INTERNATIONAL STANDARD

ISO 7937

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Microbiology of food and animal feeding stuffs — Horizontal method for enumeration of *Clostridium perfringens* — Colony-count technique

Microbiologie des aliments — Méthode horizontale pour le dénombrement de Clostridium perfringens *— Technique par comptage des colonies*

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Forword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 7937 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 9, *Microbiology*.

This second edition cancels and replaces the first edition (ISO 7937:1985), which has been technically revised.

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Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products for which it may be necessary to use different methods. Nevertheless, in all cases, every attempt should be made to apply this horizontal method as far as possible and deviations from this should only be made if absolutely necessary for technical reasons.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from it in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this International Standard so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

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Microbiology of food and animal feeding stuffs — Horizontal method for enumeration of *Clostridium perfringens* — Colony-count technique

1 Scope

This International Standard describes a horizontal method for the enumeration of viable *Clostridium perfringens* in products intended for human consumption or the feeding of animals.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of the publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations.*

3 Definitions

For the purposes of this International Standard, the following definitions apply.

3.1 ***Clostridium perfringens***: Bacteria that form characteristic colonies (surrounded by a black halo) in the specified selective medium and which give positive confirmatory reactions when the test is carried out by the method specified in this International Standard.

NOTE — For practical reasons, this definition of *Clostridium perfringens* does not exclusively describe strains of *C. perfringens*. In particular, the confirmatory tests are inadequate to distinguish between *C. perfringens* and other closely related but less commonly encountered *Clostridium* species such as *C. paraperfringens* and *C. absonum*.

3.2 enumeration of *C. perfringens*: Determination of the number of viable and confirmed *Clostridium perfringens* bacteria per millilitre or per gram of sample when the test is carried out by the method specified in this International Standard.

4 Principle

4.1 Inoculation of Petri dishes 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.

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Inoculation, under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

Mixing with a selective medium (poured-plate technique) and adding an overlay of the same medium.

4.2 Anaerobic incubation of the plates at 35 °C or 37 °C for 20 h. The temperature shall be agreed between the parties concerned and recorded in the test report.

4.3 Enumeration of the characteristic colonies.

4.4 Confirmation of the number of characteristic colonies and calculation of the number of *C. perfringens* per millilitre or per gram of sample.

5 Diluent, culture media and reagents

5.1 General

See ISO 7218.

5.2 Diluent

See ISO 6887 and any specific standard dealing with the product to be examined.

5.3 Egg-yolk-free tryptose-sulfite-cycloserine agar (SC) [1)]

5.3.1 Base

5.3.1.1 Composition

Tryptose [a)]	15,0 g
Soytone [b)]	5,0 g
Yeast extract	5,0 g
Disodium disulfite ($Na_2S_2O_5$), anhydrous	1,0 g
Ammonium iron(III) citrate [b)]	1,0 g
Agar	9,0 g to 18,0 g [c)]
Water	1 000 ml

a) The names tryptose and soytone are used at present only by certain producers of media. Any other pancreatic casein or soybean digest giving comparable results may be used.

b) This reagent should contain at least 15 % (*m*/*m*) of iron.

c) Depending on the gel strength of the agar.

1) This was originally designated EY-free TSC (Hauschild and Hilsheimer, *Appl. Microbiol.*, **27**, 1974, pp. 78-82).

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5.3.1.2 Preparation

Dissolve the components in the water by boiling.

Adjust the pH so that after sterilization it will be 7,6 ± 0,2 at 25 °C.

Dispense the base into flasks or bottles of appropriate capacity.

Sterilize for 15 min at 121 °C.

5.3.2 D-Cycloserine solution

5.3.2.1 Composition

D-Cycloserine [a]	4,0 g
Water	100 ml
[a] Use white crystalline powder only.	

5.3.2.2 Preparation

Dissolve the D-cycloserine in the water and sterilize the solution by filtration.

Store in a refrigerator at + 3 °C ± 2 °C.

Discard unused solution 4 weeks after preparation.

5.3.3 Complete medium

Immediately before use in the pour-plate method (see 9.2) add, to each 100 ml of sterile molten base (5.3.1) cooled to 47 °C ± 2 °C, 1 ml of D-cycloserine solution (5.3.2).

5.4 Fluid thioglycollate medium

5.4.1 Composition

Enzymatic digest of casein	15,0 g
L-Cysteine	0,5 g
D-Glucose	5,5 g
Yeast extract	5,0 g
Sodium chloride	2,5 g
Sodium thioglycollate (mercaptoacetate)	0,5 g
Agar	0,5 g to 2,0 g [a]
Resazurin	0,001 g
Water	1 000 ml
[a] Depending on the gel strength of the agar.	

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5.4.2 Preparation

Dissolve the components in the water by boiling.

Adjust the pH so that after sterilization it is 7,1 ± 0,2 at 25 °C.

Dispense 10 ml portions into tubes and sterilize at 121°C for 15 min.

Before use, this medium shall be de-aerated.

5.5 Lactose sulfite medium (LS)

5.5.1 Base medium

5.5.1.1 Composition

Enzymatic digest of casein	5,0 g
Yeast extract	2,5 g
Sodium chloride	2,5 g
Lactose	10 g
L-Cysteine hydrochloride	0,3 g
Water	1 000 ml

5.5.1.2 Preparation

Dissolve the components in the water by boiling (if necessary).

Adjust the pH so that after sterilization it is 7,1 ± 0,2 at 25 °C.

Dispense 8 ml portions into test tubes or bottles with inverted Durham tubes (6.7) and sterilize at 121 °C for 15 min.

The medium may be stored at + 3 °C ± 2 °C for up to 4 weeks.

5.5.2 Disodium disulfite, anhydrous solution

5.5.2.1 Composition

Disodium disulfite ($Na_2S_2O_5$), anhydrous	1,2 g
Water	100 ml

5.5.2.2 Preparation

Dissolve the disodium disulfite in the water and sterilize the solution by filtration.

Use the solution within a day.

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5.5.3 Ammonium iron(III) citrate solution

5.5.3.1 Composition

Ammonium iron(III) citrate	1 g
Water	100 ml

5.5.3.2 Preparation

Dissolve the ammonium iron(III) citrate in the water and sterilize the solution by filtration.

Use the solution within a day.

5.5.4 Complete medium

If the medium is not used on the day of the preparation, just prior to completion, de-aerate the medium by heating and then cool rapidly. If the medium is in screw-cap bottles, loosen the caps before heating and tighten them before cooling.

Then add 0,5 ml of the disodium disulfite solution (5.5.2) and 0,5 ml of the ammonium iron(III) citrate solution (5.5.3) to each 8 ml of base (5.5.1).

6 Apparatus and glassware

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

6.1 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**

See ISO 7218.

6.2 Incubator, capable of being maintained at 35 °C ± 1 °C or 37 °C ± 1 °C, depending on the temperature agreed.

6.3 Anaerobic jars or any other apparatus appropriate for anaerobic culture.

6.4 pH-meter, capable of being read to the nearest ± 0,01 pH unit at 25 °C, enabling measurements to be made which are accurate to 0,1 pH unit.

6.5 Loops, of platinium-iridium or nickel-chromium, of diameter approximately 3 mm, and **stab-inoculation needle** of the same material.

6.6 Filtration apparatus, for sterilization of solutions.

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6.7 Test tubes, **bottles** or **flasks** of appropriate capacity, in particular 16 mm × 160 mm test tubes with inverted Durham tubes, for example of length 35 mm and of diameter 7 mm.

6.8 Total-delivery graduated pipettes, of nominal capacities 1 ml and 10 ml, graduated in 0,1 ml and 0,5 ml respectively.

6.9 Petri dishes, made of glass or plastics material, of diameter 90 mm to 100 mm.

6.10 Water bath, or similar apparatus, capable of being maintained at 47 °C ± 2 °C.

6.11 Water bath, or similar apparatus, capable of being maintained at 46 °C ± 0,5 °C.

7 Sampling

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

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

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887 and any specific standard appropriate to the product concerned.

9.2 Inoculation and incubation (poured-plate technique)

Transfer, by means of a sterile pipette, 1 ml of the initial suspension, or of the test sample if the initial product is liquid, in duplicate, to the centres of empty Petri dishes. Pour 10 ml to 15 ml of the SC agar (5.3.3) maintained at 47 °C in the water bath (6.10), into the dish and mix well with the inoculum by gently rotating each dish. When the medium has solidified, add an overlayer of 10 ml of the same SC agar. Allow to solidify. Place the plates in anaerobic jars or other suitable containers (6.3) and incubate at 35 °C or 37 °C for 20 h ± 2 h. Longer incubation may result in excess blackening of the plates. The temperature shall be agreed between the parties concerned and recorded in the test report.

Follow the same procedure with the subsequent decimal dilutions.

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9.3 Counting and selection of colonies

After the specified period of incubation (see 9.2), keep the plates containing less than 150 colonies at all dilutions and, if possible, at subsequent dilutions.

Count on each plate the black colonies of presumptive *C. perfringens*.

Select 5 characteristic colonies from each plate retained for the enumeration. If less than 5 colonies are available on the plates counted, select all the characteristic colonies present. Confirm these colonies as described in 9.4.2.

NOTE — The reaction obtained in lactose sulfite medium (5.5) when incubated at 46 °C is very specific for *Clostridium perfringens* and some strains of *Clostridium paraperfringens* and *Clostridium absonum*. It is therefore not necessary to ensure that the black colonies picked from the agar are pure before inoculation into the thioglycollate broth and subsequently into the lactose sulfite medium.

9.4 Biochemical confirmation

NOTE — Commercialy available biochemical galleries may be used if in accordance with subclause 9.6.5 of ISO 7218:1996.

9.4.1 Inoculation and incubation

Inoculate each selected colony (see 9.3) into fluid thioglycollate medium (5.4).

Incubate under anaerobic conditions at 35 °C or 37 °C for 18 h to 24 h.

After incubation, transfer with no delay 5 drops of the thioglycollate culture to the LS medium by means of a sterile pipette.

Incubate at 46 °C for 18 h to 24 h in the water bath (6.11).

9.4.2 Interpretation

The tubes of LS medium are read for the production of gas and the presence of a black colour (iron sulfide precipitate). Durham tubes being more than one-quarter full of gas and tubes having a black precipitate are considered positive.

In case of doubt, when the Durham tube in a blackened medium is less than one-quarter full of gas, transfer with no delay, using a sterile pipette, 5 drops of the previous growth on LS medium (9.4.1) to another tube of LS medium. Incubate in the water bath at 46 °C for 18 h to 24 h. Read this tube as described above.

In all the other cases, the tubes will be considered as negative.

9.5 Interpretation

Bacteria which form characteristic colonies in the SC medium and which give a positive confirmation with the LS medium are considered as being *C. perfringens*.

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10 Expression of results

10.1 Counting of the colonies of *C. perfringens*

Calculate, for each of the dishes, the number a of colonies of *C. perfringens* using the following equation:

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

where

b is the number of colonies confirmed as *C. perfringens* (9.4.2);

A is the number of colonies selected for further confirmation (9.3);

C is the number of typical colonies marked on the dish (9.3).

Round off the result to a whole number of colonies.

10.2 Method of calculation

10.2.1 Dishes containing less than 150 colonies, one of which contains at least 15

Calculate the number N of *C. perfringens* present in 1 ml or 1 g of product using the following equation:

$$N = \frac{\sum a}{(n_1 + 0{,}1\, n_2)d}$$

where

$\sum a$ is the sum of the colonies of *C. perfringens* counted after identification on all the dishes of two successive dilutions, one at least of which contains 15 colonies;

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

n_2 is the number of dishes retained at the second dilution;

d is the dilution factor corresponding to the first dilution retained.

Round off the results to two significant figures.

Take as the result the number of *C. perfringens* per millilitre or per gram of product, expressed as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

A direct count (liquid product) of *C. perfringens* 37 °C on SC medium gave the following results:

- at the first dilution retained (10^{-3}): 66 colonies and 80 colonies;

- at the second dilution retained (10^{-4}): 4 colonies and 7 colonies.

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