SLOVENSKI STANDARD
SIST EN 12824:1998

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Mikrobiologija živil in krmil - Horizontalna metoda za ugotavljanje prisotnosti salmonel (ISO 6579:1993, modificiran)

Microbiology of food and animal feeding stuffs - Horizontal method for the detection of Salmonella (ISO 6579:1993 modified)

Mikrobiologie von Lebensmitteln und Futtermitteln - Horizontales Verfahren zum Nachweis von Salmonellen (ISO 6579:1993 modifiziert)

Microbiologie des aliments - Méthode horizontale pour la recherche des Salmonella (ISO 6579:1993 modifiée)

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Microbiology of food and animal feeding stuffs - Horizontal method for the detection of Salmonella (ISO 6579:1993 modified)

Microbiologie des aliments - Méthode horizontale pour la recherche des Salmonella (ISO 6579:1993 modifiée)

Mikrobiologie von Lebensmitteln und Futtermitteln - Horizontales Verfahren zum Nachweis von Salmonellen (ISO 6579:1993 modifiziert)

This European Standard was approved by CEN on 26 September 1997.

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This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Central Secretariat has the same status as the official versions.

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Contents

Foreword

This European Standard has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods" the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 1998, and conflicting national standards shall be withdrawn at the latest by May 1998.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

Endorsement notice

In June 1995 the Standard ISO 6579:1993 was submitted to Questionnaire Procedure of CEN.

In view of the Questionnaire results, the Technical Board of CEN decided not to take over ISO 6579:1993 unchanged but to publish this European Standard EN 12824:1997 with agreed common modifications as indicated by a vertical line in the left margin of the text.

The main modifications are as follows:

- the possibility to incubate the Rappaport-Vassiliadis magnesium chloride/malachite green medium for a further 24 h; and

- the introduction of specific pre-enrichment procedures for some food products.

Introduction

This European Standard is intended to provide general guidance for the examination of products not dealt with by existing European 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, this guidance 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 European Standard is next reviewed, account will be taken of all information then available regarding the extent to which the guidance have been followed and the reasons for deviation from in the case of particular products.

The harmonization of test methods cannot be immediate and, for certain groups of products, European Standards and/or national standards may already exist that do not comply with this guidance. In cases where European 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 European Standard so that, eventually, the only remaining departures from this guidance those necessary for well-established technical reasons.

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 European Standard specifies a horizontal method for the detection of *Salmonella*.

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

The incubation temperature (35 °C or 37 °C) is subject to agreement by the parties concerned and is specified in the test report.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

ISO 6887	Microbiology - General guidance for the preparation of dilutions for microbiological examination
ISO 7218	Microbiology of food and animal feeding stuffs - General rules for microbiological examinations

3 Definitions

For the purposes of this European 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 European 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 European Standard.

4 Principle

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

NOTE : *Salmonella* may be present in small numbers and are often accompanied by considerably larger numbers of other members of *Enterobacteriaceae* 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.

For certain foodstuffs, other pre-enrichment procedures shall be used. See 9.1.3.

4.2 Enrichment in selective liquid media

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

Incubation of the Rappaport-Vassiliadis 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.

Note: In some cases, the Rappaport-Vassiliadis magnesium chloride/ malachite green medium may need to be further incubated for 24 h at 42 °C. This further incubation should, in this case, be noted in the test report.

4.3 Plating out and recognition

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

- phenol red/brillant green agar, unless the European 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 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 : 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 B.1.

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

See B.2.

5.2.3 Second selective enrichment medium : Selenite/cystine medium

See 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 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. Attention is drawn to the fact that a specific European Standard relating to the product to be examined, which specifies the composition of this second medium.

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 ß-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.

5.2.10.1 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 B.11.

5.2.11.1 Tryptone-tryptophan medium

5.2.11.2 **Kovacs reagent** (N, N-dicyclohexyl-carbodiimide pentachlorophenol complex)

5.2.12 Semi-solid nutrient agar

See B.12.

5.2.13 Saline solution

See 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 : 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 for 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 : 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 European Standard. See the specific European Standard dealing with the product concerned. If there is no specific European 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 European Standard dealing with the product concerned. If there is no specific European 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 European 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).

NOTE 1 : 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 (RV medium) and 10 ml (selenite/cystine medium) portions of the pre-enrichment broths from the 10 separate test portions (see 9.3.1) may be composited for enrichment in 0,1 l and 1 l respectively of selective enrichment medium.

NOTE 2 : 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. See also the specific European 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.

9.1.3 Specific preparations of the initial suspension for certain foodstuffs

9.1.3.1 Cocoa and cocoa containing products (for example more than 20 %)

Add to the buffered peptone water (5.2.1) preferably 50 g of casein, or 100 g of skim milk powder, and 0,1 g of brillant greeen if the foodstuff is highly contaminated with Gram + flora.

9.1.3.2 Herbs and spices

In order to avoid effects due to inhibitory compounds, use the necessary quantity of pre-enrichment medium (5.2.1) to yield approximately a 1/100 dilution (mass to volume).

9.1.3.3 Foodstuffs containing swelling agents

In order to avoid an incomplete suspension, use the necessary quantity of pre-enrichment medium (5.2.1) to yield approximately a 1/100 dilution (mass to volume).

It is also possible, to ease the suspension of these foodstuffs, to add to the buffered peptone water a solution of specific enzymes (i.e. gamanase for caroube products and guar, celluclast for carboxymethylcellulose).

9.1.3.4 Foodstuffs with a high fat content (i.e. more than 20 % of fat content)

Add to the buffered peptone water (5.2.1) 1 g to 10 g of sorbitol monoleate ("Tween 80® [1)]"), depending on the fat content.

9.1.3.5 Acid and acidifying foodstuffs

Ensure that the pH value does not decrease under 4,5 during pre-enrichment.

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 hours.

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) as follows :

a) the inoculated RV medium at 42 °C for 24 h (and, if necessary, for further 24 h-see note of 4.2) ;

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

[1)] Tween 80® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

NOTE : 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 (and, if necessary, further 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/brillant 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).

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 : The following method of streaking is recommended when phenol red/brillant 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 plates (9.4.3 and 9.4.4) for the presence of typical colonies of *Salmonella*. Typical colonies of *Salmonella* grown on phenol red/brillant 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 : 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 or omnivalent *Salmonella* anti-serum may facilitate recognition of suspected colonies.

9.4.7 Indentification 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), at least 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 hours.

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)