INTERNATIONAL STANDARD

ISO 6391

Second edition
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Meat and meat products — Enumeration of *Escherichia coli* — Colony-count technique at 44 °C using membranes

Viandes et produits à base de viande — Dénombrement des Escherichia coli *— Méthode par comptage des colonies obtenues sur membranes à 44 °C*

Reference number
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Foreword

ISO (the International Organization for Standardization) is a world-wide 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.

International Standard ISO 6391 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 6, *Meat and meat products*.

This second edition cancels and replaces the first edition (ISO 6391:1988), which has been technically revised.

Annex A of this International Standard is for information only.

Introduction

Although, for well-accepted statistical reasons, the limit for the lowest number of colonies counted per plate of selective medium is set at 15, for practical purposes it is often desirable to establish an estimated count of lower numbers of *Escherichia coli*. The confidence limits of such determinations are to be found in ISO 7218.

Meat and meat products — Enumeration of *Escherichia coli* — Colony-count technique at 44 °C using membranes

1 Scope

This International Standard specifies a method for the enumeration of viable *Escherichia coli* present in all kinds of meat and meat products, including poultry.

The method will detect *Escherichia coli* (biotype 1) and lactose non-fermenting or anaerogenic variants (see reference [1]).

NOTE 1 This method may not be appropriate for the examination of processed meat having a high content of aerobic spore-forming bacteria.

NOTE 2 Some pathogenic strains of *Escherichia coli* may not be detected by this method.

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 definition applies.

3.1
Escherichia coli
Bacteria which at 44 °C form indole-positive (pink) colonies on cellulose acetate membranes overlaid on tryptone bile agar, under the conditions specified in this International Standard.

4 Principle

In general, the detection of *Escherichia coli* requires three successive stages.

4.1 Resuscitation

Inoculation of a specified quantity of the initial suspension onto cellulose acetate membranes overlaid on mineral-modified glutamate agar, and incubation at 37 °C for 4 h.

NOTE — This procedure enables *Escherichia coli* damaged by storage under frozen, dried or chill conditions or damaged by heat or chemical processes to be resuscitated. It also permits the diffusion of high concentrations of any fermentable carbohydrate, if present in the test sample, which would otherwise interfere with indole production during the subsequent isolation stage.

4.2 Isolation

Transfer of membranes from the resuscitation stage on mineral-modified glutamate agar to tryptone bile agar. Then incubation at 44 °C for 18 h to 20 h.

4.3 Detection

Demonstration of the presence of *Escherichia coli* on the membranes by the production of indole by each colony.

4.4 Calculation

Calculation of the number of *Escherichia coli* per millilitre or per gram of sample from the number of indole-positive colonies obtained on membranes at dilution levels chosen to give a significant result.

5 Diluent, culture media and reagents

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Diluent

See ISO 6887 for the preparation of dilutions.

5.3 Resuscitation media: Mineral-modified glutamate agar

5.3.1 Composition

Sodium glutamate	6,35 g
Lactose	10,0 g
Sodium formate	0,25 g
L(–)-cystine	0,02 g
L(–)-aspartic acid	0,024 g
L(+)-arginine	0,02 g
Thiamine	0,001 g
Nicotinic acid	0,001 g
Pantothenic acid	0,001 g
Magnesium sulfate ($MgSO_4.7H_2O$)	0,100 g
Ammonium iron(III) citrate [at least 15 % Fe (*m/m*)]	0,010 g
Calcium chloride ($CaCl_2.2H_2O$)	0,010 g
Dipotassium hydrogen phosphate	0,90 g
Ammonium chloride	2,5 g
Agar	12 g to 18 g [1)]
Water	1 000 ml

1) Depending on the gel strength of the agar.

5.3.2 Preparation

Dissolve the ammonium chloride in the water. Add the other components and heat to boiling. Adjust the pH so that after sterilization it is 6,7 at 25 °C. Transfer 100 ml volumes of the medium to suitable containers (6.5) and sterilize for 10 min in the autoclave (6.1) set at 121 °C.

5.3.3 Preparation of agar plates

Pour 12 ml to 15 ml of the medium, cooled to 47 °C in the water bath (6.10), into sterile Petri dishes (6.6) and allow to solidify. The plates may be stored at between 0 °C and +5 °C for up to one week.

Immediately before use, dry the agar plates carefully (e.g.in the cabinet or oven (6.4) set at 50 °C), preferably with the lids off and agar surface downwards, until any droplets have disappeared from the surface of the medium. Do not dry them any further.

NOTE — The agar should be dry enough so that 1 ml of inoculum is totally absorbed into the membrane/agar within 15 min (see 9.2.3).

5.4 Selective medium: Tryptone bile agar (see reference [3])

5.4.1 Composition

Tryptone (peptic digest of casein)	20,0 g [1]
Bile salts (refined)	1,5 g [2]
Agar	12 g to 18 g [3]
Water	1 000 ml

1) Commercial brands which favour indole formation should be used.

2) Oxoid Bile salts No. 3 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

3) Depending on the gel strength of the agar.

5.4.2 Preparation

Dissolve the components in the water and heat to boiling. Adjust the pH so that after sterilization it is 7,2 at 25 °C. Transfer 100 ml volumes of the medium to suitable containers, and sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.4.3 Preparation of agar plates

Pour 12 ml to 15 ml of the medium, cooled to 47 °C in the water bath (6.10), into sterile Petri dishes (6.6) and allow to solidify. These plates may be stored at between 0 °C and +5 °C for up to 4 days.

Immediately before use, dry the agar plates carefully (e.g. in the cabinet or oven (6.4) set at 50 °C), preferably with the lids off and agar surface downwards, until any droplets have disappeared from the surface of the medium. Do not dry them any further.

5.5 Indole detection reagent (Vracko and Sherris reagent)

5.5.1 Composition

p-Dimethylaminobenzaldehyde	5 g
Hydrochloric acid, $c(HCl) = 1$ mol/l	100 ml

5.5.2 Preparation

Dissolve the reagent in the hydrochloric acid and store at room temperature for not longer than one month.

6 Apparatus and glassware

Usual microbiological laboratory apparatus 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.

6.3 Incubator, capable of being maintained at 44 °C ± 0,5 °C.

6.4 Drying cabinet or **oven**, ventilated by convection, capable of being maintained at 50 °C ± 1 °C.

6.5 Test tubes, 18 mm x 180 mm, and **flasks** or **bottles**, 125 ml to 300 ml, for sterilization and storage of culture media.

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

6.7 Cellulose acetate membranes, of 0,45 µm to 1,2 µm pore size and of 85 mm diameter.

6.8 Pipettes, calibrated for bacteriological use, of 1 ml nominal capacity, graduated in divisions of 0,1 ml, and with an outflow opening of 2 mm to 3 mm in diameter.

6.9 Spreaders, made of plastic or glass, for example hockey sticks made from a glass rod of approximately 3,5 mm diameter, and 20 cm length, bent at right angles about 3 cm from one end and with the cut ends made smooth by heating.

6.10 Water bath, or similar apparatus, capable of being maintained at 47 °C ± 0,2 °C.

6.11 Longwave (365 nm) ultraviolet lamp, fitted with a suitable filter to remove UV radiation below 310 nm.

6.12 pH-meter, accurate to ± 0,1 pH units at 25 °C.

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 3100-1 [4].

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 sample in accordance with the specified 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.

A recommended method is given in ISO 3100-2 [5].

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887 and the specific International Standard appropriate to the product concerned.

9.2 Resuscitation procedure

9.2.1 Using sterile forceps, aseptically place a cellulose acetate membrane (6.7) onto the dried surface of each of two dishes of the mineral-modified glutamate agar (5.3.1) taking care to avoid trapping air bubbles beneath the membranes. Gently flatten the membranes with a sterile spreader (6.9).

Using a sterile pipette (6.8), add 1 ml of the initial suspension to the centre of each membrane. Using a sterile spreader (6.9), spread the inoculum evenly over the whole membrane surface, avoiding any spillage from the membrane.

9.2.2 Using another sterile pipette (6.8), inoculate similar volumes of the further diluted initial suspension onto further membranes, as specified in 9.2.1.

The time elapsing between the preparation of the test sample (clause 8) and the inoculation onto the membrane shall not exceed 45 min, unless stipulated otherwise in the relevant International Standard.

9.2.3 Leave the inoculated plates in a horizontal position at room temperature for approximately 15 min until the inocula have soaked into the membranes. Incubate the Petri dishes at 35 °C or 37 °C in the incubator (6.2) for 4 h ± 0,5 h with the membrane/agar surface uppermost.

9.3 Transfer to selective medium

9.3.1 Using sterile blunt-ended forceps, transfer membranes, inoculated side uppermost, from the mineral-modified glutamate agar (5.3.1) to the tryptone bile agar (5.4).

The moist membrane will adhere to the agar surface, avoid trapping air bubbles. Do not use a spreader as this would disperse any micro-colonies present, giving a false high count.

9.3.2 Incubate the plates at 44 °C in the incubator (6.3) for 18 h to 24 h with the membrane/agar surface uppermost. Do not stack dishes more than three high.

9.4 Detection of indole production by colonies on membranes

9.4.1 Label the lid of each plate for identification.

9.4.2 Pipette 2 ml of the indole detection reagent (5.5) into the upturned lid placed horizontally.

9.4.3 Using forceps, lift the membrane from the corresponding agar surface and lower it onto the indole reagent. If necessary, tilt the lid so that the whole of the lower membrane surface is wetted by the indole reagent. After 5 min, remove excess reagent with a pipette.

9.4.4 Place the membrane under the ultraviolet lamp (6.11) for 30 min. Indole-positive colonies develop a pink colour.

9.5 Counting

Count the indole-positive (pink) colonies on membranes containing preferably between 15 and 150 pink colonies.

10 Expression of results

Using the following criteria, determine the number of *Escherichia coli* present.

10.1 Method of calculation

10.1.1 If one or both membranes corresponding to a certain dilution have between 15 and 150 pink colonies, calculate the arithmetic mean of the number of colonies counted on the two membranes.

Retain only two significant figures, proceeding as follows:

– if the number is less than 100, round it to the nearest multiple of 5;

– if the number is greater than 100 and ends in a 5, round it to the nearest multiple of 20;

– if the number is greater than 100 and does not end in a 5, round it to the nearest multiple of 10.

Multiply this value by the reciprocal of the corresponding dilution to obtain the number of *Escherichia coli* per gram of product. Express this result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

10.1.2 If there are membranes containing between 15 and 150 pink colonies at two consecutive dilutions, calculate the number of *Escherichia coli* as specified in 9.3.5.1 of ISO 7218:1996.

10.1.3 If there are fewer than 15 pink colonies on membranes corresponding to the initial suspension, calculate the arithmetic mean, y, of the colonies counted on two dishes.

Report the result as the estimated number of *Escherichia coli* per gram, N_E:

$$N_E = y/d$$

where d is the dilution factor of the initial suspension.

10.1.4 If there are no pink colonies on membranes corresponding to the initial suspension, report the result as

– less than $1/d$ *Escherichia coli* per gram

where d is the dilution factor of the initial suspension.

10.2 Confidence limits

For calculation of the confidence interval, see ISO 7218.