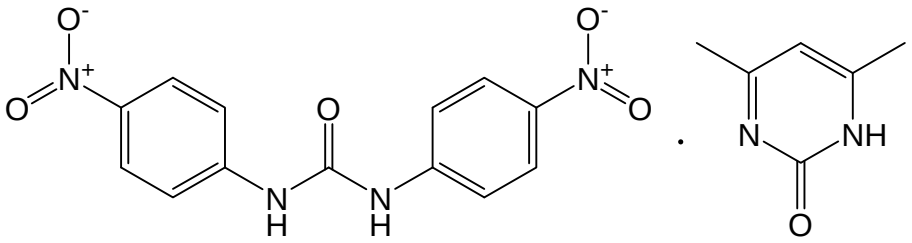
12 Nicarbazin

Nicarbazin (4,4′-dinitro carbanilid (DNC) and 2-hydroxy-4,6-dimethyl pyrimidine (HDP))



1,3-bis(4-nitrophenyl)urea, 4,6-dimethyl-1*H*-pyrimidin-2-one

$C_{13}H_{10}N_4O_5 \cdot C_6H_8N_2O$ MW: 426.38282 CAS No.: 330-95-0

【Outline of nicarbazin】

Nicarbazin is a molecular compound of above-mentioned DNC and HDP, an anticoccidial agent showing a wide preventive effect to chicken coccidosis; therefore, it mainly used as an animal drug. The residue standard value of nicarbazin has been set at 0.2-0.5 mg/kg on DNC base in Food Sanitation Act. This agent was designated to one of feed additives in 1976, and was approved to add to formula feed for growing chicks and broiler chickens in the range of 100-200 g/t to promote the effective use of nutrient components in feeds; however, recently it is approved to add only to formula feeds for broiler chickens in the prior stage at 100 g/t.

This agent is brownish yellow to greenish yellow powder without odor or with slight special odor. It is slightly soluble in *N*,*N*-dimethylformamide, and very slightly soluble in water, ethanol, ether and chloroform.

【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, 12.1.1-(1)〕

A. Reagent preparation

Nnicarbazin standard solution: Place 25 mg of nicarbazin〔$C_{13}H_{10}N_4O_5 \cdot C_6H_8N_2O$〕[1] exactly measured in a 500 mL brown volumetric flask, add 150 mL of *N*,*N*-dimethylformamide for dissolving by moderately worming, and allow being cool. Then, add the solvent up to the gauge line of the volumetric flask to prepare the nicarbazin standard stock solution (1 mL of this solution contains an amount of nicarbazin equivalent to 50 μg).

At the time of use, dilute a definite amount of the standard stock solution exactly with *N*,*N*-dimethylformamide to prepare several nicarbazin standard solutions containing amounts of nicarbazin equivalent to 5-20 μg per mL.

B. Quantification

Extraction: Place 1-2 g of the analysis sample exactly measured in a stoppered 200 mL brown Erlenmeyer flask, add 100 mL of *N*,*N*-dimethylformamide, heat while shaking and mixing at intervals in a boiling water bath for 15 min for extraction [2], and allow being cool.

Place the extracted solution in a stoppered centrifuging tube, and centrifuge at 1,500×*g* for 5 min. Dilute [3] the supernatant with *N*,*N*-dimethylformamide exactly to 50-100 fold, and filter 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 nicarbazin standard solution into a liquid chromatograph to obtain the chromatogram.

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (measurement wavelength：347 nm) [4]

Column: Octadecylsilylated silica-gel column (internal diameter: 3.9 mm, length: 300 mm, particle diameter: 10 μm) Note1 [5]

Eluent: Methanol-water (7:3)

Flow rate: 1.0 mL/min

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

Note 1. μBondapak C_{18} (Waters) or an equivalent one.

1.1.2 Formula feed 〔Feed Analysis Standards Chapter 8, Section 1, 12.1.1-(2)〕

A. Reagent preparation

Nicarbazin standard solution[6]: Prepare the solution according to (1)-A.

B Quantification

Extraction: Place 5-10 g of the analysis sample (an amount equivalent to 1 mg as nicarbazin) exactly measured in a stoppered 200 mL brown Erlenmeyer flask, add 100 mL of *N*,*N*-dimethylformamide, heat in a boiling water bath for 15 min, while moderately shaking and mixing for several seconds at intervals for extraction, and then allow being cool.

Place the extracted solution in a stoppered centrifuging tube, and centrifuge at 1,500×*g* for 5 min. Filter the supernatant through a membrane filter (pore diameter: 0.5 μm or less) to obtain a sample solution for liquid chromatography.

Liquid chromatography [7]: Process according to (1)-B, Liquid chromatography.

Calculation [7][8]: Calculate according to (1)-B, Calculation.

《Summary of analysis method》

This method is intended to determine the amount of nicarbazin 〔a molecular compound of 4,4′-dinitro carbanilid (DNC) and 2-hydroxy-4,6-dimethyl pyrimidine (HDP) 〕 in the sample by extracting with *N*,*N*-dimethylformamide by heating, and quantifying by determining DNC using a liquid chromatograph with an ultraviolet spectrophotometer, with a help of nature of DNC to be

absorbed in the ultraviolet portion (λ_{max} = 347 nm). (HDP is not detected because HDP is separated in the column, and does not be absorbed in the range of wavelength longer than approximately 300 nm)

Reference: Takayuki Koyama, Shuichi Shimada: Animal-husbandry, 41, 589 (1987)

History in the Feed Analysis Standards: 【8】new

《Validation of analysis method》

・Recovery rate and repeat accuracy (premix)

Type of sample	Concentration (g/kg)	Repeat	Recovery rate (%)	Reproducibility RSD (% or less)
Premix for starting chick	25-100	3	99.5-100.3	0.9
Premin for growing chick	25-100	3	99.6-100.0	1.1
Premix for prior stage broiler chicken	25-100	3	99.6-101.0	1.0
Premix for later stage broiler chicken	25-100	3	100.2-100.3	0.8

・Recovery rate and repeat accuracy (formula feed)

Type of sample	Concentration (mg/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (% or less)
Formula feed for starting chicken	100-200	3	99.7-99.7	0.9
Formula feed for growing chicken	100-200	3	99.1-101.3	1.4
Formula feed for prior stage broiler chicken	100-200	3	98.3-99.7	1.3
Formula feed for later stage broiler chicken	100-200	3	99.0-99.1	0.5

・Cooperative testing

Type of sample	No. of labs	ndicated amoun (g/t)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Repruducibility RSD_R (%)	HorRat
Formula feed for prior stage broiler chicken	6	125	100.6	2.1	2.4	0.44

《Notes and precautions》

[1] Store in a cold and dark place. It is preferable to use the standard stock solution within 3 months and standard solution within a week.

[2] Other than this extraction method (heating in a boiling water bath), an available one is to heat while shaking and mixing at intervals on a hot plate up to just before boiling or to boil for 2 min in an Erlenmeyer flask loosely stoppered.

[3] Dilute up to the contained amount of nicarbazin equivalent to 5-15 μg/mL.

[4] The absorption curve of nicarbazin standard solution is shown in Fig. 8.1.12-1.

[5] A column with packing material appropriately end capped is applicable.

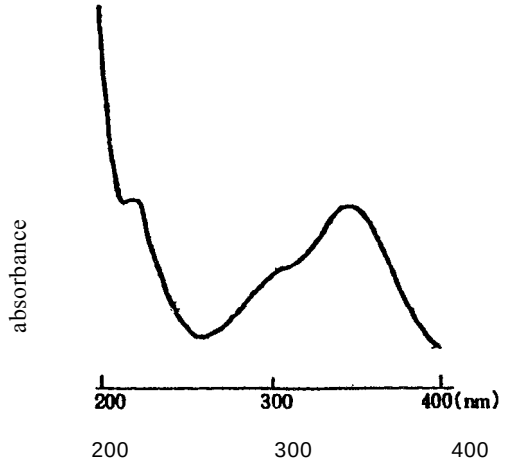


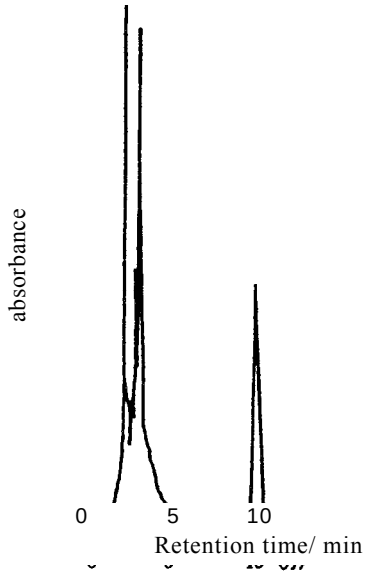
Fig. 8.1 in (in methanol-water solution (7+3), control: air))

[6] The preparation method is the same as that described under 1.1.1-A.

[7] The operation method is the same as that described under 1.1.1-B.

[8] An example of chromatogram of nicarbazin extracted from a formula feed is shown in Fig. 8.1.12-2.

Use of a degradated column for the sample containing ethoxyquin may cause double peaks of ethoxyquin and nicarbazin; therefore, it is preferable not to use a degraded column in that case. In case of using a degradated column by necessity, treat the column according to the 1.2.1 Absorptiometric method (premix)-B for removing ethoxyquin before using for liquid chromatography.



Measurement conditions

Detector: Measurement wavelength: 365 nm

Column: μBondapak C18

Eluent: Methanol-water (7:3)

Flow rate: 1.0 mL/min

Fig. 8.1.12-2 A chromatogram of nicarbazin added to a formula feed for starting chicks
(The arrow indicates the peak of nicarbazin)

1.2 Absorptiometric method

1.2.1 Premix

〔Feed Analysis Standards Chapter 8, Section 1, 12.1.2-(1)〕

A. Reagent preparation

1) Nicarbazin standard solution: Place 25 mg of nicarbazin〔$C_{13}H_{10}N_4O_5 \cdot C_6H_8N_2O$〕[1] exactly measured in a 500 mL brown volumetric flask, add 150 mL of *N*,*N*-dimethylformamide, heat moderately for dissolving, and allow being cool. Then, add the solvent up to the gauge line of the volumetric flask to prepare the nicarbazin standard stock solution (1 mL of this solution contains an amount of nicarbazin equivalent to 50 μg).

At the time of use, dilute a definite amount of the standard stock solution exactly with *N*,*N*-dimethylformamide to prepare the nicarbazin standard solution containing an amount of nicarbazin equivalent to 12.5 μg/mL.

2) Basic alumina[2]: Dry basic alumina (particle diameter: 63-200 μm (230-70mesh)) Note1 for column chromatograph at 105 °C for 3 hr.

3) Sodium hydroxide solution: Dissolve 50 g of sodium hydroxide in 50 mL of water to obtain the supernatant (stopper air tightly for storing) for use.

4) Sodium hydroxide/ethanol solution: Add ethanol to 2 mL of sodium hydroxide up to a total volume of 100 mL, and centrifuge to obtain the supernatant for use (prepare at the time of use).

B. Quantification

Extraction: Place 1-2 g of analysis sample exactly measured in a stoppered 250 mL brown Erlenmeyer flask, add 100 mL of *N*,*N*-dimethylformamide, loosely stopper the flask, heat on a hot plate [3] while shaking and mixing for several min at intervals for extraction, and then allow being cool. Place the extracted solution in a stoppered centrifuging tube to centrifuge at 650×*g* for 3 min. Dilute the supernatant exactly with *N*,*N*-dimethylformamide up to 50 to 500-fold to obtain a sample solution for column treatment.

Column treatment: Pack a column tube (internal diameter: 22 mm) with 30 g of basic alumina by dry processing, and plug the top slightly with glass wool. Place *N*,*N*-dimethylformamide in the column [4], and effuse it until the fluid level reaches 3 mm from the top of the packing material to prepare the column.

Place 25 mL of the sample solution in the column, and effuse it until the fluid level reaches 3 mm from the top of the packing material. Then, add 30 mL of *N*,*N*-dimethylformamide and 20 mL of ethanol sequentially to the column to effuse in a similar way.

Place a 50 mL volumetric flask under the column, and add 40 mL of ethanol to the column to elute nicarbazin. Then, add ethanol up to the gauge line of the volumetric flask to obtain a sample solution for measurement.

Simultaneously, treat 25 mL of the nicarbazin standard solution in a manner similar to that for the sample solution to prepare the standard solution for measurement.

Measurement: Place 15 mL of the sample solution exactly measured in respective 25 mL volumetric flasks A and B. Add 5 mL of sodium hydroxide/ethanol solution to the volumetric flask A for color-forming [5], and add ethanol up to the gauge line.

Add ethanol to the volumetric flask B up to the gauge line, mix while shaking, and measure the absorbance at a wavelength of 430 nm for the solution in the volumetric flask A using the solution in the volumetric flask B as the control.

Simultaneously, place several amounts between 10 to 20 mL of the standard solution exactly in respective 25 mL volumetric flasks, add respective 5 mL of sodium hydroxide/ethanol solutions for color-forming, and further add ethanol up to the gauge line, and measure the absorbance at a wavelength of 430 nm for each solution using ethanol as the control.

Calculation: Prepare the calibration curve from the absorbance obtained, and calculate the amount of nicarbazin in the sample.

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

1.2.2 Formula feed 〔Feed Analysis Standards Chapter 8, Section 1, 12.1.2-(2)〕

A. Reagent preparation[6]

Prepare according to (1)-A.

B. Quantification

Extraction: Place 10.0 g of the analysis sample measured in a stoppered 250 mL brown Erlenmeyer flask, add 100 mL of *N*,*N*-dimethylformamide, loosely stopper the flask, heat on a hot plate while lightly shaking and mixing for several min at intervals up to just before boiling for extraction, and then allow being cool. Place the extracted solution in a stoppered centrifuging tube, centrifuge at 650×*g* for 3 min to obtain the supernatant as a sample solution for column treatment.

Column treatment[7]: Treat the column according to (1)-B, column treatment.

Measurement[7]: Measure according to (1)-B, Measurement.

Calculation[7] Calculate according to (1)-B, Calculation.

《Summary of analysis method》

This method intended to determine the amount of nicarbazin by adsorbing nicarbazin extracted with DMF to an alumina column, rinsing out interfering substances with DMF, eluting the adsorbed incarbazin with ethanol, adding sodium hydroxide-ethanol solution, and measuring the absorbance in color-formed yellow solution at 430 nm.

References: "Official Methods of Analysis of the AOAC International" 16th Ed., 5.1.30 (1995)
Eiichi Ishiguro: Research Report of Animal Feed, 5, 127 (1979)

History in the Feed Analysis Standards: 【1】new

《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 chicken	100-200	6	96.6-96.6	1.8

・Cooperative testing

Type of sample	No. of labs	ndicated amoun (g/t)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Formula feed	6	125	93.1	4.7	4.1	0.74

《Notes and precautions》

[1] Store in a cold dark place. It is preferable to use the standard stock solution within 3 months, and standard solution within a week.

[2] Use Aluminiumoxid aktiv basisch Art. 1076 (Merck) or an equivalent one. It is preferable to pour the standard solution into an alumina column for eluting, and confirm the eluted fractions before use.

[3] Although it is better to heat on a hot plate with a stirrer, a sand-bath is also available. The place with ventilating installation is recommended.

[4] After packing a column with alumina, fill with approximately 25 mL of DMF as soon as

possible to prevent decreased alumina activity.

[5] Forms yellow-color. Measure within 10 min after color-forming.

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

[7] The operating method is the same as that described under 1.2.1-B.

2 Microquantitative test methods

2.1 Liquid chromatography 〔Feed Analysis Standards Chapter 8, Section 1, 12.2.1〕

Scope of application: Formula feed

A. Reagent preparation

1) Nicarbazin standard solution: Place 10 mg of nicarbazin 〔$C_{13}H_{10}N_4O_5 \cdot C_6H_8N_2O$〕 exactly measured in a 200 mL brown volumetric flask, add 100 mL of acetonitrile for dissolving while mildly heating [1], and allow being cool. Then, add the solvent up to the gauge line of the volumetric flask to prepare the nicarbazin standard stock solution (1 mL of this solution contains an amount of nicarbazin equivalent to 50 μg).

At the time of use, dilute a definite amount of the standard stock solution exactly with acetonitrile to prepare several nicarbazin standard solutions containing amounts of nicarbazin equivalent to 0.125-2 μg/mL.

2) Basic alumina: Dry basic alumina (particle diameter: 63-200 μm (230-70mesh)) Note2 for column chromatograph at 130 °C for 2 hr.

B. Quantification

Extraction: Place 10.0 g of the analysis sample measured in a 300 mL separating funnel, add 200 mL of acetonitrile, mix while shaking for 30 min for extraction. Filter the extracted solution through a piece of paper filter (No. 2) to obtain a sample solution for purifying.

Purification: Place exactly 100 mL of the sample solution in a 300 mL separating funnel, add 75 mL of acetonitrile-saturated hexane, mix while shaking for 10 min, leave still standing, and place the acetonitrile layer (lower layer) in a 300 mL recovery flask. Add 10 mL of butanol [2] to the acetonitrile layer, concentrate this solution under reduced pressure in a water bath at 50 °C to almost dry out, and send nitrogen gas to obtain the dry matter.

Add 20 mL of tetrahydrofuran to dissolve the residue and prepare a sample solution for column treatment.

Column treatment: Suspend 4 g of basic alumina in tetrahydrofuran. Pour this suspension into a column tube (internal diameter: 10 mm), effuse it up to the fluid level of 3 mm from the top of the packing material to prepare the column.

Place the sample solution in the column, rinse the recovery flask previously containing the sample solution twice with respective 3 mL of tetrahydrofuran, add the washings sequentially to the column, and effuse it until the fluid level reaches 3 mm from the top of the packing material. Add 10 mL of tetrahydrofuran to the column to effuse, and rinse the column in a similar way. Place a 100 mL recovery flask under the column, and add 50 mL of tetrahydrofuran-methanol (9:1) to the column to elute nicarbazin. Concentrate the eluate in a water bath at 40°C or lower to almost dry

out, and send nitrogen gas to obtain the dry matter.

Add exactly 2 mL of methanol-acetonitrile (7:3) to dissolve the residue. Filter the solution through a membrane filter [3] (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 nicarbazin standard solution into a liquid chromatograph to obtain the chromatogram.

Measurement conditions (Example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 340 nm)

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

Eluent: Water-acetonitrile (1:1)

Flow rate: 1.0 mL/min

Column temperature: 40 °C

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

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

2. Aluminiumoxid 90 aktiv basisch Art. 1076 (Merck) or an equivalent one.

《Summary of analysis method》

This method was developed to quantify nicarbazin minimally remained in formula feeds due to carry-over, etc. and intended to determine the amount of nicarbazin in the sample by extracting with acetonitrile, purifying with a basic alumina column, and quantifying using a liquid chromatograph with an ultraviolet spectrophotometer.

Reference: Takayuki Ishibashi: Research Report of Animal Feed, 19, 100 (1994)

History in the Feed Analysis Standards: 【16】new

《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	103.2-109.5	2.7
Formula feed for finishing pig	0.1-1.0	3	102.5-107.1	8.9
Formula feed for finishing beef cattle	0.1-1.0	3	101.1-107.7	6.2

・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	6	0.1	106.1	8.9	16.3	0.74

《Notes and precautions》

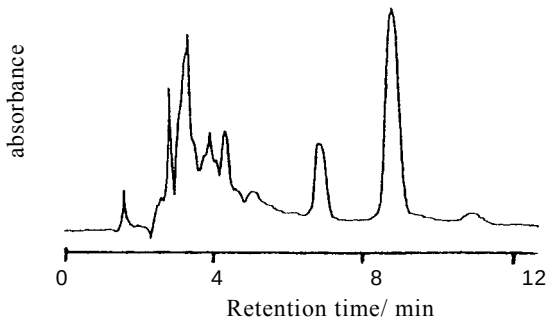
[1] Dissolve on a hot plate while shaking at intervals.

[2] Add to inhibit a sudden boiling.

[3] Products of polytetrafluoroethylene (PTFE) resin and likes, such as Millipore Filter®, Fluoro Pore Filter® and EKICRODISK®.

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

[5] An example of chromatogram is shown in Fig. 8.1.12-3.



Measurement conditions

Detector: Measurement wavelength: 340 nm

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

Eluent: Water-acetonitrile (1:1)

Flow rate: 1.0 mL/min

Column temperature: 40 °C

Fig. 8.1.12-3 A chromatogram of nicarbazin added to a formula feed for adult chickens at a level equivalent to 0.1 g/t
(The arrow indicates the peak of nicarbazin)