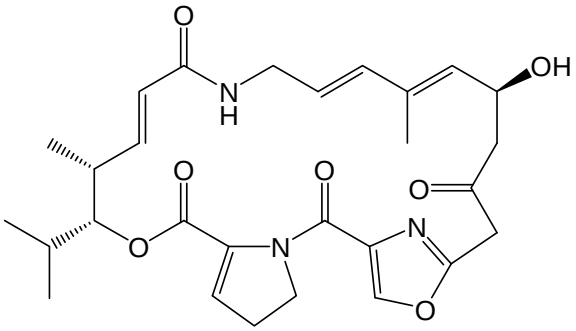
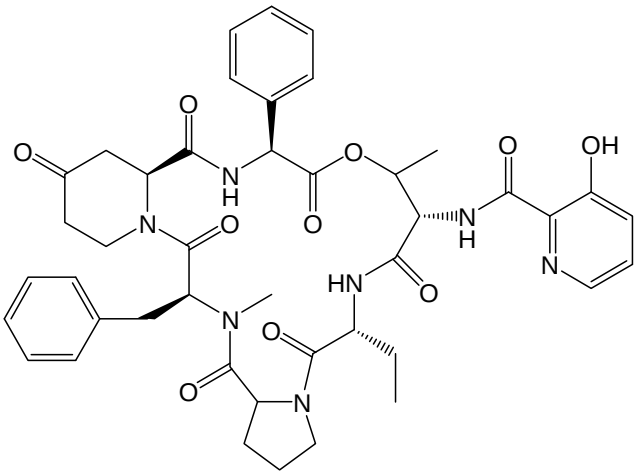
21 Virginiamycin



Virginiamycin M_1
$C_{28}H_{35}N_3O_7$ MW: 525.6
CAS No.: 21411-53-0



Virginiamycin S_1
$C_{43}H_{49}N_7O_{10}$ MW: 823.9
CAS No.: 23152-29-6

[Summary of virginiamycin]

Virginiamycin (VM) is a polypeptide antibiotic obtained by the incubation of *Streptomyces virginiae*, and composed of virginiamycin M_1 and virginiamycin S_1 (including traces of analogs).

For physicochemical properties, VM technical occurs as a light-yellow powder with a characteristic odor. It is freely soluble in chloroform, soluble in ethanol and in methanol, and very slightly soluble in water. It is extremely unstable in a basic solution, unstable in a strongly acidic solution, and most stable in a neutral solution.

VM has a strong antibacterial effect mainly on Gram-positive bacteria and an antibacterial effect on Gram-negative cocci and mycoplasma. It promotes the growth of chickens (including broilers) and pigs.

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

VM is a pure-grade antibiotic that was designated as a feed additive as of July 24, 1976. The specifications for feeds containing this ingredient are specified in Appended Table No.1, 1-(1)-C of the Standards and Specifications in the Act on Safety Assurance and Quality Improvement of Feeds.

(in g(potency)/t)

Feed of interest	For chickens (except for broilers)	For broilers	For pigs	
	Starting chicks Growing chiks	Starting period broilers Finising period broilers	Suckling piglets	Piglets
Added amount	5~15	5~15	10~20	10~20

The amount of VM added to a commercial premix is roughly 0.5 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, 21.1.1]

A. Reagent preparation

1) Buffer solution: Buffer No.2

2) Diution solvent. Adjust the pH of a mixture of Buffer No.2 and acetone (17:8) with hydrochloric acid (6 mol/L) to 5.9 to 6.1[1], and use as the dilution solvent.

3) Virginiamycin standard solution. Weigh accurately not less than 40 mg virginiamycin working standard[2], accurately add methanol and dissolve to prepare a virginiamycin standard stock solution with a concentration of 1 mg (potency)/mL[3].

At the time of use, accurately dilute a quantity of the standard stock solution with the dilution solvent to prepare high- and low-concentration standard solutions with concentrations of 2 and 0.5 µg (potency)/mL, respectively[4].

4) Culture medium: Medium F-4

5) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 9341[5] as the test organism. Add about 0.5 mL of a 10-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 acetone and citric acid monohydrate (0.5 mol/L) (4:1)[6]

B. Preparation of sample solution

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

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)[7].

Transfer accurately 20 mL of the filtrate to a 50-mL beaker, and adjust the pH to 5.9 to 6.1 with ammonia solution. Transfer the whole amount of this solution with Buffer No.2 to a 50-mL volumetric flask, add Buffer No.2 up to the marked line, and filter through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with the dilution solvent to prepare high- and low-concentration sample solutions with concentrations of 2 and 0.5 µg (potency)/mL, respectively[8].

2) When the analysis sample contains SL, MN or LS

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)[7].

Transfer accurately 20 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 with Buffer No.2 to 50-mL volumetric flask, add Buffer No.2 up to the marked line, and filter through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with the dilution solvent to prepare high- and low-concentration sample solutions with concentrations of 2 and 0.5 µg (potency)/mL, respectively[8].

C. Quantification[9]

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

«Summary of analysis method»

This method is intended to determine the amount of VM in a premix by microbiological assay using a sample solution prepared by extracting with a mixture of acetone and citric acid monohydrate (0.5 mol/L) (4:1), adjusting the pH to 5.9 to 6.1, and diluting with a mixture of Buffer No.2 and acetone (17:8) (pH 5.9 to 6.1). A premix containing SL, MN or LS, shall be treated with hydrochloric acid to remove the effect of these ingredients.

The flow sheet of this method is shown in Figure 9.2.21-1.

Sample (3.0-5.0 g)

Extract with 100 mL of acetone-citric acid monohydrate solution (0.5 mol/L) (4:1) (with a magnetic stirrer for 20 min).

Filter (through filter paper No.5A).

Collect 20 mL of the filtrate (into a 50-mL beaker).

In the presence of SL, MN or LS	In the absence of SL, MN or LS
Adjust the pH to 1.0 or less (with hydrochloric aci	
Allow to stand for 1 hr.	

Adjust the pH to 5.9-6.1 (with ammonia solution).

Add Buffer No.2 to make 50 mL in a volumetric flask.

Filter (through filter paper No.5A).

Dilute a quantity of the filtrate with Buffer No.2-acetone (17:8) (pH 5.9-6.1) to prepare high- and low-concentration sample solutions (2 and 0.5 μg

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 9.2.21-1 Quantitative test method for virginiamycin (premix)

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

Tetsuo Chihara: Research Report of Animal Feed, 11, 149 (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)/t)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Premix 1	0.5~10	3	93.8~101.3	6.7
Premix 2	0.5~10	3	97.4~103.6	5.7
Premix 3	0.5~10	3	95.7~98.3	9.0
Premix 4	0.5~10	3	96.2~108.5	5.1

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)
Pig premix	6	2	102.5	2.6	3.7

«Notes and precautions»

[1] Adjust the pH of the standard solution to that of the sample solution (around pH 6) to prevent the potential difference in pH from affecting the quantification of VM. When adjusting the pH, use the minimum amount of acid or alikali as possible to achieve almost the same concentration ofacetone in the standard solution and sample solution.

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

[3] 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 2,220 μg (potency)/mg, 50 mg of the working standard contains 111,000 μg (potency) (i.e., 50 mg × 2,220 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 111.0 mL (i.e., 111,000 μg (potency) / 1,000 μg (potency)/mL. Therefore, completely transfer 50 mg of working standard to an Erlenmeyer flask containing 111.0 mL of methanol and dissolve to prepare the standard stock solution with a concentration of 1,000 μg (potency)/mL.

[4] 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 virginiamycin standard solution is shown in Table 9.2.21-1.

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

[6] Corresponds to a mixture of acetone and citric acid monohydrate (10 w/v%) (4:1).

[7] 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.

[8] 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 9.2.21-1.

Table 9.2.21-1 Method of preparation for virginiamycin standard solution and sample solution

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

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	4	5
Amount (mL) of Buffer no.2-acetone (17:8) (pH 5.9-6.1)	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 for sample solution (premix, example)

When the analysis sample is collected in an amount equivalent to 4,000 μg (potency) of VM, the concentration of VM in the filtrate is calculated to be 16 μg (potency)/mL.

Test tube No.	1	2
Amount (mL) of sample solution	②	5
Amount (mL) of Buffer No.2-acetone (17:8) (pH 5.9-6.1)	14	15
Concentration (μg(potency)/mL)	2	0.5

Note: "②mL" means "2 mL of the filtrate (16 μg(potency)/mL)".

[9] An example standard response line for VM is shown in Figure 9.2.21-2.

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

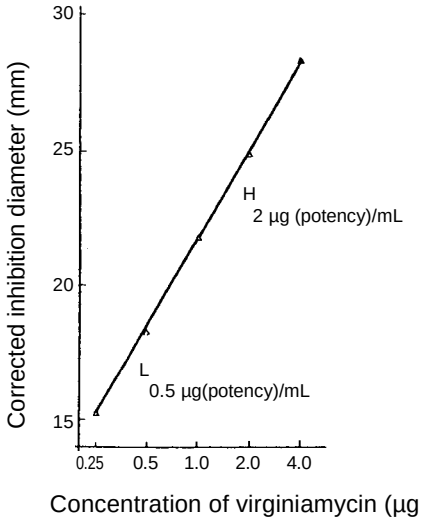


Figure 9.2.21-2 Standard response line for virginiamycin (premix, example)
(*Micrococcus luteus* ATCC 9341, Medium F-4, Agar well method)

1.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 21.2.1]

A. Reagent preparation

1) Buffer solution: Buffer No.2

2) Virginiamycin standard solution. Weigh accurately not less than 40 mg of virginiamycin working standard, accurately add methanol and dissolve to prepare a virginiamycin 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.2 and methanol (7:3) to prepare standard solutions with concentrations of 3.2, 1.6, 0.8, 0.4 and 0.2 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 0.2 mL of a 100-fold diluted 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

1) When the VM contant is not less than 10 g (potency)/t

Weigh accurately a quantity of the analysis sample (equivalent to 0.16 mg (potency) as VM), place in a 200-mL stoppered Erlenmeyer flask, add 20 mL of hexane, allow to stand for 10 minutes, add 100 mL of a mixture of Buffer No.2 and methanol (1:1), 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-methanol phase (lower phase) through filter paper (No.6)[3].

Accurately dilute a quantity of the filtrate with a mixture of Buffer No.2 and methanol (9:1) to prepare a sample solution with a concentration of 0.8 μg (potency)/mL[4].

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

Weigh accurately a quantity of the analysis sample (equivalent to 40 μg (potency) as VM), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of methanol, extract with stirring for 20 minutes, and filter the extract through filter paper (No.6).

Transfer accurately 20 mL of the filtrate to a 50-mL recovery flask, and evaporate into dryness under reduced pressure in a water bath at 50°C. Add 5 mL of hexane, shake, add accurately 3 mL of methanol to dissolve the residue, add accurately 7 mL Buffer No.2, and shake. Transfer this liquid to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and use the water-methanol phase (lower phase)[5] as the sample solution with a concentration of 0.8 μg (potency)/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 VM in a feed by microbiological assay using a samle solution prepared by each of the following procedures: when the VM content is not less than 10 g (potency)/t, extract the sample with a mixture of Buffer No.2 and methanol (1:1) and dilute with a mixture of Buffer No.2 and methanol (9:1); when the VM content is less than 10 g (potency)/t, extract the sample with methanol, purify, and dissolve in a mixture of methanol and Buffer No.2.

Of the antibacterial substances approved for combined use with VM, SL, MN and LS have strong antibacterial effects on the test organism. These ingredients, however, do not interfere with the quantification of VM because their antibacterial effects are nullified in the process of quantification.

The flow sheet of this method is shown in Figure 9.2.21-3.

i) When the analysis sample contains VM at a concentration not less than 10 g (potency)/t

Sample (equivalent to 0.16 mg (potency) as VM).
↓
Add 20 mL of hexane and allow to stand for 10 min.
↓
Extract with 100 mL of Buffer No.2-methanol (1:1)
(with a magnetic stirrer for 20 min).
↓
Centrifuge (at 1,500×g for 5 min).
↓
Remove the hexane phase and filter through filtre paper (No.6).
↓
Dilute a quantity of the filtrate with Buffer No.2-methanol (9:1) to prepare a sample solution with a concentration of 0.8 µg(potency)/mL.
↓
Dispense to agar plates (allow to satnd 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.

ii) When the analysis sample contains VM at a concentration less than 10 g (potency)/t

Sample (equivalent to 40 µg (potency) as VM).
↓
Extract with 100 mL of methanol
(with a magnetic stirrer for 20 min).
↓
Filter through filter paper (No.6).
↓
Collect 20 mL of the filtrate (into a 50-mL recovery flask).
↓
Evaporate to dryness under reduced pressure (in a water bath at 50°C).
↓
Add 5 mL of hexane and shake.
↓
Add 3 mL of methanol to dissolve the residue and add 7 mL of Buffer No.2.
↓
Centrifuge (at 1,500×g for 5 min).
↓
Collect the lower phase and use as a sample solution with a concentration of 0.8 µg (potency)/mL.
↓
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 resonse line method.

Figure 9.2.21-3 Quantitative test method for virginiamycin (feed)

References: Noriyuki Koyama: Research Report of Animal Feed, 9, 105 (1984)

History in the Feed Analysis Standards [5] 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	2~5	6	99.7~100.3	3.3
Starting period broiler formula feed	2~5	6	98.0~99.7	2.0
Starting piglet formula feed	10~20	6	100.6~101.2	1.4
Piglet grower formula feed	10~20	6	100.9~101.4	2.1

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)
Piglet formula feed	5	10	99.4	3.8	3.1

«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 virginiamycin standard solution is shown in Table 9.2.21-2.

Table 9.2.21-2 Method of preparation for virginiamycin 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.2-methanol (7:3)	23	18	15	10	10	10	5
Concentration (μg(potency)/mL)	80	8	3.2	<1.6>	0.8	0.4	0.2

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] It is permissible either to remove the hexane phase (upper phase) with a Komagome pipette etc., or to directly collect the lower phase with a Komagome pipette etc., carefully to avoid contamination with hexane.

[4] Dilute 5 mL of filtrate with 5 mL of a mixture of Buffer No.2 and methanol (9:1) to prepare a sample solution with a concentration of 0.8 μg (potency)/mL.

[5] After centrifugation, it is permissible either to gently transfer the liquid to a 50-mL separating funnel and allow the lower phase flow out of the funnel into a test tube etc., or to directly collect the lower phase with a Komagome pipette etc., carefully to avoid contamination with hexane.

[6] An example standard response line for VM is shown in Figure 9.2.21-4.

Linearity is observed in the quantification range for VM (VM concentration between 0.2 and 3.2 μg (potency)/mL).

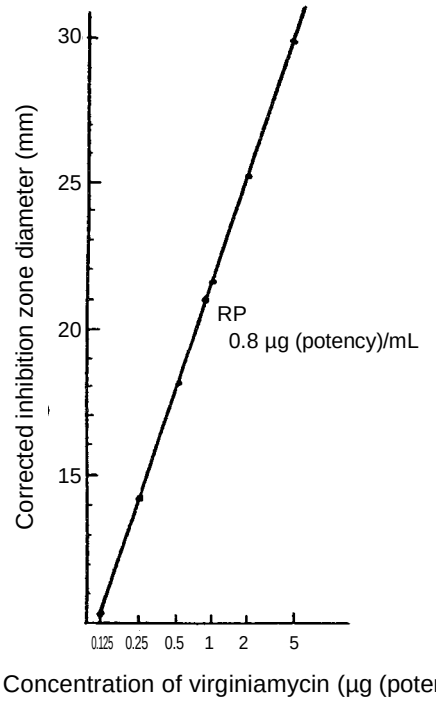


Figure 9.2.21-4 Standard response line for virginiamycin (feed, example)
(*Micrococcus luteus* ATCC 9341, Medium F-4, Agar well method)

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

2 Trace quantitative test method - Trace quantitative test method for KT, VM and TS by microbioautography (Feed)

[Feed Analysis Standards, Chapter 9, Section 2, 21.3.1]

Antibiotics of interest: KT, VM and TS

Scope of application: Feed

A. Reagent preparation

1) Kitasamycin standard solution. Weigh accurately not less than 40 mg of kitasamycin working standard[1], add 10 mL of methanol to dissolve, and further accurately add methanol to prepare a kitasamycin 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 methanol to prepare standard solutions with concentrations of 20, 10, 5, 2.5 and 1.25 µg (potency)/mL[3].

2) Virginiamycin standard solution. Weigh accurately not less than 40 mg of virginiamycin working standard[1], accurately add methanol and dissolve to prepare a virginiamycin 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 methanol to prepare standard solutions with concentrations of 20, 10, 5, 2.5 and 1.25 µg (potency)/mL[3].

3) Tylosin standard solution. Dry a suitable amount of tylosin working standard[1] under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, add a small amount of methanol to dissolve, and further accurately add Buffer No.4 to prepare a tylosin 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 methanol to prepare standard solutions with concentrations of 20, 10, 5, 2.5 and 1.25 μg (potency)/mL[3].

4) Culture medium: Medium F-111

5) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 9341[4] as the test organism. Add about 0.5 mL of a 100-diluted suspension of the test organism per 100 mL of the culture medium.

6) Developing solvent[5]

i) A mixture of hexane, ethyl acetate, acetone, and methanol (4:2:1:1)

ii) A mixture of acetonitrile and methanol (17:3)

7) Sodium sulfate (anhydrous). Dry at 110 to 120°C for 2 hours and allow to cool in a desiccator.

8) Chromogenic substrate. Dissolve 100 mg of 3-(4-iodophenyl)-2-(4-nitrophenyl)-5-phenyltetrazolium chloride in water to make 200 mL.

B. Preparation of sample solution

Extraction. Weigh 40.0 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of acetonitrile, extract with stirring for 30 minutes, and filter the extract through filter paper (No.5A). Transfer 50 mL of the filtrate to a 100-mL recovery flask, evaporate into dryness under reduced pressure in a water bath at 50°C, add 20 mL of a mixture of chloroform and ethyl acetate (9:1) to dissolve the residue, and use as the sample solution subject to column treatment.

Column treatment[6]. Wash a silica gel minicolumn (690 mg) with 10 mL of chloroform.

On the minicolumn place a funnel loaded with approximately 40 g of sodium sulfate (anhydrous)[7], pour the sample solution into the funnel, and allow to flow down until the amount in the minicolumn reservoir reaches 1 mL. Wash the recovery flask that contained the sample solution with 10 mL of a mixture of chloroform and ethyl acetate (9:1), transfer the washings to the funnel, and repeat this procedure 3 times.

Wash the sodium sulfate in the funnel with a mixture of chloroform and ethyl acetate (9:1), transfer the washings to the minicolumn, remove the funnel, and add 20 mL of a mixture of chloroform and ethyl acetate (9:1) to wash the minicolumn.

Place a 50-mL recovery flask under the minicolumn, add 30 mL of a mixture of chloroform and methanol (4:1) to the minicolumn to elute KT, VM and TS. Evaporate the eluate into dryness under reduced pressure in a water bath at 50°C, add exactly 2 mL of methanol to dissolve the residue[8], and use as the sample solution.

C. Quantification[9]

Proceed as described in Section 1, 2-C [10], except for the following procedures.

Use a thin-layer plate made of silica gel[Note 1], and develop until the ascending front of the developing solvent is 150 mm above the starting line.

Note 1. Use a TLC plate Silica gel 60 (20×20 cm) (Merck) or an equivalent after drying at 110°C for 2 hours.

«Summary of analysis method»

This method is intended to quantify and identify KT, VM or TS contamination due to carry-over etc. in a feed by microbioautography using a sample solution prepared by extracting with acetonitrile, purifying through a silica gel minicolumn, and dissolving in methanol.

The flow sheet of this method is shown in Figure 9.3.2-1.

Sample (40.0 g)
↓
Extract with 100 mL of acetonitrile (with a magnetic stirrer for 30 min).
↓
Filter (through filter paper No.5A).
↓
Collect 50 mL of the filtrate (into a 100-mL recovery flask).
↓
Evaporate into dryness (in a water bath at 50°C).
↓
Dissolve the residue with 20 mL of chloroform-ethyl acetate (9:1).
↓
Load onto a silica gel minicolumn (previously washed with 10 mL of chloroform and equipped on the reservoir with a funnel containing approximately 40 g of sodium
↓
Wash the recovery flask with 10 mL of chloroform-ethyl acetate (9:1) and load the washings onto the silica gel minicolumn (repeat 3 times).
↓
Wash the sodium sulfate with 10 mL of chloroform-ethyl acetate (9:1) and load the washings onto the silica gel minicolumn.
↓
Wash the silica gel minicolumn with 20 mL of chloroform-ethyl acetate (9:1).
↓
Elute KT, VM and TS with 30 mL of chloroform-methanol (4:1) (into a 50-mL recovery flask).
↓
↓
Evaporate into dryness under reduced pressure (in a water bath at 50°C).
↓
Dissolve the residue with 2 mL of methanol.
↓
Spot on a thin-layer plate (20 µL).
↓
Develop.
↓
Prepare agar plates (allow to stand at 10-20°C for 3 hours).
↓
Incubate (at 35-37°C for 16-24 hr).
↓
Measure the inhibition zone diameter and determine the Rf value.
↓
Calculate the potency from the calibration curve.

Figure 9.3.2-1 Trace quantitation test method for KT, VM and TS (feed)

References: Noriyuki Koyama: Research Report of Animal Feed, **17**, 96 (1992)

History in the Feed Analysis Standards [12] New, [13] Component addition (kitasamycin and virginiamycin)

«Validation of analysis method»

・Spike recovery and repeatability

Spiked component	Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Kitasamycin	Adult chicken formula feed	0.1~1	3	97.7~106.0	6.5
	Meat pig formula feed	0.1~1	3	105.0~113.3	13.5
	Dairy cattle formula feed	0.1~1	3	100.0~107.0	8.0
Virginiamycin	Adult chicken formula feed	0.1~1	3	96.0~106.0	9.1
	Meat pig formula feed	0.1~1	3	94.7~110.0	9.1
	Dairy cattle formula feed	0.1~1	3	100.0~102.3	6.5
Tylosin phosphate	Adult chicken formula feed	0.1~1	3	101.3~106.0	6.5
	Meat pig formula feed	0.1~1	3	97.7~107.0	9.8
	Dairy cattle formula feed	0.1~1	3	98.0~98.7	6.2

・Lower detection limit: 0.5 g (potency)/t for each component in the sample

«Notes and precautions»

[1] Kitasamycin working standard was deleted from the official list when the designation of kitasamycin as a feed additive was canceled. When performing the test, therefore, use a standard substance with equivalent quality.

For the definition etc. of virginiamycin, tylosin phosphate, and previously specified kitasamycin 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 970 μg (potency)/mg， 50 mg of the working standard contains 48,500 μg (potency) (i.e., 50 mg × 970 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 48.5 mL (i.e., 48,500 μg (potency) / 1,000 μg (potency)/mL). Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 10 mL of methanol to dissolve, and add 38.5 mL of water 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 the standard solution is shown in Table 9.3.2-1.

Table 9.3.2-1 Method of preparation for standard solution (trace quantitation test method, feed, example)

Test tube No.	1	2	3	4	5	6
Amount (mL) of standard solution	②	4	10	10	10	5
Amount (mL) of methanol	18	16	10	10	10	5
Concentration (μg (potency)/mL)	100	20	10	5	2.5	1.25

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

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

[5] Usually, proceed only with a mixture of acetonitrile and methanol (17:3). When an inhibition zone is observed with the sample solution, perform a re-test with a mixture of hexane, ethyl acetate, acetone, and methanol (4:2:1:1) to make a more precise identification.

[6] When the flow is slow, it is permissible to inject under pressure using the syringe plunger or a double-balloon pump.

[7] It is recommended to stuff a small amount of absorbent cotton at the top of the funnel stem on which to place sodium sulfate (anhydrous).

[8] When the residue is difficult to dissolve, apply ultrasonic waves for 2 to 3 minutes.

[9] Example standard response lines for KT, VM and TS are shown in Figure 9.3.2-2 to 4.

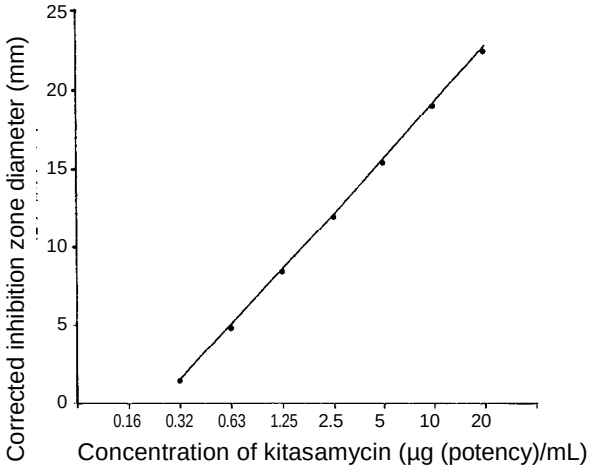


Figure 9.3.2-2 Standard response line for kitasamycin (trace quantitation test method, feed)
(*Micrococcus luteus* ATCC 9341, Medium F-111, Microbioautography)

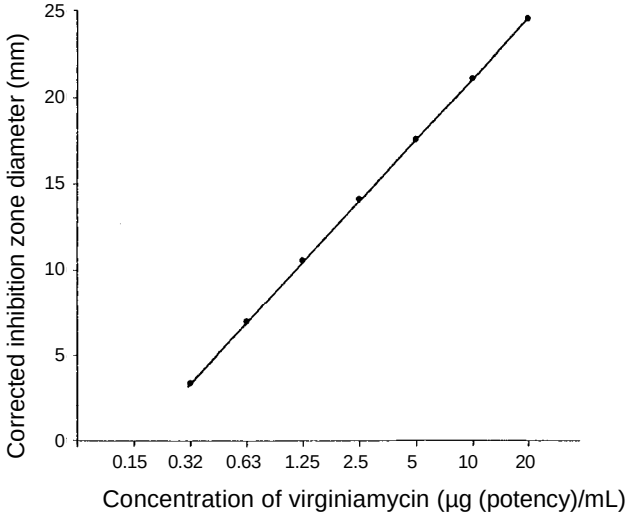


Figure 9.3.2-3 Standard response line for virginiamycin (trace quantitation test method, feed)
(*Micrococcus luteus* ATCC 9341, Medium F-111, Microbioautography)

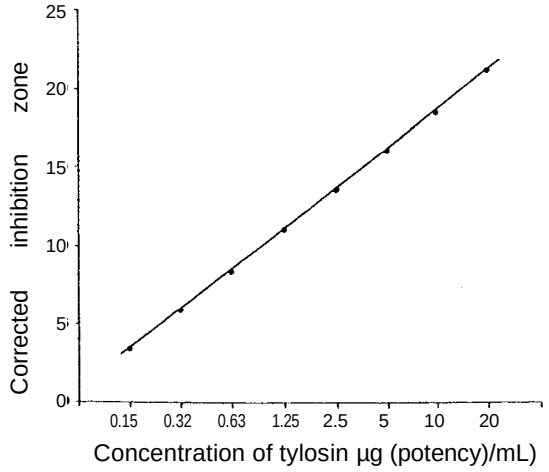


Figure 9.3.2-4 Standard response line for tylosin (trace quantitation test method, feed)
(*Micrococcus luteus* ATCC 9341, Medium F-111, Microbioautography)

[10] Refer to «Notes and precautions» [1] to [8] in Section 2 of this Chapter.