SLOVENSKI STANDARD
oSIST prEN ISO 15213-2:2022

01-november-2022

Mikrobiologija v prehranski verigi - Horizontalna metoda za ugotavljanje prisotnosti in števila Clostridium spp. - 2. del: Preštevanje Clostridium perfringens s tehniko štetja kolonij (ISO/DIS 15213-2:2022)

Microbiology of the food chain - Horizontal method for the detection and enumeration of Clostridium spp. - Part 2: Enumeration of Clostridium perfringens by colony-count technique (ISO/DIS 15213-2:2022)

Mikrobiologie der Nahrungskette - Horizontales Verfahren zum Nachweis und zur Aufzählung von Clostridium spp. - Teil 2: Zählung von Clostridium perfringens durch Koloniezählverfahren (ISO/DIS 15213-2:2022)

Microbiologie de la chaîne alimentaire - Méthode horizontale pour la recherche et le dénombrement de Clostridium spp. - Partie 2: Dénombrement de Clostridium perfringens par la technique de comptage des colonies (ISO/DIS 15213-2:2022)

Ta slovenski standard je istoveten z: **prEN ISO 15213-2**

ICS:

07.100.30	Mikrobiologija živil	Food microbiology

oSIST prEN ISO 15213-2:2022 **en,fr,de**

DRAFT INTERNATIONAL STANDARD

ISO/DIS 15213-2

ISO/TC **34**/SC **9**

Secretariat: **AFNOR**

Voting begins on:
2022-09-13

Voting terminates on:
2022-12-06

Microbiology of the food chain — Horizontal method for the detection and enumeration of *Clostridium* spp. —

Part 2:
Enumeration of *Clostridium perfringens* by colony-count technique

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche et le dénombrement de Clostridium *spp.* —

Partie 2: Dénombrement de Clostridium perfringens *par la technique par comptage des colonies*

ICS: 07.100.30

This document is circulated as received from the committee secretariat.

ISO/CEN PARALLEL PROCESSING

Reference number
ISO/DIS 15213-2:2022(E)

COPYRIGHT PROTECTED DOCUMENT

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work.

ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization. The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement. For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products,* Subcommittee SC 9, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This first edition of ISO 15213-2 cancels and replaces ISO 7937:2004. ISO 15213-1 cancels and replaces ISO 15213:2003. Both ISO 15213:2003 and ISO 7937:2004 have been technically revised. ISO 15213-3 is a new developed standard.

The main changes compared with the previous edition are as follows:

— the Scope is enlarged to samples from the primary production stage;

— the heat treatment of 10 min at 80 °C has been made optional, in case of high background flora, or for the enumeration of only spores of *Clostridium (C.) perfringens* present in the sample;

— the selective medium has been re-named from sulfite-cycloserine agar (SC) to tryptose sulfite cycloserine agar (TSC) without changes in the formulation;

— the confirmation methods described are modified according to ISO 14189;

— a flow diagram in Annex A gives a short description of the procedure;

— in Annex D (informative) two molecular methods are described for differentiation between pathogenic and non-pathogenic C. perfringens and one molecular method for the differentiation of *C. perfringens* type A strains carrying a chromosomally encoded *cpe* gene or a plasmid encoded *cpe* gene.

A list of all parts in the ISO 15213 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Clostridium (C.) perfringens is a Gram-positive, anaerobic, spore-forming bacterium. As a ubiquitous bacterium, *C. perfringens* is predominantly found in soil, but also in the intestinal tract of humans and animals. Therefore, the presence of *C. perfringens* in high numbers can be an indication of inadequate preparation or handling of food.

High numbers of *C. perfringens* in ready-to-eat-food can cause human illness, mainly diarrhoea. The strains are classified into toxin types, depending on the ability to produce different so called “major” and “minor” toxins. Food poisonings are caused by *C. perfringens* isolates with the ability to produce *C. perfringens* enterotoxin (CPE).

A characteristic feature is the heat resistance of the spores; they have the ability to germinate and multiply in ready-to-eat food after the cooking process. Ingestion of contaminated food is followed by gastrointestinal disease, when enzyme-resistant *C. perfringens* enterotoxins are set free during sporulation in the small intestine. The strains are classified into different types.

This document describes the horizontal method for the enumeration of *Clostridium (C.) perfringens* in food, feed, environmental samples, and samples from the primary production stage. The method for the enumeration of sulfite-reducing *Clostridium* spp. is described in ISO 15213-1. The method for the detection of *C. perfringens* is described in ISO 15213-3. These three parts are published as a series of International Standards because the methods are closely linked to each other. These methods are often conducted in association with each other in a laboratory and the media and their performance characteristics can be similar.

The main technical changes listed in the Foreword, introduced in this document compared with ISO 7937:2004 are considered as major (see ISO 17468).

These changes have a major impact on the performance characteristics of the method.

Microbiology of the food chain — Horizontal method for the detection and enumeration of *Clostridium* spp. —

Part 2: Enumeration of *Clostridium perfringens* by colony-count technique

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for enumeration *Clostridium perfringens* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

1 Scope

This document specifies the enumeration of *Clostridium (C.) perfringens* by colony-count technique.

This document is applicable to:

— products intended for human consumption;

— products for feeding animals;

— environmental samples in the area of food and feed production and handling;

— samples from the primary production stage.

NOTE This method has been validated in an interlaboratory study for the following food categories:

— ready-to-eat, ready-to-reheat meat products;

— eggs and egg products (derivates);

— processed fruits and vegetables;

— infant formula and infant cereals;

— multi-component foods or meal components.

It has also been validated for the following other categories:

— pet food and animal feed;

— environmental samples (food or feed production).

As this method has been validated for at least five food categories, this method is applicable for a broad range of food. For detailed information on the validation see Clause 11 and Annex C. Since the method is not commonly used for samples in the primary production stage, this category was not included in the interlaboratory study. Therefore, no performance characteristics were obtained for this category.

This horizontal method was originally developed for the examination of all samples belonging to the food chain. Based on the information available at the time of publication of this document, this method is considered to be fully suited to the examination of all samples belonging to the food chain. However, because of the large variety of products in the food chain, it is possible that this horizontal method is not

appropriate in every detail for all products. Nevertheless, it is expected that the required modifications are minimized so that they do not result in a significant deviation from this horizontal method.

This technique is suitable for, but not limited to, use for the enumeration of microorganisms in test samples and is based on a minimum of 10 colonies counted on a plate. This corresponds to a level of contamination that is expected to be higher than 10 cfu/ml for liquid samples or higher than 100 cfu/g for solid samples.

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.

ISO 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

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

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

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply. ISO and IEC maintain terminology databases for use in standardization at the following addresses:

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

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

3.1
confirmed *Clostridium (C.) perfringens*
bacteria that produce characteristic colonies in the specified selective medium under obligate anaerobic conditions and possess the enzyme acid phosphatase

3.2
human pathogenic *Clostridium (C.) perfringens*
confirmed *C. perfringens* strains which possess the ability to produce *C. perfringens* enteroxin (CPE), encoded by the *cpe* gene

Note 1 to entry: The *cpe* gene can be located either chromosomally or on plasmids. These isolates are able to produce CPE in the small intestine on sporulation and cause human illness.

3.3
presumptive *Clostridium (C.) perfringens*
spore-forming bacteria forming countable typical colonies in a specific selective medium under obligate anaerobic conditions

Note 1 to entry: Presumptive *C. perfringens* are spore-forming bacteria that are able to produce typical colonies under the conditions specified in this document.

4 Principle

4.1 General

A specified quantity of the liquid test sample, or of an initial suspension in the case of other products, is dispensed into an empty Petri dish and mixed well with a specified molten agar culture medium

to form a poured plate. Other plates are prepared under the same conditions using decimal dilutions of the test sample. If it is the intention to count only spores, a heat treatment of 10 min at 80 °C needs to be performed before plating. Additionally, a method for molecular differentiation between human pathogenic and non-pathogenic *C. perfringens* strains is described in Annex D.

When the number of cfu is expected to be at or near the limit of determination, the use of duplicate plates is preferable. If duplicate plates are used the minimum for the sum of colonies should be 10. In this case the level of contamination is expected to be higher than 5 cfu/ml for liquid samples or higher than 50 cfu/g for solid samples.

A pour-plate technique with overlay is especially suited for the enumeration of products expected to contain spreading colonies that can obscure colonies of the target microorganisms.

The enumeration of *C. perfringens* requires four successive stages as specified in Annex A.

4.2 Preparation of dilutions

For the preparation of decimal dilutions from the test portion, follow the procedure as specified in ISO 6887 (all parts).

4.3 Enumeration

Petri dishes are inoculated with a specified quantity of the test sample if the initial product is liquid, or a specified quantity of the initial suspension, in the case of other products. Additional Petri dishes are inoculated, under the same conditions, using decimal dilutions of the test sample or of the initial suspension. A selective medium is added (poured-plate technique) and then overlaid with the same medium.

The plates are incubated under anaerobic conditions at 37 °C. for 20 h. The number of colonies presumed to be *C. perfringens*, because of their characteristic appearance, is recorded.

4.4 Confirmation

Confirmatory tests are carried out. The result is calculated as the colony count of confirmed *C. perfringens* per sample volume. Additionally, the method described in Annex D may be used for molecular differentiation between human pathogenic and non-pathogenic *C. perfringens* strains.

5 Culture media and reagents

Follow current laboratory practices in accordance with ISO 7218. The composition of culture media and reagents and their preparation are specified in Annex B. For performance testing of culture media, follow the procedures in accordance with ISO 11133 and/or Annex B.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications. The usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following shall be used:

6.1 Appropriate apparatus for achieving an anaerobic atmosphere.

6.2 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**.

6.3 Drying cabinet or **oven**, ventilated, capable of operating between 25 °C and 50 °C.

6.4 Freezer, capable of operating at -20 °C ± 2 °C and at -70 °C ± 3 °C.

6.5 Incubator(s), capable of operating at 37 °C ± 1 °C.

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

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

6.8 Sterile bottles, flasks or tubes, of appropriate capacity. Bottles, flasks or tubes with non-toxic metallic or plastic screw-caps may be used.

6.9 Sterile graduated pipettes or **automatic pipettes**, of nominal capacities 10 ml, 1 ml and 0,1 ml.

6.10 Sterile loops, of approximate diameter 3 mm (10 µl volume), and of 1 µl volume, or inoculation needle or wire.

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

6.12 Thermostatically controlled water bath, capable of operating at 44 °C to 47 °C and 80 °C ± 2 °C.

7 Sampling

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

Recommended sampling techniques are given in the following documents:

— ISO/TS 17728 for food and animal feed;

— ISO 707 for milk and milk products;

— ISO 6887-3 for raw molluscs, tunicates and echinoderms from primary production areas;

— ISO 13307 for primary production stage;

— ISO 17604 for carcasses;

— ISO 18593 for surfaces.

It is important that the laboratory receives a sample that is representative of the product under consideration. The sample should not have 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 Standard dealing with the product concerned. Follow the procedures as specified in ISO 6887 (all parts). If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 General

A flow diagram of the procedure is given in Annex A.

9.2 Test portion, initial suspension and dilutions

Prepare the test sample in accordance with the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject. Prepare a single decimal dilution series from the test portion if the product is liquid, or from the initial suspension in the case of other products.

9.3 Heat pre-treatment to select spores

If it is the intention to count only spores, heat the decimal dilution series to 80 °C in a water bath (6.12) for 10 min ± 1 min. Heat treatment shall be given within 15 min after preparation of the initial suspension to avoid germination of spores. If the tube is not placed in the water bath within 15 min, it should be placed immediately in melting ice for a maximum of 2 h.

The temperature during heat-treatment should be monitored by placing an appropriate thermometer in a reference bottle of the same size as the sample bottle and containing the same volume of water at the same initial temperature as the sample being treated (6.8). The tubes should not be hermetically sealed during the heat treatment. The time taken to reach 80 °C shall not exceed 10 min and can be minimised by ensuring the water level to be at least 4 cm above the level of the sample and that water in the water bath is circulated to maximize heat exchange.

Start the time of heating (10 min) when the temperature of the reference sample has reached 80 °C. After heat treatment, the samples should be cooled immediately till approximately 20 °C.

Heat treatment should also reduce the competitive flora in some matrices containing a high level of background flora (e.g. liquid whey, silage).

9.4 Inoculation and incubation

9.4.1 Take two sterile Petri dishes with a diameter of approximately 90 mm (6.11). Transfer to each dish, by means of a sterile pipette (6.9), 1 ml of the test sample if liquid, or 1 ml of the initial suspension (10^{-1} dilution) in the case of other products. If plates from more than one dilution are prepared, this may be reduced to one dish (see ISO 7218).

9.4.2 Take one other sterile Petri dish (6.11). Use another sterile pipette (6.9) to dispense 1 ml of the 10^{-1} dilution (liquid product) or 1 ml of the 10^{-2} dilution (other products).

9.4.3 If necessary, repeat the procedure with further dilutions, using a new sterile pipette (6.9) for each decimal dilution.

9.4.4 If appropriate and possible, select only the critical dilution steps (at least two consecutive decimal dilutions) for the inoculation of the Petri dishes (6.11) that will give colony counts of between 10 and 150 colonies per plate (on 90 mm Petri dishes) or between 10 and 360 colonies per plate (on 140 mm Petri dishes).

9.4.5 Pour about 12 ml to 15 ml for 90 mm Petri dishes or 45 ml to 50 ml for 140 mm Petri dishes of the tryptose sulfite cycloserine agar (TSC) medium (see Clause B.2), molten and tempered at 44 °C to 47 °C (6.12), into each Petri dish (6.11). The time elapsed between the end of the preparation of the initial suspension (or of the 10^{-1} dilution if the product is liquid) and the moment when the medium (see Clause B.2) is poured into the dishes shall not exceed 45 min.

9.4.6 Carefully mix the inoculum with the medium by rotating the Petri dishes and allow the mixture to solidify by leaving the Petri dishes (6.11) standing on a cool horizontal surface.

9.4.7 After complete solidification, pour about 5 ml of medium (see Clause B.2) for 90 mm Petri dishes (6.11) or 10 ml for 140 mm Petri dishes (6.11) as overlay, to prevent the development of spreading colonies on the surface of the medium. Allow to solidify as specified in 9.4.6.

9.4.8 Invert the plates and incubate (6.3) the plates at 37 °C ± 1° C for 20 h ± 2 h in an anaerobic atmosphere in an anaerobic atmosphere (6.1). Longer incubation may result in excess blackening of the plates.

9.5 Enumeration of typical colonies

9.5.1 After 20 h ± 2 h of incubation, examine the plates (see 9.4.8) for presumptive *C. perfringens*.

The typical colonies, which show black or grey to yellow-brown staining (even if the colour is faint) on the TSC agar, are counted.

Upon removal of the plates from the anaerobic atmosphere, plates must be counted within 30 min as the colour of the colonies can rapidly fade and disappear upon exposure to oxygen. If anaerobic jars are used, the plates should be checked jar by jar or in small portions if the incubation was performed in an anaerobic incubator (6.1, 6.3).

9.5.2 Select the plates (see 9.5.1) containing less than 150 suspect colonies (for 90 mm Petri dishes) or less than 360 colonies (for 140 mm Petri dishes); count these colonies and record the number of presumptive colonies per dish. Then choose, at random, five such colonies from each dish for confirmation (9.6). For enumeration of plates with low or high numbers of suspect colonies, see ISO 7218.

9.6 Confirmation of *C. perfringens*

9.6.1 Selection of colonies for confirmation

9.6.1.1 For confirmation, take five presumptive colonies from each dish retained for enumeration (see 9.5.2). If more than one morphology is present among the colonies, select one of each morphology for subculture and confirmation.

9.6.1.2 Streak each of the selected colonies with a sterile loop (6.9) onto one non-selective blood agar plates, e.g. Columbia blood agar (see Clause B.3). If blood is not available, Columbia agar base or another nutrient-rich medium (e.g. Tryptone soya agar or Brain heart infusion agar) can be used with or without blood.

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 ISO 11133). Several isolates can be streaked onto identified sectors of each of the two non-selective agar plates. Streaks should obtain well isolated colonies.

Incubate the plates in an anaerobic atmosphere at 37 °C ± 1° C (6.3) for 20 h ± 2 h. Right after incubation, select well-isolated freshly grown colonies for confirmation. Confirmation may be done either by the acid phosphatase test or by the SIM agar test.

NOTE Alternative procedures can be used to confirm the isolate as *C. perfringens*, provided that the suitability of the alternative procedure has been verified (see ISO 7218).

9.6.2 Acid phosphatase test

9.6.2.1 It is known that, beside *C. perfringens*, some other *Clostridium* strains (e.g. some strains of *C. baratii*) can produce acid phosphatase, but this ability is very limited. Therefore, only a very low percentage of false positives is expected.

9.6.2.2 Colonies grown anaerobically on blood or nutrient agar plates are spread on filter paper and 2 to 3 drops of the acid phosphatase reagent (B.4) are placed onto the colonies. If a commercially available test kit is used, follow the manufacturer's instructions.

NOTE It is possible to drip acid phosphatase reagent on colonies, if no further investigation of the colonies is needed.

9.6.2.3 A purplish colour developed within 3 min to 4 min is considered as a positive reaction.

9.6.3 SIM agar test

Colonies grown anaerobically on blood agar plates are stabbed into SIM tubes (B.5). The tubes are incubated for 22 h ± 2 h at 37 °C ± 1° C. After incubation the tubes are read for:

- Sulfite production: tubes showing blackening are positive
- Motility: tubes showing growth outside the inoculation stab are positive
- Indole production: tubes giving a red coloured ring directly after adding Kovacs reagents (B.6) are positive

C. perfringens is positive for sulfite production and negative for indole production and motility.

9.6.4 Differentiation between human pathogenic and non-pathogenic *C. perfringens* strains (optional)

Additionally, the method described in Annex D can be used for molecular differentiation between human pathogenic and non-pathogenic *C. perfringens* strains.

9.6.5 Interpretation

C. perfringens produces black or grey to yellow brown colonies on TSC agar, even if the colour is faint, and possesses acid phosphatase.

10 Expression of results

For calculation of the results, follow the procedure(s) in accordance with ISO 7218. Calculate and report the results as the number of confirmed *C. perfringens* or, if the method of Annex D was also used for differentiation, of confirmed human pathogenic *C. perfringens,* in cfu per gram, per millilitre or per square centimetre. When the sampled area is not known, report as per sampling device, such as a cloth, sponge swab or stick.

If heat pre-treatment for the selection of spores (9.3) was used, the result is reported as number of confirmed *C. perfringens* spores or, if the method of Annex D was also used for differentiation, of confirmed human pathogenic *C. perfringens* spores in cfu per gram, per millilitre, per square centimetre or per sampling device.

In cases where no typical colonies of *C. perfringens* have been detected, or when no typical colonies are confirmed as *C. perfringens*, follow ISO 7218 for the expression of results for special cases.

11 Performance characteristics of the method

11.1 Interlaboratory study

Results of the interlaboratory study to determine the precision of the method are summarized in Annex C. Repeatability and reproducibility limits were determined using five food types, environmental swabs and feed silage contaminated at a low level. It is possible that the values derived from the interlaboratory study are not applicable to concentration ranges and food types other than those