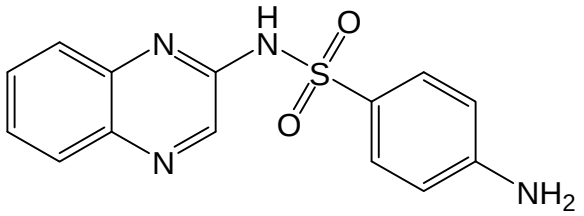
10 Sulfaquinoxaline



4-amino-*N*-quinoxalin-2-ylbenzenesulfonamide

$C_{14}H_{12}N_4O_2S$ MW: 300.33 CAS No.: 59-40-5

【Outline of sulfaquinoxaline】

Sulfaquinoxaline is light yellow to brownish yellow crystalline or crystalline powder without odor, and practically insoluble in water, ethanol or acetone.

This is one of sulfa drugs belonging to the third stage as classification of sulfonamide. Although its antibacterial potential is low, it has high anticoccidial potential, and is known to have antileucocytozoon potential.

This agent was designated to one of feed additives in 1976, and is used to promote the effective use of nutrient components in feeds (as a combination drug with amprolium, ethopabate and sulfaquinoxaline), being approved for adding to feeds at 60 g/t as sulfaquinoxaline (for starting chicks, growing chicks, and prior and later stages of broiler chickens).

【Methods listed in the Feed Analysis Standards】

1 Quantitative test methods

1.1 Liquid chromatography

1.1.1 Premix

〔Feed Analysis Standards Chapter 8, Section 1, 10.1.1-(1)〕

A. Reagent preparation

Sulfaquinoxaline standard solution: Place 20 mg of sulfaquinoxaline〔$C_{14}H_{12}N_4O_2S$〕exactly measured in a 100 mL brown volumetric flask, add acetonitrile for dissolving, further add the solvent up to the gauge line to prepare the sulfaquinoxaline standard stock solution（1 mL of this solution contains an amount of sulfaquinoxaline equivalent to 0.2 mg）.

At the time of use, exactly dilute a definite amount of the standard stock solution with acetonitrile-water (4:1) to prepare several sulfaquinoxaline standard solutions containing amounts of sulfaquinoxaline equivalent to 2.5-15 μg per mL.

B. Quantification

Extraction: Measure exactly 2 g of analysis sample [1], place it in a stoppered 200 mL brown

Erlenmeyer flask, add 100 mL of acetonitrile-water (4:1), 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 to obtain the supernatant. Dilute a definite amount of the supernatant exactly with acetonitrile-water (4:1). 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 sulfaquinoxaline standard solution into a liquid chromatograph to obtain the chromatogram.

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 240 nm)

Column: Octadecylsilylated silica-gel column (internal diameter: 4.6 mm, length: 250 mm, particle diameter: 5 μm) Note 1[2]

Eluent: Phosphate buffer solution Note 2-acetonitrile (7:3)[3]

Flow rate: 1.0 mL/min

Column temperature: 40°C

Calculation: Obtain the peak height or area from the chromatogram [4] to prepare the calibration curve, and calculate the sulfaquinoxaline amount in the sample.

Note 1. Shodex C_{18}-5B (Showa Denko) or an equivalent one

2. Dissolve 6.8 g of potassium dihydrogen phosphate in water to make a total amount of 1 L, and adjust the pH to 3.2-3.4 with phosphoric acid (1:10).

《Summary of analysis method》

This method is intended to determine the amount of sulfaquinoxaline in a premix by extracting with acetonitrile-water (4:1), diluting with the solvent, and quantifying using a liquid chromatograph with an ultraviolet spectrophotometer.

The flowsheet of analysis method is shown in Fig. 8.1.10-1.

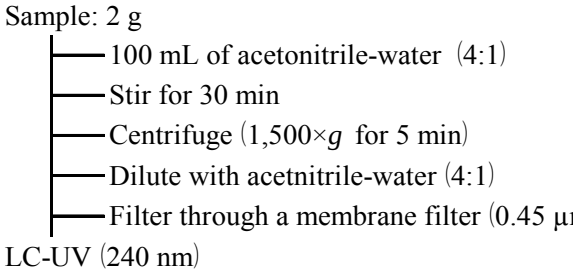


Fig. 8.1.10-1 Analysis method flowsheet of sulfaquinoxaline in a premix

Reference: Yukinobu Nakamura, Yukie Ishida, Tetsuo Chihara: Research Report of Animal Feed, 24, 72 (1999)

History in the Feed Analysis Standards: 【7】new, 【17】revision, 【21】revision

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (mg/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (% or less)
Premix for chicken 1	6-60	3	95.7-99.5	3.8
Premix for chicken 2	6-60	3	94.7-97.4	3.5
Premix for chicken 3	6-60	3	97.5-98.1	2.8

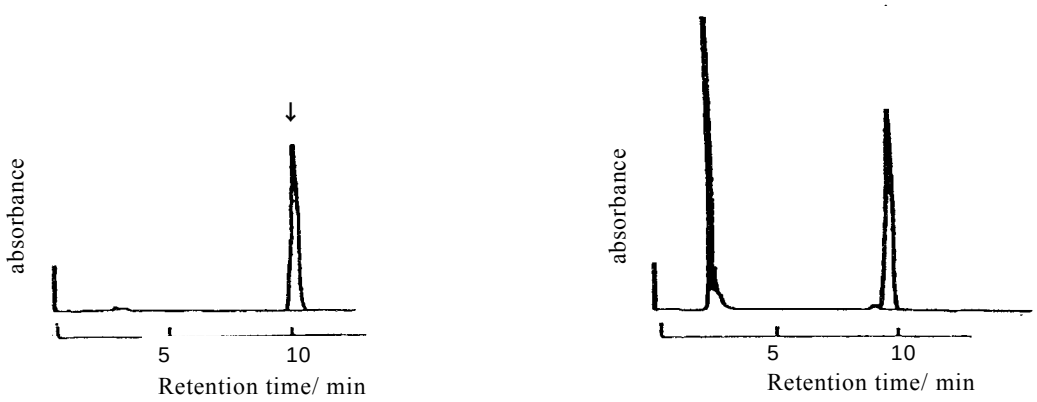
《Notes and precautions》

[1] Given the maximum content of sulfaquinoxaline in available premixes, there may be cases where sulfaquinoxaline can not sufficiently extracted when the sample is more than 2.0 g.

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

[3] 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.

[4] An example of chromatogram of sulfaquinoxaline is shown in Fig. 8.1.10-2.



（A）Standard solution (5 μg/mL)　　（B）Premix for chicken (Added 12 g/kg equivalent)

Fig. 8.1.10-2　A chromatogram of sulfaquinoxaline in a premix
(The arrow indicates the peak of sulfaquinoxaline)

Measurement conditions

Detector:　Ultraviolet spectrophotometer (measurement wavelength: 240 nm)

Column:　Shodex C_{18}-5B (internal diameter: 4.6 mm, length: 250 mm, particle diameter: 5 μm)

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

Flow rate:　1.0 mL/min

Column temperature:　40 °C

1.1.2 Formula feed 〔Feed Analysis Standards Chapter 8, Section 1, 10.1.1-(2)〕

A. Reagent preparation

Sulfaquinoxaline standard solution: Place 20 mg of sulfaquinoxaline〔$C_{14}H_{12}N_4O_2S$〕exactly measured in a 100 mL brown volumetric flask, add acetonitrile for dissolving, further add the solvent up to the gauge line to prepare the sulfaquinoxaline standard stock solution（1 mL of this solution contains an amount of sulfaquinoxaline equivalent to 0.2 mg）.

At the time of use, exactly dilute a definite amount of the standard stock solution with acetonitrile-water (4:1) to prepare several sulfaquinoxaline standard solutions containing amounts of sulfaquinoxaline equivalent to 2.5-15 μg per mL

B. Quantification

Extraction: Measure 10.0 g of analysis sample, place it in a 200 mL stoppered brown Erlenmeyer flask, add 100 mL of acetonitrile-water (4:1), and stir it for 30 min for extraction. Place the extracted solution in a stoppered brown centrifuging tube, centrifuge at 1,500×g for 5 min to obtain the supernatant. Filter the supernatant with 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 sulfaquinoxaline standard solution into a liquid chromatograph to obtain the chromatogram.

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 240 nm)

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

Eluent: Phosphate buffer solution Note 2-acetonitrile (7:3)[2]

Flow rate: 1.0 mL/min

Column temperature: 40°C

Calculation: Obtain the peak height or area from the chromatogram [3] to prepare the calibration curve, and calculate the sulfaquinoxaline amount in the sample.

Note 1. Shodex C_{18}-5B (Showa Denko) or an equivalent one

2. Dissolve 6.8 g of potassium dihydrogen phosphate in water to make a total amount of 1 L, and adjust the pH to 3.2-3.4 with phosphoric acid（1:10）

《Summary of analysis method》

This method is intended to determine the amount of sulfaquinoxaline by extracting with acetonitrile-water (4:1), and quantifying using a liquid chromatograph with an ultraviolet spectrophotometer.

The flowsheet of analysis method is shown in Fig. 8.1.10-3.

Sample: 10 g
- 100 mL of acetonitrile-water (4:1)
- Stir for 30 min
- Centrifuge (1,500×*g* for 5 min)
- Filter through a membrane filter (0.5 μm
- LC-UV (240 nm)

Fig. 8.1.10-3 Analysis method flowsheet of sulfaquinoxaline in a formula feed

Reference: Tetsuo Chihara, Yukie Ishida, Akira Shimizu: Research Report of Animal Feed, 23, 100 (1998)

History in the Feed Analysis Standards: 【7】new,【20】revision

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (mg/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (%)
Formula feed for starting chick (mush)	30-120	3	96.9-97.7	1.5
Formula feed for growing chick (mush)	30-120	3	96.8-98.7	1.5
Formula feed for later stage broiler chicken (mush)	30-120	3	96.4-97.9	1.8
Formula feed foe growing chick (pellet)	30-120	3	103.0-108.2	2.3

・Cooperative testing

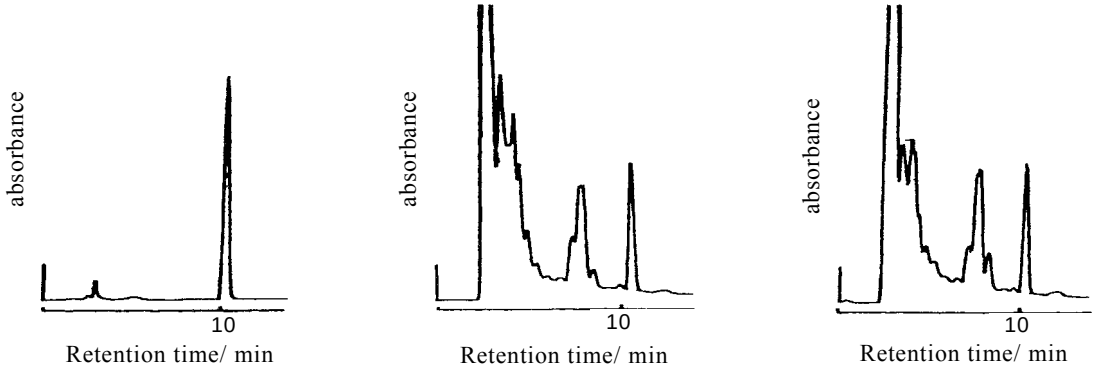
Type of sample	No. of labs	Concentration (mg/kg)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Formula feed for growing chick	6	60	101.2	1.2	2.8	0.33

《Notes and precautions》

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

[2] 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.

[3] An example of chromatogram of sulfaquinoxaline is shown in Fig. 8.1.10-4.



(A) Standard solution (5 μg/mL)

(B) Formula feed for later stage broiler chickens (added 60 g/t equivalent)

(C) Formula feed for growing chicks (added 60 g/t equivalent)

Fig. 8.1.10-4 A chromatogram of sulfaquinoxaline in a formula feed
(The arrow indicates the peak of sulfaquinoxaline)

Measurement conditions

Detector: Ultraviolet spectrophotometer (measurement wavelength: 240 nm)

Column: Shodex C_{18}-5B (internal diameter: 4.6 mm, length: 250 mm, particle diameter: 5 μm)

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

Flow rate: 1.0 mL/min

Column temperature: 40 °C

2 Microquantitative test method

2.1 Liquid chromatography 〔Feed Analysis Standards Chapter 8, Section 1, 10.2.1〕

A. Reagent preparation

1) Sulfaquinoxaline standard solution: Place 25 mg of sulfaquinoxaline〔$C_{14}H_{12}N_4O_2S$〕exactly measured in a 250 mL brown volumetric flask, add acetonitrile for dissolving, further add the solvent up to the gauge line to prepare the sulfaquinoxaline standard stock solution（1 mL of this solution contains an amount of sulfaquinoxaline equivalent to 0.1 mg）.

At the time of use, exactly dilute a definite amount of the standard stock solution with phosphate buffer solution-acetonitrile (9:1) to prepare several sulfaquinoxaline standard solutions containing amounts of sulfaquinoxaline equivalent to 2.5-20 μg per mL

2) Phosphate buffer solution: Dissolve 9 g of disodium hydrogenphosphate·12-water and 3.4 g of potassium dihydrogen phosphate in water to make a total amount of 1 L.

3) Fluorescamine solution: Dissolve 100 mg of fluorescamine[1] in acetone to make a total amount of 25 mL.

B. Quantification

Extraction: Measure 10.0 g of analysis sample [2], place it in a stoppered 200 mL brown Erlenmeyer flask, add 100 mL of acetonitrile, and stir for 30 min for extraction. Place the extracted solution in a stoppered brown centrifuging tube, centrifuge at 650×g for 5 min to obtain the supernatant as a sample solution for column treatment.

Column treatment: Place the sample solution in a octadecylsilylated silica-gel minicolumn (360 mg), and discard 5 mL of the first effluent. Place 5 mL of subsequent effluent exactly in a 50 mL brown volumetric flask, and add phosphate buffer solution up to the gauge line to prepare the sample solution [3] for fluoresceinization.

Fluoresceinization: Place 5 mL of the sample solution exactly in a test tube, exactly add and mix 0.5 mL of fluorescamine fluid, and allow still standing for 1 min [4] . Filter this solution through a membrane filter (pore diameter: 0.5 μm or less) to prepare the sample solution for liquid chromatography.

Simultaneously, place exactly 5 mL of each sulfaquinoxaline standard solution in a test tube, and fluoresceinize similarly to the sample solution to obtain the standard solution for liquid chromatography.

Liquid chromatography: Inject respective 20 μL of the sample solution and each standard

solution into a liquid chromatograph to obtain the chromatograms.

Measurement conditions (example)

Detector: Fluorescence detector (excitation wavelength: 410 nm, fluorescence wavelength: 490 nm)

Column: Octadecylsilylated silica-gel column[5](internal diameter: 4.0 mm, length: 250 mm, particle diameter: 5 μm) Note 1

Eluent: Phosphate buffer solution-methanol (1:1) [6]

Flow rate: 0.7 mL/min

Column temperature: 40 °C

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

Note 1. Nucleosil $5C_{18}$ (Macherey-Nagel) or an equivalent one.

《Summary of analysis method》

This method is intended to determine a minute amount of sulfaquinoxaline residue in a formula feed or premix caused by carry-over or others, by extracting with acetonitrile, purifying with a C_{18} minicolumn, fluoresceinizing with phosphate buffer solution and fluorescamine solution, and quantifying using a liquid chromatograph with a fluorescence detector.

References: Masayuki Shimomura, Shinji Kawaguchi,: Research Report of Animal Feed, 16, 96 (1991)

Shinji Kawaguchi: Research Report of Animal Feed, 17, 52 (1992)

History in the Feed Analysis Standards: 【13】new

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (mg/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (% or less)
Premix for chicken	0.5-1	3	95.0-101.3	2.5
Premix for pig	0.5-1	3	97.0-98.3	6.4
Premix for cattle	0.5-1	3	97.7-102.7	6.5
Formula feed for adult chicken	0.5	3	90.3	5.0
Formula feed for growing pig	0.5	3	97.0	5.2
Formula feed for beef cattle	0.5	3	91.0	3.8

・Cooperative testing

Type of sample	No. of labs	Concentration (mg/kg)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Premix for chicken	6	0.5	95.7	5.3	6.2	0.49
Formula feed for adult chicken	6	0.5	89.0	6.7	9.8	0.77

《Notes and precautions》

[1] Commercially available from Wako Pure Chemical Industries as Fluram (Fluka Chemie AG, Swiss).

[2] The amount of sulfaquinoxaline extractable by this method is approximately 40 μg; therefore, when the amount of quantified sulfaquinoxaline is approximately 4 mg/kg, it is better to re-analyze a smaller amount of sample

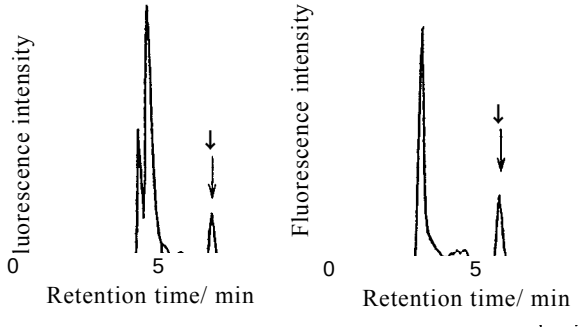
[3] Note that some samples produce precipitation after left still standing for 2-3 hr after adding phosphate buffer solution, which may cause decreased fluorescence intensity.

[4] The time required for fluoresceinization is very short. Stability of the fluoresceinized liquid is confirmed up to approximately 4 hr; however, in some samples, the fluorescence intensity may decrease. Therefore, it is better not to leave it for a long time.

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

[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 is shown in Fig. 8.1.10-5.



Measurement conditions

Detector: Measurement wavelength: Em 410 nm, Ex 490 nm

Column: Nucleosil 5 C_{18}

Eluent: Phosphate buffer solution-methanol (1:1)

Flow rate: 0.7 mL/min

Column temperature: 40 °C

Fig. 8.1.10-5 A chromatogram of sulfaquinoxaline
(The arrow indicates the peak of a sulfaquinoxaline fluorescent derivatization agent)

【Other analysis methods】

3 Quantitative test method by absorptiometric method

Scope of application：Formula feeds and premixes other than those forming-processed such as pellets

A. Reagent preparation

1) Sulfaquinoxaline standard solution: Place 40 mg of sulfaquinoxaline〔$C_{14}H_{12}N_4O_2S$〕exactly measured in a 50 mL brown volumetric flask, add *N*,*N*-dimethylformamide for dissolving, further add the solvent up to the gauge line to prepare the sulfaquinoxaline standard stock solution[1]（1 mL of this solution contains an amount of sulfaquinoxaline equivalent to 0.8 mg）.

At the time of use, exactly dilute a definite amount of the standard stock solution with *N*,*N*-dimethylformamide（1 mL of this solution contains an amount of sulfaquinoxaline equivalent to 8 μg）.

2) Basic alumina[2]: Basic alumina for a column chromatograph (with a standard sifter [40~150 μm])

3) Sodium chloride-sodium hydroxide solution: Dissolve 2 g of sodium hydroxide and 100 g of sodium chloride in 500 mL of water.

4) *N*-1-naphthylethylenediamine solution: Dissolve 50 mg of *N*-1-naphthylethylenediamine dihydrochloride in 27 mL of hydrochloric acid (2:25) (prepare at the time of use).

5) Zirconium oxychloride solution: Dissolve 5 g of zirconium oxychloride in 100 mL of water.

6) Sodium nitrite solution: Dissolve 100 mg of sodium nitrite in 100 mL of water (prepare at the time of use).

7) Ammonium sulphamate solution: Dissolve 0.5 g of ammonium sulphamate in 100 mL of water.

B. Quantification

Extraction: Measure 8 g of analysis sample [3], place it in a stoppered 200 mL brown Erlenmeyer flask, add 100 mL of *N*,*N*-dimethylformaldehyde, loosely stopper, and extract in a boiling water bath while shaking at intervals for 20 min. After cooling, place it in a stoppered centrifuging tube, and centrifuge [3] at 1,000×g for 5 min to obtain the supernatant as a sample solution for column treatment.

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

Place exactly 10 mL of the extracted solution in the column, and flow down spontaneously to a fluid level of 5 mm from the surface of the packing material. Then, effuse it while washing the inside wall of the column twice with respective 5 mL of chloroform (discard the effluent).

Fix a suction machine [4] at the top of the column, and let air through it until the alumina dries and the column returns to room temperature.

Place a 25 mL volumetric flask under the column, and add 25 mL of sodium chloride-sodium hydroxide solution to the column to elute sulfaquinoxaline. Add 1 mL of hydrochloric acid to the eluate, and add water up to the gauge line to prepare the sample solution for color-forming.

Simultaneously, treat the columns for 10 mL of sulfaquinoxaline standard solution and 10 mL of *N*,*N*-dimethylformamide in a similar way to the sample extract to obtain the standard solution and blank test solution.

Color-forming and measurement: Place exactly 10 mL of sample solution in a stoppered 50 mL centrifuging tube, add and mix 2 mL of zyconium oxychloride solution, further add and mix 1 mL of sodium nitrite solution and leave it still standing for 2 min. Then, add and mix 1 mL of ammonium sulphamate solution and leave it still standing for 2 min. Additionally add and mix 1 mL of *N*-1-naphthylethylenediamine solution [5] and leave it still standing for 10 min, to which add 2 g of sodium chloride and 10 mL of 1-butanol-hexane (4:1), stopper the tube, vigorously shake it until sodium chloride dissolved [5], and centrifuge at 650×g. Collect the butanolhexane layer (upper layer) to measure the absorbance at wavelength of 550 nm using 1-butanol-hexane (4:1) as the control.

Simultaneously, treat 10 mL of standard solution and 10 mL of standard blank test solution in a similar way to the sample solution to obtain the absorbance [7].

Calculation: Calculate the amount of sulfaquinoxaline in the sample using the formula mentioned

below.

Amount of sulfaquinoxaline in the sample (mg/kg) $= \frac{A}{A'} \times 100$

A : The absorbance of the sample solution after deducting the absorbance of the blank test.

A' : The absorbance of the sample solution after deducting the absorbance of the blank test.

《Summary of analysis method》

This method is intended to determine the amount of sulfaquinoxaline in the sample by extracting with *N*,*N*-dimethylformamide in a boiling water bath, purifying with a basic alumina column, color-forming by adding 1 mol/L of hydrochloric acid, sodium nitrite solution, ammonium sulphamate solution and *N*-1-naphthylethylenediamine-dihydrochloric acid solution, measuring the absorbance at 545 nm, and quantifying by comparing it with the absorbance of the standard solution.

The recovery rates of sulfaquinoxaline added to feeds forming-processed such as pellets are as low as 40-50 %. Therefore, we set a scope of application for the quantification method.

Reference: "Official Methods of Analysis of the AOAC International", 14th Ed., 42 171 (1984)

《Notes and precautions》

[1] The stability can be kept for approximately 1 month by storing in an airtight container in a dark place.

[2] Use Basic Alumina No. 1076 (Merck) or an equivalent one.

[3] As for premix, take 1-2 g of sample, extract, centrifuge, and dilute to approximately 50 to 200-fold for column treatment.

[4] Suction using a mini-pump such as Uni pump (NIPPON RIKAGAKU KIKAI)

[5] Diazo coupling method was used. An almost constant absorbance was obtained up to the coupling time of 20 min. However, decreased absorbance was observed thereafter, being considered caused by change of the products obtained by the coupling. Therefore, the coupling time in this method was set to 10 min.

[6] A shaking apparatus with an attachment for centrifuging tube is useful for shaking.

[7] Absorption curves of sulfaquinoxaline is shown in Fig. 8.1.10-6.

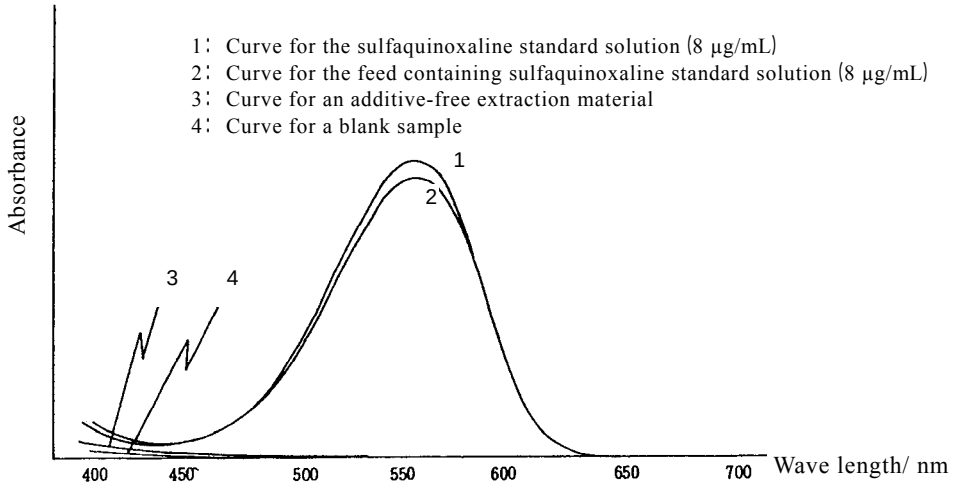


Fig. 8.1.10-6　Absorption curves of sulfaquinoxaline