BRITISH STANDARD

BS EN 15782:2009

Animal feeding stuffs — Determination of nicarbazin — High-performance liquid chromatographic method

ICS 65.120,

BSI
British Standards

National foreword

This British Standard is the UK implementation of EN 15782:2009.

The UK participation in its preparation was entrusted to Technical Committee AW/10, Animal feeding stuffs.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Futtermittel - Bestimmung von Nicarbazin - Hochleistungsflüssigchromatographisches Verfahren

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Contents

Page

Foreword

This document (EN 15782:2009) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by February 2010, and conflicting national standards shall be withdrawn at the latest by February 2010.

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1 Scope

This European Standard specifies a method for the determination of additive use of nicarbazin in animal feeding stuffs and premixtures (maximum concentration 2,5% nicarbazin) using high performance liquid chromatography. Nicarbazin is a 1:1 equimolar mixture of 4,4'-dinitrocarbanilide (DNC) and 4,6-dimethyl-2-pyriminol (HDP). Nicarbazin is generally determined by using DNC as the target compound. In this method the DNC moiety of nicarbazin is detected.

The limit of quantitation is 20 mg/kg. The limit of detection is 0,5 mg/kg

NOTE A lower limit of quantitation may be achievable but should be validated by the user.

2 Principle

Samples are extracted using an acetonitrile/methanol mixture. For feeding stuffs, water is added additionally. An aliquot of the extract is assayed using a reverse phase isocratic HPLC method which measures the 4,4'dinitrocarbanilide moiety at a wavelength of 350 nm.

3 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

3.1 Water, resistance > 10 MOhm.cm$^{-1.}$

3.2 Acetonitrile (CH_3CN), HPLC grade.

3.3 Methanol (CH_3OH), HPLC grade.

3.4 Extraction solvent

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

3.5 Eluent for liquid chromatography

Mix 650 ml acetonitrile (3.2) with 350 ml of purified water (3.1). Mix well using a magnetic stir plate and stir bar and degas (e.g. with helium) before use.

3.6 Nicarbazin reference standard

3.7 Standard solutions

3.7.1 Nicarbazin stock standard solution, 100 µg/ml

Dissolve 10 mg, weighed to the nearest 0,1 mg, of nicarbazin reference standard (3.6) in 100 ml extraction solvent (3.4). To aid with dissolution, sonication for approximately 5 min is recommended. Mix well. This solution is stable for 24 h when stored in subdued light at ambient or refrigerated storage conditions (see remark 3.7.1).

NOTE 1 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 µg/ml by diluting the prepared stock standard solution (3.7.1) with acetonitrile. Record a UV-Vis spectrum between 220 nm and 450 nm using a mixture of methanol/acetonitrile (5:95 v/v) as a reference solution. The maximum

absorbance measured between 340 nm 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.

NOTE 2 The susceptibility of nicarbazin to photon-degradation is well known. To avoid degradation during sample preparation and analysis, protect the samples and extracts from daylight at all times.

3.7.2 Nicarbazin working standard solutions

Prepare a range of calibration working standards containing 0 μg/ml; 0,25 μg/ml; 0,5 μg/ml; 1 μg/ml; 2 μg/ml; 5 μg/ml and 10 μg/ml nicarbazin by diluting the stock standard solution (3.7.1) with HPLC eluent (3.5). Working standards must be prepared daily.

4 Apparatus

Usual 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 HPLC column, 250 mm x 4,6 mm packed with RP C18, 5 µm material, or equivalent.

NOTE 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.

4.2 Mechanical shaker (e.g. Gyratory shaker, wrist action shaker or equivalent).

4.3 Micro filters for sample filtration, 0,2 μm to 0,5 μm.

4.4 Ultrasonic bath

4.5 Waterbath, 50°C ± 2°C.

5 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this European Standard. A recommended sampling method is given in EN ISO 6497.

6 Preparation of test sample

6.1 General

The milling and mixing of compound feed samples prior to assay is obligatory. Grind feed samples through a

mill 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.

NOTE The susceptibility of nicarbazin to photon-degradation is well known. To avoid degradation during sample preparation and analysis, protect the samples and extracts from daylight at all times.

6.2 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 sec. Put the container in an upright position and wait a few seconds for settlement of the generated dust.

6.3 Spiked feed samples; 100 mg/kg

Transfer 2,5 ml of the stock standard solution (3.7.1) 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 min before starting the extraction procedure by adding water for swelling (see 7.2.2).

7 Procedure

7.1 General

Complete each assay within one working day.

7.2 Extraction

7.2.1 Premixtures

Accurately weigh 1 g to the nearest 0,01 g of the test sample directly into a volumetric flask of 200 ml.

Add 80 ml of extraction solvent (3.4), close the flask and mix manually by swirling.

Put the flasks in a waterbath of 50°C (4.5) for at least 15 min with intermediate swirling at 8 min.

Mix thoroughly for 15 min using a mechanical means (4.2).

Put the flasks in an ultrasonic bath (4.4) and sonicate for 15 min.

Cool down to room-temperature, adjust to volume with HPLC eluent (3.5) and mix.

Allow sample solids to settle (minimum 30 min).

If additional dilutions are required, dilute the samples with HPLC eluent (3.5) to a final nicarbazin concentration which falls within the standard curve levels (3.7.2).

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

7.2.2 Animal feeding stuffs

Accurately weigh 2,5 g to the nearest 0,01 g of the test sample in a 50 ml disposable centrifuge tube or directly in a volumetric flask of 100 ml.

Add 5 ml of water. Take care that the whole sample becomes wet.

Wait at least 10 min.

Add 35 ml of extraction solvent (3.4), close the tube or flask and mix manually by swirling.

Put the tubes or flasks in a waterbath of 50°C (4.5) for 15 min with intermediate swirling at 8 min.

Mix thoroughly for 15 min using a mechanical means (4.2).

Put the tubes or flasks in an ultrasonic bath (4.4) and sonicate for 15 min.

If using disposable centrifuge tubes, transfer the sample extract quantitatively into a 100 ml volumetric flask with HPLC eluent (3.5), adjust to volume and mix.

If additional dilutions are required, allow samples to settle (minimum 30 min) and dilute the samples with HPLC eluent (3.5) 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.

NOTE 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.

7.3 HPLC determination

7.3.1 HPLC conditions

The following conditions are offered for guidance, other conditions may be used provided that they give equivalent results.

analytical column	as in 4.1.3
mobile phase	as in 3.5
flow rate	1,0 ml/min
detection wavelength	350 nm
injection volume	20 µl

Check the stability of the chromatographic system, injecting several times the calibration solution (3.7.2) containing 1,0 µg/ml, until constant peak areas and retention times are achieved.

7.3.2 Calibration graph

Inject each calibration solution (3.7.2) to determine the peak area/height for each concentration. Plot a calibration graph using the peak areas/heights of the calibration solutions as the ordinate and the corresponding concentrations in µg/ml as the abscissae.

7.3.3 Sample solution

Inject the sample extract (7.2.1 or 7.2.2) at least 2 times using the same volume as taken for the calibration solutions and determine the peak area/height of the DNC peaks.

8 Calculation and expression of results

Calculate the mass fraction of nicarbazin in the test sample by the equation:

$$w_E = \frac{V_e \cdot \rho_n}{m} \cdot f_d \quad (1)$$

where

w_E is the numerical value for the mass fraction of nicarbazin in the test sample in mg/kg;

V_e is the extraction volume, in ml, viz. 200 for premixtures (7.2.1) and 100 for feeding stuffs (7.2.2);

ρ_n is the numerical value of the nicarbazin mass in concentration in the sample extract in μg/ml;

m is the numerical value of the mass of the test sample, in g;

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

9 Precision

9.1 Collaborative study

Details of the collaborative study of the method are summarized in Annex A. The values derived from this collaborative study may not be applicable to concentration ranges and matrices other than those given.

9.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5% of the cases exceed the repeatability limit r .

9.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment will in not more than 5% of the cases exceed the reproducibility limit R.

9.4 Recovery

The recovery obtained for compound feeds should be in the range of 90% to 110% at spike levels between 20 mg/kg and 200 mg/kg.

10 Test report

The test report shall specify:

a) information necessary for the complete identification of the test sample;

b) the sampling method used, if known;

c) the test method used, with reference to this European Standard;

d) operating details not specified in this European Standard, or regarded as optional, together with details of any incidents which may have influenced the test results(s);

e) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Results of an interlaboratory study

An international interlaboratory study involving 19 laboratories in 8 countries was carried out on:

Sample A: broiler feed I, 6% fat

Sample B: broiler feed II, 1% fat

Sample C: broiler feed II, 11% fat

Sample D: broiler feed I, 6% fat

Sample E: premixture for broiler feed

The test was organized by the State Institute for Quality Control of Agricultural Products in the Netherlands (RIKILT) in 2 000 and the results obtained were subject to statistically analyses. Precision data are given in Table A.1.

Table A.1 — Precision data

	Sample				
	A	**B**	**C**	**D**	**E**
Number of laboratories participating	19	19	19	19	17
Number of laboratories after eliminating outliers	17	18	18	16	10
Mean nicarbazin mass fraction, mg/kg	22,0	45,0	117	260	7308
Repeatability standard deviation, s_r, mg/kg	2,2	3,2	7,0	6,8	417
Coefficient of variation of repeatability, r; %	10,2	7,1	6,0	2,6	5,7
Repeatability limit, r (2,8 s_r), mg/kg	6,3	9,0	19,7	18,9	1166
Reproducibility standard deviation, s_R, mg/kg	2,7	4,1	9,2	12,5	628
Coefficient of variation of reproducibility, R;%	12,3	9,1	7,9	4,8	8,6
Reproducibility limit, R (2,8 s_R), mg/kg	7,6	11,5	25,9	34,9	1760
Horrat value[a]	1,22	1,00	1,01	0,70	1,95

[a] Horrat is the ratio between established RSD_R and predicted RSD_R.

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