

Stofnaam	Maduramycine
Type methode	HPLC
Te onderzoeken in	Diervoeders en voormengsels
Minimum bepaalbaarheidsgrens	2 mg/kg
Herhaalbaarheid	RSD <sub>r</sub> (relatieve standaarddeviatie) voor diervoeders en premixen op de volgende niveaus: Broiler feed 2.5 mg/kg: 8.5% Broiler feed 4.5 mg/kg: 8.2% Turkey feed 5 mg/kg: 3.3% Broiler feed 9 mg/kg: 6.2% Premix 450 mg/kg: 3.2%
Reproduceerbaarheid	RSD <sub>R</sub> voor diervoeders en premixen op de volgende niveaus: Broiler feed 2.5 mg/kg: 15% Broiler feed 4.5 mg/kg: 25% Turkey feed 5 mg/kg: 16% Broiler feed 9 mg/kg: 17% Premix 450 mg/kg: 11%
Categorie	A
Titel	Determination of maduramycin-ammonium in feedingstuffs and premixtures by High Performance Liquid Chromatography CANFAS/SMT4-CT98-2216/ final method maduramicin/2002-10-08

# BEPALING VAN MADURAMYCINE

## 1. Purpose and scope

The method is for the determination of maduramicin in feedingstuffs and premixtures. The usual concentration of maduramicin in feedstuffs is 5 mg/kg, in premixtures 500 mg/kg. The limit of determination is 2 mg/kg. The limit of detection is 0,5 mg/kg

## 2. Principle

The sample is extracted with methanol. The content of maduramicin is determined by reversed-phase-high-performance-liquid chromatography (HPLC) with post column derivatisation with vanillin using a Vis-detector.

## 3. Reagents

- 3.1 Methanol, HPLC-grade
- 3.2 1,5-Dimethylhexylamine
- 3.3 Sulfuric acid, 95-97%, p.a.
- 3.4 Ortho-phosphoric acid, appr. 85%, p.a.
  - 3.4.1 Diluted o-phosphoric acid:  
Dissolve 10 ml of o-phosphoric acid (0) to 100 ml with demineralised water
- 3.5 Potassium dihydrogen phosphate, p.a.
- 3.6 Phosphate buffer solution 10 mmol/l, pH=4,0:  
Dissolve 1.36 g of potassium dihydrogen phosphate (0) in 500 ml of demineralised water. Add 3.0 ml of o-phosphoric acid (0) and 10 ml of 1,5-dimethyl hexylamine (0) . Adjust the pH to 4.0 with diluted o-phosphoric acid (0) and fill with demineralised water to 1000 ml.  
The solution can be stored some weeks, but if fungus grow, prepare a new one.
- 3.7 Mobile phase:  
Dilute 100 ml of phosphate-buffer solution 10 mmol/l, pH=4 (0) with methanol (0) to 1000 ml.
- 3.8 Vanillin  $\geq$  98% (HPLC)
  - 3.8.1 Vanillin reagent:  
Dissolve 10 g of vanillin (0) in a mixture of 250 ml of methanol (0) and 5.0 ml of sulfuric acid (0). Mix well and sonicate for some min under vacuum at room temperature. This solution has to be prepared daily prior to use and has to be cooled with ice water during use.
- 3.9 Maduramicin K<sup>+</sup>- salt (0)  
The purity-grade accounted as NH<sub>4</sub><sup>+</sup>-salt has to be taken into consideration at the following operations

- 3.9.1 Stock-standard-solution 100 µg/ml:  
Dissolve in a 100 ml volumetric flask to the nearest 0.1 mg 10 mg of reference standard (0) with methanol (0) to 100 ml. This solution should be stored at 4°C not longer than 1 month.
- 3.9.2 Standard solution 10 µg/ml:  
Dilute 10.0 ml of the stock-standard-solution (0) to 100 ml with methanol (0) in a 100 ml volumetric flask  
Standard solutions should be stored at 4°C not longer than 1 week.
- 3.9.3 Standard solution 1 µg/ml  
Dilute 2.0 ml of the stock-standard-solution (0) to 200.0 ml with methanol (0) in a 200 ml volumetric flask.  
Standard solution should be prepared freshly
- 3.9.4 Calibration solutions  
Into a series of 50 ml graduated flasks transfer 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0 ml of the intermediate standard solution (0). Make up to the mark with methanol and mix. These solutions correspond to 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, and 2.0 µg of maduramicin per ml respectively.  
Calibration solutions should be prepared freshly

#### 4. Apparatus

- 4.1 Centrifuge
- 4.2 Ultrasonic bath
- 4.3 HPLC-equipment  
with pump and column oven
- 4.3.1 Autosampler with injection system,  
suitable for injection of 50 µl
- 4.3.2 (UV)-Vis-detector (0 and 8.6)
- 4.3.3 Liquid chromatographic column  
250 x 4.6 mm, 5µm, Hypersil BDS C 18 or equivalent
- 4.3.4 Reagent pump
- 4.3.5 PEEK mixing chamber (8.6)
- 4.3.6 PTFE reaction coil (knitted) (0 and 8.6)  
0.5 mm x 10 m (volume ≈2.0 ml)
- 4.3.7 Reactor oven for the PTFE-reaction coil,  
suitable to 100°C (or suitable water bath)
- 4.4 Freezer
- 4.5 Membrane-filter, PTFE , 0.22µm

## 5. Procedure

### 5.1 General

#### 5.1.1 Blank feed

For the performance of the recovery test (0) a blank feed should be analysed to check that neither maduramicin nor interfering substances are present. The blank feed should be similar in type to that of the sample and maduramicin 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 maduramicin, similar to that present in the sample. To fortify at a level of 5 mg/kg transfer 500 µl stock-standard solution (100 µg/ml (0)) to the flask. Add 10 g of the blank feed, mix thoroughly and leave for 10 min, mixing again several times before proceeding with the extraction step (0). Alternatively, if a blank feed similar in type to that of the sample is not available ( see 0 ), 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 maduramicin 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.

### 5.2 Extraction

#### 5.2.1 Feedingstuffs

Weigh to the nearest 0.01 g 10 g of the  $\leq 1$  mm ground sample into a 250 ml volumetric flask and add 50 ml methanol (0) . Close the flask with a suitable method, and place in a ultrasonic bath at 50° C for 20 minutes. Shake vigorously, store and cool down to room temperature appr. 15 min, decant the clear supernant and place in freezer for 2 - 3 hours to settle down fat. Then centrifuge an aliquote for 1-2 min. After membrane (0) filtration 50 µl of this solution are injected into the HPLC-apparatus.

#### 5.2.2 Premixes

Weigh to the nearest 0.01 g 1 g of the  $\leq 0.5$  mm ground sample into a 250 ml volumetric flask and add 50 ml methanol (0) . Close the flask with a suitable method, and place in an ultrasonic bath at 50° C for 20 minutes. Cool down to room temperature, shake vigorously , store some minutes and dilute an aliquote 1 : 10 of the clear supernatant with methanol and place in freezer for 2-3 hours to settle down fat. Then centrifuge an aliquote for 1-2 min. After membrane (0) filtration 50 µl of this solution are injected into the HPLC-apparatus.

### 5.3 HPLC determination

#### 5.3.1 Parameters:

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

Analytical column (0)	250 x 4.6 mm, 5µm , Hypersil BDS C 18
Mobile phase (0)	Dilute 100 ml of phosphate-buffer solution 10 mmol/l, pH=4 (0) with methanol (0) to 1000 ml.
Column oven:	40°C
Flow rate:	0.4 ml/min
Post-column reagent (0)	vanillin reagent (8.7)
Flow rate reagent pump:	0.4 ml/min
Reactor temperature:	95°C (0)

Detection wavelength: 520 nm  
Injection volume: 50 µl  
Retention time: approx. 25 min  
Run time: 30 –35 min

Check the stability of the chromatographic system, injecting several times the calibration solution (0) containing 1.0 µg/ml, until constant peak areas and retention times are achieved.

Working with the described conditions there is baseline separation from other ionophores like salinomycin, narasin, monensin, semduramicin.

### 5.3.2 Calibration graph

Inject each calibration solution (0) several times and determine the mean peak areas for each concentration. Plot a calibration graph using the mean peak areas of the calibration solutions as the ordinate and the corresponding concentrations in µg/ml as the abscissae.

### 5.3.3 Sample solution

Inject the sample extract (0) at least 2 times using the same volume as taken for the calibration solutions and determine the mean peak area of the maduramicin peaks.

## 6. Calculation of the results

From the mean area (0) of the maduramicin peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (0)

The maduramicin content in mg/kg of the sample is given by the following formula:

$$\text{mg maduramicin/kg} = \frac{C \cdot 50 \cdot F}{M}$$

C = maduramicin concentration of the sample extract (0) in µg/ml

M = mass of the test portion in g

F = dilution factor according to (0)

## 7. Validation of the results

### 7.1 Identity

The identity of maduramicin can be confirmed by co-chromatography.

A sample extract (0) is fortified by addition of an appropriate amount of calibration solution. The amount of added maduramicin should be similar to the amount of maduramicin found in the sample extract.

Only the height of the maduramicin-peak should be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of the height, must be within  $\pm 10\%$  of the original width of the maduramicin peak of the unfortified sample extract.

## 8. Observations

8.1 The detector is used in visual scope at 520 nm and should give sufficient light energy. Noise preferably should be  $< 1 \cdot 10^{-5}$  AU (250nm, 600nm)

8.2 A temperature of 92°C to 98°C is possible, high stability ( $\pm 1^\circ\text{C}$ ) should be guaranteed

8.3 The length of the teflon tube (e.g. 1m ID 0.25 mm) between reagent-pump and mixing chamber and the length of the teflon tube (e.g. 3m ID 0.17 mm) between reactor and detector should be optimized if there are problems with bubbles

8.4 Maduramicin is very toxic. LD50 = 33mg/kg (rat).

8.5 Only area is allowed for calculation

8.6 The use of stainless steel tubing in the post-column reactor and detector should be avoided.

8.7 Dimethylaminobenzaldehyde (DMAB) is also suitable as a reagent for post-column derivatisation although a full validation of this reagent has not been performed.