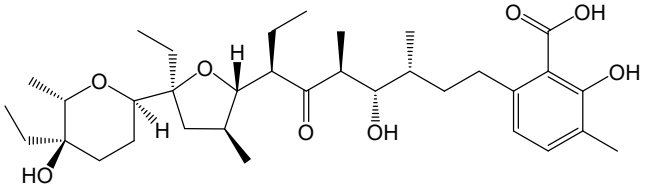
28 Lasalocid sodium



Lasalocid

$C_{34}H_{54}O_8$ MW: 590.8 CAS No.: 25999-31-9

[Summary of lasalocid sodium]

Lasalocid is a polyether antibiotic obtained by the incubation of *Streptomyces lasaliensis* and has the chemical structure shown above. The one used as a feed additive is its sodium salt (LS). The difference between LS and the other polyether antibiotics (SL, SD, NR and MN) is that LS 1) has a benzene ring in its chemical structure, 2) emits fluorescence, and 3) is negative in chromogenic reaction with vanillin.

For physicochemical properties, LS technical occurs as a white to brownish white powder with a characteristic odor. It is slightly soluble in acetone, in ethanol, in chloroform, in ethyl acetate, and in methanol, and practically insoluble in water.

LS (excluding forumulations with lasalocid content not more than 2%) is designated as deleterious substance under the Cabinet Order for the Designation of the Poisonous and Deleterious Substances (Cabnet Order No.2, 1965). For the handing of this substance, make sure to conform to the procedures specified in the Poisonous and Deleterious Substances Control Act (Act No.303, 1950).

LS 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»

LS is a pure-grade antibiotic that was designated as a feed additive as of July 6, 1983. 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	Fattening cattle
Added amount	75	75	33

The amount of LS added to a commercial premix is roughly 15 to 60 g (potency)/kg.

As excessive consumption of LS 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 seurce safety.

For this reason, feed manufacturers are required to control the feeds containing LS according to the separately specified control test methods (for chicken feed (58 Chiku B No.1676, notified by the Head of the Livestock Industry Bureau, Ministry of Agriculture, Forestry and Fisheries, as of July 6, 1983), chicken feed and cattle feed (3 Chiku B No.1113, notified by the Head of the Livestock Industry Bureau and Head of the Fisheries Agency, the Ministry of Agriculture, Forestry and Fisheries, as of June 3, 1991), and for feed other than powder feed for chickens (6 Chiku B No.1012, notified by the Head of the Livestock Industry Bureau, 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, 28.1.1]

A. Reagent preparation

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

2) Lasalocid standard solution. Weigh accurately not less than 40 mg of lasalocid working standard[1], accurately add methanol and dissolve to prepare a lasalocid 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 4 and 1 μg (potency)/mL, respectively[3].

3) Culture medium: Medium F-18

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

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

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 methanol, 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 4 and 1 μg (potency)/mL, respectively[4].

C. Quantification[5]

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

«Summary of analysis method»

This method is intended to determine the amount of LS in a premix by microbiological assay using a sample solution prepared by extractingwith methanol and diluting with a mixture of water and methanol (3:1). None of the antibacterial substances approved for combined use with LS interfere with the quantification of LS.

The flow sheet of this method is shown in Figure 9.2.28-1.

Sample (3.0-5.0 g)
↓
Extract with 100 mL of methanol (with a magnetic stirrer for 20 min).
↓
Filter (through filter paper No.5A).
↓
Dilute a quantity of the filtrate with water-methanol (3:1) to prepare high- and low-concentration sample solutions (4 and 1 μg (potency)/mL, respectively).
↓
Despense 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 9.2.28-1 Quantitative test method for lasalocid sodium (premix)

References: Kiyoshi Kanno: Research Report of Animal Feed, 10, 76 (1985)
History in the Feed Analysis Standards [6] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Premix 1	7.5~75	6	95.6~99.3	1.6
Premix 2	7.5~75	6	96.2~99.7	2.7
Premix 3	7.5~75	6	93.3~98.8	3.0

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Broiler plemix	6	8	100.3	2.2	2.6
Broiler premix	6	15	102.4	2.0	4.2

«Notes and precautions»

[1] For the definition etc. of lasalocid 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 1,022 μg (potency)/mg, 50 mg of the

working standard contains 51,100 μg (potency) (i.e, 50 mg × 1,022 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 51.1 mL (i.e., 51,100 μg (potency) / 1,000 μg (potency)/mL). Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 51.1 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 for lasalocid standard solution is shown in Table 9.2.28-1.

[4] 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 9.2.28-1.

Table 9.2.28-1 Method of preparation for lasalocid standard solution and sample solution

1) Method of preparation for lasalocid 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 (3:1)	23	16	15	15
Concentration (μg (potency)/mL)	80	16	4	1

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 80,000 μg (potency) of LS, the concentration lf LS in the filtrate is calculated to be 800 μg (potency)/mL.

Test tube No.	1	2	3	4
Amount (mL) of sample solution	②	4	5	5
Amount (mL) of water-methanol (3:1)	18	16	15	15
Concentration (μg (potency)/mL)	80	16	4	1

Note: "②mL" means "2 mL of the filtrate (800 μg (potency)/mL)".

[5] An example standard response line for LS is shown in Figure 9.2.28-2.

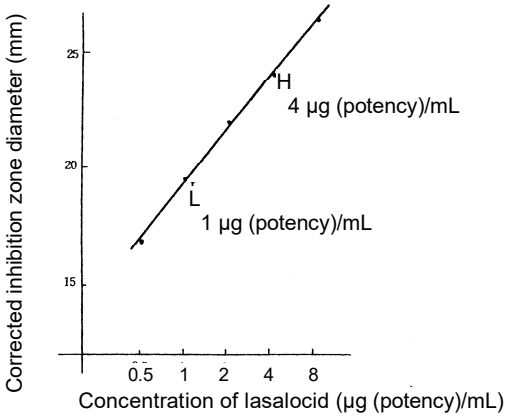


Figure 9.2.28-2 Standard response line for lasalocid (premix, example)

(*Bacillus subtilis* ATCC 6633, Medium F-18, Agar well method)

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

1.2 Chiken feed

[Feed Analysis Standards, Chapter 9, Section 2, 28.2.1]

Scope of application: Chicken feed

A. Reagent preparation

1) Lasalocid standard solution. Weigh accurately not less than 40 mg of lasalocid working standard, accurately 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 standard stock solution with a mixture of water and methanol (3:1) to prepare high- and low-concentration standard solutions with concentrations of 1 and 0.5 µg (potency)/mL, respectively[1].

2) Culture medium: Medium F-18

3) Spore suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.4 mL of 1×10^7 spores/mL per 100 mL of the culture medium.

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

5) Silica gel. Dry silica gel for colum chromatography[Note 1] (particle size: 63 to 200 µm (230 to 70mesh)) at 110°C for 2 hours.

B. Preparation of sample solution

Extraction. Weigh accurately a quantity of the analysis sample (equivalent to 1 mg (potency) as LS)[2], place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of chloroform, extract with stirring for 20 minutes, and filter the extract through filter paper (No.5A). Transfer 25 mL of the filtrate to a 50-mL stoppered test tube, dehydrate with sodium sulfate (anhydrous), filter through filter paper (No.5A), and use the filtrate as the sample solution subject to column treatment.

Column treatment. Suspend 2.5 g of silica gel in methanol, pour into a column tube (10 mm in internal diameter), and wash with 20 mL of methanol and 50 mL of chloroform in this order to prepare the column.

Load accurately 5 mL of the sample solution onto the column, allow to flow out at a rate of 2 to 3 mL/min until the liquid level is 3 mm above the top of the packing material, and add 30 mL of chloroform and allow to flow out in the same manner[3].

Place a 50-mL recovery flask under the column, add 15 mL methanol to the column to elute LS, evaporate the eluate into dryness under reduced pressure in a water bath at 50°C, and add accurately 5 mL methanol to dissolve the residue. Accurately dilute a quantity of this solution with a mixture of water and methanol (5:1) to prepare a high-concentration sample solution with a concentration of 1 µg (potency)/mL[4]. Further, accurately dilute this solution with a mixture of water and methanol (3:1) to prepare a low-concentration sample solution with a concentration of 0.5 µg (potency)/mL[5].

C. Quantification[6]

Proceed by the 2-2 dose method[7].

Note 1. Use a Silica gel 40 (Merck) or an equivalent.

«Summary of analysis method»

This method is intended to determine the amount of LS in a chicken feed by microbiological assay using a sample solution prepared by extracting with chloroform, adsorbing to a silica gel column, and removing fat and oil. None of the antibacterial substances approved for combined use with LS interfere with the quantification of LS.

The flow sheet of this method is shown in Figure 9.2.28-5.

Sample (13.33 g, equivalent to 1 mg (potency) as LS)
|
Extract with 100 mL of chloroform (with a magnetic stirrer for 20 min).
|
Filter (through filter paper No.5A).
|
Dehydrate with with sodium sulfate (anhydrous).
|
Filter (through filter paper No.5A).
|
Load 5 mL of the filtrate onto a silica gel column (Merck Silica gel 40) (at a flow rate of 2-3 mL/min).
|
Wash the column with 30 mL of chloroform.
|
Elute LS with 15 mL of methanol (into a 50-mL recovery flask).
|
Evaporate into dryness under reduced pressure (in a water bath at 50°C).
|
Dissolve the residue in 5 mL of methanol.
|
Dilute with water-methanol (5:1) to prepare a high-concentration sample solution (1 μg (potency)/mL).
|
Dilute a quantity of the high-concentration sample solution with water-methanol (3:1) to prepare a low-concentration sample solution (0.5 μg
|
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 9.2.28-5 Quantitative test method for lasalocid sodium (chicken feed)

References: Kiyoshi Kanno: Livestock Research, 37(5), 8 (1983)

History in the Feed Analysis Standards [5] 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 grower formula feed	75~125	6	99.3~102.3	2.1
Finishing period broiler formula feed	75~125	6	98.3~100.7	3.6

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Broiler formula feed	7	75	100.8	2.7	5.6

«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 lasalocid standard solution is shown in Table 9.2.28-2.

Table 9.2.28-2 Method of preparation for lasalocid standard solution (chicken feed, example)

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

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

[2] Usually corresponds to 13.33 g.

[3] The flow rate for the washing shall be 2 to 3 mL/min.

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

Solution of dried residue	2 mL
Water-methanol (5:1)	18 mL

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

High-concentration sample solution	10 mL
Water-methanol (3:1)	10 mL

[6] An example standard response line for LS is shown in Figure 9.2.28-6.

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

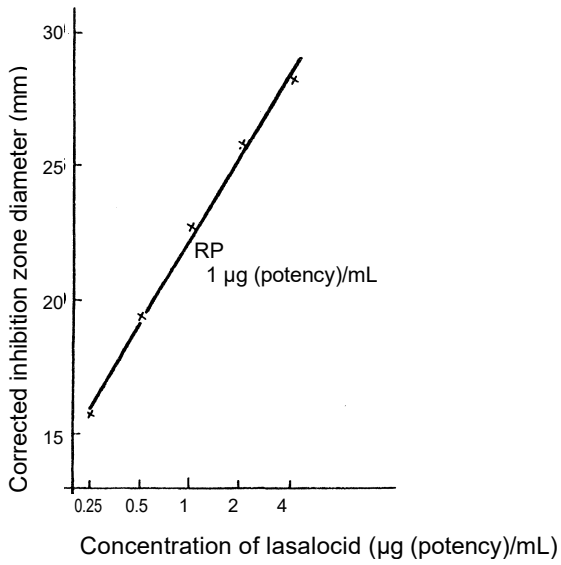


Figure 9.2.28-6 Standard response line for lasalocid (chicken feed, example)
(*Bacillus subtilis* ATCC 6633, Medium F-18, Agar well method)

1.3 Cattle feed

[Feed Analysis Standards, Chapter 9, Section 2, 28.2.2]

Scope of application: Cattle feed

A. Reagent preparation

1) Lasalocid standard solution. Weigh accurately not less than 40 mg of lasalocid working standard, accurately 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 a mixture of water and methanol (3:1) to prepare high- and low-concentration standard solutions with concentrations of 2 and 1 µg (potency)/mL, respectively[1].

2) Culture medium: Medium F-18

3) Spore suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.4 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 agar well method.

5) Enzyme solution. Dissolve 4 g of diastase in water to make 100 mL.

B. Preparation of sample solution

Extraction. Weigh 18.2 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 15 mL of the enzyme solution, stir, and allow to stand for 10 to 20 minutes at room temperature. Further, add 85 mL of acetonitrile, extract with stirring for 30 minutes, filter the extract through filter paper (No.5A), and use the filtrate as a sample solution subject to purification[2].

Purification. Transfer exactly 25 mL of the sample solution to a 200-mL separating funnel, add 25 mL of water[3], further add 50 mL of hexane, shake for 10 minutes, and allow to stand. Transfer the hexane phase (upper phase) to a 200-mL recovery flask. To the residual liquid add 50 mL of hexane, shake for 1

minutes, and transfer the hexane phase to the recovery flask. Further, to the residual liquid add 50 mL of hexane and proceed in the same manner. Evaporate the hexane phase into dryness under reduced pressure in a water bath at 50°C, add 10 mL of hexane to dissolve the residue, and use as a sample solution subject to column treatment.

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

Load the sample solution onto a minicolumn and allow to flow down until the amount in the minicolumn reservoir is 1 mL. Wash the recovery flask that contained the sample solution with 10 mL of hexane, add the washings to the minicolumn, and repeat this procedure 2 times. Then, load 20 mL of hexane and 20 mL of chloroform in this order to wash the minicolumn.

Place a 50-mL recovery flask under the minicolumn, and load 30 mL of a mixture of chloroform and methanol (4:1) onto the minicolumn to elute LS. Evaporate the eluate into dryness under reduced pressure in a water bath at 50°C, add exactly 10 mL of methanol to dissolve the residue, and filter this solution through filter paper (No.5A).

Accurately dilute a quantity of the filtrate with a mixture of water and methanol (45:7) to prepare a high-concentration sample solution with a concentration of 2 μg (potency)/mL[4]. Further, accurately dilute this solution with a mixture of water and methanol (3:1) to prepare a low-concentration sample solution with a concentration of 1 μg (potency)/mL[5].

C. Quantification[6]

Proceed by the 2-2 dose method[7].

«Summary of analysis method»

This method is intended to determine the amount of LS in a cattle feed by microbiological assay using a sample solution prepared by extracting with acetonitrile, separating by liquid-liquid partition, and purifying through a silica gel minicolumn.

The flow sheet of this method is shown in Figure 9.2.28-7.

Sample (18.2 g)
|
Add 15 mL of enzyme solution, stir, and allow to stand for 10-20 min.
|
Add 85 mL of acetonitrile and stir (with a magnetic stirrer for 30 min).
|
Filter (through filter paper No.5A).
|
To 25 mL of the riltrate add 25 mL of water and 50 mL of hexane and (in a 200-mL separating funnel) and shake for 10 min.
|
Collect the hexane phase (upper phase) (into a 200-mL recovery flask).
|
To the separating funnel add 50 mL of hexane, shake for 1 min, and transfer to a recovery flask (repeat 2times).
|
Evaporate into dryness under reduced pressure (in a water bath at 50°C).
|
Dissolve the residue with 10 mL of hexane.
|
Load on a silica gel minicolumn (previously washed with 10 mL of hexane).
|
Wash the round-bottom flask with 10 mL of hexane and load the washings onto the silica gel minicoumn (repeat 2 times).
|
Wash the silica gel minicolumn with 20 mL of hexane and 20 mL of chloroform in this order.
|
Elute LS with 30 mL of chloroform-methanol (4:1) (into a 50-mL recovery flask).
|
Evaporate into dryness under reduced pressure (in a water bath at 50 °C).
|
Dissolve the residue in 10 mL of methanol.
|
Filter (through filter paper No.5A).
|
Dilute a quantity of the filtrate with water-methanol (45:7) to prepare a high-concentration sample solution (2 μg (potency)/mL).
|
Dilute a quantity of the high-concentration sample solution with water-methanol (3:1) to prepare a low-concentration sample solution (1 μ
|
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 9.2.28-7 Quantitative test method for lasalocid sodium (cattle feed)

References: Ayako Shirato, Noriyuki Koyama: Research Report of Animal Feed, 16, 172 (1991)

History in the Feed Analysis Standards [13] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Meat cattle fattener formula feed 1	33	3	100.8~100.8	0.9
Meat cattle fattener formula feed 2	33	3	98.9~98.9	3.5

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Meat cattle fattener formula feed	11	33	95.7	3.5	6.0

«Notes and precautions»

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

An example method of preparation for lasalocid standard solution is shown in Table 9.2.28-3.

Table 9.2.28-3 Method of preparation for lasalocid standard solution (cattle feed, example)

Test tube No.	1	2	3	4
Amount (mL) of standard solution	②	2	5	5
Amount (mL) of water-methanol (3:1)	23	18	15	5
Concentration (μg (potency)/mL)	80	8	2	1

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

[2] In the case of pellet feed, the constituents can be altered as a result of heat-processing, making it difficult to extract LS with an ordinary solvent. For this reason, this method employs the use of an enxyme for decomposing a substance that can interfere with the extraction of LS with acetonitrile.

[3] The addition of water is intended to facilitate the transfer of LS to the hexane phase during liquid-liquid partition.

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

Filtrate	4 mL
Water-methanol (45:7)	26 mL

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

High-concentration sample solution	8 mL
Water-methanol (3:1)	8 mL

[6] An example standard response line for LS is shown in Figure 9.2.28-8.

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

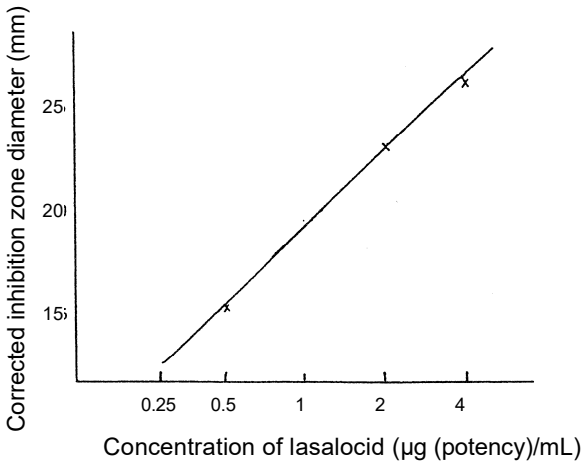


Figure 9.2.28-8 Standard response line for lasalocid (cattle feed, example)
(*Bacillus subtilis* ATCC 6633, Medium F-18, Agar well method)

2 1 Quantitative test method - Liquid chromatography

2.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 28.1.2]

A. Reagent preparation

Lasalocid sodium standard solution. Weigh accurately a quantity of lasalocid working standard equivalent to 50 mg (potency), place in a 100-mL volumetric flask, add methanol and dissolve, and further add methanol to the marked line to prepare a lasalocid sodium standard stock solution (1 mL of this solution contains an amount equivalent to 0.5 mg (potency) as lasalocid sodium).

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

B. Quantification

Extraction. Weigh accurately 2 to 5 g of the sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of methanol, and extract with stirring for 30 minutes. Transfer the extract to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and accurately dilute a quantity of the supernatant liquid with methanol. Further filter this solution 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 the lasalocid sodium standard solutions into a chromatograph to obtain chromatograms.

Example operating conditions

Detector: Fluorescence detector (excitation wavelength: 310 nm; emission wavelength: 420 nm)

Column: Octadecylsilanized silica gel column (4.6 mm in internal diameter, 250 mm in length, 5 μm in particle size)[Note 1] [1]

Eluent: A mixture of methanol and phosphoric acid buffer solution[Note2] (9:1)

Flow rate: 1.0 mL/min

Column temperature: 40°C

Calculation. Calculate the peak height or peak area from the obtained chromatograms[2] to prepare the

calibration curve, and estimate the amount of lasalocid sodium in the sample.

Note 1. Use a Shodex Silica C18M 4E (Showa Denko K.K.) or an equivalent

2. Disolve 2.72 g of potassium dihydrogen phosphate in water to make 1 L and adjust the pH to 2.9 to 3.1 with phosphoric acid (1:10).

«Summary of analysis method»

This method is intended to determine the amount of LS in a premix by liquid chromatography using a sample solution prepared by extracting with methanol and diluting with methanol.

The flow sheet of this method is shown in Figure 9.2.28-3.

2.0-5.0 g of the sample
↓
Add 100 mL of methanol and extract with stirring for 30 min.
↓
Centrifuge (at 1,500×g for 5 min).
↓
Filter through membrane filter (pore size not exceeding 0.5 μm).
↓
LC-FL (Ex: 310 nm, Em: 420 nm)

Figure 9.2.28-3 Quantitative test method for lasalocid sodium (premix)

References: Toru Hashimoto: Research Report of Animal Feed, 25, 29 (2000)

History in the Feed Analysis Standards [22] New

«Validation of analysis method»

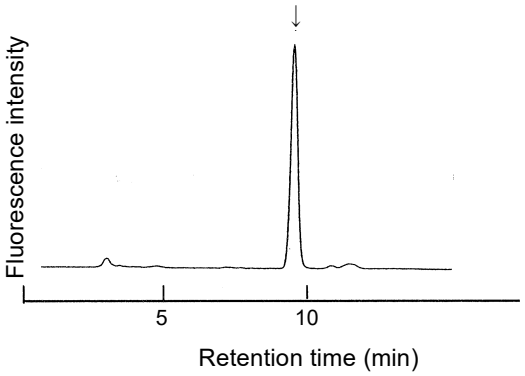
・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/kg)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Chicken premix 1	18.25~75	3	98.5~101.5	1.2
Chicken premix 2	18.25~75	3	95.8~100.1	2.6
Cattle premix	18.25~75	3	98.2~100.8	1.4

«Notes and precautions»

[1] Any column with an equivalent end-capped packing material is applicable.

[2] An example chromatogram for LS is shown in Figure 9.2.28-4.



Operating conditions

Detector: Measured wavelength: Em 310 nm; Ex 420 nm

Column: Shodex C18-5B

Eluent: A mixture of methanol and phosphoric acid buffer solution (9:1)

Flow rate: 1.0 mL/min

Column temperature: 40°C

Figure 9.2.28-4 Chromatogram for lasalocid sodium added to a chiken pemix
(The arrow indicates the peak of lasalocid)

2.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 28.2.3]

Scope of application: Chicken feed

A. Reagent preparation

1) Lasalocid sodium standard solution. Weigh accurately a quantity of lasalocid working standard equivalent to 50 mg (potency), place in a 100-mL volumetric flask, add methanol to dissolve, further add ethanol up to the marked line to prepare a lasalocid sodium standard stock solution (1 mL of this solution contains an amount of lasalocid sodium equivalent to 0.5 mg (potency)).

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

2) Enzyme solution. Dissolve 2.5 g of diastase in water to make 100 mL.

B. Quantification

Extraction

1) When the analysis sample is a heat-processed feed such as pellets

Weigh 10.0 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 20 mL of the enzyme solution, mix thoroughly[1], and allow to stand in a water bath at 40°C for 20 minutes. Further, add 80 mL of methanol, and extract with stiring for 10 minutes[2]. Transfer 50 mL of the extract to a centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, filter the supernatant liquid through membrane filter (pore size not exceeding 0.5 µm), and use the filtrate as a sample solution subject to liquid chromatography.

2) When the analysis sample is a feed other than heat-processed feeds such as pellets

Weigh 10.0 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of methanol, and extract with stirring for 30 minutes. Transfer the extract to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and filter the supernatant liquid through membrane filter (pore size not exceeding 0.5 µm), and use the filtrate as a sample solution subject to liquid chromatography.

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

Example operating conditions

Detector: Fluorescence detector (excitation wavelength: 310 nm, emission wavelength: 420 nm)

Column: Octadecylsilanized silica gel column (4.6 mm in internal diameter, 250 mm in length, 5 μm in particle size)[Note 1] [3]

Eluent: A mixture of methanol and phosphoric acid buffer solution[Note 2] (9:1)

Flow rate: 1.0 mL/min

column temperature: 40°C

Calculation. Calculate the peak height or peak area from the obtained chromatograms[4] to prepare the calibration curve, and estimate the amount of lasalocid sodium in the sample.

Note 1. Use Shodex Silica C18M 4E (Showa Denko K.K.) or an equivalent.

2. Dissolve 2.72 g of potassium dihydrogen phosphate in water to make 1 L and adjust the pH to 2.9 to 3.1 with phosphoric acid (1:10).

«Summary of analysis method»

This method is intended to determine the amount of LS in a chicken feed by liquid chromatography equipped with a fluorescence detector using a sample solution prepared by extraction and purification. When the analysis sample is a pellet feed, it shall be treated with an enzyme solution and heated before being extracted with methanol. When the analysis sample is a feed other than pellets, it shall be extracted with ethanol.

The flow sheet of this method is shown in Figure 9.2.28-9.

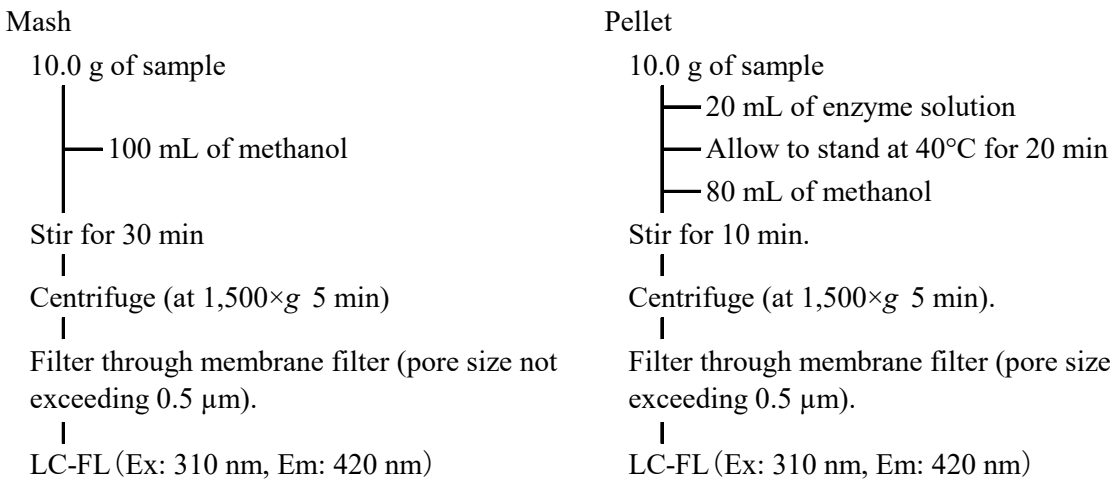


Figure 9.2.28-9 Quantitative test method for lasalocid sodium (chicken feed)

References: Eiichi Ishiguro: Research Report of Animal Feed, 14, 96 (1988)

Yuzo Ono, Jun Noguchi: Research Report of Animal Feed, 24, 91 (1999)

History in the Feed Analysis Standards [21] 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 grower formula feed 1 (not heat-processed)	37.5~112.5	3	97.3~99.7	5.2
Starting chick grower formula feed 2 (not heat-processed)	37.5~112.5	3	95.6~100.4	5.5
Broiler fattener starter formula feed (not heat-processed)	37.5~112.5	3	98.8~103.2	4.9
Starting chick grower formula feed (heat-processed)	37.5~112.5	3	104.4~107.4	4.0

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr (%)	Inter-lab reproducibility RSDR (%)
Starting chick grower formula feed (not heat-processed)	6	75	96.9	1.6	2.1
Starting chick grower formula feed (heat-processed)	6	75	106.3	1.7	4.0

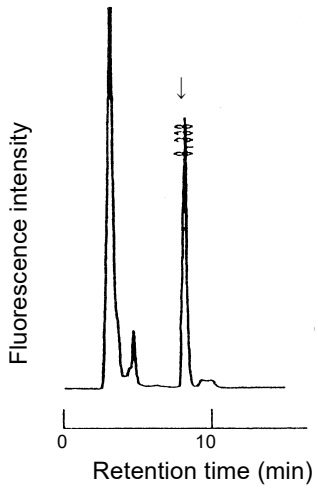
«Notes and precautions»

[1] Moisten the whole analysis sample.

[2] In case of pellet feed, the constituents can be altered as a result of heat-processing, making it difficult to extract LS with methanol. For this reason, this method employs the use of an enzyme for decomposing a substance that can interfere with the extraction of LS with ethanol.

[3] Any column with an equivalent end-capped packing material is applicable.

[4] An example chromatogram is shown in Figure 9.2.28-10.



Operating conditions

Detector: Measured wavelength: Ex 310 nm; Em 420 nm

Column: Shodex C18-5B

Eluent: A mixture of methanol and phosphoric acid buffer solution (9:1)

Flow rate: 1.0 mL/min

Figure 9.2.28-10 Chromatogram for a chicken feed (other than pellets) spiked with lasalocid sodium

(The arrow indicates the peak of lasalocid)

3 Trace quantitative test method (feed)

3.1 Trace quantitative test method for polyether antibiotics by microbioautography [Feed Analysis Standards, Chapter 9, Section 2, 28.3.1]

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.

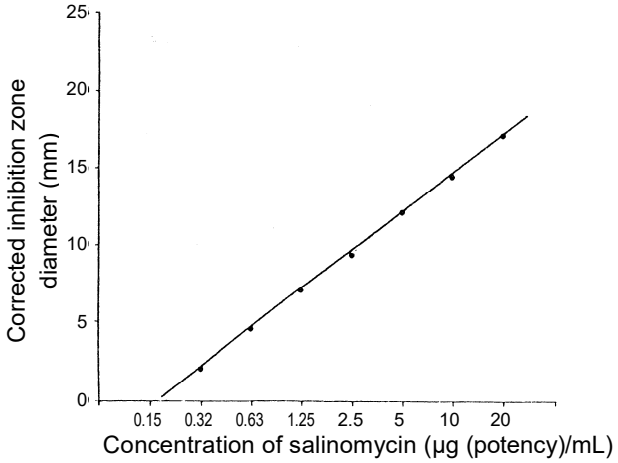


Figure 9.3.3-2 Standard response line for salinomycin (trace quantitation test method, feed)
(*Bacillus subtilis* ATCC 6633, Medium F-22, Microbioautography)

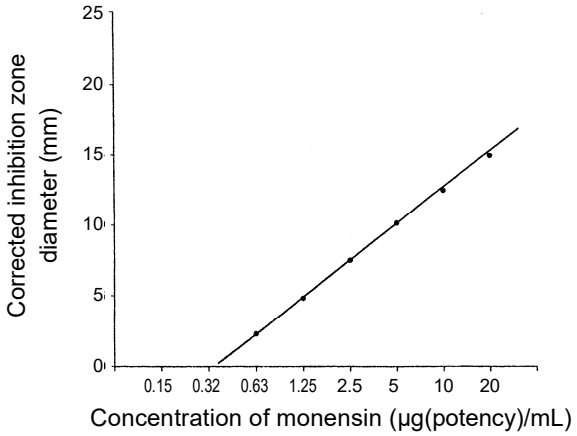


Figure 9.3.3-3 Standard response line for monensin (trace quantitation test method, feed)
(*Bacillus subtilis* ATCC 6633, Medium F-22, Microbioautography)

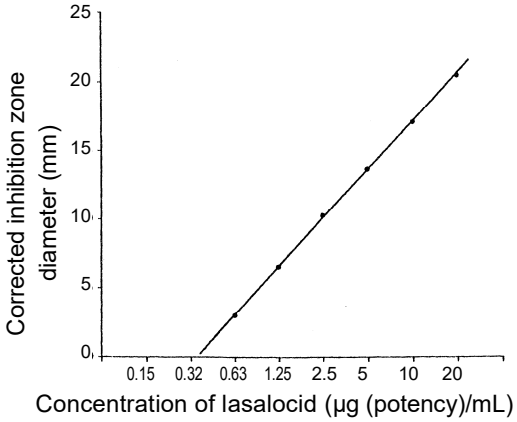


Figure 9.3.3-4 Standard response line for lasalocid (trace quantitation test method, feed)
(*Bacillus subtilis* ATCC 6633, Medium F-22, Microbioautography)

[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, 28.3.2]

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.

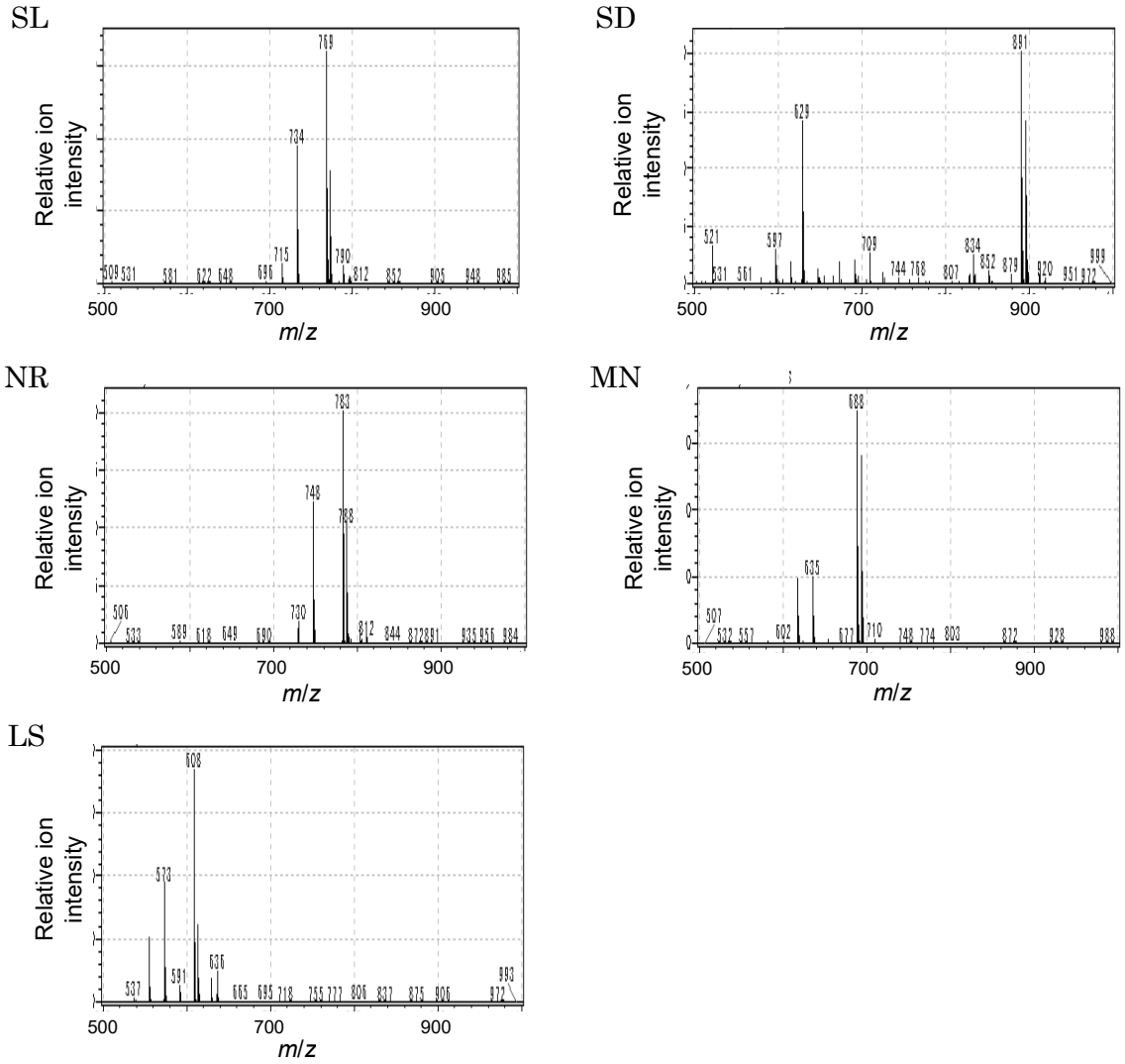
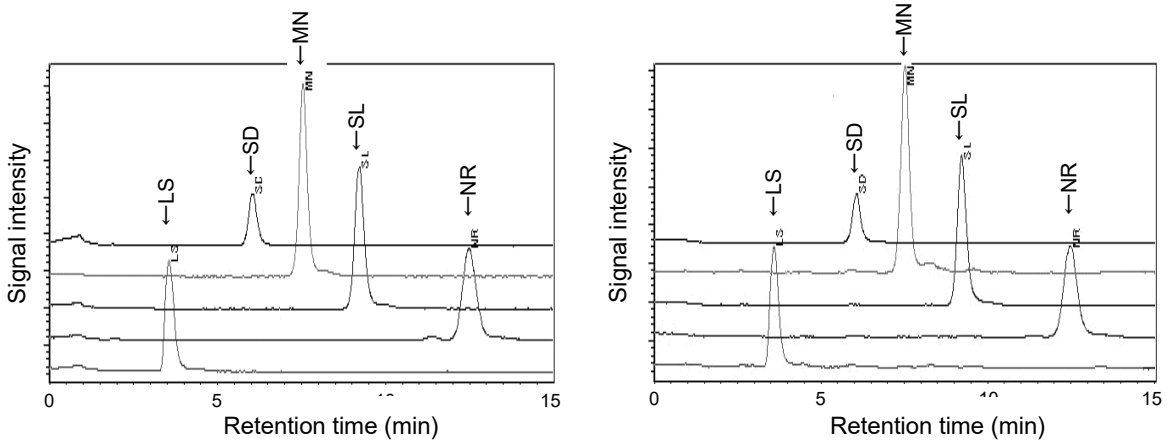


Figure 9.3.4-2 Mass spectrum for each antibiotic

[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, 28.4.1]

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, 28.5.1]

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 Absorption spectrometry (chicken feed)

[58 Chiku B No.1676, notified by the Head of the Livestock Industry, Ministry of Agriculture, Forestry and Fisheries, as of July 6, 1983]

1. Reagents and reagent preparation

(1) Methanol (guaranteed grade)

(2) Diethyl ether (guaranteed grade)

(3) Sodium hydroxide (guaranteed grade)

(4) Ethyl acetate (guaranteed grade)

(5) *p*-Nitrobenzene diazonium fluoroborate solution. Dissolve 50 mg of *p*-nitrobenzene diazonium fluoroborate in water to make 100 mL (prepare at the time of use).

(6) Lasalocid sodium standard solution. Weigh accurately 40 mg of lasalocid sodium working standard (potency), place in a 100-mL volumetric flask, add methanol to dissolve, and further add methanol up to the marked line to prepare a lasalocid sodium standard stock solution (1 mL of this solution contains 400 μg (potency) of lasalocid sodium).

At the time of use, dilute the stock solution exactly 10-fold with methanol to prepare a lasalocid sodium standard solution[Note 1] (1 mL of this solution contains 40 μg (potency) of lasalocid sodium).

2. Quantification[Note2]

Extraction. Weigh accurately 10 g of the analysis sample, place in a 100-mL stoppered Erlenmeyer flask, add accurately 30 mL of diethyl ether[Note 3], stir for 10 minutes with a magnetic stirrer, allow to stand for 5 minutes, filter the supernatant liquid under pressure[Note 4], and use the filtrate as the sample solution.

Development and measurement. Immediately transfer exactly 4 mL of the sample solution[Note 5] to a 10-mL stoppered test tube, evaporate into dryness in a water bath at 50°C [Note 6], add accurately 6 mL of methanol, shake for 1 minute[Note 7], and filter through dry filter paper[Note 8]. Transfer exactly 3 mL of the filtrate to a 10-mL stoppered test tube, dissolve under ice-cooling, add exactly 1 mL of *p*-nitrobenzene diazonium fluoroborate and exactly 1 mL of 4 % sodium hydroxide solution, and determine the

absorbance at a wavelength of 560 nm using methanol as the blank[1] Note 9.

Preparation of calibration curve[Note 10]. Weigh accurately 20 g of a lasalocid sodium-unspiked sample (with the same composition as the analysis sample except for the absence of lasalocid sodium), place in a 100-mL stoppered Erlenmeyer flask, add accurately 60 mL of diethyl ether[Note 11], stir for 10 minutes with a magnetic stirrer, allow to stand for 5 minutes, and filter the supernatant liquid by injection under pressure[Note 4]. Transfer exactly 4 mL of the filtrate[Note 12] to each of 10-mL stoppered test tubes A, B, C, D and E, and evaporate into dryness in a water bath at 50°C[Note 6]. To the respective test tubes add 0, 1, 2, 3 and 4 mL of the lasalocid sodium standard solution[Note 13], further add methanol to make exactly 6 mL in each tube[Note 14], shake for 1 minute[Note 7], and filter [Note 15] through dry filter paper[Note 8]. Then proceed in the same manner as described for the sample solution and determine the absorbance to prepare the calibration curve.

Calculation. Calculate the amount of lasalocid sodium in the sample solution from the calibration curve and estimate the amount of lasalocid sodium in the sample according to the following equation.

$$\text{Amount (g (potency)/t) of lasalocid sodium in the sample} = L \times \frac{30}{W} \times \frac{1}{4}$$

L: Amount (μg (potency)) of lasalocid sodium in the sample solution calculated from the calibration curve

W: Weight (g) of the sample used for analysis (g)

Note 1. The standard solution can be stored for about 1 week in a refrigerator.

2. Avoid direct sunlight during quantification.

3. Ethyl acetate can be used in lieu of diethyl ether.

4. Cut out a piece of Toyo filter paper No.6 or an equivalent and set it in a membrane filter holder equipped with a syringe etc.

   Instead of filtering by injection under pressure, it is permissible to transfer the extract to a stoppered centrifuge tube and centrifuge (at 3,000 rpm for 5 minutes). When using ether, care should be taken not to catch fire.

5. When using ethyl acetate, the amount of the sample solution shall be 2 mL, and the amount of methyl alcohol after evaporating into dryness shall be 3 mL. The filtration procedure can be skipped to proceed to the step of cooling in ice.

6. Evaporation shall be performed with a ventilation system, such as draft chamber. Care should be taken not to catch fire. When using ethyl acetate, evaporate the sample solution into dryness in a water bath at 90°C or higher. When the sample is rich in fat, it can evaporate into a tarry mass but not into dryness, which however, is acceptable as long as there is no ether (ethyl acetate) odor left.

7. Shake vigorously with the hand. It is permissible to shake in a shaker or an ultraviolet washer. Undissolved residues can be left on the test tube wall, but do not affect the quantification.

8. Use Toyo filter paper No.6 or an equivalent.

9. Determine the absorbance within 1 hour after development.

10. When proceeding under the same conditions (standard solution, evaporating temperature, time

of operation, etc.), the slope of the calibration curve is not affected but the blank value is affected by the composition of raw materials of feed. As long as the test is performed under the same conditions, therefore, a previously prepared calibration curve can be used after correcting for the blank value (absorbance of the sample solution − absorbance of the unspiked feed).

11. Use ethyl acetate if ethyl acetate is used in Note 3.
12. When using ethyl acetate, the amount shall be 2 mL.
13. When using ethyl acetate, the amounts shall be 0, 0.5, 1.0, 1.5 and 2.0 mL, respectively.
14. When using ethyl acetate, the amount shall be 3 mL.
15. When using ethyl acetate, this process can be skipped.

«Summary of analysis method»

This method is intended to determine the absorbance of LS in a feed at a wavelength of 560 nm using a sample solution prepared by extracting with diethyl ether, changing the solvent to methanol, and developing with *p*-nitrobenzene diazonium fluoroborate.

The flow sheet of this method is shown in Figure 9.2.28-11.

Analysis sample (10 g)	Unspiked sample (20 g)				
Extract with 30 mL of diethyl ether (with a magnetic stirrer for 30 min).	Extract with 60 mL of diethyl ether (with a magnetic stirrer for 30 min).				
Filter (through filter paper No.6) by injection under pressure or centrifuge (at 3,000 rpm (1,500×g) for 5 min).	Filter (through filter paper No.6) by injection under pressure or centrifuge (at 3,000 rpm (1,500×g) for 5 min).				
4 mL of filtrate or supernatant	4 mL (A) of filtrate or supernatant	4 mL (B) of filtrate or supernatant	4 mL (C) of filtrate or supernatant	4 mL (D) of filtrate or supernatant	4 mL (E) of filtrate or supernatant
Evaporate into dryness under reduced pressure (at 50°C)	Same as left	Same as left	Same as left	Same as left	Same as left
6 mL of methanol	6 mL of methanol	5 mL of methanol	5 mL of methanol	5 mL of methanol	5 mL of methanol
		1 mL of standard solution (40 µg (potency)/mL)	1 mL of standard solution (80 µg (potency)/mL)	1 mL of standard solution (120 µg (potency)/mL)	1 mL of standard solution (160 µg (potency)/mL)
Shake for 1 min.	Same as left	Same as left	Same as left	Same as left	Same as left
Filter (through filter paper No.6).	Same as left	Same as left	Same as left	Same as left	Same as left
3 mL of filterate (10-mL stoppered test tube)	Same as left	Same as left	Same as left	Same as left	Same as left
Cool in ice.	Same as left	Same as left	Same as left	Same as left	Same as left
1 mL of diazotization solution[Note]	Same as left	Same as left	Same as left	Same as left	Same as left
1 mL of 4% NaOH solution	Same as left	Same as left	Same as left	Same as left	Same as left
Determine the absorbance (at 560 nm).	Same as left	Same as left	Same as left	Same as left	Same as left

Note: *p*-Nitrobensene diazonium fluoroborate solution

Figure 9.2.28-11 Flow sheet of rapid quantitative method for lasalocid sodium in feed (ether extraction method)

References: Yoshitsugu Tanaka, Tomoyuki Toyota, Shigetaka Miyoshi: Research Report of Animal Feed, 9, 140 (1984)

«Notes and precautions»

[1] After development, allow the sample solution to stand for 5 to 10 minutes before determining the absorbance.

5.2 Liquid chromatography (chicken feed and cattle feed (including pellets))

[3 Chiku B No.1113, notified by the Head of the Livestock Industry Bureau, Ministry of Agriculture, Forestry and Fisheries, as of June 3, 1991]

1. instruments and equipments

(1) Stoppered Erlenmeyer flask
(2) Volumetric flask
(3) Pipette
(4) Volmetric cylinder
(5) Microsyringe
(6) Magnetic stirrer
(7) Water bath
(8) High-performance liquid chromatograph

2. Reagents

(1) Methanol (guaranteed grade)
(2) Monophosphate dihydrogen phosphate (guaranteed grade)
(3) Phosphoric acid (guaranteed grade)
(4) Takadiastase. An enzyme obtained by the extraction and pufirication of a cultured filtrate of *Aspergillus oryzae*, mainly containing amylase, a digestive enzyme for carbohydrate.
(5) Lasalocid sodium standard for feed analysis

3. Preparation of reagents

(1) Lasalocid sodium working standard solution

Weigh accurately 50 mg (potency) of lasalocid sodium standard, place in a 100-mL volumetric flask, add methanol to dissolve, further add methanol up to the marked line to prepare a lasalocid sodium standard stock solution (1 mL of this solution contains lasalocid sodium 500 µg (potency)).

At the time of use, accurately dilute this stock solution with methanol to prepare lasalocid sodium standard solutions containing amounts equivalent to 2, 4 and 6 µg (potency)/mL in the case of cattle feed, or amounts equivalent to 6, 8 and 10 µg (potency)/mL in the case of chicken feed[Note 1]

(2) Enzyme solution [Note 1].

Dissolve 2.5 of of takadiastase in water to make 100 mL.

4 Quantification

(1) Extraction[Note 2]

Weigh 10 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 20 mL

of the enzyme solution, mix thoroughly, and allow to stand in a water bath at 40°C for 20 minutes. Then, add 80 mL of methanol, stir for 10 minutes with a magnetic stirrer, filter the supernatant liquid through membrane filter (pore size not exceeding 0.5 μm), and use the filtrate as the sample solution.

(2) High-performance liquid chromatography

Inject 20 μL each of the sample solution and lasalocid sodium standard solutions into a high-performance liquid chromatograph using a microsyringe to obtain chromatograms[1].

Example operating conditions[Note 3]

Detector:	Fluorescence detector (excitation wavelength: 310 nm, emission wavelength: 419 nm)
Column:	A stainless steel tube 4.6 mm in internal diameter and 250 mm in length, packed with octadecylsilanized silica gel 5 μm in particle size.
Eluent:	A mixture of methanol and 10 mmol/L monophosphate dihydrogen phosphate solution (adjust the pH to 2.1 with phosphoric acid) (9:1)
Flow rate:	1.0 mL per minute

(3) Calculation

Calculate the amount of lasalocid sodium in the sample according to the following equiation.

$$\text{Amount (g (potency/t) of lasalocid sodium in the sample} = \frac{A}{2}$$

A: Weight (ng (potency)) of lasalocid sodium calculated from the calibration curve

Note 1. When it is difficult to dissolve, apply ultrasonic waves.

2. In the case of powder feed, the expression “add 20 mL of the enzyme solution, mix thoroughly, and allow to stand in a water bath at 40°C for 20 minutes. Then, add 80 mL of methanol” shall be replaced with “add 100 mL of methanol”.

3. The methanol used as eluent shall be a regent for high-performance liquid chromatography or an equivalent.

«Summary of analysis method»

This method is intended to determine the amount of LS in a chicken feed or cattle feed (including pellets) by liquid chromatography equipped with a fluorescence detector using a sample solution prepared by extraction.

The flow sheet of this method is shown in Figure 9.2.28-12.

10 g of the analysis sample

↓

Add 20 mL of enzyme solution and allow to stand at 40°C for 20 min.

↓

Add 80 mL of methanol and extract (with a magnetic sitter for 10 min)

↓

Supernatant liquid.

↓

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

↓

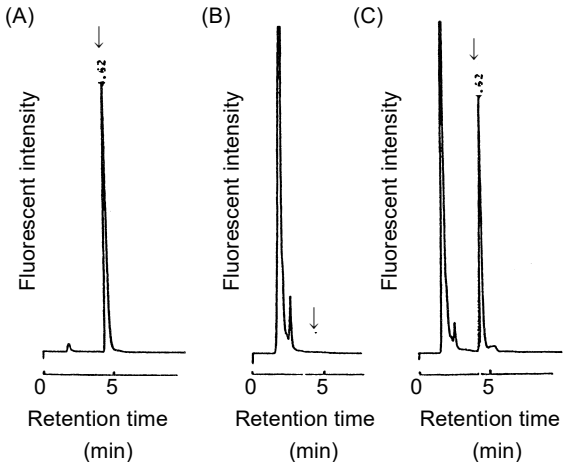
LC-FL (Ex: 310 nm, Em: 419 nm)

Figure 9.2.28-12 Rapid quantitative method for lasalocid sodium in a feed

References: Yoshitsugu Tanaka, Tomoyuki Toyota, Shigetaka Miyoshi: Research Report of Animal Feed, 9, 140 (1984)

«Notes and precautions»

[1] An example chromatogram is shown in Figure 9.2.28-13.



A: LS standard solution (150 ng)

B: Finishing period broiler formula feed (unspiked)

C: Finishing period broiler formula feed (spiked with LS equivalent to 75 mg/kg)

Column: Wakosil 5C18, Wako Pure Chemical Industries, Ltd.

Figure 9.2.28-13 Chromatogram for lasalocid sodium

(The arrow indicates the peak of lasalocid)

5.3 Spectrophotometry (feed other than powder feed for chickens)

[6 Chiku B No.1012, 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) Volumetric flask

(3) Separating funnel

(4) Recovery flask

(5) Stoppered centrifuge tube

(6) Syringe

(7) Stoppered test tube

(8) Magnetic stirrer

(9) Centrifuge

(10) Water bath

(11) Spectrophotometer

(12) Chemical balance

(13) Ultrasonic cleaner

2. Reagents

(1) Acetonitrile (guaranteed grade)

(2) Anhydrous ethanol (guaranteed grade)

(3) Methanol (guaranteed grade)

(4) Acetone (guaranteed grade)

(5) Hexane (guaranteed grade)

(6) Chloroform (guaranteed grade)

(7) Potassium hydroxide (guaranteed grade)

(8) Silica gel cartridge[Note 1]

(9) Takadiastase. An enzyme obtained by extraction and purification of a culture filtrate of *Aspergillus oryzae*, mainly containing amylase, a digestive enzyme for carbohydrate. It is a hygroscopic light-yellow powder.

(10) *p*-Nitrobenzene diazonium fluoroborate

(11) Lasalocid working standard

(12) Nitrogen gas

3. Preparation of reagents

(1) Lasalocid sodium standard solution. Weigh 100 mg (potency) of lasalocid standard, place in a 100-mL volumetric flask, add ethanol up to the marked line and dissolve to prepare a lasalocid sodium standard stock solution[1] (1 mL of this solution contains lasalocid sodium equivalent to 1 mg (potency)).

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

(2) Enzyme solution. Dissolve 4 g of takadiastase in 100 mL of water. Prepare at the time of use.

(3) *p*-Nitrobenzenediazonium fluoroborate solution. Dissolve 50 mg of *p*-nitrobenzenediazonium fluoroborate in 100 mL of water. Prepare at the time of use.

4. Quantification

(1) Extraction

Weigh 15 g of the analysis sample, place in a 200-mL stoppered Erlenmeyer flask, add 15 mL of the enzyme solution, mix thoroughly, and allow to stand in a water bath at 40°C for 20 minutes. Then, add 85 mL of acetonitrile, stir for 30 minutes with a magnetic stirrer, and filter the supernatant liquid through filter paper (No.5A). Transfer exactly 50 mL of the filtrate to a 300-mL separating funnel, add 50 mL of water, mix, add 100 mL of hexane, shake for 10 minutes, allow to stand, and transfer the hexane phase (upper phase) to a 300-mL recovery flask. Add 100 mL of hexane to the

separating funnel, shake for 1 to 2 minutes, allow to stand, and transfer the hexane phase to the recovery flask. Evaporate this solution into dryness under reduced pressure in a water bath at approximately 50°C. Add 10 mL of hexane to dissolve the residue and use as the sample solution subject to cartridge column chromatography.

(2) Cartridge column chromatography

Connect a silica gel cartridge with a syringe and wash with 10 mL of hexane[Note2]. Transfer the sample solution to the syringe, wash the recovery flask with 2 to 3 mL of hexane, and transfer the washings to the syringe. Further, repeat this washing procedure 2 times, allow to flow down until the amount is 1 mL or less[Note2], and add 30 mL of chloroform to wash the syringe[Note2]. Place a 50-mL stoppered centrifuge tube under the silica gel cartridge, and elute lasalocid sodium with 10 mL of a mixture of acetone and methanol (4:1)[Note2]. Evaporate the eluate into dryness with nigrogen gas in a water bath at 50°C, to the residue add exactly 20 mL of anhydrous ethanol, dissolve with ultrasonic waves for 1 minutes, and use as the sample solution.

(3) Development

Transfer exactly 4 mL of the sample solution to a 25- to 30-mL stoppered test tube, add accurately 6 mL of anhydrous ethanol, mix, allow to cool in an ice water bath for 5 minutes, add accurately 1 mL of *p*-nitrobenzenediazonium fluoroborate solution, and mix. Allow the stoppered test tube to stand in an ice water bath for 10 minutes, add 1 mL of sodium hydroxide (4 w/v%) solution, mix, and develop. Allow the stoppered test tube to return to room temperature and determine the absorbance at the wavelength of maximum absorption of about 560 nm, using anhydrous ethanol as the blank.

(4) Preparation of calibration curve

Proceed as described in (1) and (2) with 15 g of a lasalocid sodium-unspiked sample (with the same composition as the analysis sample except for the absence of lasalocid sodium) to prepare an unspiked sample solution.

Transfer exactly 4 mL of the unspiked sample solution to each of 25- to 30-mL stoppered test tubes A, B, C and D. Add 6 mL each of the lasalocid standard solutions with concentrations of 5, 10, 15 µg (potency)/mL or anhydrous ethanol to the stoppered test tubes, develop as directed in (3), and determine the absorbance to prepare the calibration curve [2].

(5) Calculation

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

$$\text{Amount (g (pitency)/t) of lasalocid sodium in the sample} = A \times 4$$

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

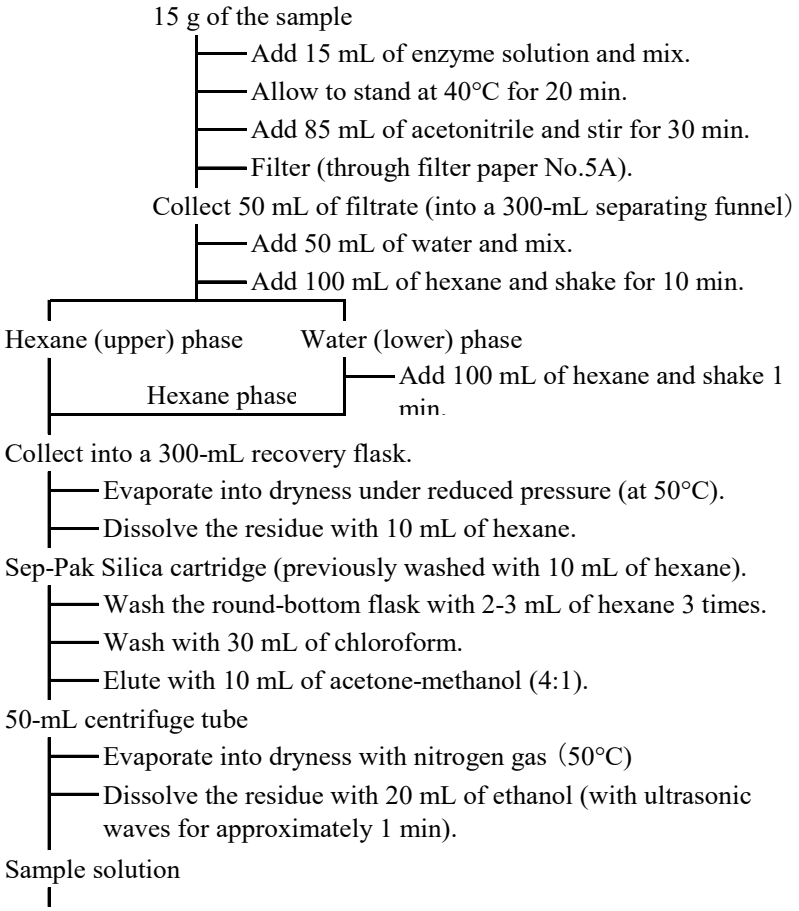
Note 1. Use a Sep-Pak Plus Silica cartridge (Waters) or an equivalent.

2. Inject under pressure at a rate of 2 to 3 mL/min.

«Summary of analysis method»

This method is intended to determine the absorbance of LS in a feed (mainly pellet feed for cattle) at a wavelength of 560 nm using a sample solution prepared by extracting with an enzyme solution and acetonitrile, changing the solvent to hexane, purifying through a silica gel minicolumn, and developing by diazotization.

The flow sheet of this method is shown in Figure 9.2.28-14.



Spiked sample solution	Unspiked sample solution			
4 mL (test tube)	4 mL (test tube A)	4 mL (test tube B)	4 mL (test tube C)	4 mL (test tube D)
6 mL of ethanol	6 mL of ethanol	6 mL of Standard solution 5 μg(potency)/mL	6 mL of Standard solution 10 μg(potency)/mL	6 mL of Standard solution 15 μg(potency)/mL
Mix.	Same as left	Same as left	Same as left	Same as left
Allow to stand in a cold water bath (at 2-4°C for 5 min).	Same as left	Same as left	Same as left	Same as left
Add 1 mL of diazotization solution[Note] and mix	Same as left	Same as left	Same as left	Same as left
Allow to stand in a cold water bath (at 2-4°C for 10 min).	Same as left	Same as left	Same as left	Same as left
Add 1 mL of 4% NaOH solution and mix.	Same as left	Same as left	Same as left	Same as left
Allow to return to room temperature	Same as left	Same as left	Same as left	Same as left
Determine absorbance (at 560 nm).	Same as left	Same as left	Same as left	Same as left

Note: *p* -Nitrobenzene diazonium fluoroborate solution

Figure 9.2.28-14 Rapid quantitative method for lasalocid sodium

References: Noriyuki Koyama, Atsushi Kito, Shigetaka Suzuki: Research Report of Animal Feed , 20, 124 (1995)

«Notes and precautions»

[1] The standard stock solution is stable for approximately 3 when stored at 4°C.

[2] An example calibration curve for lasalocid sodium is shown in Figure 9.2.28-15.

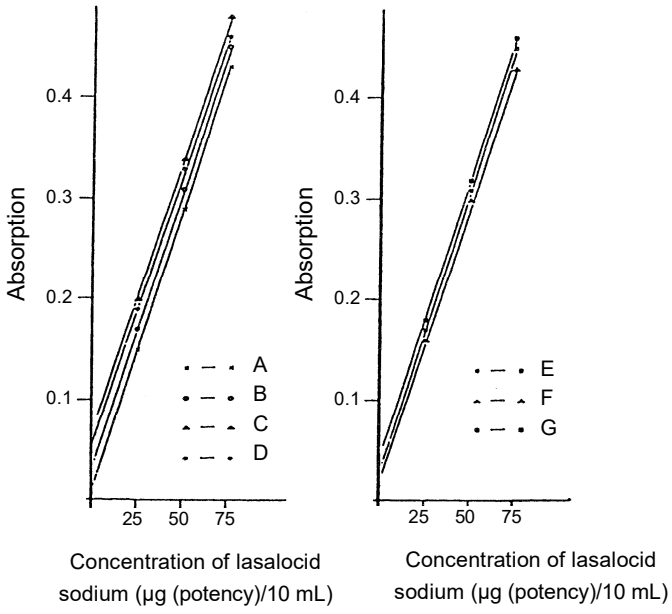


Figure 9.2.28-15 Calibration curves for 7 kinds of feeds