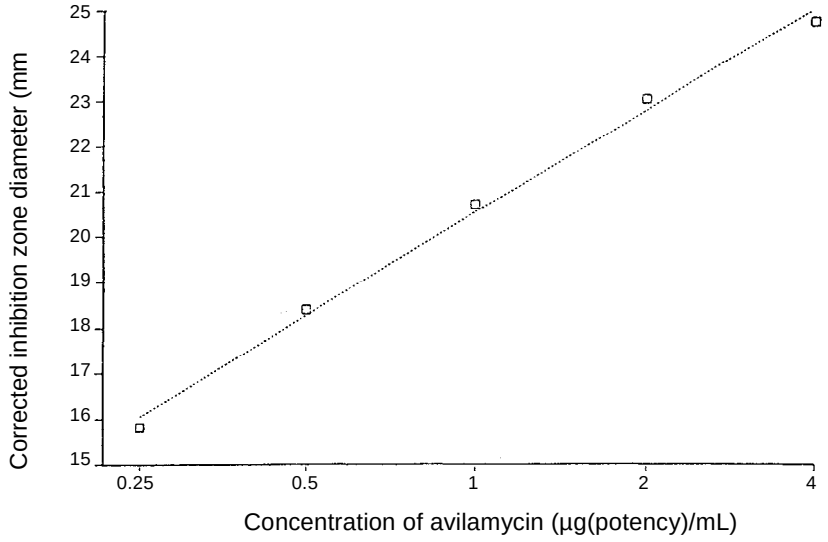


Figure 9.2.2-2 Standard response line for avilamycin
(premix not containing SL, MN or LS, example)
(*Micrococcus luteus* ATCC 10240, Medium F-8, Agar well method)

1.2 Premix containing SL or LS [Feed Analysis Standards Chapter 9, Section 2, 2.1.2]

Scope of application: Premix containing SL or LS

A. Reagent preparation

1) Buffer solution: Buffer No.7

2) Dilution solvent: A mixture of Buffer No.7 and acetone (4:1)

3) Avilamycin standard solution. Dry a suitable amount of avilamycin working standard under reduced pressure (not exceeding 2.67 to 3.33 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, add accurately acetone and dissolve to prepare an avilamycin standard stock solution with a concentration of1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of standard stock solution with the dilution solvent to prepare high- and low-concentration standard solutions with concentrations of 0.4 and 0.1 μg (potency)/mL, respectively[1].

4) Culture medium: Medium F-25[2].

5) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 10240[3] as the test organism. Add about 0.2 mL of the suspension of the test organism to 100 mL of the culture medium.

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

B. Preparation of sample solution

Extraction. Weigh accurately 3 to 5 g of the analysis sample[Note 1], place in a 200-mL stoppered Erlenmeyer flask, add 20 mL of Buffer No.7, and stire for 5 minutes. Further, add 80 mL of acetone, extract[4] with stirring for 20 minutes, and filter the extract through filter paper (No.5A). Dilute a quantity of the filtrate with Buffer No.7 so that the concentration of acetone is 45 v/v%[5], filter through filter paper (No.5A), and use the supernatant as a sample solution subject to column treatment.

Column treatment. Wash an octadecylsilanized silica gel minicolumn (360 mg) with 10 mL of acetone and 10 mL of a mixture of Buffer No.7 and acetone (11:9) in this order.

Place a 50-mL one-mark flask under the minicolumn, place accurately 10 mL of the sample solution in the minicolumn, inject under pressure[Note 2] to force AVM to flow out. Add 5 mL of a mixture of Buffer No.7 and acetone (11:9) to the minicolumn, inject under pressure[Note 2] to force to flow out in the same manner, and repeat this procedure twice. Add a mixture of Buffer No.7 and acetone (11:9) to the one-mark flask up to the marked line, dilute a quantity of this liquid with Buffer No.7 so that the concentration of acetone is 20 v/v%, and further dilute with a mixture of Buffer No.7 and acetone (4:1) to prepare high- and low-concentration sample solutions (0.4 and 0.1 μg (potency)/mL, respectively) [6].

C. Quantification[7]

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

Note 1 Pulverize the analysis sample so that the particles pass through a 0.5-mm sieve [9].

2 Set the flow rate to 2 to 3 mL/min.

(Additional notes) When the pH of an extract of the premix of interest is 4.5 or lower, proceed as directed below.

Weigh accurately 3 to 5 g of the analysis sample, place in a 50-mL beaker, add 20 mL of Buffer No.7, stir for 5 minutes, add sodium hydroxide solution (10 mol/L) until the pH becomes 4.5 to 5.0, and determine the amount required.

Separately, weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 20 mL of Buffer No.7, add the previously determined required amount of sodium hydroxide solution (10 mol/L), and stir for 5 minutes. Then add an amount of acetone calculated by subtracting the already added amount of sodium hydroxide solution (10 mol/L) from 80 mL, and extract with stirring for 20 minutes.

«Summary of analysis method»

This method is intended to determine the amount of AVM in a premix containing SL or LS by microbiological assay using a sample solution prepared by extracting with Buffer No.7 and acetone and purifying with a C_{18} minicolum. This method is not applicable to a premix containing MN.

The flow sheet of the analysis method is shown in Figure 9.2.2-3.

Sample (3.0-5.0 g)
↓
Add 20 mL of Buffer No.7 and stir (using a magnetic stirrer for 5 min).
↓
Add 80 mL of acetone and stir (using a magnetic stirrer for 20 min).
↓
Filter (filter paper: No.5A).
↓
Dilute a quantity of the filtrate with Buffer No.7 so that acetone concentration is 45 v
↓
Filter (filter paper: No.5A).
↓
Load 10 mL of the filtrate on a C_{18} minicolumn (previously washed with 10 mL of acetone and with Buffer No.7-acetone (11:9).
↓
Elute AVM 3 times with 5 mL of Buffer No.7-acetone (11:9) (into a 50-mL flask with pressurized injection).
↓
Add Buffer No.7-acetone (11:9) to make 50 mL in a one-mark flask.
↓
Dilute with Buffer No.7 so that acetone concentration is 20 v/v%.
↓
Dilute a quantity with Buffer No.7 or Buffer No.7-acetone (4:1) to prepare high-and low-concentration sample solutions (0.4 and 0.1 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 9.2.2-3 Quantitative test method for avilamycin (premix containing SL or LS)

References: Seishi Araki, Suzuko Kazama: Research Report of Animal Feed, 22, 97 (1997)

History in the Feed Analysis Standards [19] 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 (2 types) (SL content is 20 times the AVM content.)	0.2~1	3	100.3~109.7	10.6
Chicken premix (2 types) (LS content is 30 times the AVM content.)	0.2~1	3	90.3~105.0	7.6

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/kg)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Chicken premix	8	1	100.5	2.3	5.1

«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 avilamycin standard solution is shown in Table 9.2.2-2.

[2] It is permissible to prepare a medium by adding 1,000 mL of water to 45 g of Antibiotic Medium 12 (Difco or an equivalent medium) and 30 g of sodium chloride, dissolving by heating, adjusting the pH to 7.9 to 8.1, and autoclaving at 121°C for 15 minutes.

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

[4] When the pH of the extract is significantly high, the quantified amount of AVM can be low. In this case, proceed as directed in Additional notes.

[5] An example amounts for diluting the filtrate is as follows:

Filtrate	9 mL
Buffer No.7	7 mL

[6] 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 9.2.2-2.

Table 9.2.2-2 Method of preparation for avilamycin standard solution and sample solution

1) Method of preparation for avilamycin standard solution (SL or Premix containing MN, example)

Test tube No.	1	2	3	4	5
Amount (mL) of standard solution	②	2	4	4	5
Amount (mL) of Buffer No.7-acetone (4:1)	18	18	16	16	15
Concentration (μg(potency)/mL)	100	10	2	0.4	0.1

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

2) Method of preparation for the sample solution (SL or premix containing MN, example)

When the analysis sample is collected in an amount equivalent to 800 μg (potency) of AVM, the concentration of avilamycin in the sample solution in the one-mark flask is calculated to be 0.9 μg (potency)/mL.

Test tube No.	1	2
Amount (mL) of sample solution	⑧	5
Amount (mL) of Buffer No.7	10	
Amount (mL) of Buffer No.7-acetone (4+1)		15
Concentration (μg(potency)/mL)	0.4	0.1

Note: "⑧mL" means "8 mL of the solution in the one-mark flask (0.9 μg(potency)/mL

When the analysis sample is collected in an amount equivalent to 8,000 μg (potency) of AVM, the concentration of avilamycin in the sample solution in the one-mark flask is

calculated to be 9 µg (potency)/mL.

Test tube No.	1	2	3
Amount (mL) of sample solution	⑧	2	5
Amount (mL) of Buffer No.7	10		
Amount (mL) of Buffer No.7-acetone (4:1)		18	15
Concentration (µg(potency)/mL)	4	0.4	0.1

Note: ⑧ "8 mL" means "8 mL of the solution in the one-mark flask (9 µg(potency)/mL)"

[7] An example standard response line for AVM is shown in Figure 9.2.2-4.

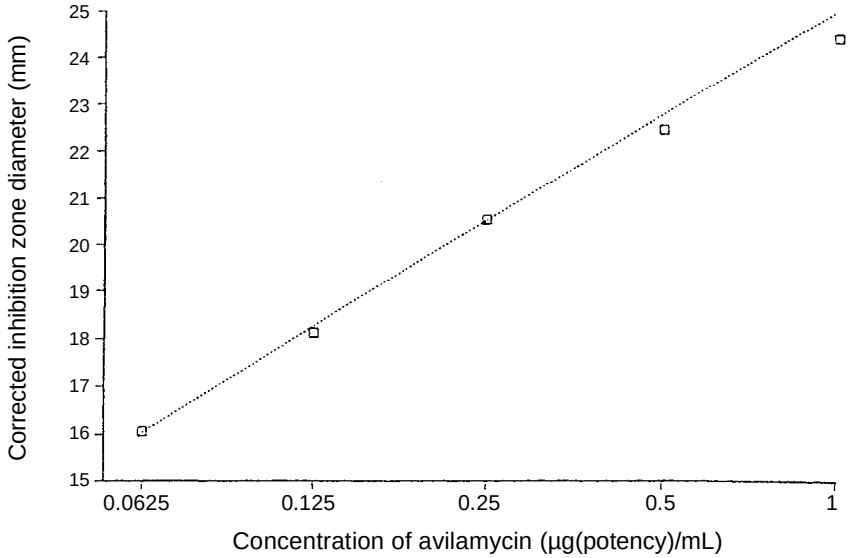


Figure 9.2.2-4 Standard response line for avilamycin
(premix containing SL or LS, example)
(*Micrococcus luteus* ATCC 10240, Medium F-25, agar well method)

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

[9] As the particle size of AVM formulation is coarse, pulverize the analysis sample into particles not exceeding 0.5 mm to reduce the variability of quantified results.

1.3 Feed

[Feed Analysis Standards Chapter 9, Section 2, 2.2.1]

Scope of application: Feeds for pigs

A. Reagent preparation

1) Buffer solution: Buffer No.7

2) Dilution solvent: A mixture of Buffer No.7 and acetone (4:1)

3) Avilamycin standard solution. Dry a suitable amount of avilamycin working standard under reduced pressure (not exceeding 2.67 to 3.33 kPa) at 60 °C for 3 hours, weigh accurately not less than 40 mg, add accurately acetone and dissolve to prepare an avilamycin 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 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-25 [2]

5) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 10240[3] as the test organism. Add about 0.5 mL of a 10-fold diluted suspension of the test organism to 100 mL of the culture medium.

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

B. Preparation of the sample solution

Weigh 10.0 g of the analysis sample[Note 1], place in a 200-mL stoppered Erlenmeyer flask, add 100 mL acetone solution (4:1), extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A).

Accurately dilute a quantity of filtrate with the dilution solvent and a mixture of Buffer No.7 and acetone (19:1) so that the concentration of acetone in the sample solution is 20 v/v%, to prepare a sample solution with a concentration of 0.2 µg (potency)/mL[4].

C. Quantification [5]

Proceed by the standard response line method[6].

Note 1 Pulerize the analysis sample so that the particles pass through a 0.5-mm sieve[7].

«Summary of analysis method»

This method is intended to determine the amount of avilamycin (AVM) in a feed for pigs by microbiological assay using a sample solution prepared by extracting with acetone solution (4:1) and diluting with a mixture of Buffer No.7 and acetone (4:1) and a mixture of Buffer No.7 and acetone (19:1).

The flow sheet of the analysis method is shown in Figure 9.2.2-5.

Sample (10.0 g)
↓
Dilute with 100 mL of acetone-water (4:1).
(magnetic stirrer, for 20 min)
↓
Filter (filter paper: No.5A).
↓
Dilute a quantity of the filtrate with dilution solvent or with Buffer No.7-acetone (19:1) so that acetone concentration is 20 v/v% to prepare a sample solution (0.2 µ
↓
Dispense to agar plates (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 9.2.2-5 Quantitative test method for avilamycin (feeds for pigs)

References: Shinji Oshima, Naoki Shinoda, Yoshiyasu Hashimoto, Tetsuo Chihara: Research Report of Animal Feed, 32, 61 (2007)

History in the Feed Analysis Standards [31] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Pig formula feed (3 types)	10~40	3 each	94.1~112.7	7.8

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Piglet grower formula feed	8	20	101.3	4.0	5.5

«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 avilamycin standard solution is shown in Table 9.2.2-3.

[2] It is permissible to use a medium prepared by adding 1,000 mL of water to 45 g of Antibiotic Medium 12 (Difco or an equivalent medium) and 30 g of sodium chloride, dissolving by heating, adjusting the pH to 7.9 to 8.1, and autoclaving at 121℃ for 15 minutes.

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

[4] 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 9.2.2-3.

Table 9.2.2-3 Method of preparation for avilamycin standard solution and sample solution

1) Method of preparation for avilamycin standard solution (feeds for pigs, example)

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

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

2) Method of preparation for the sample solution (feeds for piga, example)

When the added concentration of AVCM in the analysis sampleis is 10 g (potency)/t, the concentration of AVM in the filtrate is calculated to be 1 μg (potency)/mL.

Test tube No.	1
Amount (mL) of sample solution	④
Amount (mL) of Buffer No.7-acetone (19:1)	16
Concentration (μg(potency)/mL)	0.2

Note: ④"4 mL" means "4 mL of the filtrate (1 μg(potency)/mL)".

When the spike concentration of AVM in the analysis sample is 20 g (potency)/t, the

concentration of AVM in the filtrate is calculated to be 2 µg (potency)/mL.

Test tube No.	1	2
Amount (mL) of sample solution	④	10
Amount (mL) of Buffer No.7-acetone (19:1)	16	
Amount (mL) of Buffer No.7-acetone (4:1)		10
Concentration (µg(potency)/mL)	0.4	0.2

Note: "④ mL" means "4 mL of the filtrate (2 µg(potency)/mL)".

When the added concentration of AVM in the analysis sample is 40 g (potency)/t, the concentration of AVM in the filtrate is calculated to be 4 µg (potency)/mL.

Test tube No.	1	2
Amount (mL) of sample solution	④	5
Amount (mL) of Buffer No.7-acetone (19:1)	16	
Amount (mL) of Buffer No.7-acetone (4:1)		15
Concentration (µg(potency)/mL)	0.8	0.2

Note: ④mL" means "4 mL of the filtrate (4 µg(potency)/mL)".

[5] An example standard response line for AVM is shown in Figure 9.2.2-6.

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

[7] As the particle size of AVM formulation is coarse, pulverize the analysis sample to particles not exceeding 0.5 mm to reduce the variability of quancified values.

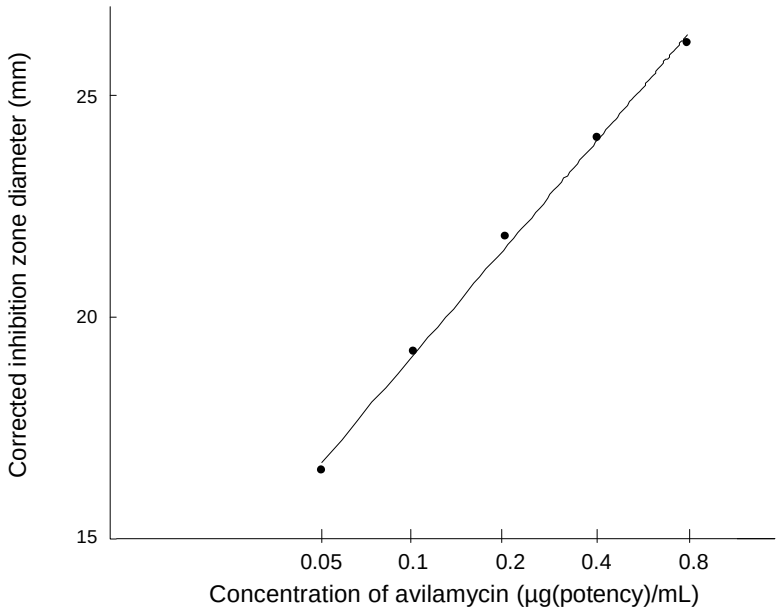


Figure 9.2.2-6 Standard response line for avilamycin (feeds for pigs, example)

(*Micrococcus luteus* ATCC 10240, Medium F-25, Agar well method)

1.4 Feed not containing SL or LS [Feed Analysis Standards Chapter 9, Section 2, 2.2.2]

Scope of application: Feeds not containing SL or LS

A. Reagent preparation

1) Buffer solution: Buffer No.7

2) Dilution solvent. A mixture of Buffer No.7 and acetone (4:1)

3) Avilamycin standard solution. Dry a suitable amount of avilamycin working standard under reduced pressure (not exceeding 2.67 to 3.33 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, add accurately acetone and dissolve to prepare an avilamycin 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 dilution solvent to prepare[1] standard solution with concentrations of 2 μg (potency)/mL, 1 μg (potency)/mL, 0.5 μg (potency)/mL, 0.25 μg (potency)/mL, and 0.125 μg (potency)/mL.

4) Culture medium: Medium F-8

5) Bacterial suspension and amount of addition Use *Micrococcus luteus* ATCC 10240[2] as the test organism, and add about 0.4 mL of a 10-fold diluted suspension of the test organism to 100 mL of the culture medium.

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

B. Preparation of the sample solution

Extraction. Weigh 10.0 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of chloroform, extract with stirring for 20 minutes, filter the extract through filter paper (No.5A), and use the supernatant as the sample solution subject to column treatment.

Column treatment. Wash the silica gel minicolumn (690 mg) with 10 mL of chloroform.

Place accurately 2.5 to 10 mL of the sample solution[3] (equivalent to 2.5 to 10 μg (potency) as AVM) in the minicolumn, allow to flow down until the remaining amount in the reservoir reaches 1 mL, and add 30 mL of a mixture of chloroform and acetone (17:3) to wash the minicolumn. Place a 50-mL round-bottom flask under the minicolumn, add 20 mL of acetone to the minicolumn to elute AVM.

Evaporate the eluate into dryness under reduced pressure in a water bath at 50°C, add accurately 5 to 20 mL of the dilution solvent [3] to dissolve the residue[4] to prepare a sample solution with a concentration of 0.5 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 AVM in a feed by microbiological assay using a sample solution prepared by extracting with chloroform, purifying with silica gel, and dissolving with a mixture of Buffer No.7 and acetone (4:1). Under the conditions of this quantitative method, when the feed of interest contains SL or LS in combination, the presence of these ingredients leads to a larger zone of growth inhibition and thus affects the quantitative results for AVM (Table 9.2.2-4).

The flow sheet of the analysis method is shown in Figure 9.2.2-7.

Sample (10.0 g)
|
Extract with 100 mL of chloroform.
(magnetic stirrer, 20 min)
|
Filter (filter paper: No.5A).
|
Load a quantity of the filtrate on the silica gel minicolumn
(previously washed with chloroform).
|
Wash with 30 mL ofchloroform-acetone (17:3) .
|
Elute AVM with 20 mL of acetone (into a 50-mL round-bottom flask).
|
Evaporate into dryness (in a water bath at 50 °C).
|
Dissolve the residue with a quantity of Buffer No.7-
acetone (4:1) to prepare a sample solution (0.5 µ
|
Dispense to agar plates (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 9.2.2-7 Quantitative test method for avilamycin (feed)

References: Kiyosi Kanno, Takashi Sasaki: Research Report of Animal Feed, 17, 83 (1992)

History in the Feed Analysis Standards [14] New

Table 9.2.2-4 Recovery of AVM from a feed containing SL or LS in combination.

Sample	Recovery (%)
1) AVM 2.5 g(potency)/t	100
2) AVM 2.5 g(potency)/t + SL 50 g(potenc	207
3) AVM 2.5 g(potency)/t + LS 75 g(potenc	202

Note: The edge of the inhibition zone is not clear for 2) a

«Validation of analysis method»

- Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Staring Chik formula feed	2.5~40	3	98.3~106.5	6.5
Finishing broiler formula feed	2.5~40	3	102.1~107.2	6.9
Sucking piglet formula feed	2.5~40	3	93.7~107.0	9.0

- Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Starting broiler formula feed	11	13	100.8	4.5	3.7

«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 avilamycin standard solution is shown in Table 9.2.2-5.

Table 9.2.2-5 Method of preparation for avilamycin standard solution (feed, 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.7-acetone (4:1)	18	18	16	10	10	10	5
Concentration (μg(potency)/ml	100	10	2	1	<0.5>	0.25	0.125

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 Secton 1, 1 of this Chapter.

[3] Example amounts of the sample solution loaded on the silica gel minicolumn and of the dilution solvent used to dissolve the residue resulting from drying under reduced pressure are shown in Table 9.2.2-6.

Table 9.2.2-6 Amount of the sample solution loaded on the silica gel cartridge and of the dilution solvent used to dissolve the residue

Spike concentration (g(potency)/t) of AVM in sample solution	Amount of sample solution loaded on silica gel minicolumn	Amount of dilution solvent for dissolving residue
2.5	10 mL	5 mL
5	10 mL	10 mL
10	10 mL	20 mL
20	5 mL	20 mL
40	3 mL	20 mL

[4] Plug and stir vigorously for 2 to 3 minutes, and further with ultrasonic waves for about 1 minute.

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

[6] An example standard response line for AVM is shown in Figure 9.2.2-8.

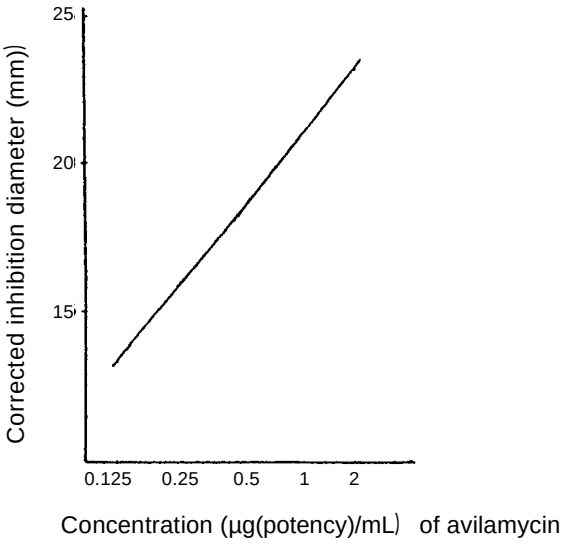


Figure 9.2.2-8 Standard response line for avilamycin (feed, example)
(*Micrococcus luteus* ATCC 10240, Medium F-8, Agar well method)