SLOVENSKI STANDARD
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Mikrobiologija - Splošno navodilo o metodah za ugotavljanje prisotnosti salmonel

Microbiology -- General guidance on methods for the detection of Salmonella

Microbiologie -- Directives générales concernant les méthodes de recherche des Salmonella

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INTERNATIONAL STANDARD

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Microbiology — General guidance on methods for the detection of *Salmonella*

Microbiologie — Directives générales concernant les méthodes de recherche des Salmonella

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Contents

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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.

International Standard ISO 6579 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 9, *Microbiology*.

This third edition cancels and replaces the second edition (ISO 6579:1990), of which it constitutes a technical revision.

Annexes A, B and C form an integral part of this International Standard. Annex D is for information only.

Introduction

This International Standard is intended to provide general guidance for the examination of products not dealt with by existing International Standards and to be taken into account by organizations preparing microbiological methods of test for application to foods or to animal feeding stuffs. Because of the large variety of products within this field of application, these guidelines may not be appropriate in every detail for certain products, and for other products it may be necessary to use different methods. Nevertheless, it is hoped that in all cases every attempt will be made to apply the provided guidelines as far as possible and that deviations from them will 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 the guidelines have been followed and the reasons for deviation from them 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 these guidelines. In cases where International Standards already exist for the product to be tested, they should be followed, but 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 these guidelines will be those necessary for well-established technical reasons.

Microbiology — General guidance on methods for the detection of *Salmonella*

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella* 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.

1 Scope

This International Standard gives general guidance on methods for the detection of *Salmonella*.

Subject to the limitations discussed in the introduction, this International Standard is applicable to products intended for human consumption or feeding of animals.

The incubation temperature (35 °C or 37 °C) shall be agreed by the parties concerned and shall be specified in the test report.

2 Normative references

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

ISO 7218:1985, *Microbiology — General guidance for microbiological examinations.*

3 Definitions

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

3.1 ***Salmonella*:** Microorganisms which form typical colonies on solid selective media and which display the biochemical and serological characteristics described when tests are carried out in accordance with this International Standard.

3.2 detection of *Salmonella*: Determination of the presence or absence of these microorganisms, in a particular mass of product, when tests are carried out in accordance with this International Standard.

4 Principle

The detection of *Salmonella* necessitates four successive stages (see also annex A).

NOTE 1 *Salmonella* may be present in small numbers and are often accompanied by considerably larger numbers of other members of *Enterobacteriaceæ* or of other families. Therefore, selective enrichment is necessary; furthermore, pre-enrichment is often necessary to permit detection of injured *Salmonella*.

4.1 Pre-enrichment in non-selective liquid medium

Inoculation of buffered peptone water (also used as diluent) with the test portion, and incubation at 35 °C or 37 °C (as agreed) for 16 h to 20 h.

4.2 Enrichment in selective liquid media

Inoculation of magnesium chloride/malachite green medium and of a selenite/cystine medium with the culture obtained in 4.1.

Incubation of the magnesium chloride/malachite green medium at 42 °C for 24 h and incubation of the

selenite/cystine medium at 35 °C or 37 °C (as agreed) for 24 h and a further 24 h.

4.3 Plating out and recognition

From the cultures obtained in 4.2, inoculation of two selective solid media:

— phenol red/brilliant green agar, unless the International Standard appropriate to the product to be examined, or other specific considerations (for example the isolation of lactose-positive *Salmonella*), require substitution of some other medium as the one for obligatory use;

— any other solid selective medium (see 5.2.4.2).

Incubation at 35 °C or 37 °C (as agreed), and examination after 24 h and, if necessary, after 48 h to check for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

4.4 Confirmation

Subculturing of colonies of presumptive *Salmonella*, plated out as described in 4.3, and confirmation by means of appropriate biochemical and serological tests.

5 Culture media, reagents and sera

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Culture media and reagents

NOTE 2 Because of the large number of culture media and reagents, it has been considered preferable, for the clarity of the text, to give their composition and preparation in annex B.

5.2.1 Non-selective pre-enrichment medium: Buffered peptone water

See clause B.1.

5.2.2 First selective enrichment medium: Rappaport-Vassiliadis magnesium chloride/malachite green medium (RV medium)

See clause B.2.

5.2.3 Second selective enrichment medium: Selenite/cystine medium

See clause B.3.

5.2.4 Solid selective plating-out media

5.2.4.1 First medium: Phenol red/brilliant green agar (Edel and Kampelmacher)

See clause B.4.

This first medium is compulsory unless otherwise stated (see 4.3).

5.2.4.2 Second medium

The choice of the second medium is left to the discretion of the testing laboratory, unless there is a specific International Standard relating to the product to be examined, which specifies the composition of this second medium.

5.2.5 Nutrient agar

See clause B.5.

5.2.6 Triple sugar/iron agar (TSI agar)

See clause B.6.

5.2.7 Urea agar (Christensen)

See clause B.7.

5.2.8 L-Lysine decarboxylation medium

See clause B.8.

5.2.9 Reagent for detection of β-galactosidase (or prepared paper discs, used in accordance with the manufacturer's instructions)

See clause B.9.

5.2.10 Reagents for Voges-Proskauer (VP reaction)

See clause B.10.

5.2.10.1 VP medium

5.2.10.2 Creatine solution (*N*-amidinosarcosine)

5.2.10.3 1-Naphthol, ethanolic solution

5.2.10.4 Potassium hydroxide solution

5.2.11 Reagents for indole reaction

See clause B.11.

5.2.11.1 Tryptone-tryptophan medium

5.2.11.2 Kovacs reagent (*N,N*-dicyclohexylcarbodiimide pentachlorophenol complex)

5.2.12 Semi-solid nutrient agar

See clause B.12.

5.2.13 Saline solution

See clause B.13.

5.3 Sera

Several types of agglutinant sera containing antibodies for one or several O-antigens are available commercially, i.e. anti-sera containing one or more "O" groups (called monovalent or polyvalent anti-O sera), anti-Vi sera, and anti-sera containing antibodies for one or several H-factors (called monovalent or polyvalent anti-H sera).

Every attempt should be made to ensure that the anti-sera used are adequate to provide for the detection of all *Salmonella* serotypes. Assistance towards this objective may be obtained by using anti-sera prepared by a supplier recognized as competent (for example, by an appropriate government agency).

6 Apparatus and glassware

NOTE 3 Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

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

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

See ISO 7218.

6.2 Drying cabinet or **oven**, ventilated by convection, capable of operating between 37 °C ± 1 °C and 55 °C ± 1 °C.

6.3 Incubator, capable of operating at 35 °C ± 1 °C or 37 °C ± 1 °C, depending on the temperature agreed.

6.4 Water bath, capable of operating at 42,0 °C ± 1 °C or **incubator**, capable of operating at 42,0 °C ± 0,5 °C.

6.5 Water baths, capable of operating at 45 °C ± 1 °C, 55 °C ± 1 °C and 70 °C ± 1 °C.

6.6 Water bath, capable of operating at 35 °C ± 1 °C or 37 °C ± 1 °C, depending on the temperature agreed.

6.7 Loops, made of platinum/iridium or nickel/ chromium, of diameter approximately 3 mm.

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

6.9 Culture bottles or flasks

NOTE 4 Bottles or flasks with non-toxic metallic or plastic screw-caps may be used.

6.10 Culture tubes, 8 mm in diameter and 160 mm in length.

6.11 Measuring cylinders

6.12 Graduated pipettes, of nominal capacities 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml divisions.

6.13 Petri dishes, of small size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

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. See 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.

8 Preparation of the test sample

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.

9 Procedure

(See diagram in annex A.)

9.1 Test portion and initial suspension

9.1.1 See ISO 6887 and the specific International Standard dealing with the product concerned.

For preparation of the initial suspension, use as dilution fluid the pre-enrichment medium specified in 5.2.1.

9.1.2 In general, to prepare the initial suspension, add a 25 g test portion to 225 ml of pre-enrichment medium (5.2.1), which is the ratio of test portion to pre-enrichment medium specified in this method.

If the prescribed test portion is other than 25 g, use the necessary quantity of pre-enrichment medium to yield approximately a 1/10 dilution (mass to volume).

NOTES

5 To reduce the examination workload when more than one 25 g test portion from a specified lot of food has to be examined, and when evidence is available that compositing (pooling the test portions) does not affect the result for that particular food, the test portions may be composited. For example, if 10 test portions of 25 g are to be examined, combine the 10 units to form a composite test portion of 250 g and add 2,25 litres of pre-enrichment broth. Alternatively, the 0,1 ml (RV medium) and 10 ml (selenite/cystine medium) portions of the pre-enrichment broths from the 10 separate test portions (9.3.1) may be composited for enrichment in 0,1 litre and 1 litre respectively of selective enrichment medium.

6 Dried or powdered food products may need a special rehydration procedure to enhance the recovery of *Salmonella*. Two techniques may be used for this purpose, that of immersion and that of agitation. Refer for this purpose to the specific International Standard dealing with the product under examination. If such a standard is not available, it is recommended that the parties concerned come to an agreement on this subject.

9.2 Non-selective pre-enrichment

Incubate the initial suspension at 35 °C or 37 °C (as agreed) for not less than 16 h and not more than 20 h.

9.3 Selective enrichment

9.3.1 Transfer 0,1 ml of the culture obtained in 9.2 to a tube containing 10 ml of the RV medium (5.2.2); transfer 10 ml of the culture obtained in 9.2 to a flask containing 100 ml of selenite/cystine medium (5.2.3).

9.3.2 Incubate the two inoculated media (9.3.1) for 18 h to 24 h as follows:

a) the inoculated RV medium at 42 °C for 24 h;

b) the inoculated selenite/cystine medium at 35 °C or 37 °C (as agreed) for 24 h and a further 24 h.

NOTE 7 For the selenite/cystine medium, it may, in some cases, be advantageous to raise the incubation temperature to 42 °C. This modification should be indicated in the test report.

9.4 Plating out and identification

9.4.1 Using the culture obtained in the RV medium, after incubation for 24 h, inoculate, by means of a loop (6.7), the surface of one large-size Petri dish (6.13) containing the first selective plating-out medium (generally the phenol red/brilliant green agar, see 5.2.4.1), so that well-isolated colonies will be obtained.

In the absence of large dishes, use two small dishes, one after the other, using the same loop (see note 8).

Proceed in the same way with the second selective plating-out medium (5.2.4.2) using a new loop and Petri dishes of appropriate size.

NOTE 8 The following method of streaking is recommended when phenol red/brilliant green agar is used. Use one loop (6.7) for two dishes. Take a droplet from the edge of the surface of the fluid. Inoculate both dishes according to the two diagrams in annex D . Use the whole dish; loop streaks should be spaced about 0,5 cm apart. (Do not flame the loop or recharge it after making the first streak, nor when passing to the second dish.) When only one large dish is used, the method of streaking should be as indicated for the first dish in annex D.

9.4.2 Using the culture obtained in the selenite/cystine medium after incubation for 24 h, repeat the procedure described in 9.4.1 with the two selective plating-out media.

9.4.3 Invert the dishes (9.4.1) and (9.4.2) so that the bottom is uppermost, and place them in the incubator (6.3) set at 35 °C or 37 °C (as agreed).

9.4.4 After a total incubation period of 48 h of the selenite/cystine medium (see 9.3.2 and 9.4.3), repeat the procedure described in 9.4.2 and 9.4.3.

9.4.5 After incubation for 20 h to 24 h, examine the dishes (9.4.3 and 9.4.4) for the presence of typical colonies of *Salmonella*. Typical colonies of *Salmonella* grown on phenol red/brilliant green agar cause the colour of the medium to change from pink to red.

9.4.6 If growth is slight or if no typical colonies of *Salmonella* are present, reincubate at 35 °C or at 37 °C (as agreed) for a further 18 h to 24 h.

Re-examine the plates for the presence of typical colonies of *Salmonella*.

NOTE 9 Any typical or suspect colony should be subjected to a confirmation (9.5); the recognition of colonies of *Salmonella* is to a large extent a matter of experience, and their appearance may vary somewhat, not only from species to species, but also from batch to batch of medium. In this respect, agglutination, at this stage, of colonies with polyvalent *Salmonella* anti-serum may facilitate recognition of suspected colonies.

9.4.7 Identification kits currently available commercially and permitting the identification of *Salmonella* may be used.

9.5 Confirmation

9.5.1 Selection of colonies for confirmation

For confirmation, take from each dish of each selective medium (see 9.4.5 and 9.4.6), five colonies considered to be typical or suspect.

If on one dish there are fewer than five typical or suspect colonies, take for confirmation all the typical or suspect colonies.

Streak the selected colonies onto the surface of predried nutrient agar plates (5.2.5), in a manner which will allow well-isolated colonies to develop.

Incubate the inoculated plates at 35 °C or 37 °C (as agreed) for 18 h to 24 h.

Use pure cultures for biochemical and serological confirmation.

9.5.2 Biochemical confirmation

By means of an inoculating wire, inoculate the media specified in 9.5.2.1 to 9.5.2.6 with each of the cultures obtained from the colonies selected in 9.5.1.

9.5.2.1 TSI agar (5.2.6)

Streak the agar slope surface and stab the butt.

Incubate at 35 °C or 37 °C (as agreed) for 24 h.

Interpret the changes in the medium as follows:

Butt

yellow:	glucose positive (fermentation of glucose)
red or unchanged:	glucose negative (no fermentation of glucose)
black:	formation of hydrogen sulfide
bubbles or cracks:	gas formation from glucose

Slant surface

yellow:	lactose and/or sucrose positive (lactose and/or sucrose used)
red or unchanged:	lactose and sucrose negative (neither lactose nor sucrose used)

Typical *Salmonella* cultures show alkaline (red) slants with gas formation and acid (yellow) butts, with (in about 90 % of the cases) formation of hydrogen sulfide (blackening of the agar).

When a lactose-positive *Salmonella* is isolated (see 4.3), the TSI slant is yellow. Thus, preliminary confirmation of *Salmonella* cultures shall not be based on the results of the TSI agar test only (see 9.5.3).

9.5.2.2 Urea agar (5.2.7)

Streak the agar slope surface.

Incubate at 35 °C or 37 °C (as agreed) for 24 h and examine at intervals.

If the reaction is positive, splitting of urea liberates ammonia, which changes the colour of phenol red to rose-pink and later to deep cerise. The reaction is often apparent after 2 h to 4 h.

9.5.2.3 L-Lysine decarboxylation medium (5.2.8)

Inoculate just below the surface of the liquid medium.

Incubate at 35 °C or 37 °C (as agreed) for 24 h.

A purple colour after incubation indicates a positive reaction.

A yellow colour indicates a negative reaction.

9.5.2.4 Detection of β-galactosidase (5.2.9)

Suspend a loopful of the suspected colony in a tube containing 0,25 ml of the saline solution (5.2.13).

Add 1 drop of toluene and shake the tube.

Put the tube in a water bath (6.6) set at 35 °C or 37 °C (as agreed) and leave for several minutes.

Add 0,25 ml of the reagent for detection of β-galactosidase and mix.

Replace the tube in the water bath set at 35 °C or 37 °C (as agreed), leave for 24 h, examining the tube at intervals.

A yellow colour indicates a positive reaction. The reaction is often apparent after 20 min.

If prepared paper discs (5.2.9) are used, follow the manufacturer's instructions.

9.5.2.5 Medium for Voges-Proskauer (VP) reaction (5.2.10)

Suspend a loopful of the suspected colony in a sterile tube containing 0,2 ml of the VP medium (5.2.10.1).

Incubate at 35 °C or 37 °C (as agreed) for 24 h.