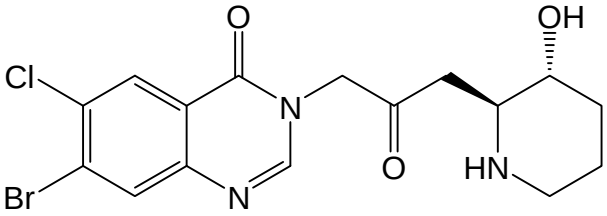
13 Calcium halofuginone polystyrenesulfonate (HPS)

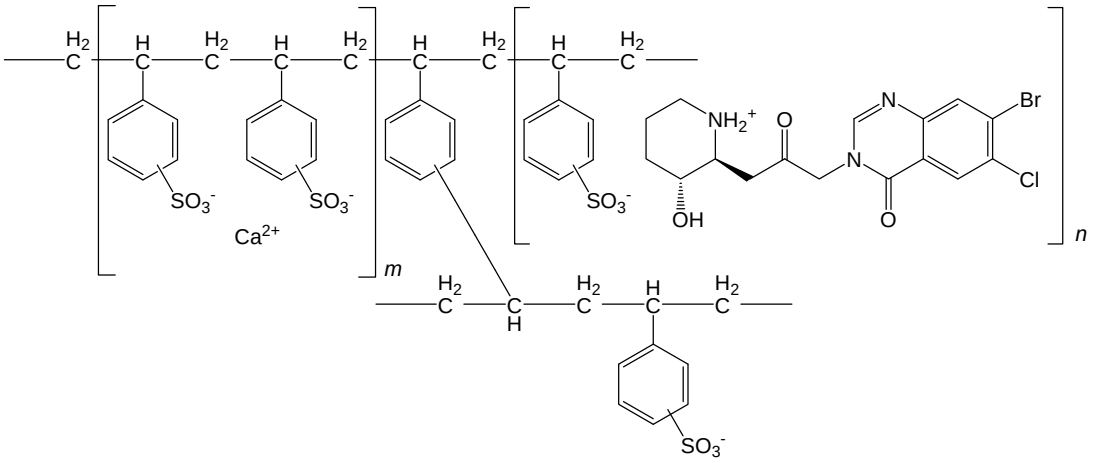
Halofuginone



7-bromo-6-chloro-3-[3-[(2*S*,3*R*)-3-hydroxy-2-piperidinyl]-2-oxopropyl]-4-quinazolinone

$C_{16}H_{17}BrClN_3O_3$ MW: 414.68 CAS No.: 55837-20-2

HPS



【Outline of calcium halofuginone polystyrenesulfonate】

Halofuginone is a synthetic alkaloid developed in 1964 by American Cyanamid (USA), also called chlorobromofebrifugine.

A dextrorotatory compound of halofuginone was synthesized by Roussel-Uclaf (France) in 1976, which was found to have anticoccidial action, and reported to be effective to prevent avian leucocytozoon.

However, halofuginone showed acute toxicity at 3.5 mg/kg (mice, p.o.), being in the category of poisonous substances in Japan; therefore, halofuginone derivatives with lower toxicity was going to be developed.

Calcium halofuginone polystyrenesulfonate (HPS) is a new halofuginone derivative with low acute toxicity, synthesized in 1979 by Sankyo Kasei Kogyo, and was approved in 1987 to add to feeds for chickens at 40 g/t intending to promote the effective use of nutrient components in feeds.

HPS, if excessively received by chickens, cause development disorders. Therefore, the additive amount (40 g/ton) must be followed strictly, and the additive must be mixed evenly to ensure the safety.

For these reasons, feed makers were required to control the feeds containing HPS according to the appended methods (Notification of Livestock Industry Bureau and Fisheries Agency, MAFF, 62 Chiku B 3099, December 25, 1987)

(Appended items)

Control methods of feeds containing calcium halofuginone polystyrenesulfonate

1. Feed makers must determine the amount of calcium halofuginone polystyrenesulfonate in each production lot by the quantification method given below (3. Rapid quantification method), and can sell only feeds with the quantitative value within the control limit specified under 2.
2. The control limit is 100±25 % of the amount of calcium halofuginone polystyrenesulfonate permitted to be contained in feeds.
3. The production lot described under 1 means each brand lot continuously manufactured within one day, and sampling of feed from the lot should be performed according to the MAFF Notification No. 757-2-1-(1)-A, July 24, 1976.
4 Feed makers must record the state of manufacture/control for the feed containing calcium halofuginone polystyrenesulfonate, including the name of the feed, date of manufacture, lot no., manufacture amount, analytical data and judgment, and must store the records for 1 year.

【Methods listed in the Feed Analysis Standards】

1 Quantitative test methods

1.1 Liquid chromatography (premix)

〔Feed Analysis Standards Chapter 8, Section 1, 13.1.1-(1)〕

A. Reagent preparation

Halofuginone hydrobromide standard solution: Place 20 mg of halofuginone hydrobromide 〔$C_{16}H_{18}Br_2ClN_3O_3$〕 exactly measured in a 200 mL volumetric flask, add hydrochloric acid (0.1 mol/L) for dissolving [1], and further add the solution up to the gauge line to prepare the halofuginone hydrobromide standard stock solution (1 mL of this solution contains an amount of halofuginone hydrobromide equivalent to 0.1 mg).

At the time of use, dilute a definite amount of the standard stock solution exactly with hydrochloric acid (0.1 mol/L) to prepare several halofuginone hydrobromide standard solutions containing amounts of halofuginone hydrobromide equivalent to 0.5-5 μg/mL [2].

B. Quantification

Extraction: Place 2 g of the analysis sample exactly measured in a stoppered 100 mL Erlenmeyer flask, add 25 mL of butyl acetate and 50 mL of nitric acid (2:3), and stir for 30 min for extraction. Place the nitric acid layer (lower layer) in a centrifuging tube to centrifuge at 1,500×*g* for 5 min. Add exactly 2 mL of the supernatant in a 50 mL volumetric flask containing 0.5 g of calcium carbonate and 15 mL of water [3], and allow still standing for 30 min. Then, add water up to the gauge line of the volumetric flask, and filter this solution through a membrane filter (pore diameter: 0.5 μm or less) to obtain a sample solution for liquid chromatography.

Liquid chromatography: Inject respective 20 μL of the sample solution and each halofuginone hydrobromide standard solution into a liquid chromatograph to obtain the chromatogram.

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 243 nm)

Column: Octadecylsilylated silica-gel column (internal diameter: 4.6 mm, length: 250 mm, particle diameter: 5 μm) Note1 [4]

Column temperature: 40 °C

Eluent: Acetic acid buffer solution Note 2-methanol (11:9) [5][6]

Flow rate: 1.0 mL/min

Calculation: Obtain the peak height or area from the chromatogram [7], and calculate the amount of calcium halofuginone polystyrenesulfonate (C (g/kg)) using the formula given below.

$$C = \frac{A \times 3{,}125}{8.5}$$

A : The weight of halofuginone hydrobromide (ng) obtained from the calibration curve.

Note 1. Wakosil $5C_{18}$-200 (Wako Pure Chemical Industries) or an equivalent one.

2. Dissolving 4.85 g of ammonium acetate and 7.5 mL of acetic acid in water to make a total amount of 1 L, and adjust the pH to 4.3 with acetic acid.

《Summary of analysis method》

This method is intended to determine the amount of halofuginone by making halofuginone free from calcium halofuginone polystyrenesulfonate by adding nitric acid (2:3) and *n*-butyl acetate to a premix, extracting it in the nitric acid layer, sampling a part of the layer to neutralize with calcium carbonate, and quantifying using a liquid chromatograph with an ultraviolet spectrophotometer.

Reference: Kazuo Izumi, Eiichi Ishiguro: Research Report of Animal Feed 15, 36 (1990)

History in the Feed Analysis Standards: 【9】new, 【12】revision

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (g/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (% or less)
Premix for starting chick	5-50	3	92.7-93.9	5.1
Premix for growing chick	5-50	3	89.9-95.0	8.5
Premix for prior stage broiler chicken	5-50	3	87.4-94.4	4.8

・Cooperative testing

Type of sample	No. of labs	Concentration (g/kg)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Premix for prior stage broiler chicken	7	25	97.4	3.0	4.1	0.58

《Notes and precautions》

[1] Being difficult to dissolve, dissolve via ultrasonic treatment.

[2] The halofuginone hydrobromide content in HPS is 8.5 %; therefore, the amount of halofuginone hydrobromide at the peak height (or area) when a sample solution of premix

containing 25 g/kg of HPS is injected in the high performance liquid chromatograph is 1.7 μg/mL.

[3] Add by little and little to avoid bubbling.

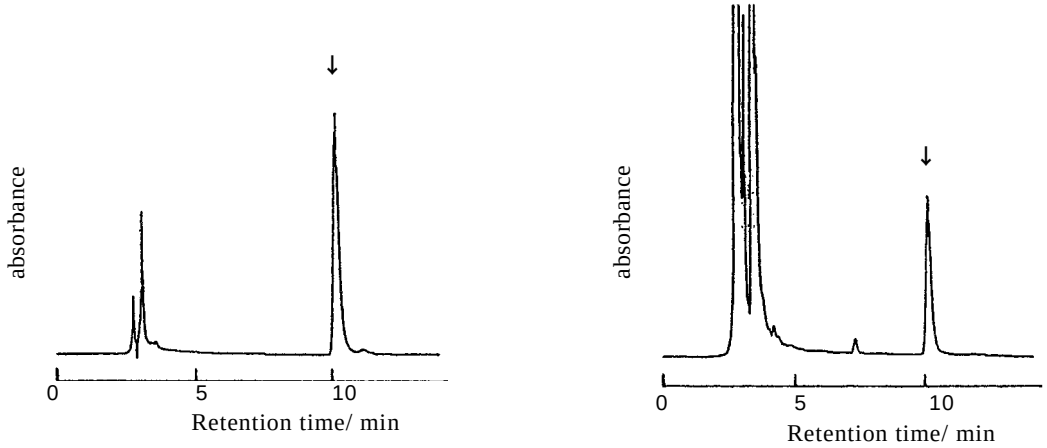
[4] Any column with an equivalent end-capped packing material is applicable. **The column used at the time of discussing about development of this analysis method was** Wakosil $5C_{18}$-200**.**

[5] Since the eluent contains buffer solution, LC apparatus and columns used should be washed enough, and the eluent should be replaced entirely with methanol, acetonitrile or others before storing. At the time of use, give eluent after replacing with water.

[6] When the separation is not enough, it may be improved by use of methanol in place of acetonitrile.

It is good to analyze at approximately 40 °C, in the case where a chromatograph attached with a column oven is used.

[7] An example of chromatogram is shown in Fig. 8.1.13-1.



Hhalofuginone hydrobromide standard solution (100 ng)

Premix for growing chicks (added at 2.5 %)

Fig. 8.1.13-1 A chromatogram of halofuginone hydrobromide
(The arrow indicates the peak of halofuginone)

Measurement conditions

Detector: Measurement wavelength: 243 nm

Column: Wakosil $5C_{18}$-200

Eluent: Acetic acid buffer solution-methanol（11:9）

Flow rate: 1.0 mL/min

Column temperature: 40 °C

1.2 Liquid chromatography (Formula feed)

〔Feed Analysis Standards Chapter 8, Section 1, 13.1.1-(2)〕

A. Reagent preparation

Halofuginone hydrobromide standard solution: Prepare the halofuginone hydrobromide standard stock solution according to (1)-A [1].

At the time of use, dilute exactly a definite amount of the standard stock solution with hydrochloric acid (0.1 mol/L) to prepare several halofuginone hydrobromide standard solutions containing amounts of halofuginone hydrobromide equivalent to 0.4-1.2 μg/mL [2].

B. Quantification

Extraction: Place 5 g of the analysis sample exactly measured in a stoppered 50 mL centrifuging tube, add 20 mL of ethyl acetate and 10 mL of sodium carbonate solution (1 w/v%) for extraction while shaking for 15 min [3]. Centrifuge the extracted solution at 1,500×*g* for 10 min [4], and place the ethyl acetate layer (upper layer) in a 300 mL separating funnel.

Add 20 mL of ethyl acetate to that centrifuging tube, process in a similar way, and add the ethyl acetate layer to that separating funnel. Repeat this procedure 4 times [5], and add respective ethyl acetate layers to that separating funnel to prepare a sample solution for purifying.

Purification: Add 10 mL of hydrochloric acid (0.1 mol/L) to the sample solution, mix them while shaking for 5 min, leave still standing, and place the water layer (lower layer) in a 200 mL recovery flask. Add 10 mL of hydrochloric acid (0.1 mol/L) to the residual solution, process in a similar way, and add the water layer to that recovery flask. Concentrate the water layer to approximately 5 mL in a water bath at 50°C under reduced pressure, and place it in a 20 mL volumetric flask. Wash that recovery flask with a small amount of hydrochloric acid (0.1 mol/L), and add the washings to that volumetric flask. Then, add hydrochloric acid (0.1 mol/L) up to the gauge line of the volumetric flask, and filter this solution through a membrane filter (pore diameter: 0.5 μm or less) to obtain a sample solution for liquid chromatography.

Liquid chromatography: Inject respective 20 μL of the sample solution and each halofuginone hydrobromide standard solution into a liquid chromatograph to obtain the chromatogram.

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 243 nm)

Column: Octadecylsilylated silica-gel column[6] (internal diameter: 4.6 mm, length: 250 mm, particle diameter: 5 μm) Note1

Column temperature: 40 °C

Eluent: Acetic acid buffer solution Note2-methanol (11:9) [7][8]

Flow rate: 1.0 mL/min

Calculation: Obtain the peak height or area from the chromatogram [9], and calculate the amount of calcium halofuginone polystyrenesulfonate (*C* (g/t)) using the following formula.

$$C = A \times \frac{20}{8.5}$$

A : The weight of halofuginone hydrobromide (ng) obtained from the calibration curve.

《Summary of analysis method》

This method is intended to determine the amount of HPS by separating halofuginone from calcium halofuginone polystyrenesulfonate (HPS) in the sample under a mildly alkalinized condition (0.1 mol/L sodium carbonate), repeating shaking extraction with ethyl acetate, inversely extracting the ethyl acetate layer with hydrochloric acid (1:100), injecting this solution into the silica gel of the liquid chromatograph chemically binding to the octadecyl group, measuring the absorbance at 243 nm, and quantifying HPS from the peak height or area.

Reference: Eiichi Ishiguro, Yasuhiro Sato: Research Report of Animal Feed, 12, 36 (1987)

History in the Feed Analysis Standards: 【9】new

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (g/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (%)
Formula feed for prior stage broiler chicken	40	3	80.2	7.3
Formula feed for later stage broiler chicken	40	3	81	5.6
Formula feed for starting chick	40	3	83.1	7.9

・Cooperative testing

Type of sample	No. of labs	Concentration (g/kg)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Formula feed for later stage broiler chicken	7	40	78.2	4.5	5.4	0.80

《Notes and precautions》

[1] The preparation method is the same as that described under 1.1-A.

[2] The halofuginone hydrobromide content in HPS is 8.5 %; therefore, the amount of halofuginone hydrobromide at the peak height (or area) when a sample solution of premix containing 40 g/t of HPS is injected in the high performance liquid chromatograph is 0.85 μg/mL.

[3] It is preferable to use a shaking apparatus attached with a centrifuging tube holder capable of horizontal shaking.

[4] Some samples may be hard to be separated by the first shaking and centrifuging. In that case, add 20 mL of ethyl acetate directly to the centrifuging tube, and secondly shake and centrifuge to achieve good separation.

Perform the second centrifuge or later for approximately 3 min.

[5] Halofuginone in a sample is very hard to be extracted, with achievable recovery rates of approximately 35 % for single extraction with ethyl acetate, and 60 % for double extraction.

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

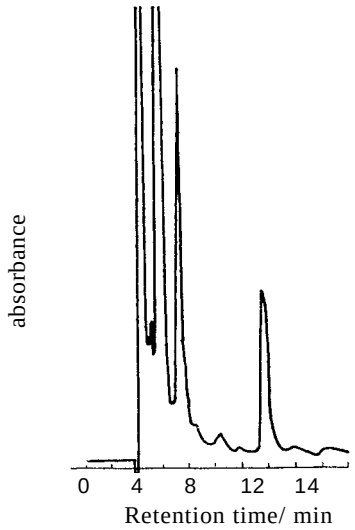
[7] Since the eluent contains buffer solution, LC apparatus and columns used should be washed enough, and the eluent should be replaced entirely with methanol, acetonitrile or others before storing. At the time of use, give eluent after replacing with water.

[8] When the separation is not enough, it may be improved by use of methanol in place of

acetonitrile.

It is good to analyze at approximately 40 °C, in the case where a chromatograph attached with a column oven is used.

[9] An example of chromatogram is shown in Fig. 8.1.13-2.



Measurement conditions

Detector: Measurement wavelength: 243 nm

Column: μBondapak C_{18}

Eluent: Acetic acid buffer solution-Methanol (11:9)

Flow rate: 1.0 mL/min

Column temperature: 40 °C

Fig. 8.1.13-2 A chromatogram of halofuginone hydrobromide added to a feed for starting chicks (HPS: 40 g /t)
(The arrow indicates the peak of halofuginone)

2 Microquantitative test methods

2.1 Liquid chromatography 〔Feed Analysis Standards Chapter 8, Section 1, 13.2.1〕

Scope of application: Formula feed

A. Reagent preparation

1) Halofuginone hydrobromide standard solution: Place 20 mg of halofuginone hydrobromide 〔$C_{16}H_{18}Br_2ClN_3O_3$〕 exactly measured in a 200 mL volumetric flask, add hydrochloric acid (0.1 mol/L) for dissolving [1], and further add the solution up to the gauge line to prepare the halofuginone hydrobromide standard stock solution (1 mL of this solution contains an amount of halofuginone hydrobromide equivalent to 0.1 mg).

At the time of use, dilute a definite amount of the standard stock solution exactly with hydrochloric acid (0.1 mol/L) to prepare several halofuginone hydrobromide standard solutions containing amounts of halofuginone hydrobromide equivalent to 0.1-0.8 μg/mL [2].

2) Acetic acid buffer solution: Dissolve 4.9 g of ammonium acetate and 7.5 mL of acetic acid in water to make a total amount of 1 L, and adjust the pH to 4.3 with acetic acid.

B. Quantification

Extraction[3]: Place 5 g of the analysis sample exactly measured in a stoppered 50 mL centrifuging tube, add 20 mL of ethyl acetate and 10 mL of sodium carbonate (1 w/v%), mix while shaking for 15 min [4], centrifuge at 1,500×*g* for 10 min[5] , and place the ethyl acetate layer (upper layer) in a 300 mL separating funnel.

Add 20 mL of ethyl acetate to that centrifuging tube, process in a similar way, and add the ethyl acetate layer to that separating funnel. Repeat this procedure 4 times in a similar way [6], and add respective ethyl acetate layers to that separating funnel to prepare a sample solution for purification.

Purification: Add 10 mL of hydrochloric acid (0.1 mol/L) to the sample solution, mix them while shaking for 5 min, allow still standing, and place the water layer (lower layer) in a 100 mL recovery flask. Add 10 mL of hydrochloric acid (0.1 mol/L) to the residual solution and process in a similar way. Concentrate the water layer in a water bath at 50 °C to approximately 5 mL of amount under reduced pressure to prepare a sample solution for column treatment.

Column treatment[7]: Wash sequentially an octadecylsilylated silica-gel minicolumn (1,000 mg) Note1 with 10 mL of methanol and 10 mL of water.

Place the sample solution in the column, effuse it up to the fluid level reaching 1 mm of height from the top of the packing material. Then, wash twice the recovery flask which contained the sample solution with respective 5 mL of water, add the washings sequentially to the column, and effuse it in a similar way. Add 5 mL of water, respective 5 mL (twice) of methanol solution (3:2), and respective 5 mL (4 times) of hydrochloric acid (0.1 mol/L) sequentially to the column for washing.

Place a 50 mL recovery flask under the column, and add 10 mL of methanol-chloroform (9:1) to the column to elute halofuginone. Concentrate the eluate in a water bath at 50 °C to almost dry out under reduced pressure, and send nitrogen gas to obtain the dry matter.

Add exactly 1 mL of water-acetonitrile-acetic acid buffer solution (62:23:15) to dissolve the residue. Filter this solution through a membrane filter (pore diameter: 0.5 μm or less) to obtain a sample solution for liquid chromatography.

Liquid chromatography: Inject respective 20 μL of sample solution and each halofuginone hydrobromide standard solution into a liquid chromatograph to obtain the chromatogram.

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 243 nm)

Column: Octadecylsilylated silica-gel column (internal diameter: 3.9 mm, length: 300 mm, particle diameter: 10 μm) Note 2[8]

Eluent: Water-acetonitrile-acetic acid buffer solution (62:23:15) [9]

Flow rate: 1.0 mL/min

Column temperature: 40°C

Calculation: Obtain the peak height or area from the chromatogram [10] to prepare the calibration curve, and calculate the amount of calcium halofuginone polystyrenesulfonate (C (g/t)) using the following formula.

$$C = A \times \frac{1}{8.5}$$

A : The weight (ng) of halofuginone hydrobromide obtained from the calibration curve.

Note 1. Mega Bond Elut C18 (Varian, reservoir volume: 6 mL) or an equivalent one.

2. μBondapak C_{18} (Waters) or an equivalent one.

《Summary of analysis method》

This method was developed to quantify calcium halofuginone polystyrenesulfonate (HPS) minimally remained in formula feeds due to carry-over, etc.

This method is intended to determine the amount of HPS by separating halofuginone from calcium halofuginone polystyrenesulfonate (HPS) in the sample under a mildly alkalinized condition (0.1 mol/L sodium carbonate), repeating shaking extraction with ethyl acetate, inversely extracting the ethyl acetate layer with hydrochloric acid (1:100), purifying with a C_{18} cartridge, and quantifying HPS using a liquid chromatograph with an ultraviolet spectrophotometer.

Reference: Takayuki Ishibashi, Masayuki Shimomura, Eiichi Ishiguro: Research Report of Animal Feed, 17, 60 (1992)

History in the Feed Analysis Standards: 【14】new

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (mg/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (%)
Formula feed for adult chicken	0.3-1.0	3	86.0-92.5	6.0
Formula feed for later stage broiler chicken	0.3-1.0	3	85.9-92.6	8.4
Formula feed for finishing pig	0.3-1.0	3	87.2-96.1	7.4

・Cooperative testing

Type of sample	No. of labs	Concentration (mg/kg)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Formula feed for adult chicken	7	0.5	99.8	12.8	14.8	1.18

《Notes and precautions》

[1] Being difficult to dissolve, dissolve via ultrasonic treatment.

[2] The halofuginone hydrobromide content in HPS is 8.5 %; therefore, the amount of halofuginone hydrobromide at the peak height (or area) when a sample solution of formula feed containing 0.5 g/t of HPS is injected in the high performance liquid chromatograph is approximately 0.21 μg/mL.

[3] The procedure is the same as that described under 1.2-B.

[4] It is preferable to use a shaking apparatus attached with a centrifuging tube holder capable of horizontal shaking.

[5] Some samples may be hard to be separated by the first shaking and centrifuging. In that case, add 20 mL of ethyl acetate directly to the centrifuging tube, and secondly shake and centrifuge to achieve good separation.

Perform the second centrifuge or later for approximately 3 min.

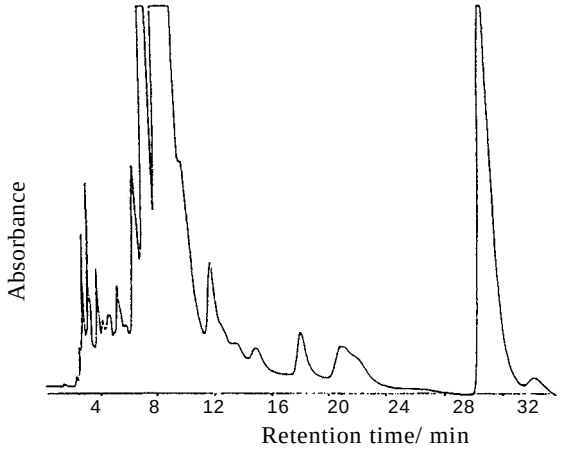
[6] Halofuginone in a sample is very hard to be extracted, with achievable recovery rates of approximately 35 % for single extraction with ethyl acetate, and 60 % for double extraction.

[7] Since natural falling requires time, add pressure by something like double-balls to control the flow rate between approximately 1 and 2 mL/min.

[8] Since the eluent contains buffer solution, LC apparatus and columns used should be washed enough, and the eluent should be replaced entirely with methanol, acetonitrile or others before storing. At the time of use, give eluent after replacing with water.

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

[10] An example of chromatogram is shown in Fig. 8.1.13-3.



Measurement conditions

Detector: Measurement wavelength: 243 nm

Column: μBondapak C_{18}

Eluent: Water-acetonitrile-acetic acid buffer solution（62:23:15）

Flow rate: 1.0 mL/min

Column temperature: 40 °C

Fig. 8.1.13-3 A chromatogram of halofuginone hydrobromide added to a formula feed for adult chickens (0.5 g/t HPS)
(The arrow indicates the peak of halofuginone)

【Control analysis method】

3. Fast determination method

〔Notification of Livestock Industry Bureau and Fisheries Agency, MAFF, 62 Chiku B 3099, December 25, 1987〕

Quantification

Place 15.0 g of the analysis sample measured in a stoppered 100 mL Erlenmeyer flask, add 60 mL of ethyl acetate and 30 mL of sodium carbonate (10 w/v%), stir for 45 min with a magnetic stirrer for extraction [Note1], and place the extracted solution in a stoppered 50 mL centrifuging tube to centrifuge at 3,000 rpm for 5 min [Note2].

Place 30 mL of the ethyl acetate layer in a 100 mL separating funnel, add 6 mL of hydrochloric acid (1:99) while shaking for 3 min for mixing, and allow still standing. Then, transfer the hydrochloric acid layer (lower layer) in a 50 mL beaker, add 6 mL of hydrochloric acid (1:99) to the residual solution, process in a similar way, and add the hydrochloric acid layer (lower layer) to that beaker. Heat this solution on a water bath at 80 °C for 30 min for volatilizing ethyl acetate to prepare the sample solution to be applied to the cartridge [Note 3] for pretreatment of the sample.

Place the sample solution in a cartridge for sample-pretreatment previously attached with a syringe and washed with 8 mL of methanol and 5mL of water [Note 4], wash out the solution in that beaker with a small amount of water, add the washings to the cartridge, wash the cartridge sequentially with 5 mL of water and 5 mL of water-methanol (9:1) [Note 5], and elute the solution with 8 mL of methanol-water (6:4) into a 20 mL beaker to obtain a sample eluate.

Add 0.3 mL of sodium hydroxide (32 w/v%) solution to the sample eluate, hydrolyze on a water bath at 80 °C for 45 min or more [Note 6], allow being cool to the room temperature, add 1 mL of hydrochloric acid (1:2), transfer to a stoppered measuring test tube, wash that container with a small

amount of water, and add the washings to the sample solution [Note 7]. Cool the test tube for 5 min in ice, add 1 mL of sodium nitrite (0.1 w/v%) solution [Note 8], mix while shaking, and allow still standing for 2 min. Then, take out the sample from the ice, add 1 mL of ammonium sulphamate (1 w/v%) solution, mix while shaking vigorously for 30 sec, add 1 mL of *N*-1-naphthylethylenediamine dihydrochloride (0.1 w/v%) solution for color-forming, add water up to a total amount of 10 mL, allow still standing for 15 min, and determine the absorbance at the maximum wavelength near the wavelength of 545 nm using water as the control [Note 9].

Differently, place respective 15 g of additive-free samples without adding calcium halofuginone polystyrenesulfonate with the same component as the sample in stoppered 100 mL Erlenmeyer flasks, add 45, 60 and 75 mg of the standard calcium halofuginone polystyrenesulfonate for feed analysis [Note 10] (containing amounts of calcium halofuginone polystyrenesulfonate equivalent to 450 µg, 600 µg and 750 µg, respectively) to flasks, respectively, and measure the absorbance in the same manner as given above to prepare the calibration curve.

The amount of calcium halofuginone polystyrenesulfonate in the sample (g/t)=(A / 600) ×40

A ：the weight of calcium halofuginone polystyrenesulfonate obtained from the calibration curve (µg)

Note 1. A shaking apparatus can be used for extraction in place of the stirrer. In case of stirrer extraction, use a rotor with length of approximately 35 mm.

2. When no centrifugal machine is available, a cotton-plugged infundibulum or the like can be used for filtering the extracted supernatant.
3. Use Sep-Pak C_{18}cartridge (Waters) or an equivalent one.
4. Set the effusing rate from the cartridge to approximately 3 mL/min.
5. Discard the washings.
6. Take care not to dry.
7. The liquid volume must be 7 mL or less.
8. Store at 4 °C and use within a week.
9. Finish the measurement within 60 min.

Since the maximum wavelength differs by the feed components, obtain preliminarily the maximum wavelength of the sample feed.

10. The standard calcium halofuginone polystyrenesulfonate diluted by 100-fold with calcium carbonate (1 % preparation). Since calcium halofuginone polystyrenesulfonate was very hard to extract, the standard addition method was selected, where the quantification is performed by adding the standard calcium halofuginone polystyrenesulfonate to an additive-free sample.