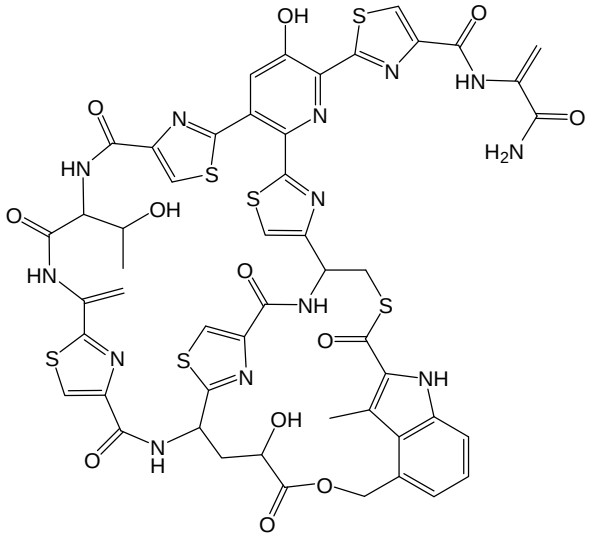
19 Nosiheptide



$C_{51}H_{43}N_{13}O_{12}S_6$ MW: 1222 CAS No.: 56377-79-8

[Summary of nosiheptide]

Nosiheptide (NH) is a polypeptide antibiotic obtained by the incubation of *Streptomyces actuosus*.

For physicochemical properties, NH technical occurs as light-yellow grayish white to greenish light-yellow brown crystals or powder, and has no odor or slightly has a characteristic odor. It is soluble in cyclohexane, sparingly soluble in tetrahydrofuran, slightly soluble in acetone and in chloroform, very slightly soluble in ethanol, and practically insoluble in water.

NH has a strong antibacterial effect on most part of Gram-positive bacteria and part of Gram-negative 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»

NH is a pure-grade and feed-grade antibiotic that was designated as a feed additive as of December 25, 1987. 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 (excluding broilers)	For broilers	For pigs	
	Stating chicks Growing chicks	Finishing period broilers Growing period broilers	Suckling piglets	Piglets
Added amount	2.5~10	2.5~10	2.5~20	2.5~20

The amount of NH added to a commercial premix is roughly 1 to 10 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, 19.1.1]

Scope of application: Premix with copper sulfate content of 17 g/kg or lower

A. Reagent preparation

1) Buffer solution: Buffer No.4
2) Diution solvent: Buffer No.4-acetone (4:1)
3) Nosiheptide standard solution. Dry a suitable amount of nosiheptide 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 *N*,*N*-dimethylformamide and dissolve to prepare a nosiheptide 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 the dilution solvent to prepare high- and low-concentration standard solutions with concentrations of 0.1 and 0.025 µg (potency)/mL, respectively[3].
4) Culture medium: Medium F-7
5) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 9341[4] as the test organism. Add about 0.2 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) Potassium dihydrogen phosphate solution. Dissolve 52.5 g of potassium dihydrogen phosphate in 1,000 mL of water and adjust the pH to 10.4 to 10.6 with a saturated solution of sodium hydroxide.
8) Extracting solvent: A mixture of *N*,*N*-dimethylformamide and potassium dihydrogen phosphate solution (7:3)

B. Preparation of sample solution

Weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 2 g of ethylenediaminetetraacetic acid tetrasodium salt[5] and 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer the extract to a 50-mL stoppered centrifuge tube, and centrifuge at 650×*g* for 15 minutes. Accurately dilute a quantity of the supernatant liquid with the dilution solvent to prepare high- and low-concentration sample solutions with concentrations of 0.1 and 0.025 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 NH in a premix by microbiological assay using a sample solution prepared by extracting with a mixture of *N*,*N*-dimethylformamide and potassium dihydrogen phosphate solution (7:3) containing ethylenediaminetetraacetic acid tetrasodium salt and diluting with a mixture of Buffer No.4 and acetone (4:1).

This method is not applicable to a premix spiked with copper sulfate at a concentration exceeding

17 g/kg.

The flow sheet of this method is shown in Figure 9.2.19-1.

Sample (3.0-5.0 g)

↓

Add 2 g of ethylenediaminetetraacetic acid tetrasodium salt and extract with 100 mL of *N*,*N*-dimethylformamide-potassium dihydrogen phosphate solution (7:3) (with a magnetic stirrer for 20 min).

↓

Centrifuge (at 650×g for 15 min).

↓

Dilute a quantity of the filtrate with Buffer No.4-acetone (4:1) to prepare high- and low-concentration sample solutions (0.1 and 0.025 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 9.2.19-1 Quantitative test method for nosiheptide (premix)

References: Shoichi Yamatani, Kyoko Akimoto: Research Report of Animal Feed, 22, 116 (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 1	0.4~10	3	93.3~98.7	3.4
Chicken premix 2	0.4~10	3	94.7~101.0	2.2
Chicken premix 3	0.4~10	3	93.3~99.3	2.1

・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	2	99.9	1.9	5.8

«Notes and precautions»

[1] For the definition etc. of nosiheptide 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 945 μg (potency)/mg, 50 mg of the working standard contains 47,250 μg (potency) (i.e., 50 mg × 945 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 47.25 mL (i.e., 47,250 μg (potency) / 1,000 μg (potency)/mL).

Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 47.25 mL of *N*,*N*-dimethylformamide and dissolve 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 nosiheptide standard solution is shown in Table 9.2.19-1.

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

[5] Ethylenediaminetetraacetic acid tetrasodium salt is intended as a chelating agent to remove the effect of minerals contained in the premix.

[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 9.2.19-1.

Table 9.2.19-1 Method of preparation for nosiheptide standard solution and sample solution

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

Test tube No.	1	2	3	4	5
Amount (mL) of standard solution	②	2	2	2	5
Amoun (mL) of Buffer No.4-acetone (4:1)	18	18	18	18	15
Concentraiton (μg(potency)/mL)	100	10	1	0.1	0.025

Note: "② mL" 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 10,000 μg (potency) of NH, the concentration of NH in the filtrate is calculated to be 100 μg (potency)/mL.

Test tube No.	1	2	3	4
Amount (mL) of sample solution	②	2	2	5
Amount (mL) of Buffer No.4-acetone (4:1)	18	18	18	15
Concentration (μg(potency)/mL)	10	1	0.1	0.025

Note: "② mL" means "2 mL of the supernatant (100 μg(potency)/mL)".

[7] An example standard response line for NH is shown in Figure 9.2.19-2.

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

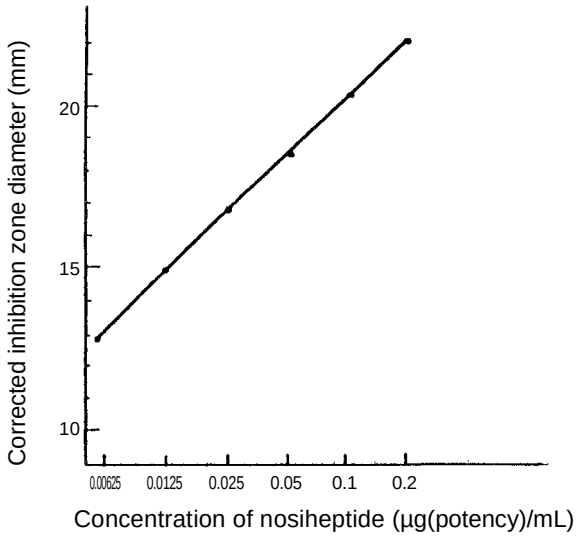


Figure 9.2.19-2 Standard response line nosiheptide (premix, example)
(*Micrococcus luteus* ATCC 9341, Medium F-7, Agar well method)

2.1 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 19.2.1]

Scope of application: Feeds excluding pelleted feeds, feeds for suckling period or feeds with NH contant less than 5 g (potency)/t

A. Reagent preparation

1) Buffer solution: Buffer No.4

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

3) Nosiheptide standard solution. Dry a suitable amount of nosiheptide 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 *N*,*N*-dimethylformamide and dissolve to prepare a nosiheptide 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 nosiheptide standard solutions with concentrations of 0.2, 0.1, 0.05, 0.025 and 0.0125 μg (potency)/mL[1].

4) Culture medium: Medium F-7

5) 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.

6) Agar plate. Proceed by the agar well method[3].

B. Preparation of sample solution

Extraction. Weigh 10.0 g of the analysis sample, place in a 200-mL stoppered amber Erlenmeyer flask, add 100 mL of a mixture of Buffer No.4 and acetone (1:1)[4], extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A). Transfer accurately 10 mL of the filtrate to a 50-mL amber volumetric flask[5], add water up to the marked line, and use as the sample solution subject to column treatment.

Folumn treatment. Cover with aluminum foil the reservoir of an octadecylsilanized silica gel (trifunctional) minicolumn (400 mg)[Note 1], and wash the minicolumn with 10 mL acetone and with 10 mL of water in this order.

Transfer accurately 10 mL of the sample solution to the minicolumn, and inject under pressure to force to flow out [Note 2]. Add 20 mL of water[6] to the minicolumn, and inject under pressure to force to flow out [Note 2] in the same manner.

Place a 100-mL amber recovery flask under the minicolumn, add 10 mL of acetone and 20 mL of a mixture of acetone and chloroform (1:1) to the minicolumn in this order, and allow to flow out to elute NH. Condense the eluate to approximately 1 mL under reduced pressure in a water bath at 50°C, introduce nitrogen gas to evaporate into dryness, and add accurately 20 mL of the dilution solvent to dissolve the residue. Accurately dilute a quantity of this solution with the dilution solvent to prepare a sample solution with a concentration of 0.05 μg (potency)/mL.

C. Quantification[7]

Proceed by the standard response line method[8].

Note 1. Use a Sep-Pak Plus tC_{18} Cartridge (Waters) connected with a reservoir with a sitable capacity or an equivalent.

2. Set the flow rate to 0.6 to 0.8 mL/min.

«Summary of analysis method»

This method is intended to determine the amount of NH (pure-grade and feed-grade) in a feed by microbiological assay using a sample solution prepared by extracting with a mixture of Buffer No.4 and acetone (1:1) and purifying through an octadecylsilanized silica gel (trifunctional) (tC_{18}) minicolumn. None of the antibacterial substances approved for combined use with NH interfere with the quantification of NH.

This method is not applicable to pelleted feed, feed for suckling period or feeds with NH contant less than 5 g (potency)/t.

The flow sheet of this method is shown in Figure 9.2.19-3.

Sample (10.0 g)

↓

Extract with 100 mL of Buffer No.4-acetone (1:1) (with a magnetic stirrer for 20 min).

↓

Filter (through filter paper No.5A).

↓

Add water to 10 mL of filtrate to make 50 mL in an amber volumetric flask.

↓

Load 10 mL onto a tC_{18} minicolumn (previously washed with 10 mL of acetone and 10 mL of water) (inject under pressure at a flow rate of 0.6-0.8 mL/min).

↓

Wash the tC_{18} minicolumn with 20 mL of water
(inject under pressure at a flow rate of 0.6-0.8 mL/min).

↓

Elute NH with 10 mL of acetone and 20 mL of acetone-chloroform (1:1)
(allow to flow down into a recovery flask).

↓

Condense under reduced pressure (in a water bath at 50°C).

↓

Introduce nitrogen gas to evaporate into dryness.

↓

Dissolve the residue with 20 mL of Buffer No.4-acetone (4:1).

↓

Dilute a quantity of the sample stock solution with Buffer No.4-acetone (4:1) to prepare a sample solution with a concentration of 0.5 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 response line method.

Figure 9.2.19-3 Quantitative test method for nosiheptide (feed)

References: Kyoko Akimoto, Yuji Fukumoto: Research Report of Animal Feed, 20, 81 (1995)

History in the Feed Analysis Standards [17] 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	5~20	3	94.7~103.7	8.9
Starting period broiler formula feed	5~20	3	93.0~95.7	5.8
Piglet formula feed	5~20	3	94.7~100.7	8.3

・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	7	10	98.3	3.3	6.0

«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 nosiheptide standard solution is shown in Table 9.2.19-2.

Table 9.2.19-2 Method of preparation for nosiheptide standard solution (feed, example)

Test tube No.	1	2	3	4	5	6	7	8
Amount (mL) of standard solution	②	2	2	4	10	10	10	10
Amount (mL) of Buffer No.4-acetone (4:1)	18	18	18	16	10	10	10	10
Concentration (μg(potency)/mL	100	10	1	0.2	0.1	<0.05>	0.025	0.0125

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] The agar well method is more sensitive to NH than the cylinder plate method.

[4] Prepare at the time use. Stir for 30 minutes or longer with a stirrer before use.

[5] As NH is unstable in light, use an amber container or cover the container with aluminum foil.

[6] The addition of water shall be made by two 5-mL portions and one 10-mL portion, a total of 20 mL.

[7] An example standard response line for NH is shown in Figure 9.2.19-4.

Linearity is observed in the quantification range for NH (NH concentrations between 0.125 and 2 μg (potency)/mL).

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

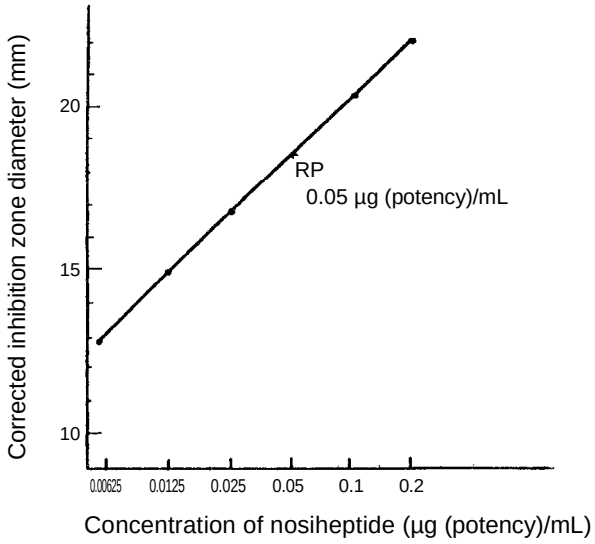


Figure 9.2.19-4 Standard response line for nosiheptide (feed, example)
(*Micrococcus luteus* ATCC 9341, Medium F-7, Agar well method)

2 Trace quantitative test method - Microbioautography (Feed)

[Feed Analysis Standards, Chapter 9, Section 2, 19.3.1]

Scope of application: Feed

A. Reagent preparation

1) Nosiheptide standard solution. Dry a suitable amount of nosiheptide 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 *N*,*N*-dimethylformamideand dissolve to prepare a nosiheptide 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 methanol to prepare standard solutions with concentrations of 2, 1, 0.5, 0.25 and 0.125 μg (potency)/mL[1].

2) Culture medium: Medium F-111

3) Bacterial suspension and amount of addition. Use *Micrococcus luteus* ATCC 9341[2] as the test organism. Add about 0.5 mL of a 100-fold diluted suspension of the test organism per 100 mL of the culture medium.

4) Developing solvent: A chloroform-methanol-ammonia reagent (20:13:5)Note 1

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

6) Chromigenic 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. Wash a silica gel minicolumn (690 mg) with 10 mL of chloroform. Place a funnel loaded with approximately 40 g of sodium sulfate (anhydrous)[3] on the minicolumn, pour the sample solution into the funnel, and allow to flow down until the amount in the reservoir of the minicolumn reaches 1 mL[4]. Wash the recovery flask that contained the sample solution with 10 mL of a mixture of chloroform and ethyl acetate (9:1), add the washings to the funnel, and repeat this process three times.

Wash the sodium sulfate remaining in the funnel with 10 mL of a mixture of chloroform and ethyl acetate (9:1), add the washings to the minicolumn, remove the funnel, add 20 mL of a mixture of chloroform and ethyl acetate (9:1) to wash the minicolumn.

Place a 100-mL recovery flask under the minicolumn, add 30 mL of a mixture of chloroform and methanol (4:1) to elute NH. 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[5], and use as the sample solution.

C. Quantification[6]

Proceed as described in Section 1, 2-C[7], except that the developing solvent shall be placed in the developing chamber and allowed to stand for not less than 1 hour. The thin-layer plate shall be of silica gelNote 2, and developed at 20 to 25°C to a distance of 150 mm from the starting line.

Note 1. Dilute ammonia solution with water to prepare a solution with ammonia content of 17%.

2. 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 a trace of NH in a feed resulting from

contamination due to carry-over etc., by microbioautography using a sample solution prepared by extracting with acetonitrile, purifying through a silica gel column, and dissolving in methanol.

The flow sheet of this method is shown in Figure 9.2.19-5.

Sample (40 g)
↓
Extract with 100 mL of acetonitrile (with a magnetic stirrer for 30 min).
↓
Filter (through filter peper No.5A).
↓
Collect 50 mL of the filtrate (into a 100-mL recovery flask).
↓
Evaporate into dryness under reduced pressure (in a water bath at 50°C).
↓
Dissolve the residue with 20 mL of chloroform-ethyl acetate (9:1).
↓
Load onto a silica gel column (previsouly washed with 10 mL of chloroform and place on the reservoir a funnel loaded with about 40 g of sodium sulfate).
↓
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 NH 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 paltes (allow to stand at 10-20°C for 3 hr).
↓
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 9.2.19-5 Trace quantitative test method for nosiheptide (feed)

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

History in the Feed Analysis Standards [14] New, [17] Revision

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Adult chicken formula feed	0.1~1	3	89.3~104.0	2.4
Meat pig formula feed	0.1~1	3	94.7~102.7	8.8
Dairy cattle formula feed	0.1~1	3	89.3~106.0	5.5

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)
Adult chicken formula fe	11	1	108.5	6.2	8.7

«Notes and precautions»

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

An example method of preparation for nosiheptide standard solution is shown in Table 9.2.19-3.

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

[3] It is recommended to stuff a small plug of absorbent cotton at the top of the funnel stem and on which to place sodium sulfate (anhydrate).

[4] When the flow is slow, it is permissible to inject under pressure useing the plunger of the syringe or a double-baloon pump.

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

Table 9.2.19-3 Method of preparation for nosiheptide standard solution (trace quantitative test method, feed, example)

Test tube No.	1	2	3	4	5	6	7
Amount (mL) of standard solution	②	4	2	10	10	10	5
Amount (mL) of methanol solution	18	16	18	10	10	10	5
Concentration (μg(potency)/mL)	100	20	2	1	0.5	0.25	0.125

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

[6] An example standard response line for NH is shown in Figure 9.2.19-6.

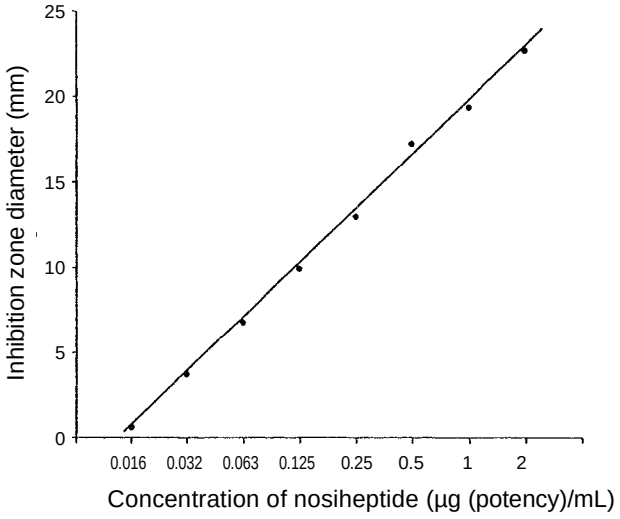


Figure 9.2.19-6 Standard response line for nosiheptide (trace quantitative test method, feed, example)

(*Micrococcus luteus* ATCC 9341, Medium F-111, Microbioautography)

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