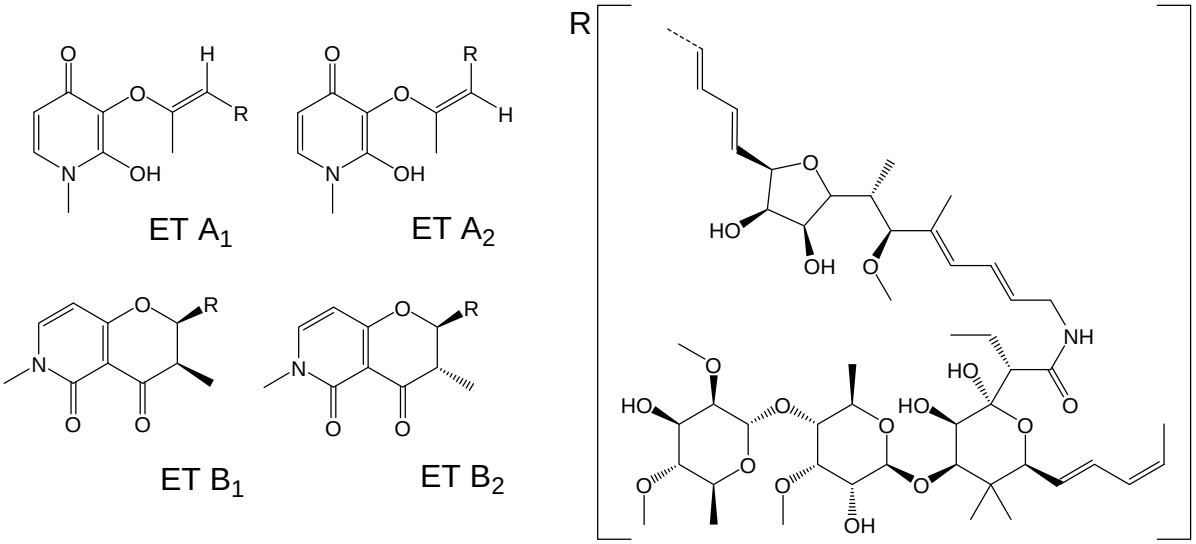
5 Efrotomycin



$C_{59}H_{88}N_2O_{20}$ MW: 1145

[Summary of efrotomycin]

Efrotomycin (ET) is an antibiotic obtained by the incubation of *Nocardia lacamduranns*, and composed of 4 components: Efrotomycin A_1, Efrotomycin A_2, Efrotomycin B_1, and Efrotomycin B_2.

For physicochemical properties, ET occurs as a light yellow-brown to yellow-brown powder. It is freely soluble in methanol or in isopropanol, slightly soluble in acetonitrile, very slightly soluble in water, and practically insoluble in hexane.

Although its antibacterial spectrum is narrow, ET has an antibacterial effect on part of the Gram-positive bacteria and on treponema, and has a growth promoting effect on pigs etc.

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

ET is a pure-grade antibiotic that was designated as a feed additive as of June 3, 1991. The supecifications for feeds containing this ingredient are specificed 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	
	Sucking piglets	Piglets
Added amount	2~16	2~16

[Methods listed in the Feed Analysis Standards]

1 Quantitative test methods - Plate method

1.1 Premix

[Feed Analysis Standards Chapter 9, Section 2, 5.1.1]

A. Reagent preparation

1) Buffer solution

i) Buffer No.2

ii) Buffer No.12

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

3) Efrotomycin standard solution. Weigh accurately no less than 40 mg of efrotomycin working standard [1], accurately add a mixture of Buffer No.12 and acetonitrile (4:1) and dissolve to prepare[2] an efrotomycin standard stock solution with a concentration of 0.4 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 1.6 and 0.4 µg (potency)/mL, respectively[3].

4) Culture medium: Medium F-6

5) Spore suspension and amount of addition. Use *Bacillus cereus* ATCC 19637[4] as the test organism. Add 1.0 mL of the spore suspension with a concentration of 1×10^7 spores/mL to 100 mL of culture medium.

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

7) Extracting solvent: A mixture of acetone, water, and ammonia solution (200:49:1) [5]

B. Preparation of sample solution

Weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, extract with stirring for 20 minutes, and filter the extract 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 1.6 µg and 0.4 µg (potency)/mL, respectively[6] .

C. Quantification [7]

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

However, each agar plate shall be incubated at 27 to 29°C for 16 to 24 hours.

«Summary of analysis method»

This method is intended to determine the amount of ET in a premix by microbiological assay using a sample solution prepared by extracting with a mixture of acetone, water, and ammonia solution (200:49:1) and diluting with a mixture of Buffer No.2 and methanol (4:1).

The flow sheet of the analysis method is shown in Figure 9.2.5-1.

Sample (3.0-5.0 g)

↓

Extract with 100 mL of acetone-water-ammonia solution (200:49:1).
(magnetic stirrer, 20 min)

↓

Filter (filter paper: No.5A).

↓

Dilute a quantity of the filtrate with Buffer No.2-methanol (4:1) to prepare
high- and low-concentration sample solutions (1.6 and 0.4 μg(potency)/mL,

↓

Dispense to agar plates (allow to stand at 10-20 °C for 2 hr).

↓

Incubate (at 27-29 °C for 16-24 hr).

↓

Measure the inhibition zone diameter.

↓

Calculate the potency by the 2-2 dose method.

Figure 9.2.5-1 Quantitative test method for efrotomycin (premix)

References: Yukiko Mitstui, Masayuki Shimomura: Research Report of Animal Feed, 29, 42 (2004)

History in the Feed Analysis Standards [27] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD (% or less)
Pig premix	1~8	3	97.6~102.3	2.9
Pig premix	1~8	3	99.0~102.0	5.4
Pig premix	1~8	3	100.5~102.5	5.0

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/kg	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Pig premix	7	3	98.5	1.8	4.0

«Notes and precautions»

[1] For the definition etc. of efrotomycin 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 817 μg (potency)/mg, 50 mgof the working standard contains a 40,850 μg (potency) (i.e., 50 mg × 817 μg (potency)/mg. To prepare a standard stock solution with a concentration of 400 μg (potency)/mL, therefore, the required amount of solvent is calculated to be 102.13 mL (i.e., 40,850 μg (potency) / 400 μg (potency)/mL. Therefore, completely transfer 50 mg of working standard to an Erlenmeyer flask containing 102.13 mL of a mixture of Buffer No.12 and acetonitrile (4:1) and dissolve to prepare a standard stock solution with a concentration of 400 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 efrotomycin standard solution is shown in Table 9.2.5-1.

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

[5] Corresponds to a mixture of ammonia solution (1:49) and acetone (1:4).

[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 9.2.5-1.

Table 9.2.5-1 Method of preparation for efrotomycin standard solution and sample solution

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

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	4	4	5
Amount (mL) of Buffer No.2-methanol (4:1)	18	16	16	15
Concentration (μg(potency)/mL)	40	8	1.6	0.4

Note: "②mL" means "2 mL of standard solution (0.4 mg(potency)/mL)".

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

When the analysis sample is collected in an amount equivalent to 12,000 μg (potency) of ET, the concentration of ET in the filtrate is calculated to be 120 μg (potency)/mL.

Test tube No.	1	2	3	4
Amount (mL) of sampe solution	⑤	4	4	5
Amount (mL) of Buffer No. 2-methanol(4:1)	10	16	16	15
Concentration (μg(potency)/mL)	40	8	1.6	0.4

Note: "⑤mL" means "5 mL of the filtrate (120 μg(potency)/mL)".

[7] An example standard response line for ET is shown in Figure 9.2.5-2.

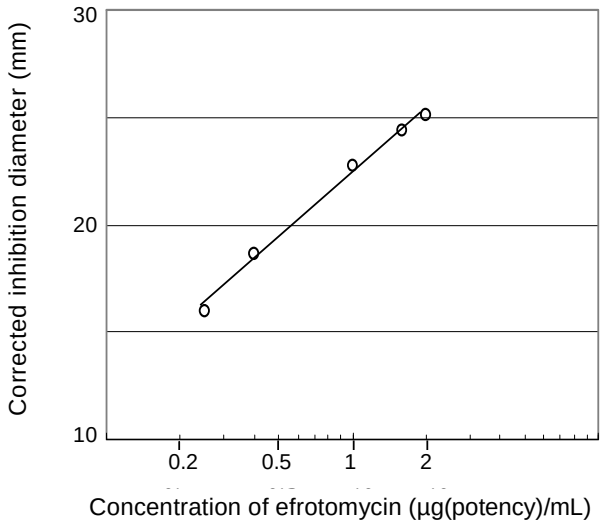


Figure 9.2.5-2 Standard response line for efrotomycin (premix, example)
(*Bacillus cereus* ATCC 19637, Medium F-6, Agar well method)

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

1.2 Feed

[Feed Analysis Standards Chapter 9, Section 2, 5.2.1]

A. Reagent preparation

1) Buffer solution
   i) Buffer No.2
   ii) Buffer No.12
2) Dilution solvent: A mixture of Buffer No.2 and methanol (4:1)
3) Efrotomycin standard solution. Weigh accurately not less than 40 mg of efrotomycin working standard, accurately add a mixture of Buffer No.12 and acetonitrile (4:1) and dissolve to prepare 0.4 mg (potency)/mL of efrotomycin standard stock solution.

   At the time of use, accurately dilute a quantity of standard stock solution to prepare standard solutions with concentrations at 1.6, 0.8, 0.4, 0.2, and 0.1 μg (potency)/mL[1].
4) Culture medium. Use Medium F-6
5) Spore suspension and amount of addition. Use *Bacillus cereus* ATCC 19637[2] as the test organism, and add about 1 mL of a spore suspension with a concentration of 1×10^7 spores/mL to 100 mL of the culture medium.
6) Agar plate. Proceed by the agar well method.

B. Preparation of the sample solution

Extraction. Weigh accurately a quantity ofthe analysis sample[3] (equivalent to 0.04 to 0.32 mg (potency) as ET), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of dichloromethane, extract with stirring for 20 minutes. Place the extract in a 50-mL stoppered centrifuge tube, centrifuge at 1,000×*g* for 10 minutes, filtrate supernatant liquid through a filter paper (No.5A), and use the supernatant as a

sample solution subject to column treatment.

Column treatment. Wash the silica gel minicolumn (690 mg) with 20 mL of dichloromethane.

Place 10 mL of the sample solution in the minicolumn, allow to flow down until the remaining amount in the reservoir of the microcolumn reaches 1 mL[Note 1]. Further, add 20 mL of a mixture of ethyl acetate and ammonia solution (180:1) to wash the minicolumn. Place a 50-mL round-bottom flask under the microcolumn, add 20 mL of methanol to the minicolumn to elute ET.

Evaporate the eluate into dryness under reduced pressure in a water bath at 50°C, add accurately 2 mL of methanol to dissolve the residue, add accurately 8 mL of Buffer No.2 and stir, and, if necessary, accurately dilute a quantity of the liquid with the dilution solvent, to prepare a sample solution with a concentration of 0.4 μg (potency)/mL[4].

C. Quantification [5]

Proceed by the standard response line method[6].

However, each agar plate shall be incubate at 27 to 29°C for 16 to 24 hours.

Note 1 When it is difficult for the sample solution to flow down freely, inject under pressure at a flow rate of approximately 1 mL/min, and then wash and elute in the same manner.

«Summary of analysis method»

This method is intended to determine the amount of ET in a feed by a microbiological assay using a sample solution prepared by extracting with dichloromethane and refining with silica gel minicolumn.

The flow sheet of the analysis method is shown in Figure 9.2.5-3.

Sample (an amount equivalent to 0.04-0.32 mg(potency) as ET)
↓
Extract with 100 mL of dichloromethane (magnetic stirrer, 20 min).
↓
Centrifuge (at 1,000×g for 10 min)
↓
Filter (filter paper: No.5A).
↓
Load 10 mL of the filtrate on a silica gel minicolumn (previously washed with20 mL
↓
Wash the silica gel minicolumn with 20 mL of ethyl acetate-ammonia water (180:1).
↓
Elute ET with 20 mL of methanol (into a round-bottom flask).
↓
Evaporate into dryness under reduced pressure (in a water bath at 50 °).
↓
Add 2 mL of methanol to dissolve the residue and add 8 mL of Buffer No.2 and stir.
↓
If necessary, dilute with Buffer No.2-methanol (4:1) to prepare a sample solution at a concentration of 0.4 μg(potency)/mL.
↓
Dispense to agar plates (allow to stand at 10-20 °C for 2 hr).
↓
Inubate (at 27-29 °C for 16-24 hr).
↓
Measure the inhibition zone diameter.
↓
Calculate the potency by the standard response line method.

Figure 9.2.5-3 Quantitative test method for efrotomycin (feed)

References: Kiyoshi Kanno: Research Report of Animal Feed, 24, 102 (1999)

History in the Feed Analysis Standards [13] New, [21] Revision

«Validation of analysis method»

・Spike recovery and repeatability

Type of sample	Spike concentration (g(potency)/t)	Spike recovery (%)
Pig formula feed (mash)	6	92.0
Pig formula feed (crumble)	6	99.0
Pig formula feed (mash)	8	95.0

・Collaborative study

Type of sample	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Sucking piglet formula feed	8	8	103.8	3.4	5.2

«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 efrotomycin standard solution is shown in Table 9.2.5-2.

Table 9.2.5-2 Method of preparation for efrotomycin standard solution (feed, example)

Ttest tube No.	1	2	3	4	5	6	7
Amount (mL) of standard solution	②	4	4	10	10	10	5
Amount (mL) of Buffer No.2-methanol (4:1)	18	16	16	10	10	10	5
Concentration (μg(potency)/mL)	40	8	1.6	0.8	<0.4>	0.2	0.1

Note: "②mL" means "2 mL of the standard stock solution (0.4 mg(potency)/mL".

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

[3] In principle, the analysis sample is collected in an amount of 20 g.

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

Example dilution factors for the analysis sample collected in an amount of 20 g are shown in Table 9.2.5-3.

Table 9.2.5-3 Labeled potency of ET and dilution factors for a sample collected in an amount of 20 g (example)

Labeled potency (g(potency)/t)	Dilution factor
2	Not diluted
4	2-fold
8	4-fold
16	8-fold

[5] An example standard response line for ET is shown in Figure 9.2.5-4.

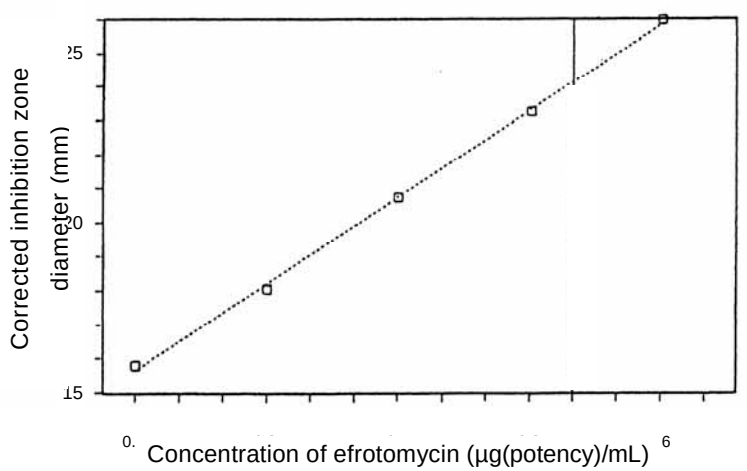


Figure 9.2.5-4 Standard response line for efrotomycin (feed, example)
(*Bacillus cereus* ATCC 19637, Medium F-6, Agar well method)

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