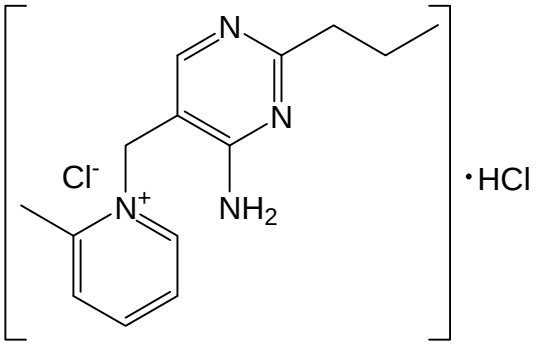
1 Amprolium



1-(4-amino-2-propylpyrimidin-5-ylmethyl)-2-methylpyridinium chloride hydrochloride

$C_{14}H_{20}Cl_2N_4$ MW: 315.24 CAS No.: 137-88-2

【Outline of amprolium】

Amprolium is an antithiamine series anticoccidial agent reported in 1960 by Rogers *et al*, which is mainly effective on cecal coccidiosis.

This agent is white to light yellow powder without smell or with picoline-like smell, and freely soluble in water, soluble in methanol, very slightly soluble in ethanol, and practically insoluble in ether or chloroform.

Amprolium is allowed to add to feed for chickens in the range of 40-250 g/t (40-250 g/t for two-combination with ethopabate, and 100 g/t for three-combination with ethopabate and sulfaquinoxaline)

【Methods listed in Feed Analysis Standards】

1 Assay

1.1 Liquid chromatography

1.1.1 Premix 〔Feed Analysis Standards Chapter 8, Section 1, 1.1.1-(1)〕

A. Reagent preparation

Amprolium standard solution: Place 25 mg of amprolium〔$C_{14}H_{19}ClN_4 \cdot HCl$〕exactly measured in a 50 mL brown volumetric flask, add methanol to dissolve, further add the solvent up to the gauge line to prepare an amprolium standard stock solution (1 mL of this solution contains an amount of amprolium equivalent to 0.5 mg).

Exactly dilute a definite amount of standard stock solution with methanol at the time of use to prepare several amprolium standard solutions containing amounts of amprolium equivalent to 0.5-4μg/mL.

B. Quantification

Extraction: Place 2-4 g of the analysis sample exactly measured in a 200 mL brown stoppered Erlenmeyer flask, add 100 mL of ethanol [1], and stir for 30 min for extraction. Place the extracted solution in a

stoppered brown centrifuging tube, centrifuge at 1,500×*g* for 5 min, and exactly dilute a definite amount of the supernatant with methanol [note 1] to obtain a sample solution for column-treatment

Column treatment: Place the sample solution in a neutral alumina mini-column (1,710 mg) [2], discard the first 5 mL of the outflow, and filter the second 5 mL of outflow through a membrane filter (0.5 μm or less pore diameter) to obtain a sample solution for liquid chromatography.

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

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 265 nm)

Column: Octadecylsilylated silica-gel column (inner diameter: 4.6 mm, length: 250 mm, particle diameter: 5 μm) [note 3][3].

Eluent: Phosphate buffer solution [note 4]-methanol（7:3） [4]

Flow rate: 0.7 mL/min

Calculation: Obtain the peak height or area from the chromatogram [5] to prepare the calibration curve, and calculate the amprolium amount in the sample.

Note 1 Prepare the sample solution containing an amount of amprolium equivalent to 2 μg/mL.

2 Flow rate is 2-3 mL/min.

3 Puresil 5μC_{18} 120Å (Waters) or an equivalent one.

4 Dilute 9.71 g of potassium dihydrogen phosphate and 1.35 g sodium 1-hexane sulfonate in water up to the total amount of 1 L, and adjust the pH to 3.4-3.5 with phosphoric acid.

《Summary of analysis method》

This method is intended to determine the amount of amprolium in a premix by extracting with methanol, diluting with the solvent, removing interfering substances with a neutral alumina minicolumn, and quantifying by using a liquid chromatograph with ultraviolet spectrophotometer. This is an improved method of one listed in Feed Analysis Standards in 1988, based on the method of Fukumoto *et al*.

References: Yuji Fukumoto, Shuichi Shimada: Research Report of Animal Feed, 12, 53 (1987)

Takayuki Koyama, Yuji Shirai: Research Report of Animal Feed, 21, 95 (1996)

History in the Feed Analysis Standards: 【9】new,【17】revision,【18】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 chicken 1	10-50	3	99.1-101.8	1.7
Premix for chicken 2	10-50	3	100.3-100.5	1.3
Premix for chicken 3	10-50	3	99.7-100.5	2.3

・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 chicken	7	25	96.3	2.4	4.2	0.19

《Notes and cautions》

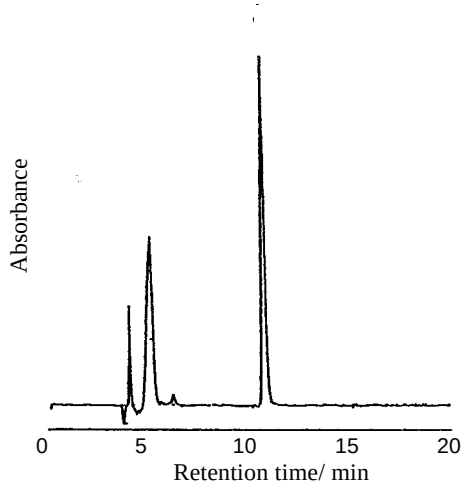
[1] Methanol-water (7:3) may be used similarly to the formula feed described under 1.1.2.

[2] Use a new neutral alumina minicolumn.

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

[4] 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.

[5] An example of chromatogram of amprolium is shown in Fig. 8.1.1-1.



Measurement conditions

Detector: Measurement wavelength: 265 nm

Column: Puresil 5μ C18 120Å

Eluent: Phosphate buffer solution－methanol (7:3)

Flow rate: 0.7 mL/min

Fig. 8.1.1-1 A chromatogram of amprolium added to a premix for chickens
(The arrow indicates a peak of amprolium)

1.1.2 Formula feed 〔Feed Analysis Standards Chapter 8, Section 1, 1.1.1 (2)〕

A. Reagent preparation

Amprolium standard solution: Place 25 mg of amprolium〔$C_{14}H_{19}ClN_4 \cdot HCl$〕exactly measured in a 50 mL brown volumetric flask, add methanol for dissolving, further add the solvent up to the gauge line to prepare an amprolium standard stock solution (1 mL of this solution contains an amount of amprolium equivalent to 0.5 mg).

At the time of use, exactly dilute a definite amount of the standard stock solution with methanol-water (7:3) to prepare several amprolium standard solutions containing amounts of amprolium equivalent to 1.0-30 μg per mL.

B. Quantification

Extraction: Place 10.0 g of analysis sample in a stoppered 200 mL brown Erlenmeyer flask, add 30 mL of water and mix them well [1], ultrasonicate at water temperature of 30-40 °C for 10 min [2], further add 70 mL of methanol to this solution, and stir for 30 min to obtain the extract. Place the extract in a stoppered centrifuging tube, centrifuge at 1,800×*g* for 5 min to obtain the supernatant as a sample solution for column treatment.

Column treatment: Place the sample solution in a neutral alumina minicolumn (1,710 mg)[3], discard the first 3 mL of naturally out flowed solution, and take the second 4 mL[4] of solution in a 10 mL test tube.

Filter a definite amount of effluent through a membrane filter (pore diameter: 0.5 μm or less) to obtain a sample solution for liquid chromatography.

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

Measurement conditions (an example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 265 nm)

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

Eluent: Methanol-citric acid buffer solution Note2 (13:7)[6]

Flow rate: 1.0 mL/min

Calculation: Obtain the peak height or area from the chromatogram[7] to prepare the calibration curve, and calculate the amount of amprolium in the sample.

Note 1 Mightysil RP-18 (Kanto Chemical) or equivalent one

2 Dissolve 19.2 g of citric acid monohydrate and 2.07 g of *n*-sodium dodecyl sulfate in water up to total volume of 1L, and adjust the pH at 3.5 with sodium hydroxide solution (10 mol/L).

《Summary of analysis method》

This method is intended to determine the amount of amprolium in formula feeds by extracting with water and methanol, removing the interfering substances with a neutral alumina minicolumn, and quantifying by using a liquid chromatograph with an ultraviolet spectrophotometer. This is an improved method of one listed in Feed Analysis Standards in 1996, based on the method of Koyama *et al.*

References: Yuji Fukumoto, Shuichi Shimada：Research Report of Animal Feed, 12, 53 (1987)
Takayuki Koyama, Yuji Shirai：Research Report of Animal Feed, 21, 80 (1996)
Tomotaro Yoshida, Manabu Matsuzaki：Research Report of Animal Feed, 31, 88 (2006)

History in the Feed Analysis Standards: 【9】new,【17】revision,【18】revision, 【29】reversion

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (mg/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (% or less)
Formula feed for starting chick	40-250	3	99.9-103.0	2.0
Formula feed for growing chick	40-250	3	101.6-103.3	0.7
Formula feed for prior stage broiler chicken	40-250	3	100.0-101.6	1.4

・Cooperative testing

Type of sample	No. of labs	Concentration (mg/kg)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Forumula feed for starting chick	9	100	100.0	0.9	2.7	0.30

《Notes and precautions》

[1] Stir with a magnetic stirrer for 1-2 min.

[2] It may be allowed still standing in thermostat at 40 °C for 10 min.

[3] Use a new one for the neutral alumina minicolumn.

[4] Well mix the effluent before the use in processing afterwards.

[5] 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 Mightysil RP-18GP (Kanto Chemical).

[6] 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.

[7] An example of chromatogram of amprolium is shown in Fig. 8.1.1-2.

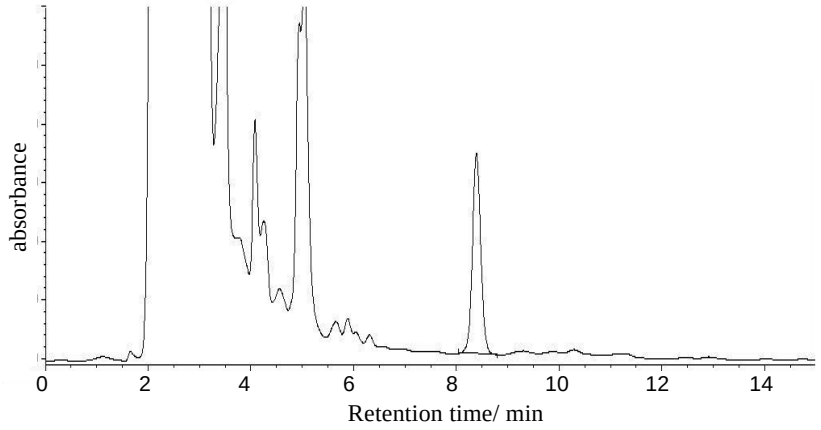


Fig. 8.1.1-2 A chromatogram of amprolium (100 g/t) added to a formula feed for starting chicks (The arrow indicates a peak of amprolium)

Measurement conditions are as noted under “measurement conditions (example)”.

1.2 Absorptiometric method

1.2.1 Premix 〔Feed Analysis Standards Chapter 8, Section 1, 1.1.2-(1)〕

A. Reagent preparation

1) Extraction solvent: Methanol-water（2:1）

2) Amprolium standard solution: Place 25 mg of amprolium〔$C_{14}H_{19}ClN_4 \cdot HCl$〕exactly measured in a 100 mL brown volumetric flask, add methanol-water (2:1) for dissolving, further add the solvent up to the gauge line to prepare an amprolium standard stock solution (1 mL of this solution contains an amount of amprolium equivalent to 0.25 mg).

At the time of use, exactly dilute a definite amount of the standard stock solution with methanol-water (2:1) to prepare the amprolium standard solution containing an amount of amprolium equivalent to 2.5μg per mL.

3) Sodium hydroxide solution: Add methanol to 15 mL of sodium hydroxide solution (1.1 w/v%) to make a total amount of 200 mL.

4) Naphthalenediol solution: Dissolve 25 mg of 2,7-naphthalenediol[1] in methanol to make 1 L.

5) Coloring test solution: Place 90 mLof naphthalenediol solution in a 250 mL volumetric flask, add 5mL of potassium ferricyanide solution (0.2 w/v%) to mix while shaking, to which add 5 mL of potassium cyanide solution[2](1 w/v%) to mix while shaking, and allow it still standing for 30 min. Then, add 100 mL of sodium hydroxide solution to this solution to mix while shaking, and filter through a glass filter (G2). Prepare this coloring test solution at the time of use, and use within 75 min after preparation.

B. Quantification

Extraction: Place 1-3 g of an analysis sample exactly measured in a 250 mL separating funnel, to which add 100 mL of methanol-water (2:1) to mix while shaking for 1 hr for extraction[3], and allow it still standing. Filter the extracted solution with a paper filter（No. 5 A） [4], dilute with methanol-water (2:1) exactly 50 to 500-fold [5], and use it as a sample solution for measurement.

Measurement: Place 4 mL of the sample solution exactly measured in a stoppered centrifuging tube, to which add 10 mL of coloring test solution to mix while shaking. Then, allow it still standing for 20 min, and centrifuge at 650×*g* for 3 min. Place the supernatant in a cell, immediately, cover it with a lid to measure the absorbance at the wavelength of 530 nm using a blank test solution (4 mL of methanol-water (2:1) treated similarly to the sample solution) as the control solution.

At the sane time, place 4 mL of amprolium standard solution exactly measured in a stoppered centrifuging tube, and measure the absorbance in the same conditions as the sample solution.

Calculation: Calculate the amount of amprolium in the sample from the absorbance obtained.

1.2.2 Formula feed 〔Feed Analysis Standards Chapter 8, Section 1, 1.1.2-(2)〕

A. Reagent preparation

1) Extraction solvent[6]: Conform to (1)-A-1).

2) Amprolium standard solution[6]: Conform to (1)-A-2).

3) Basic alumina: Dry basic alumina (particle diameter: 63-200 μm (230-70 mesh)) Note 1 for column chromatograph at 100 °C for 3 hr.

4) Sodium hydroxide solution[6]: Conform to (1)-A-3).

5) Naphthalenediol solution[6]: Conform to (1)-A-4).

6) Coloring test solution[6]: Conform to (1)-A-5).

B. Quantification

Extraction: Measure 15.0 g or less of analysis sample (1.5-2.5 mg amprolium equivalent), place it in a 250 mL separating funnel, add 100 mL of methanol-water (2:1), mix while shaking for 1 hr for extraction[3], and allow it still standing. Filter the extracted solution through a paper filter (No. 5A） [4], and use it as a sample solution for column treatment.

Column treatment: Pack 5 g of basic alumina in a column tube (internal diameter:10 mm) in a dry processing to prepare the column.

Place 25 mL of sample solution [7] in the column, discard the first 5 mL of effluent, and use the subsequent effluent as a sample solution for measurement.

Measurement [8]: Conform to (1)-B “Measurement”.

Calculation [8]: Conform to (1)-B “Calculation”.

Note 1: Aluminiumoxid aktiv basisch Art. 1076 (Merck) or an equivalent one.

《Summary of analysis method》

In the analysis methods described under 1.2.1 and 1.2.2 are intended to determine the amount of, amprolium in a feed or a premix by extracting with methanol-water (2:1), and, after removing the interfering substances by filtering with a basic alumina column in the case of amprolium in a feed（in the case of amprolium in a premix, however, this procedure possibly causes a decreased quantitative value due to partial adsorption of amprolium to the column; therefore, this procedure is skipped, quantifying by measuring the absorbance at 530 nm with coloring reaction to 2,7-naphthalenediol, potassium ferricyanide, potassium cyanide, and sodium hydroxide/aqueous methanol solution.

Reference: “Official Methods of Analysis of the AOAC International”, 18th Ed., 5.1.08 (2005).

History in the Feed Analysis Standards: 【2】new, 【9】partial revision,【17】premix and formula feed are separated.

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (mg/kg)）	Repeat	Recovery rate （%）	Repeat accuracy RSD (% or less)
Formula feed for adult chicken	50-100	6	95.5-97.3	4.1

・Cooperative testing

Type of sample	No. of test room	(mg/kg) (mg/kg)	(%) (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Formula feed	6	100	94.0	4.0	12.4	2.17

《Notes and precautions》

[1] Alias: 2,7-dihydroxynaphthalene

[2] The solution is stable for approximately 2 weeks.

[3] Shaking extraction with a shaking apparatus or strong stirring extraction with a magnetic stirrer is also applicable.

[4] Centrifuge before filtering, if it is hard to filter.

[5] Dilute to approximately 15 to 25 mg/mL.

[6] The preparation method is the same as the method described under 1.2.1.

[7] An approximate volume may be applicable.

[8] The operation method is the same as the method described under 1.2.1.

2 Microquantitative test method

2.1 liquid chromatography 〔Feed Analysis Standards Chapter 8, Section 1, 1.2.1〕

Scope of application: Formula feed

A. Reagent preparation

Amprolium standard solution: Place 25 mg of amprolium〔$C_{14}H_{19}ClN_4 \cdot HCl$〕exactly measured in a 50 mL brown volumetric flask, add methanol for dissolving, further add the solvent up to the gauge line to prepare an amprolium standard stock solution (1 mL of this solution contains an amount of amprolium equivalent to 0.5 mg).

At the time of use, exactly dilute a definite amount of the standard stock solution with methanol [1] to prepare several amprolium standard solutions containing amounts of amprolium equivalent to 0.1-1 μg per mL.

B. Quantification

Extraction: Measure 20.0 g of analysis sample, place it in a 200 mL stoppered brown Erlenmeyer flask, add 100 mL of acetonitrile methanol（4:1）, stir it for 45 min for extraction. Place the extracted solution in a stoppered brown centrifuging tube, centrifuge at 1,500×*g* for 5 min, and use the supernatant as a sample solution for column treatment.

Column treatment I: Place the sample solution in a neutral alumina minicolumn（1,710 mg）[2], and flow out approximately 30 mL of the solution with pressure injection Note 1. Place exactly 25 mL of effluent in a 100 mL recovery flask, concentrate it under reduced pressure in water bath at 45-50 °C until almost dry out, and then send nitrogen gas to obtain the dry matter. Dissolve the residues by adding 10 mL of acetonitrile-methanol (99:1) [3] to obtain the sample solution for column treatment II.

Column treatment II: Place the sample solution in a neutral alumina minicolumn（1,710 mg）[2] and flow out with pressure injection Note 1. Wash 3 times the recovery flask which previously contained

sample solution with respective 10 mL of acetonitrile-methanol (99:1), and add the washings sequentially to the minicolumn to effuse in an similar way. Then, add 10 mL of the solvent to the minicolumn to effuse in a similar way for washing. Place a 100 mL recovery flask under the minicolumn to which add 20 mL of acetonitrile-methanol (9:1) to elute amprolium with pressure injection Note 1. Concentrate the eluate under reduced pressure in a water bath at 45-50 °C to almost dry out, and then send nitrogen gas to obtain the dry matter.

Dissolve [5] the residues by exactly adding 5 mL of methanol [1], and filter this solution through a membrane filter (pore diameter: 0.5 μm or less) to obtain the sample solution for liquid chromatography.

Liquid chromatography [6]: Inject respective 20 μL of the sample solution and each amprolium standard solution into the liquid chromatograph to obtain the chromatogram.

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 265 nm)

Column: Octadecylsilylated silica-gel column (internal diameter: 4.6 mm, length: 250 mm, particle diameter: 5 μm) Note 2[7]

Eluent: Phosphate buffer solution Note 3—methanol (7:3)[8]

Flow rate: 0.7 mL/min

Calculation: Obtain the peak height or area from the chromatogram [9] to prepare the calibration curve, and calculate the amprolium amount in the sample.

Note 1. Flow rate is 2-3 mL/min.

2. Puresil 5μC_{18} 120Å (Waters) or an equivalent one.

3. Dissolve 9.71 g of potassium dihydrogen phosphate and 1.35 g of sodium 1-hexane sulfonate in water to make a total amount of 1 L, and adjust the pH to 3.4-3.5 with phosphoric acid.

《Summary of analysis method》

This method was developed to quantify a minute amount of amprolium remaining in a formula feed caused by carrying over, etc. This method is intended to determine the amount of amprolium in a feed by extracting with acetonitrile-methanol (4:1), cleaning up with a neutral alumina minicolumn, and quantifying using a liquid chromatograph with an ultraviolet spectrophotometer. This is an improved method of one listed in Feed Analysis Standards in 1991, based on the method of Suzuki.

References: Akira Suzuki: Research Report of Animal Feed, 12, 72 (1991)

Takayuki Koyama, Yuji Shirai：Research Report of Animal Feed, 21, 102 (1996)

History in the Feed Analysis Standards: 【13】new,【18】revision

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (mg/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD(% or less)
Formula feed for adult chicken	0.1-1.0	3	100.6-107.3	5.7
Formula feed for growing pig	0.1-1.0	3	100.3-107.0	7.8
Formula feed for growing beef cattle	0.1-1.0	3	99.0-108.7	3.8

・Cooperative testing

Type of sample	No. of test rooms	Concentration (mg/kg)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Formula feed for adult chicken	7	0.5	81.0	4.9	12.7	0.58

《Notes and precautions》

[1] Methanol-water（7:3）may be used similarly to the formula feed described under 1.1.2. However, use a same solvent for the standard solution and sample solution.

[2] For a neutral alumina minicolumn, use a new one. Since this column alone can clean up completely for usual samples, the column treatment II may be omitted.

[3] When the residue can not be dissolved, apply ultrasonic treatment for 1-2 min.

[4] A suction manifold（Waters, Sep-pak vacuum manifold, etc.）is convenient for use in place of a pressure injection.

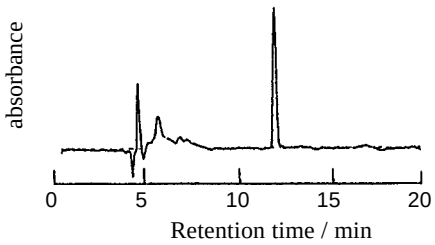
[5] When the residue can not be dissolved, apply ultrasonic treatment for 1-2 min.

[6] This method is the same as that described in the paragraph of liquid chromatography under 1.1.1.

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

[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] An example of chromatogram of amprolium is shown in Fig. 8.1.1-3.



Measurement conditions

Detector: Wavelength: 265 nm

Column: Puresil 5μC18 120Å

Eluent: Phosphate buffer solution-methanol (7:3)

Flow rate: 0.7 mL/min

Fig. 8.1.1-3. A chromatogram of amprolium added to a formula feed for growing beef cattle
（The arrow indicates the peak of amprolium）