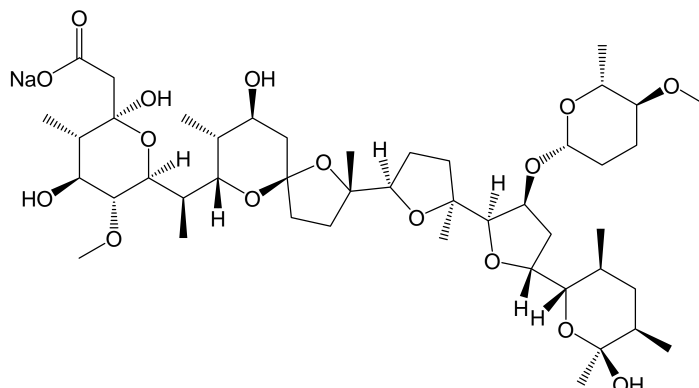
15 Semduramicin sodium



Semduramicin sodium	$C_{45}H_{75}O_{16}Na$	MW: 895.1	CAS No.: 119068-77-8
Semduramicin	$C_{45}H_{76}O_{16}$	MW: 873.1	CAS No.: 113378-31-7

[Summary of semduramicin sodium]

Semduramicin is a polyether antibiotic obtained by the incubation of *Actinomadura roseorufa*, and has the chemical structure shown above. The one used as a feed additive is its sodium salt (SD).

For physicochemical properties, SD technical occurs as white to gray-white crystalline powder. It is soluble in methanol, sparingly soluble in ethanol, slightly soluble in dichloromethane and in ether, and practically insoluble in water and in isooctane.

Formulations containing not less than 0.5 % of semduramicin are designated as deleterious substances under the Cabinet Order for the Designation of the Poisonous and Deleterious Substances (Cabnet Order No.2, 1965). For the handling of these substances, make sure to comform to the procedures specified in the Poisonous and Deleterious Substances Act (Act No.303, 1950).

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

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

SD is a pure-grade antibiotic that was designated as a feed additive as of July 18, 1994. The specifications for feeds containing this ingredient are specified in Appended Table 1, 1-(1)-C of the Ministerial Ordinance Concerning the Ingredient Specifications for Feeds and Feed Additives.

(in g(potency)/t)

Feed of interest	For chickens (except for broilers)	For broilers
	Starting chicks Growing chicks	Starting broilers Growing broilers
Added amount	25	25

Like SL, NR, MN and LS, excessive consumption of SD can cause growth disturbace in chickens. It is therefore necessary to strictly conform to the specified amounts of addition (25 g (potency) per 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 chicken feeds that contain SD according to the separately described control test method (6 Chiku B No.1012, notified by the Head of the Livestock Industry Bureau, the Ministry of Agriculture, Forestry and Fisheries, as of July 18, 1994).

[Methods listed in the Feed Analysis Standards]

1 Quantitative test method - Plate method

1.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 15.1.1]

Scope of application: Premix not containing TP

A. Reagent preparation

1) Dilution solvent: A mixture of water and methanol (7:3)
2) Semduramicin standard solution. Dry a suitable amount of semduramicin working standard[1] under reduced pressure (not exceeding 0.67 kPa) at 100°C for 3 hours, weigh accurately not less than 40 mg, accurately add methanol and dissolve to prepare a semduramicin 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 2.5 and 1.25 µg (potency)/mL, respectively[3].
3) Culture medium: Medium F-15
4) Bacterial suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.1 mL of a spore suspension with a concentration of 1×10^9 spores/mL per 100 mL of the culture medium 100 mL.
5) Agar plate. Proceed by the agar well method.
6) Extracting solvent: A mixture of dichloromethane[4] and 2,2,4-trimethylpentane (1:1)

B. Preparation of sample solution

Extraction. 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). Dilute a quantity of the filtrate 2- to 10-fold with the extracting solvent[5], and use as the sample solution subject to column treatment.

Column treatment. Wash a silica gel minicolumn (690 mg) with 5 mL of methanol and with 5 mL of dichloromethane 5 mL in this order.

Accurately transfer a quantity of the sample solution (equivalent to 0.1 to 0.5 mg (potency) as SD) to the minicolumn and allow to flow down until the amount in the minicolumn reservoir reaches 1 mL. Add accurately 20 mL of a mixture of dichloromethane[4] and acetone (9:1) and accurately 8 mL of a mixture of dichloromethane[4] and acetone (4:1)[6] to the minicolumn, and allow to flow out in this order to wash the minicolumn.

Place a 100-mL round-bottom flask under the minicolumn, and add 10 mL of a mixture of acetone and methanol (4:1) to the minicolumn to elute SD. Evaporate the eluate into dryness in a water bath at 50°C, and accurately add a quantity of the dilution solvent to dissolve the residue. Accurately dilute a

quantity of of this liquid with the dilution solvent to prepare high- and low-concentration sample solutions with concentrations of 2.5 and 1.25 µg (potency)/mL, respectively.

C. Quantification[7]

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

«Summary of analysis method»

This method is intended to determine the amount of SD in a premix by microbiological assay (2-2 dose method) using a sample solution prepared by extracting with a mixture of dichloromethane and 2,2,4-trimethylpentane (1:1) and purifying through a silica gel minicolumn. The ratio of the concentration of the high-concentration standard solution to that of the low-concentration standard solution is set to 2 because the slope of the standard response line is relatively steep.

Except for TP, none of the antibacterial substances approved for combined use with SD interfere with the quantification of SD.

The flow sheet of this method is shown in Figure 9.2.15-1.

Sample (3.0-5.0 g)
|
Extract with 100 mL of dichloromethane-2,2,4-trimethylpentane (1:1).
(magnetic sitter, 20 min)
|
Filter (filter paper: No.5A).
|
Load a quantity of the filtrate onto a silica gel minicolumn (previously washed with 5 mL of methanol and 5 mL of dichloromethane).
|
Wash the minicolumn with 20 mL of dichloromethane-acetone (9:1).
|
Wash the minicolumn with 8 mL of dichloromethane-acetone (4:1).
|
Elute SD with 10 mL of acetone-methanol (4:1) (into a 200-mL round-bottom
|
Evaporate into dryness under reduced pressure (in a water bath at 5°C).
|
Dissolve the residue with 20-25 mL of water-methanol (7:3).
|
Dilute a quantity of the solution with water-methanol (7:3) to prepare high- and low-concentration sample solutions (2.5 and 1.25 µg(potency)/mL,
|
Dispense to agar plates (allow to stand at 10-20°C).
|
Incubate (35-37°C for 16-24 hr).
|
Measure the inhibition zone diameter.
|
Calculate the potency by the 2-2 dose method.

Figure 9.2.15-1 Quantitative test methods for semduramicin sodium (premix)

References: Shoichi Yamatani: Research Report of Animal Feed, 21, 113 (1996)

History in the Feed Analysis Standards [18] 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	100.7~103.7	7.0
Chicken premix 2	0.4~10	3	98.0~100.3	1.8
Chicken premix 3	0.4~10	3	98.3~101.0	1.7

・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	99.2	2.1	3.6

«Notes and precautions»

[1] For the difinition etc. of semduramicin 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)

If the labeled potency of the working standard is 951 µg (potency)/mg, 50 mg of the working standard contains 47,550 µg (potency) (i.e., 50 mg × 951 µg (potency)/mg. To prepare a standard stock solution with a concentration of 1,000 µg (potency)/m, the required amount of solvent is thus calculated to be 47.55 mL (i.e., 47,550 µg (potency) / 1,000 µg (potency)/mL. Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 47.55 mL of methanol and dissolve to prepare a standard stock solution with a concentration of 1,000 µg (potency)/mL.

[3] For 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 semduramicin standard solution is shown in Table 9.2.15-1.

Table 9.2.15-1 Method of preparation for semduramicin standard solution (premix, example)

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	5	5
Amount (mL) of water-methanol (7:3)	18	18	15	5
Concentration (µg(potency)/mL)	100	10	2.5	1.25

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

[4] The dichloromethane shall be of JIS Guaranteed grade. Although there are some dichloromethane formulations spiked with about 0.3% methanol as a stabilizing agent for testing residual pesticides and PCB, the use of such grades affect the retention of the column and results in a poor recovery.

[5] Examples of the dilution factor for the filtrate, the amount loaded onto the column , etc. are shown in Table 9.2.15-2.

[6] The amount of the mixture of dichloromethane and acetone (4:1) shall be precisely 8 mL. The larger the amount, the lower the recovery.

Table 9.2.15-2 Dilution factor for the filtrate, amount loaded onto the column, etc.

Amount of sample collected (equivalent to mg(potency))	Dilution factor for filtrate (-fold)	Amount loaded onto column (mL)	Amount of dissolved residue (mL)	Concentration of solution (μg(potency)/mL)
2	2	10	20	5
10	4	10	25	10
50	10	10	25	20

Note: the concentration of the residue in the solution prepared by diluting the residue with dilution solvent in a flask. Dilute this solution further with dilution solvent to prepare high and low-concentration sample solutions with concentrations of 2.5 and 1.25 μg(potency)/mL, respectively.

[7] An example standard response line for SD is shown inFigure 9.2.15-2.

The ratio of the concentration of the high-concentration standard solution to that of the low-concentration standard solution is set to 2 because the slope of the standard resonse line is relatively steep.

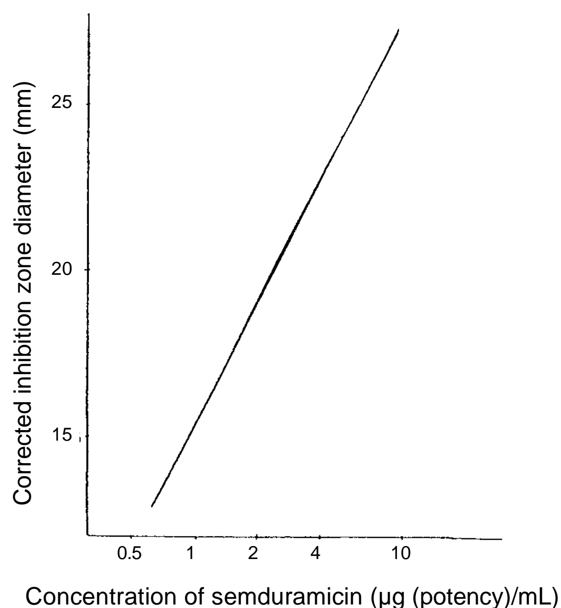


Figure 9.2.15-2 Standard response line for semduramicin (premix, example)
(*Bacillis subtilis* ATCC 6633, Medium F-15, Agar well method)

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

1.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 15.2.1]

Scope of application : Feed not containing TP

A. Reagent preparation

1) Dilution solvent: A mixture of water and methanol (7:3)

2) Semduramicin standard solution. Dry a suitable amount of semduramicin working standard under reduced pressure (not exceeding 0.67 kPa) at 100°C for 3 hours, weigh accurately not less than 40 mg, accurately add methanol and dissolve to prepare a semduramicin 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 dilution solvent to prepare high- and low-concentration standard solutions with concentrations of 2.5 and 1.25 µg (potency)/mL, respectively[1].

3) Culture medium: Medium F-15

4) Bacterial 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 1×10^9 spores/mL per 100 mL of the culture medium.

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

B. Preparation of sample solution

Extraction. Weigh accurately a quantity of the analysis sample (equivalent to 0.5 mg (potency) as SD)[2], place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of a mixture of dichloromethane[3] and 2,2,4-trimethylpentane (1:1), and extract with stirring for 20 minutes. Transfer the extract to a 50-mL stoppered centrifuge tube, centrifuge at 650×*g* for 15 minutes, and use as the sample solution subject to column treatment.

Column treatment. Wash a silica gel minicolumn (690 mg) with 5 mL of methanol and 5 mL of dichloromethane in this order.

Transfer accurately 25 mL of the sample solution to the minicolumn, and allow to flow down until the amount remaining in the reservoir reaches 1 mL. Accuraetly add 20 mL of a mixture of dichloromethane[3] and acetone (9:1) and 8 mL of a mixture of dichloromethane and acetone (4+1)[4] to the minicolumn, and allow to flow out in this order to wash the minicolumn.

Place a 100-mL round-bottom flask under the minicolumn, add 10 mL of a mixture of acetone and methanol (4:1) to the minicolumn to elute SD. Evaporate the eluate into dryness in a water bath at 50°C, and accurately add 25 mL of the dilution solvent to dissolve the residue. Accurately dilute a quantity of this liquid with the dilution solvent to prepare high- and low-concentration sample solutions with concentrations of 2.5 and 1.25 µg (potency)/mL, respectively.

C. Quantification[5]

Proceed by the 2-2 dose method[6].

«Summary of analysis method»

This method is intended to determine the amount of SD in a feed by microbiological assay using a sample solution prepared by extracting with a mixture of dichloromethane and 2,2,4-trimethylpentane (1:1) and purifying through a silica gel minicolumn. The ratio of the concentration of the high-concentration standard solution to that of the low-concentration standard solution is set to 2 as the slope

of the standard response line is relatively steep. This method is almost the same as the quantitative test method for SD in a premix.

Except for TP, none of the antibacterial substances approved for combined use with SD interfere with the quantification of SD.

The flow sheet of this method is shown in Figure 9.2.15-3.

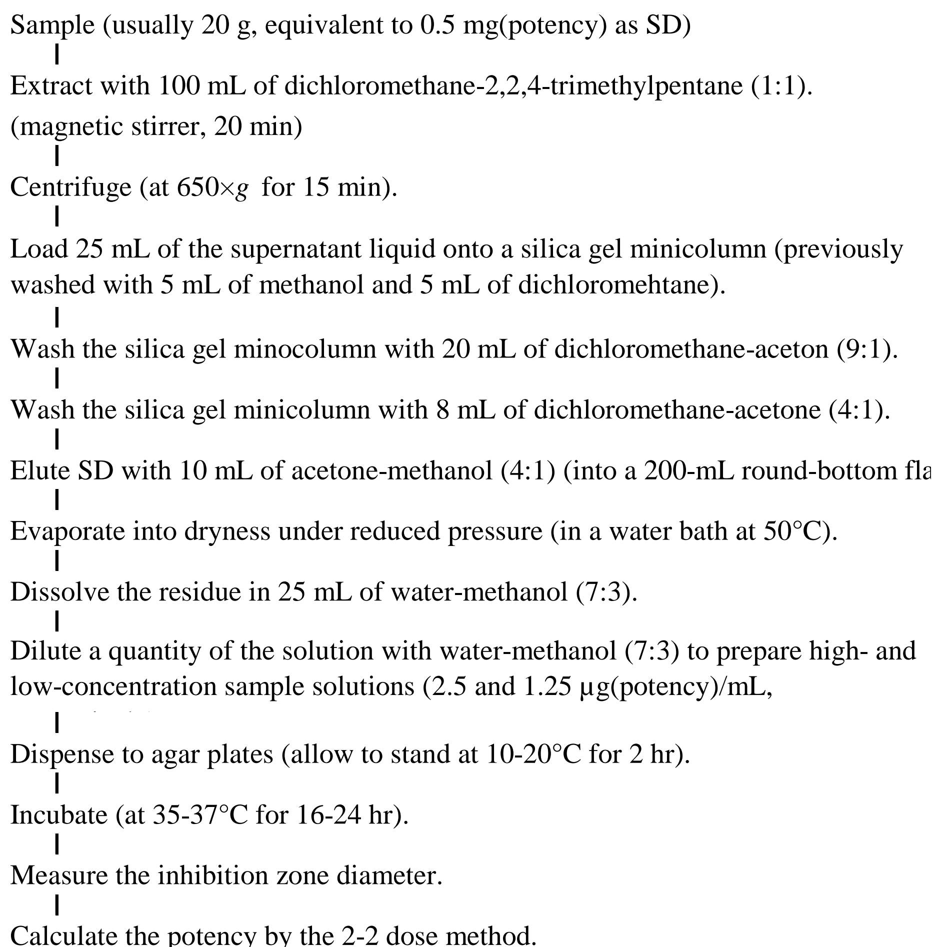


Figure 9.2.15-3 Quantitative test methods for semduramicin sodium (feed not containing TP)

References: Shoichi Yamatani: Research Report of Animal Feed, 19, 155 (1994)

History in the Feed Analysis Standards [18] 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	25~50	3	99.3~107.0	11.0
Starting broiler formula feed	25~50	3	97.7~105.0	3.4
Growing broiler formula feed	25~50	3	98.3~99.7	8.5

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)
Growing broiler formula feed	7	25	100.3	3.8	5.2

«Notes and precautions»

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

An example method of preparation for the semduramicin standard solution is shown in Table 9.2.15-3.

Table 9.2.15-3 Method of preparation for semduramicin standard solution (feed that does not contain TP, example)

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	5	5
Amount (mL) of water-methanol (7:3)	18	18	15	5
Concentration (μg(potency)/mL)	100	10	2.5	1.25

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

[2] Usually corresponds to 20 g.

[3] The dichloromethane shall be of JIS Guaranteed grade. Although there are some dichloromethane formulations spiked with about 0.3% methanol as a stabilizing agent for testing residual pesticides and PCB, the use of such grades affect the retention of the column and results in a poor recovery.

[4] The amount of the mixture of dichloromethane and acetone (4:1) shall be accurately 8 mL. The larger the amount, the lower the recovery.

[5] An example standard response line for SD is shown in Figure 9.2.15-4.

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

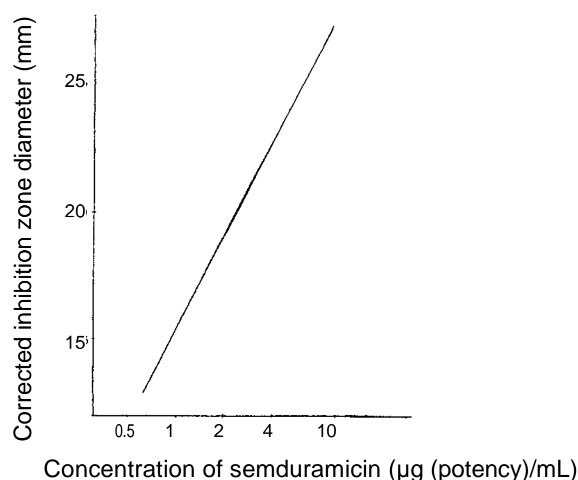


Figure 9.2.15-4 Standard response line for semduramicin (feed that does not contain TP, example)

(*Bacillis subtilis* ATCC 6633, Medium F-15, Agar well method)

2 Quantitative test method for polyether antibiotics by liquid chromatography

2.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 15.1.2]

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[1] 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)[1], 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)[1], 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)[1], 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)Note 1 [2]

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

Reaction solutionNote 2: 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[3] to prepare the calibration curve, and estimate the amount of each antibioticNote 3, 4.

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.

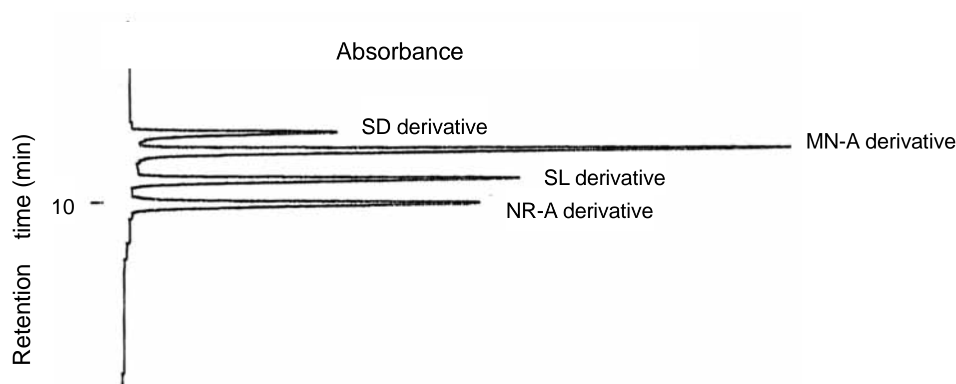


Figure 9.3.1-1 Chromatogram for a mixed standard solution

(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.

2.0-5.0 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).

|

Dilute a quantity of the filtrate with methanol-water (9:1).

|

Filter through membrane filter (pore size not exceeding 0.5 μm).

|

LC-UV (520 nm)

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

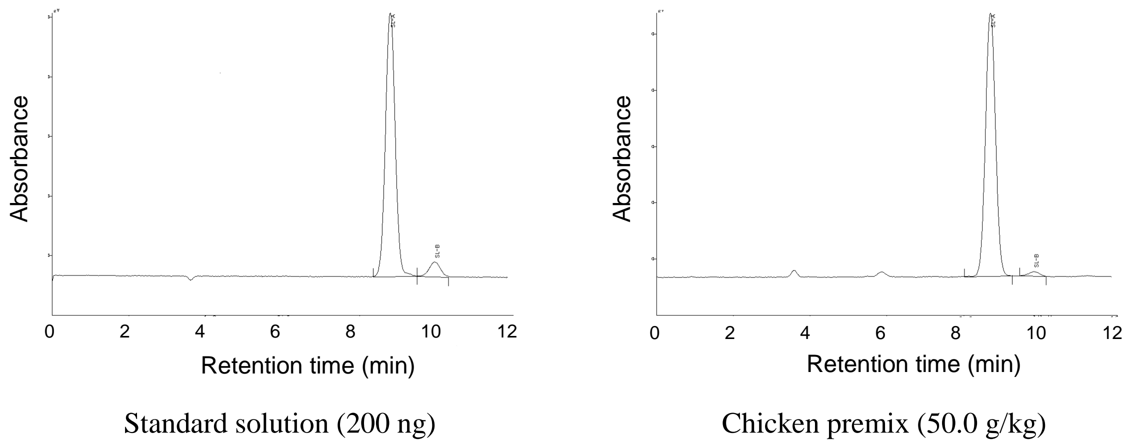
Spiked component	Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Salinomycin sodium	Starting chick grower premix	12.5~85.0	3	99.3~102.1	3.8
	Broiler fattener finisher premix	12.5~85.0	3	96.3~102.7	3.0
	Meat cattle fattener premix	12.5~85.0	3	98.0~100.8	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.



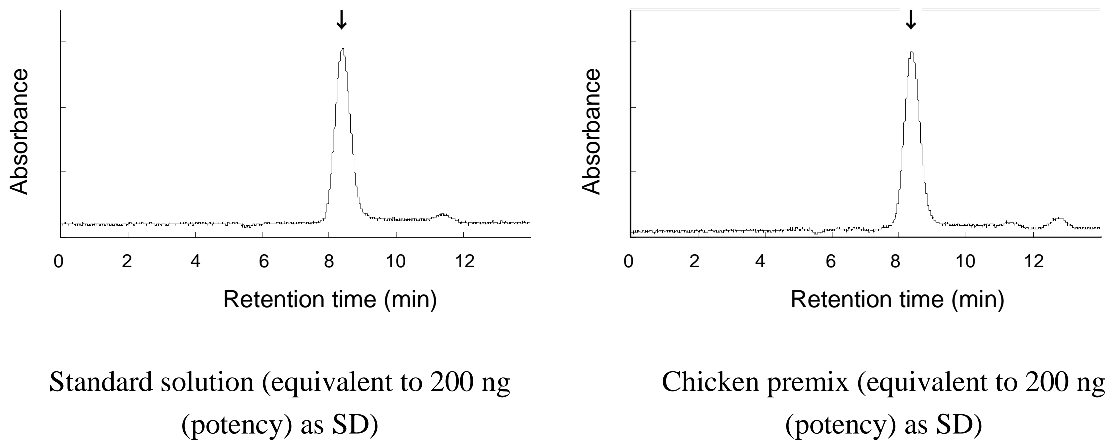
Standard solution (200 ng)

Chicken premix (50.0 g/kg)

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.



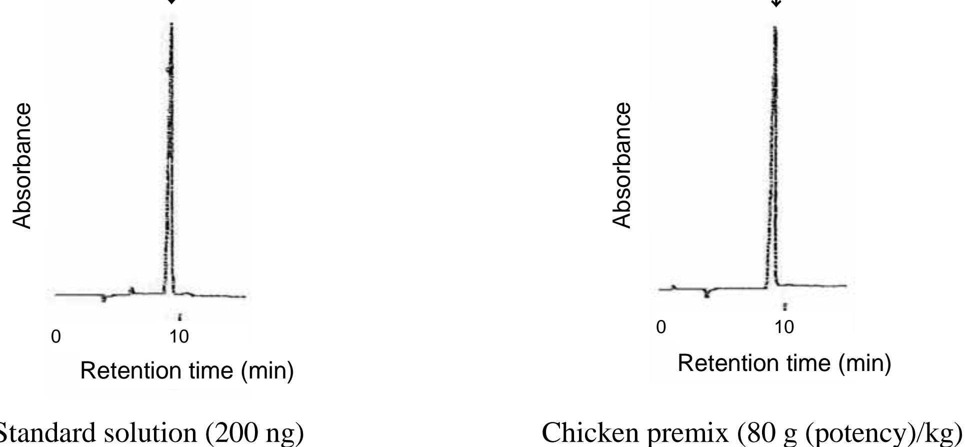
Standard solution (equivalent to 200 ng (potency) as SD)

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.

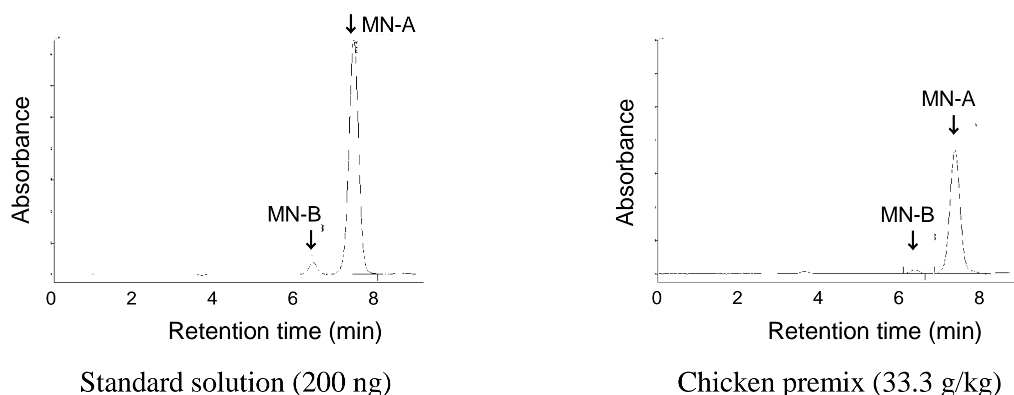


Standard solution (200 ng) Chicken premix (80 g (potency)/kg)

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, 15.2.2]

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[1]. 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[2].

Calculation. Proceed as directed in 1.1-B Calculation[3].

«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 Sectoin. 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.

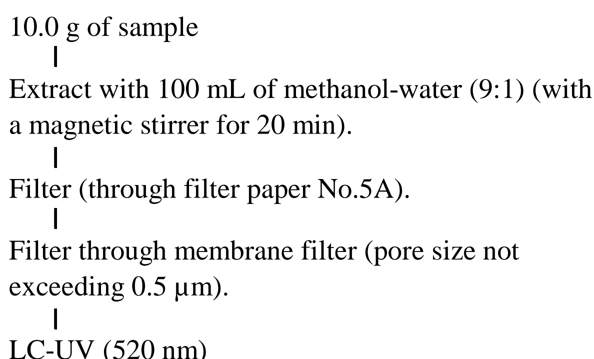


Figure 9.3.1-7 Quantitation test method for salinomycin sodium, semduramicin sodium, narasin and monensin sodium by liquid chromatography (feed)

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

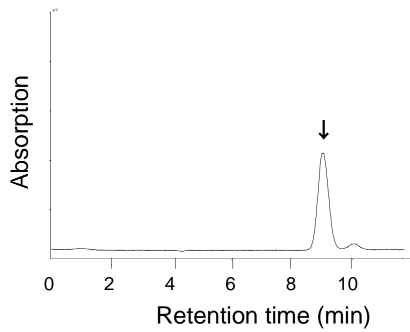
Spiked component	Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)	HorRat
Salinomycin sodium	Chicken formula feed	7	50	94.4	2.7	2.0	0.22
Semduramicin sodium	Broiler finisher formula feed	7	25	97.9	1.8	1.8	0.18
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»

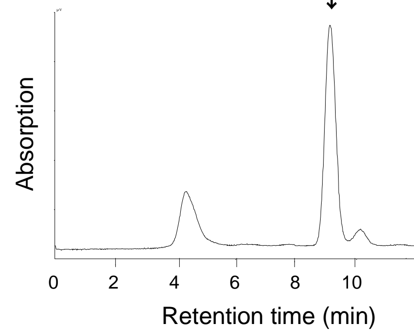
[1] As low concentrations of the standard solution are likely to change over time, make sure to prepare immediately before analysis. The peak that appears at a retention time approximately 1.5 times greater than the main peak is that of hydroxyl semduramicin, a degraded substance of the standard substance.

[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)

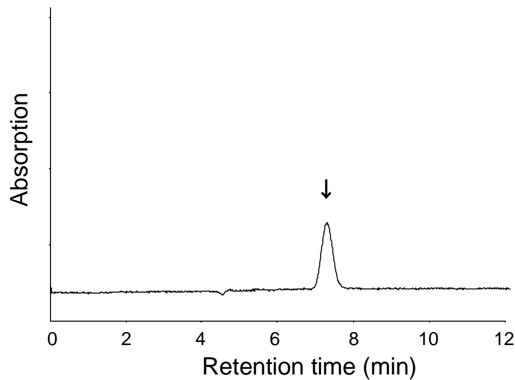


Starting chick grower formula feed (50 g (potency)/t)

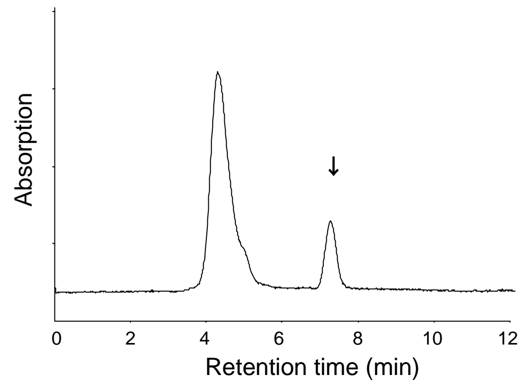
Figure 9.3.1-8 Chromatograms for salinomycin

(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)



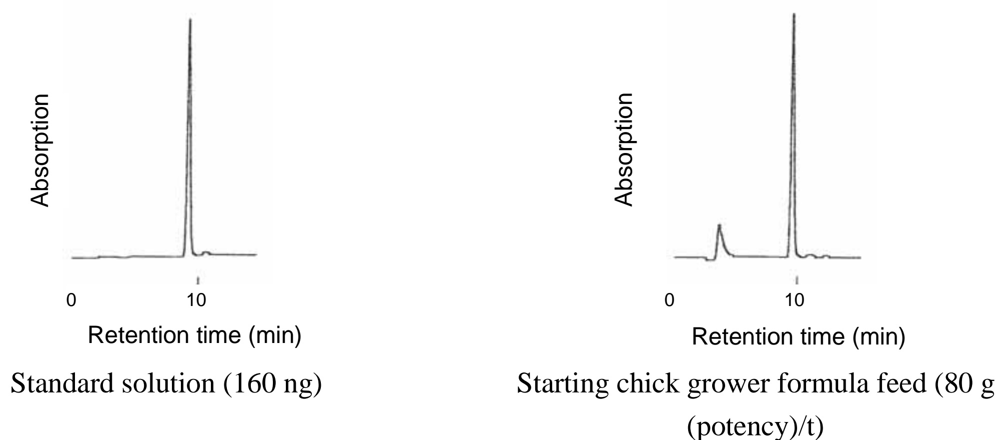
Chicken formula feed (25 g (potency)/t)

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.

↓ ↓

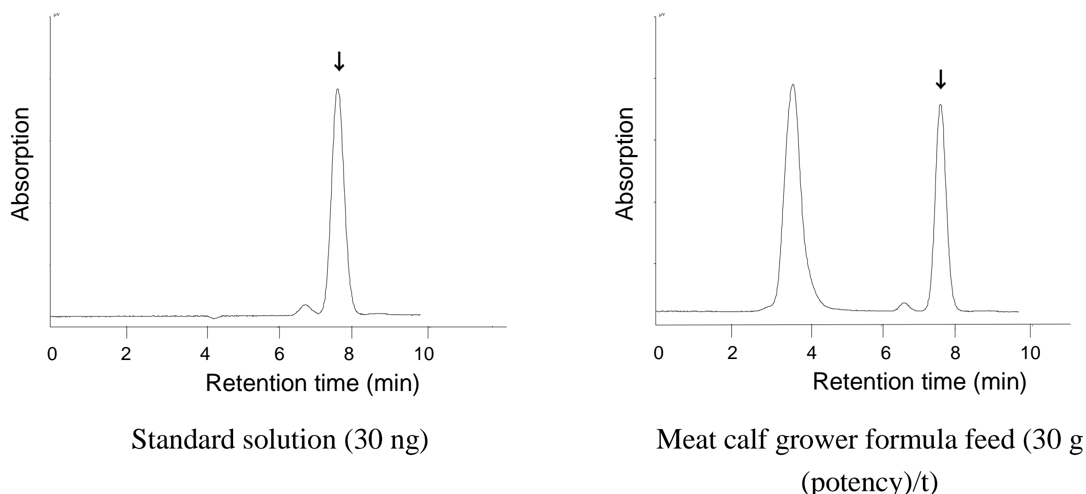


Standard solution (160 ng)   Starting chick grower formula feed (80 g (potency)/t)

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.



Standard solution (30 ng)   Meat calf grower formula feed (30 g (potency)/t)

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 spectrometry (Feed)

[Feed Analysis Standards, Chapter 9, Section 2, 15.3.1]

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

Scope of application: Formula feeds

A. Reagent preparation

1) Standard stock solution of each antibiotic[1]. Weigh accurately a quantity equivalent to 20 mg (potency) each of salinomycin working standard[Note 1], 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)[2].

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)[Note 2]

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[Note3]

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

Nebulizer gas: N_2 (1.5 L/min)

CDL temperature: 250°C

Heat block temperature: 200°C

Monitored ions[3]: *m/z* 769 (salinomycin)

m/z 891 (semduramicin)

m/z 783 (narasin 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[4] to prepare a calibration curve, and estimate the amount of each antibiotic in the sample solution[Note 4].

Note 1. Prepared by drying a suitable amount under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours

2. Gemini 5μ C18 110A (Phenomenex; the retention times of salinomycin, semduramicin, narasin 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 narasin, the calculated amount of narasin A shall be regarded as the amount of narasin. 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.

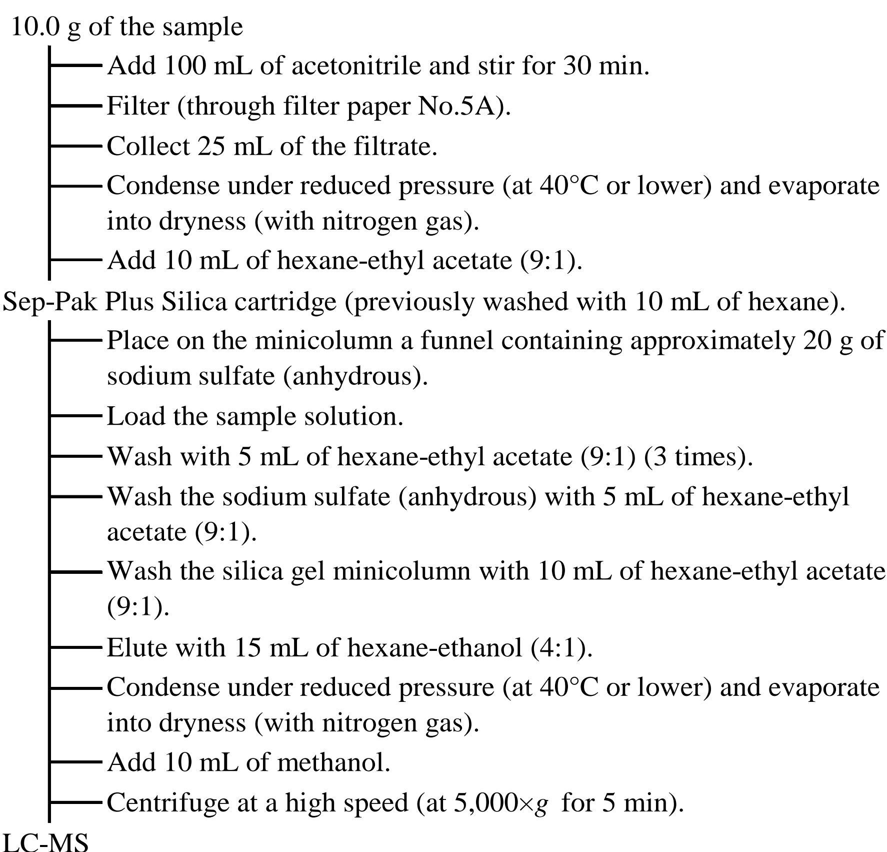


Figure 9.3.4-1 Method of collective trace quantitation for polyether antibiotics by liquid chromatography-mass spectrometry

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.

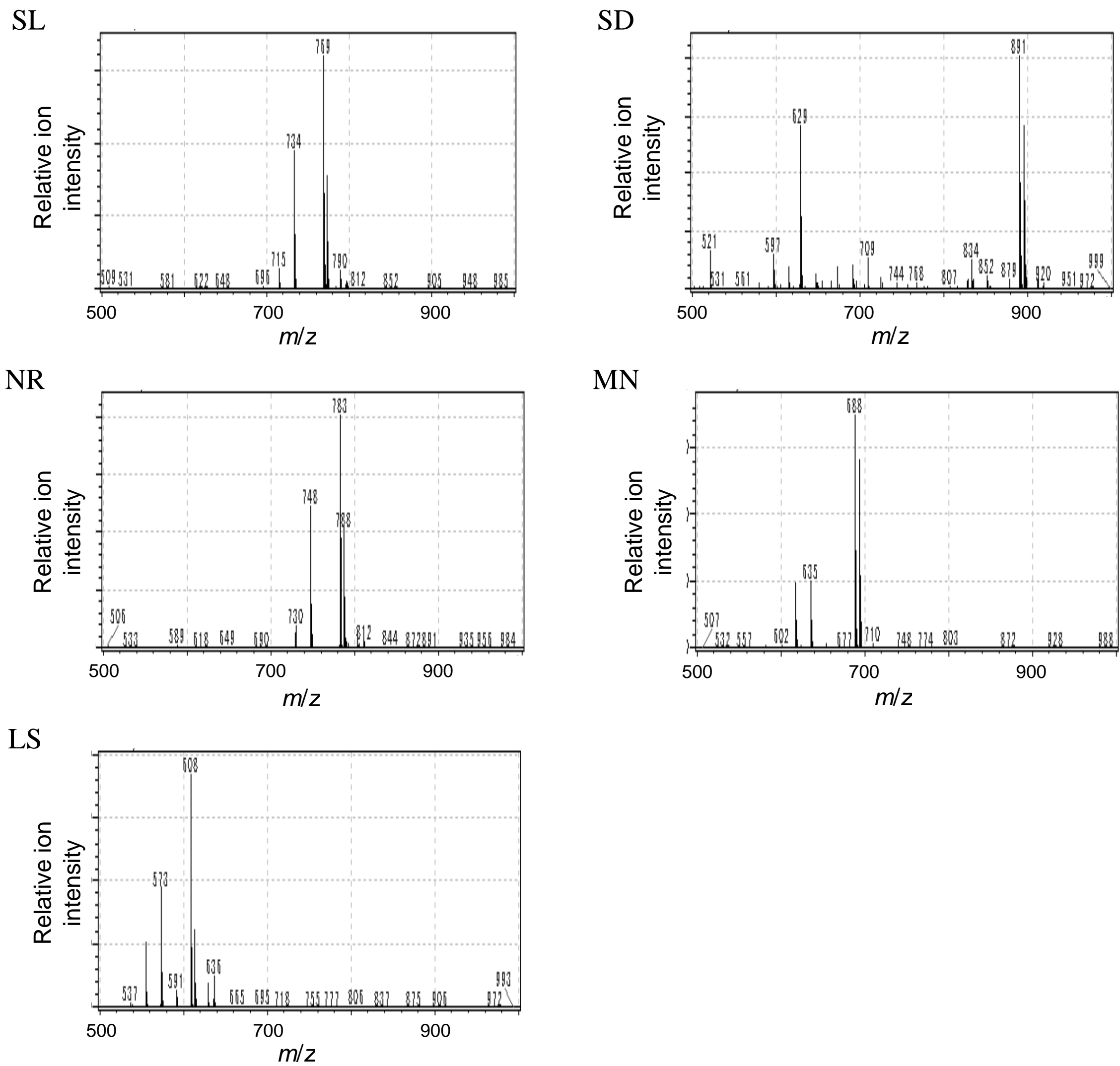
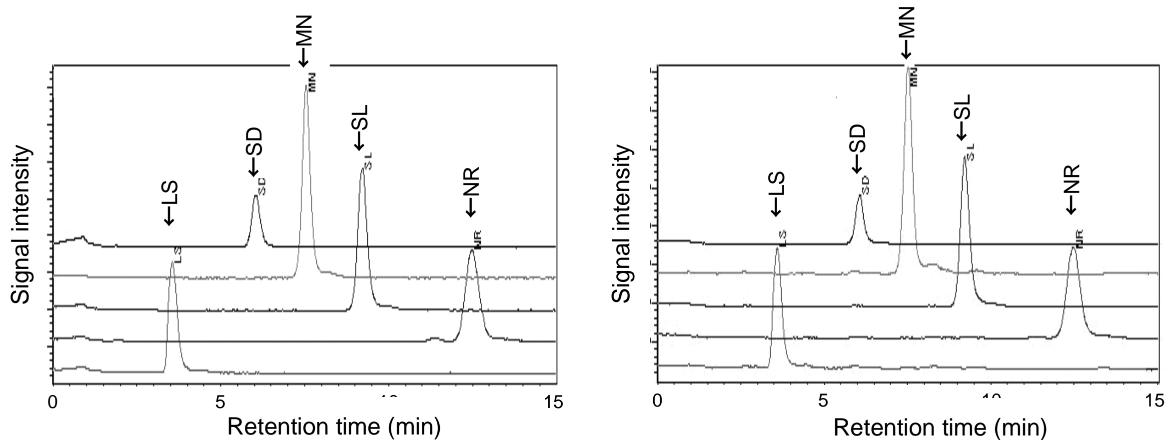


Figure 9.3.4-2 Mass spectrum for each antibiotic

[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 Identification test method - Microbioautography

4.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 15.4.1]

A. Reagent preparation

1) Semduramicin standard solution. Dry a suitable amount of semduramicin working standard under reduced pressure (not exceeding 0.67 kPa) at 100°C for 3 hours, weigh accurately not less than 40 mg, accurately add methanol and dissolve to prepare a semduramicin 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 a standard solution with a concentration of 10 µg (potency)/mL[1].

2) Culture medium: Medium F-15

3) Spore suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.2 mL of the spore suspension with a concentration of 1×10^9 spores/mL per 100 mL of the culture medium.

4) Extracting solvent: A mixture of dichloromethane and 2,2,4-trimethylpentane (1:1)

5) Developing solvent

   i) A mixture of ethyl acetate, hexane, acetone and methanol (20:8:1:1)

   ii) A mixture of acetonitrile, ethyl acetate and acetone (2:1:1)

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

B. Preparation of sample solution

Extraction. Weigh accurately 3 to 5 g of 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). Dilute a quantity of the filtrate 2- to 10-fold with extracting solvent, and use as the sample solution for column treatment.

Column treatment. Wash a silica gel minicolumn (690 mg) with 5 mL of methanol and 5 mL of dichloromethane in this order.

Accuraetly transfer a quantity of sample solution (equivalent to 0.1 to 0.5 mg (potency) as SD) to the minicolumn, allow to flow down until the amount in the reservoir of the minicolumn reaches 1 mL[2], add accurately 20 mL of a mixture of dichloromethane and acetone (9:1) and accurately 8 mL of a mixture of dichloromethane and acetone (4:1) to the minicolumn, and allow to flow out in this order to wash the minicolumn.

Place a 100-mL round-bottom flask under the minicolumn, and add 10 mL of a mixture of acetone and methanol (4:1) to the minicolumn to elute SD. Evaporate the eluate into dryness in a water bath at 50°C, and accurately add a quantity of methanol to dissolve the residue[3] to prepare a sample solution with a concentration of 10 µg (potency)/mL.

C. Identification

Proceed as directed in the Thin-layer chromatography, Preparation of agar plates, Incubation, and

Identification, described in Section 1, 2. Microbioautograph, C. Quantification [4].

Use a thin-layer plate made of silica gel[Note 1], spot 100 µL each of the standard solution and the sample solution, and develop until the front of the developing solvent reaches the top of the thin-layer plate.

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

«Summary of analysis method»

This method is intended to identify SD in a premix by microbioautography using a sample solution prepared by extracting with a mixture of dichloromethane and 2,2,4-trimethylpentane (1:1) and purifying though a silica gel minicolumn.

The flow sheet of this method is shown in Figure 9.2.15-5.

Sample (3.0-5.0 g)

|

Extract with 100 mL of dichloromethabe-2,2,4-trimethylpentane (1:1).
(magnetic stirrer, 20 min)

|

Filter (filter paper: No.5A).

|

Load a quantity of the filtrate onto a silica gel minicolumn (previously washed with 5 mL of methanol and 5 mL of dichloromethane).

|

Wash the silica gel minicolumn with 20 mL of dichloromethane-acetone (9:1).

|

Wash the silica gel minicolumn with 8 mL of dichloromethane-acetone (4:1).

|

Elute SD with 10 mL of acetone-methanol (4:1) (into a 100-mL round-bottom fla

|

Evaporate into dryness under reduced pressure (in a water bath at 50°C).

|

Dissolve the residue in a quantity of methanol to prepare a sample solution (10 µg(potency)/mL).

|

Spot the thin-layer plate (100 µL).

|

Develop.

|

Prepare agar plates (allow to stand at 10-20°C for 3 hr).

|

Incubate (at 35-37°C for 16-24 hr).

|

Determine the Rf value.

Figure 9.2.15-5 Identification test method for semduramicin sodium (premix)

References: Shoichi Yamatani: Research Report of Animal Feed, 21, 121 (1996)

History in the Feed Analysis Standards [18] New

«Notes and precautions»

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

An example method of preparation for the semduramicin standard solution is shown in Table 9.2.15-4.

Table 9.2.15-4 Method of preparation for semduramicin standard solution (identification test method, premix, example)

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

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

[2] If the flow is slow, it is permissible to use a syringe plunger or a double-balloon pump to inject under pressure.

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

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

4.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 15.5.1]

A. Reagent preparation

1) Ssemduramicin standard solution. Dry a suitable amount of semduramicin working standard under reduced pressure (not exceeding 0.67 kPa) at 100°C for 3 hours, weigh accurately not less than 40 mg, accurately add methanol and dissolve to prepare a semduramicin 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 a standard solution with a concentration of 10 μg (potency)/mL[1].

2) Culture medium: Medium F-15

3) Spore suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.2 mL of the spore suspension with a concentration of 1×10^9 spores/mL per 100 mL of the culture medium.

4) Extracting solvent: A mixture of dichloromethane and 2,2,4-trimethylpentane (1:1)

5) Developing solvent

i) A mixture of ethyl acetate, hexane, acetone and methanol (20:8:1:1)

ii) A mixture of acetonitrile, ethyl acetate and acetone (2:1:1)

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

B. Preparation of sample solution

Extraction. Weigh accurately a quantity of the analysis sample (equivalent to 0.5 mg (potency) of SD), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of a mixture of dichloromethane and 2,2,4-trimethylpentane (1:1), and extract with stirring for 20 minutes. Transfer 50 mL of the extract to a stoppered centrifuge tube, centrifuge at 650×*g* for 15 minutes, and use as the sample solution subject to column treatment.

Column treatment. Wash a silica gel minicolumn (690 mg) with 5 mL of methanol and 5 mL of dichloromethane in this order.

Transfer accurately 25 mL of the sample solution to the minicolumn, and allow to flow down until the amount in the reservoir of the minicolumn reaches 1 mL[2]. Add accurately 20 mL of a mixture of dichloromethane and acetone (9:1) and accureately 8 mL of a mixture of dichloromethane and acetone (4:1) to the minicolumn and elute in this order to wash the minicolumn.

Place a 100-mL round-bottom flask under the minicolumn, add 10 mL of a mixture of acetone and methanol (4:1) to the minicolumn to elute SD. Evaporate the eluate into dryness in a water bath at 50°C, and accurately add a quantity of methanol to dissolve the residue[3] to prepare a sample solution with a concentration of 10 µg (potency)/mL.

C. Identification

Proceed as directed in the Thin-layer chromatography, Preparation of agar plates, Incubation, and Identification, described in Section 1, 2. Microbioautograph, C. Quantification[4].

Use a thin-layer plate made of silica gel[Note 1]. Spot 100 µL each of the standard solution and the sample solution, and develop until the front of the developing solvent reaches the top of the thin-layer plate.

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

«Summary of analysis method»

This method is intended to identify SD in a feed by microbioautography using a sample solution prepared by extracting with a mixture of dichloromethane and 2,2,4-trimethylpentane (1:1) and purifying though a silica gel minicolumn.

The flow sheet of this method is shown in Figure 9.2.15-6.

Sample (usually 20.0 g, equivalent to 0.5 mg(potency) as SD).
|
Extract with 100 mL of dichloromethane-2,2,4-trimethylpentane.
(magnetic stirrer, 20 min).
|
Centrifuge (at 650×*g* for 15 min).
|
Load 25 mL of the supernatant liquid onto a silica gel minicolumn (previously washed with 5 mL of methanol and 5 mL of dichloromethane).
|
Wash the silica gel minicolumn with 20 mL of dichloromethane-acetone (9:1).
|
Wash the silica gel minicolumn with 8 mL of dichloromethane-acetone (4:1).
|
Elute SD with 10 mL of acetone-methanol (4:1) (into a 100-mL round bottom fla
|
Evaporate into dryness under reduced pressure (in a water bath at 50°C).
|
Dissolve the residue in a quantity of methanol to prepare a sample solution (10 μg(potency)/mL).
|
Spot on the thin-layer plate (100 μL).
|
Develop.
|
Prepare agar plates (allow to stand at 10-20°C for 3 hr).
|
Incubate (at 35-37°C for 16-24 hr).
|
Determine the Rf value.

Figure 9.2.15-6 Identification test method for semduramicin sodium (feed)

References: Shoichi Yamatani: Research Report of Animal Feed, 21, 121 (1996)

History in the Feed Analysis Standards [18] New

«Notes and precautions»

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

An example method of preparation for semduramicin standard solution is shown inTable 9.2.15-5.

Table 9.2.15-5 Method of preparation for semduramicin standard solution (example)

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

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

[2] If the flow is slow, it is permissible to use a syringe plunger or a double-balloon pump to inject under pressure.

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

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

5 Control analysis method - Rapid quantitative method for chicken feed

[6 Chiku B No.1021, notified by the Head of the Livestock Industry Bureau, Ministry of Agriculture, Forestry and Fisheries, as of July 18, 1994]

1 Instruments and equipments

(1) Stoppered Erlenmeyer flask

(2) One-mark flask

(3) Stoppered centrifuge tube

(4) Syringe

(5) Stoppered test tube

(6) Magnetic stirrer

(7) Centrufuge

(8) Water bath

(9) Spectrophotometer

(10) Chemical balance

(11) Ultrasonic cleaner

2 Reagents

(1) Dichloromethane (guaranteed grade)

(2) 2,2,4-trimethylpentane (guaranteed grade)

(3) Anhydrous ethanol (guaranteed grade)

(4) Methanol (guaranteed grade)

(5) Acetone (guaranteed grade)

(6) *p*-Dimethylaminobenzaldehyde (guaranteed grade)

(7) Semduramicin working standard

(8) Silica gel cartridge[Note 1]

(9) Nitrogen gas

3 Preparaton of reagents

(1) Semduramicin sodium standard solution. Place accurately 100 mg (potency) of semduramicin working standard to a 100-mL one-mark flask, add anhydrous ethanol[1] up to the marked line and dissolve to prepare a semduramicin sodium standard stock solution (1 mL of this liquid contains an amount equivalent to 1 mg (potency) as semduramicin sodium)[2].

At the time of use, accurately dilute this stock solution with anhydrous ethanol to prepare semduramicin sodium standard solutions that contain amounts equivalent to 5, 10 and 15 µg (potency)/mL of semduramicin sodium.

(2) *p*-Dimethylaminobenzaldehyde solution[3]. Dissolve 600 mg of *p*-dimethylaminobenzaldehyde in approximately 50 mL of anhydrous ethanol, gradually add 8 mL of sulfuric acid, and add anhydrous ethanol to make 100 mL. Prepare at the time of use.

4 Quantification

(1) Extraction

Weigh 20 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL

of a mixture of dichloromethane and 2,2,4-trimethylpentane (1:1), and extract with stirring for 30 minutes with a magnetic stirrer. Transfer the extract to a stoppered centrifuge tube, centrifuge at 3,000 rpm for 10 minutes, and use the supernatant liquid as the sample solution subject to cartridge column chromatography.

(2) Cartridge column chromatography

Connect the silica gel cartridge to a syringe, and wash with 10 mL of methanol and 10 mL of dichloromethane in this order[Note 2]. Measure accurately 20 mL of the sample solution and place in the syringe, and force to flow down until the amount goes down to 1 mL or less[Note 2]. Place 10 mL of dichloromethane and 20 mL of a mixture of dichloromethane and acetone (9:1) in the syringe, and force to flow down in this order to wash the cartridge[Note 2]. Place a stoppered test tube[4] under the silica gel cartridge, and elute semduramicin sodium with 10 mL of a mixture of acetone and methanol (4:1)[Note 2]. Evaporate the eluate into dryness in a water bath at 69 to 71°C by introducing nitrogen gas.

(3) Development

Add accurately 10 mL of anhydrous ethanol to the stoppered test tube, dissolve the residue with the aid of ultrasonic waves for approximately 1 minute, and use the solution as the sample solution. To this solution add accurately 5 mL of *p*-dimethylaminobenzaldehyde solution, mix, develop by heating in a water bath at 69 to 71°C for 40 minutes, and allow to cool rapidly in an ice water bath down to room temperature. Determine the absorbance at the wavelength of maximum absorption at about 535 nm, using anhydrous ethanol as the blank[5].

(4) Preparation of calibration curve

Take 20 g of an unspiked sample (with the same composition as the analysis sample except for the absence of semduramicin soium), and proceed as directed in (1) to prepare the unspiked sample solution subject to cartridge colum chromatography. Take 20-mL portions of the semduramicin sodium-free sample solution and proceed as directed in (2) using cartridges A, B and C. To each of the stoppered test tubes add accurately 10 mL each of the semduramicin sodium standard solutions with concentrations of 5, 10 and 15 µg (potency)/mL, respectively. Then, develop and determine the absorbance as directed in (3), and prepare a calibration curve [6].

(5) Calculation

Calculate the concentration of semduramicin sodium in the sample solution from the calibration curve, and estimate the amount of semduramicin sodium in the sample according to the following equation.

Amount (g (potency)/t) of semduramicin sodium in the sample $= A \times 2.5$

A : Concentration (µg (potency)/mL) of semduramicin sodium in the sample solution estimated from the calibration curve.

A flow injector[7] is applicable to the quantification procedure.

Note 1. Sep-Pak Plus Silica (Waters) or an equivalent.

2. Inject under pressure at a rate of 2 to 3 mL per minute [8].

«Summary of analysis method»

This method is intended to determine the amount of semduramicin sodium in a feed by extracting the analysis sample with a mixture of dichloromethane and 2,2,4-trimethylpentane (1:1), purifying through a silica gel minicolumn, adding *p*-dimethylaminobenzaldehyde (chromogen), developing by heating in a water bath at 70°C for 40 minutes, and determine the absorbance at the wavelength about 535 nm.

The flow sheet of this method is shown in Figure 9.2.15-7.

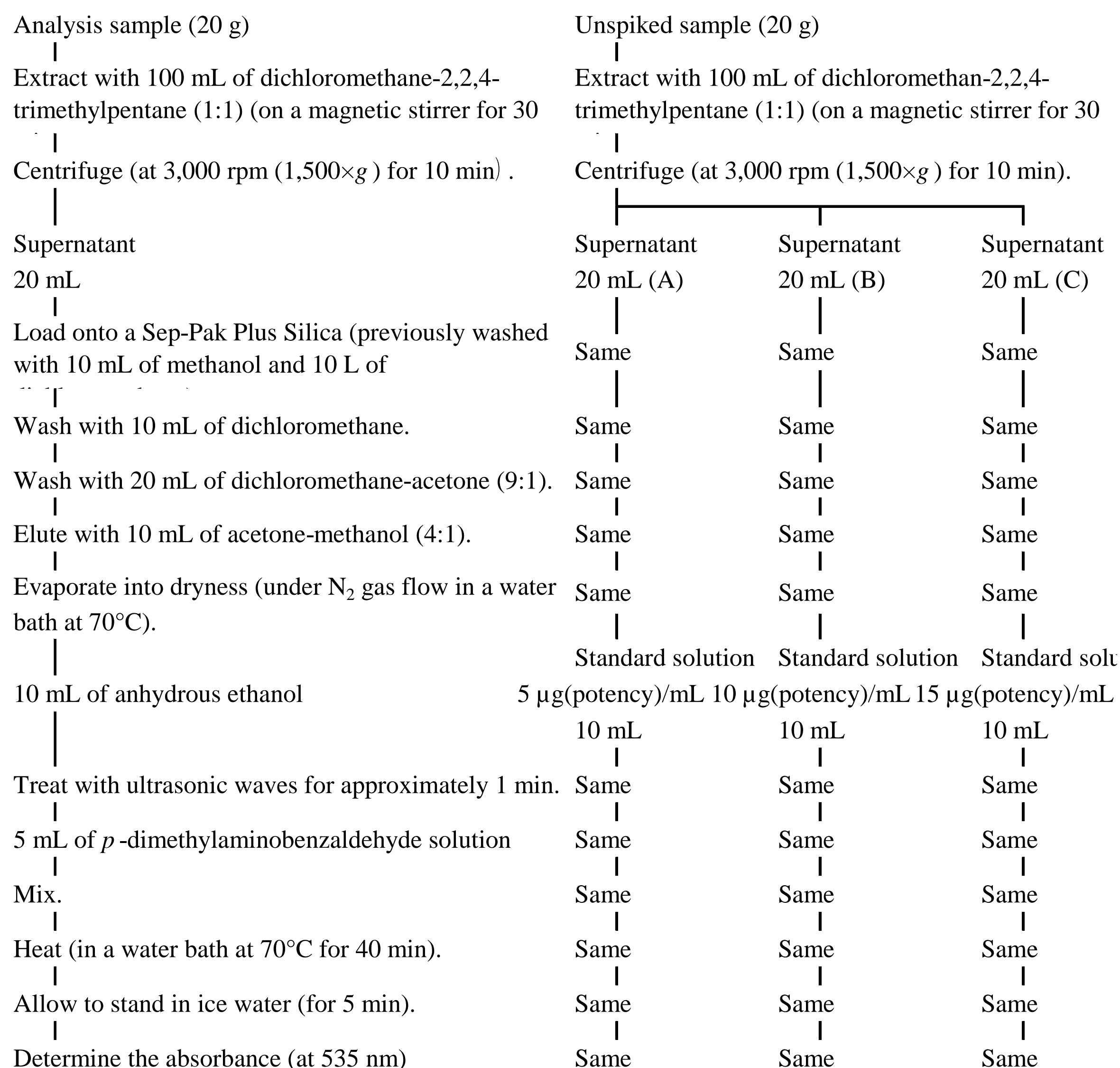


Figure 9.2.15-7 Rapid quantitative method for semduramicin sodium (chicken feed)

References: Noriyuki Koyama , Atsushi Kito: Research Report of Animal Feed, 20, 115 (1995)

Yamaguchi, Suzuki, Kawabata, Takebayashi, Tsuchihashi: Test of Feed, 540, 17 (2008)

«Notes and precautions»

[1] The anhydrous ethanol shall be of a guaranteed grade. If the purity of the ethanol is low, the residual 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 is stable for approximately 3 months when stored at 4°C.

[3] Usually, *p*-dimethylaminobenzaldehyde is used as an Ehrlich reagent and reacted with hydroxyl groups etc. in an acid-ethanol solution to develop color.

[4] The use of stoppeerd 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 advisable to use rubber bands etc. to hold the stopper in place.

[5] An example absorption spectrum for semduramicin sodium is shown in Figure 9.2.15-8.

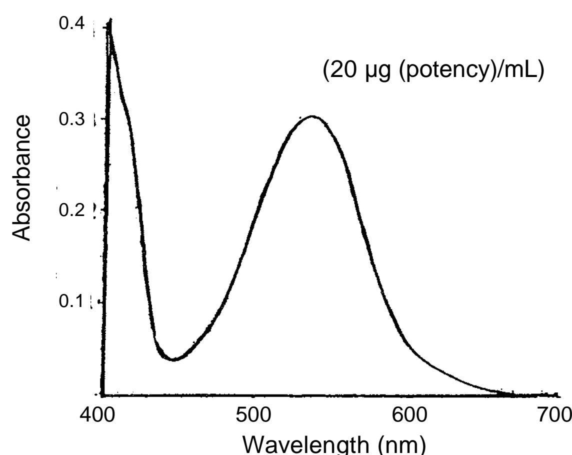


Figure 9.2.15-8 Absorption spectrum of semduramicin sodium standard solution

[6] Example calibration curves for semduramicin sodium are shown in Figure 9.2.15-9.

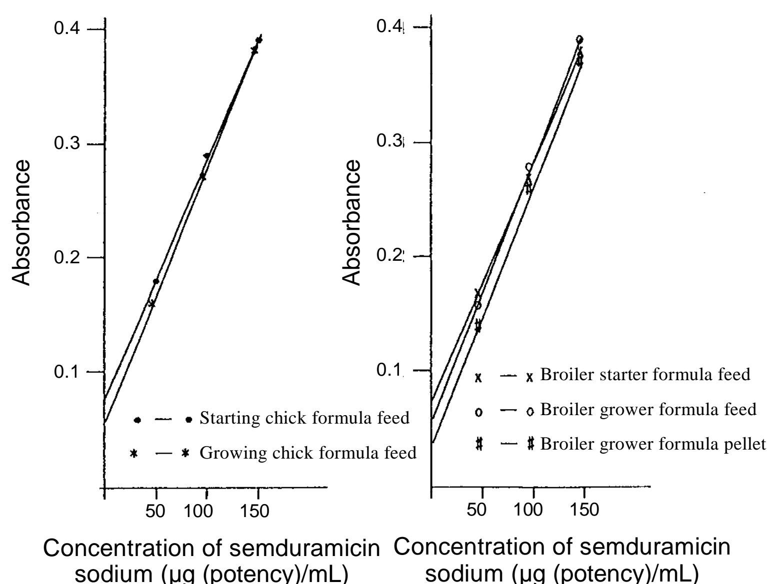


Figure 9.2.15-9 Calibration curves for semduramicin sodium obtained from 5 different kinds of feeds.

[7] For the summary of the Flow Injection Analysis, Refer to 13. Salinomycin sodium 6.1 «Notes and

precautions» [9] (p.****) of this Section.

[8] Use a syringe plunger or a double-balloon pump to inject under pressure.