SLOVENSKI STANDARD
kSIST-TS FprCEN/TS 17780:2021

01-december-2021

Organska, organsko-mineralna in anorganska gnojila - Ugotavljanje prisotnosti vrst Salmonella (Salmonella spp.)

Organic, organo-mineral and inorganic fertilizers - Detection of Salmonella spp.

Organische, organisch-mineralische und mineralische Düngemittel - Nachweis von Salmonella spp

Engrais organiques, organo-minéraux et inorganiques - Recherche des Salmonella spp.

Ta slovenski standard je istoveten z: **FprCEN/TS 17780**

ICS:

65.080 Gnojila Fertilizers

kSIST-TS FprCEN/TS 17780:2021 **en,fr,de**

TECHNICAL SPECIFICATION

SPÉCIFICATION TECHNIQUE

TECHNISCHE SPEZIFIKATION

FINAL DRAFT FprCEN/TS 17780

October 2021

ICS 65.080

English Version

Organic, organo-mineral and inorganic fertilizers - Detection of Salmonella spp.

Engrais organiques, organo-minéraux et inorganiques - Recherche des Salmonella spp.

Organische, organisch-mineralische und mineralische Düngemittel - Nachweis von Salmonella spp

This draft Technical Specification is submitted to CEN members for Vote. It has been drawn up by the Technical Committee CEN/TC 260.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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Ref. No. FprCEN/TS 17780:2021 E

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European foreword

This document (FprCEN/TS 17780:2021) has been prepared by Technical Committee CEN/TC 260 “Fertilizers and liming materials”, the secretariat of which is held by DIN.

This document is currently submitted to the Vote on TS.

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

Introduction

This document describes a method for the detection of *Salmonella* spp. 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.

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 method for the detection of *Salmonella* spp. in organic, organo-mineral and inorganic fertilizers. The method is based on EN ISO 6579-1 and its validated alternative methods for the detection of *Salmonella* spp. in food and feeding stuff.

It requires three successive steps: A selective enrichment, an isolation on a chromogenic agar, and if positive a confirmation with a serological test (and if required, a selective media).

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:

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

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

3.1
***Salmonella* spp.**
microorganism which forms typical colonies on solid selective media described and which displays the morphological, physiological and biochemical characteristics described when the analysis is carried out in accordance with this document

3.2
detection of *Salmonella*
determination of the detection or not detection of *Salmonella* (3.1), in 25 g or 25 mL of product, when tests are carried out in accordance with this document

3.3
laboratory sample
sample intended for laboratory inspection or testing

3.4
test sample
sample prepared from the laboratory sample (3.3) and from which test portions (3.5) will be taken

3.5
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

4 Principle

4.1 General

The detection of *Salmonella* spp. requires three successive steps as specified in normative Annex A. The three steps are the selective enrichment, the isolation on a chromogenic agar, and the confirmation with a serological test (and if required, a selective media).

NOTE 1 *Salmonella* can be present in small numbers and are often accompanied by considerably larger numbers of other bacteria such as Enterobacteriaceae or of other families. Enrichment is used to allow the detection of low numbers of *Salmonella* or stressed *Salmonella*.

Stressed microorganisms are defined in this document as those present in the environment that may be injured or that may have developed in harsh environments. Such organisms may be difficult to detect because they struggle to grow on selective media. However, under suitable conditions, they may repair the cellular damages and recover their normal properties.

Alternative methods based on molecular biology can be used if giving the same results as those given in this document (see EN ISO 7218).

4.2 Enrichment in selective liquid medium

Buffered peptone water (BPW) containing 10 mg/l novobiocin at room temperature is inoculated with the test portion (refer to 9.1), then incubated between 34 °C and 38 °C for 18 h ± 2 h.

For large quantities (e.g. 1 l or more), it is recommended to pre-warm the BPW to 34 °C to 38 °C before mixing it with the test portion.

4.3 Plating out on selective solid media

From the enrichment obtained in 4.2, the chromogenic solid media (5.2) is inoculated.

This selective agar is incubated between 34 °C and 38 °C for 24 h ± 3 h (or according to the manufacturer's instructions if explicitly recommended).

4.4 Confirmation

Colonies of presumptive *Salmonella* are confirmed by means of appropriate serological test. If the serological test gives a negative result, the inoculation of a selective agar (Table C.2) is required. If the test gives a negative result, up to 4 other presumptive colonies will be tested (if possible and up to 5 colonies in total).

5 Culture media and reagents

5.1 General

For current laboratory practice, see EN ISO 7218 and EN ISO 11133.

Composition of culture media and reagents and their preparation are described in Annex B.

5.2 Isolation chromogenic agar

This isolation medium is chosen by the testing laboratory and shall highlight the C8-esterase enzymatic activity. For examples of isolation media, see Table C.1.

5.3 Non-selective agar

General purpose agar supporting the growth of a wide range of non-fastidious strains is chosen by the testing laboratory. For example, for non-selective agar, see B.4.

5.4 Confirmation selective agar

The confirmation medium is chosen by the testing laboratory and shall highlight the production of hydrogen sulphide (H_2S) by the strains (see B.5). For examples of isolation media, see Table C.2.

6 Equipment and consumables

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

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

6.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave), see EN ISO 7218.

6.2 Drying cabinet or oven, capable of operating between 25 °C and 50 °C.

6.3 Incubator(s), capable of operating in the range 34 °C to 38 °C or at 37 °C ± 1 °C.

6.4 Water bath, capable of operating at 47 °C to 50 °C, containing an antibacterial agent.

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

6.6 Freezer, capable of operating at -20 °C ± 5 °C.

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

6.8 pH-meter, with a reading to the nearest 0,1 pH unit from 20 °C to 25 °C.

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

6.10 Sterile filter, with a 0,2 µm porosity.

6.11 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 (3.3) which is representative and has not been damaged or changed during transport or storage.

8 Preparation of test sample

The sample is prepared in accordance to the European or International Standard that is specific to the concerned matrix [see EN ISO 6887 (all parts)]. 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)

9.1 Test portion and initial suspension

For preparation of the initial suspension, use as diluent the enrichment medium specified in Annex B, B.2 (buffered peptone water). Pre-warm the BPW supplemented with Novobiocin (nBPW) to room temperature before use.

A test portion (3.5) of 25 grams or 25 millilitres is weighed and nBPW (refer to 4.2) is added to yield a tenfold dilution (mass or volume) (see EN ISO 6887-1).

NOTE 1 If the samples are suspected to exhibit extreme pH values, double buffered peptone water (supplemented with novobiocin) can be used as diluent for the initial suspension.

NOTE 2 If the initial suspension is used for other microbiological tests, novobiocin can be added subsequently.

9.2 Selective pre-enrichment

Incubate the initial suspension (9.1.) between 34 °C and 38 °C (6.3.) for 18 h ± 2 h.

It is permissible to store the pre-enrichment culture at 5 °C (6.5.) for a maximum of 72 h after the incubation.

9.3 Isolation

From the selective enrichment (9.2.), inoculate the surface of the chromogenic isolation agar (5.2) by means of a 10 µl loop (6.7.) so that well-isolated colonies will be obtained.

Allow the plates to equilibrate at room temperature if they were stored at a lower temperature. If necessary, dry the surface of the plates before use (see EN ISO 11133).

Incubate the selective plating-out medium between 34 °C and 38 °C (6.3.) for 24 h ± 3 h (or according to the manufacturer's instructions if explicitly recommended).

Typical colonies exhibiting the C8-esterase activity are smooth (or rarely rough), round and magenta.

After incubation, check the chromogenic medium for the presence of colonies, which, from their characteristics, are considered to be suspect colonies.

9.4 Confirmation

9.4.1 General

The combination of enzymatic activity and serological test results indicate whether an isolate belongs to the genus *Salmonella* or not. For the characterization of *Salmonella* strains, full serotyping is needed. Guidance for serotyping is given in CEN ISO/TR 6579-3:2014.

For a clear distinction between positive and negative serological reactions, it is helpful to verify the reactions of the media of each biochemical test with well-characterized positive and negative control strains.

9.4.2 Selection of colonies for confirmation

Mark suspect colonies on each plate (9.3.). Select one suspect colony for confirmation. If this is negative, select up to four more suspect colonies (testing up to 5 colonies in total).

If well-isolated colonies are available on the selective plating media (9.3.), the serological and biological confirmation can be performed directly on a suspect colony. It is recommended to subculture this colony on a non-selective agar (5.3) to get a pure culture with enough material to work with.

9.4.3 Serological testing

9.4.3.1 General

The suspect colonies (9.4.2.) are tested for auto-agglutination. Strains that are auto-agglutinable cannot be tested for the presence of *Salmonella* antigens and will have to be confirmed by test for H_2S production (9.4.4). The suspect colonies are tested for the presence of *Salmonella* O- and H-antigens by slide agglutination using polyvalent antisera (B.7). Use the antisera according to the manufacturer's instructions if different from the method described below to detect the presence of *Salmonella* O- and H-antigens.

The following tests (9.4.3.2 to 9.4.3.5) are the minimum required for serological testing of *Salmonella* spp. Further guidance on serological confirmation and on serotyping is given in CEN ISO/TR 6579-3.

9.4.3.2 Elimination of auto-agglutinable strains

Place one drop of saline solution (B.6) on a clean glass slide. Using a loop, disperse part of the colony to be tested in the saline solution to obtain a homogeneous and turbid suspension.

Rock the slide gently for 5 s to 60 s (depending on the manufacturer's instructions). Observe the suspension, preferably against a dark background. If the bacteria have formed granules in the suspension, this indicates auto-agglutination and serological confirmation will become complicated. Additional information on the treatment of auto-agglutinating strains can be found in CEN ISO/TR 6579-3.

9.4.3.3 Examination for O-antigens

Using one non-auto-agglutinating pure colony, proceed according to 9.4.3.2 using one drop of polyvalent anti-O sera (B.7) in place of the saline solution.

If agglutination occurs, this is considered a positive reaction.

9.4.3.4 Examination for H-antigens

Using one non-auto-agglutinating pure colony, proceed according to 9.4.3.2 using one drop of polyvalent anti-H sera (B.7) in place of the saline solution.

If agglutination occurs, this is considered a positive reaction.

9.4.3.5 Interpretation of serological reactions

Table 1 gives the interpretation of the confirmatory tests (9.4) carried out on the colonies used (9.4.2).

Table 1 — Interpretation of confirmatory tests

C-8 esterase activity	Auto-agglutination	Serological reactions	Interpretation
Typical	No	O- and/or H-antigens positive	Detection
Typical	No	O- and H-antigens negative	Presumptive *Salmonella* test for H_2S production (9.4.4)
Typical	Yes	Not tested because of auto-agglutination (see 9.4.3.2)	Presumptive *Salmonella* test for H_2S production (9.4.4)
No typical reactions	—	—	Non detection