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Animal feeding stuffs — Determination of semduramicin content — Liquid chromatographic method using a “tree” analytical approach

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Foreword

This document (EN 16158:2012) 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of an identical text or by endorsement, at the latest by August 2012, and conflicting national standards shall be withdrawn at the latest by August 2012.

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1 Scope

This European standard specifies a high-performance liquid chromatographic (HPLC) method for the determination of the semduramicin content at authorized level in animal feeding stuffs [2], using mass spectrometry detection or post-column derivatization and (UV)-VIS detection (hereinafter UV detection). This method is applicable to poultry feed. The limit of quantitation is 1,0 mg/kg when mass spectrometry is used for detection and 3,0 mg/kg when the detection is performed by UV with post-column derivatization. Lower limits of quantitation are achievable but this is to be validated by the user.

The method allows the discrimination of semduramicin from monensin, salinomycin, narasin, maduramicin and lasalocid.

2 Normative references

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

prEN ISO 6498, *Animal feeding stuffs — Guidelines for sample preparation (ISO/DIS 6498)*

3 Principle

Semduramicin is extracted using acetonitrile with mechanical shaking during 30 min. The extracts are filtered through 0,2 µm Nylon filters. Semduramicin is determined by reverse-phase liquid chromatography using electrospray (ESI) single quadrupole mass spectrometry detection in single ion monitoring (SIM) mode (LC-MS) [4] or using post-column derivatization with dimethylaminobenzaldehyde (DMAB) and spectrophotometric detection at 598 nm (LC-PCD-UV) [5]. If the detection used is ESI-MS the quantitation is performed through a standard addition approach. When LC-PCD-UV is used the quantitation is performed through external standard calibration.

4 Reagents

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

4.1 LC-MS.

4.1.1 Water, HPLC grade, or equivalent (e.g. Milli-Q purified water).

4.1.2 Acetonitrile, HPLC gradient grade, minimum 99,9 % purity.

4.1.3 Methanol, HPLC grade or hypergrade LC-MS.

4.1.4 Ammonium formate, HPLC grade.

4.1.5 Mobile phase.

4.1.5.1 Ammonium formate solution, c = 20 mmol/l.

Accurately weigh 1,25 g to the nearest 0,01 g of ammonium formate (4.1.4) into a 1 000 ml volumetric flask. Dissolve in water (4.1.1) and make up to 1 000 ml of volume with water. Prepare fresh solutions monthly.

4.1.5.2 HPLC mobile phase.

Mix methanol (4.1.3) and ammonium formate solution (4.1.5.1) in proportion of 90+10 (v+v). Filter under vacuum using a solvent filtration system (5.11) and Nylon filters (5.13).

4.2 LC-PCD-UV.

In addition to the reagents 4.1.1, 4.1.2, 4.1.3 and 4.1.4:

4.2.1 Sulphuric acid, minimum 98 % purity.

4.2.2 Dimethylaminobenzaldehyde (DMAB), minimum 99 % purity.

4.2.3 Formic acid, minimum 98 % purity.

4.2.4 Mobile phase.

4.2.4.1 Post-column reaction reagent.

In a 500 ml volumetric flask (5.7) add first about 250 ml cold methanol (4.1.3) then 15 ml sulphuric acid (4.2.1). Dissolve 15 g DMAB (4.2.2) in the mixture. Cool down and make up to 500 ml with methanol (4.1.3). Filter under vacuum using the equipment in (5.11) and a membrane filter (5.12). Store in a refrigerator (from +2 °C to + 8 °C). This reagent is stable for 28 days.

NOTE The methanol used for preparing the post-column reaction reagent should be kept refrigerated (from +2 °C to +8 °C).

4.2.4.2 Ammonium formate solution, c = 100 mmol/l at pH = 3.

Accurately weigh 6,30 g to the nearest 0,01 g of ammonium formate (4.1.4) into a 1 000 ml volumetric flask (5.7). Dissolve in 900 ml water (4.1.1). Adjust the pH to 3,0 using formic acid (4.2.3) and make up to 1 000 ml with purified water. Prepare fresh monthly.

4.2.4.3 HPLC mobile phase (solvent blank).

Mix methanol (4.1.3) and ammonium formate solution (4.2.4.2) in proportion of 90+10 (v+v). Filter under a vacuum using a solvent filtration system (5.11) and Nylon filters (5.13).

4.3 Reference standards LC-PCD-UV method.

WARNING — Avoid inhalation of and exposure to the toxic standard materials and solutions thereof. Work in a fume-hood when handling the solvents and solutions. Wear safety glasses and protective clothing.

Declaration of purity is required for each lot of reference standard.

4.3.1 Semduramicin sodium standard, minimum 93 % purity expressed as semduramicin.

NOTE Available from Phibro Animal Health Corporation, Third Floor 65 Challenger Road Ridgefield Park, NJ 07660-2103 USA. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

4.4 Reference standards LC-MS method.

In addition to the reference standard 4.3.1:

4.4.1 Nigericin sodium standard, minimum 98 % purity to be used as internal standard (I.S.).

NOTE Available from Calbiochem, A Brand of EMD Biosciences, Inc. 10394 Pacific Center Court, San Diego, CA 92121 USA. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results."

4.5 Standard solutions.

Protect all standard solutions from daily light.

4.5.1 Semduramicin stock standard solution, ca. 1 mg/ml.

Accurately weigh 10 mg to the nearest 0,1 mg of semduramicin sodium standard (4.3.1) into a 10 ml volumetric flask (5.7). Note down the exact weight of semduramicin sodium. Dissolve in methanol (4.1.3) and make up to 10 ml with methanol. Store at -20 °C and protected from light. Prepare freshly every 3 months.

Determine the experimental concentration of the semduramicin stock solution using the reference standard purity value provided by the supplier expressed as semduramicin using Equation (1).

$$C_s = \frac{m}{10} \times P \qquad (1)$$

where

C_s is the experimental concentration of semduramicin in the stock standard in mg/ml;

P is the purity of the semduramicin standard expressed as semduramicin given by the supplier in percent e.g. 0,934;

m is the weighed mass of semduramicin sodium standard (4.3.1) in mg.

4.5.2 Nigericin sodium stock standard solution (for the LC-MS method), ca. 1 mg/ml.

Accurately weigh 10 mg to the nearest 0,1 mg of nigericin sodium standard (4.4.1) into a 10 ml volumetric flask (5.7). Note down the exact weight of nigericin sodium. Dissolve in methanol (4.1.3) and make up to 10 ml with methanol. Store at -20 °C and protected from light. Prepare freshly every 3 months.

Determine the experimental concentration of the nigericin sodium stock solutions using the reference standard purity value provided by the supplier using Equation (2).

$$C_n = \frac{m}{10} \times P \qquad (2)$$

where

C_n is the experimental concentration of nigericin sodium in the stock standard in mg/ml;

P is the purity of the nigericin sodium standard given by the supplier in % e.g. 0,98;

m is the weighed mass of nigericin sodium standard (4.4.1) in mg.

4.5.3 Semduramicin intermediate standard solution (for the LC-MS method), ca. 100 µg/ml.

Accurately pipette 1,0 ml of the semduramicin stock standard solution (4.5.1) into a 10 ml volumetric flask (5.7). Make up to 10 ml with acetonitrile (4.1.2). Store at -20 °C and protected from light. Prepare fresh intermediate solutions monthly.

4.5.4 Nigericin sodium intermediate standard solution (for the LC-MS method), ca. 100 µg/ml.

Accurately pipette 1,0 ml of the nigericin sodium stock standard solution (4.5.2) into a 10 ml volumetric flask (5.7). Make up to 10 ml of with acetonitrile (4.1.2). Store at -20 °C and protected from light. Prepare fresh intermediate solutions monthly.

4.5.5 Semduramicin spiking solution (for the LC-MS method), ca. 2 µg/ml.

Accurately pipette 200 µl of the semduramicin intermediate solution (4.5.3) with an appropriate automatic pipette (5.6) into a 10 ml volumetric flask (5.7). Make up to 10 ml with acetonitrile (4.1.2). Store at -20 °C and protected from light. Prepare fresh spiking solutions weekly.

4.5.6 Nigericin sodium spiking solution (for the LC-MS method), ca. 2 µg/ml.

Accurately pipette 200 µl of the nigericin sodium intermediate solution (4.5.4) with an appropriate automatic pipette (5.6) into a 10 ml volumetric flask. Make up to 10 ml with acetonitrile (4.1.2). Store at -20 °C and protected from light. Prepare fresh spiking solutions weekly.

5 Apparatus

Usual laboratory apparatus and, in particular, the following:

5.1 LC-MS method HPLC system consisting of the following:

5.1.1 Pump, pulse free, flow capacity 0,1 ml/min to 2,0 ml/min.

5.1.2 Injection system, manual or autosampler, with a loop suitable for 10 µl injections.

5.1.3 Single quadrupole mass spectrometer or triple quadrupole mass spectrometer used in the MS configuration able to operate in a mass range at least between 200 m/z to 1 000 m/z.

NOTE For the MS detector the vacuum should be as stable as possible to ensure satisfactory repeatability. The system should therefore be pumped at least 48 h before starting the measurements.

5.1.4 Computer data system.

5.1.5 Analytical column, Alltima HP C18, 5 µm ,150 mm x 2,1 mm, 190 Å, 12 % C load and 200 m^2/g or equivalent.

5.1.6 Guard column, Alltima HP C18, 5 µm, 7,5 mm x 2,1 mm, 190 Å, 12% C load and 200 m^2/g or equivalent.

5.2 LC-PCD-UV method HPLC system consisting of the following:

5.2.1 Pump, pulse free, flow capacity 0,1 ml/min to 2,0 ml/min.

5.2.2 Injection system, manual or autosampler, with a loop suitable for 100 µl injections.

5.2.3 UV/VIS detector, variable wavelength, suitable for reliable measurements at 598 nm, or UV/VIS photodiode array detector (DAD).

5.2.4 Computer data system.

5.2.5 Post-column reactor, with a 1,5 ml to 2,0 ml reaction coil, for operation at 92 °C.

The coil may be a commercially available coil or it may be made using 1/16” 316 SS tubing, 0,020" <> 0,5 mm ID (between 8 m and 10 m length) folded to fit the reactor heating chamber. To ensure

effective mixing of reagent and column effluent, use a vortex or static mixing tee (not a regular tee) before the reaction coil.

5.2.6 Post column reagent pump, pulse free, flow capacity from 0,5 ml/min to 2,0 ml/min.

5.2.7 Analytical column, Inertsil ODS-3 C18, 5 µm, 150 x 4,6 mm 100 Å 15% carbon load and 450m^2/g or equivalent.

5.2.8 Guard column, Prevail C18, 5 µm, 7,5 x 4,6 mm or equivalent.

5.3 Glass vials, 1,5 ml HPLC glass vials.

5.4 Shaker, head-over-head or equivalent.

5.5 Balances, one analytical, of 10 g capacity or greater with 0,1 mg readability.

5.6 Variable-volume positive displacement piston pipettes, suitable for pipetting volumes ranged from 20 µl to 1 000 µl.

5.7 Volumetric flasks, 10 ml, 20 ml, 500 ml and 1 000 ml.

5.8 Glass graduated cylinders, 100 ml and 1 000 ml.

5.9 Polypropylene centrifuge tubes, 50 ml capacity, stoppered (Falcon® or equivalent).

5.10 Disposable syringes, 20 ml.

5.11 Solvent filtration system, all glass filter apparatus suitable for 47 mm filters.

5.12 Membrane filters, Durapore® membrane filters, PVDF, hydrophobic, of 47 mm diameter and pore size 0,45 µm or equivalent.

5.13 Nylon filters, 47 mm diameter and pore size 0,22 µm or equivalent.

5.14 Syringe filters Nylon 0,2 µm or equivalent i.e. full chemical compatibility with acetonitrile.

5.15 pH meter.

5.16 Grinding instrument.

5.17 Sieve, with 1 mm apertures.

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 [1].

7 Preparation of test sample

7.1 General

Prepare the test sample in accordance with prEN ISO 6498.

7.2 Laboratory sample

Grind the laboratory sample (usually 50 g) so that it passes completely through a sieve with 1 mm apertures. Mix thoroughly.

7.3 Test sample

The test sample consists of a representative and homogenised aliquot of the ground laboratory sample of at least 20 g.

7.4 Test portion

Accurately weigh 5,0 g ± 0,1 g of the thoroughly mixed test sample into a 50 ml polypropylene stoppered tube (Falcon® tube or equivalent). Note down the mass expressed in mg (W_{tp}). Submit it to the analysis procedure (8).

8 Procedure

8.1 Preparation of positive and negative control samples

The use of quality control samples and quality control charts is recommended.

With each set of samples, include a positive control sample (PCS) and a negative control sample (NCS) on a daily basis. For the PCS, weigh 5,0 g ± 0,1 g of a poultry blank feed. Accurately pipette, with an appropriate automatic pipette (5.6), 130 µl of the semduramicin stock standard solution (4.5.1) and add to the poultry blank. Mix thoroughly to ensure maximum contact with the feed. Allow penetration for at least 1 hour before extraction. The NCS is constituted by 5,0 g ± 0,1 g of non-spiked poultry blank feed. Both PCS and NCS will be submitted to the same treatment as unknown samples.

8.2 Samples extraction

Accurately weigh 5,0 g ± 0,1 g test portion into a polypropylene centrifuge tube (5.9). Add 15 ml acetonitrile (4.1.2), close tightly the polypropylene centrifuge tube (5.9) and check that there is no solvent leak through the cap. Shake vigorously to ensure appropriate suspension of the feed into the extraction solvent. Perform a solid-liquid extraction by shaking the mixture for 30 min on the shaker (5.4).

8.3 Filtration

Filter the supernatants (8.2) through a 0,2 µm Nylon syringe filter (5.14) into a clean polypropylene tube (5.9).

8.4 HPLC analysis

8.4.1 LC-MS

8.4.1.1 Dilution

Accurately pipette with an appropriate automatic pipette (5.6), 75 µl of the filtered extract (8.3) into a 10 ml volumetric flask (5.7) Make up to 10 ml volume with acetonitrile (4.1.2).

8.4.1.2 Standard additions

Accurately pipette with an appropriate automatic pipette (5.6), four 900 µl aliquots of the diluted extract (8.4.1.1) into 1,5 ml glass vials (5.3) and label as S0, S1, S2 and S3 respectively.

Accurately pipette with an appropriate automatic pipette (5.6), 25 µl of the nigericin sodium spiking solution (4.5.6) and add to each of the vials labelled as S0, S1, S2 and S3 respectively.

Accurately pipette with an appropriate automatic pipette (5.6), 0 µl, 15 µl, 45 µl and 75 µl of the semduramicin spiking solution (4.5.5) and add to the vials labelled as S0, S1, S2 and S3 respectively.

Accurately pipette with an appropriate automatic pipette (5.6), 75 µl, 60 µl, 30 µl and 0 µl of acetonitrile (4.1.2) and add to the vials labelled as S0, S1, S2 and S3 respectively.

Cap all vials and shake vigorously.

NOTE When using highly sensitive mass spectrometers an appropriate further dilution of all the solutions (e.g. 1:10) from the vials labelled as S0, S1, S2 and S3 is recommended in order to prevent source saturation.

8.4.1.3 Analytical conditions

a) HPLC separation parameters:

1) column: as in (5.1.5);

2) guard column: as in (5.1.6);

3) mobile phase: as in (4.1.5.2)

4) flow rate: 0,25 ml/min;

5) injection volume: 10 µl;

6) injection mode: full loop;

7) column temperature: 25 °C;

8) autosampler temperature: 4 °C.

In these conditions the retention times are approximately 4,0 min and 7,0 min for semduramicin and nigericin respectively. The total run time is 15 min.

NOTE 1 If longer retention times are observed, increase appropriately the total run time.

b) MS parameters

1) ionisation mode: ESI^+ ;

2) acquisition mode: single ion monitoring (SIM);

3) probe temperature: 250 °C;

4) cone voltage: 60 V;

5) needle voltage: 2,7 kV;

6) ammonium adducts (used for identification and for quantification):

i) semduramicin m/z > 890,5;

ii) nigericin (I.S.) m/z > 742,5;

NOTE 2 All conditions mentioned were optimised for the LC-MS used at EC-JRC-IRMM for the optimisation and validation of this protocol being therefore only indicative values and have to be optimised for the used equipment.

7) sodium adducts (used only for identification):

    i) semduramicin m/z > 895,5;

    ii) nigericin (I.S.) m/z > 747,5;

8) mass span: ± 0,25;

9) dwell time: 0,50 s.

NOTE 3 The quantification is based on the signal of the correspondent ammonium adducts of the molecular ions. If a MS/MS instrumentation is used, it shall be used in MS configuration.

NOTE 4 Optimize the instrumental parameters in order to have a similar absolute signal of the semduramicin and nigericin ammonium adducts in a solution containing both substances at the same concentration level, e.g. prepare a sample containing 25 µl solution (4.5.5) + 25 µl solution (4.5.6) + 950 µl acetonitrile and check that in the optimized conditions the obtained absolute signal for the respective ammonium adducts are of the same order of magnitude.

NOTE 5 After a maximum of 50 injections a cleaning step is strongly recommended. This cleaning step involves flushing the column with pure methanol (4.1.3) at 0,25 ml/min during 30 min followed by re-conditioning of the column with an acetonitrile (4.1.2) injection with the mobile phase (4.1.5.2) used for the analysis for the HPLC column. It is advised to include the cleaning step always after the full standard addition of one sample.

8.4.2 LC-PCD-UV

8.4.2.1 System set up

Start up the system as follows. Switch on the oven of the post column derivatization system. Turn on the HPLC pump with a low flow (e.g. 0,1 ml/min) before starting the post-column reagent flow to prevent back flow of reagent into the analytical column. Start then the post-column reagent flow with a low flow (e.g. 0,1 ml/min). Slowly increase alternatively the two flows until reaching 0,7 ml/min and 0,9 ml/min respectively for the HPLC flow and the post-column reagent flow. Wait until the temperature is stabilized at 92 °C.

8.4.2.2 External calibration curve

8.4.2.2.1 General

Accurately pipette with an appropriate automatic pipette (5.6), one aliquot of the filtered extract (8.3) into 1,5 ml glass vials (5.3) and label it as S0.

Cap the vial and shake vigorously.

Prepare the respective HPLC calibrants as follows:

8.4.2.2.2 HPLC standard 1, ca. 2,5 µg/ml

Accurately pipette 50 µl of semduramicin stock standard solution (4.5.1) with an automatic pipette (5.6) into a 20 ml volumetric flask. Make up to 20 ml with acetonitrile (4.1.2). Mix well. Store at -20 °C. Prepare fresh weekly.

8.4.2.2.3 HPLC standard 2, ca. 6,5 µg/ml

Accurately pipette 130 µl of semduramicin stock standard solution (4.5.1) with an automatic pipette (5.6) into a 20 ml volumetric flask. Make up to 20 ml with acetonitrile (4.1.2). Mix well. Store at -20 °C. Prepare fresh weekly.

8.4.2.2.4 HPLC standard 3, ca. 10,5 µg/ml

Accurately pipette 210 µl of semduramicin stock standard solution (4.5.1) with an automatic pipette (5.6) into a 20 ml volumetric flask. Make up to 20 ml with acetonitrile (4.1.2). Mix well. Store at -20 °C. Prepare fresh weekly.

8.4.2.2.5 HPLC standard 4, ca. 14,5 µg/ml

Accurately pipette 290 µl of semduramicin stock standard solution (4.5.1) with an automatic pipette (5.6) into a 20 ml volumetric flask. Make up to 20 ml with acetonitrile (4.1.2). Mix well. Store at -20 °C. Prepare fresh weekly.

8.4.2.2.6 HPLC standard 5, ca. 18,5 µg/ml

Accurately pipette 370 µl of semduramicin stock standard solution (4.5.1) with an automatic pipette (5.6) into a 20 ml volumetric flask. Make up to 20 ml with acetonitrile (4.1.2). Mix well. Store at -20 °C. Prepare fresh weekly.

8.4.2.3 Analytical conditions

a) HPLC separation parameters:

   1) analytical column (5.2.7);

   2) guard column (5.2.8);

   3) column temperature: 38 °C;

   4) mobile phase: as in (4.2.4.3);

   5) flow rate: 0,7 ml/min;

   6) wavelength: 598 nm;

b) Post-column reaction parameters:

   1) post-column reactor: as in (5.2.5);

   2) mobile phase: as in (4.2.4.1);

   3) flow rate: 0,9 ml/min;

   4) reactor temperature: 92 °C.

NOTE During the series of measurements, the post column reaction reagent (4.2.4.1) shall be kept cool and protected from the light by means of ice blocks or in an ice bath.

Using the analytical column (5.2.7) guard column (5.2.8) combination, with the above conditions, the retention time for semduramicin is approximately 10 min. The total run is set at e.g. 25 min to allow the elution of all the other coccidiostats, if present.

8.4.2.4 System shutdown

Shut down the system as follows. Turn off the post-column reagent flow before stopping HPLC pump to prevent back flow of reagent into the analytical column. Wash the column and reactor for 30 min to 45 min with methanol (4.1.3) at the same flow rate as the analysis i.e. 0,9 ml/min.

8.5 HPLC determination

8.5.1 LC-MS method

Perform the injections from each vial labelled as S0, S1, S2 and S3 as prepared in (8.4.1.2) in duplicate in the sequence S0, S1, S2 and S3 followed by the injection of a solvent blank (4.2.4.3). Inject the control samples prepared as described in (8.1) and each of the samples prepared as in (8.4.1.2).

8.5.2 LC-PCD-UV method

Perform consecutive injections of each HPLC calibrant as prepared in (8.4.2.2) from the lowest to the highest concentration. Inject the control samples prepared as described in (8.1). Inject each of the samples prepared as in (8.3) in duplicate. Re-inject each HPLC calibrant.

8.5.3 System suitability

8.5.3.1 Run acceptance

To accept the experiments the following criteria shall be met:

— The signal that may be present in the negative control sample (NCS) corresponding to semduramicin shall not exceed the equivalent of 0,9 mg/kg;

— The lowest fortified sample or the lowest calibrant shall present a clear quantification signal corresponding to semduramicin (S/N > 10);

— The spiked samples and/or the lowest HPLC standard shall pass the identification criteria (8.5.3.2).

For quantitative results to be acceptable:

— The recovery of the semduramicin in the PCS shall be between 80 % and 120 %;

— The external calibration line and the standard addition line shall have a determination coefficient (R^2) of 0,995 or a correlation coefficient (r) of 0,998 or better for the LC-PCD-UV method and (R^2) of 0,990 or a correlation coefficient (r) of 0,995 or better for the LC-MS method.

8.5.3.2 Identification of the analyte

— The retention time of the signal of semduramicin shall be identical to the retention time recorded in the positive control sample within a margin of ± 5 %. In case of doubt, standard addition (standard material added to the sample) shall be performed;

— The signal of semduramicin shall increase with the different fortified samples and/or with the different HPLC standards;

— The presence of the signal corresponding to semduramicin at a S/N > 10 shall be confirmed;

— For the LC-MS method, the presence of the correspondent sodium adduct at the identical retention time of the equivalent ammonium adduct within a margin of ± 2 % shall be verified;

— For the LC-PCD-UV method, if other signals are present in the sample, the resolution between the semduramicin signal (SEM) and the adjacent signals shall be at least 0,8.

9 Calculation

9.1 LC-MS method

Perform an automatic integration (baseline mode) of the peaks obtained for the respective ammonium adducts of semduramicin and nigericin i.e. at m/z > 890,5 (retention time ~ 3,5 min) and m/z > 742,5 (retention time ~ 6,7 min.) and calculate the signal ratio (3).

$$Ratio = \frac{ApSEM}{ApNIG(I.S.)} \quad (3)$$

where

ApSEM is the absolute signal of the semduramicin ammonium adduct;

ApNIG(I.S.) is the absolute signal of the nigericin ammonium adduct.

Determine the equivalent added concentration of semduramicin in feed for S0, S1, S2 and S3 using Equation (4).

$$Csi = \frac{Vs \times Cs \times 200}{675} \times \frac{15000}{Wtp} \quad (4)$$

where

Csi is the equivalent added concentration of semduramicin in feed for S0, S1, S2 and S3 in mg/kg, with i = 0, 1, 2 or 3 respectively;

Vs is the volume of the semduramicin spiking solution (4.5.5) added respectively in S0, S1, S2 and S3 in µl;

Cs is the concentration of semduramicin in the stock standard solution (4.5.1) in mg/ml;

Wtp is the weighed mass of the test portion (7.4) in mg.

Plot each of the measured area ratios calculated using Equation (3) (y-axis) versus the correspondent equivalent added concentration of semduramicin in feed in S0; S1; S2; and S3 (x-axis).

The unknown concentration in mg semduramicin per kilogram of compound feed (C*sx*) is obtained by extrapolation of the obtained regression line at y = 0.

9.2 LC-PCD-UV method

Determine the equivalent concentration of semduramicin for each of the standard solutions of the HPLC range using Equation (5):

$$Cstd = \frac{V \times Cs}{20} \quad (5)$$

where

Cstd is the exact concentration of each HPLC standard in µg/ml;

Cs is the concentration of semduramicin in the stock standard solution (4.5.1) in mg/ml;

V is the volume pipetted from the semduramicin stock standard solution (4.5.1) in µl.

Plot each of the individual measured area (y-axis) versus the respective calculated equivalent concentration of semduramicin of each HPLC standard C*std* (5).

Use the following Equation (6) for the calculation of the equivalent semduramicin concentration in feed (*Csx*) of the unknown sample in mg/kg.

$$Csx = \frac{15000 \times (A - b)}{a \times Wtp} \tag{6}$$

where

A is the area of semduramicin in the unknown sample;

b is the intercept of the calibration curve obtained from the standard solutions (8.4.2.2);

a is the slope coefficient of the calibration curve obtained from the standard solutions (8.4.2.2);

Wtp is the weighed mass of the test portion (7.4) in mg.

10 Precision

10.1 Collaborative study

Details of the collaborative studies [6] [7]are summarized in Annex A. The values derived from this test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, expressed as a percentage of the mean determined value, 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 cases exceed the repeatability limit (*r*) derived from the following Equation (7):

$$r = 2 \times 2^{1/2} \times RSD(r) = 2{,}8 \times RSD(r) \tag{7}$$

where

RSD(r) is the repeatability relative standard deviation.

10.3 Reproducibility

The absolute difference between two single test results, expressed as a percentage of the mean determined value, obtained using the same method on identical test material on different days with the same operator using the same equipment, will in not more than 5 % of cases exceed the reproducibility limit (*R*) derived from the following Equation (8):

$$R = 2{,}8 \times RSD(R) \tag{8}$$

where

RSD(R) is the reproducibility relative standard deviation.

11 Test report

The test report shall specify the following information:

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

b) the sampling method used, if known;

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

d) all 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);

e) the test result obtained;

f) if the repeatability has been checked, the final quoted result obtained;

g) if the trueness has been checked, the final quoted result obtained.

Annex A
(informative)

Results of collaborative study

A.1 Procedure

A collaborative study was organized and carried out according to the recognized international guidelines [8]. Positive test materials have been prepared by an external company under "real manufacturing conditions". The test materials contained the target analyte semduramicin (SEM) at three different concentration levels, ranging from half (10 mg/kg) to twice (50 mg/kg) the maximum authorized concentration (MAC) (from 20 mg/kg to 25 mg/kg). Additionally, potential interferents, namely monensin, lasalocid, maduramicin, narasin and salinomycin, at their respective cross contamination levels specified by the European Commission [3], were included in the materials.

Two different poultry feedingstuffs diets (starters and finishers) were tested within the study and for one of the tested methods (LC-PCD-UV) the laboratories have received the same materials in two different forms i.e. pelleted and ground.

Each material (pelleted and ground) has been submitted to a re-homogenisation procedure by the organising laboratory using a Retsch Laboratory Sample divider PT100, followed by a subdivision into approximately 50 g aliquots. Homogeneity of the materials was assessed by analyzing, in duplicate, 10 randomly selected aliquots using one of the tested methodologies (LC-PCD-UV) that had been previously single laboratory validated. Test materials were considered homogeneous if the ANOVA single factor lead to an F value below the corresponding $Fcrit$.

The whole assay involved two different phases. The first one (training phase) was intended to allow the participants to familiarize with the methods as well as to identify potential unintentional deviations from the stated procedures. The training phase included the analyses of unknown samples at the maximum authorized concentration (MAC) of both diets (starters and finishers) and both forms (ground and pelleted) as well as spiked samples. The samples tested within the training phase can be classified as follows:

a) Training phase samples

   1) Unknown samples (all dispatched as blind samples):

      i) 1 ground compound feed for poultry (starters) in duplicate;

      ii) 1 ground compound feed for poultry (finishers) in duplicate;

      iii) 1 pelleted compound feed for poultry (starters) in duplicate.

   2) Disclosed sample intended to be used for the control samples:

      i) 1 ground compound feed for poultry (starters) blank in single.

The laboratories selected from the training phase carried out the validation phase of the study. The training phase included the analyses of unknown samples at three concentration levels of both diets (starters and finishers) as well as spiked samples. Participants performing the analysis using the LC-PCD-UV method were requested to perform the analyses of ground and pelleted samples whereas participants performing the analysis using the LC-MS method were requested to perform the analysis of the ground samples using the standard addition quantification approach and an alternative quantification approach (external standard calibration). The samples tested within the validation phase for each of the methods can be classified as follows:

b) Validation phase samples (LC-PCD-UV):

1) Unknown samples (all dispatched as blind samples):

i) 3 ground compound feeds for poultry (starters) (A1, A2 and A3) in duplicate;

ii) 3 ground compound feeds for poultry (finishers) (A4, A5 and A6) in duplicate;

iii) 3 pelleted compound feeds for poultry (starters) (C1, C2 and C3) in duplicate;

iv) 3 pelleted compound feeds for poultry (starters) (C4, C5 and C6) in duplicate.

2) Disclosed samples intended to be used for the control samples:

i) 1 ground compound feed for poultry (starters) blank in single;

ii) 1 pelleted compound feed for poultry (starters) blank in single.

Fifteen laboratories reported results for the validation phase of the LC-PCD-UV method within a reasonable timeframe.

NOTE Experience has shown that the combination Inertsil ODS-3 C18 – Prevail C18 column – guard column to provide better results. Alternatively, the following columns have also led to a suitable resolution among the monensin (Mon A and Mon B) and semduramicin (SEM) signals within the collaborative study:

– Purosfer STAR RP18e, 150x4,6mm 5µm 120 Å pore size; 330 m^2/g surface area; 17% carbon load;

– Luna C18 (2) 150x4,6mm 5µm 100 Å pore size; 400 m^2/g surface area; 17,5% carbon load;

– Zorbax Eclipse XDB-C18 150x4,6mm 5µm 80 Å pore size; 180 m^2/g surface area; 10% carbon load.

c) Validation phase samples (LC-MS)

1) Unknown samples (all dispatched as blind samples)

i) 3 ground compound feeds for poultry (starters) (A1, A2 and A3) in duplicate

ii) 3 ground compound feeds for poultry (finishers) (A4, A5 and A6) in duplicate

2) Disclosed sample intended to be used for the control samples

i) 1 ground compound feed for poultry (starters) blank in single

Fifteen laboratories reported results for the validation phase of the LC-MS method within a reasonable timeframe.

A.2 Statistical analysis of results

NOTE The trial results were examined for evidence of individual systematic error using Cochran and Grubbs tests sequentially, by procedures described in the international guidelines [8]. Calculations for repeatability (r) and reproducibility (R) limits as defined by the guidelines were carried out after removal of outliers. In particular, HORRAT values were calculated and were within the performance acceptability limits (0,5-2) [8].The calculated method performance figures (repeatability, reproducibility and HORRAT values) are presented in Tables A.1 to A.4.

Table A.1 — Precision data LC-PCD-UV method (ground materials)

Parameter	Samples					
	A1	A2	A3	A4	A5	A6
Number of laboratories retained after elimination of outliers	14	13	13	13	14	13
Mean semduramicin content, mg/kg	9,5	19,5	39,1	9,4	18,9	37,8
Repeatability standard deviation (s_r,), mg/kg	1,0	0,3	1,8	0,2	0,8	1,9
Repeatability relative standard deviation (RSD_r), %	10	2	5	2	4	5
Repeatability limit (r), mg/kg	2,7	0,9	5,1	0,6	2,3	5,4
Reproducibility standard deviation (s_R), mg/kg	1,5	2,6	5,6	1,4	2,7	4,4
Reproducibility relative standard deviation (RSD_R), %	16	13	14	15	14	11
Reproducibility limit (R), mg/kg	4,2	7,2	15,6	3,9	7,5	12,2
HORRAT value	1,4	1,3	1,5	1,3	1,4	1,2

Table A.2 — Precision data LC-PCD-UV method (pelleted materials)

Parameter	Samples					
	C1	C2	C3	C4	C5	C6
Number of laboratories retained after elimination of outliers	14	14	13	13	13	14
Mean semduramicin content, mg/kg	9,5	18,6	37,7	9,5	18,7	36,8
Repeatability standard deviation (s_r,), mg/kg	0,5	0,9	0,6	0,3	1,3	1,3
Repeatability relative standard deviation (RSD_r), %	6	5	2	4	7	4
Repeatability limit (r), mg/kg	1,5	2,6	1,8	1,0	3,6	3,7
Reproducibility standard deviation (s_R), mg/kg	1,2	2,7	4,8	1,4	2,5	5,0
Reproducibility relative standard deviation (RSD_R), %	12	14	13	15	13	14
Reproducibility limit (R), mg/kg	3,3	7,5	13,3	4,0	6,9	14,1
HORRAT value	1,1	1,4	1,4	1,3	1,3	1,5

Table A.3 — Precision data LC-MS method (Standard Addition)

Parameter	Samples					
	A1	A2	A3	A4	A5	A6
Number of laboratories retained after elimination of outliers	13	12	11	13	13	13
Mean semduramicin content, mg/kg	10,2	21,9	40,7	10,8	20,1	41,9
Repeatability standard deviation (s_r,), mg/kg	0,4	0,9	1,6	1,0	1,4	3,6
Repeatability relative standard deviation (RSD_r), %	4	4	4	9	7	9
Repeatability limit (r), mg/kg	1,0	2,4	4,4	2,8	3,9	10,1
Reproducibility standard deviation (s_R), mg/kg	1,5	1,8	5,2	2,0	2,9	5,9
Reproducibility relative standard deviation (RSD_R), %	15	8	13	18	14	14
Reproducibility limit (R), mg/kg	4,1	5,0	14,5	5,5	8,1	16,4
HORRAT value	1,3	0,8	1,4	1,6	1,4	1,5

Table A.4 — Precision data LC-MS method (External Standard Calibration)[1)]

Parameter	Samples					
	A1	A2	A3	A4	A5	A6
Number of laboratories retained after elimination of outliers	13	13	13	13	13	13
Mean semduramicin content, mg/kg	9,1	18,9	35,7	9,4	18,0	37,4
Repeatability standard deviation (s_r,), mg/kg	0,4	0,4	1,3	0,5	0,8	1,0
Repeatability relative standard deviation (RSD_r), %	5	2	4	5	4	3
Repeatability limit (r), mg/kg	1,2	1,1	3,5	1,3	2,2	2,7
Reproducibility standard deviation (s_R), mg/kg	1,2	2,0	4,0	1,2	2,0	4,1
Reproducibility relative standard deviation (RSD_R), %	14	10	11	12	11	11
Reproducibility limit (R), mg/kg	3,5	5,5	11,2	3,2	5,7	11,4
HORRAT value	1,2	1,0	1,2	1,1	1,1	1,2

[1)] The use of this quantification approach is restricted to those cases where the matrix effect will not be relevant.

A.3 Example chromatogram

An example of a chromatogram and their correspondent spectra when using the LC-MS method (SIM acquisition) for a compound feed containing semduramicin, monensin, salinomycin, narasin, maduramicin and lasalocid is shown in Figure A.1.

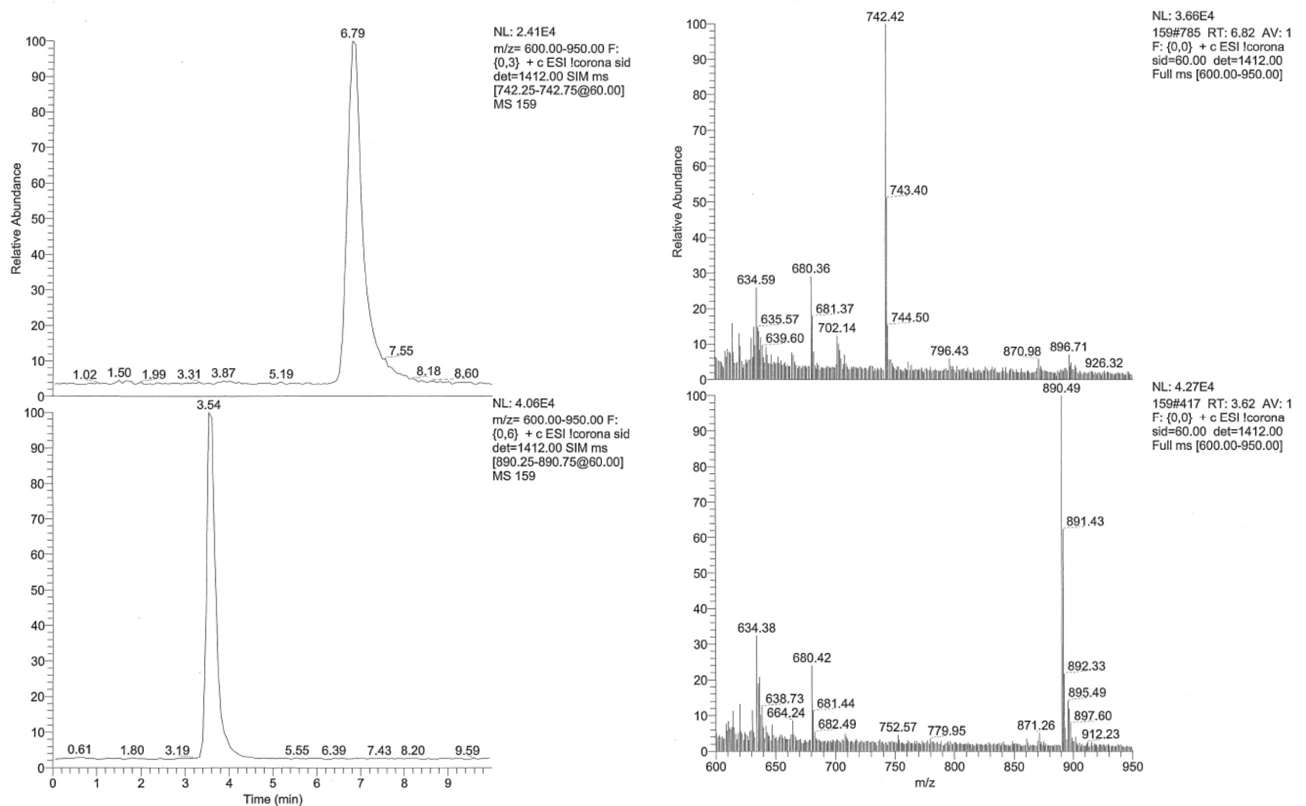

Figure A.1 — Chromatograms (a) and spectra (b) of the internal standard nigericin (A) and semduramicin (B) in a typical compound feed for poultry containing also monensin, salinomycin, narasin, maduramicin and lasalocid at their cross contamination levels

An example of a chromatogram when using the LC-PCD-(UV)-VIS method for a compound feed containing semduramicin, monensin, salinomycin, narasin, maduramicin and lasalocid is shown in Figure A.2.

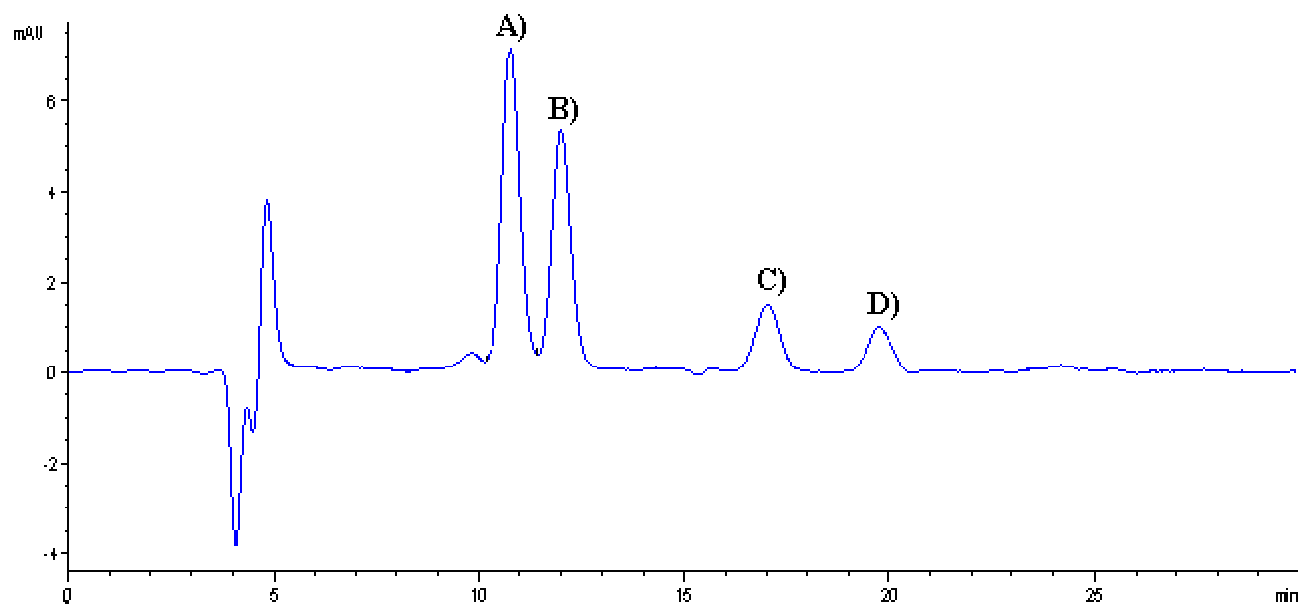

Figure A.2 — Chromatogram of a typical compound feed for poultry containing semduramicin (A) at authorized level and containing also monensin (B), salinomycin (C), narasin (D), maduramicin (C) and lasalocid at their respective cross contamination levels

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