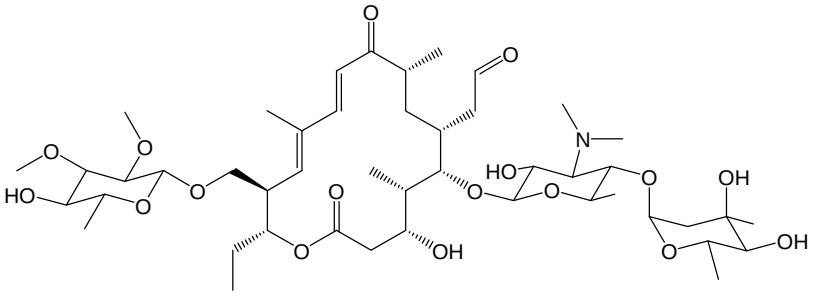
32 Tylosin phosphate



Tylosin A

$C_{46}H_{77}NO_{17}$ MW: 916.1 CAS No.: 1401-69-0 (tylosin), 1405-53-4 (tylosin phosphate)

[Summary of tylosin phosphate]

Tylosin is a macrolide antibiotic, intended exclusively for animal use, obtained by the incubation of *Streptomyces fradiae*. The one used as a feed additive is its phosphoric acid salt (TS).

For physicochemical properties, TS technical occurs as a light yellow to yellow liquid substance with a characteristic odor.

TS has an antibacterial effect mainly on Gram-positive bacteria and partly on Gram-negative bacteria and mycoplasma, and has a growth-promoting effect on pigs.

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

TS 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 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 pigs
	Suckling piglets
Added amount	11~44

The amount of TS added to a commercial premix is roughly 4.4 to 88 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, 32.1.1]

A. Reagent preparation

1) Buffer solution: Buffer No.4

2) Tylosinstandard solution. Dry a suitable amout 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, accurately add a small amount of methanol to dissolve, further accurately add Buffer No.4 to prepare a tylosinstandard 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.4 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-7

4) Suspension of test organism and amount of addition. Use *Micrococcus luteus* ATCC 9341[4] as the test organism. Add about 0.1 mL of a 10-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

Weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 50 mL of water, stir for 20 minutes, further add 50 mL of methanol, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A)[5].

Accurately dilute a quantity of the filtrate with Buffer No.4 to prepare high- and low-concentration sample solutions with concentrations of 2 and 0.5 μg (potency)/mL, respectively[6].

C. Quantification[7]

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

«Summary of analysis method»

This method is intended to determine the amount of TS in a premix by microbiological assay using a sample solution prepared by extracting with a mixture of water and methanol and diluting with Buffer No.4.

The flow sheet of this method is shown in Figure 9.2.32-1.

Sample (3.0-5.0 g)
↓
Add 50 mL of water and stir (with a magnetic stirrer for 20 min).
↓
Add 50 mL of methanol and extract (with a magnetic stirrer for 20 min).
↓
Filter (through filter paper No.5A).
↓
Dilute a quantity of the filtrate with Buffer No.4 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 9.2.32-1 Quantitative test method for tylosin phosphate (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

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Vitamin premix	2~10	3	98.2~99.9	0.7
Vitamin/mineral premix	2~10	3	96.8~99.5	0.9

«Notes and precautions»

[1] For the definition etc. of tylosin 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 potncy of the working standard is 1,053 μg (potency)/mg, 50 mg of the working standard contains 52,650 μg (potency) (i.e., 50 mg × 1,053 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 52.65 mL (i.e., 52,650 μg (potency) / 1,000 μg (potency)/mL). Therefore, completely transfer 50 mg of the working standard to a Erlenmeyer flask containing 3 mL of methanol, dissolve, and add 49.65 mL of Buffer No.4 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 tylosinstandard solution is shown in Table 9.32-1.

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

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

[6] For the method of preparation for 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 9.2.32-1.

Table 9.2.32-1　Method of preparation for tylosin standard solution and sample solution

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

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	4	5
Amount (mL) of Buffer No.4	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 20,000 µg (potency) of TS, the concentration of TS in the filtrate is calculated to be 200 µg (potency)/mL.

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

Note: "②mL" means "2 mL of filtrate (200 µg(potency)/mL)".

[7] An example standard response line for TS is shown in Figure 9.2.32-2.

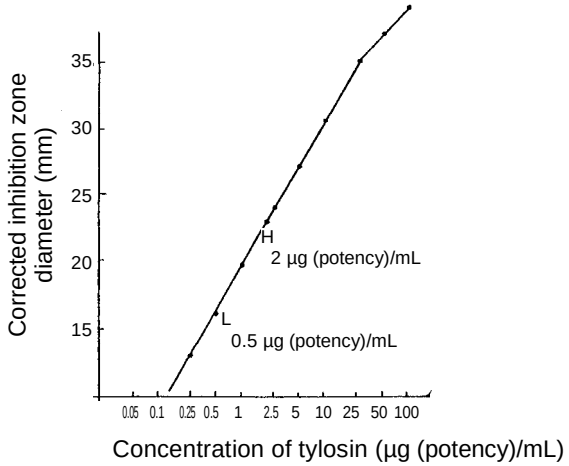


Figure 9.2.32-2 Standard response line for tylosin (premix, example)
(*Micrococcus luteus* ATCC 9341, Medium F-7, Agar well method)

[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, 32.2.1]

A. Reagent preparation

1) Buffer solution: Buffer No.4
2) Tylosin standard solution. Dry a suitable amount of tylosin 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 a small amount of methanol to dissolve, further accurately add Buffer No.4 to prepare a tylosin 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.4 and methanol (4:1) 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-7
4) Suspension of test organism and amount of addition. Use *Micrococcus luteus* ATCC 9341[2] as the test organism. Add about 0.1 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 TS content is not less than 40 g(potency)/t

Weigh accurately a quantity of the analysis sample (equivalent to 0.4 mg (potency) as TS), place in a 200-mL stoppered Erlenmeyer flask, add 50 mL of water, and stir for 20 minutes. Further add 50 mL of methanol, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with a mixture of Buffer No.4 and methanol (7:1)[3] to prepare a sample solution with a concentration of 0.8 μg (potency)/mL[4].

2) When the TS content is not less than 10 g(potency)/t and less than 40 g(potency)/t

Weigh accurately a quantity of the analysis sample (equivalent to 0.2 mg (potency) as TS), place in a 200-mL stoppered Erlenmeyer flask, add 50 mL of water , and stir for 20 minutes. Further add 50 mL of methanol , extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A)

Accurately dilute a quantity of the filtrate with Buffer No.4[3] to prepare a sample solution with a concentration of 0.8 μg (potency)/mL[4].

3) When the TS content is less than 10 g (potency)/t

Weigh accurately a quantity of the analysis sample (equivalent to 80 μg (potency) as TS), place in a 200-mL stoppered Erlenmeyer flask, add 50 mL of water, and stir for 20 minutes. Further add 50 mL of methanol, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A).

Transfer exacctly 20 mL of the filtrate to a 50-mL recovery flask, evaporate under reduced pressure into dryness in a water bath at 50°C, and add accurately 8 mL of water to dissolve the residue. To this solution add accurately 12 mL of a mixture of Buffer No.4 and methanol (2:1) and shake to prepare a sample solution with a concentration of 0.8 μg (potency)/mL[4].

C. Quantification[5]

Proceed by the standard response line method[6].

«Summary of analysis method»

This method is intended to determine the amount of TS in a feed by microbiological assay using a sample solution prepared by extracting with a mixture of water and methanol and diluting with Buffer No.4 or a mixture of Buffer No.4 and methanol. None of the antibacterial substances approved for combined use with TS interfere with the quantification of TS.

The flow sheet of this method is shown in Figure 9.2.32-3.

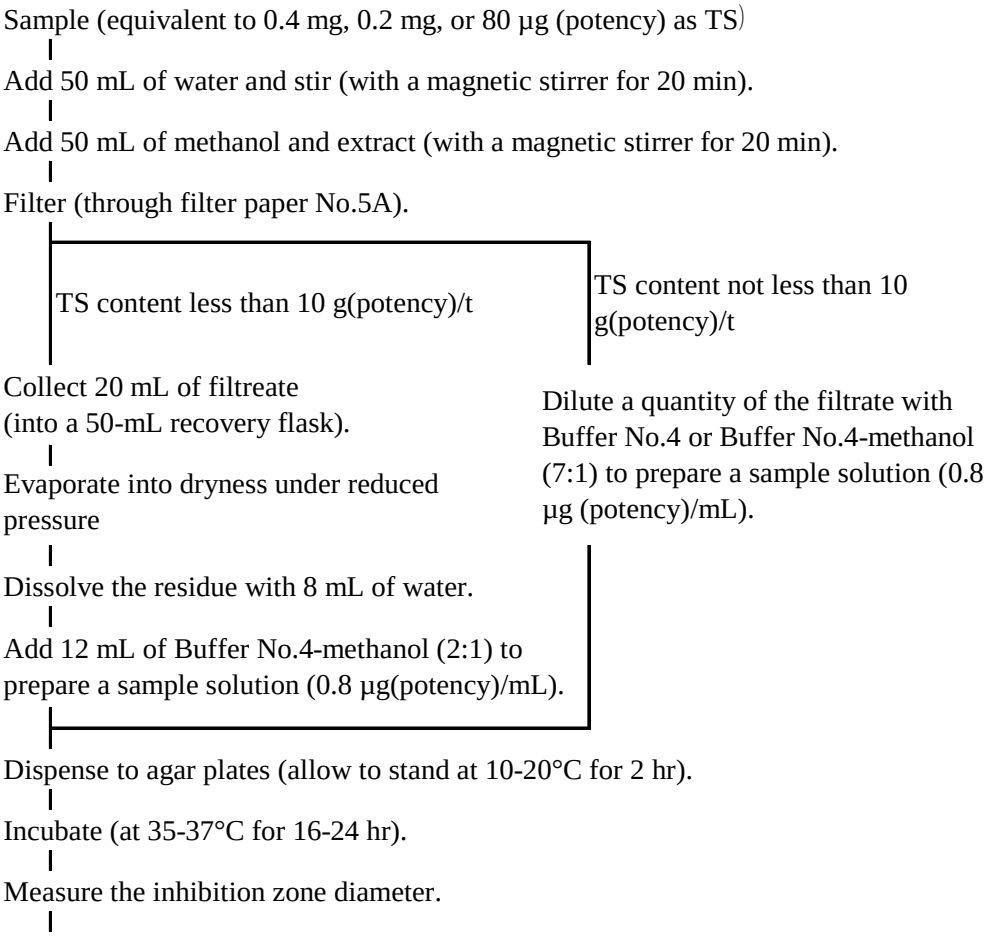


Figure 9.2.32-3 Quantitative test method for tylosin phosphate (feed)

References: Noriyuki Koyama: Research Report of Animal Feed, 7, 129 (1981)

History in the Feed Analysis Standards [4] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Starting chick formula feed	4.4~22	6	98.7~100.7	2.7
Growing chick formula feed	4.4~22	6	99.3~101.1	2.8
Suckling piglet formula feed	4.4~22	6	99.7~100.4	1.6

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Suckling piglet formula feed	5	88	101.2	4.8	5.2

«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 tylosin standard solution is shown in Table 9.2.32-2.

Table 9.2.32-2 Method of preparation tylosin standard solution (feed, example)

Test tube No.	1	2	3	4	5	6	7
Amount (mL) of standard solution	②	2	8	10	10	10	5
Amount (mL) of Buffer No.4-methanol (4:1)	23	18	12	10	10	10	5
Concentration (μg(potency)/mL)	80	8	3.2	1.6	<0.8>	0.4	0.2

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

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

[3] An example method for preparation for the filtrate is shown in Table 9.2.32-3.

Table 9.2.32-3 Method of preparation for filtrate (example)

	Amount of sample (as TS)	
	equivalent to 0.4 mg	equivalent to 0.2 mg
Amount (mL) of filtrate	④	⑧
Amount (mL) of Buffer No.4-methanol (7:1)	16	
Amount (mL) of Buffer No.4		12

Note: "④ and ⑧ mL mean the collected amounts of filtrate".

[4] The content of methanol is 20 v/v%.

[5] An example standard response line for TS is shown in Figure 9.2.32-4.

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

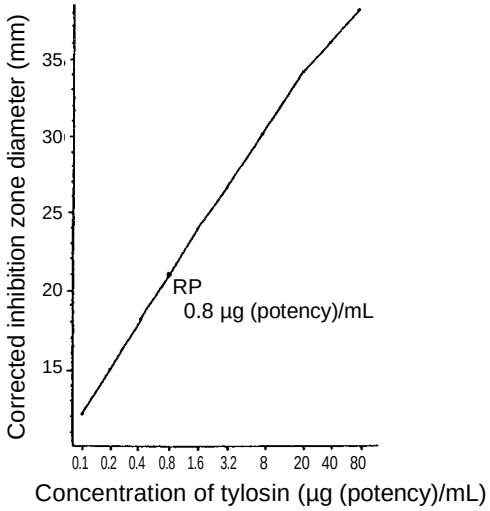


Figure 9.2.32-4 Standard response line for tylosin (feed, example)

(*Micrococcus luteus* ATCC 9341, Medium F-7, Agar well method)

[6] 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, 32.3.1]

Antibiotics of interest: KT, VM and TS (3 components)

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.

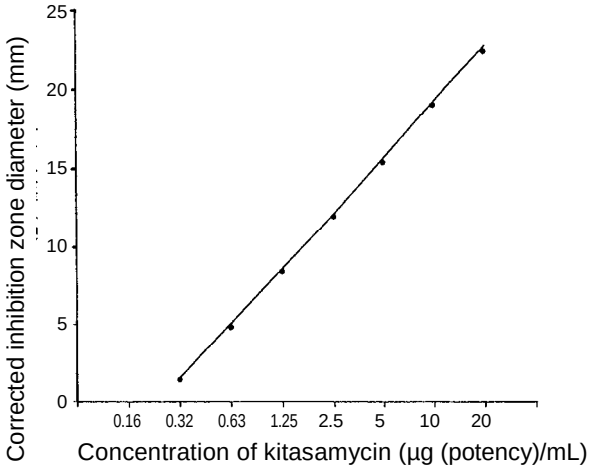


Figure 9.3.2-2 Standard response line for kitasamycin (trace quantitation test method, feed)
(*Micrococcus luteus* ATCC 9341, Medium F-111, Microbioautography)

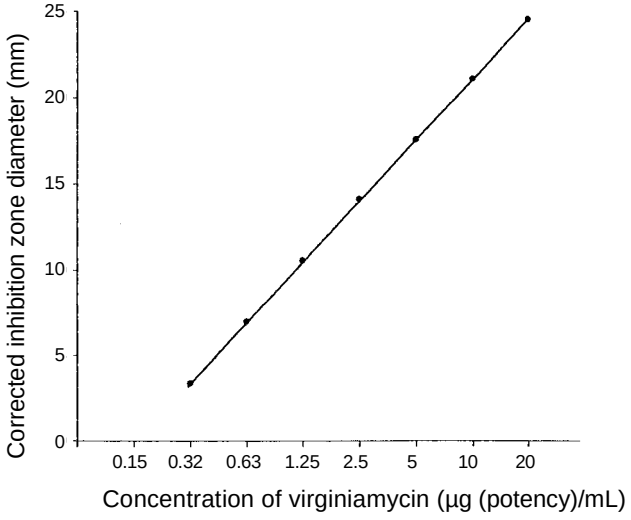


Figure 9.3.2-3 Standard response line for virginiamycin (trace quantitation test method, feed)
(*Micrococcus luteus* ATCC 9341, Medium F-111, Microbioautography)

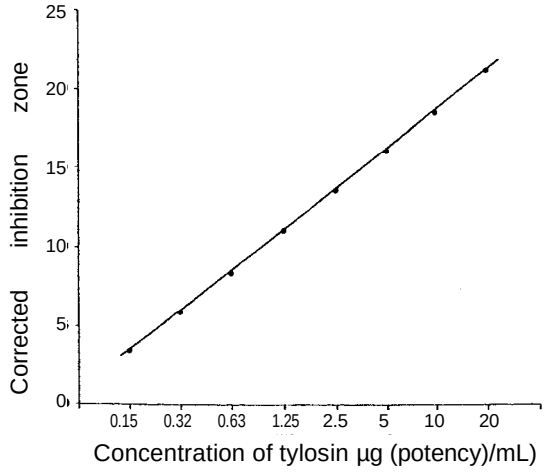


Figure 9.3.2-4 Standard response line for tylosin (trace quantitation test method, feed)
(*Micrococcus luteus* ATCC 9341, Medium F-111, Microbioautography)

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

3 Identification test method - Microbioautography

3.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 32.4.1]

A. Reagent preparation

1) Tylosin standard solution. Dry a suitable amount of tylosin 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 a small amount of methanol to dissolve, further accurately add Buffer No.4 to prepare a tylosin 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 methanol to achive a concentration of 10 µg (potency)/mL, and add an equal amount of ammonia solution (25%) to prepare a tylosin standard solution with a concentration of 5 µg (potency)/mL[1].

2) Culture medium: Medium F-111

3) Suspension of test organism and amount of addition. Use *Micrococcus luteus* ATCC 9341[2] as the test organism. Add about 0.1% of a 10-fold diluted suspension of the test organism per 100 mL of the culture medium.

4) Developing solvent: A mixture of acetonitrile and methanol (17:3)

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

B. Preparation of sample solution

Weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 50 mL of water, stir for 20 minutes, further add 50 mL of methanol, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A).

Dilute a quantity of the filtrate with methanol to achieve a concentration of 10 µg (potency)/mL, and add an equal amount of ammonia solution (25%) to prepare a sample solution with a concentration of

5 µg (potency)/mL.

C. Identification

Proceed as directed in the Thin-layer chromatography, Preparation of agar plates, Incubation, and Identification in Section 1, 2-C [3], 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 reaches the top of the thin-layer plate.

Note 1. Use TLC plate Silica gel 60 (20×20 cm) (Merck) or an equivalent.

«Summary of analysis method»

This method is intended to identify TS in a premix by microbioautography using a sample solution prepared for quantification.

The flow sheet of this method is shown in Figure 9.2.32-5.

Sample (3.0-5.0 g)
↓
Add 50 mL of water and stir (with a magnetic stirrer for 20 min).
↓
Add 50 mL of methanol and extract (with a magnetic stirrer for 20 min).
↓
Filter (through fulter paper No.5A).
↓
Dilute a quantity of the filtrate with methanol to achieve a concentration of 10 µg (potency)/mL.
↓
Add an equal amount of ammonia solution (25%) to prepare a sample solution (5 µg (potency)/mL).
↓
Spot on thin-layer plates (25 µL).
↓
Develop.
↓
Prepare agar plates (allow to stand at 10-20°C for 3 min).
↓
Incubate (at 35-37°C for 16-24 hr).
↓
Determine the Rf value.

Figure 9.2.32-5 Identification test method for tylosin phosphate (premix)

References: Yoshinori Omuro, Yumi Takahashi: Research Report of Animal Feed, 22, 126 (1997)

History in the Feed Analysis Standards [19] New

«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 tylosinstandard solution is shown in Table 9.2.32-4.

Table 9.2.32-4 Method of preparation for tylosinstandard solution (identification test method, premix, example)

Test tube No.	1	2	3
Amount (mL) of standard solution	②	2	5
Amount (mL) of methanol	18	18	
Amount (mL) of ammonia solution (25%)			5
Concentration (μg (potency)/mL)	100	10	5

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

3.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 32.5.1]

A. Reagent preparation

1) Tylosin standard solution. Dry a suitable amount of tylosin 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 a small amount of methanol to dissolve, further accurately add Buffer No.4 to prepare a tylosin 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 methanol to achieve a concentration of 10 μg (potency)/mL, and add an equal amount of ammonia solution (25%) to prepare a tylosin standard solution with a concentration of 5 μg (potency)/mL[1].

2) Culture medium: Medium F-111

3) Suspension of test organism and amount of addition. Use *Micrococcus luteus* ATCC 9341 as the test organism[2]. Add about 0.1% of a 10-fold diluted suspension of the test organism per 100 mL of the culture medium.

4) Developing solvent: A mixture of acetonitrile and methanol (17:3)

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

B. Preparation of sample solution

Weigh accurately a quantity of the analysis sample (equivalent to 0.4 mg (potency) as TS), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of acetonitrile, and extract with stirring for 20 minutes. Filter the extract through filter paper (No.5A), to the filtrate add an equal amount of ammonia solution (25 %) to prepare a sample solution with a concentration of 2 μg (potency)/mL.

C. Identification

Proceed as directed in the Thin-layer chromatography, Preparation of agar plates, Incubation, and Identification in Section 1, 2-C [3], except the following procedures.

Use a thin-layer plate made of silica gel [Note 1], spot 50 μL each of the standard solution and sample solution, and develop until the ascending front of the developing solvent reaches the top of the thin-layer plate.

Note 1. Use TLC plate Silica gel 60 (20×20 cm) (Merck) or an equivalent.

«Summary of analysis method»

This method is intended to identify TS in a feed by microbioautography using a sample solution prepared for quantification.

The flow sheet of this method is shown in Figure 9.2.32-6.

Sample (equivalent to 0.4 mg (potency as TS)
↓
Extract with 100 mL of acetonitrile (with a magnetic stirrer for 20 min).
↓
Filter (through filter paper No.5A).
↓
To a quantity of filtrate add the equal amount of ammonia solution (25%) to prepare a sample solution with a concentration of 2 µg (potency)/mL.
↓
Spot on a thin-layer plate (50 µL).
↓
Develop.
↓
Prepare agar plates (allow to stand at 10-20°C for 3 hr).
↓
Incubate (at 35-37°C for 16-24 hr).
↓
Determin the Rf value.

Figure 9.2.32-6 Identification test method for tylosin phosphate (feed)

References: Yoshinori Omuro, Yumi Takahashi: Research Report of Animal Feed, 22, 126 (1997)

History in the Feed Analysis Standards [19] New

«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 tylosinstandard solution is shown in Table 9.2.32-5.

Table 9.2.32-5 Method of preparation for tylosinstandard solution (identification test method, feed, example)

Test tube No.	1	2	3
Amount (mL) of standard solution	②	2	5
Amount (mL) of methanol	18	18	
Amount (mL) of ammonia solution (25%)			5
Concentration (µg (potency)/mL)	100	10	5

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