

Stofnaam	Nicarbazine
Type methode	HPLC
Te onderzoeken in	Diervoeders; voormengsels
Minimum bepaalbaarheidsgrens	20 mg/kg
Herhaalbaarheid	RSD _r (Relatieve standaarddeviatie) voor diervoeders en premixen op de volgende niveaus: 20 mg/kg: 10% 45 mg/kg: 7.1% 110 mg/kg: 6.0% 240 mg/kg: 2.6% 7500 mg/kg: 5.7%
Reproduceerbaarheid	RSD _r voor diervoeders en premixen op de volgende niveaus: 20 mg/kg: 12% 45 mg/kg: 9.1% 110 mg/kg: 7.9% 240 mg/kg: 4.8% 7500 mg/kg: 8.2%
Categorie	A
Titel	Determination of nicarbazin in animal feedingstuffs and premixtures by High Performance Liquid Chromatography CANFAS / SMT4-CT98-2216 / final method nicarbazin / 2003-01-31

1 SCOPE

This operating procedure specifies a method for the determination of the nicarbazin content in animal feedingstuffs and premixtures (maximum concentration 2.5 % nicarbazin) using high performance liquid chromatography. The limit of quantitation (LOD) determined in the pre-validation study was 20 mg/kg.

2 PRINCIPLE

Samples are extracted by heating in a waterbath, mechanical shaking and sonoration using an acetonitrile/methanol mixture. For feeding stuffs, also water is added. The mixture is transferred in a volumetric flask. After settlement of the solids, an aliquot is filtered and assayed using a reverse-phase isocratic method which measures the 4,4'-dinitrocarbanilide (DNC) moiety at a wavelength of 350 nm.

3 REAGENTS

Use only reagents of recognised analytical grade. Use water complying with at least grade 3 in accordance with ISO 3696.

3.1 Acetonitrile, HPLC grade

3.2 Methanol, HPLC grade

3.3 Extraction solvent. Mix 500 ml of acetonitrile (3.1) with 500 ml of methanol (3.2). Mix well using a magnetic stir plate and stir bar.

3.4 Eluent for liquid chromatography. Mix 650 ml acetonitrile (3.1) with 350 ml of purified water (resistance $> 10 \text{ M}\Omega\cdot\text{cm}^{-1}$). Mix well using a magnetic stir plate and stir bar and degas (e.g. with helium) before use.

3.5 Nicarbazin reference standard.

4 APPARATUS

Using laboratory apparatus and, in particular, the following:

4.1 High performance liquid chromatography system consisting of the following:

4.1.1 An autosampler or manual injector set to inject a volume of 20 μl .

4.1.2 A pump set to deliver a constant eluent flow rate of 1,0 ml/min

4.1.3 A column, length 300 mm, internal diameter 3.9 mm, packed with a stationary phase consisting of C-18 material. The particle size should not be smaller than 5 μm and not greater than 10 μm . (A Nova-Pak or Bonda-Pak column is recommended, but also other columns can be used provided that a satisfactory separation of DNC is achieved).

4.1.4 A detector allowing the measurement of absorbance of UV light at a wavelength of 350 nm, with integrator/recorder.

- 4.2 Mechanical shaker (e.g. Gyrotory shaker, wrist action shaker)
- 4.3 Micro filters for sample filtration, 0.2 - 0.5 μm
- 4.4 Mill to prepare laboratory samples with a maximum particle size of 1 mm
- 4.5 Ultrasonic bath
- 4.6 Waterbath, 50 °C
- 4.7 Disposable centrifuge tubes of 50 ml with a screw cap

5 PREPARATION OF THE SAMPLES

5.1 Test samples

The milling and mixing of compound feed samples prior to assay is obligatory. Grind feed samples through a mill (4.4) equipped with a 1 mm screen. After milling, mix the entire sample thoroughly. Store the sample at room temperature in subdued light. Premix samples are not milled.

5.1.1 Mixing of the test samples before weighing

The container should be filled to a maximum of 50 % of the total volume. Bring the container in a horizontal position and rotate bottom and top of the container in circles moving the container up and down along the virtual centre of the container for 30 seconds. Put the container in an upright position and wait a few seconds for settlement of the generated dust.

5.2 Spiked feed samples; 100 mg/kg

Transfer 2.5 ml of the stock standard solution (6.4.1.1) in the sample tube or flask. Evaporate to a small volume (less than 0.5 ml) with a gentle stream of nitrogen, add 2.5 g blank feed, mix thoroughly and wait 10 minutes before starting the extraction procedure by adding water for swelling (see 6.2.2).

6 PROCEDURE

6.1 General

Complete each assay within one working day.

6.2 Extraction

6.2.1 Premixtures

Weigh to the nearest 0.001 g, approximately 1.0 g of the test sample directly into a wide neck volumetric flask of 200 ml.

Add 80 ml of extraction solvent (3.3), close the flask and mix manually by swirling.

Put the flasks in a waterbath of 50 °C for 15 minutes with intermediate swirling at 8 minutes.

Mix thoroughly 15 minutes using a mechanical means (4.2).

Put the flasks in an ultrasonic bath (4.5) and sonorate for 15 minutes.

Cool down to room-temperature, adjust to volume with HPLC eluent (3.4) and mix.

Allow sample solids to settle (minimum 30 minutes).

If additional dilutions are required, dilute the samples with HPLC eluent (3.4) to a final nicarbazin concentration of ca 5 µg/ml.

Filter an aliquot of the final dilution through a micro filter (4.3) for analysis by HPLC.

6.2.2 Animal feedingstuffs

Weigh to the nearest 0.01 g, approximately 2.5 g (see remark 9.1) of the test sample into a 50 ml disposable centrifuge tube (4.7) or directly into a wide neck volumetric flask of 100 ml.

Add 5 ml of water. Take care that the whole sample is wetted.

Wait at least 10 minutes.

Add 35 ml of extraction solvent (3.3), close the tube or flask and mix manually by swirling.

Put the tubes or flasks in a waterbath of 50 °C (4.6) for 15 minutes with intermediate swirling at 8 minutes.

Mix thoroughly 15 minutes using a mechanical means (4.2).

Put the tubes or flasks in an ultrasonic bath (4.5) and sonorate for 15 minutes.

Transfer the sample extract if necessary quantitatively in a 100 ml volumetric flask with HPLC eluent (3.4), adjust to volume and mix.

If additional dilutions are required, dilute the samples with HPLC eluent (3.4) to a final nicarbazin concentration which falls within the standard curve levels.

Filter an aliquot of the final dilution through a micro filter (4.3) for analysis by HPLC.

6.3 Determination

6.3.1 Inject 20 µl of the sample extract on to the column of the liquid chromatograph (4.1) and measure the area/height of the DNC peak.

6.3.2 Determine the nicarbazin concentration of the extract by reference to the mean of a calibration curve prepared as described in 6.4 and analysed before and after the sample extracts.

6.4 Calibration

6.4.1 Preparation of nicarbazin standard solutions

6.4.1.1 Nicarbazin stock standard solution, 100 µg/ml

Dissolve 10 mg, weighed to the nearest 0.1 mg, of nicarbazin reference standard (3.5) in 100 ml extraction solvent (3.3). To aid with dissolution, sonication for approximately 5 minutes is recommended. Mix well. This solution is stable for 24 hours when stored in subdued light at ambient or refrigerated storage conditions (see remark 9.2).

6.4.1.2 Nicarbazin working standard solutions for feedingstuffs containing 50-250 mg/kg nicarbazin and for premixtures

Prepare a range of calibration working standards containing 0, 1, 2, 3, 4, 5 and 10 µg/ml nicarbazin by diluting the stock standard solution (6.4.1.1) with HPLC eluent (3.4). Working standards must be prepared daily.

6.4.1.3 Nicarbazin working standard solutions for feedingstuffs containing 20-50 mg/kg nicarbazin

Prepare a range of calibration working standards containing 0; 0.25; 0.5; 1; 2 and 2.5 µg/ml nicarbazin by diluting the stock standard solution (6.4.1.1) with HPLC eluent (3.4). Working standards must be prepared daily.

7 EXPRESSION OF RESULTS

Calculate the nicarbazin content of the test sample by the equation:

$$W_E = \frac{V_e \times c}{M} \times f$$

Where:

W_E is the numerical value for the nicarbazin content of the test sample in mg/kg

V_e is the extraction volume, in ml, viz. 200 for premixtures (6.2.1) and 100 for feedingstuffs (6.2.2)

C is the numerical value of the nicarbazin concentration of the sample extract in µg/ml

M is the numerical value of the mass of the test sample, in g

F is the dilution factor introduced to prepare final sample extracts fitting with the standard curve levels

8 RECOVERY

The recovery obtained for compound feeds should be higher than 90 % at spike levels between 20 and 200 mg/kg.

9 REMARKS

9.1 Homogeneity

For relatively inhomogeneous compound feed samples, the weighed sample amount should be increased to 10 gram with simultaneous up-scaling of the volume of extraction solvent used.

9.2 Solubility

The solubility of the nicarbazin reference standard in extraction solvent is critical. The nicarbazin concentrations in the prepared stock solutions must be monitored by use of a cuvet spectrophotometer as follows. Prepare a solution of 10 $\mu\text{g/ml}$ by diluting the prepared stock standard solution (6.4.1.1) with acetonitrile. Record a UV-Vis spectrum between 220 and 450 nm using a mixture of methanol/acetonitrile (5:95 v/v) as a reference solution. The maximum absorbance measured between 340 and 350 nm should be within a margin of +/- 5 % of the default value. The default value should be established in your own laboratory by preparing a stock standard solution in duplicate and monitoring the UV-Vis spectra as described above. The default value is the mean result of the duplicates.

9.3 Method characteristics