SLOVENSKI STANDARD
SIST EN 16939:2017

01-oktober-2017

Krma: metode vzorčenja in analize - Določevanje tilozina, spiromicina in virginiamicina - Tenkoplastna kromatografija in bioavtografija

Animal feeding stuffs: Methods of sampling and analysis - Detection of tylosin, spiramycin and virginiamycin - Thin Layer Chromatography and bioautography

Futtermittel - Probenahme- und Untersuchungsverfahren - Nachweis von Tylosin, Spiramycin und Virginiamycin - Dünnschichtchromatographie und Bioautographie

Aliments pour animaux : Méthodes d'échantillonnage et d'analyse - Détection de tylosine, spiramycine et virginiamycine - Chromatographie sur couche mince et bioautographie

Ta slovenski standard je istoveten z: **EN 16939:2017**

ICS:

65.120	Krmila	Animal feeding stuffs

SIST EN 16939:2017 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 16939

August 2017

ICS 65.120

English Version

Animal feeding stuffs: Methods of sampling and analysis - Detection of tylosin, spiramycin and virginiamycin - Thin Layer Chromatography and bioautography

Aliments pour animaux : Méthodes d'échantillonnage et d'analyse - Détection de tylosine, spiramycine et virginiamycine - Chromatographie sur couche mince et bioautographie

Futtermittel - Probenahme- und Untersuchungsverfahren - Nachweis von Tylosin, Spiramycin und Virginiamycin - Dünnschichtchromatographie und Bioautographie

This European Standard was approved by CEN on 24 April 2017.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

Ref. No. EN 16939:2017 E

Contents

Page

European foreword

This document (EN 16939:2017) 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 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 2018, and conflicting national standards shall be withdrawn at the latest by February 2018.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

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

WARNING — The use of this protocol involves hazardous materials, operations and equipment. This protocol does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

According to the CEN-CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

1 Scope

The method makes it possible to detect and identify spiramycin, tylosin and virginiamycin in animal feeding stuffs (feed raw materials of mainly plant origin and compound feeds) excluding mineral feeds and premixtures. The limit of detection is about 2 mg/kg for spiramycin, 1 mg/kg for tylosin and 1 mg/kg for virginiamycin. In some milk replacers, it can be slightly higher than 1 mg/kg for virginiamycin.

Reported limits of detection are probably little overestimated but were fully validated during the collaborative study (see Annex B). In each laboratory, each day of analysis, spiked blank samples at 1 mg/kg for spiramycin and virginiamycin and at 0,5 mg/kg for tylosin are analysed for checking lower detection limits (see 9.2 and 9.3). These lower limits of detection are achievable, but should be established with an in-house validation first.

Some other antibiotics can interfere in the detection of these 3 specific macrolide antibiotics. The known interferences are specified in Annex A of the method.

That method should be used as a qualitative screening and/or a post-screening method (after microbiological plate test, for example). The follow-up of the antibiotics presence may be done by other analytical technics (LC and/or LC-MS technics) ([4], [10]). For confirmatory purposes, LCMS is required.

2 Normative references

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

EN ISO 6498, *Animal feeding stuffs - Guidelines for sample preparation (ISO 6498)*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
microbiological activity of antibiotics
ratio of the dose that inhibits the growth of a suitable susceptible microorganism to the dose of an International Chemical Reference Substance/Antibiotic that produces similar inhibition

Note 1 to entry Microbiological activity is a property measured by a microbiological assay. The activity (potency) of an antibiotic product is expressed as the ratio of the dose that inhibits the growth of a suitable susceptible microorganism to the dose of an International Chemical Reference Substance/Antibiotic that produces similar inhibition.

Note 2 to entry The microbiological activity is expressed as International Unit/mg or µg/mg with possibility to have microbiological activities higher or lower than 1 000 µg/mg.

3.2
retardation factor
Rf
ratio of the distance which the product travelled by the distance which the solvent front travelled using the initial spotting site as reference

Note 1 to entry These values depend on the solvent used and the type of TLC plate and are not physical constants, see Figure 1.

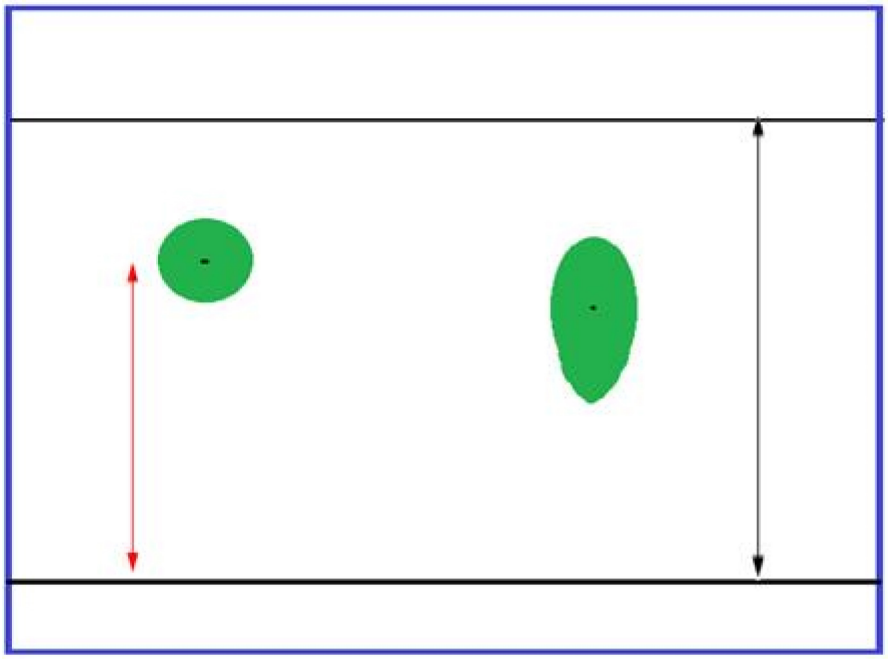

Figure 1 — TLC plate with 2 inhibition zones

3.3
sensitivity of a method
SE
ability of the method to classify a positive sample as positive

3.4
specificity of a method
SP
ability to classify a negative sample as negative

3.5
spiramycin
macrolide antibiotic and often a mixture of different cofactors (spiramycin I, II, III..)

Note 1 to entry One mg of spiramycin base is considered to be equivalent to 3200 International Unit.

3.6
tylosin
macrolide antibiotic and mixture of four macrolide antibiotics produced by a strain of Streptomyces fradiae and depending on the manufacturing source

Note 1 to entry The main component of the mixture (>80 %) is tylosin A. Tylosin B (desmycosin), tylosin C (macrocin) and tylosin D (relomycin) may also be present. All four components contribute to the potency of tylosin, which is not less than 900 IU/mg, calculated with reference to the dried substance (European Pharmacopoeia). Relative antimicrobial activities of tylosin derivatives are: tylosin A – 1,0, tylosin B – 0,83, tylosin C – 0,75 and tylosin D – 0,35.

3.7
virginiamycin
macrolide antibiotic and mixture of 2 major synergistic cofactors: virginiamycin components M1 and S1

4 Principle

The sample is extracted with a mixture of methanol and water. The extracts are purified by a liquid-liquid partition with chloroform. The chloroformic phase is concentrated. The concentrated feed extracts and reference spiked blank feeds extracts are subjected to thin-layer chromatography (TLC) on silica gel. The antibiotics are detected and identified by comparing their Rf values with those of the standard substances by bioautography with agar media inoculated with Micrococcus luteus.

5 Reagents and culture media

5.1 General

All the reagents shall be of analytical grade.

5.2 Microorganism: Bacterial suspension of *Micrococcus luteus* ATCC 9341 (reclassified to *Kocuria rhizophila* ATCC 9341).

See Annex C for preparation of bacterial suspensions.

Other techniques for the preparation and the storage of the bacterial suspension may be used if the detection limits specified can be reached.

5.3 Culture media:

5.3.1 Antibiotic medium 1.

Use the water bath (6.6) to liquefy media just before inoculating Micrococcus Luteus.

Beef extract:	1,5 g
Yeast extract:	3,0 g
Pancreatic digest of casein:	4,0 g
Meat peptone:	6,0 g
Glucose:	1,0 g
Agar:	10 g to 20 g
Water:	1 000 ml

Autoclave at 121 °C for 15 min

Final pH: 6,5 ± 0,2

Antibiotic medium 1 may be conserved at least 6 months at 5 °C ± 3 °C.

5.3.2 Antibiotic medium 1 supplemented with tylosin.

Just before inoculating with *Micrococcus luteus*, add to the antibiotic medium 1 (5.3.1), 0,2 % (v/v) of the solution of tylosin at 4 µg/ml (5.20.10).

Adjust the pH to 6,5 ± 0,1.

5.3.3 Antibiotic medium 11.

Use the water bath (6.6) to liquefy media just before inoculating *Micrococcus Luteus*.

The composition is the same than that of antibiotic medium 1 (5.3.1) but the final pH is: 8,0 ± 0,2.

Antibiotic medium 11 may be conserved at least 6 months at 5 °C ± 3 °C.

5.3.4 Antibiotic medium 11 supplemented with methanol, pH 8 buffer and tylosin.

Just before inoculating the medium with *Micrococcus luteus*, add to the antibiotic medium 11 (5.3.3):

— 4 % (v/v) of methanol (5.4);

— 10 % (v/v) of pH 8 buffer (5.15);

— 0,2 % (v/v) of the tylosin solution at 4 µg/ml (5.21.10).

Adjust the pH to 7,6 ± 0,1.

NOTE The addition of methanol allows a better diffusion of spiramycin in the agar. The addition of tylosin allows a better identification and exacerbate detection in bioautography (9.6).

5.4 Methanol.

5.5 Mixture of methanol (5.4) and water 1/1 (v/v).

5.6 Chloroform.

5.7 Ethyl acetate.

5.8 Dichloromethane.

5.9 Acetone.

5.10 Glycerol.

5.11 2, 3, 5 triphenyltetrazolium chloride (TTC).

5.12 Silicon anti-foaming agent.

Silicon anti-foaming agent type SE2 ® or equivalent.

5.13 Mixture of methanol and phosphate buffer solution pH 8,0 for the stock solution of spiramycin.

Phosphate buffer solution:

Dipotassium hydrogen phosphate K_2HPO_4:	16,7 g
Potassium dihydrogen phosphate KH_2PO_4:	0,5 g
Sodium hydrogen carbonate $NaHCO_3$:	20,0 g
Water to:	1 000 ml
pH:	8,0

Mix one volume of methanol with one volume of phosphate buffer solution.

5.14 Phosphate buffer solution pH 7,0 for the stock solution of tylosine.

Potassium dihydrogen phosphate KH_2PO_4:	5,5 g
Dipotassium hydrogen phosphate K_2HPO_4:	13,6 g
Water to:	1 000 ml
pH:	7,0

5.15 Phosphate buffer solution pH 8,0 for supplementing the antibiotic medium 11.

Dipotassium hydrogen phosphate K_2HPO_4:	1,41 g
Disodium hydrogen phosphate Na_2HPO_4, 2 H_2O:	57,5 g
Water to:	1 000 ml

Sterilize for 15 min at 121 °C.

pH:	8,0

5.16 Eluent 1 for spiramycin and tylosin detection.

Methanol (5.4).

5.17 Eluent 2 for spiramycin and tylosin detection.

Dichloromethane (5.8)/methanol (5.4)/acetone (5.9)/glycerol (5.10): 49/30/20/1 (v/v/v/v).

Prepare freshly.

5.18 Eluent 3 for virginiamycin detection.

Ethyl acetate saturated with water. In a separating funnel, shake ethyl acetate (5.7) with an excess of water. Discard the lower phase. Prepare freshly.

5.19 Contrast solution.

Dissolve 0,1 g of TTC = 2, 3, 5 triphenyltetrazolium chloride (5.11) in 100 ml of water.

5.20 Blank feed sample (Commercial feed or internal manufactured feed), free of antibiotic.

5.21 Reference standard substances and standard stock solutions.

5.21.1 Standard substance of spiramycin.

Spiramycin of known microbiological activity (in µg/mg or equivalent).

To convert UI/mg to µg/mg: 1 mg of spiramycin is considered to be equivalent to 3200 International Unit.

5.21.2 Standard substance of tylosin.

Tylosin of known microbiological activity (in µg/mg or equivalent).

5.21.3 Standard substance of virginiamycin.

Virginiamycin of known microbiological activity (in µg/mg or equivalent).

5.21.4 Stock solution of spiramycin (1 000 µg/ml).

Dissolve an accurately weighed quantity of the standard substance (5.21.1) in the mixture (5.13) and dilute with the same mixture to give a solution of 1 000 µg/ml of microbiological activity (equivalent to 3 200 IU/ml).

5.21.5 Stock solution of tylosin (500 µg/ml).

Dry the standard substance (5.21.2) for 3 h at 60 °C in a vacuum oven.