SLOVENSKI STANDARD
SIST-TS CEN/TS 17710:2023

01-februar-2023

Rastlinski biostimulanti - Ugotavljanje prisotnosti Listeria monocytogenes

Plant biostimulants - Detection of Listeria monocytogenes

Pflanzen-Biostimulanzien - Nachweis von Listeria monocytogenes

Biostimulants des végétaux - Détection de Listeria monocytogenes

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Ta slovenski standard je istoveten z: **CEN/TS 17710:2022**

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ICS:

65.080 Gnojila Fertilizers

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TECHNICAL SPECIFICATION

SPÉCIFICATION TECHNIQUE

TECHNISCHE SPEZIFIKATION

CEN/TS 17710

March 2022

ICS 65.080

English Version

Plant biostimulants - Detection of *Listeria monocytogenes*

Biostimulants des végétaux - Détection de *Listeria monocytogenes*

Pflanzen-Biostimulanzien - Nachweis von *Listeria monocytogenes*

This Technical Specification (CEN/TS) was approved by CEN on 3 January 2022 for provisional application.

The period of validity of this CEN/TS is limited initially to three years. After two years the members of CEN will be requested to submit their comments, particularly on the question whether the CEN/TS can be converted into a European Standard.

CEN members are required to announce the existence of this CEN/TS in the same way as for an EN and to make the CEN/TS available promptly at national level in an appropriate form. It is permissible to keep conflicting national standards in force (in parallel to the CEN/TS) until the final decision about the possible conversion of the CEN/TS into an EN is reached.

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European foreword

This document (CEN/TS 17710:2022) has been prepared by Technical Committee CEN/TC 455 “Plant Biostimulants”, the secretariat of which is held by AFNOR.

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.

Any feedback and questions on this document should be directed to the users’ national standards body. A complete listing of these bodies can be found on the CEN website.

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

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Introduction

This document was prepared by the experts of CEN/TC 455 “Plant Biostimulants”. The European Committee for Standardization (CEN) was requested by the European Commission (EC) to draft European standards or European standardization deliverables to support the implementation of Regulation (EU) 2019/1009 of 5 June 2019 laying down rules on the making available on the market of EU fertilizing products (“FPR” or “Fertilising Products Regulation”).

This standardization request, presented as M/564, also contributes to the Communication on “Innovating for Sustainable Growth: A Bio economy for Europe”. The Working Group 5 “Labelling and denominations”, was created to develop a work program as part of this request. The technical committee CEN/TC 455 “Plant Biostimulants” was established to carry out the work program that will prepare a series of standards. The interest in biostimulants has increased significantly in Europe as a valuable tool to use in agriculture. Standardization was identified as having an important role in order to promote the use of biostimulants. The work of CEN/TC 455 seeks to improve the reliability of the supply chain, thereby improving the confidence of farmers, industry, and consumers in biostimulants, and will promote and support commercialisation of the European biostimulant industry.

Biostimulants used in agriculture can be applied in multiple ways: on soil, on plant, as seed treatment, etc. A microbial plant biostimulant consists of a microorganism or a consortium of microorganisms, as referred to in Component Material Category 7 of Annex II of the EU Fertilising Products Regulation.

This document is applicable to all microbial biostimulants in agriculture.

The Table 1 below summarizes many of the agro-ecological principles and the role played by biostimulants.

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Table 1 — Agro-ecological principles and the role played by biostimulants

Increase biodiversity
By improving soil microorganism quality/quantity
Reinforce biological regulation and interactions
By reinforcing plant-microorganism interactions
- symbiotic exchanges i.e. Mycorrhizae
- symbiotic exchanges i.e. Rhizobiaceae/Faba
- secretions mimicking plant hormones (i.e. Trichoderma)
By regulating plant physiological processes
- for e.g. growth, metabolism, plant development…
Improve biogeochemical cycles
- improve absorption of nutritional elements
- improve bioavailability of nutritional elements in the soil
- stimulate degradation of organic matter

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WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably trained staff.

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1 Scope

This document provides a method for the detection of *Listeria monocytogenes* in microbial plant biostimulants for verifying that the concentration of this human pathogen does not exceed the respective limits outlined in the EU Regulation on Fertilising Products [1].

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.

CEN/TS 17724, *Plant biostimulants — Terminology*

CEN/TS 17708, *Plant biostimulants — Preparation of sample for microbial analysis*

EN ISO 11290-1:2017, *Microbiology of the food chain — Horizontal method for the detection and enumeration of Listeria monocytogenes and of Listeria spp. — Part 1: Detection method (ISO 11290-1:2017)*

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

EN ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations (ISO 7218)*

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3 Terms and definitions

For the purposes of this document, the terms and definitions are given in CEN/TS 17724 and the following apply.

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3.1
Listeria monocytogenes
microorganisms which form typical colonies on solid selective media described and which display the morphological, physiological and biochemical characteristics described when the analysis is carried out in accordance with this document

[SOURCE: EN ISO 11290-1:2017, 3.1]

4 Principle

4.1 General

The detection of *Listeria monocytogenes* requires four successive stages as specified in Annex A.

NOTE *L. monocytogenes* can be present in small numbers and is often accompanied by considerably larger numbers of bacteria belonging to different taxonomic groups or different *Listeria* species. Pre-enrichment is used to permit the detection of low numbers of *L. monocytogenes* or injured *L. monocytogenes*.

4.2 Pre-enrichment in non-selective liquid medium

Half-Fraser broth (225 ml) at ambient temperature is inoculated with the test portion sample (25 g or 25 ml), then incubated at 30 °C ± 1 °C for 24 h to 26 h.

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

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For large quantities (e.g. 1-L or more), it is recommended to pre-warm the broth to 30 °C before mixing it with the test portion.

4.3 Enrichment in/on selective media

Fraser broth is inoculated at 37°C (0,1 ml of culture (4.2) in 10 ml of Fraser broth) and incubated at 37°C ± 1°C for 24 h ± 2 h.

4.4 Plating out on selective solid media

Streak both primary AND secondary enrichments onto:

— Agar *Listeria* according to Ottaviani and Agosti (ALOA) [3];

— A second selective agar of choice, e.g. PALCAM agar, Oxford agar[2].

The agar prepared according to Ottaviani and Agosti is incubated 24 h ± 2 h, 37 °C ± 1 °C and additionally 24 h ± 2 h, 37 °C ± 1 °C, then examined. The second selective agar is incubated as specified by the manufacturer.

4.5 Confirmation

Colonies of presumptive *L. monocytogenes* are subcultured and their identity is confirmed by means of appropriate morphological and biochemical tests.

5 Culture media, reagents, antisera

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For current laboratory practices CEN/TS 17708 and EN ISO 11133 shall be used.

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

6 Equipment and consumables

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

Usual microbiological laboratory equipment according to EN ISO 7218 shall be used and, in particular, the following.

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

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

6.3 Incubators, capable of operating at 30 °C ± 1 °C, 37 °C ± 1 °C, and at 25 °C ± 1 °C (optional).

6.4 Water bath, capable of operating at 47 °C to 50 °C.

6.5 Sterile loops, approximately 3 mm in diameter or 10 µl, and inoculating needle or wire.

6.6 pH-meter, having an accuracy of calibration of ± 0,1 pH unit at 25 °C.

6.7 Sterile graduated pipettes or automatic pipettes of nominal capacities of 1 ml, and 10 ml.

[2] PALCAM agar and Oxford agar are examples of a suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of these products. https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam

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

6.9 Microscope, preferably with phase-contrast, and with slides and cover slips.

6.10 Refrigerator, capable of operating at 5 °C ± 3 °C.

6.11 Peristaltic blender (stomacher) with 400 ml sterile bags.

6.12 Blender motor and jars or vortex.

7 Sampling

Sampling is not part of the method specified in this document (see the specific European Standard dealing with the product concerned). If there is no specific International or European Standard, it is recommended that the parties concerned come to an agreement on this subject.

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

8 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International or European Standard dealing with the product concerned (see EN ISO 6887-1).

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9 Preparation procedure

9.1 Test portion and initial suspension

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9.1.1 General

To ensure a truly representative analytical unit, agitate liquids or free flowing materials until the contents are homogeneous. If the sample unit is a solid, obtain the analytical unit by taking a portion from several locations within the sample unit. To reduce the workload, the analytical units may be combined for analysis. It is recommended that a composite contains no more than five analytical units.

General rules for the preparation of the initial suspension for microbiological examination are described in CEN/TS 17708. The initial suspension of the microbial biostimulant product samples can be prepared according to EN ISO 11290-1:2017 using, as dilution fluid, the selective primary enrichment medium, half-Fraser broth, described in B.1.

A representative sample of the product should be prepared taking into consideration the different formulations of the biostimulant-based products.

Add 25 g or 25 ml of the product (the analytical unit) to 225 ml of half-Fraser broth in a 400 ml stomacher bag (6.11) or a blender jar (6.12). For composite samples, analytical units may be combined up to 125 g or ml (e.g. 125 g or ml of food to 1 125 ml of half-Fraser broth). Pre-warm the half-Fraser broth to room temperature before use.

If alternate analytical units are required, maintain a ratio of 1 part sample material to 9 parts half-Fraser broth.

9.1.2 Liquid – water based– formulations

Aseptically add 25 ml of the product (the analytical unit) to 225 ml of half-Fraser broth in a 400 ml sterile stomacher bag (6.11) or a blender jar (6.12). Pre-warm the half-Fraser broth to room temperature before use. Blend, stomach or vortex as required for thorough mixing.

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9.1.3 Liquid – oil based (emulsifiable concentrate – EC) formulations

Aseptically add 25 g of the product (the analytical unit) to 225 ml of half-Fraser broth in a 400 ml sterile stomacher bag (6.11) or a blender jar (6.12). Pre-warm the half-Fraser broth to room temperature before use. Blend, stomach or vortex as required for thorough mixing.

9.1.4 Solid – Wettable Powder (WP) formulations

Aseptically add 25 g of the product (the analytical unit) to 225 ml of half-Fraser broth in a 400 ml sterile stomacher bag (6.11). Pre-warm the half-Fraser broth to room temperature before use. Homogenize the mixture 2 min at higher speed with a stomacher (6.11).

9.1.5 Solid – Water dispersible granules (WDG) formulations

Aseptically add 25 g of the product (the analytical unit) to 225 ml of half-Fraser broth in a 400 ml sterile stomacher bag (6.11). Pre-warm the half-Fraser broth to room temperature before use. Homogenize the mixture 2 min at higher speed with a stomacher (6.11).

9.1.6 Solid – Pellets, granules, microgranules (slow release) formulations

Aseptically add 25 g of the product (the analytical unit) to 225 ml of half-Fraser broth in a 400 ml sterile stomacher bag (6.11). Pre-warm the half-Fraser broth to room temperature before use. Homogenize the mixture for 2 min at higher speed with a stomacher (6.11).

9.1.7 Solid substrates

Aseptically add 25 g of the product (the analytical unit) to 225 ml of half-Fraser broth in a 400 ml sterile stomacher bag (6.11). Pre-warm the half-Fraser broth to room temperature before use. Homogenize the mixture 2 min at higher speed with a stomacher (6.11).

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9.2 Non-selective pre-enrichment

Prepare the test portion sample (25 g or 25 ml) in half-Fraser broth (225 ml). Incubate for 24 h to 26 h at 30 °C.

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NOTE 1 A black coloration can develop during incubation.

NOTE 2 It is possible to store at 5 °C (6.10) the pre-enriched sample after incubation before transfer to Fraser broth for a maximum of 72 h.

9.3 Selective enrichment

9.3.1 After incubation of the initial suspension (primary enrichment in half-Fraser broth) for 24 h to 26 h (9.2), transfer 0,1 ml of the culture obtained in 9.2 to a tube or bottle containing 10 ml of secondary enrichment medium (Fraser Broth) (described in B.2).

9.3.2 Incubate the inoculated medium (9.3.1) for 24 h ± 2 h at 37 °C (6.3).

Half-Fraser broth and Fraser broth may be refrigerated before transfer or isolation on selective agar for a maximum of 72 h. Refrigeration provides for greater laboratory productivity and analytical flexibility. Following the period of refrigeration, the secondary enrichment broth is resuspended before transfer or plating onto agar media.

9.4 Plating out

9.4.1 General

9.4.1.1 From the primary enrichment culture (9.2) incubated for 25 h ± 1 h at 30 °C (6.3), inoculate, by means of a loop (6.5), the surface of the first selective plating medium, Agar *Listeria* according to Ottaviani and Agosti (ALOA) (described in B.3), to obtain well-separated colonies.

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Proceed in the same way with the second selective plating-out medium of choice (B.4).

NOTE Half-Fraser broth and Fraser broth can be refrigerated at 5 °C (6.10) before isolation on selective agar for a maximum of 72 h [4].

9.4.1.2 From the secondary enrichment medium incubated for 24 h ± 2 h at 37 °C (6.3) (9.3.2), repeat the procedure described in 9.4.1.1 with the two selective plating-out media.

9.4.1.3 Invert the Petri dishes obtained in 9.4.1.1 and 9.4.1.2 and place them in an incubator set at 37 °C (6.3) for Agar *Listeria* according to Ottaviani and Agosti (B.3). For the second selective medium (B.4), follow the manufacturer's instructions.

9.4.1.4 For Agar *Listeria* according to Ottaviani and Agosti (B.3), incubate for a total of 48 h ± 2 h. If colonies of presumptive *L. monocytogenes* are evident at 24 h ± 2 h the incubation may be stopped at this stage. For second selective agar incubate for the appropriate time. Examine the dishes (9.4.1.3) for the presence of presumptive colonies of *L. monocytogenes*.

NOTE After incubation plates can be refrigerated at 5 °C (6.10) for a maximum of 48 h before reading.

9.4.2 Agar *Listeria* according to Ottaviani and Agosti (B.3)

Consider as presumptive *L. monocytogenes* the blue-green colonies surrounded by an opaque halo (typical colonies). Colonies of *Listeria ivanovii* are also blue-green and surrounded by an opaque halo.

NOTE 1 Some strains of *L. monocytogenes* exposed to stress conditions, particularly acid stress, can show a very weak halo (or even no halo).

NOTE 2 Some rare *L. monocytogenes* are characterized by a slow PIPLC (phosphatidyl inositol phospholipase C) activity. Such bacteria are detected when the total duration of incubation is more than, for example, four days. Some of these strains could be pathogenic [5]. No *L. monocytogenes* strains have been described as PIPLC negative.

NOTE 3 Some organisms other than *Listeria* spp. can produce blue colonies on this medium [6].

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9.4.3 Second selective medium

After the appropriate time, examine the plates for the presence of colonies which are considered to be presumptive *L. monocytogenes*, based on their characteristics for the type of medium used (B.4).

9.5 Confirmation of *L. monocytogenes*

9.5.1 General

Confirmation tests are to be conducted in accordance with the standard EN ISO 11290-1:2017; in the recent revision and validation of this standard, catalase test and Camp-test became optional for *L. monocytogenes*. The microscopic aspect of confirmation remains mandatory, with an agar allowing distinction of pathogenic *Listeria* spp. used. For the haemolysis test or CAMP test, blood agar is now extended from defibrinated sheep blood only, to calf or bovine blood. For the haemolysis test, blood agar is to be inoculated by stabbing or by streaking (only if positive at purification step) [2].

Appropriate positive and negative control strains for each of the confirmatory tests shall be used.

9.5.2 Selection of colonies for confirmation

For confirmation of presumptive *L. monocytogenes*, take at least one colony presumed to be *L. monocytogenes* (see 9.4.2 and 9.4.3). One confirmed isolate per sample is sufficient. If the first colony is negative take further colonies presumed to be *L. monocytogenes* from selective medium (up to a maximum of five colonies from each plate of each selective medium).