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Djurfoder: Metoder för provtagning och analys – Screening av förekomst av tylosin, virginiamycin, spiramycin, zink-bacitracin och avoparcin i foder vid halter under tillsatsnivå genom mikrobiologiskt platt-test

Animal feeding stuffs: Methods of sampling and analysis – Screening on the antibiotics tylosin, virginiamycin, spiramycin, bacitracin-zinc and avoparcin at sub-additive levels in compound feed by a microbiological plate test

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Denna standard är framtagen av kommittén för Livsmedel och foder, SIS/TK 435.

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EUROPEAN STANDARD
NORME EUROPÉENNE
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EN 16936

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English Version

Animal feeding stuffs: Methods of sampling and analysis - Screening on the antibiotics tylosin, virginiamycin, spiramycin, bacitracin-zinc and avoparcin at sub-additive levels in compound feed by a microbiological plate test

Aliments pour animaux : Méthodes d'échantillonnage et d'analyse - Dépistage des antibiotiques tylosine, virginiamycine, spiramycine, bacitracine-zinc et avoparcine à des niveaux sous-additifs dans les aliments composés par essai sur plaque microbiologique

Futtermittel - Probenahme- und Untersuchungsverfahren - Screening auf die Antibiotika Tylosin, Virginiamycin, Spiramycin, Bacitracin-Zink und Avoparcin in Konzentrationen unterhalb von Zusatzstoffen in Mischfuttermitteln mittels mikrobiologischem Plattentest

This European Standard was approved by CEN on 6 February 2017.

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European foreword

This document (EN 16936:2017) 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of an identical text or by endorsement, at the latest by November 2017, and conflicting national standards shall be withdrawn at the latest by November 2017.

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1 Scope

This European Standard presents a method describing the screening on the antibiotics tylosin, virginiamycin, spiramycin, bacitracin-zinc and avoparcin at sub-additive levels in complete feeding stuffs and milk replacers by a microbiological 3-plate test.

The limit of detection of the method is 1 mg/kg for avoparcin, tylosin, spiramycin and virginiamycin, and 5 mg/kg for zinc bacitracin. The presence of other (veterinary) antibiotics may interfere with the method.

Furthermore, high concentrations of metals (Cu, Zn) may interfere. The method should be used as a qualitative screening method. Positive results can be analysed further by TLC; for confirmatory purposes LC-MS is required [1].

A lower limit of detection for zinc bacitracin (3 mg/kg) is achievable (see Table 2), but should be established with an in house validation first.

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 13969, *Milk and milk products - Guidelines for a standardized description of microbial inhibitor tests (ISO 13969)*

3 Principle

The feed sample is extracted with a mixture of acetone, hydrochloric acid (HCl) and water. Neutralized extract is dispensed into wells in three different test plates. Each of these test plates holds a different composition with respect to culture medium, indicator bacterium and/or pH. After a 16-18 h incubation period, the presence of antibiotic residues is indicated by the appearance of a zone of growth inhibition around the sample. Comparison of the inhibition pattern with a reference set (Table 1) may yield a presumptive identification of the antibiotic.

4 Reagents and materials

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.

4.1 Test organisms:

Kocuria rhizophila ATCC 9341 (formerly: *Micrococcus luteus*)

Micrococcus luteus ATCC 10240

Bacillus megaterium ATCC 10778

See Annex A for the preparation of the bacterial suspensions.

4.2 Culture media:

In order to improve the reproducibility of the method, it is recommended to use dehydrated basic components or dehydrated complete media for the preparation of culture media. Follow the manufacturers' instructions.

4.2.1 Culture medium for Kocuria rhizophila ATCC 9341.

Meat peptone	6	g
Tryptone	4	g
Yeast extract	3	g
Meat extract	1,5	g
Glucose	1	g
Agar	15	g
Dipotassium hydrogen phosphate (K_2HPO_4)	20	g (to be added after sterilization)
Demineralized water	1000	ml

The basic dehydrated medium is commercially available as Antibiotic medium No. 1.

Dissolve the components in the water by heating. Autoclave the medium at 121 °C ± 1 °C for 15 min.

Adjust the pH to 8,0 ± 0,1.

4.2.2 Culture medium for Micrococcus luteus ATCC 10240.

Meat peptone	6	g
Tryptone	4	g
Yeast extract	3	g
Meat extract	1,5	g
Glucose	1	g
Agar	15	g
Demineralized water	1000	ml

The basic dehydrated medium is commercially available as Antibiotic medium No. 1.

Dissolve the components in the water by heating. Autoclave the medium at 121 °C ± 1 °C for 15 min. Adjust the pH to 6,5 ± 0,1.

4.2.3 Culture medium for Bacillus megaterium ATCC 10778.

Pancreatic digest of casein	5	g
Yeast extract	2,5	g
Glucose	1	g
Agar	15	g
Demineralized water	1000	ml

The basic dehydrated medium is commercially available as Plate Count Agar.

Dissolve the components in the water by heating. Autoclave the medium at 121 °C ± 1 °C for 15 min. Adjust the pH to 6,0 ± 0,1.

4.3 Water, demineralized or deionized or at least equivalent.

4.4 Sodium chloride (NaCl).

4.5 Acetone, analytical grade.

4.6 Hydrochloric acid (HCl).

4.7 Dipotassium hydrogen phosphate (K_2HPO_4).

4.8 Potassium dihydrogen phosphate (KH_2PO_4).

4.9 Sodium hydroxide (NaOH).

4.10 Methanol, analytical grade.

4.11 Extraction solvent: acetone/ hydrochloric acid/water mixture (475/25/500 v/v/v):

Transfer 500 ml of water (4.3) into a 1000 ml volumetric flask, ad 25 ml of hydrochloric acid (4.6) and mix. Make up to 1000 ml with acetone (4.5).

4.12 Phosphate buffer pH 6,5:

Dissolve 18,6 g potassium dihydrogen phosphate (4.8) and 14,8 g dipotassium hydrogen phosphate (4.7) in water (4.3). Adjust the pH to 6,5 ± 0,1 and fill up to 1000 ml with water (4.3).

4.13 Hydrochloric acid solution: 0,1 M:

Transfer 500 ml of water (4.3) into a 1000 ml volumetric flask. Slowly add 8,212 ml hydrochloric acid (4.6). Make up to a final volume of 1000 ml with water (4.3).

4.14 Standard solvent:

Transfer 400 ml of 0,1 M hydrochloric acid (4.13) into a 1000 ml volumetric flask, ad 600 ml of acetone (4.5) and mix.

4.15 Standard solutions and control samples:

Correct all weighing for purity and salt contents in accordance with EN ISO 13969.

4.15.1 Neomycin standard stock solution.

Dissolve 50,0 mg ± 0,1 mg of neomycin in 100 ml water (4.3) and mix. The thus-prepared neomycin standard stock solution contains 500 µg/ml of neomycin. The neomycin standard stock solution may be kept for one month if stored at 0 °C to + 5 °C.

4.15.2 Tylosin solutions and control samples:

4.15.2.1 Tylosin standard stock solution:

Dissolve 50,0 mg ± 0,1 mg of tylosin in 100 ml water (4.3) and mix. The thus-prepared tylosin standard stock solution contains 500 µg/ml of tylosin. The tylosin standard stock solution may be kept for one month if stored at 0 °C to + 5 °C.

4.15.2.2 Tylosin working solution:

Dilute 2 ml of the tylosin standard stock solution (4.15.2.1) with water (4.3) to 100 ml and mix. The thus-prepared tylosin working solution contains 1 µg/ml of tylosin.

4.15.2.3 Tylosin control sample:

Weigh 10,0 g of feed sample and spike with 0,2 ml of the tylosin standard stock solution (4.15.2.1). Mix and leave at room temperature for 30 min, before starting the analysis. This positive control sample contains 1 mg/kg of tylosin.

4.15.3 Avoparcin standard stock solution and control samples:

4.15.3.1 Avoparcin standard stock solution:

Dissolve 5,0 mg ± 0,1 mg of avoparcin in standard solvent (4.14), and fill up to 100 ml. The thus-prepared avoparcin standard stock solution contains 50 µg/ml of avoparcin. The avoparcin standard stock solution may be kept for one month if stored at 0 °C to + 5 °C.

4.15.3.2 Avoparcin control sample:

Weigh 10,0 g of feed sample and spike with 0,2 ml of the avoparcin standard stock solution (4.15.3.1). Mix and leave at room temperature for 30 min, before starting the analysis. This positive control sample contains 1 mg/kg of avoparcin.

4.15.4 Spiramycin standard stock solution and control samples:

4.15.4.1 Spiramycin standard stock solution:

Dissolve 5,0 mg ± 0,1 mg of spiramycin in 5 ml of methanol (4.10). Dilute to 100 ml with water (4.3) and mix. The thus-prepared spiramycin standard stock solution contains 50 µg/ml of spiramycin. The spiramycin standard stock solution may be kept for one month if stored at 0 °C to + 5 °C.

4.15.4.2 Spiramycin control sample:

Weigh 10,0 g of feed sample and spike with 0,2 ml of the spiramycin standard stock solution (4.15.4.1). Mix and leave at room temperature for 30 min, before starting the analysis. This positive control sample contains 1 mg/kg of spiramycin.

4.15.5 Virginamycin standard stock solution and control samples:

4.15.5.1 Virginamycin standard stock solution:

Dissolve 5,0 mg ± 0,1 mg of virginiamycin in 5 ml of methanol (4.10). Dilute to 100 ml with water (4.3) and mix. The thus-prepared virginiamycin standard stock solution contains 50 µg/ml of virginiamycin. The virginiamycin standard stock solution may be kept for one month if stored at 0 °C to + 5 °C.

4.15.5.2 Virginiamycin control sample:

Weigh 10,0 g of feed sample and spike with 0,2 ml of the virginiamycin standard stock solution (4.15.5.1). Mix and leave at room temperature for 30 min, before starting the analysis. This positive control sample contains 1 mg/kg of virginiamycin.

4.15.6 Zinc bacitracin standard stock solution and control samples:

4.15.6.1 Zinc bacitracin standard stock solution:

Dissolve 5,0 mg ± 0,1 mg of zinc bacitracin in 5 ml of 0,1 M hydrochloric acid (4.13). Add 5 ml of phosphate buffer pH 6,5 (4.12), mix and dilute to 50 ml with water (4.3). The thus-prepared zinc bacitracin standard stock solution contains 100 µg/ml of zinc bacitracin. The zinc bacitracin standard stock solution may be kept for one month if stored at 0 °C to + 5 °C.