BRITISH STANDARD

BS EN 15781:2009

Animal feeding stuffs — Determination of maduramicin-ammonium by reversed-phase HPLC using post-column derivatisation

ICS 65.120

BSI British Standards

National foreword

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

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Aliments des animaux - Détermination de la maduramicine ammonium par HPLC en phase inverse à l'aide de la dérivation post-colonne

Futtermittel - Bestimmung von Maduramicin-Ammonium durch Umkehrphasen HPLC-Verfahren mittels Nachsäulenderivatisierung

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Foreword

This document (EN 15781:2009) 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of an identical text or by endorsement, at the latest by February 2010, and conflicting national standards shall be withdrawn at the latest by February 2010.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 high performance liquid chromatography (HPLC) method for the determination of the content of maduramicin in feeding stuffs and premixtures.

The usual concentration of maduramicin in feedstuffs is 5 mg/kg, in premixtures 500 mg/kg. The limit of quantification is 2 mg/kg. The limit of detection is 0,5 mg/kg.

NOTE A lower limit of quantification may be achievable but shall be validated by the user.

2 Normative reference(s)

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696, *Water for analytical laboratory use - Specification and test methods* (ISO 3696:1987)

3 Principle

The sample is extracted with methanol. Maduramicin is determined by reversed-phase HPLC using post-column derivatization with vanillin and detection at 520 nm.

4 Reagents

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

WARNING — Use all solvents and solutions in a fume hood. Wear safety glasses, protective clothing and avoid skin contact.

4.1 Water, complying with EN-ISO 3696, grade 1

4.2 Methanol (CH_3OH), HPLC grade

4.3 1,5-Dimethylhexylamine ($CH_3(CH_2)_4CH_2N(CH_3)_{2)}$

4.4 Sulfuric acid (H_2SO_4), purity 95% to 97% by volume

4.5 Ortho-phosphoric acid, (H_3PO_4), purity approximately 85% by volume

4.5.1 Diluted o-phosphoric acid

Dissolve 10 ml of ortho-phosphoric acid (4.5) to 100 ml with water (4.1).

4.6 Potassium dihydrogen phosphate (KH_2PO_4)

4.7 Phosphate buffer solution, (KH_2PO_4) = 10 mmol/l, pH 4,0

Dissolve 1,36 g of potassium dihydrogen phosphate (4.6) in 500 ml of water (4.1). Add 3,0 ml of ortho-phosphoric acid (4.5) and 10 ml of 1,5-dimethylhexylamine (4.3). Adjust the pH to 4,0 with diluted ortho-phosphoric acid (4.5.1) and fill with demineralised water to 1 000 ml. The solution can be stored for some weeks, but in case of fungal growth, prepare a new one.

4.8 Mobile phase

Dilute 100 ml of phosphate buffer solution (4.7) with methanol (4.2) to 1 000 ml.

4.9 Vanillin, 4-hydroxy-3-methoxybenzaldehyde, minimum 98% purity by volume (HPLC grade)

4.9.1 Vanillin reagent

Dissolve 10 g of vanillin (4.9) in a mixture of 250 ml of methanol (4.2) and 5,0 ml of sulphuric acid (4.4). Mix well and sonicate for some minutes 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.

NOTE Dimethylaminobenzaldehyde (DMAB) is also suitable as a reagent for post-column derivatisation (details are given in Annex B) although a full validation of this reagent has not been performed.

4.10 Maduramicin

WARNING — Maduramicin is very toxic. Avoid inhalation and exposure to the toxic standard material and solutions thereof.

4.11 Standard solutions

4.11.1 Stock-standard-solution, 100 µg/ml

Accurately weigh 10 mg to the nearest 0,1 mg maduramicin (4.10) into a 100 ml volumetric flask. Dissolve in methanol (4.2) and dilute to volume. Store below 4°C. Prepare fresh every month.

4.11.2 Standard solution, 10 µg/ml

Dilute 10 ml of the stock-standard-solution (4.11.1) to 100 ml with methanol (4.2) in a 100 ml volumetric flask. Store below 4°C. Prepare fresh every week.

4.11.3 Calibration solutions

The interlaboratory study was performed with 8 calibration solutions. Into a series of 50 ml volumetric flasks transfer 1,0 ml, 2,0 ml, 3,0 ml, 4,0 ml, 5,0 ml, 6,0 ml, 8,0 ml and 10,0 ml of the intermediate standard solution (4.11.2). Dilute to volume with methanol (4.2) and mix. These solutions correspond to 0,2 µg, 0,4 µg, 0,6 µg, 0,8 µg, 1,0 µg, 1,2 µg, 1,6 µg, and 2,0 µg of maduramicin per ml respectively. Alternatively, you may use 5 calibration solutions with maduramicin concentrations of 0,4 µg, 0,7 µg, 1,0 µg, 1,5 µg and 2,0 µg per ml respectively. Calibration solutions should be prepared on the day of analysis.

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Centrifuge

5.2 Ultrasonic bath

5.3 HPLC system consisting of the following

5.3.1 Pump, pulse free, flow capacity 0,4 ml/min

5.3.2 Injection system, manual or autosampler, with loop suitable for 50 µl injection

5.3.3 UV/VIS detector, suitable for measurements at 520 nm

NOTE Noise preferably should be < $1 \cdot 10^{-5}$ AU (250 nm, 600 nm)

5.3.4 Integrator, or computer data system.

5.3.5 Post column reactor consisting of the following

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

5.3.5.1 PEEK mixing chamber

5.3.5.2 PTFE reaction coil, 1,5 ml to 2,0 ml reaction coil, for operating at 95°C

The coil may be a commercially available knitted coil or it may be made using 7,5 m to 10 m of 316 SS tubing, 0,5 mm ID, coiled in a format to fit the reactor heating chamber (a suggestion is to wrap the coil in enough aluminium foil to make it fit snugly in the heater and to provide good heat transfer to the coil). A knitted coil is preferable. To ensure effective mixing of reagent and column effluent, use a vortex or static mixing tee (not a regular tee) before the reaction coil.

NOTE 1 The length of the polytetrafluoroethylene (PTFE) tube (e.g. 1 m ID 0,25 mm) between reagent-pump and mixing chamber and the length of the Teflon tube (e.g. 3 m ID 0,17 mm) between reactor and detector should be optimized if there are problems with bubbles.

NOTE 2 A temperature of 92°C to 98°C is possible, high stability (1°C) should be guaranteed.

5.3.5.3 Reactor oven or water bath for the PTFE-reaction coil, suitable for operating at 95°C

5.3.6 Post column reagent pump, pulse free, flow capacity 0,4 ml/min

5.3.7 Liquid chromatographic column, 250 mm x 4,6 mm, 5 µm material, Hypersil BDS C18 or equivalent

5.3.8 Column oven, suitable for operating at 40°C

5.4 Shaker, rotary or wrist-action shaker

5.5 Freezer

5.6 Membrane-filter, PTFE, pore size within the range of 0,20 µm – 0,45 µm

6 Sampling

It is important that the laboratory receives a sample that is truly representative and has not 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.

7 Preparation of test sample

Sample preparation is not part of the method specified in this European Standard. A recommended method that can be used for sample preparation is given in ISO 6498.

8 Procedure

8.1 Preparation of quality control sample

8.1.1 Blank feed

For the performance of the recovery test (8.1.2) a blank feed should be analyzed 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.

8.1.2 Recovery test

A recovery test should be carried out by analyzing the blank feed which has been fortified by the 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 (4.11.1) to a 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 (8.2)

Alternatively, if a blank feed similar in type to that of the sample is not available (8.1.1), a recovery test can be performed by means of the standard addition method. In this case, the sample to be analyzed is fortified with a quantity of maduramicin similar to that already present in the sample. This sample is analyzed together with the unfortified sample and the recovery can be calculated by subtraction.

Acceptable recovery is between 90% and 110%.

8.2 Extraction

8.2.1 Feeding stuffs

Accurately weigh 10 g to the nearest 0,01 g of the ground sample (with particles of ≤ 1mm) into a 250 ml volumetric flask and add 50 ml methanol (4.2). Close the flask with a suitable method, and place it in an ultrasonic bath (5.2) at 50°C for 20 min. Shake vigorously (5.4), store and cool down to room temperature in approximately 15 min, decant the clear supernatant and place in a freezer (5.5) for 2 h to 3 h to settle down fat. Then centrifuge an aliquot for 1 min to 2 min. After membrane (5.6) filtration, 50 µl of this solution is injected into the HPLC-apparatus.

8.2.2 Premixes

Accurately weigh 1 g to the nearest 0,01 g of the ground sample (with particles of ≤ 0,5 mm) into a 250 ml volumetric flask and add 50 ml methanol (4.2). Close the flask with a suitable method, and place in an ultrasonic bath (5.2) at 50°C for 20 min. Cool down to room temperature, shake vigorously (5.4), store some min and dilute an aliquot of the clear supernatant 1:10 with methanol and place in a freezer (5.5) for 2 h to 3 h to settle down fat. Then centrifuge an aliquot for 1 min to 2 min. After membrane filtration (5.6), 50 µl of this solution is injected into the HPLC-apparatus.

NOTE A larger sample amount may be used, but shall be validated by the user. In the case a larger sample amount is used the volume of the extraction solvent has to be adjusted accordingly.

WARNING — Maduramicin is very toxic. Avoid inhalation and exposure to the toxic material when grinding the sample.

8.3 HPLC determination

8.3.1 HPLC conditions

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

analytical column	as in 5.3.7
mobile phase	as in 4.8
column oven	40°C
flow rate	0,4 ml/min
detection wavelength	520 nm
injection volume	50 µl
post-column reagent	vanillin reagent (4.9.1)
flow rate reagent pump	0,4 ml/min
reactor temperature	95°C
retention time	approx. 25 min
run time	30 min to 35 min

Check the stability of the chromatographic system, injecting several times the calibration solution (4.11.3) 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 and semduramycin.

8.3.2 Calibration graph

Inject each calibration solution (4.11.3) 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.

8.3.3 Sample solution

Inject the sample extract (8.2) at least 2 times using the same volume as taken for the calibration solutions and determine the mean peak area of the maduramicin peaks.

9 Calculation of results

From the mean area[1)] of the maduramicin peaks of the sample solution, determine the concentration of the sample solution in µg/ml by reference to the calibration graph (8.3.2).

1) Only area that is allowed for calculation.

Use the following equation for the calculation of the mass fraction of maduramicin (w_m) in the sample in mg per kg.

$$w_m = \frac{\rho_m \cdot 50 \cdot f_d}{m} \quad (1)$$

ρ_m is the mass concentration of maduramicin in the sample extract (8.2) in µg/ml;

m is the mass of the test portion in g;

f_d is the dilution factor according to (8.2).

10 Interpretation of confirmation data

The identity of maduramicin can be confirmed by co-chromatography. A sample extract (8.2) 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 ± 10% of the original width of the maduramicin peak of the unfortified sample extract.

11 Precision

11.1 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.

11.2 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*.

11.3 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.*

12 Test report

The test report shall specify:

a) information necessary for complete identification of the sample;

b) the sampling method used, if known;

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

d) the test result obtained, or, if the repeatability has been checked, the final quoted result obtained,

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

Annex A
(informative)

Results of the interlaboratory study

An European interlaboratory study involving 10 laboratories in 9 countries was carried out on 5 different samples:

Sample A: broiler feed, 4% fat, content of maduramicin 2,5 mg/kg

Sample B: broiler feed, 8% fat, content of maduramicin 4,5 mg/kg

Sample C: turkey feed, content of maduramicin 5 mg/kg

Sample D: broiler feed, 4% fat, content of maduramicin 9 mg/kg

Sample E: premixture for broiler feed, content of maduramicin 450 mg/kg

NOTE 1 Broiler feed contains wheat, broken rice, soya extruded, corn gluten feed and pig fat in the usual industrial quantities.

NOTE 2 Turkey feed contained wheat, corn, soya expellers, rape, peas, potato protein and pig fat in the usual industrial quantities.

The test was organized by the State Institute for Quality Control of Agricultural Products in the Netherlands (RIKILT) in 2002 and the results obtained were subjected to statistical analysis in accordance with ISO 5725-1 and ISO 5725-2 to give the precision data shown in Table A.1.

Table A.1 — Results of the collaborative study for maduramicin

parameter	Sample				
	A	B	C	D	E
Number of laboratories participating	10	10	10	10	10
Number of laboratories after eliminating outliers	10	10	9	10	9
Number of test results from remaining laboratories	20	20	18	20	17
Mean maduramicin mass fraction, mg/kg	2,62	4,44	5,05	9,37	464
Repeatability standard deviation, s_r, mg/kg	0,22	0,36	0,17	0,58	14,6
Coefficient of variation of repeatability, r, %	8,53	8,19	3,29	6,18	3,15
Repeatability limit r ($2{,}8 \cdot s_r$), mg/kg	0,63	1,02	0,47	1,62	40,9
Reproducibility standard deviation, s_R, mg/kg	0,40	1,13	0,81	1,57	52,9
Coefficient of variation of reproducibility, R, %	15,3	25,4	16,1	16,8	11,4
Reproducibility limit R ($2{,}8 \cdot s_R$), mg/kg	1,12	3,16	2,28	4,41	148
HorRat value[a]	1,10	1,98	1,29	1,47	1,80

[a] HorRat is the ratio between the established RSD_R and the predicted RSD_R

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Annex B
(informative)

Conditions for post-column derivatisation with DMAB

B.1 Reagents

B.1.1 Methanol, CH_3OH, HPLC-grade

B.1.2 Sulfuric acid, H_2SO_4, 95% to 97%

B.1.3 4-(dimethylamino) benzaldehyde, DMAB, $C_9H_{11}NO$

B.1.4 Methanol-sulphuric acid

40 ml sulphuric acid (B.1.2) is stirred cautiously into 950 ml methanol (B.1.1). The solution is degassed prior to use in an ultrasonic bath (5.2) for 15 min.

B.1.5 DMAB-solution

Dissolve 60,0 g 4-(dimethylamino)benzaldehyde (B.1.3) in 950 ml methanol (B.1.1). The solution is degassed prior to use in an ultrasonic bath (5.2) for 15 min.

B.2 Apparatus

B.2.1 Post-column reactor (double pump or two single pumps) with mixing chamber, reaction coil of inert material (for example Teflon® or Peek®) for operation at 95˚C, 7.0 m with 0.33 mm ID and water bath or reactor oven for operation at 95˚C.

Flow rate of methanol-sulphuric acid mixture (B.1.4)	0,4 ml/min
Flow rate of DMAB-solution (B.1.5)	0,4 ml/min
Temperature of the post-column reaction	95˚C
VIS-detector after post-column reaction	600 nm
Volume of injections	100 µl

NOTE If only one pump is available for the post-column reaction, the reagents B.1.4 and B.1.5 may be mixed (1/1 v/v). Since DMAB undergoes quick auto-oxidation resulting in darkening of the solution it has to be kept protected from light in an ice-bath and has to be used within 24 h.

B.3 Other conditions

Other conditions are not changed compared to the use of vanillin reagent (see 5.3.5 and 8.3.1).

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[4] EN ISO 6497, *Animal feeding stuffs – Sampling (ISO 6497:2002)*

[5] ISO 6498, *Animal feeding stuffs – Preparation of test samples*

[6] ISO 5725 (all parts), *Accuracy (trueness and precision) of measurement methods and results*

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