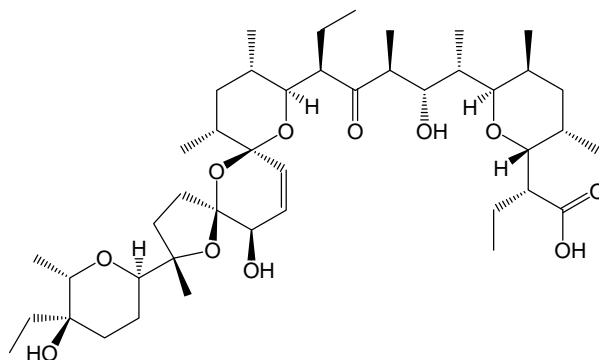


# 18 Narasin



$C_{43}H_{72}O_{11}$  MW: 765.0 CAS No.: 55134-13-9

## [Summary of narasin]

Narasin (NR) is a polyether antibiotic obtained by the incubation of an NR-producing strain of *Streptomyces aureofaciens*. It is a derivative of salinomycin having an additional methyl group with the chemical structure shown above, alternatively called “methyl salinomycin”.

For physicochemical properties, feed-grade NR technical occurs as grayish brown to dark brown powder or particles. It is very soluble in ethyl acetate, in chloroform, in acetone, in benzene and in dimethylsulfoxide, sparingly soluble in hexane and in petroleum ether, and practically insoluble in water.

Formulations with NR content exceeding 10% are designated as poisonous substances and those with NR content exceeding 1%, as deleterious substances, under the Cabinet Order for the Designation of the Poisonous and Deleterious Substances (Cabinet Order No.2, 1965). For the handling of these substances, make sure to conform to the procedures specified in the Poisonous and Deleterious Substances Control Act (Act No.303, 1950).

NR has an antibacterial effect on part of the Gram-positive bacteria, an anticoccidial effect, and a growth promoting effect on chickens (including broilers).

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

NR is a feed-grade antibiotic that was designated as a feed additive as of December 19, 2001. 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
		Starting chicks Growing chicks
Added amount	80	80

Like SL, MN, and LS, excessive consumption of NR can cause growth disturbance in chickens. It is

therefore necessary to strictly conform to the specified amounts of addition (80 g (potency)/1 ton of the feed of interest) and to achieve homogeneous mixture to secure the safety.

For this reason, feed manufacturers are required to control the feeds containing NR according to the separately described control test methods (13 SeiChiku No.4573, notified by the Head of the Livestock Division of the Production Bureau as of December 19, 2001).

## [Methods listed in the Feed Analysis Standards]

### 1 Quantitative test methods - Plate method (premix)

#### 1.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 18.1.1]

##### A. Reagent preparation

- 1) Sodium hydrogen carbonate-methanol solution. Dissolve 50 mg of sodium hydrogen carbonate in 200 mL of methanol<sup>[1]</sup>, and filter through filter paper (No.5A).
- 2) Dilution solvent: A mixture of water and methanol (7:3)
- 3) Narasin standard solution. Weigh accurately not less than 40 mg of narasin working standard<sup>[2]</sup>, accurately add sodium hydrogen carbonate-methanol solution<sup>[3]</sup> and dissolve to prepare a narasin standard stock solution with a concentration of 1 mg (potency)/mL<sup>[4]</sup>.  
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<sup>[5]</sup>.
- 4) Culture medium: Medium F-22
- 5) Spore suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.1 mL of the spore suspension with a concentration of  $1 \times 10^9$  spores/mL per 100 mL of the culture medium.
- 6) Agar plate. Proceed by the cylinder plate method, respectively<sup>[6]</sup>.
- 7) Extracting solvent. A mixture of methanol and water (9:1)

##### B. Preparation of sample solution

Weigh accurately 2 to 4 g of the analysis sample (equivalent to not more than 240 mg (potency)<sup>[7]</sup> as NR), 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 2 and 0.5 µg (potency)/mL, respectively<sup>[8]</sup>.

##### C. Quantification<sup>[9]</sup>

Proceed by the 2-2 dose method<sup>[10]</sup>.

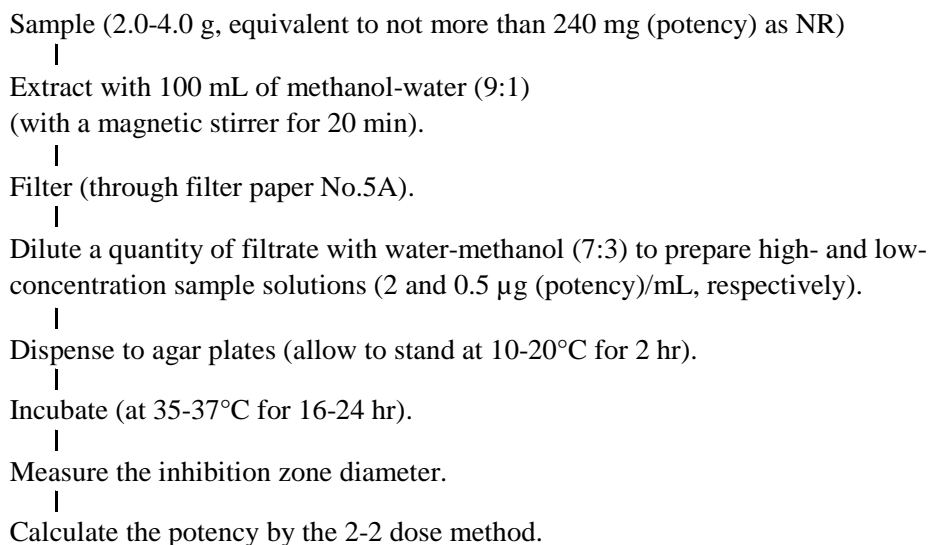
### «Summary of analysis method»

This method is intended to determine the amount of NR in a premix by microbiological assay using a sample solution prepared by extracting with a mixture of methanol and water (9:1) and diluting with a mixture of water and methanol (7:3).

None of the antibacterial substances approved for combined use with NR interfere with the

quantification of NR.

The flow sheet of this method is shown in Figure 9.2.18-1.



**Figure 9.2.18-1 Quantitative test method for narasin (premix)**

References: Tetsuo Chihara, Katsumi Yamamoto: Research Report of Animal Feed, 28, 82 (2003)

History in the Feed Analysis Standards [26] 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	8~80	3	98.9~100.4	2.6
Chicken premix 2	8~80	3	98.1~101.4	2.5
Chicken premix 3	8~80	3	96.6~100.7	2.4

## «Notes and precautions»

- [1] As sodium hydrogen carbonate is difficult to dissolve in methanol, it is recommended to apply ultrasonic waves for about 10 minutes after stirring for 30 minutes with a stirrer.
- [2] For the definition etc. of narasin working standard, refer to «Notes and precautions» [9] in Section 1, 1 of this Chapter.
- [3] Used to neutralize methanol. When the narasin working standard has been dissolved only with methanol, white suspended solids can result from diluting the solution with a dilution solvent with water content not less than 60%.
- [4] 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 963 µg (potency)/mg, 50 mg of the working standard contains 48,150 µg (potency) (i.e., 50 mg × 963 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 48.15 mL (i.e., 48,150 µg (potency) / 1,000 µg (potency)/mL).

Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 48.15 mL of methanol and dissolve to prepare a standard stock solution with a concentration of 1,000 µg (potency)/mL.

[5] 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 narasin standard solution is shown in Table 9.2.18-1.

[6] When using Medium F-22, the cylinder plate method is less sensitive to NR than the agar plate method. However, taking into consideration the slope of the standard response line, the concentration of the spore suspension, the spiked amount of NR, and the amount of the sample to be collected, the cylinder plate is recommended for preparing agar plates.

[7] Good recoveries resulted in most of the cases except when an analysis sample with a spiked concentration of 80 g (potency)/kg is collected in an amount of 5.0 g (equivalent of 400 mg (potency) as NR), which resulted in a poor recovery. Accordingly, the amount of the sample to be collected shall be in the range from 2.0 to 4.0 g, equivalent to not more than 240 mg (potency) as NR.

[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 9.2.18-1.

**Table 9.2.18-1 Method of preparation for narasin standard solution and sample solution**

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

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	4	5
Amount (mL) of water-methanol (7:3)	18	18	16	15
Concentration (µg(potency)/mL)	100	10	2	0.5

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 weighed in an amount equivalent to NR 200,000 µg (potency) of NR, the concentration of NR in the filtrate is calculated to be 2,000 µg (potency)/mL.

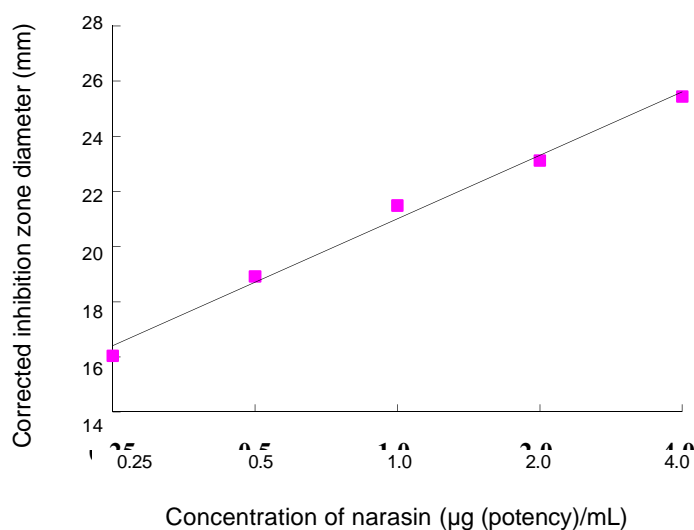
Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	2	5
Amount (mL) of water-methanol (7:3)	18	18	18	15
Concentration (µg(potency)/mL)	200	20	2	0.5

Note: "② mL" means "2 mL of the filtrate (2,000 µg(potency)/mL)".

[9] An example standard response line for NR is shown in Figure 9.2.18-2.

Linearity is observed in the quantification range for NR (NR concentrations between 0.5 and 2.0 µg (potency)/mL).

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



**Figure 9.2.18-2 Standard response line for NR (premix, example)**  
(*Bacillus subtilis* ATCC 6633, Medium F-22, Cylinder method)

## 1.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 18.2.1]

### A. Reagent preparation

- 1) Sodium hydrogen carbonate-methanol solution. Dissolve 50 mg of sodium hydrogen carbonate in 200 mL of methanol<sup>[1]</sup>, and filter through filter paper (No.5A).
- 2) Dilution solvent: A mixture of water and methanol (7:3)
- 3) Narasin standard solution. Weigh accurately not less than 40 mg of narasin working standard, accurately add the sodium hydrogen carbonate-methanol solution and dissolve to prepare a narasin 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 the dilution solvent to prepare high- and low-concentration standard solution with concentrations of 2 and 0.5 µg (potency)/mL.
- 4) Culture medium: Medium F-22
- 5) Spore suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.1 mL of the spore suspension with a concentration of  $1 \times 10^9$  spores/mL per 100 mL of the culture medium.
- 6) Agar plate. Proceed by the cylinder plate method<sup>[2]</sup>.
- 7) Extracting solvent: A mixture of methanol and water (9:1)

### B. Preparation of sample solution

Weigh accurately a quantity of the analysis sample (equivalent to 0.8 mg (potency)<sup>[3]</sup> as NR), 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).

Load the filtrate onto a column (column tube (14 mm in internal diameter) dry-packed with 12 g<sup>[4]</sup> of basic alumina for column chromatography (particle size: 74 to 177 µm (200 to 80 mesh))<sup>[5]</sup>), and

discard the first 5 mL of the filtrate<sup>[6]</sup>.

Accurately dilute a quantity of the subsequent filtrate<sup>[7]</sup> with a mixture of water and methanol (9:1) to prepare a high-concentration sample solution with a concentration of 2 µg (potency)/mL<sup>[8]</sup>, and accurately dilute this solution with the dilution solvent to prepare a low-concentration sample solution with a concentration of 0.5 µg (potency)/mL<sup>[9]</sup>.

### C. Quantification

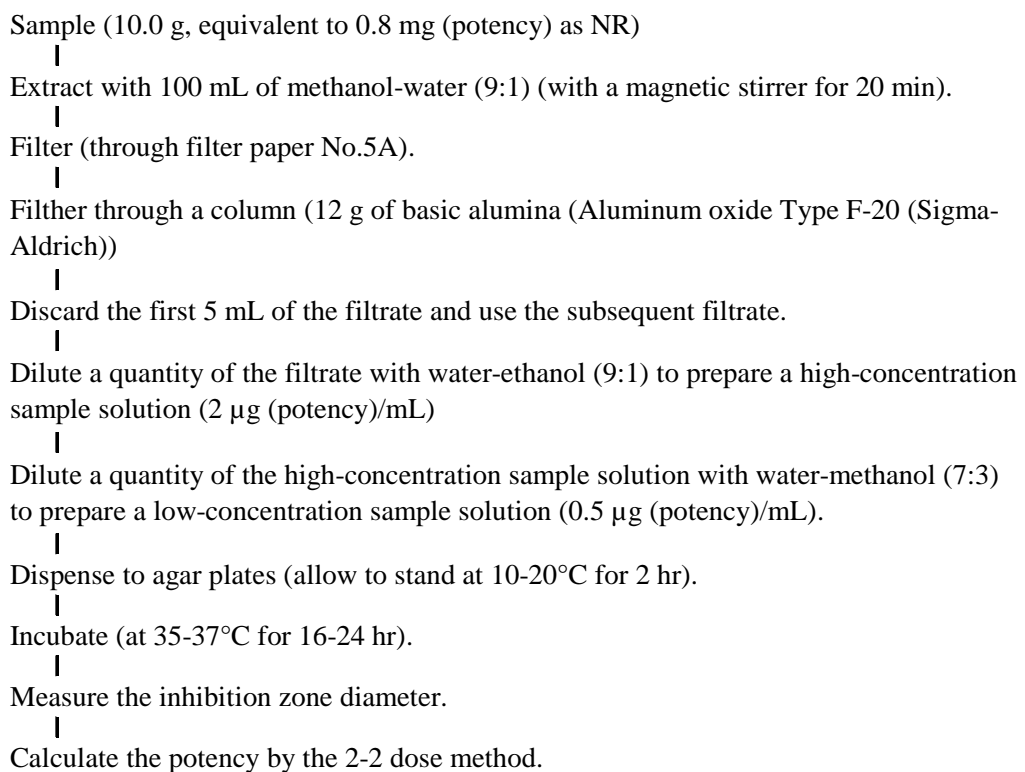
Proceed by the 2-2 dose method<sup>[10]</sup>.

## «Summary of analysis method»

This method is intended to determine the amount of NR in a chicken feed by microbiological assay using a sample solution prepared by extracting with a mixture of methanol and water (9:1) and filtering through a column packed with basic alumina.

None of the antibacterial substances approved for combined use with NR interfere with the quantification of NR.

The flow sheet of this method is shown in Figure 9.2.18-3.



**Figure 9.2.18-3 Quantitative test method for narasin (chicken feed)**

References: Tetsuo Chihara: Research Report of Animal Feed, 27, 80 (2002)

History in the Feed Analysis Standards [24] New

## «Validation of analysis method»

### • Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD(% or lower)
Starting chick formula feed	40~120	3	98.9~101.2	2.4
Fattening broiler starter formula feed	40~120	3	99.5~101.7	3.4
Fattening broiler finisher formula feed	40~120	3	97.9~99.1	4.3

### • Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Intra-lab reproducibility RSDR(%)
Starting chick formula feed	7	80	102.3	2.4	2.2

## «Notes and precautions»

[1] As sodium hydrogen carbonate is difficult to dissolve in methanol, it is recommended to stir for 30 minutes with a stirrer and apply ultrasonic waves for about 10 minutes.

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

An example method of preparation for the narasin standard solution is shown in Table 9.2.18-2.

**Table 9.2.18-2 Method of preparation for narasin standard solution (chicken feed, example)**

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	4	5
Amount (mL) of water-methanol (7	18	18	16	15
Concentration (µg(potency)/mL)	100	10	2	0.5

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

[3] When using Medium F-22, the cylinder plate method is less sensitive to NR than the agar plate method. However, taking into consideration the slope of the standard response line, the concentration of the spore suspension, the spiked amount of narasin, and the amount of the sample to be collected, the cylinder plate method is recommended for preparing agar plates.

[4] Usually corresponds to 10 g.

[5] It is recommended to use a vibrator etc. to compact the packing material tightly.

[6] As the first portion of the filtrate is turbid, discard the first 5 mL and use the subsequent filtrate.

[7] The quantified value is constant in the 5- to 30-mL fraction.

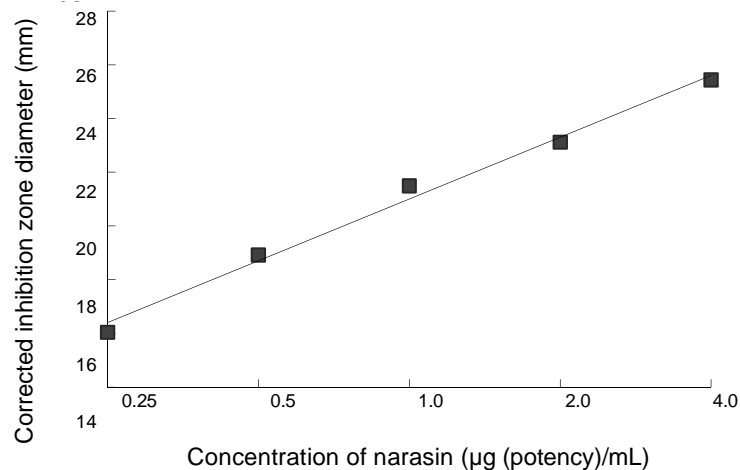
[8] An example method of preparation for the high-concentration sample solution is shown below.

Filtrate	5 mL
Water-methanol (9:1)	15 mL

[9] An example method of preparation for the low-concentration sample solution is shown below.

High-concentration sam	5 mL
Water-methanol (7:3)	15 mL

[10] An example standard response line for narasin is shown in Figure 9.2.18-4.



**Figure 9.2.18-4 Standard response line for narasin (chicken feed, example)**  
(*Bacillus subtilis* ATCC 6633, Medium F-22, Cylinder plate method)

## 2 Quantitative test method - Quantitative test method for polyether antibiotics by liquid chromatography

### 2.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 18.1.2]

**Antibiotics of interest:** SL, SD, NR and MN (4 components)

#### A. Reagent preparation

1) Salinomycin sodium standard solution. Dry a suitable amount of salinomycin working standard<sup>[1]</sup> under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours, weigh accurately a quantity equivalent to 20 mg (potency), place in a 100-mL volumetric flask, add methanol to dissolve, and further add methanol up to the marked line to prepare a salinomycin sodium standard stock solution (1 mL of this solution contains an amount equivalent to 0.2 mg (potency) as salinomycin sodium).

At the time of use, accurately dilute a quantity of standard stock solution with a mixture of methanol and water (9:1) to prepare several salinomycin sodium standard solutions containing salinomycin sodium in amounts equivalent to 2.5 to 20 µg (potency) in 1 mL.

2) Semduramicin sodium standard solution. Weigh accurately a quantity of semduramicin working standard equivalent to 20 mg (potency)<sup>[1]</sup>, place in a 100-mL volumetric flask, add methanol to dissolve, and further add methanol up to the marked line to prepare a standard stock solution (1 mL of this solution contains an amount equivalent to 0.2 mg (potency) as semduramicin sodium).

At the time of use, accurately dilute a quantity of the standard stock solution with methanol to prepare several semduramicin sodium standard solutions containing semduramicin sodium in amounts equivalent to 2.5 to 20 µg (potency) in 1 mL.

3) Narasin standard solution. Weigh accurately a quantity of narasin working standard equivalent to 20 mg (potency)<sup>[1]</sup>, place in a 100-mL volumetric flask, add methanol to dissolve, and further add methanol up to the marked line to prepare a narasin standard stock solution (1 mL of this solution contains narasin in an amount equivalent to 0.2 mg (potency)).



At the time of use, accurately dilute a quantity of the standard stock solution with a mixture of methanol and water (9:1) to prepare several narasin standard solutions containing narasin in amounts equivalent to 0.5 to 20 µg (potency) in 1 mL.

- 4) Monensin sodium standard solution. Weigh accurately a quantity of monensin working standard equivalent to 20 mg (potency)<sup>[1]</sup>, place in a 100-mL volumetric flask, add methanol to dissolve, and further add methanol up to the marked line to prepare a monensin sodium standard stock solution (1 mL of this solution contains an amount equivalent to 0.2 mg (potency) as monensin sodium).

At the time of use, accurately dilute a quantity of the standard stock solution with a mixture of methanol and water (9:1) to prepare several monensin sodium standard solutions containing monensin sodium in amounts equivalent to 2.5 to 20 µg (potency) in 1 mL.

### B. Quantification

Extraction. Weigh accurately 2 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of a mixture of methanol and water (9:1), 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 methanol and water (9:1), filter through membrane filter (pore size not exceeding 0.5 µm), and use the filtrate as the sample solution subject to liquid chromatography.

Liquid chromatography. Inject 20 µL each of the sample solution and antibiotic standard solutions into a liquid chromatograph to obtain chromatograms.

#### Example operating conditions

Detector: Ultraviolet-visible absorption detector (measured wavelength: 520 nm)

Column: Octadecylsilanized silica gel column (4.6 mm in internal diameter, 150 mm in length, 5 µm in particle size)<sup>Note 1 [2]</sup>

Eluent: A mixture of methanol, water and acetic acid (940:60:1)

Reaction solution<sup>Note 2</sup>: Gradually add 10 mL of sulfuric acid to 475 mL of methanol while stirring, add 15 g of vanillin and dissolve (prepare at the time of use).

Flow rate: 0.6 mL/min for the eluent; 0.6 mL/min for the reaction solution

Reaction vessel temperature: 95°C

Calculation. Calculate the peak height or peak area from the obtained chromatogram<sup>[3]</sup> to prepare the calibration curve, and estimate the amount of each antibiotic<sup>Note 3, 4</sup>.

Note 1. Use a Mightysil RP-18 GP (Kanto Chemical Co., Inc.) or an equivalent.

2. Develop by allowing the eluate from the column to react with the reaction solution through the reaction coil (0.5 mm in internal diameter, 5 mm in length (10 m )) in the reaction vessel, and immediately transfer to the ultraviolet-visible absorption detector. The reaction solution shall be used in a light-resistant container.

3. For monensin sodium, the calculated amount of monensin A shall be regarded as the amount of monensin sodium. The peak of monensin A appears as the main peak on the chromatogram from each monensin sodium standard solution. On the chromatogram of the sample solution, the peak of monensin A appears at the same retention time as the peak of monensin A from the standard solution.

4. For narasin, the calculated amount of narasin A shall be regarded as the amount of narasin. The peak of narasin A appears as the main peak on the chromatogram of each narasin standard solution. On the chromatogram from the sample solution, the peak of narasin A appears at the same retention time as the peak of narasin A from the standard solution.

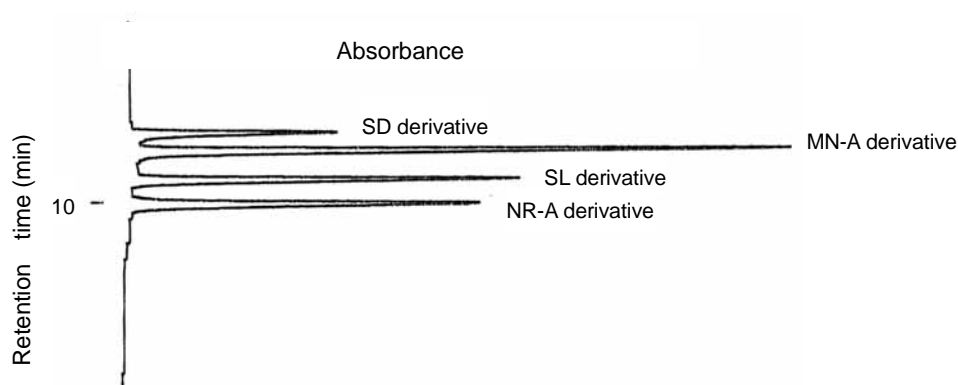
### «Summary of analysis method»

This method is intended to determine the amount of salinomycin, semduramicin, narasin A, and monensin A in a premix by determining the absorbances of their derivatives produced by extracting with a mixture of methanol and water (9:1), separating by liquid chromatography using an octadecylsilanized silica gel (ODS) column, and allowing to react with vanillin. It is also called the post-column derivatization method.

The principle of this derivatization (chromogenic) reaction depends on the so-called Komarowsky reaction, which involves aldol condensation of the alcoholic hydroxyl groups of salinomycin, semduramicin, narasin A and monensin A with the benzaldehyde group of vanillin, in an acidic solution containing sulfuric acid, to produce derivatives of these antibiotics that have wavelengths of maximal absorption of about 520 nm.

This method allows for simultaneous quantification of salinomycin sodium (SL), semduramicin sodium (SD), narasin (NR), and monensin sodium (MN). Care should be taken that, of the peaks of monensin sodium, the peak of monensin B can appear at the same retention time as the peak of semduramicin sodium, and thus interfere with the quantification.

For reference, the nature of separation of the mixed standard solution is shown in Figure 9.3.1-1. Because of the above-mentioned possibility of interference from peaks other than those of interest, it is preferable to use a single-component standard solution rather than a mixed standard solution for the preparation of the calibration curve.



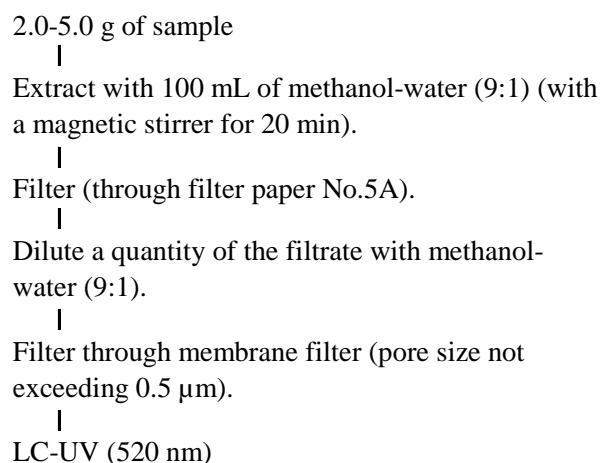
**Figure 9.3.1-1 Chromatogram for a mixed standard solution**  
(SL: 100 ng, SD: 200 ng, NR-A: 100 ng, MN-A: 50 ng)

Narasin is a mixture of narasin A, narasin B, narasin D and narasin I, and the “narasin” designated as a feed additive is defined as containing narasin A as the main ingredient. Monensin is a mixture of monensin A, monensin B, monensin C and monensin D, and the “monensin” designated as a feed additive is defined as containing monensin A as the main ingredient. In the test method described here,

the quantified amounts of narasin A and of monensin A are regarded as the amounts of narasin and of monensin, respectively, based on the premises that commercial narasin and monensin formulations contain narasin and monensin at a concentration of not less than 95%, respectively. It should be borne in mind, therefore, that the “narasin” and “monensin” quantified by this method are not exactly the same as the “narasin” and “monensin” quantified by microbiological assay.

For more details, refer to «Notes and precautions» [1] of General Notice 13 in Chapter 1.

The flow sheet of this method is shown in Figure 9.3.1-2.



**Figure 9.3.1-2 Quantitation test method for salinomycin sodium, semduramicin sodium, narasin, and monensin sodium by liquid chromatography (premix)**

References: Daisaku Makino: Research Report of Animal Feed, 27, 57 (2002)

Daisaku Makino: Research Report of Animal Feed, 27, 64 (2002)

Mayumi Nishimura: Research Report of Animal Feed, 28, 69 (2003)

Katsumi Yamamoto, Tetsuo Chihara: Research Report of Animal Feed, 28, 82 (2003)

History in the Feed Analysis Standards [25] New, [26] Component addition (semduramicin sodium, narasin)

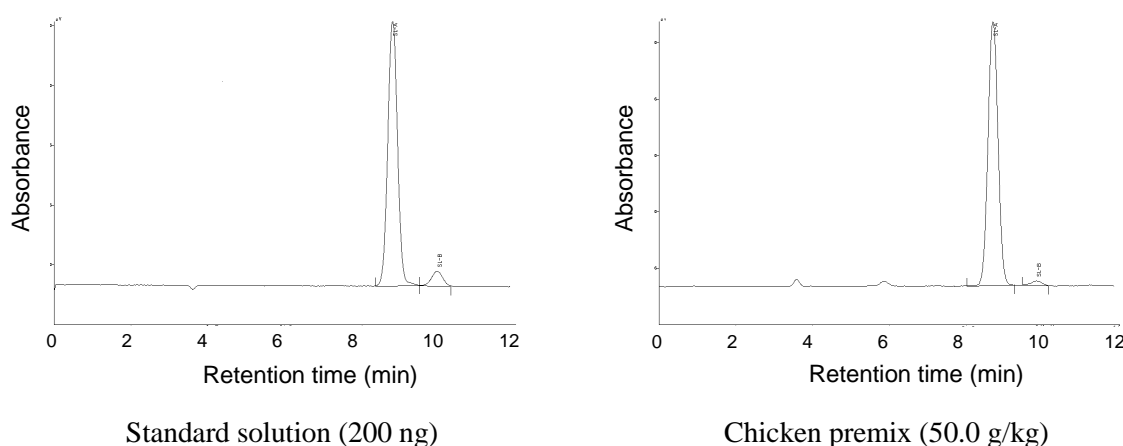
## «Validation of analysis method»

### • Spike recovery and repeatability

Spiked component	Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Salinomycin sodium	Starting chick grower premix	12.5~85.0	3	99.3~102.1	3.8
	Broiler fattener finisher premix	12.5~85.0	3	96.3~102.7	3.0
	Meat cattle fattener premix	12.5~85.0	3	98.0~100.8	2.8
Semduramicin sodium	Chicken premix 1	8~42	3	99.8~101.8	2.4
	Chicken premix 2	8~42	3	98.5~102.4	2.8
	Chicken premix 3	8~42	3	98.2~100.7	2.7
Narasin	Chicken premix 1	8~80	3	98.7~103.8	0.9
	Chicken premix 2	8~80	3	96.0~ 99.4	0.8
	Chicken premix 3	8~80	3	96.6~ 99.8	0.5
Monensin sodium	Starting chick grower premix	5~80	3	98.2~102.4	2.1
	Broiler fattener finisher premix	5~80	3	101.4~102.5	2.0
	Meat cattle fattener premix	5~80	3	96.6~99.5	4.7

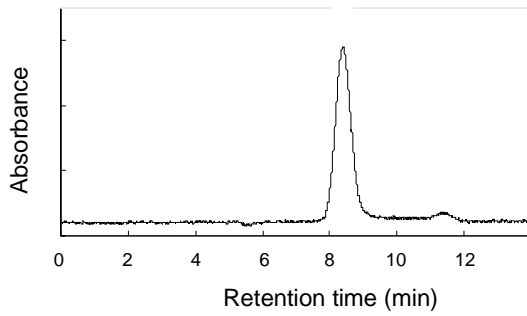
## «Notes and precautions»

- [1] For the definition etc. of the working standards for salinomycin, semduramicin, narasin, and monensin, refer to «Notes and precautions» [9] in Section 1, 1 of this Chapter.
- [2] Any column is applicable as long as it is packed with an equivalent end-capped material. The columns used for the validation of this method were Shodex C18M 4D for narasin and Mightysil RP-18 GP for salinomycin, semduramicin, and monensin.
- [3] Example chromatograms for salinomycin are shown in Figure 9.3.1-3.

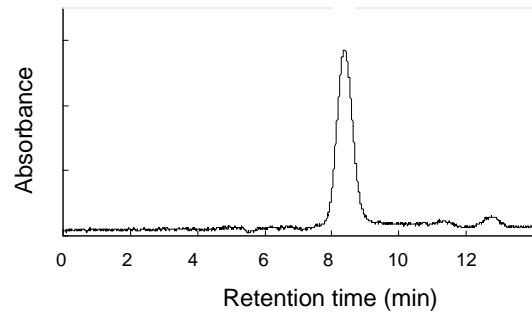


**Figure 9.3.1-3 Chromatograms for salinomycin**  
(The arrow indicate the peak of the SL derivative)

Example chromatograms for semduramicin are shown in Figure 9.3.1-4.



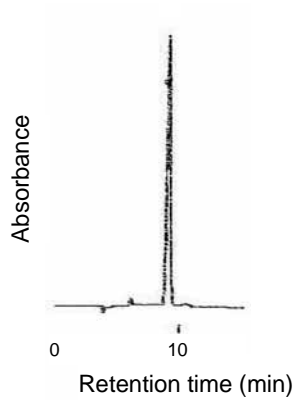
Standard solution (equivalent to 200 ng (potency) as SD)



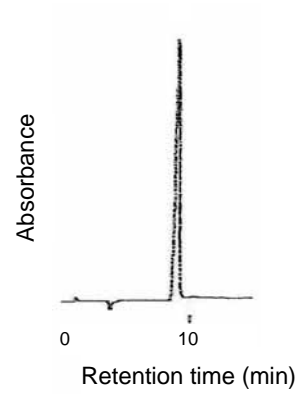
Chicken premix (equivalent to 200 ng (potency) as SD)

**Figure 9.3.1-4 Chromatograms for semduramicin**  
(The arrow indicate the peak of the SD derivative)

Example chromatograms for narasin are shown in Figure 9.3.1-5.



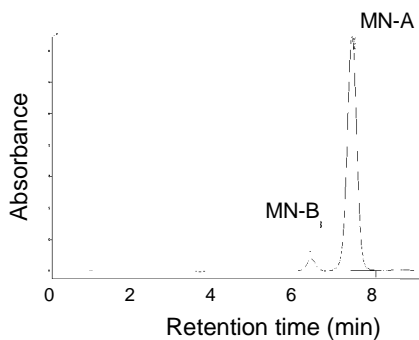
Standard solution (200 ng)



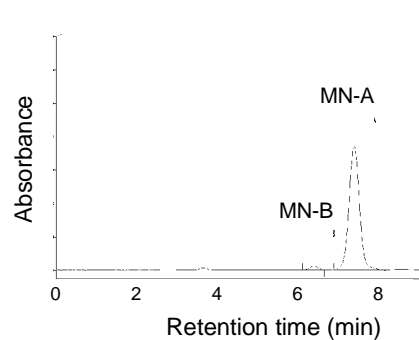
Chicken premix (80 g (potency)/kg)

**Figure 9.3.1-5 Chromatograms for narasin**  
(The arrow indicate the peak of the NR-A derivative)

Example chromatograms for monensin are shown in Figure 9.3.1-6.



Standard solution (200 ng)



Chicken premix (33.3 g/kg)

**Figure 9.3.1-6 Chromatograms for monensin**

(The arrows indicate the peaks of the MN-A derivative (main peak) and MN-B derivative)

## 2.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 18.2.2]

**Antibiotics of interest:** SL, SD, NR and MN (4 components)

### A. Reagent preparation

1) Salinomycin sodium standard solution. Prepare a salinomycin sodium standard stock solution as directed in 1.1-A.

At the time of use, accurately dilute a quantity of the standard stock solution with a mixture of methanol and water (9:1) to prepare several salinomycin sodium standard solutions containing salinomycin sodium in amounts equivalent to 0.5 to 8 µg (potency) in 1 mL.

2) Semduramicin sodium standard solution<sup>[1]</sup>. Prepare a semduramicin standard stock solution as directed in 1.1-A.

At the time of use, accurately dilute a quantity of standard stock solution with methanol to prepare several semduramicin sodium standard solutions containing semduramicin sodium in amounts equivalent to 0.5 to 10 µg (potency) in 1 mL.

3) Narasin standard solution. Proceed as directed in 1.1-A.

4) Monensin sodium standard solution. Prepare a monensin standard stock solution as directed in 1.1-A.

At the time of use, accurately dilute a quantity of the standard stock solution with a mixture of methanol and water (9:1) to prepare several monensin sodium standard solutions containing monensin sodium in amounts equivalent to 0.5 to 15 µg (potency) in 1 mL.

### B. Quantification

Extraction. Weigh 10.0 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of a mixture of methanol and water (9:1), extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A). Filter the filtrate through membrane filter (pore size not exceeding 0.5 µm) and use as a sample solution subject to liquid chromatography.

Liquid chromatography. Proceed as directed in 1.1-B Liquid chromatography<sup>[2]</sup>.

Calculation. Proceed as directed in 1.1-B Calculation<sup>[3]</sup>.

## «Summary of analysis method»

This method is intended to determine the amount of salinomycin, semduramicin, narasin A and monensin A in a chicken feed or cattle feed by post-column derivatization liquid chromatography using a sample solution prepared by extracting with a mixture of methanol and water (9:1) as described in 1.1. Quantification test method for polyether antibiotics by liquid chromatography (premix) in this Section. For the principle of the measurement etc., refer to 1.1 «Summary of analysis method».

In this method, none of the antibacterial substances approved for combined use with semduramicin sodium interfere with the quantification of semduramicin sodium. Of the monensin sodium that are not approved for combined use, however, monensin B was found to interfere with the quantification of semduramicin sodium.

For the nature of separation of the mixed standard solution, refer to Figure 9.3.1-1.

The flow sheet of this method is shown in Figure 9.3.1-7.

10.0 g of sample  
 |  
 Extract with 100 mL of methanol-water (9:1) (with  
 a magnetic stirrer for 20 min).  
 |  
 Filter (through filter paper No.5A).  
 |  
 Filter through membrane filter (pore size not  
 exceeding 0.5 µm).  
 |  
 LC-UV (520 nm)

**Figure 9.3.1-7 Quantitation test method for salinomycin sodium, semduramicin sodium, narasin and monensin sodium by liquid chromatography (feed)**

References: Toshiaki Hayakawa, Masato Funatsu: Research Report of Animal Feed, 26, 51 (2001)

Ikumi Kobayashi: Research Report of Animal Feed, 27, 71 (2002)

Tetsuo Chihara: Research Report of Animal Feed, 27, 94 (2002)

Toshiaki Hayakawa, Daisaku Makino: Research Report of Animal Feed, 26, 60 (2001)

History in the Feed Analysis Standards [23] New, [24] Component addition (narasin), [25] Component addition (semduramicin sodium)

## «Validation of analysis method»

### • Spike recovery and repeatability

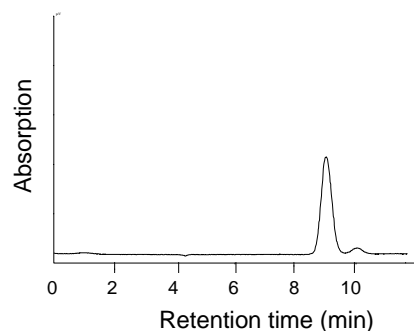
Spiked component	Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Salinomycin sodium	Starting chick grower formula feed	25~50	3	96.7~101.7	4.6
	Broiler fattener starter formula feed	25~50	3	96.0~ 98.7	2.1
	Broiler fattener finisher formula feed	25~50	3	97.7~101.3	4.0
	Calf grower formula feed	7.5~22.5	3	97.0~100.7	4.6
	Meat cattle fattener starter formula feed	7.5~22.5	3	98.3~103.3	4.6
	Meat cattle fattener finisher formula feed	7.5~22.5	3	97.7~103.0	4.0
Semduramicin sodium	Starting chick grower formula feed	12.5~37.5	3	95.6~97.8	1.3
	Broiler fattener starter formula feed	12.5~37.5	3	97.5~98.7	1.9
	Broiler fattener finisher formula feed	12.5~37.5	3	97.7~98.3	1.5
Narasin	Starting chick grower formula feed	40~120	3	97.8~102.2	2.7
	Broiler fattener starter formula feed	40~120	3	99.4~102.5	2.7
	Broiler fattener finisher formula feed	40~120	3	96.3~99.8	1.9
Monensin sodium	Starting chick grower formula feed	40~120	3	99.0~100.3	1.0
	Broiler fattener starter formula feed	40~120	3	99.3~99.7	1.2
	Broiler fattener finisher formula feed	40~120	3	98.7~100.0	1.0
	Calf grower formula feed	15~45	3	100.3~102.0	1.2
	Meat cattle fattener starter formyla feed	15~45	3	98.0~99.7	1.7
	Meat cattle fattener finisher formula feed	15~45	3	100.7~102.0	1.7

• Collaborative study

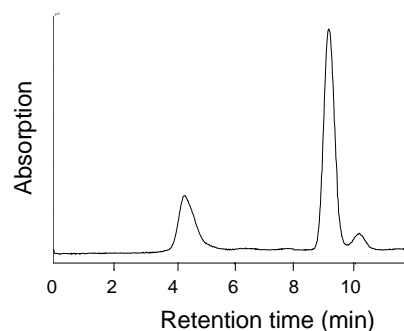
Spiked component	Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)	HorRat
Salinomycin sodium	Chicken formula feed	7	50	94.4	2.7	2.0	0.22
Semduramicin sodium	Broiler finisher formula feed	7	25	97.9	1.8	1.8	0.18
Narasin	Starting chick grower formula feed	7	80	99.7	2.9	2.1	0.25
Monensin sodium	Cattle formula feed	6	30	98.0	2.0	2.6	0.27

## «Notes and precautions»

- [1] As low concentrations of the standard solution are likely to change over time, make sure to prepare immediately before analysis. The peak that appears at a retention time approximately 1.5 times greater than the main peak is that of hydroxyl semduramicin, a degraded substance of the standard substance.
- [2] The columns used for validation of this method are Shodex C18M4D for narasin and Mightysil RP-18 GP for salinomycin, semduramicin, and monensin.
- [3] Example chromatograms for salinomycin are shown in Figure 9.3.1-8.



Standard solution (40 ng)

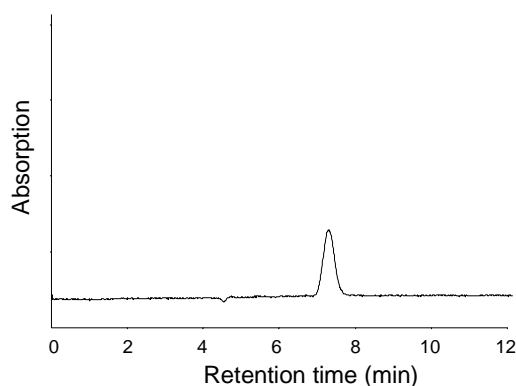


Starting chick grower formula feed (50 g (potency)/t)

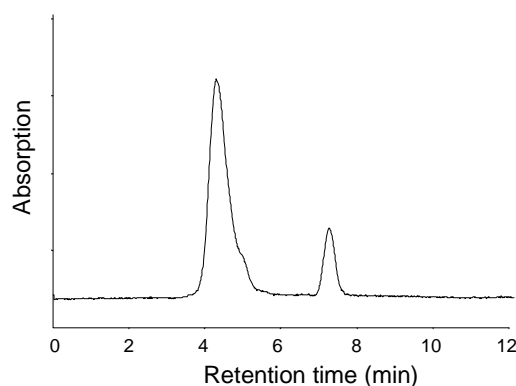
**Figure 9.3.1-8 Chromatograms for salinomycin**

(The arrow indicate the peak of the SL derivative)

Example chromatograms for semduramicin are shown in Figure 9.3.1-9.



Standard solution (50 ng)



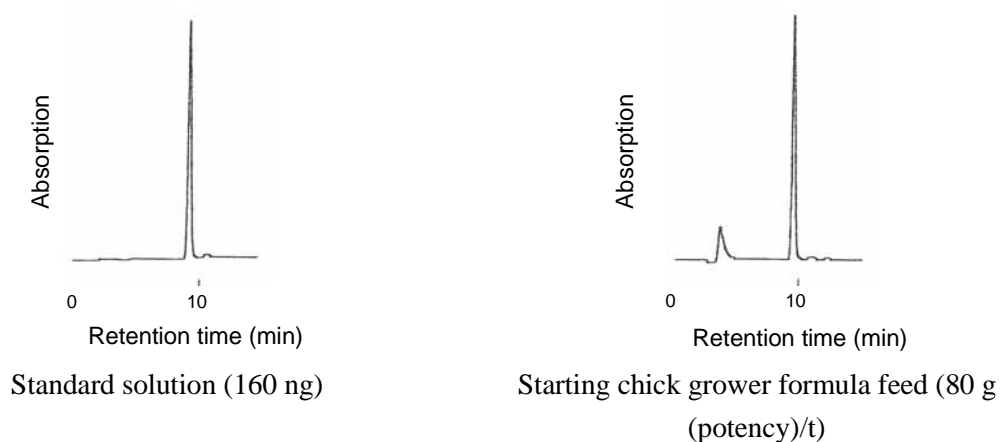
Chicken formula feed (25 g (potency)/t)

**Figure 9.3.1-9 Chromatograms for semduramicin**

(The arrow indicate the peak of an SD derivative)

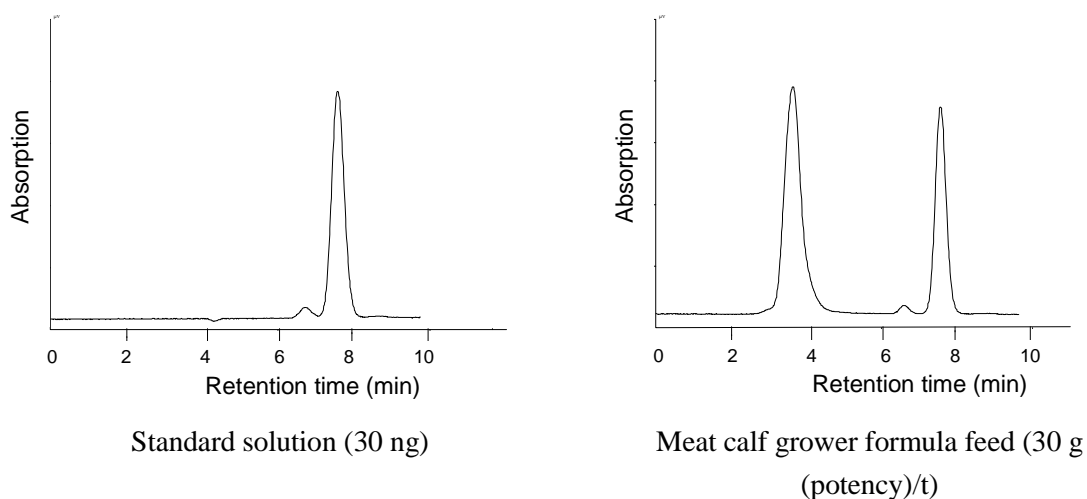


Example chromatograms for narasin are shown in Figure 9.3.1-10.



**Figure 9.3.1-10 Chromatograms for narasin**  
(The arrow indicate the peak of NR-A derivative)

Example chromatograms for monensin are shown in Figure 9.3.1-11.



**Figure 9.3.1-11 Chromatograms for monensin**  
(The arrow indicate the peak of the MN-A derivative)

### 3 Trace quantitative test method - Trace quantitative test method for polyether antibiotics by liquid chromatography mass spectroscopy (Feed)

[Feed Analysis Standards, Chapter 9, Section 2, 18.3.1]

**Antibiotics of interest:** SL, SD, NR, MN and LS (5 components)

**Scope of application:** Formula feed

#### A. Reagent preparation

- 1) Standard stock solution of each antibiotic<sup>[1]</sup>. Weigh accurately a quantity equivalent to 20 mg (potency) each of salinomycin working standard<sup>Note 1</sup>, semduramicin working standard, narasin working standard, monensin working standard, and lasalocid working standard, place each in a 100-mL volumetric flask,

add methanol to dissolve, and further add methanol up to the marked line to prepare respective standard stock solutions (1 mL each of these solutions contains an amount equivalent to 0.2 mg (potency) as salinomycin sodium, semduramicin sodium, narasin, monensin sodium, and lasalocid sodium, respectively).

- 2) Mixed standard solution. At the time of use, mix quantities of the standard stock solutions of salinomycin sodium, semduramicin sodium, narasin, monensin sodium, and lasalocid sodium. Accurately dilute the mixture with methanol to prepare several mixed standard solutions containing amounts equivalent to 0.1 to 2 µg (potency) as each antibiotic in 1 mL.

### B. Quantification

Extraction. Weigh 10.0 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 exactly 25 mL of the filtrate to a 100-mL recovery flask, condense under reduced pressure almost into dryness in a water bath at 40°C, and evaporate into dryness by introducing nitrogen gas.

Add 10 mL of a mixture of hexane 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 hexane, and on the minicolumn reservoir place a funnel previously loaded with approximately 20 g of sodium sulfate (anhydrous)<sup>[2]</sup>.

Pour the sample solution into the funnel, and allow to flow down until the liquid level reaches the top of the column packing material. Wash the recovery flask that contained the sample solution 3 times with 5 mL of a mixture of hexane and ethyl acetate (9:1), transfer the washings each time to the funnel, and allow to flow down in the same manner. Further, wash the sodium sulfate in the funnel with 5 mL of a mixture of hexane and ethyl acetate (9:1), allow to flow down in the same manner, remove the funnel, and add 10 mL of a mixture of hexane and ethyl acetate (9:1) to wash the minicolumn.

Place a 50-mL recovery flask under the minicolumn, and add 15 mL of a mixture of hexane and ethanol (4:1) to the minicolumn to elute each antibiotic. Condense the eluate almost into dryness under reduced pressure in a water bath at 40°C, and evaporate into dryness by introducing nitrogen gas.

Add exactly 10 mL of methanol to dissolve the residue, centrifuge at 5,000×g for 5 minutes, and use the supernatant liquid as the sample solution subject to liquid chromatography-mass spectrometry.

Measurement by liquid chromatography-mass spectrometry. Inject 5 µL each of the sample solution and mixed standard solutions into a liquid chromatograph-mass spectrometer to obtain selected ion detection chromatograms.

#### Example operating conditions

Column: Octadecylsilanized silica gel column (2 mm in internal diameter, 50 mm in length, 5 µm in particle size)<sup>Note 2</sup>

Eluent: A mixture of 5 mmol/L ammonium acetate solution and acetonitrile (1:4)

Flow rate: 0.2 mL/min

Column temperature: 40°C

Detector: Quadrupole mass spectrometer<sup>Note 3</sup>

Ionization method: Electrospray ionization (ESI) (positive ion mode)

Nebulizer gas: N<sub>2</sub> (1.5 L/min)

CDL temperature: 250°C

Heat block temperature: 200°C

Monitored ions<sup>[3]</sup>:

- m/z* 769 (salinomycin)
- m/z* 891 (semduramicin)
- m/z* 783 (narsin A)
- m/z* 688 (monensin A)
- m/z* 608 (lasalocid)

Calculation. Calculate the peak height or peak area from the obtained selected ion detection chromatogram<sup>[4]</sup> to prepare a calibration curve, and estimate the amount of each antibiotic in the sample solution<sup>Note 4</sup>.

- Note 1. Prepared by drying a suitable amount under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours
2. Gemini 5 $\mu$  C18 110A (Phenomenex; the retention times of salinomycin, semduramicin, narsin A, monensin A and lasalocid are approximately 9, 6, 13, 8 and 4 minutes, respectively, under the operating conditions of this method) or an equivalent
  3. Operating conditions for LCMS-2010EV (Shimadzu)
  4. For narsin, the calculated amount of narsin A shall be regarded as the amount of narsin. For monensin, the calculated amount of monensin A shall be regarded as the amount of monensin sodium.

## «Summary of analysis method»

This method is intended to determine the amounts of SL, SD, NR, MN and LS in a feed at the same time by liquid chromatography-mass spectrometry using electrospray ionization (ESI) (positive ion mode) 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 9.3.4-1.

- 10.0 g of the sample
- Add 100 mL of acetonitrile and stir for 30 min.
  - Filter (through filter paper No.5A).
  - Collect 25 mL of the filtrate.
  - Condense under reduced pressure (at 40°C or lower) and evaporate into dryness (with nitrogen gas).
  - Add 10 mL of hexane-ethyl acetate (9:1).
- Sep-Pak Plus Silica cartridge (previously washed with 10 mL of hexane).
- Place on the minicolumn a funnel containing approximately 20 g of sodium sulfate (anhydrous).
  - Load the sample solution.
  - Wash with 5 mL of hexane-ethyl acetate (9:1) (3 times).
  - Wash the sodium sulfate (anhydrous) with 5 mL of hexane-ethyl acetate (9:1).
  - Wash the silica gel minicolumn with 10 mL of hexane-ethyl acetate (9:1).
  - Elute with 15 mL of hexane-ethanol (4:1).
  - Condense under reduced pressure (at 40°C or lower) and evaporate into dryness (with nitrogen gas).
  - Add 10 mL of methanol.
  - Centrifuge at a high speed (at 5,000×g for 5 min).
- LC-MS

**Figure 9.3.4-1 Method of collective trace quantitation for polyether antibiotics by liquid chromatography-mass spectrometry**

References: Daisaku Makino, Miho Yamada: Research Report of Animal Feed, 33, 62 (2008)

History in the Feed Analysis Standards [31] New

### «Validation of analysis method»

• Spike recovery and repeatability

Spiked component	Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Salinomycin sodium	Adult chicken grower formula feed	0.5~5	3	95.0~96.2	2.4
	Meat pig fattener formula feed	0.5~5	3	95.5~98.4	2.3
	Meat cattle fattener formula feed	0.5~5	3	89.7~98.8	2.9
Semduramicin sodium	Adult chicken grower formula feed	0.5~5	3	89.4~89.5	1.2
	Meat pig fattener formula feed	0.5~5	3	80.0~84.6	10
	Meat cattle fattener formula feed	0.5~5	3	88.7~90.0	3.9
Narasin	Adult chicken grower formula feed	0.5~5	3	86.8~88.9	7.6
	Meat pig fattener formula feed	0.5~5	3	83.0~88.3	6.6
	Meat cattle fattener formula feed	0.5~5	3	83.4~89.7	13
Monensin sodium	Adult chicken grower formula feed	0.5~5	3	104.3~108.7	1.5
	Meat pig fattener formula feed	0.5~5	3	104.1~104.5	0.9
	Meat cattle fattener formula feed	0.5~5	3	103.7~107.5	1.1
Lasarosid sodium	Adult chicken grower formula feed	0.5~5	3	91.6~94.5	2.8
	Meat pig fattener formula feed	0.5~5	3	86.0~91.4	4.5
	Meat cattle fattener formula feed	0.5~5	3	85.2~89.4	3.8

• Collaborative study

Spiked component	Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)	HorRat
Salinomycin sodium	Adult chicken grower formula feed	8	0.5	95.0	2.7	6.4	0.36
Semduramicin sodium	Adult chicken grower formula feed	8	0.5	98.6	2.6	8.0	0.45
Narasin	Adult chicken grower formula feed	8	0.5	88.5	3.5	5.7	0.31
Monensin sodium	Adult chicken grower formula feed	8	0.5	101.0	3.6	5.0	0.28
Lasarosid sodium	Adult chicken grower formula feed	8	0.5	93.3	3.8	8.2	0.46

• Lower detection limit\*: 0.5 g (potency)/t for each component

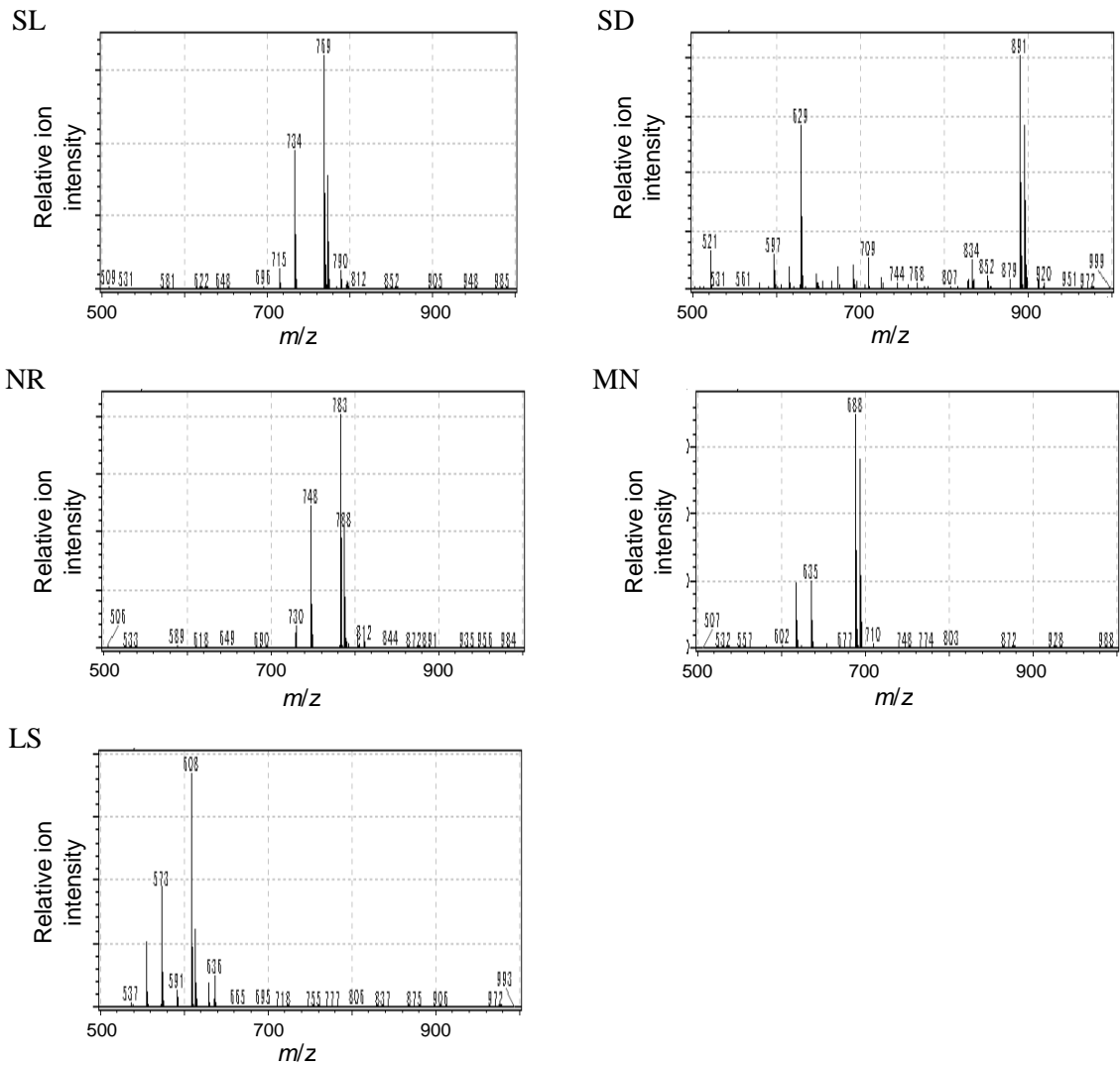
## «Notes and precautions»

- [1] For the definition etc. of each working standard, refer to «Notes and precautions» [9] in Section 1, 1 of this Chapter.
- [2] 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). Alternatively, a reservoir with an appropriate frit packed with sodium sulfate (anhydrous) is applicable.
- [3] Ammonium adduct ion  $[M+NH_4]^+$  of each antibiotic shall be used as monitored ions.

The mass spectra for salinomycin, semduramicin, narasin A, monensin A and lasalocid are shown in Figure 9.3.4-2.

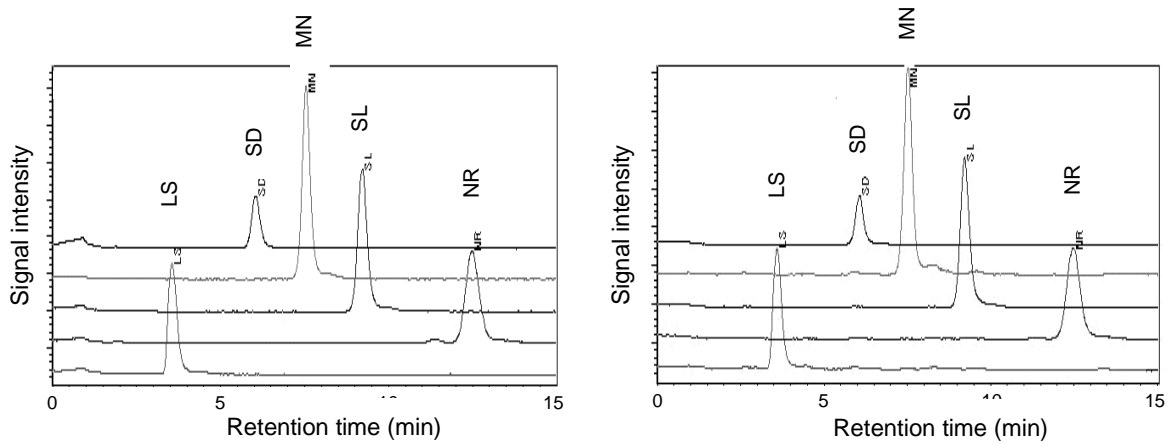
Under the example operating conditions mentioned above, fragment ions were detected other than the monitored ions of interest for each antibiotic. It is therefore necessary to confirm in advance the possible production of these fragment ions and their charge/mass ratios, as they can differ depending on the operating conditions and the type of the liquid chromatograph-mass spectrometer. Typical fragment ions produced under the operating conditions of this test include  $m/z$  734, 629, 748, 635 (or 618) and 573 (or 555) for salinomycin, semduramicin, narasin A, monensin A and lasalocid, respectively.

When these antibiotics are detected by this test method, it is recommended not only to quantify by monitoring the ions of interest but to confirm that the same fragment ions are detected in the sample solution as in the standard solutions under the operating conditions employed.



**Figure 9.3.4-2 Mass spectrum for each antibiotic**

[4] Example selected ion detection (SIM) chromatograms obtained from a mixed standard solution and sample solution are shown in Figure 9.3.4-3.



Mixed standard solution (equivalent to 0.6 ng (potency))

Adult chicken grower formula feed (equivalent to 0.5 g (potency)/t)

**Figure 9.3.4-3 SIM chromatograms for the mixed standard solution and sample solution**

(The arrow indicates the peak of each antibiotic)

## 4 Control test method - Rapid quantitative method for chicken feed

[13 SeiChiku No.4573, notified by the Head of Production Bureau, Ministry of Agriculture, Forestry and Fisheries, as of December 19, 2001]

### 1 Instruments and equipments

- (1) Stoppered Erlenmeyer flask
- (2) Volumetric flask
- (3) Volumetric cylinder
- (4) Test tube
- (5) Pipette
- (6) Thermostat
- (7) Magnetic stirrer
- (8) Spectrophotometer
- (9) Chemical balance

### 2 Reagents

- (1) Anhydrous ethanol (guaranteed grade)
- (2) *p*-Dimethylaminobenzaldehyde (guaranteed grade)
- (3) Sulfuric acid (guaranteed grade)
- (4) Narasin working standard

### 3 Preparation of reagents

- (1) Standard narasin solution. Place accurately 8 mg (potency) of narasin working standard in a 100-mL volumetric flask, add anhydrous ethanol<sup>[1]</sup> and dissolve to make 100 mL, and use as the narasin standard stock solution<sup>[2]</sup> (1 mL of this solution contains an amount equivalent to 80 µg (potency) as narasin).

At the time of use, dilute a quantity of of this stock solution accurately 10-fold with anhydrous ethanol (1 mL of this solution contains an amount equivalent to 8 µg (potency) as narasin).

- (2) *p*-Dimethylaminobenzaldehyde solution<sup>[3]</sup>. Dissolve 600 mg of *p*-dimethylaminobenzaldehyde in approximately 50 mL of anhydrous ethanol, gradually add 1 mL of sulfuric acid, and add anhydrous ethanol to make 100 mL (prepare at the time of use).
- (3) Sulfuric acid-anhydrous ethanol solution. Gradually add 1 mL of sulfuric acid to approximately 30 mL of anhydrous ethanol, and add anhydrous ethanol to make 100 mL (prepare at the time of use).

### 4 Quantification<sup>Note 1</sup>

Place 5 g of the analysis sample<sup>Note 2</sup> in a 200-mL stoppered Erlenmeyer flask, add 100 mL of anhydrous ethanol, stir with a magnetic stirrer for 10 minutes to extract narasin, immediately filter, and use the filtrate as the sample solution.

Dispense accurately 10 mL of the sample solution to each of 50 mL test tubes A, B and C<sup>[4]</sup>. Add accurately 5 mL of anhydrous ethanol to each of test tubes A and B and accurately 5 mL of the narasin standard solution to test tube C. Further, add accurately 5 mL of the sulfuric acid-anhydrous ethanol

solution to test tube A and accurately 5 mL of the *p*-dimethylaminobenzaldehyde solution to each of test tubes B and C<sup>Note 3</sup>, mix, and develop by heating in a thermostat at 70±1°C for 20 minutes<sup>[5]</sup>. After allowing to cool<sup>[6]</sup>, determine absorbances *a*, *b* and *c* of solutions A, B and C, using anhydrous ethanol as the blank, at a wavelength of about 600 nm<sup>[7]</sup>. Separately, proceed in the same manner with an unspiked control sample (with the same composition as the analysis sample except for the absence of narasin), and determine absorbances *a'*, *b'* and *c'*<sup>Note 4</sup>.

Calculate the content of narasin in the sample according to the following equation.

$$\text{Content of narasin in the sample (g (potency)/t)} = \frac{b-a}{c-b} \times 80 - \frac{b'-a'}{c'-b'} \times 80$$

Note 1. Make sure to avoid direct sunlight and water contamination during the process of quantification.

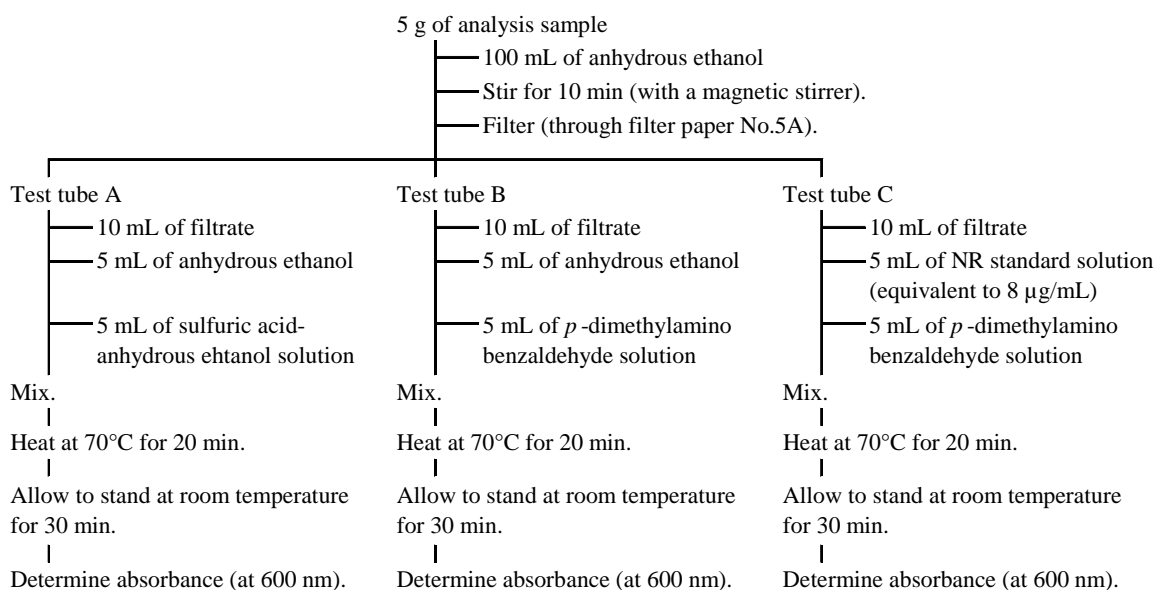
2. Pulverize and mix the whole amount of the collected sample before being used as the analysis sample.
3. The sulfuric acid-ethanol solution and *p*-dimethylaminobenzaldehyde solution shall be added last, and the subsequent procedure performed promptly.
4. For the blank value of the unspiked control sample<sup>[8]</sup>, a previously measured one can be used as long as the analysis sample has the same composition as the control sample.

A flow injector is applicable to the quantification procedure<sup>[9]</sup>.

## «Summary of analysis method»

This method is intended to determine the amount of narasin in a feed by extracting the sample with ethanol, adding sulfuric acid- *p*-dimethylaminobenzaldehyde (chromogenic agent), developing by heating in a water bath at 70°C for 20 minutes, and measuring absorbance at the wavelength of about 600 nm.

The flow sheet of this method is shown in Figure 9.2.18-5.



**Figure 9.2.18-5 Rapid quantitative method for narasin (chicken feed)**

References: Tetsuo Chihara, Haruko Suefuji, Yukie Ishida: Livestock research, 57, 373 (2003)



## «Notes and precautions»

[1] The anhydrous ethanol shall be of a guaranteed grade. If the purity of the ethanol is low, the contained water can interfere with the chromogenic reaction with *p*-dimethylaminobenzaldehyde and thus affect the quantified results.

By the same token, make sure to avoid water contamination during the process of quantification.

[2] The standard stock solution shall be stored in a cool, dark place and used within 2 weeks after preparation. Alternatively, the standard stock solution can be used for about 6 months as long as it has been prepared at a high concentration about 400 µg (potency)/mL and stored in 2-mL portions in hermetic containers at a temperature not more than -20°C. At the time of use, accurately dilute this solution with anhydrous ethanol to prepare a standard stock solution at a specified concentration.

[3] The principle of this chromogenic reaction depends on so-called Komarowsky reaction, which involves aldol condensation between the alcoholic hydroxyl group of narasin and the benzaldehyde group of *p*-dimethylaminobenzaldehyde under acidic conditions with sulfuric acid, producing a narasin derivative having a maximum absorption at about 600 nm.

[4] The use of stoppered test tubes is intended to prevent ethanol volatilization and moisture contamination in the subsequent process of heating in a water bath for development.

Care should be taken that the stopper can pop out when the tube is heated. It is recommended to use rubber bands etc. to hold the stopper in place.

[5] The chromogenic substrate develops a blue-purple color when reacted with narasin. It can develop a light yellow to light red color when reacted with the blank for the unspiked control sample.

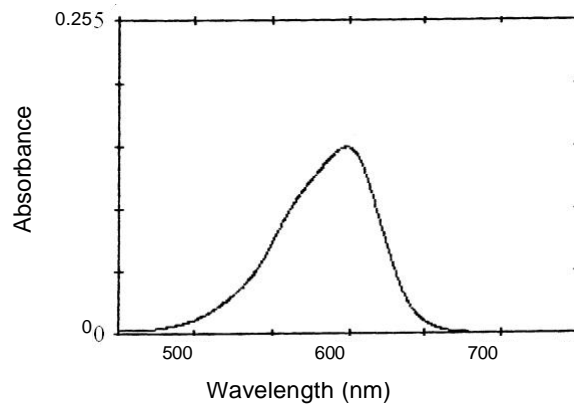
The absorbance of narasin was stabilized when developed at 70°C for 15 minutes or longer.

[6] The absorbance of narasin was stabilized when allowed to cool at room temperature for 25 minutes or longer. It is permissible to cool to room temperature in a water bath etc. before determining the absorbance.

[7] The absorption spectrum of the chromogenic substrate is shown in Figure 9.2.18-6. The wavelength of maximum absorption is 598.5 nm.

[8] In the spike recovery study, the average blank value for the narasin-unspiked control sample was found to be equivalent to about 4.2 to 4.8 g (potency)/t as narasin. It should be noted, however, that higher values can be observed for some types of raw materials for feed (milo, fish meal, alfalfa, etc.).

[9] For the summary of the flow injection analysis, refer to «Notes and precautions» [9] in 13. Salinomycin sodium 6.1 of this Section.



**Figure 9.2.18-6 Absorption spectrum of a narasin derivative**