INTERNATIONAL STANDARD

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Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp.

Microbiologie des aliments — Méthode horizontale pour la recherche des Salmonella *spp.*

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 6579 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This fourth edition cancels and replaces the third edition (ISO 6579:1993), which has been technically revised.

Annexes A and B form a normative part of this International Standard. Annex C is for information only.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products, may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

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 this method 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.

Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp.

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella*, and especially *Salmonella* Typhi and *Salmonella* Paratyphi, 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 specifies a horizontal method for the detection of *Salmonella*, including *Salmonella* Typhi and *Salmonella* Paratyphi.

Subject to the limitations discussed in the Introduction, this International Standard is applicable to

— products intended for human consumption and the feeding of animals;

— environmental samples in the area of food production and food handling.

WARNING — The method may not recover all *Salmonella* Typhi and Paratyphi.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

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

ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

3 Terms and definitions

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

3.1
Salmonella
microorganisms which form typical or less 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 *Salmonella* (3.1), in a particular mass or volume of product, when tests are carried out in accordance with this International Standard

4 Principle

4.1 General

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

NOTE The *Salmonella* may be present in small numbers and are often accompanied by considerably larger numbers of other *Enterobacteriaceæ* or other families. Furthermore, pre-enrichment is necessary to permit the detection of low numbers of *Salmonella* or injured *Salmonella*.

4.2 Pre-enrichment in non-selective liquid medium

Buffered peptone water is inoculated at ambient temperature with the test portion, then incubated at 37 °C ± 1 °C for 18 h ± 2 h.

For certain foodstuffs the use of other pre-enrichment procedures is necessary. See 9.1.2.

For large quantities, the buffered peptone water should be heated to 37 °C ± 1 °C before inoculation with the test portion.

4.3 Enrichment in selective liquid media

Rappaport-Vassiliadis medium with soya (RVS broth) and Muller-Kauffmann tetrathionate/novobiocin broth (MKTTn broth) are inoculated with the culture obtained in 4.2.

The RVS broth is incubated at 41,5 °C ± 1 °C for 24 h ± 3 h, and the MKTTn broth at 37 °C ± 1 °C for 24 h ± 3 h.

4.4 Plating out and identification

From the cultures obtained in 4.3, two selective solid media are inoculated:

— xylose lysine deoxycholate agar (XLD agar);

— any other solid selective medium complementary to XLD agar and especially appropriate for the isolation of lactose-positive *Salmonella* and *Salmonella* Typhi and *Salmonella* Paratyphi strains; the laboratory may choose which medium to use.

The XLD agar is incubated at 37 °C ± 1 °C and examined after 24 h ± 3 h. The second selective agar is incubated according to the manufacturer's recommendations.

NOTE For information, Brilliant green agar (BGA), bismuth sulfite agar, etc., could be used as the second plating-out medium.

4.5 Confirmation of identity

Colonies of presumptive *Salmonella* are subcultured, then plated out as described in 4.4, and their identity is confirmed 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 Because of the large number of culture media and reagents, it is considered preferable, for clarity, to give their compositions and preparations in annex B.

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

See B.1.

5.2.2 First selective enrichment medium: Rappaport-Vassiliadis medium with soya (RVS broth)

See B.2.

5.2.3 Second selective enrichment medium: Muller-Kauffmann tetrathionate novobiocin broth (MKTTn broth)

See B.3.

5.2.4 Solid selective plating-out media

5.2.4.1 First medium: Xylose lysine deoxycholate agar (XLD agar)

See B.4.

5.2.4.2 Second medium

The choice of the second appropriate medium is left to the discretion of the testing laboratory. The manufacturer's instructions should be precisely followed regarding its preparation for use.

5.2.5 Nutrient agar

See B.5.

5.2.6 Triple sugar/iron agar (TSI agar)

See B.6.

5.2.7 Urea agar (Christensen)

See B.7.

5.2.8 L-Lysine decarboxylation medium

See B.8.

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

See B.9.

5.2.10 Reagents for Voges-Proskauer (VP) reaction

See B.10.

5.2.11 Reagents for indole reaction

See B.11.

5.2.12 Semi-solid nutrient agar

See B.12.

5.2.13 Physiological saline solution

See B.13.

5.3 Sera

Several types of agglutinating 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 only anti-sera prepared by a supplier recognized as competent (for example, by an appropriate government agency).

6 Apparatus and glassware

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

Usual microbiological laboratory 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 Drying cabinet or **oven**, ventilated by convection, capable of operating between 37 °C and 55 °C.

6.3 Incubator, capable of operating at 37 °C ± 1 °C.

6.4 Water bath, capable of operating at 41,5 °C ± 1 °C, or **incubator**, capable of operating at 41,5 °C ± 1 °C.

6.5 Water baths, capable of operating at 44 °C to 47 °C.

6.6 Water bath, capable of operating at 37 °C ± 1 °C.

It is recommended to use a water bath (6.4, 6.5 and 6.6) containing an antibacterial agent because of the low infective dose of *Salmonella*.

6.7 Sterile loops, of diameter approximately 3 mm or 10 µl, or **sterile pipettes**.

6.8 pH-meter, having an accuracy of calibration of ± 0,1 pH unit at 20 °C to 25 °C.

6.9 Test tubes or **flasks,** of appropriate capacity.

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

6.10 Graduated pipettes or **automatic pipettes**, of nominal capacities 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml divisions.

6.11 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 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 General

See ISO 6887-1 and the specific International Standard dealing with the product concerned. See ISO 8261 for milk and milk products.

For preparation of the initial suspension, in the general case use as diluent the pre-enrichment medium specified in 5.2.1 and 4.2 (buffered peptone water).

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

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 l of pre-enrichment broth. Alternatively, the 0,1 ml (in 10 ml of RVS broth) and 1 ml (in 10 ml of MKTTn broth) portions of the pre-enrichment broth from the 10 separate test portions (see 9.3.1) may be composited for enrichment in 100 ml of selective enrichment media.

9.1.2 Specific preparations of the initial suspension for certain foodstuffs

NOTE The following specific preparations concern only the case of *Salmonella*. Specific preparations applicable for the determination of any microorganisms are described in ISO 6887-2, ISO 6887-3, ISO 6887-4 and ISO 8261.

9.1.2.1 **Cocoa and cocoa-containing products** (e.g. more than 20 %)

Add to the buffered peptone water (5.2.1) preferably 50 g/l of casein (avoid the use of acid casein), or 100 g/l of sterile skim milk powder and add, after about 2 h incubation, 0,018 g/l of Brilliant green if the foodstuff is likely to be highly contaminated with Gram-positive flora.

9.1.2.2 Acidic and acidifying foodstuffs

Ensure that the pH does not fall to below 4,5 during pre-enrichment.

NOTE The pH of acidic and acidifying foodstuffs is more stable if double-strength buffered peptone water is used.

9.2 Non-selective pre-enrichment

Incubate the initial suspension (9.1) at 37 °C ± 1 °C for 18 h ± 2 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 RVS broth (5.2.2); transfer 1 ml of the culture obtained in 9.2 to a tube containing 10 ml of MKTTn broth (5.2.3).

9.3.2 Incubate the inoculated RVS broth (9.3.1) at 41,5 °C ± 1 °C for 24 h ± 3 h and the inoculated MKTTn broth at 37 °C ± 1 °C for 24 h ± 3 h. Care should be taken that the maximum allowed incubation temperature (42,5 °C) is not exceeded.

9.4 Plating out and identification

9.4.1 After incubation for 24 h ± 3 h, using the culture obtained in the RVS broth (9.3.2), inoculate by means of a loop (6.7) the surface of one large-size Petri dish (6.11) containing the first selective plating-out medium (XLD 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.

Proceed in the same way with the second selective plating-out medium (5.2.4.2) using a sterile loop and Petri dishes as above.

9.4.2 After incubation for 24 h ± 3 h, using the culture obtained in the MKTTn broth (9.3.2), 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 37 °C for the first plating-out medium (5.2.4.1). The manufacturer's instructions shall be followed for the second plating-out medium (5.2.4.2).

9.4.4 After incubation for 24 h ± 3 h, examine the plates (9.4.3) for the presence of typical colonies of *Salmonella* and atypical colonies that may be *Salmonella* (see Note). Mark their position on the bottom of the dish.

Typical colonies of *Salmonella* grown on XLD agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

NOTE *Salmonella* H_2S negative variants (e.g. *S.* Paratyphi A) grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening.

Incubate the second selective solid medium at the appropriate temperature and examine after the appropriate time to check for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

9.5 Confirmation

9.5.1 General

If shown to be reliable, commercially available identification kits for the biochemical examination of *Salmonella* may be used. The use of identification kits concerns the biochemical confirmation of colonies. These kits should be used following the manufacturer's instructions.

NOTE The recognition of colonies of *Salmonella* is to a large extent a matter of experience, and their appearance may vary somewhat, not only from serovar to serovar, but also from batch to batch of the selective culture medium used.