INTERNATIONAL STANDARD

ISO 13722

First edition
1996-12-15

Meat and meat products — Enumeration of *Brochothrix thermosphacta* — Colony-count technique

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Viande et produits à base de viande — Dénombrement des Brochothrix thermosphacta *— Technique par comptage des colonies obtenues*

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https://standards.iteh.ai/catalog/standards/sist/ee68f956-8278-42fa-af5d-2301c4464a9a/iso-13722-1996

Reference number
ISO 13722:1996(E)

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.

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.

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International Standard ISO 13722 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 6, *Meat and meat products*.

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International Organization for Standardization
Case Postale 56 • CH-1211 Genève 20 • Switzerland

Printed in Switzerland

Meat and meat products — Enumeration of *Brochothrix thermosphacta* — Colony-count technique

1 Scope

This International Standard specifies a method for the enumeration of viable *Brochothrix thermosphacta* in all kinds of meat and meat products, including poultry, by means of a colony-count technique.

2 Normative references

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ISO 13722:1996

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of 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 3100-2:1988, *Meat and meat products — Sampling and preparation of test samples — Part 2: Preparation of test samples for microbiological examination.*

ISO 6887:1993, *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 Definition

For the purposes of this International Standard, the following definition applies.

3.1 ***Brochothrix thermosphacta***: Gram-positive bacteria which form characteristic oxidase-negative colonies on a solid selective medium [streptomycin sulfate/thallium acetate/actidione (STAA) agar] under the test conditions specified in this International Standard.

4 Principle

4.1 Surface plating, on a solid selective culture medium contained in Petri dishes, of a specified quantity of the test sample if the initial product is liquid, or of a specified quantity of the initial suspension in the case of other products.

Preparation of other plates, under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

4.2 Incubation of the plates between 22 °C and 25 °C for 48 h ± 4 h.

4.3 Subjection of the colonies to a confirmation test.

4.4 From the number of colonies confirmed, calculation of the number of *Brochothrix thermosphacta* per millilitre or per gram of sample from colonies obtained on plates at dilution levels chosen so as to give the most reliable result (see 9.3).

5 Dilution fluid, culture media and reagents

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Dilution fluid

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For the preparation of dilutions, see ISO 6887.

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5.3 Solid selective medium: Streptomycin sulfate/thallium acetate/actidione (STAA) agar (see reference [2])

ISO 13722:1996
https://standards.iteh.ai/catalog/standards/sist/ee68f956-8278-42fa-af5d-2301c4464a9a/iso-13722-1996

5.3.1 Base medium

5.3.1.1 Composition

Peptone	20,0 g
Yeast extract	2,0 g
Glycerol	15,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	1,0 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	1,0 g
Agar	9 g to 18 g[1)]
Water	900 ml

1) Depending on the gel strength of the agar.

5.3.1.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling. Adjust the pH so that after sterilization it is 7,0 at 25 °C.

Sterilize for 15 min in an autoclave (6.1) set at 121 °C.

5.3.2 Streptomycin sulfate solution

5.3.2.1 Composition

Streptomycin sulfate	1,0 g
Water	100 ml

5.3.2.2 Preparation

Dissolve the streptomycin sulfate in the water. Sterilize by filtration.

5.3.3 Actidione solution

WARNING — Actidione and thallium acetate are both toxic. Take appropriate procedures to prevent contamination of the operator and the environment when using these chemicals and their solutions.

5.3.3.1 Composition

Actidione (cycloheximide)	150 mg
Water	90 ml

5.3.3.2 Preparation

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Dissolve the actidione in the water. Sterilize by filtration.

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5.3.4 Thallium acetate solution

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5.3.4.1 Composition

Thallium acetate	250 mg
Water	100 ml

5.3.4.2 Preparation

Dissolve the thallium acetate in the water. Sterilize by filtration.

5.3.5 Complete medium

5.3.5.1 Composition

Base medium (5.3.1)	900 ml
Streptomycin sulfate solution (5.3.2)	50 ml
Actidione solution (5.3.3)	30 ml
Thallium acetate solution (5.3.4)	20 ml

5.3.5.2 Preparation

Melt the base medium and cool it in a water bath (6.9) set at 47 °C. Using sterile conditions, warm an aliquot of the other liquids (5.3.2, 5.3.3 and 5.3.4) to 47 °C in the water bath. Add the stipulated volumes (5.3.5.1) of these solutions to the cooled medium, mixing well between each addition.

5.3.6 Preparation of agar plates for enumeration

Pour 15 ml to 20 ml portions of the complete medium (5.3.5) into sterile Petri dishes (6.4) and allow to solidify.

The plates may be stored prior to drying at between 0 °C and 5 °C for up to 1 week.

Immediately before use, dry the agar plates, preferably with the lids removed and with the agar surfaces facing downwards, in the incubator (6.2) set at a temperature between 37 °C and 50 °C, until the droplets have disappeared from the surface of the medium. Do not dry them any further. The agar plates can also be dried in a drying cabinet for 30 min with half-open lids, or overnight with the lids in place.

Ready-prepared agar plates are available commercially. Store and use them according to the manufacturer's instructions.

5.4 Oxidase reagent

5.4.1 Composition

N,N,N′,N′-Tetramethyl-*p*-phenylenediamine dihydrochloride	1,0 g
Distilled water	100 ml

5.4.2 Preparation

Dissolve the reagent in the cold water. The reagent shall be prepared immediately prior to use.

Commercially available disks or sticks may be used. In this case, follow the manufacturer's recommendations.

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ISO 13722:1996
https://standards.iteh.ai/catalog/standards/sist/ee68f956-8278-42fa-af5d-2301c4464a9a/iso-13722-1996

6 Apparatus

Usual microbiological laboratory equipment and, in particular, the following.

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

See ISO 7218.

6.2 Incubator or **drying cabinet,** ventilated by convection, capable of operating between 37 °C ± 1 °C and 50 °C ± 1 °C.

6.3 Incubator, capable of operating between 22 °C ± 1 °C and 25 °C ± 1 °C.

6.4 Petri dishes, made of glass or plastic, of diameter 90 mm to 100 mm.

6.5 Total delivery (blow-out) pipettes, having wide openings, of nominal capacities 10 ml and 1 ml, graduated in 0,1 ml divisions.

6.6 Rubber bulbs, or any other type of safety device suitable for use with the graduated pipettes.

6.7 Culture bottles or **flasks,** of suitable capacities.

6.8 Spreaders (hockey-stick type) made of glass, of diameter approximately 3,5 mm and length 20 cm, bent at right angles about 3 cm from one end; the cut ends shall be made smooth by heating.

Disposal sterile plastic spreaders may also be used.

6.9 Water baths, capable of being maintained at 47 °C ± 2 °C.

6.10 Colony-counting equipment, consisting of an illuminated base with a dark background and a mechanical or electronic digital counter.

6.11 pH-meter, accurate to within ± 0,1 pH unit at 25 °C.

6.12 Wires, made of platinium, or **rods**, made of glass or plastic, approximately 3 mm in diameter.

7 Sampling

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

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 3100-1.

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ISO 13722:1996
https://standards.iteh.ai/catalog/standards/sist/ee68f956-8278-42fa-af5d-2301e4464a9a/iso-13722-1996

8 Preparation of test sample

Take, in accordance with the method described in ISO 3100-2, a representative test sample. Start the examination of the pretreated sample as soon as possible. It may be stored, if necessary, at a temperature between 0 °C and + 2 °C, but for not longer than 24 h.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887 and ISO 3100-2.

9.2 Inoculation and incubation

9.2.1 Transfer, by means of a sterile pipette (6.5), 0,1 ml of the test sample if the product is liquid, or of the initial suspension in the case of other products, to each of two agar plates (5.3.6). Repeat the procedure using further decimal dilutions.

9.2.2 Carefully spread the liquid as quickly as possible over the surface of the agar plate without touching the sides of the dish with the spreader (6.8). Use a fresh sterile spreader for each plate. Leave the plates with the lids on for about 15 min at ambient temperature for the liquid to be absorbed into the agar.

9.2.3 Invert the prepared plates (9.2.2) and incubate them for 48 h ± 4 h in the incubator (6.3) set between 22 °C and 25 °C.

9.3 Counting of the colonies

After the specified period of incubation (see 9.2.3), count, using the colony-counting equipment (6.10), the characteristic colonies on each dish containing 15 to 300 colonies. Characteristic colonies are shiny, round or circular colonies of diameter 0,75 mm or larger, have an off-white colour, and are oxidase negative (see 9.4).

9.4 Confirmation

Pseudomonads are able to grow on STAA agar (5.3). They shall be differentiated from *B. thermosphacta* by performing an oxidase test as follows.

Moisten a piece of filter paper with the oxidase reagent (5.4). Take a sample of the bacterial culture obtained from the STAA agar using a platinum wire or glass or plastic rod (6.12) (a nickel/chrome wire gives false positives) and deposit it on the moistened filter paper. Oxidase-positive colonies appear within 15 s as purple colonies. *B. thermosphacta* is oxidase negative.

10 Expression of results

10.1 Count of *B. thermosphacta*

10.1.1 If at least 80 % of the selected colonies are confirmed (9.4), take as the number of *B. thermosphacta* the number given by the count made as in 9.3.

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10.1.2 In all other cases, calculate the number of *B. thermosphacta* from the percentage of *B. thermosphacta* obtained as in 9.3 which are confirmed (9.4).

Round the result to a whole number of colonies.

ISO 13722:1996
https://standards.iteh.ai/catalog/standards/sist/ee68f956-8278-42fa-af5d-2301c4464a9a/iso-13722-1996

10.2 Method of calculation

10.2.1 General case: Dishes containing between 15 and 300 characteristic colonies

Retain dishes containing not more than 300 characteristic colonies at two consecutive dilutions. It is necessary that one of these dishes contains at least 15 characteristic colonies.

Calculate the number N of *B. thermosphacta* per millilitre or per gram of product, depending on the case, using the following equation:

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

where

$\sum a$ is the sum of the characteristic colonies counted on all the dishes retained from two successive dilutions, at least one of which contains 15 colonies;

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

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

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

d is the dilution factor corresponding to the first dilution.

Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is 5 or more, the preceding figure is increased by one unit. Proceed stepwise until two significant figures are obtained.

Take as the result the number of *B. thermosphacta* per millilitre (liquid products) or per gram (other products), expressed as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

A count of *B. thermosphacta* between 22 °C and 25 °C gave the following results:

— at the first dilution retained: 83 and 97 characteristic colonies

— at the second dilution retained: 13 and 8 characteristic colonies

$$N = \frac{\sum a}{V(n_1 + 0{,}1 n_2)\, d} = \frac{83 + 97 + 13 + 8}{0{,}1[2 + (0{,}1 \times 2)] \times 10^{-1}} = \frac{201}{0{,}022} = 9\,136$$

Rounding the result as specified above gives 9 100 or $9{,}1 \times 10^3$ *B. thermosphacta* per millilitre or per gram of product.

10.2.2 Estimation of small numbers

If the two dishes at the level of the test sample (liquid products) or the initial suspension (other products) contain between 0 and 15 characteristic colonies, report the result as follows:

— less than 15 *B. thermosphacta* per millilitre (liquid products), or

— less than $15 \times 1/d$ *B. thermosphacta* per gram (other products),

where d is the dilution factor of the initial suspension.

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If the two dishes at the level of the test sample (liquid products) or of the initial suspension (other products) do not contain any characteristic colonies, express the result as follows:

ISO 13722:1996
https://standards.iteh.ai/catalog/standards/sist/ee68f956-8278-42fa-af5d-2301c4464a9a/iso-13722-1996

— less than 1 *B. thermosphacta* per millilitre (liquid products), or

— less than $1/d$ *B. thermosphacta* per gram (other products),

where d is the dilution factor of the initial suspension.

11 Precision

The confidence interval of this method varies from – 7 % to 8 % for 300 colonies and from – 29 % to 41 % for 15 colonies.

12 Test report

The test report shall specify the method used and the results obtained.

It shall also mention all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the result(s).

The test report shall include all information necessary for the complete identification of the sample.