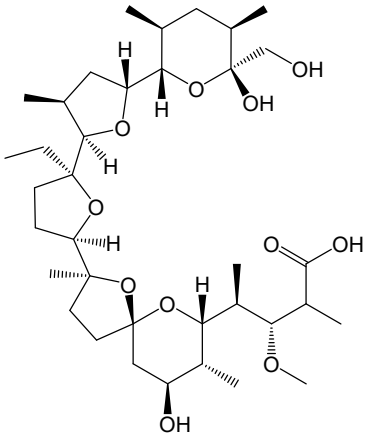
27 Monensin sodium



Monensin A

$C_{36}H_{62}O_{11}$ MW: 670.9 CAS No.: 17090-79-8 (monensin A), 22373-78-0 (monensin Na)

[Summary of monensin sodium]

Monensin is a polyether antibiotic obtained by the incubation of *Streptomyces cinnamonensis* and has the chemical structure shown above. The one used as a feed additive is its sodium salt (MN). Although MN is a mixture of MN-A, MN-B, MN-C, and MN-D, the one designated as a feed additive is labeled as "antibiotic containing MN-A as the main ingredient".

For physicochemical properties, MN technical occurs as a light brownish white to light orange-yellow powder or crystalline powder, and slightly has a characteristic odor. It is freely soluble in ethanol, in chloroform and in methanol, sparingly soluble in acetone, and practically insoluble in water. It is stable in a neutral or basic solution and unstable in an acid solution.

Formulations with monensin content exceeding 8% 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 handing of these substances, make sure to conform to the procedures specified in the Poisonous and Deleterious Substances Control Act (Act No.303, 1950).

MN has an antibacterial effect on part of the Gram-positive bacteria and a coccidial effect. It promotes growth of chickens (including broilers) and improves feed efficiency in fattening cattle.

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

MN is a pure-grade antibiotic that was designated as a feed additive as of September 5, 1978. 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	For cattle	
	Starting chicks Growing chicks	Starting period broilers Finishing period broilers	Calves	Fattening cattle
Added amount	80	80	30	30

The amount of MN added to a commercial premix is roughly 5 to 40 g (potency)/kg.

As excessive consumption of MN can cause growth disturbance in chickens and cattle, it is necessary to strictly conform to the added amount specified in the above table and achieve homogeneous mixture to secure the safety.

For this reason, feed manufacturers are required to control the feeds containing monensin sodium according to the separately specified control test methods (for chicken feed (53 Chiku B No.2173 and 53 SuiShin No.464, notified by the Head of the Livestock Industry Bureau and Head of the Fisheries Agency, the Ministry of Agriculture, Forestry and Fisheries, as of September 5, 1978) and for cattle feed (60 Chiku B No. 2928, notified by the Head of the Livestock Industry Bureau and Head of the Fisheries Agency, the Ministry of Agriculture, Forestry and Fisheries, as of October 15, 1985)).

[Methods listed in the Feed Analysis Standards]

1 Quantitative test method - Plate method

1.1 Premix [Feed Analysis Standards, Chapter 9, Section 2, 27.1.1]

A. Reagent preparation

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

2) Monensin standard solution. Weigh accurately not less than 40 mg of monensin working standard[1], accurately add methanol and dissolve to prepare a monensin 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 5 and 1.25 μg (potency)/mL, respectively[3].

3) Culture medium: Medium F-16[4]

4) 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 1×10^8 spores/mL per 100 mL of the culture medium.

5) Agar plate. Proceed by the cylinder plate method[5].

6) Extracting solvent: A mixture of methanol and water (9:1)

B. Preparation of sample solution

1) When the analysis sample does not contain OTC or CTC

Weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask,

add 100 mL of the extracting solvent, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with the dilution solvent to prepare high- and low-concentration sample solutions with concentrations of 5 and 1.25 μg (potency)/mL, respectively[6].

2) When the analysis sample contains OTC or CTC

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).

Load the filtrate onto a column (column tube (14 mm in internal diameter) dry-packed[7] with 12 g of basic alumina for column chromatography (pore size: 74 to 177 μm (200 to 80 mesh))), and discard the first 5 mL of the filtrate.

Accurately dilute a quantity of the subsequent filtrate with the dilution solvent to prepare high- and low-concentration sample solutions with concentrations of 5 and 1.25 μg (potency)/mL, respectively[6].

C. Quantification[8]

Proceed by the 2-2 dose method[9].

«Summary of analysis method»

This method is intended to determine the amount of MN 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). Except for OTC and CTC, none of the antibacterial substances approved for combined use with MN interfere with the quantification of MN. When OTC or CTC is used in combination with MN, the extract shall be purified with a column packed with basic alumina.

The flow sheet of this method is shown in Figure 9.2.27-1.

Sample (3.0-5.0 g)

↓

Extract with 100 mL of methanol-water (9:1) (with a magnetic stirrer for 20 min).

↓

Filter (through filter paper No.5A).

In the presence of OTC or CTC	In the absence of OTC or CTC
Filter through a column (packed with 12 g of basic alumina (Aluminum oxide Type F-20, Sigma-Aldrich)).	

↓

Discard the first 5 mL of the filtrate and use the subsequent filtrate.

↓

Dilute a quantity of the filtrate with water-methanol (7:3) to prepare high- and low-concentration sample solutions (5 and 1.25 μg (potency)/mL, respectively).

↓

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

↓

Incubate (at 35-37°C for 16-24 hr).

↓

Measure the inhibition zone diameter.

↓

Calculate the potency by the 2-2 dose method.

Figure 9.2.27-1 Quantitative test method for monensin sodium (premix)

References: Noriyuki Koyama: Research Report of Animal Feed, 6, 163 (1980)

History in the Feed Analysis Standards [3] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Vitamin premix	20~80	3	101.0~101.2	2.6
Vitamin/mineral premix	20~80	3	99.4~100.8	2.4

«Notes and precautions»

[1] For the definition etc. of monensin working standard, refer to «Notes and precautions» [9] in Section 1, 1 of this Chapter.

[2] For the method of preparation for the standard stock solution, refer to «Notes and precautions» [10] in Section 1, 1 of this Chapter.

Method of preparation: Example (when the weighed amount is 50 mg)

When the labeled potency of the working standard is 960 μg (potency)/mg, 50 mg of the working standard contains 48,000 μg (potency) (i.e., 50 mg × 960 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 48.0 mL (i.e., 48,000 μg (potency) / 1,000 μg (potency)/mL. Therefore, completely transfer 50 mL of the working standard to an Erlenmeyer flask containing 48.0 mL of methanol, and dissolve to prepare the standard stock solution with a concentration of 1,000 μg (potency)/mL.

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

An example method of preparation monensin standard solution is shown in Table 9.2.27-1.

[4] In some cases, Medium F-18 produces a better inhibition zone than Medium F-16. In such a case, 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.

[5] The cylinder plate method is more sensitive to low concentrations of MN than the agar well method and results in better linearity of the standard response line.

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

An example method of preparation is shown in Table 9.2.27-1.

Table 9.2.27-1 Method of preparation for monensin standard solution and sample solution

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

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	4	5	5
Amount (mL) of water-methanol (7:3)	18	16	15	15
Concentration (μg (potency)/mL)	100	20	5	1.25

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

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

When the analysis sample is collected in an amount equivalent to 50,000 μg (potency) of MN, the concentration of MN in the filtrate is calculated to be 500 μg (potency)/mL.

Test tube No.	1	2	3
Amount (mL) of sample solution	②	2	5
Amount (mL) of water-methanol (7:3)	18	18	15
Concentration (μg (potency)/mL)	50	5	1.25

Note: "②mL" means "2 mL of the filtrate (500 μg (potency)/mL)".

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

[8] An example standard response line for MN is shown in Figure 9.2.27-2.

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

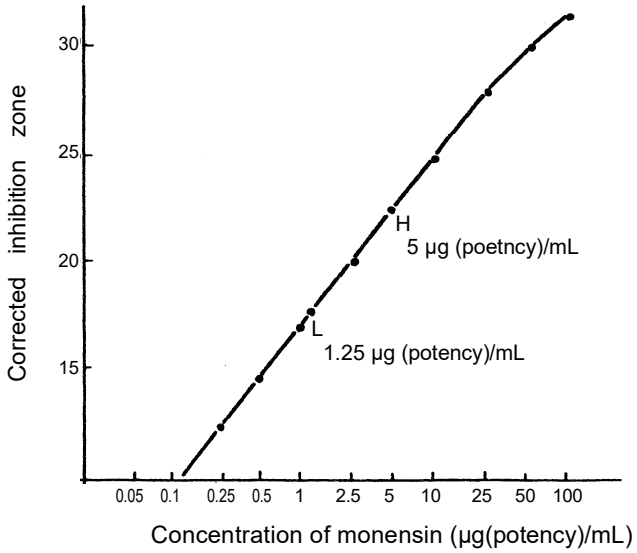


Figure 9.2.27-2 Standard response line for monensin (premix, example)
(*Bacillus subtilis* ATCC 6633, Medium F-16, Cylinder plate method)

1.2 Chicken feed

[Feed Analysis Standards, Chapter 9, Section 2, 27.2.1]

Scope of application: Chicken feed

A. Reagent preparation

1) Monensin standard solution. Weigh accurately not less than 40 mg of monensin working standard, accurately add methanol and dissolve to prepare a monensin 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 a mixture of water and methanol (7:3) to prepare high- and low-concentration standard solutions with concentrations of 4 and 1 μg (potency)/mL, respectively[1].

2) Culture medium[2]: Medium F-16

3) 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 1×10^7 spores/mL per 100 mL of the culture medium.

4) Agar plate. Proceed by the cylinder plate method[3].

5) Extracting solvent: A mixture of methanol and water (9:1)

B. Preparation of sample solution

Weigh accurately a quantity of the analysis sample[4] (equivalent to 0.8 mg (potency) as MN), and place in a 100-mL stoppered Erlenmeyer flask, add 50 mL of the extracting solvent, extract with stirring for 20 minutes, and filter the extract with filter paper (No.5A).

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

Accurately dilute a quantity of the subsequent filtrate with a mixture of water and methanol (9:1) to prepare a high-concentration sample solution with a concentration of 4 μg (potency)/mL[6]. Further,

accurately dilute this solution with a mixture of water and methanol (7:3) to prepare a low-concentration sample solution with a concentration of 1 μg (potency)/m [7].

C. Quantification[8]

Proceed by the 2-2 dose method[9].

«Summary of analysis method»

This method is intended to determine the amount of MN 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 MN interfere with the quantification of MN.

The flow sheet of this method is shown in Figure 9.2.27-3.

Sample (10.0 g, equivalent to 0.8 mg (potency) as MN)
↓
Extract with 50 mL of methanol-water (9:1) (with a magnetic stirrer for 20 min).
↓
Filter (through filter paper No.5A).
↓
Filter through a column (packed with 12 g of basic alumina (Aluminum oxide Type F-20, Sigma-Aldrich)).
↓
Discard the first 5 mL of the filtrate and use the subsequent filtrate.
↓
Dilute a quantity of the filtrate with water-methanol (9:1) to prepare a high-concentration sample solution (4 μg (potency)/mL).
↓
Dilute a quantity of the high-concentration sample solution with water-methanol (7:3) to prepare a low-concentration samplesolution (1 μg (potency)/mL).
↓
Dispense to agar plates (allow to stand at 10-20°C for 2 hr).
↓
Incubate (at 35-37°C for 16-24 hr).
↓
Measure the inhibition zone diameter.
↓
Calculate the potency by the 2-2 dose method.

Figure 9.2.27-3 Quantitative test method for monensin sodium (chicken feed)

References: Toyoko Abe, Toshitake Kono: Research Report of Animal Feed, 6, 114 (1980)
History in the Feed Analysis Standards [3] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Starting chick formula feed	60~100	6	99.5~100.2	1.3
Growing chick formula feed	60~100	6	99.5~100.8	1.2

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Growing chick formula feed	4	80	100.1	2.0	2.9

«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 monensin standard solution is shown in Table 9.2.27-2.

Table 9.2.27-2 Method of preparation for monensin standard solution (chicken feed, example)

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	4	4	5
Amount (mL) of water-methanol (7:3)	18	16	16	15
Concentration (μg (potency)/mL)	100	20	4	1

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

[2] When the analysis sample contains no antibacterial substances other than MN, it is permissible to use Medium F-22 in lieu of Medium F-16 and perform the test as described in 2. Plate method (Part 2).

[3] The cylinder plate method is more sensitive to low concentrations of MN than the agar well method and with better linearity of the standard response line.

[4] Usually corresponds to 10.0 g.

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

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

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

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

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

[8] An example standard response line for MN is shown in Figure 9.2.27-4.

Linearity is observed in the quantification range for MN (MN concentrations between 1 and 4 μg (potency)/mL).

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

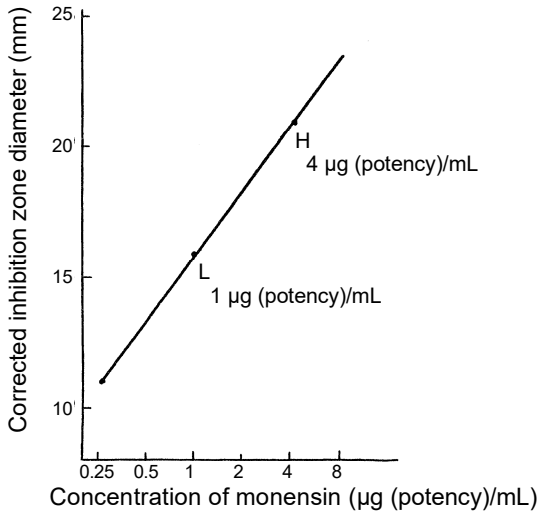


Figure 9.2.27-4 Standard response line for monensin (chicken feed, example)
(*Bacillus subtilis* ATCC 6633, Medium F-16, Cylinder plate method)

1.3 Cattle feed

[Feed Analysis Standards, Chapter 9, Section 2, 27.2.2]

Scope of application: Cattle feed

A. Reagent preparation

1) Monensin standard solution. Weigh accurately not less than 40 mg of monensin working standard, accurately add methanol and dissolve to prepare a monensin standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with a mixture of water and methanol (7:3) to prepare high- and low-concentration standard solutions with concentrations of 2 and 0.5 μg (potency)/mL, respectively[1].

2) Culture medium: Medium F-22 [2]

3) Spore suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.5 mL of the spore suspension with a concentration of 1×10^7 spores/mL per 100 mL of the culture medium.

4) Agar plate. Proceed by the cylinder plate method [3].

5) Extracting solvent: A mixture of methanol and water (9:1)

B. Preparation of sample solution

Weigh accurately a quantity of the analysis sample[4] (equivalent to 0.3 mg (potency) as MN), place in a 100-mL stoppered Erlenmeyer flask, add 50 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[5] with 12 g of basic alumina for column chromatography (particle size: 74 to 177 μm (200 to 80 mesh))), and discard the first 5 mL of the filtrate.

Accurately dilute a quantity of the subsequent filtrate to prepare a high-concentration sample solution with a concentration of 2 μg (potency)/mL[6]. Further, accurately dilute this solution with a

mixture of water and methanol (7:3) to prepare a low-concentration sample solution with a concentration of 0.5 μg (potency)/mL[7].

C. Quantification[8]

Proceed by the 2-2 dose method[9].

«Summary of analysis method»

This method is intended to determine the amount of MN in a cattle 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. As the amount of MN added to the feed is smaller for callte feed than chicken feed, Medium F-22 shall be used alternatively as it is more sensitive to MN.

The flow sheet of this method is shown in Figure 9.2.27-5.

Sample (10.0 g, equivalent to 0.3 mg (potency) as MN)
↓
Extract with 50 mL of methanol-water (9:1) (with a magnetic stirrer for 20 min).
↓
Filter (through filter paper No.5A).
↓
Filter through a column (packed with 12 g of basic alumina (Aluminum oxide Type F-20 (Sigma-Aldrich))).
↓
Discard the first 5 mL of the filtrate and use the subsequent filtrate.
↓
Dilute a quantity of the filtrate to prepare a high-concentration sample solution (2 μg (potency)/mL).
↓
Dilute a quantity of the high-concentration sample solution with water-methanol (7:3) to prepare a low-concentration sample solution (0.5 μg(potency)/mL).
↓
Dispense to agar plates (allow to stand at 10-20°C for 2 hr).
↓
Incubate (at 35-37°C for 16-24 hr).
↓
Measure the inhibition zone diameter.
↓
Calculate the potency by the 2-2 dose method.

Figure 9.2.27-5 Quantitative test method for monensin sodium (cattle feed)

References: Toyoko Kusama: Research Report of Animal Feed, 11, 107 (1986)

History in the Feed Analysis Standards [7] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Cattle formula feed 1	15~45	3	101.6~108.7	4.5
Cattle formula feed 2	15~45	3	102.3~110.8	10.4
Cattle formula feed 3	15~45	3	105.9~110.9	5.5

«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 monensin standard solution is shown in Table 9.2.27-3.

Table 9.2.27-3 Method of preparation for monensin standard solution (cattle feed, 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: "②mL" means "2 mL of standard stock solution (1 mg (potency)/mL)".

[2] Medium F-22 contains 50 g of magnesium sulfate in 1,000 mL and is more sensitive to MN than Medium F-16.

[3] The cylinder plate method is more sensitive to low concentrations (not more than 0.5 μg (potency)/mL) of MN than the agar well method and with better linearity of the standard resonse line.

[4] Usually corresponds to 10.0 g.

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

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

Filtrate	6 mL
Water	12 mL

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

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

[8] An example standard response line for MN is shown in Figure 9.2.27-6.

Linearity is observed in the quantification range for MN (MN concentrations between 0.5 and 2 μg (potency)/mL).

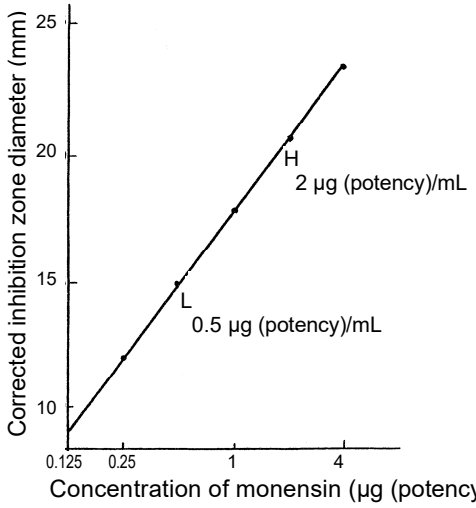


Figure 9.2.27-6 Standard response line for monensin (cattle feed, example)
(*Bacillus subtilis* ATCC 6633, Medium F-22, Cylinder plate method)

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

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

2.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 27.1.2]

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 solution[Note 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 antibiotic[Note 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.

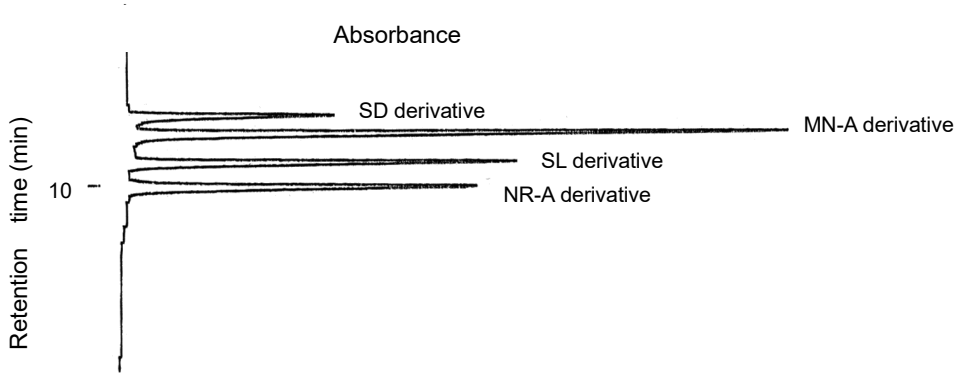


Figure 9.3.1-1 Chromatogram for a mixed standard solution
(SL: 100 ng, SD: 200 ng, NR-A: 100 ng, MN-A: 50 ng)

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

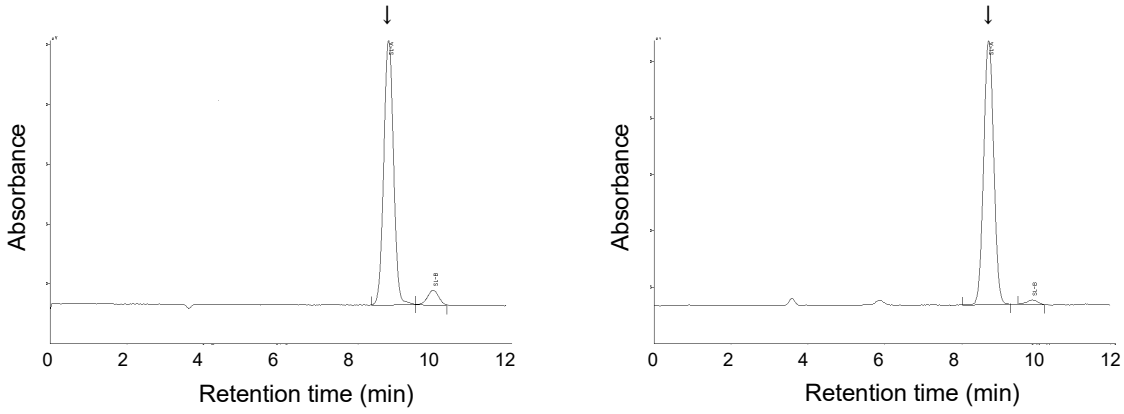
«Notes and precautions»

[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.

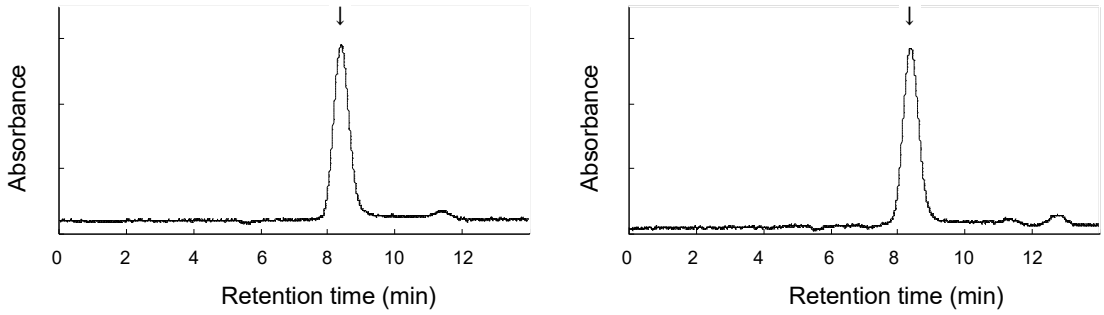


Standard solution (200 ng) Chicken premix (50.0 g/kg)

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.



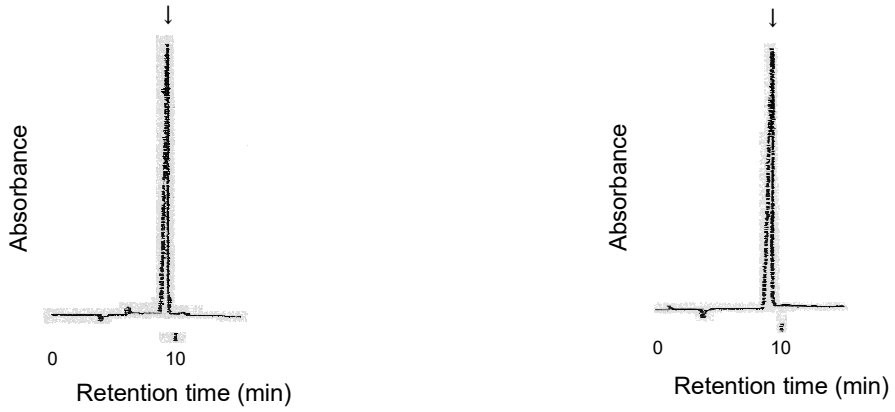
Standard solution (equivalent to 200 ng (potency) as SD)

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.

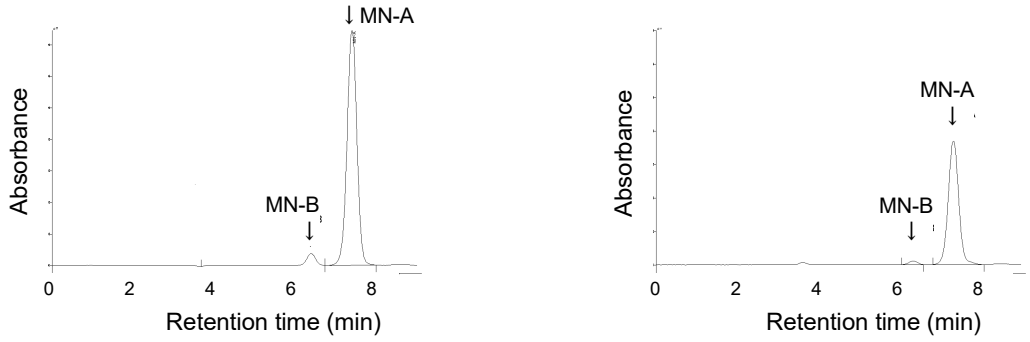


Standard solution (200 ng) Chicken premix (80 g (potency)/kg)

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, 27.2.3]

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.

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

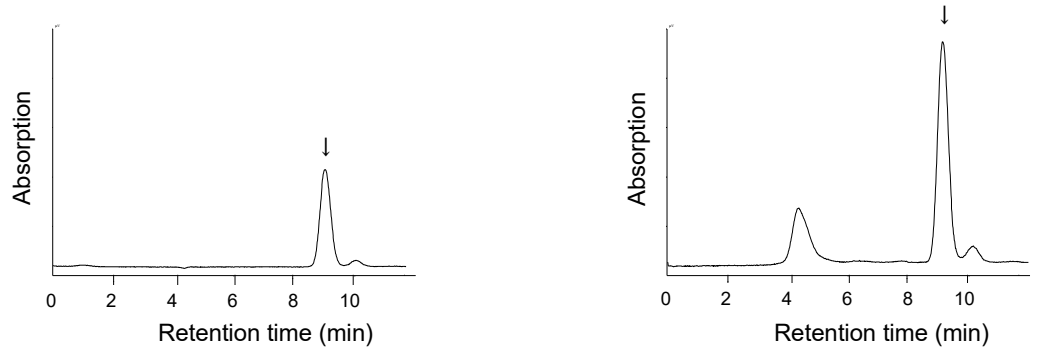
«Notes and precautions»

[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.

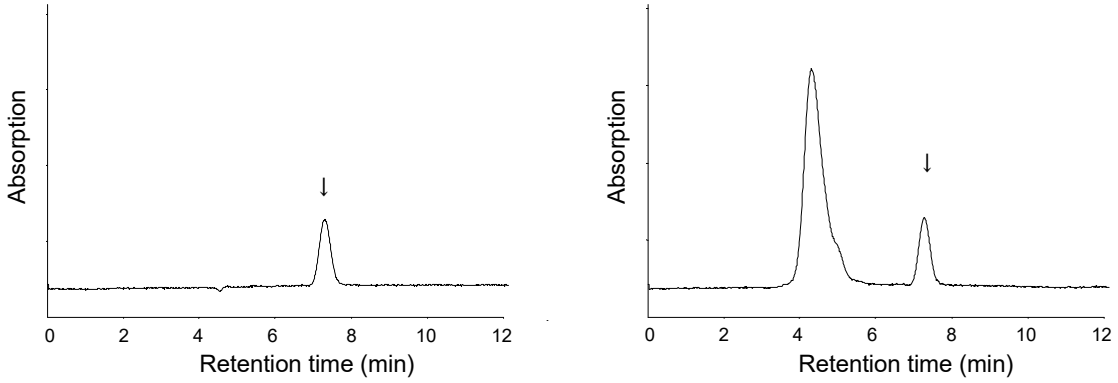


Standard solution (40 ng) Starting chick grower formula feed (50 g (potency)/t)

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.

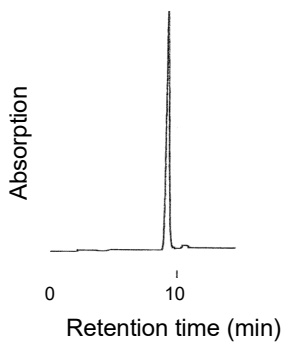


Standard solution (50 ng) Chicken formula feed (25 g (potency)/t)

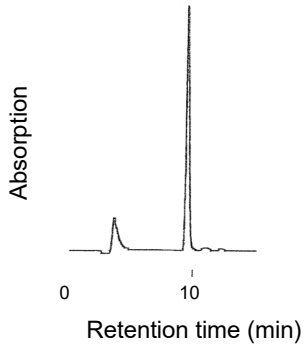
Figure 9.3.1-9 Chromatograms for semduramicin

(The arrow indicate the peak of an SD derivative)

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



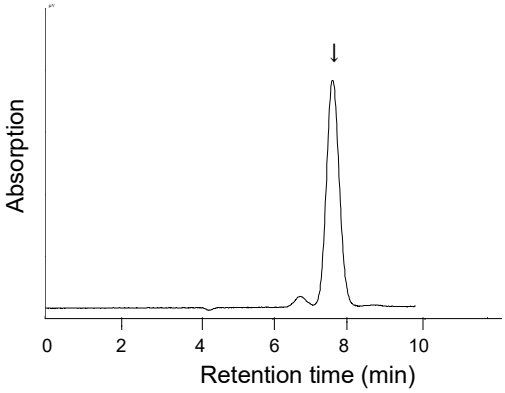
Standard solution (160 ng)



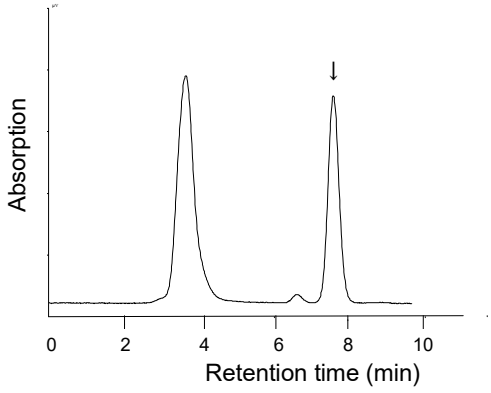
Starting chick grower formula feed (80 g (potency)/t)

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 (Feed)

3.1 Trace quantitative test method for polyether antibiotics by microbioautography [Feed Analysis Standards, Chapter 9, Section 2, 27.3.1]

Antibiotics of interest: SL, MN and LS (3 components)

Scope of application: Feed

A. Reagent preparation

1) Salinomycin standard solution. Dry a suitable amount of salinomycin working standard under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, add methanol and dissolve to prepare a salinomycin standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with methanol to prepare standard solutions with concentrations of 20, 10, 5, 2.5 and 1.25 μg (potency)/mL[1].

2) Monensin standard solution. Weigh accurately not less than 40 mg of monensin working standard, add methanol and dissolve to prepare a monensin standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with methanol to prepare standard solutions with concentrations of 20, 10, 5, 2.5 and 1.25 μg (potency)/mL[1].

3) Lasalocid standard solution. Weigh accurately not less than 40 mg of lasalocid working standard, add methanol and dissolve to prepare a lasalocid standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with methanol to prepare standard solutions with concentrations of 20, 10, 5, 2.5 and 1.25 μg (potency)/mL[1].

4) Culture medium: Medium F-22

5) Pore suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.1 mL of the pore suspension with a concentration of 1×10^7 spores/mL per 100 mL of the culture medium.

6) Developing solvent[2]

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

ii) A mixture of ethyl acetate and ammonia solution (180:1)

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

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

B. Preparation of sample solution

Extraction. Weigh 40.0 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of acetonitrile, extract with stirring for 30 minutes, and filter the extract through filter paper (No.5A). Transfer 50 mL of the filtrate to a 100-mL recovery flask, evaporate into dryness under reduced pressure in a water bath at 50°C, add 20 mL of a mixture of chloroform and ethyl acetate (9:1) to dissolve the residue, and use as the sample solution subject to column treatment.

Column treatment. Wash a silica gel minicolumn (690 mg) with 10 mL of chloroform.

On the minicolumn place a funnel loaded with approximately 40 g of sodium sulfate (anhydrous)[3], pour the sample solution into the funnel, and allow to flow down until the amount in the minicolumn reservoir reaches 1 mL[4]. Wash the recovery flask that contained the sample solution with 10 mL of a mixture of chloroform and ethyl acetate (9:1), transfer the washings to the funnel, and repeat this procedure 3 times.

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

Place a 50-mL recovery flask under the minicolumn, and add 30 mL of a mixture of chloroform and methanol (4:1) to the minicolumn to elute SL, MN and LS. Evaporate the eluate into dryness under reduced pressure in a water bath at 50°C, accurately add 2 mL of methanol to dissolve the residue[5], and use as the sample solution.

C. Quantification[6]

Proceed as described in Section 1, 2-C [7] except for the following procedures.

Use a thin-layer plate made of silica gel[Note 1] and develop until the ascending front of the developing solvent reaches the top of the thin-layer plate.

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

«Summary of analysis method»

This method is intended to quantify and identify SL, MN or LS contamination due to carry-over etc. in a feed by microbioautography using a sample solution prepared by extracting with acetonitrile, purifying through a silica gel minicolumn, and dissolving in methanol.

The flow sheet of this method is shown in Figure 9.3.3-1.

Sample (40.0 g)
|
Extract with 100 mL of acetonitrile (with a magnetic stirrer for 30 min).
|
Filter (through filter paper No.5A).
|
Collect 50 mL of the filtrate (into a 100-mL recovery flask).
|
Evaporate into dryness under reduced pressure (in a water bath at 50°C).
|
Dissolve the residue with 20 mL of chloroform-ethyl acetate (9:1).
|
Load onto a silica gel minicolumn (previously washed with 10 mL of chloroform and equipped on the reservoir with a funnel containing approximately 40 g of sodium sulfate).
|
Wash the recovery flask with 10 mL of chloroform-ethyl acetate (9:1) and load the washings onto the silica gel minicolumn (repeat 3 times).
|
Wash the sodium sulfate with 10 mL of chloroform-ethyl acetate (9:1) and load the washings onto the silica gel minicolumn.
|
Wash the silica gel minicolumn with 20 mL of chloroform-ethyl acetate (9:1).
|
Elute SL, MN and LS with 30 mL of chloroform-ethanol (4:1) (into a 50-mL recovery flask).
|
Evaporate into dryness under reduced pressure (in a water bath at 50°C).
|
Dissolve the residue with 2 mL of methanol.
|
Spot on a thin-layer plate (20 μL).
|
Develop.
|
Prepare agar plates (allow to stand at 10-20°C for 3 hr).
|
Incubate (at 35-37°C for 16-24 hr).
|
Measure the inhibition zone diameter and determine the Rf value.
|
Calculate the potency from the calibration curve.

Figure 9.3.3-1 trace quantitation test method for salinomycin sodium, monensin sodium and lasalocid sodium (feed)

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

History in the Feed Analysis Standards [12] New

«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	Adult chicken formula feed	0.1~1	3	102.0~110.0	8.9
	Meat pig formula feed	0.1~1	3	106.7~120.0	8.3
	Dairy cattle formula feed	0.1~1	3	104.7~116.7	9.9
Monensin sodium	Adult chicken formula feed	0.1~1	3	97.3~106.7	5.4
	Meat pig formula feed	0.1~1	3	99.3~106.0	11.5
	Dairy cattle formula feed	0.1~1	3	98.7~110.0	5.2
Lasarosid sodium	Adult chicken formula feed	0.1~1	3	94.0~116.0	18.6
	Meat pig formula feed	0.1~1	3	91.3~112.0	21.7
	Dairy cattle formula feed	0.1~1	3	94.7~112.0	21.7

・Lower detection limit: 0.5 g (potency)/t each in the sample for each component

«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 standard solution is shown in Table 9.3.3-1.

Table 9.3.3-1 Method of preparation for standard solution (trace quantitation test method, feed, example)

Test tube No.	1	2	3	4	5	6
Amount (mL) of standard s	②	4	10	10	10	5
Amount (mL) of methanol	18	16	10	10	10	5
Concentration (μg (potency)/mL)	100	20	10	5	2.5	1.25

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

[2] Usually, proceed only with a mixture of ethyl acetate, hexane, acetone, and methanol (20:8:1:1). When an inhibition zone is observed with the sample solution, perform a re-test with a mixture of ethyl acetate and ammonia solution (180:1) to make a more precise identification.

[3] 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).

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

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

[6] Example standard response lines for SL, MN and LS are shown in Figure 9.3.3-2 to 4.

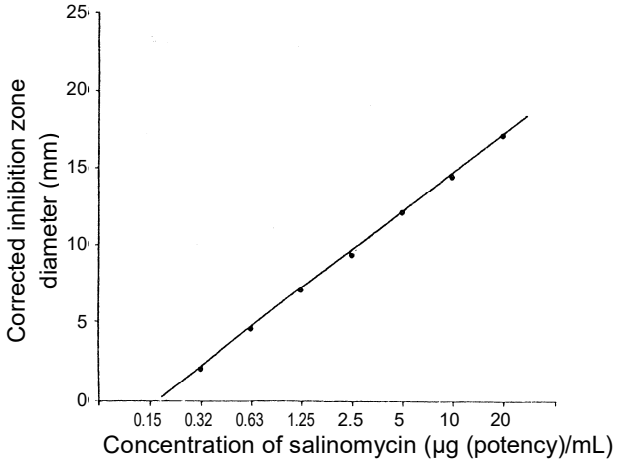


Figure 9.3.3-2 Standard response line for salinomycin (trace quantitation test method, feed)
(*Bacillus subtilis* ATCC 6633, Medium F-22, Microbioautography)

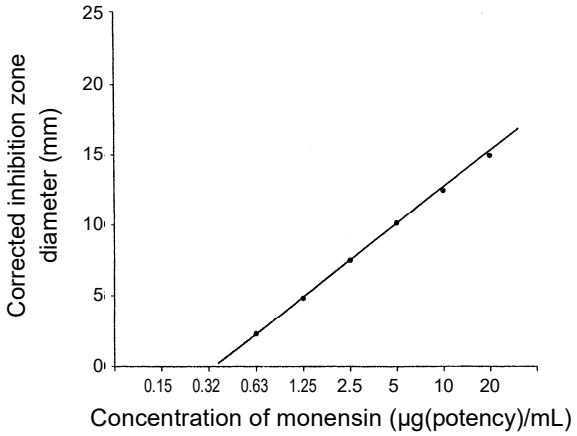


Figure 9.3.3-3 Standard response line for monensin (trace quantitation test method, feed)
(*Bacillus subtilis* ATCC 6633, Medium F-22, Microbioautography)

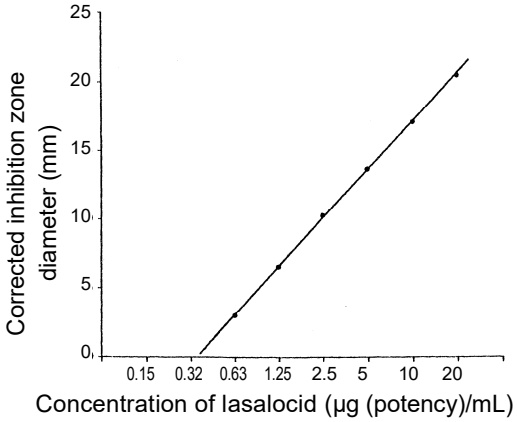


Figure 9.3.3-4 Standard response line for lasalocid (trace quantitation test method, feed)
(*Bacillus subtilis* ATCC 6633, Medium F-22, Microbioautography)

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

3.2 Trace quantitative test method for polyether antibiotics by liquid chromatography mass spectrometry [Feed Analysis Standards, Chapter 9, Section 2, 27.3.2]

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[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.

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.

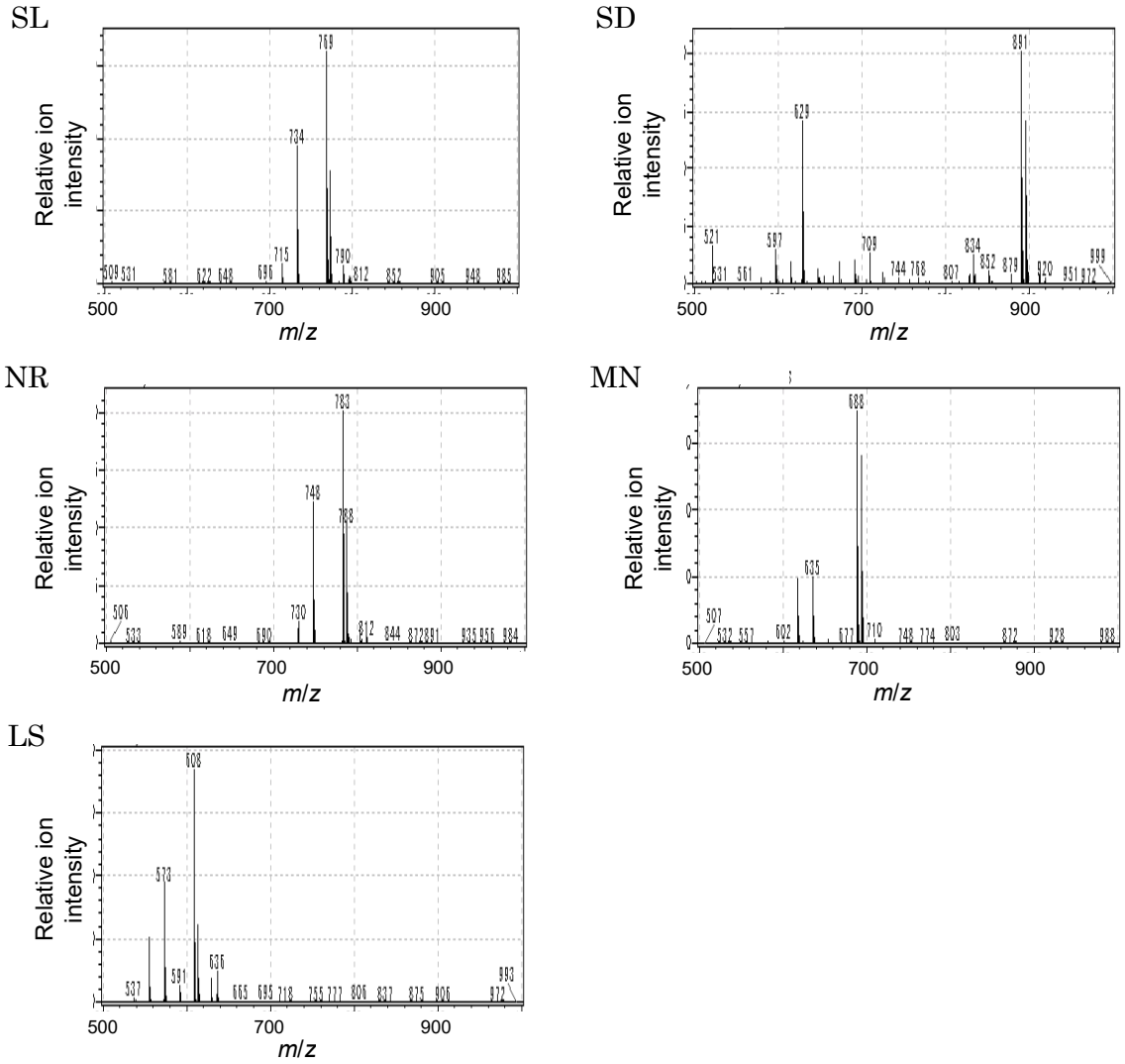
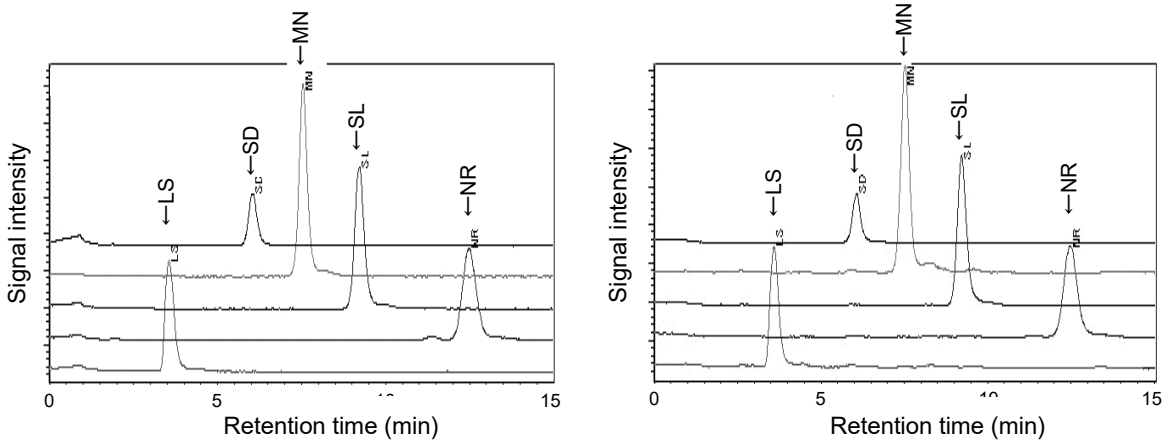


Figure 9.3.4-2 Mass spectrum for each antibiotic

[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 Identification test method - Identification test method for polyether antibiotics by microbioautography

4.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 27.4.1]

Antibiotics of interest: SL, MN and LS

Scope of application: Feed

A. Reagent preparation

1) Salinomycin standard solution. Dry a suitable amount of salinomycin working standard under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, add methanol and dissolve to prepare a salinomycin standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with methanol to prepare a standard solution with a concentration of 10 μg (potency)/mL[1].

2) Monensin standard solution. Weigh accurately not less than 40 mg of monensin working standard, add methanol and dissolve to prepare a monensin standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with methanol to prepare a standard solution with a concentration of 10 μg (potency)/mL[1].

3) Lasalocid standard solution. Weigh accurately not less than 40 mg of lasalocid working standard, add methanol and dissolve to prepare a lasalocid standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with methanol to prepare a standard solution with a concentration of 10 μg (potency)/mL[1].

4) Culture medium: Medium F-22

5) Pore suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.2 mL of a pore suspension with a concentration of 1×10^7 pores/mL per 100 mL of the culture medium.

6) Extraction solvent. A mixture of methanol and water (9:1) (use methanol in the case of LS.)

7) Developing solvent. A mixture of ethyl acetate, hexane, acetone, and methanol (20:8:1:1)

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

B. Preparation of sample solution

Weigh accurately 3 to 5 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extraction 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 extraction solvent 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 in Section 1, 2-C[2] except for the following procedures.

Use a thin-layer plate made of silica gel[Note 1], spot 25 µL each of the standard solution and sample solution, and develop until the ascending front of the developing solvent reaches the top of the thin-layer plate.

Note 1. Use 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 AL, MN and LS in a premix by microbioautography using a sample solution prepared for quantification.

The flow sheet of this method is shown in Figure 9.3.5-1.

Sample (3.0-5.0 g)
↓
Extract with 100 mL of methanol-water (9:1) (use methanol in the case of LS) (with a magnetic stirrer for 20 min).
↓
Dilute a quantity of the filtrate with methanol-water (9:1) (use methanol in the case of LS) to prepare the sample soluiton (10 µg (potency)/mL).
↓
Spot on a thin-layer plate (25 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 9.3.5-1 Identification test method for SL, MN and LS (premix)

References: Hisaaki Hiraoka: Research Report of Animal Feed, 21, 159 (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 standard solution is shown in Table 9.3.5-1.

Table 9.3.5-1 Example method of preparation for standard solution (identification test method, premix, example)

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

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

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

4.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 27.5.1]

Antibiotics of interest: SL, MN and LS

Scope of application: Feed

A. Reagent preparation

Proceed as described in 5.1 (2) A[1].

B. Preparation of sample solution

Weigh accurately a quantity of the analysis sample (equivalent to 0.5 mg (potency) as SL or MN, or equivalent to 1 mg (potency) as LS), place in a 200-mL stoppered Erlenmeyer flask, add 50 mL of the extraction solvent (100 mL of chloroform in the case of LS), extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A) to prepare a sample solution with a concentration of 10 µg (potency)/mL.

C. Identification

Proceed as described in 5.1 (2)-C [2].

«Summary of analysis method»

This method is intended to identify SL, MN and LS in a feed by microbioautography using a sample solution prepared for quantification.

The flow sheet of this method is shown in Figure 9.3.5-2.

Sample (equivalent to 0.5 mg (potency) as SL or MN, or 1 mg (potency) as LS).
|
SL or MN: Extract with 50 mL of methanol-water (9:1) (with a magnetic stirrer for 20 mi
LS: Extract with 100 mL of chloroform (with a magnetic stirrer for 20 min).
|
Use the filtrate as the sample solution (10 µg (potency)/mL).
|
Spot on a thin-layer plate (25 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 9.3.5-2 Identification test method for salinomycin sodium, monensin sodium and lasalocid sodium (feed)

References: Hisaaki Hiraoka: Research Report of Animal Feed, 21, 15 (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 standard solution is shown in Table 9.3.5-2.

Table 9.3.5-2 Method of preparation for standard solution (identification test method, feed, example)

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

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

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

5 Control test method - Rapid quantitative method

5.1 Chicken feed

[53 Chiku B No.2173 and 53 SuiShin No.464, notified by the Head of the Livestock Industry Bureau and Head of the Fisheries Agency, Ministry of Agriculture, Forestry and Fisheries, as of September 5, 1978]

1 Instruments and equipments

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

2 Reagents and standard substances

(1) Anhydrous ethanol: guaranteed grade
(2) *p*-Dimethylaminobenzaldehyde
(3) Sulfuric acid: guaranteed grade
(4) Monensin sodium standard for feed analysis

3 Preparation of reagents

(1) Monensin sodium standard solution

Place accurately 8 mg (potency) of monensin sodium standard for feed analysis in a 100-mL volumetric flask, add anhydrous ethanol[1] and dissolve to make 100 mL, and use this solution as the monensin sodium standard stock solution (1 mL of this solution contains 80 μg (potency) of monensin sodium).

At the time of use, dilute a quantity of this stock solution exactly 10-fold with anhydrous ethanol (1 mL of this solution contains 8 μg (potency) of monensin sodium).

The standard stock solution shall be stored in a cool, dark place, and use within 2 weeks after

preparation[2].

(2) *p*-Dimethylaminobenzaldehyde solution[3]

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[Note 1]

Place 5 g of the analysis sample[Note 2] in a 200-mL stoppered Erlenmeyer flask, add 100 mL of anhydrous ethanol[4], stir for 10 minutes with a magnetic stirrer to extract monensin sodium, immediately filter, and use the filtrate as the sample solution.

Transfer accurately 10 mL of the sample solution to each of 50-mL test tubes A, B and C[5]. Add 5 mL of ethanol to each of test tubes A and B, and accurately 5 mL of the standard solution to test tube C. Further, add accurately 5 mL of sulfuric acid-anhydrous ethanol solution to test tube A, and accurately 5 mL of *p*-dimethylaminobenzaldehyde solution to each of test tubes B and C[Note 3], mix, and develop by heating in a thermostat at 70±1°C for 20 minutes[6]. After allowing to cool, determin absorbances *a*, *b* and *c* of solutions A, B and C at a wavelength of about 578, using anhydrous ethanol as the blank[7]. Separately, proceed in the same manner with a monensin sodium-unspiked control samle (with the same composition as the analysis sample except for the absence of monensin sodium[8], and determine absorbances *a*′, *b*′ and *c*′ [Note 4].

Calculate the content of monensin sodium in the sample according to the following equation.

$$\text{Content (g (potency)/t)of monensin sodium in the sample} = \frac{b-a}{c-b} \times 50 - \frac{b'-a'}{c'-b'} \times 50$$

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

2. Distribute the whole amount of the collected sample uniformly over a piece of paper, divide into 8 equal areas, from each area collect an equal amount to a total of 30 to 40 g, mix homogeneously, and use the mixture as the analysis sample.
3. The sulfuric acid-ethanol solution and *p*-dimethylaminobenzaldehyde solution shall be added last, and the subsequent procedure performd promptly.
4. A previously determined value can be used as the blank value for the monensin sodium-unspiked control sample as long as the composition of the sample does not differ from that of the control sample.

A flow injectior [9] is applicable to the quantification procedure.

«Summary of analysis method»

This method is intended to determine the absorbance of MN in a feed at a wavelength of 600 or 578 nm using a sample solution prepared by extracting with ethanol, adding sulfuric acid or *p*-dimethylaminobenzaldehyde (chromogenic substrate), and developing in a water bath at 70°C for 20 minutes.

The flow sheet of this method is shown in Figure 9.2.27-7.

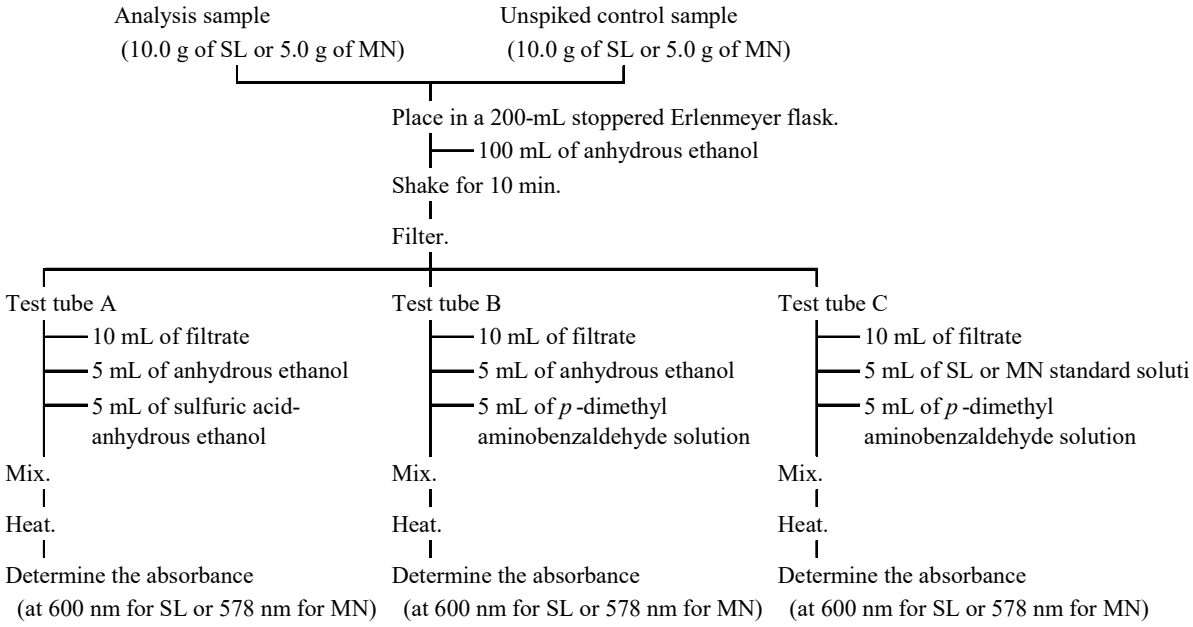


Figure 9.2.27-7 Rapid quantitative method for salinomycin and monensin (feed)

References: Toshitake Kono, Akitsugu Yamamoto: Research Report of Animal Feed, 5, 174 (1979)
Noriyuki Koyama: Research Report of Animal Feed, 11, 209 (1986)
Tadashi Suhara: Research Report of Animal Feed , 11, 214 (1986)

«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 raction 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 can be used for 6 months as long as it is prepared at a high concentration (400 μg (potency)/mL) and stored in 2-mL portions in small test tubes at -20°C or lower. At the time of use, accurately dilute the stock solution with anhydrous ethanol.

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

[4] MN is freely soluble in ethanol, in methanol and chloroform, slightly soluble in acetone, and practically insoluble in water.

[5] The use of stoppered test tubes is intended to avoid ethanol volatilization and vapor contamination when developed in a thermostat.

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

[6] The chromogenic substrate for MN develops a blue color. The blank for the unspiked control sample

can develop a light yellow to light red color.

As MN is unstable in an acid solution and so is its chromogenic substrate, make sure to avoild direct sunlight and determine the absorbance promptly after development.

[7] The absorption spercrum for MN is shown in Figure 9.2.27-8. The maximum absorption occurs at a wavelength of about 578 nm.

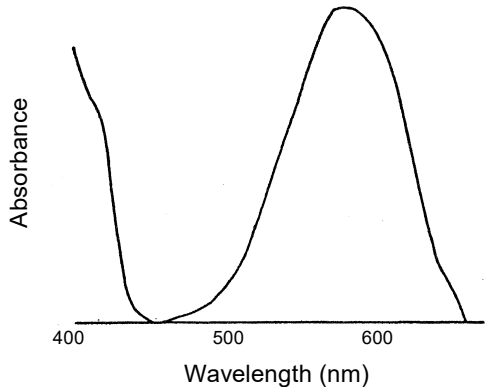


Figure 9.2.27-8 Absorption spectrum of chromogenic substrate for monensin (solvent: ethanol)

[8] The MN-unspiked sample also has an appearant quantified value (blank value), which therefore shall be subtracted.

When the feed factory performs quality control, a previously determined value can be used as the blank value as long as the composition of the feed is constant.

The blank value is higher for such raw feed materials as Argentine milo (100 ppm), fish meal (70 ppm), alfalfa (30 ppm), and meat-and-bone meal (10 ppm).

When the absorbance of the unspiked sample is abnormally high, it can be attributed to the effect of an interfering substance. In this case, the extracted sample solution shall be passed through a column packed with alumina as directed in Section 2, 13.1.1 in Chapter 9 of the Feed Analysis Standards, and the resulting solution subjceted to color development.

・Alumina column

The alumina column shall be prepared by dry-packing 12 g of basic alumina for cholumn chromatography (Sigma-Aldrich, Aluminum oxide Type F-20, particle size: 74 to 177 μm, 200 to 80 mesh) in a chromatography tube (14 mm in internal diameter, 300 mm in length) previously stuffed with glass wool in the bottom.

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

6.2 Cattle feed

[60 Chiku B No.2928, notified by the Head of the Livestock Industry Bureau, Ministry of Agriculture, Forestry and Fisheries, as of October 15, 1985]

1. Instruments and equipments

(1) Stoppered Erlenmeyer flask

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

2. Reagents and standard substances

(1) Anhydrous ethanol: guaranteed grade
(2) *p*-Dimethylaminobenzaldehyde
(3) Sulfuric acid: guaranteed grade
(4) Monensin sodium standard for feed analysis

3. Preparation of reagents

(1) Monensin sodium standard solution

Place accurately 12 mg (potency) of monensin sodium standard for feed analysis in a 100-mL volumetric flask, add anhydrous ethanol and dissolve to make 100 mL, and use this solution as the monensin sodium standard stock solution (1 mL of this solution contains 120 μg (potency) of monensin sodium).

At the time of use, dilute a quantity of this stock solution exactly 20-fold with anhydrous ethanol (1 mL of this solution contains 6 μg (potency) of monensin sodium).

The standard stock solution shall be stored in a cool, dark place and used within 2 weeks after preparation.

(2) *p*-Dimethylaminobenzaldehyde solution

Dissolve 600 mg of *p*-dimethylaminobenzaldehyde in approximately 50 mL of anhydrous ethanol, gradually add 1 mL of sulfuric acid, further 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 further add anhydrous ethanol to make 100 mL (prepare at the time of use).

4. Quantification[Note 1]

Place 10 g of analysis sample[Note2] in a 200-mL stoppered Erlenmeyer flask, add 100 mL of anhydrous ethanol, stir for 10 minutes with a magnetic stirrer to extract monensin sodium, and immediately filter to prepare a sample solution.

Transfer exactly 10 mL of the sample solution to each of 50-mL test tubes A, B and C. Add accurately 5 mL of anhydrous ethanol to each of test tubes A and B and accurately 5 mL of the standard solution to test tube C. Further, add accurately 5 mL of sulfuric acid-anhydrous ethanol solution to test tube A and accurately 5 mL of *p*-dimethylaminobenzaldehyde solution[Note 3] to each of test tubes B and C, mix, and develop by heating in a thermostat at 70±1°C for 20 minutes[6]. After allowing to cool,

determine absorbances a, b and c of solutions A, B and C at a wavelength of about 600 nm, using anhydrous ethanol as the blank. Separately, proceed in the same manner with a monensin sodium-unspiked control sample and determine absorbances a', b' and c' Note 4.

Calculate the content of monensin sodium in the sample according to the following equation.

$$\text{Content (g (potency)/t) of monensin sodium in the sample} = \frac{b-a}{c-b} \times 15 - \frac{b'-a'}{c'-b'} \times 15$$

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

2. Distribute the whole amount of the collected sample uniformly over a piece of paper, divide into 8 equal areas, collect about an equal amount from each area to a total of 30 to 40 g, mix, and use the mixture as the analysis sample.
3. The sulfuric acid-ethanol solution and *p*-dimethylaminobenzaldehyde solution shall be added last and the subsequent process performed rapidly.
4. A previously determined value can be used as the blank value for the monensin-sodium unspiked control sample as long as the composition of the sample does not differ from that of the control sample.

A flow injector is applicable to the quantification procedure.

«Summary of analysis method»

The procedures of this method are the same as those of the rapid quantitative method for chicken feed except that (1) the monensin sodium standard solution shall be prepared by diluting the stock solution with a concentration of 120 μg (potency)/mL exactly 20-fold to achieve a concentration of 6 μg (potency)/mL, and that (2) the analysis sample for monensin sodium shall be collected in an amount of 10 g.

For more details of this method, refer to 6.1 (1) Chicken feed.