

Stofnaam	Narasin
Type methode	HPLC
Te onderzoeken in	Diervoeder, premixen en concentraten
Minimum bepaalbaarheids grens	20 mg/kg
Herhaalbaarheid	RSD _r (relatieve standaarddeviatie) voor diervoeders en premixen op de volgende niveaus: 20 mg/kg: 7.6% 45 mg/kg: 2.2% 70 mg/kg: 4.1% 120 mg/kg: 3.4% 10000 mg/kg: 4.4%
Reproduceerbaarheid	RSD _R voor diervoeders en premixen op de volgende niveaus: 20 mg/kg: 9.2% 45 mg/kg: 7.3% 70 mg/kg: 6.8% 120 mg/kg: 6.1% 10000 mg/kg: 6.2%
Categorie	A
Titel	CANFAS/ SMT4-CT98-2216/ final method narasin/ 2003-01-31

1 Scope

The method serves for the quantitative determination of narasin sodium in feedstuffs, premixtures and concentrates. The limit of determination is 20 mg/kg, the limit of detection 1 mg/kg.

2 Principle

Narasin is extracted using a mixture of methanol and phosphate buffer (90+10) with mechanical shaking. After dilution and filtration through a membrane filter narasin is determined by reverse phase HPLC using post column derivatisation with dimethylaminobenzaldehyde in a solution containing sulphuric acid and detection at 600 nm.

3 Reagents

- 3.1 Methanol - HPLC grade
- 3.2 Di-potassiumhydrogenphosphate, anhydrous
- 3.3 Di-potassiumhydrogenphosphate solution, $c(\text{K}_2\text{HPO}_4) = 0.05 \text{ mol/l}$ water
- 3.4 Potassiumdihydrogenphosphate, anhydrous
- 3.5 Potassiumdihydrogenphosphate solution, $c(\text{KH}_2\text{PO}_4) = 0.01 \text{ mol/l}$ water
- 3.6 1,5-Dimethylhexylamine (6-methyl-2-heptylamine, $\text{C}_8\text{H}_{19}\text{N}$)
- 3.7 Ortho-phosphoric acid, $w(\text{H}_3\text{PO}_4) = 85 \%$
- 3.8 Sulphuric acid, $w(\text{H}_2\text{SO}_4) = 95-97 \%$
- 3.9 4-(Dimethylamino)-benzaldehyde (DMAB, $\text{C}_9\text{H}_{11}\text{NO}$)
- 3.10 Extraction solvent: 900 ml methanol (3.1) are mixed with 100 ml di-potassiumhydrogenphosphate solution (3.3).
- 3.11 Phosphate buffer: To 500 ml solution of potassiumdihydrogenphosphate (3.5) 3.0 ml o-phosphoric acid (3.7) and 10.0 ml 1,5-dimethylhexylamine (3.6) are added. The pH is adjusted to 4.0 with o-phosphoric acid, and the solution is made up to 1000 ml with water.
- 3.12 Mobile phase: 900 ml methanol (3.1) are mixed with 100 ml phosphate buffer (3.11). The solution is degassed prior to use in an ultrasonic bath (4.3) during 15 min.
- 3.13 Methanol-sulphuric acid: 40 ml sulphuric acid (3.8) are given cautiously while stirring to 950 ml methanol (3.1). The solution is degassed prior to use in an ultrasonic bath (4.3) during 15 min.
- 3.14 DMAB-solution: 60.0 g dimethylaminobenzaldehyde (3.9) are solved in 950 ml methanol (3.1). The solution is degassed prior to use in an ultrasonic bath (4.3)

during 15 min.

- 3.15 Narasin-sodium reference standard (monocarboxylic acid-polyether-sodium salt, $C_{43}H_{71}NaO_{11}$) with defined microbiological activity and factor composition.
- 3.16 Narasin-stock solution, 250 $\mu\text{g/ml}$
An amount of narasin-sodium (3.15) equivalent to 25.00 mg microbiological activity is solved in 100 ml methanol (3.1). The solution is stable for 4 weeks if kept at $>0 - < 10$ $^{\circ}\text{C}$.

4 Apparatus

- 4.1 HPLC-system consisting of:
 - 4.1.1 Pump - pulse free, flow capacity 0.1-2.0 ml/min
 - 4.1.2 Injection system, manual or autosampler with loop suitable for 100 μl injections
 - 4.1.3 Post-column reactor (double pump or two single pumps) with mixing chamber, reaction coil of inert material (f.e. Teflon or Peek) for operation at 93 $^{\circ}\text{C}$, 7.0 m with 0.33 mm ID and water bath or reactor oven for operation at 93 $^{\circ}\text{C}$
 - 4.1.4 VIS-detector, variable wavelength, suitable for measurements at the wavelength of 600 nm
 - 4.1.5 Analytical column - 4 μm C18 Hypersil ODS, 250 x 4 mm f.e. Shandon or equivalent (7.2)
- 4.2 Magnetic stirrer or mechanical shaker
- 4.3 Ultrasonic water bath
- 4.4 Membrane filter of Teflon, pore diameter 0.45 μm
Commercially available equipment

5 Procedure

- 5.1 General
 - 5.1.1 Blank feed
For the performance of the recovery test (5.1.2) a blank feed should be analysed to check that neither narasin nor interfering substances are present. The blank feed should be similar in type to that of the sample and narasin or interfering substances should not be detected.
 - 5.1.2 Recovery test
A recovery test should be carried out by analysing the blank feed which has been fortified by addition of a quantity of narasin, similar to that present in the sample. To fortify at a level of 50 mg/kg transfer 4 ml of the stock solution (3.16) to a conical flask and evaporate the solution to approximately 0.5 ml. Add 20 g of the blank feed,

mix thoroughly and leave for 10 minutes mixing again several times before processing with the extraction step (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (5.1.1), a recovery test can be performed by means of the standard addition method. In this case, the sample to be analysed is fortified with a quantity of narasin similar to that already present in the sample. This sample is analysed together with the unfortified sample and the recovery can be calculated by subtraction.

5.1.3 Mixing of the test sample before weighing

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

5.2 Extraction

5.2.1 Premixtures

Weigh 5.0 g into a 500-ml-Erlenmeyer flask, add 200 ml extraction solvent (3.10) and place the flask for 5 min in the ultrasonic water bath (4.3). Remove the flask from the ultrasonic water bath and stir on a magnetic stirrer, or shake the flask on a mechanical shaker (4.2) for 1 h. Let settle the coarse particles. The extract is diluted subsequently with mobile phase (3.12) to a final concentration of ca. 4 µg/ml and filtered through a membrane filter (4.4).

5.2.2 Final feeds

Weigh 20.0 g into a 250-ml-Erlenmeyer flask, add 100 ml extraction solvent (3.10) and place the flask for 5 min in the ultrasonic water bath (4.3). Remove the flask from the ultrasonic water bath and stir on a magnetic stirrer, or shake the flask on a mechanical shaker (4.2) for 1 h. Let settle the coarse particles. The extract is diluted subsequently to a final concentration of ca. 1 µg/ml with mobile phase (3.12) and filtered through a membrane filter (4.4).

5.3 HPLC procedure

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

Narasin is separated on a reversed phase column (4.1.5), detected and its concentration measured after post-column reaction (4.1.3) with a VIS-Detector (4.1.4) at 600 nm.

An aliquot of the sample solution (5.2), f.e. 100 µl is injected on the separation column and eluted with the mobile phase (3.12). The mean heights of the peaks resp. the areas of several injections of the calibration solutions (5.4.2) are measured.

HPLC-conditions

Column (4.1.5)	Hypersil ODS, 250 x 4 mm, 5 µm
Mobile phase (3.12)	Mixture of 900 ml methanol (3.1) + 100 ml phosphate buffer (3.11)
Flow rate of mobile phase	0.7 ml/min
Flow rate of methanol-sulphuric acid-mixture (3.13)	0.4 ml/min
Flow rate of DMAB-solution (3.14)	0.4 ml/min
Temperature of the post-column reaction	93 °C
VIS-Detector after post-column reaction	600 nm

Volume of injections	100 µl
Calculation	Height or area of peak

5.4 Calibration curve

5.4.1 Preparation of the working standard solution: 10 ml the stock solution (3.16) are diluted with the extraction solvent (3.10) to 100 ml. The concentration of narasin-sodium is $w = 25 \mu\text{g/ml}$. The solution is stable for 4 weeks if kept at $>0 - < 10 \text{ }^\circ\text{C}$.

5.4.2.1 Preparation of the calibration solution for concentrations $\geq 200 \text{ mg/kg}$: 5.0, 10.0, 20.0 and 40.0 ml of the working standard solution (5.4.1) is pipetted into a 100-ml-volumetric flask each, filled up with mobile phase (3.12) and mixed. The concentration of narasin-sodium corresponds to $= 1.25; 2.50; 5.00$, and $10.00 \mu\text{g/ml}$.
The calibration solutions have to be prepared daily.

5.4.2.2 Preparation of the calibration solution for concentrations $\leq 200 \text{ mg/kg}$: 1.0, 2.0, 4.0 and 8.0 ml of the working standard solution (5.4.1) is pipetted into a 100-ml-volumetric flask each, filled up with mobile phase (3.12) and mixed. The concentration of narasin-sodium corresponds to $= 0.25, 0.50, 1.00$ and $2.00 \mu\text{g/ml}$.
The calibration solutions have to be prepared daily

5.4.3 Preparation of the calibration curve
100 µl each of the calibration solutions (5.4.2) is injected and the mean height or area of the peaks of several injections measured. Under the above conditions the retention time of narasin is approximately 19 min.

6 Calculation

The concentration of narasin-sodium is calculated in mg/kg microbiological activity from the mean height or area of the peak of factor A in sample solution (5.3) and the calibration curve (5.4.3) based on the assumption that the relation of microbiological activity to content of factor A is the same in the feed additive and in the standard. The content w in the sample is calculated from the concentration received respecting weigh and dilution by means of the following formula:

$$w = \frac{V * b * F}{E} \quad \text{mg/kg.}$$

V = volume of extractant in ml (200 ml for premixtures (see 5.2.1) and 100 ml for final feeds (see 5.2.2))

b = concentration of the sample solution in $\mu\text{g/ml}$ microbiological activity of narasin-sodium

E = weigh of the sample in g

F = factor of dilution

7 Remarks

7.1 Extraction

Due to the addition of di-potassiumhydrogenphosphate to the extractant solvent with most of the samples it is possible to let stand the extracts over night at room temperature performing dilution and chromatography the following day. Since it may occur - especially in premixtures and mineral feeds - that there is a slight breakdown of narasin the analysis has to be repeated with shaking of the extract for not more than 1 hour before chromatography.

In a few feedstuffs it was observed that unknown compounds interfered with the retention time and peak shape in chromatograms when low concentrations (< 20 mg/kg) of narasin were present. To overcome this difficulty 10 g of Alumina 90 (Merck 1.01097 or equivalent) were added to the weigh.

If interfering pharmaceutical agents are present the following procedure may be applied:

Weigh 20.0 g sample into 250 ml Erlenmeyer flask. Add 100 ml hexane, stopper and shake for at least one hour on a wrist-action shaker. Filter sample solutions through 42 Whatman filter or equivalent into 125-ml-Erlenmeyer flask. Pipet 20.0 ml of extract and evaporate to dryness on the nitrogen evaporator. Dissolve the residue in 20.0 ml of extraction solvent. Introduce this solution into a prepared column with 10 g Alumina 90. Filter a portion of the eluate before proceeding to the HPLC analysis.

7.2 Separation material

Baseline separation between narasin factor A and salinomycin must be obtained.

Hypersil ODS 5 mm in a 250 x 4 mm steel column has been proven as the best one. It is possible to separate narasin from other polyether antibiotics and to get the peaks of the 4 main factors. Inertsil and Purospher can be recommended if there is doubt whether narasin is separated from other compounds. The retention times are longer than with Hypersil.

7.3 Protection against corrosion

All fittings, which come in contact to the methanol-sulphuric acid-mixture (3.13), should be made from Teflon, Peek or comparable material.

7.4 Post-column reaction

If only one pump for the post-column reaction is available the reagents 3.13 and 3.14 may be mixed. Since DMAB undergoes quick auto-oxidation resulting in darkening of the solution this has to be kept protected from light in an ice bath and has to be used within 24 h.

8 Literature

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