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SLOVENSKI STANDARD
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Ugotavljanje tilozina, spiramicina, virginiamicina, karbadoksa in olakvindoksa pri koncentracijah, manjših od vsebnosti dodatkov v krmnih mešanicah - Potrditvena analiza z LCMS

Identification of tylosin, spiramycin, virginiamycin, carbadox and olaquindox at sub-additive levels in compound feed - Confirmatory analysis by LCMS

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Bestimmung von Tylosin, Spiramycin, Virginiamycin, Carbadox und Olaquindox in Konzentrationen unterhalb von Zusatzstoffen in Mischfuttermitteln - Bestätigungsanalyse mittels LC-MS

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Identification de la tylosine, spiramycine, virginiamycine, du carbadox et de l’olaquindix dans les aliments composés pour animaux à des concentrations inférieures à celles des additifs - Analyse de confirmation par CL-SM

Ta slovenski standard je istoveten z: prEN 17049

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65.120	Krmila	Animal feeding stuffs

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Identification of tylosin, spiramycin, virginiamycin, carbadox and olaquindox at sub-additive levels in compound feed - Confirmatory analysis by LCMS

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This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 327.

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European foreword

This document (prEN 17049:2016) has been prepared by Technical Committee CEN/TC 327 "Animal feeding stuffs: Methods of sampling and analysis", the secretariat of which is held by NEN.

This document is currently submitted to the CEN Enquiry.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

WARNING — The method described in this standard implies the use of reagents that pose a hazard to health. The standard does not claim to address all associated safety problems. It is the responsibility of the user of this standard to take appropriate measures for the health and safety protection of the personnel prior to use of the standard and to ensure that regulatory and legal requirements are complied with.

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1 Scope

This European standard specifies a high performance liquid chromatography – mass spectrometry (LC-MS/MS) method for the identification of tylosin, spiramycin, virginiamycin, carbadox and olaquindox in animal feeds.

The method is suitable for the identification of low concentrations of tylosin, spiramycin, virginiamycin, carbadox and olaquindox in compound animal feeds. A limit of identification of 1 mg/kg for tylosin, spiramycin and virginiamycin, 4 mg/kg for carbadox and 3 mg/kg for olaquindox should be obtained by using the described method. The method was fully validated during a collaborative study (see Annex A).

Since tylosin, spiramycin and virginiamycin are fermentation products consisting of a mixture of several closely related compounds, the analysis is based on detection and identification of the most abundant constituents. For tylosin the marker is tylosin A, for spiramycin the marker is spiramycin I and II and for virginiamycin the marker is virginiamycin M1 and S1. The other isomers and forms can be readily detected with the same method but adjustment of the MS parameters according to the molecular mass of precursor and product ions need to be made. Carbadox and olaquindox are analysed as such.

2 Normative references

No normative references apply to this document.

3 Principle

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The compounds are extracted from the feed with a mixture of water and methanol. An aliquot of the liquid phase is diluted and applied to a pre-conditioned SPE column. After washing of the SPE column, compounds of interest are eluted with methanol. The obtained extract is evaporated and re-dissolved in dilute formic acid. The resulting extract is analysed by LC-MS/MS. Separation is carried out on a silica-based C18 bonded phase column and detection is performed by mass spectrometry in multiple reaction monitoring mode.

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The validation of this method was performed at concentration levels that were calculated on a weight (w/w) basis. Expression of working ranges in terms of w/w concentration is common practice in residue analysis of veterinary drugs, in fact Maximum Residue Limits (MRL) are exclusively expressed on a w/w basis. For feed additives however, tolerances have been expressed traditionally as microbiological activity. To translate the validation experiments concerning the level at which they were performed, to units expressed as microbiological activity, the w/w concentrations should be corrected for the microbiological potency of the preparation used for spiking experiments.

4 Reagents and materials

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

4.1 General

All reagents are of 'Analytical reagent' grade or better unless otherwise stated. Throughout this method, “water” means demineralised water with a conductivity of at least 10 Mohm.cm-1. Guaranteed purity is required for each lot of reference standard.

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4.2 Reagents and materials

4.2.1 Acetonitrile (LC-MS grade)

4.2.2 Methanol (LC-MS grade)

4.2.3 Formic acid (LC-MS grade)

4.2.4 Tylosin

4.2.5 Spiramycin

4.2.6 Virginiamycin

4.2.7 Carbadox

4.2.8 Olaquindox

4.3 Solutions

4.3.1 HPLC Mobile phase A: Formic acid 5mM

Measure 200 µl formic acid (4.2.3) and transfer to a volumetric flask of 1 000 ml, make up to the mark with water. Filter and degas before use.

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4.3.2 HPLC Mobile phase B: Formic acid 50 mM/ acetonitrile (10/90, v/v)

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Measure 200 µl formic acid (4.2.3) and transfer to a volumetric flask of 1 000 ml, add 100 ml water and make up to the mark with acetonitrile (4.2.1). Filter and degas before use.

4.4 Standard solutions

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4.4.1 Stock solution tylosin (500 µg/ml)

Weigh between 10 and 50 mg of tylosin standard substance (4.2.4) and transfer to a brown glass bottle. Calculate the required amount of methanol (4.2.2) and add that amount (on a weight basis) to obtain a standard solution of 500 µg/ml. Store this stock solution in the dark at 4-8 °C. Under these conditions it is stable for at least one month.

4.4.2 Stock solution spiramycin (500 µg/ml)

Weigh between 10 and 50 mg of spiramycin standard substance (4.2.5) and transfer to a brown glass bottle. Calculate the required amount of methanol (4.2.2) and add that amount (on a weight basis) to obtain a standard solution of 500 µg/ml. Store this stock solution in the dark at 4-8 °C. Under these conditions it is stable for at least one month.

4.4.3 Stock solution virginiamycin (500 µg/ml)

Weigh between 10 and 50 mg of virginiamycin standard substance (4.2.6) and transfer to a brown glass bottle. Calculate the required amount of methanol (4.2.2) and add that amount (on a weight basis) to obtain a standard solution of 500 µg/ml. Store this stock solution in the dark at 4-8 °C. Under these conditions it is stable for at least one month.

4.4.4 Stock solution carbadox (500 µg/ml)

Weigh between 10 and 50 mg of carbadox standard substance (4.2.7) and transfer to a brown glass bottle. Calculate the required amount of methanol (4.2.2) and add that amount (on a weight basis) to

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obtain a standard solution of 500 µg/ml. Store this stock solution in the dark at 4-8 °C. Under these conditions it is stable for at least one month.

4.4.5 Stock solution olaquindox (500 µg/ml)

Weigh between 10 and 50 mg of olaquindox standard substance (4.2.8) and transfer to a brown glass bottle. Calculate the required amount of methanol (4.2.2) and add that amount (on a weight basis) to obtain a standard solution of 500 µg/ml. Store this stock solution in the dark at 4-8 °C. Under these conditions it is stable for at least one month.

4.4.6 Mixed stock solution 1

Measure 1,0 ml of stock solutions 4.4.1, 4.4.2 and 4.4.3 and transfer into a 25 ml volumetric flask. Accurately measure 4,0 ml of stock solution 4.4.4 and transfer to the same volumetric flask. Accurately measure 3,0 ml of stock solution 4.4.5 and transfer to the same volumetric flask. Make up to the mark with water and mix. The concentration of tylosin, spiramycin, virginiamycin, carbadox and olaquindox in this stock solution is 20, 20, 20, 80 and 60 mg/l respectively. Store the stock solution in the dark at 4-8 °C. Under these conditions it is stable for at least one week.

4.4.7 Mixed stock solution 2

Mix equal volumes of mixed stock solution 1 (4.4.6) and water. Store the stock solution in the dark at 4-8 °C. The concentration of tylosin, spiramycin, virginiamycin, carbadox and olaquindox in this stock solution is 10, 10, 10, 40 and 30mg/l respectively. Prepare this stock solution fresh daily.

4.4.8 Calibration standard

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Measure 50 µl of mixed stock solution 1 (4.4.6) and transfer to a volumetric flask of 10 ml. Make up to the mark with water. The concentration of tylosin, spiramycin, virginiamycin, carbadox and olaquindox in this calibration standard is 100, 100, 100, 400 and 300 µg/l respectively. Prepare this calibration standard fresh daily.

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5 Apparatus

Usual laboratory equipment and, in particular, the following:

5.1 Analytical balance suitable to accurately weigh between 0 and 10 g with an accuracy of 0,1 mg

5.2 Balance suitable to accurately weigh between 0 and 1 500 g with an accuracy of 0,1 g

5.3 Centrifuge

5.4 Ultrasonic bath

5.5 Evaporation unit

5.6 Centrifuge tubes of different volumes, adapted to the centrifuge

5.7 SPE Vacuum manifold

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5.8 Oasis HLB® cartridges polymer phase, 60 mg, 3 ml (Waters WAT094226 or equivalent)[1]

5.9 Sample vials suitable for the auto-sampler system that is used (5.11.1)

5.10 Head-over-head shaker

5.11 LC-MS/MS equipment

5.11.1 LC-MS/MS equipment comprised of gradient HPLC system

5.11.2 LC-MS/MS equipment comprised of analytical column Symmetry® 300 C18 150 × 3 mm, 5 µm particle size (Waters WAT106154 or comparable)[2]

NOTE During the method validation, this recommended LC column has proved to be fit for purpose.

5.11.3 LC-MS/MS equipment comprised of mass spectrometer suitable for tandem MS measurement (triple quadrupole or ion trap) and equipped with an electrospray interface.

6 Sampling

The laboratory should receive a sample that is truly representative and has not been damaged or changed during transport or storage.

NOTE 1 Sampling is not part of the method specified in this European standard. Sampling is described in Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. [7]

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NOTE 2 For quantification of the content multi-level standard addition is applied to account for the considerable variability of feed composition. This procedure is described in Annex C.

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7 Sample preparation

7.1 Sample pre-treatment

Weigh 5,0 g ± 0,1 g each test sample and transfer in a 50 ml centrifuge tube (5.6) and proceed with the procedure at 7.3.

7.2 Quality control samples

A known negative sample, preferably of approximately the same composition, is included in each series (code S0). Weigh 5,0 g ± 0,1 g of the known negative sample as indicated in 7.1 and proceed with the procedure at 7.3.

Also in each series a confirmation control sample is prepared by spiking an aliquot of the extract of the negative control sample S0 obtained after sample extraction (see 7.6).

[1] Oasis HLB® cartridges polymer phase, 60 mg, 3 ml is an example of a suitable product available commercially. This information is given for the convenience of users of this European standard and does not constitute an endorsement by CEN of this product.

[2] Symmetry® 300 C18 150 × 3 mm, 5 µm particle size is an example of a suitable product available commercially. This information is given for the convenience of users of this European standard and does not constitute an endorsement by CEN of this product.

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7.3 Sample extraction

To each sample add 10 ml water, close the tubes and shake vigorously by hand. Add 10 ml of methanol (4.2.2), close the tubes and mix manually. Place the tubes in a head-over-head shaker (5.10) during 20 min. Centrifuge (5.3) the tubes at 3500 rpm for 10 min.

Accurately measure 0,5 ml of the supernatant and transfer to a clean centrifuge tube (5.6). Add 3,5 ml water and mix.

NOTE For the preparation of the confirmation control sample proceed as described in 7.6.

7.4 Sample purification

Condition an Oasis HLB® cartridge (5.8) with subsequently 3 ml methanol (4.2.2) and 5 ml water. Apply the extract obtained in 7.3 (7.6 in case of the recovery and confirmation control samples) to the cartridge and apply mild vacuum to transfer at a flow rate of approximately 1 ml/min. Wash the cartridge with 3 ml of water. Place a clean centrifuge tube (5.6) under the cartridge and subsequently elute the analytes of interest with 3 ml of methanol (4.2.2).

7.5 Sample preparation for LC-MS/MS

Evaporate the extract obtained in 7.4 TO dryness at 40 °C under a mild stream of nitrogen (5.5). Redissolve the residue in 1,0 ml of 5 mM formic acid (4.3.1).

Transfer the extract to a sample vial (5.9). Proceed as described in Clause 8.

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7.6 Confirmation control

Take an aliquot of 0,5 ml of the extract of sample S0 obtained in 7.3 and transfer to a clean centrifuge tube (5.6). Add 3,5 ml water and 12,5 µl of the mixed stock solution 2 (4.4.7) and mix. The concentration in the extract is comparable to the addition of 1 mg/kg of virginiamycin, spiramycin and tylosin, 4 mg/kg carbadox and 3 mg/kg olaquindox before sample preparation. Proceed as described in 7.4.

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8 LC-MS/MS analysis

8.1 General

Quantification of the content is performed by multi-level standard addition to account for the considerable variability of feed composition. This procedure is described hereunder.

The procedure is fully validated in-house and is described in Annex C 'Quantitative analysis' for information. Other conditions may be used provided that they give equivalent results.

8.2 LC-MS/MS experimental conditions

— Mount the analytical column in the HPL system, flush the solvent lines and adjust the gradient settings. See Table C.2 for guidance;

— Mount the electrospray interface and connect the analytical column exit to the flow splitter and subsequently the flow splitter transfer line to the MS interface;

— Make sure the mass spectrometer is properly calibrated according to the manufacturer's instructions;

— Optimise the mass spectrometer for the compounds of interest according to the manufacturer's instructions.