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Organic, organo-mineral and inorganic fertilisers — Enumeration of *Escherichia coli*

Einführendes Element — Haupt-Element — Ergänzendes Element

Élément introductif — Élément central — Élément complémentaire

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European foreword

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

This is a working document.

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 Standardization Request given to CEN by the European Commission and the European Free Trade Association.

This document shall be used in combination with prEN 17803, *Detection of specific pathogens.*

Introduction

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

— PFC 1(A): Organic fertiliser;

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

— PFC 1(C): Inorganic fertiliser, 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.

This methodology has been developed to detect and enumerate *Escherichia coli* in organic, organo-mineral and inorganic fertilisers in order to control certain hygienic requirements imposed by Regulation (EU) 2019/1009 [1]. The method described in this document is based on ISO 16649-2 [4] for the enumeration of β-D-glucuronidase-positive *E. coli*.

The species *Escherichia coli* is a Gram-negative bacterium of faecal origin and consists of a large number of different strains. Consequently, it can be used as an indicator of faecal contamination and as a parameter to monitor the effectiveness of pasteurization or disinfection treatments during the manufacturing process of organic, organo-mineral and inorganic fertilisers. The presence or absence of *E. coli* does not reflect the presence or absence of other pathogens in the material tested.

Because of the large variety of fertilisers, this method may not be appropriate in every detail for certain products. 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 fertilisers can have a negative impact on the survivability of microorganisms. In addition to an unfavourable shift in the pH value, these components can have a strong osmotic effect or be toxic to the microorganisms (e.g. copper). Therefore, it may be necessary to investigate the inhibitory effects of these components in a pre-test.

1 Scope

This document specifies a method for the enumeration of *Escherichia coli* (*E. coli*) in organic fertilisers classified as PFC 1(A) in Regulation (EU) 2019/1009 [1], organo-mineral fertilisers classified as PFC 1(B), inorganic fertilisers classified as PFC 1 (C) which contain 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; and blends classified as PFC 7 in which the most abundant component by mass is PFC 1(A) or PFC 1(B).

This document is also applicable to the blends of fertilising products where a blend is a mix of at least two of the following component EU fertilising products: fertilisers, liming materials, soil improvers, growing media, inhibitors or plant biostimulants, and where organic, organo-mineral or inorganic fertilisers as indicated above are the highest % in the blend by mass or volume, or in the case of liquid form by dry mass. If organic, organo-mineral or inorganic fertilisers as indicated above are not the highest % in the blend, the European Standard for the highest % of the blend applies. In case a blend of fertilising products is composed of components in equal quantity, or in case the component EU fertilising products used for the blend have identical formulations, the user decides which standard to apply.

Strains of *E. coli* which do not grow at 44 °C ± 1 °C and, in particular, those that are β-D-glucuronidase negative, such as *E. coli* O157, will not be detected. Some genera within the family *Enterobacteriaceae*, in particular *Shigella* spp. and *Salmonella* spp., can also show β-D-glucuronidase activity at 44 °C ± 1 °C.

NOTE This method has been validated in an interlaboratory study with specific products that were present on the market during the study (Annex C).

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

prEN 17803, *Detection of specific pathogens*

EN 1482 (all parts), *Fertilisers and liming materials — Sampling and sample preparation*

prEN ISO/DIS 7218, *Microbiology of food chain — General requirements and guidance for microbiological examinations*

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:

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

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

3.1
Escherichia coli
bacteria which form typical blue colonies on tryptone-bile-X-glucuronide (TBX) agar (β-D-glucuronidase-positive) at 44 °C ± 1 °C under the conditions specified in this method

3.2
enumeration of *Escherichia coli*
determination of the number of colony-forming units (CFU) of β-D-glucuronidase-positive *Escherichia coli* per gram or per millilitre of product or per sample device, when the analysis and calculation is carried out in accordance with this document

4 Principle

From the initial suspension, aliquots of appropriate dilutions are used to inoculate the selective medium tryptone-bile-X-glucuronide (TBX) agar through pour plating, which are incubated for 21 h ± 3 h at 44 °C ± 1 °C. After incubation, β-D-glucuronidase-positive *E. coli* are enumerated and, if necessary, confirmed by a positive indole reaction.

5 Culture media and reagents

Current laboratory practices in accordance with standards comparable to prEN ISO/DIS 7218 shall be followed. The composition of culture media and reagents and their preparation are specified in Annex B. Use either dehydrated complete media or constituents of uniform quality and chemicals of recognized analytical grade for uniformity of results during the preparation of media. For performance testing of culture media, it is recommended to follow the procedures in accordance with standards comparable to EN ISO 11133:2014 [3] and Annex B.

6 Equipment and consumables

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

Usual microbiological laboratory equipment as described in prEN ISO/DIS 7218 shall be used and, in particular, the following.

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

6.2 Incubator(s), capable of maintaining a temperature of 36 °C ± 2 °C, 44 °C ± 1 °C and 44 °C to 47 °C.

6.3 Homogenising equipment, as described in prEN 17803.

6.4 Mechanical stirrer, such as a Vortex Mixer facilitates the homogenous mixing of decimal dilutions.

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

6.6 Water bath, capable of maintaining temperatures of 44 °C to 47 °C

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

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 1 ml and 10 ml.

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

7 Sampling

Sampling is not included in this document. Sampling should be performed carefully, following the principles described in EN 1482 (all parts) with the appropriate adaptations to account for the specificities of organic, organo-mineral and mineral fertilisers, and for the microbiological quality of the samples.

8 Preparation of test sample

The preparation of the test sample is not included in this document. The test sample shall be prepared following prEN 17803.

9 Procedure

9.1 General

The procedure as given in Annex A shall be followed.

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 control plates.

9.2 Preparation of the initial suspension and decimal dilutions

Preparation of the initial suspension shall be performed according to prEN 17803.

To prepare a first decimal dilution, transfer 1 ml of the initial suspension using a sterile pipette (6.11) into a tube (6.10) containing 9 ml of sterile phosphate buffer solution (B.2 or B.3) brought to room temperature, and mix with a mechanical stirrer (6.4).

To prepare further decimal dilutions, using a sterile pipette (6.11) transfer 1 ml of the first dilution into a tube containing 9 ml of sterile phosphate buffer (B.2 or B.3) and mix with a mechanical stirrer (6.4).

Repeat this procedure until the dilution corresponds to an appropriate number of cells for enumeration.

9.3 Inoculation of TBX agar plates

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

For enumeration, one plate per dilution shall be used with at least two successive dilutions. To improve the reliability of the results, two plates per dilution are recommended.

If only one dilution is used, then two plates of this dilution shall be used to improve reliability of the results.

For laboratories that do not operate under quality assurance principles, two plates per dilution shall be used to improve reliability of the results.

NOTE Where greater dilutions are made and low counts are expected, the number of inoculated plates for enumeration tests should be increased to ensure that an inoculation volume corresponding to at least 0,1 g of the

sample is distributed on the plates. If the colony counts are expected to be close to the given limit value (for example the limit value specified in the Fertilising Products Regulation [1]), it is recommended to increase the accuracy of the method by adding 10 ml distributed into two pour-plated Petri dishes with a diameter of 140 mm, or at least three pour-plated Petri dishes with a diameter of 90 mm.

Approximately 15 ml (for 90 mm Petri dishes) or 45 ml (for 140 mm Petri dishes) of TBX agar (5.3.1), cooled down to 44 °C to 47 °C in a water bath (6.6) or an incubator (6.2), shall be poured into each Petri dish containing 1 ml of the test sample or an appropriate dilution.

The inoculum shall be carefully mixed with the medium and the mixture shall be allowed to solidify, with the Petri dishes standing on a cool horizontal surface.

The time which elapses between the inoculation of the empty Petri dish and pouring of the medium shall not exceed 15 min.

The inoculated dishes shall be incubated upside down in an incubator (6.2) set at 44 °C ± 1 °C for 21 h ± 3 h.

9.4 Enumeration of colonies

After incubation, all plates showing less than 150 typical colonies and less than 300 total (typical and atypical) colonies shall be used for the enumeration. Atypical colonies shall not be considered for the enumeration.

When embedded in TBX agar, typical *E. coli* colonies have a diameter greater than 1 mm and appear as dark to light blue as a result of the β-D-glucuronidase reaction.

On the surface of the TBX agar, typical colonies appear blue in the centre with a white edge.

9.5 Confirmation (optional)

9.5.1 General

Confirmation is required in case of any doubt on whether *E. coli* colonies are present on the primary agar medium (TBX) [5, 6].

Confirmation is recommended if the calculated result will be above the limit value specified in Regulation (EU) 2019/1009 [1].

NOTE Any other method capable of unambiguously identifying *E. coli* than the one described in this document [e.g., commercially available biochemical test kits, serological tests, polymerase chain reaction (PCR) analyses or Matrix-Assisted Laser Desorption Ionization - Time-of-Flight Mass Spectrometry (MALDI-TOF)] may be used for confirmation if giving the same results as the method specified in this document.

If typical β-D-glucuronidase-positive *E. coli* colonies on TBX agar have been confirmed, that shall be stated in the results.

9.5.2 Subculturing

If it is not possible to isolate a single colony due to high background flora, a pure culture shall be established by subculturing. With a sterile loop (6.9) streak plates from up to 5 typical colonies from each countable plate (9.4) shall be prepared on poured TBX agar (B.2) or MacConkey agar No. 3 (B.3), yielding single colonies after incubation.

NOTE Any other medium giving a clear distinction between *E. coli* and other bacteria is applicable.

TBX agar (B.2) shall be incubated at 44 °C ± 1 °C for 21 h ± 3 h, or MacConkey agar No. 3 (B.3) at 36 °C ± 2 °C (6.2) for 21 h ± 3 h. On TBX agar, typical colonies show a blue centre with a white edge. On MacConkey agar No. 3, non-lactose fermenters form colourless colonies, coliform bacteria appear as

intense violet-red colonies and colonies typical for *E. coli* additionally show bile precipitation. Typical colonies may be subjected to a subsequent indole reaction.

9.5.3 Indole reaction

The indole reaction, indole test or tryptophan test can be used to differentiate *Salmonella* and *Enterobacter* (indole-negative) from *E. coli* (indole-positive) [5, 6].

At least 5 typical single colonies shall be picked from each countable plate (9.4), or pure typical single colonies shall be picked from each isolation (9.5.2) with a sterile loop (6.9). For each suspected colony, 3 ml to 5 ml of tryptone/tryptophan medium (B.4) shall be inoculated. If less than 5 typical colonies are present, all colonies shall be tested.

The inoculated tryptone/tryptophan medium shall be incubated at 44 °C ± 1 °C (6.2) for 21 h ± 3 h. After incubation, 1 ml of the Kovacs reagent (B.5) shall be added.

The formation of a red ring indicates a positive reaction. A yellow-brown ring indicates a negative reaction.

NOTE Commercially available kits for the detection of the indole reaction can be used if they show equivalent results to the method specified in this document.

10 Expression of results

The number of colony forming units (CFU) of *E. coli* per gram or per ml of test portion (N) shall be calculated using Formula 1. In special cases (e.g. no colonies detected, more than 150 typical colonies, etc.) results shall be expressed according to the instructions stated in prEN ISO/DIS 7218:2022, Clause 11.2.6.4.

$$N = \frac{\sum C}{V\left(n_1 + 0{,}1 n_2\right) d} \tag{1}$$

where

ΣC is the number of colonies counted on all Petri dishes considered from one or two successive dilutions;

V is the volume of inoculum applied to each Petri dish, in ml;

n_1 is the number of dishes retained at the first countable dilution;

n_2 is the number of dishes retained at the second countable dilution (n_2 = 0 if only one dilution is considered);

d is the dilution factor corresponding to the more concentrated dilution retained (d = 1 for undiluted liquid product).

When confirmation is necessary, a given number A (generally 5 per plate) of β-D-glucuronidase-positive *E. coli* colonies shall be confirmed from each of the dishes retained for counting.

After confirmation, the number of colonies (a) complying with identification criteria shall be calculated for each of the dishes using Formula 2.

$$a = \frac{b}{A} \times C \tag{2}$$

where

A is the number of colonies selected for confirmation from each Petri dish;

b is the number of colonies confirmed from A;

C is the total number of colonies in the Petri dish.

And replace Σ*C* by Σ*a* in Formula (1) to account for the ratio of verified colonies.

NOTE 1 Σ*a* is the total number of colonies complying with identification criteria from all of the dishes selected.

NOTE 2 If Σ*a* is < 10, the final result is expressed as an estimated value.

11 Method validation

The method was validated in an interlaboratory study for the following products in Table 1 (*Escherichia coli* was investigated in both native and spiked test material):

Table 1 — Product groups and matrices for which the methods described in this document are applicable and tested in a validation trial

Product group	**Matrix**
Organic fertilisers	to be determined at an international ring trial
Organo-mineral fertilisers	to be determined at an international ring trial
Inorganic fertilisers	to be determined at an international ring trial

International ring trials will be conducted on the basis of this document.

12 Test report

The test report shall contain at least the following information:

— all information necessary for the complete identification of the sample;

— the sampling method used (EN 1482), with an indication of all deviations from the sampling method, if known;

— the size of the test portion and/or the nature of the object examined;

— the test method used, with reference to this document;

— any deviation in the media or the incubation conditions or confirmation used;

— any unusual features observed;

— all operating conditions not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the result(s);

— the results obtained, indicating clearly the method of expression used;

— if repeatability has been checked, the final result obtained; and

— the date(s) of start and end of the test.

Annex A
(normative)

Diagram of the procedure

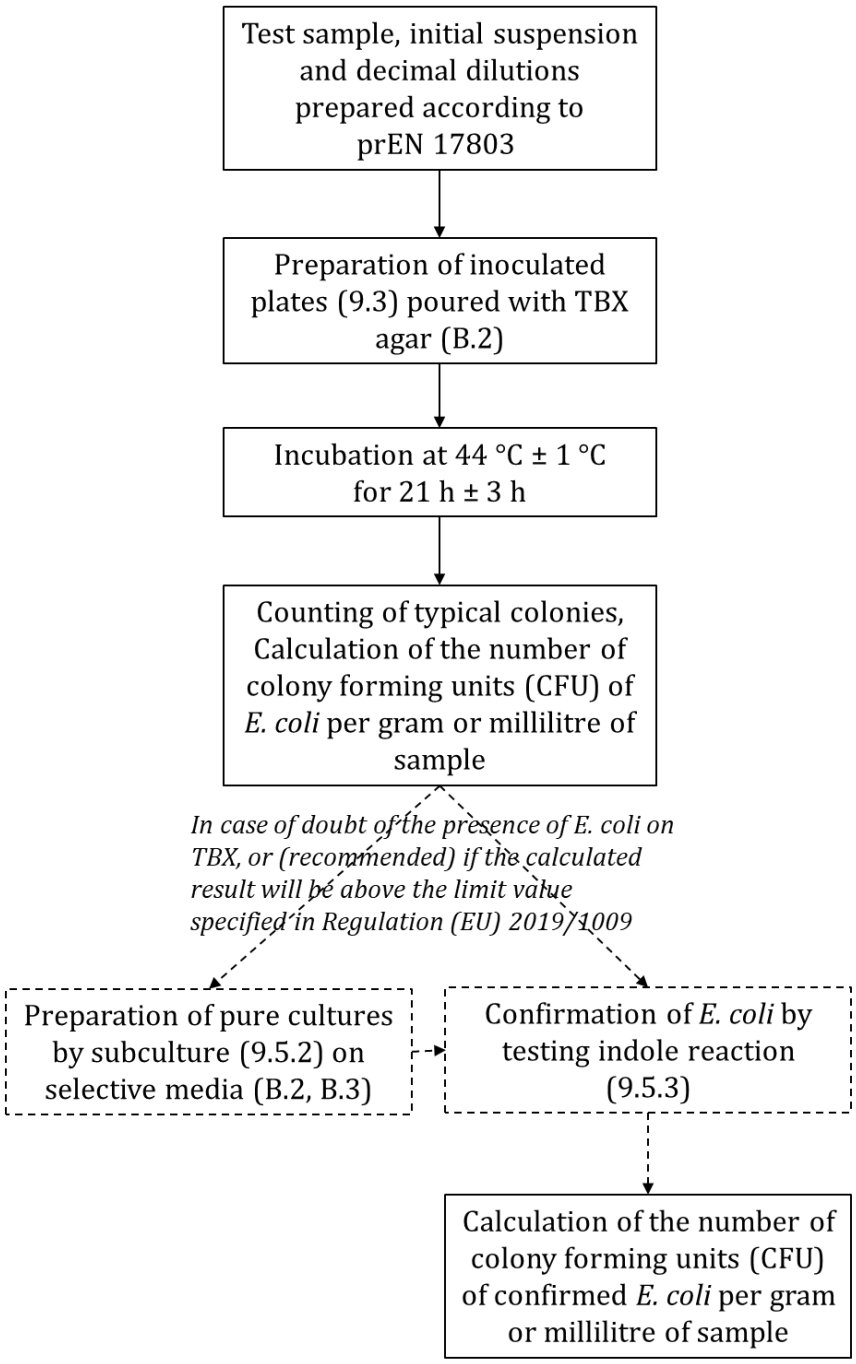

Figure A.1 — Flow diagram of the method for the enumeration of *Escherichia coli* in organic, organo-mineral and inorganic fertilisers

Annex B
(normative)

Composition and preparation of culture media and reagents

B.1 General

If media or reagents are prepared from dehydrated complete media/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf lives of the media indicated in this annex have been shown in some studies. Is is recommended that the users verify this under their own storage conditions according to EN ISO 11133 [3].

The preparation and performance of culture media is a fundamental step to ensure the integrity of microbiological examination. When ready-to-use media are used, the manufacturers of this available media should have a quality programme that ensure the quality of the media they supply, according to EN ISO 11133 [3]. Under this condition, the user/laboratory does not need to run additional testing on such media, but shall ensure the storage condition according to the ones recommended by the manufactures.

The performance for diluents and media prepared by the user/laboratory directly from commercially available dehydrated formulations and/or from basic individual components should be evaluated according to EN ISO 11133 [3].

B.2 Basic phosphate buffer

B.2.1 Composition

Peptone[a]	1,0 g
Sodium chloride (NaCl)	5,0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)‡	9,0 g
Potassium dihydrogen phosphate (KH_2PO_4)‡	1,5 g
Water	1 000 ml

[a] For example, enzymatic digest of casein.

‡ See B.3.

Commercially available, ready-to-use diluent is suitable.

B.2.2 Preparation

Dissolve the components in water in flasks, bottles or test tubes (6.10), by heating if necessary.

If necessary, adjust the pH (6.8) to 7,0 ± 0,2 at 25 °C after sterilization.

B.3 Double-buffered phosphate buffer

This buffer contains twice the amount of the two buffer components (marked ‡ in B.2.1) with respect to the basic phosphate buffer.

Commercially available, ready-to-use diluent is suitable.

B.4 Tryptone-bile-X-glucuronide (TBX) agar

B.4.1 Composition

Enzymatic digest of casein	20,0 g
Bile salts No. 3	1,5 g
5-Bromo-4-chloro-3-indolyl β-D-glucuronide acid (BCIG), Cyclo hexylammonium salt monohydrate (CAS N° 114162-64-0)	144 µmol[a]
Agar	9 g to 18 g[b]
Water	1 000 ml

[a] E.g. 0,075 g of cyclohexylammonium salt.

[b] Depending on the gel strength of the agar.

B.4.2 Preparation

Dissolve the BCIG in the dimethyl sulfoxide or in the diluent recommended by the manufacturer. Dissolve all components in the water and heat to boiling.

If necessary, adjust the pH (6.8) to 7,2 ± 0,2 at 25 °C after sterilization.

Sterilize the medium in the autoclave (6.1) set at 121°C ± 3°C for 15 min. Immediately cool the medium in the water bath (6.6) at 44 °C to 47 °C up to the use in pour plate method. For the production of plates for the confirmation reaction cool the medium to 44 °C to 47 °C in a water bath (6.6), mix and pour into sterile Petri dishes (6.12). Allow to solidify.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) until the surface of the agar is dry.

Store the poured plates, protected for drying, at 5 °C ± 3 °C (6.7) for up to 4 weeks.

B.5 MacConkey Agar No. 3 (optional)

B.5.1 General

A selective medium giving excellent differentiation between coliforms and non-lactose fermenters with inhibition of Gram-positive enterococci.

B.5.2 Composition

Peptone	20,0 g
Lactose	10,0 g
Bile salts	1,5 g
Sodium chloride (NaCl)	5,0 g
Neutral red	0,03 g
Crystal violet	0,001 g
Agar	9 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar

B.5.3 Preparation

Dissolve all components in the water and heat to boiling.

If necessary, adjust the pH (6.8) to 7,1 ± 0,2 after sterilization.

Sterilize the medium in the autoclave (6.1) set at 121°C ± 3°C for 15 min. Immediately cool the medium in the water bath (6.6) at 44 °C to 47 °C, mix and pour into sterile Petri dishes (6.12). Allow to solidify.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) until the surface of the agar is dry.

Store the poured plates, protected for drying, at 5 °C ± 3 °C (6.7) for up to 4 weeks.

B.6 Tryptone/tryptophan medium (optional)

B.6.1 Composition

Tryptone	10,0 g
Sodium chloride (NaCl)	5,0 g
DL-Tryptophan	1,0 g
Water	1 000 ml

B.6.2 Preparation

Dissolve the components in water and heat to boiling whilst stirring continuously to dissolve all ingredients completely.

If necessary, adjust the pH (6.8) to 7,5 ± 0,2 at 25 °C after sterilization.

Dispense 3m to 5 ml of the medium into each of several tubes (6.10).

Sterilize for 15 min in the autoclave (6.1) set at 121 °C ± 3 °C. Store the poured tubes at 5 °C ± 3 °C (6.7) for up to 3 months.

B.7 Kovacs reagent (optional)

B.7.1 Composition

4-Dimethylaminobenzaldehyde	5,0 g
Hydrochloric acid, mass concentration ρ = 1,18 g/ml to 1,19 g/ml	25 ml
2-Methylbutan-2-ol	75 ml

B.7.2 Preparation

Mix the components. Store the complete reagent, in closed flasks in the dark, at 5 °C ± 3 °C (6.7) for up to 6 months.

Annex C
(informative)

Method validation studies and performance characteristics

To be filled in after validation studies.

This method has been validated in an interlaboratory study for the following organic, organo-mineral and mineral fertilisers, as well as blends:

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Validations have been performed with specific materials that were present on the market during the validation study. Materials/formulation can change over time regarding their composition.

Bibliography

[1] Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003

[2] EN 1482 (all parts), *Fertilisers and liming materials — Sampling and sample preparation*

[3] EN ISO 11133:2014[1], *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media (ISO 11133)*

[4] ISO 16649-2:2001, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli — Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide*

[5] Kilian M. & Bulow P. Rapid diagnosis of Enterobacteriaceae. Detection of bacterial glycosidases. Acta Pathol Microbiol. Scand. Sect. B. 1976, 84 pp. 245–251

[6] Le Minor L., Buissière J., Novel G., Novel M. Relation entre le sérotype et l'activité β-glucuronidasique chez les Salmonella. Ann Microbiol (Paris). 1978, Aug–Sep; 129B (2) pp. 155-165

[1] As impacted by EN ISO 11133:2014/A1:2018 and EN ISO 11133:2014/A2:2020.