SLOVENSKI STANDARD
kSIST-TS FprCEN/TS 17781:2021

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Organska, organsko-mineralna in anorganska gnojila - Ugotavljanje prisotnosti Escherichia coli

Organic, organo-mineral and inorganic fertilizers - Detection of Escherichia coli

Organische, organisch-mineralische und mineralische Düngemittel - Nachweis von Escherichia coli

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Engrais organiques, organo-minéraux et inorganiques - Recherche des Escherichia coli

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Ta slovenski standard je istoveten z: FprCEN/TS 17781

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65.080 Gnojila Fertilizers

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Organic, organo-mineral and inorganic fertilizers - Detection of *Escherichia coli*

Engrais organiques, organo-minéraux et inorganiques – Recherche des *Escherichia coli*

Organische, organisch-mineralische und anorganische Düngemittel - Nachweis von *Escherichia coli*

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European foreword

This document (FprCEN/TS 17781:2021) has been prepared by Technical Committee CEN/TC 260 “Fertilizers and liming materials”, the secretariat of which is held by DIN.

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Introduction

This document describes a method for the detection and enumeration of *Escherichia coli* in fertilizers of the following Product Function Categories (PFCs) of EU fertilizing products, as described in the Regulation (EU) 2019/1009 [1]:

— PFC 1(A): Organic fertilizer;

— PFC 1(B): Organo-mineral fertilizer;

— PFC 1(C): Inorganic fertilizer, which contains more than 1 % by mass of organic carbon, other than organic carbon from chelating or complexing agents, nitrification inhibitors, denitrification inhibitors or urease inhibitors, coating agents, urea or calcium cyanamide. The present method was validated on products known as present on the market in April 2021 and conform to Regulation (EU) 2019/1009 [1] that are inorganic fertilizers with more than 1 % of organic carbon such as struvite with low level of organic matter. In case that other products would be developed having other physical and chemical characteristics, it might become necessary to develop different methods to correctly account for pathogenic microorganisms they might contain.

This methodology has been developed to detect and enumerate *Escherichia coli* in organic, organo-mineral and inorganic fertilizers in order to be able to control certain hygienic requirements in the Regulation (EU) 2019/1009 [1].

Escherichia coli is a Gram negative bacterium with a faecal origin. Consequently, it can be used as an indicator of faecal contamination. It can also be used to monitor the effectiveness of pasteurization or disinfection treatments but it is comparatively sensitive (to heat, high pH) and therefore cannot reflect the behaviour of all pathogens in fertilizers.

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Because of the large variety of fertilizers, this method may not be appropriate in every detail for certain products. In this case, different methods which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this method as far as possible.

Mineral components in fertilizers can have a negative impact on the survivability of microorganisms when they go into solution. In addition to an unfavourable shift in the pH value, the products can have a strong osmotic effect or be toxic to cells themselves (e.g. copper). Therefore, it may be necessary to test the inhibitory effect of the fertilizers to be investigated in a pre-test.

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1 Scope

This document is applicable to fertilizing products, which are classified as PFC 1(A) and PFC 1(B) or the PFC 1(A) and PFC 1(B) component in PFC 7 of Regulation (EU) 2019/1009 [1]. However, the present method was not validated for blends.

This document specifies a colony-count technique at 44 °C on a solid medium containing a chromogenic ingredient for the detection of the enzyme ß-glucuronidase. The method is based on ISO 16649-2 [4].

Strains of *Escherichia coli* which do not grow at 44 °C and, in particular, those that are ß-glucuronidase negative, such as *Escherichia coli* O157, will not be detected. Detected microorganisms are presumptively determined β-glucuronidase-positive *Escherichia coli*, since some Enterobacteriaceae, in particular *Shigella* and *Salmonella*, can also show β-glucuronidase activity at 44 °C.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

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— IEC Electropedia: available at https://www.electropedia.org/

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— ISO Online browsing platform: available at https://www.iso.org/obp

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3.1
laboratory sample
sample intended for laboratory inspection or testing

3.2
test sample
sample prepared from the laboratory sample (3.1) and from which test portions (3.3) will be taken

3.3
test portion
quantity of material taken from the test sample (or if both are the same, from the laboratory sample) and on which the test is carried out

3.4
glucuronidase-positive presumptive *Escherichia coli*
bacteria which at 44 °C form typical blue colony on tryptone-bile-glucuronide medium (TBX) under the conditions specified in this method

[SOURCE: ISO 16649-2:2001, 3.1, modified – The reference to ISO 16649-2 has been replaced with a reference to this document]

3.5
confirmed ß-glucuronidase-positive Escherichia coli
ß-glucuronidase-positive presumptive *Escherichia coli* showing a positive indole reaction in tryptophan broth under the conditions specified in this method

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3.6
enumeration of ß-glucuronidase-positive *Escherichia coli*
determination of the number of colony-forming units (CFU) of ß-glucuronidase-positive *Escherichia coli*, per milliliter or per gram of sample, when test, confirmation and calculations are carried out in accordance with this method

[SOURCE: ISO 16649-2:2001, 3.2, modified – The reference to ISO 16649-2 has been replaced with a reference to this document]

3.7
initial suspension
primary dilution obtained after a weighed or measured quantity of the product under examination (or of a test sample prepared from the product) has been mixed with, normally, a nine-fold quantity of diluent

Note 1 to entry: A closer ratio between the diluent and the quantity of product is often not recommended because of possible inhibiting influences of the matrix.

3.8
further dilution
suspension or solution obtained by mixing a measured volume of the initial suspension (3.7) with an x-fold volume of diluent and by repeating this operation with further dilutions until a dilution series, suitable for the inoculation of culture media, is obtained.

Note 1 to entry: Ten-fold dilutions are normally used to produce a decimal dilution series, but other ratios may be required for specific purposes.

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[SOURCE: EN ISO 6887-1:2017, 3.7]

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4 Principle

a) Preparation of sterile liquid tryptone-bile-glucuronide medium (TBX) tempered at 44 °C to 47 °C;

b) Drawing a representative test sample under aseptic conditions;

c) Preparation of the initial suspension with a tempered diluent to obtain a homogeneous distribution of bacterial cells from the test portion;

d) Preparation of further dilutions of the initial suspension in order to reduce the number of microorganisms per unit volume or to reduce the cell inhibitory properties of the initial suspension. To allow, after incubation, the counting of colonies;

e) Inoculation of blank plates with an aliquot of the optimum dilutions and pouring of the molten agar medium into each plate, mixing and solidification;

f) Incubation of inverted plates at 44 °C ± 1 °C for 18 h to 24 h;

g) Counting of typical blue colonies, considering the specific properties of *Escherichia coli*

h) Biochemical verification of isolates during detection of indole if necessary;

i) Calculation of the number of colony-forming units (CFU) of ß-glucuronidase-positive *Escherichia coli* per gram or per milliliter of sample.

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5 Diluents, culture media and reagents

5.1 General

For standard laboratory practice, EN ISO 7218 and EN ISO 11133 can be used.

Composition of culture media and reagents and their preparation are specified in Annex B (normative).

For uniformity of results, in the preparation of media, either use a dehydrated complete medium or use constituents of uniform quality and chemicals of recognized analytical grade.

5.2 Diluents

5.2.1 General

Fertilizers with a high mineral content can significantly change the pH value of the initial suspension, which can negatively affect the viability of the microorganisms under investigation. In general (organic fertilizers), a basic phosphate buffer is sufficient to prepare the initial suspension. When testing organo-mineral or inorganic fertilizers, the pH value of the substrate in solution should be determined in a preliminary test. The general use of a double-buffered phosphate buffer is recommended. If the substrate is simultaneously tested for the presence of *Salmonella* (FprCEN/TS 17780), the initial suspension for enrichment can be used with buffered or double-buffered peptone water (with 10 g peptone). In this case, rapid processing of the initial suspension is necessary.

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5.2.2 basic phosphate buffer

See B.1.

5.2.3 double-buffered phosphate buffer

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See B.2.

5.3 Culture media

5.3.1 Tryptone-bile-glucuronic medium (TBX)

See B.3.

5.3.2 MacConkey Agar No. 3 (optional)

See B.4.

5.3.3 Medium and Reagent for indole reaction (optional)

5.3.3.1 Tryptone/tryptophan medium

See B.5.

5.3.3.2 Kovacs reagent

See B.6.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment (EN ISO 7218 can be used) and, in particular, the following.

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6.1 Equipment for dry sterilization (oven) and wet sterilization (autoclave).

EN ISO 7218 can be used.

6.2 Incubator.

Capable of maintaining a temperature of 44 °C ± 1 °C. Optionally also capable of maintaining a temperature of 37 °C ± 1 °C and/or 35 °C ± 1 °C and/or 44 °C to 47 °C.

6.3 Blending equipment.

The following apparatus may be used:

— a peristaltic homogenizer with sterile bags (paddle homogenizer), possibly with the option to adjust blending speed and time; or

— a laboratory shaker with sterile bags.

6.4 Mechanical stirrer.

A mechanical stirrer e.g. Vortex Mixer facilitates the homogenous mixing of decimal dilutions.

6.5 Balance.

Balances of the required range and accuracy for the different products to be weighed.

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6.6 Water bath.

Capable of maintaining temperatures of 44 °C to 47 °C

6.7 Cooling unit, adjustable at 5 °C ± 3°C.

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6.8 pH-meter, capable of reading to the nearest 0,1 pH unit at 20 °C to 25 °C.

6.9 Sterile loops of approximate diameter, 3 mm (10 µl volume).

6.10 Sterile tubes, bottles, or flasks with caps of appropriate capacity.

6.11 Pipettes or pipettor and sterile tips of nominal capacities of 10 ml and 1 ml.

6.12 Sterile Petri dishes with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm).

7 Sampling

Sampling should be performed carefully, following the principles described in EN 1482 (all parts) with appropriate adaptations, required to account for specificities of organic and organo-mineral fertilizers and to microbiological quality of the samples.

It is important that the laboratory receives a laboratory sample which is representative and has not been contaminated, damaged or changed during transport or storage.

Liquid, semi-liquid and moist fertilizers shall be stored at 5 °C ± 3 °C after receipt of the sample until testing. They shall not be frozen under any circumstances.

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8 Preparation of test sample

The sample is prepared in accordance to the European or International Standard that is specific to the concerned matrix (EN ISO 6887 (all parts) and EN ISO 18593 may both be used). If there is no specific European or International Standard, it is advised that the different parties agree on this subject.

9 Procedure (see Figure A.1 in Annex A (normative))

9.1 Preparation of the initial suspension and decimal dilutions

Weigh 10 g (wet mass) of the material into a sterile plastic bag and add 90 ml pre-warmed phosphate buffer (5.2.2 or 5.2.3).

If the initial suspension is also used for *Salmonella* analysis, the amount of sample has to be adapted, for example weigh 26 g into a sterile plastic bag and add 234 ml buffered peptone water or double-buffered peptone water.

The amount of sample shall adequately represent the material to be tested. In case of examination of very coarse inhomogeneous material, the sample quantity should be increased.

It is recommended to check the pH of the initial suspension and to correct it to a pH between 6 and 8.

Paddle the sample in a paddle blender (6.3) for 1 min on high speed. If the sample material contains hard, sharp parts that can damage the bag, first mix the sample manually and then shake the bag on a laboratory shaker for 10 min.

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Prepare a first dilution from the treated initial suspensions. Use a 1 ml pipette (6.11). Transfer 1 ml initial suspension into a tube containing 9 ml of sterile phosphate buffer solution (5.2.2 or 5.2.3) brought to room temperature and mix with a mechanical stirrer (6.4).

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Prepare further decimal dilutions. Transfer 1 ml of the first dilution into a tube containing 9 ml of sterile phosphate buffer (5.2.2 or 5.2.3) and mix with a mechanical stirrer. Repeat this procedure until the appropriate estimate for the number of cells is obtained.

9.2 Inoculation and incubation

9.2.1 General

The time elapsing between the start of the preparation of the initial suspension and the moment when the plates are inoculated shall not exceed 45 min.

To check sterility, prepare a control pour plate, respectively.

Cultivation of an aliquot of the initial suspension is only possible if no toxic or inhibiting substances are expected in the sample. For organo-mineral and inorganic fertilizers, it is recommended to use aliquots from the first dilution (10^{-2}) and higher. Liquid fertilizers can be used undiluted for cultivation if they do not contain any toxic or inhibiting substances.

9.2.2 Procedure

Using a sterile pipette or a micropipette (6.11), transfer to a sterile Petri dish (6.12) 1 ml of the test sample (if liquid), or 1 ml of the initial dilution (10^{-1}) in the case of other products.

Inoculate two plates per dilution.

Repeat the procedure with the further decimal dilutions, if necessary, using a new sterile pipette for each dilution.