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Organic, organo-mineral and inorganic fertilisers — Detection of *Salmonella* spp.

Einführendes Element — Haupt-Element — Ergänzendes Element

Élément introductif — Élément central — Élément complémentaire

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European foreword

This document (CEN/prEN 17780) has been prepared by Technical Committee CEN/TC 260 “Fertilisers and liming materials”, the secretariat of which is held by DIN.

This is a working document.

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This document has been prepared under a Standardization Request given to CEN by the European Commission and the European Free Trade Association.

This document shall be used in combination with prEN 17803.

Introduction

This document describes a method for the detection of *Salmonella* spp. in fertilisers of the following Product Function Categories (PFCs) of EU fertilising products, as described in Regulation (EU) 2019/1009 [1]:

— PFC 1(A): Organic fertiliser;

— PFC 1(B): Organo-mineral fertiliser;

— PFC 1(C): Inorganic fertiliser, which contains more than 1 % by mass of organic carbon, other than organic carbon from chelating or complexing agents, nitrification inhibitors, denitrification inhibitors or urease inhibitors, coating agents, urea or calcium cyanamide.

This methodology has been developed to detect *Salmonella* spp. in organic, organo-mineral and inorganic fertilisers in order to control certain hygienic requirements imposed by Regulation (EU) 2019/1009 [1]. The method described in this document is based on EN ISO 6579-1 [4,5].

Salmonella spp. are rod-shaped Gram-negative bacteria of faecal/intestinal origin within the family *Enterobacteriaceae*. Consequently, presence of *Salmonella* spp. can be used as an indicator of faecal contamination and presence/absence of *Salmonella* spp. can be used as a parameter to evaluate the sanitation measures during the manufacturing process. The presence or absence of *Salmonella* spp. does not reflect the presence or absence of other pathogens in the examined product.

Because of the large variety of fertilisers, this method may not be appropriate in every detail for certain products. 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 method as far as possible.

Mineral components in fertilisers can have a negative impact on the survivability of microorganisms. In addition to an unfavourable shift in the pH value, these components can have a strong osmotic effect or be toxic to the microorganisms (e.g. copper). Therefore, it may be necessary to investigate the inhibitory effects of these components in a pre-test.

1 Scope

This document specifies a method for the detection of *Salmonella* spp. in organic fertilisers classified as PFC 1(A) in Regulation (EU) 2019/1009 [1], organo-mineral fertilisers classified as PFC 1(B), inorganic fertilisers classified as PFC 1 (C) which contain more than 1 % by mass of organic carbon, other than organic carbon from chelating or complexing agents, nitrification inhibitors, denitrification inhibitors or urease inhibitors, coating agents, urea or calcium cyanamide; and blends classified as PFC 7 in which the most abundant component by mass is PFC 1(A), PFC 1(B) or PFC 1(C) with the conditions stated above.

This document is also applicable to the blends of fertilising products where a blend is a mix of at least two of the following component EU fertilising products: fertilisers, liming materials, soil improvers, growing media, inhibitors or plant biostimulants, and where organic, organo-mineral or inorganic fertilisers as indicated above are the highest % in the blend by mass or volume, or in the case of liquid form by dry mass. If organic, organo-mineral or inorganic fertilisers as indicated above are not the highest % in the blend, the European Standard for the highest % of the blend applies. In case a blend of fertilising products is composed of components in equal quantity, or in case the component EU fertilising products used for the blend have identical formulations, the user decides which standard to apply.

Most of the *Salmonella* serovars are detected with the method described in this document. For the detection of some specific *Salmonella* serovars (e.g., *Salmonella* Typhi and *Salmonella* Paratyphi), additional cultivation steps might be necessary.

NOTE This method has been validated in an interlaboratory study with specific products that were present on the market during the study (Annex C). Products can change over time regarding their microbial composition and other characteristics, and the formulation of the products may also change in the future.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

prEN 17803, *Detection of specific pathogens*

EN 1482 (all parts), *Fertilisers and liming materials — Sampling and sample preparation*

EN ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations (ISO 7218)*.

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at https://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

3.1
***Salmonella* spp.**
bacteria which form typical colonies on the solid selective media described in this document and which display the morphological, physiological and biochemical characteristics described when the analysis is carried out in accordance with this document

3.2
detection of Salmonella
analysis for the presence or absence of *Salmonella* spp. (3.1), in 25 g or 25 ml of product, when tests are carried out in accordance with this document

4 Principle

The detection of *Salmonella* spp. requires four successive steps as specified in Annex A. The four steps are the non-selective pre-enrichment, the selective enrichment, the isolation of presumptive colonies after cultivation on two selective media, and the confirmation of presumptive colonies with a serological or biochemical test.

From the initial suspension on buffered peptone water (BPW), a pre-enrichment is obtained by incubation at 36 °C ± 2 °C for 18 h ± 2 h. From this non-selective pre-enrichment obtained, Rappaport-Vassiliadis Soya (RVS) broth is inoculated and incubated at 41,5°C ± 1 °C for 24 h ± 3 h. From the selective enrichment obtained, two different selective solid media based on different enzymatic reactions are inoculated and incubated at 36 °C ± 2 °C for 24 h ± 3 h (or according to the manufacturer's instructions if explicitly recommended). After incubation, and depending on the media chosen, it is determined if presumptive *Salmonella* spp. are present on the plates. Colonies of presumptive *Salmonella* spp. are subcultured on a non-selective agar (if needed). The identity of presumptive *Salmonella* spp. colonies is confirmed by means of appropriate serological or biochemical tests.

NOTE 1 *Salmonella* spp. can be present in small numbers and are often accompanied by considerably larger numbers of other bacteria of the same family (*Enterobacteriaceae*) or of other families. Enrichment is used to allow the detection of low numbers of *Salmonella* spp. or stressed *Salmonella* spp.

NOTE 2 Stressed microorganisms are defined here as those present in the environment that can be injured or that can have developed in harsh environments. Such microorganisms can be difficult to detect because they struggle to grow on selective media. However, under suitable conditions, they can repair the cellular damages and recover their normal properties.

NOTE 3 Validated alternative methods to detect *Salmonella* spp. based on molecular biology may be used if giving the same results as those given in this document (or e.g. comparable to prEN ISO/DIS 7218).

5 Culture media and reagents

Current laboratory practices in accordance with standards comparable to prEN ISO/DIS 7218 shall be followed. The composition of culture media and reagents and their preparation are specified in Annex B. Use either dehydrated complete media or constituents of uniform quality and chemicals of recognized analytical grade for uniformity of results during the preparation of media. In case of performance testing of the culture media and reagents, it is recommended to follow procedures in accordance with standards comparable to EN ISO 11133:2014 [3] and Annex B.

6 Equipment and consumables

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

Usual microbiological laboratory equipment as described in prEN ISO/DIS 7218 shall be used and, in particular, the following.

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

6.2 Drying cabinet or oven, capable of operating between 25 °C and 50 °C.

6.3 Incubator(s), capable of operating in the range 36 °C ± 2 °C and 41,5 °C ± 1 °C .

6.4 Water bath, capable of operating at 47 °C to 50 °C, containing an antibacterial agent.

6.5 Cooling unit, adjustable at 5 °C ± 3 °C.

6.6 Freezer, capable of operating at -20 °C ± 5 °C.

6.7 Sterile loops, of approximate diameter, 3 mm (10 µl volume).

6.8 pH-meter, with a reading to the nearest 0,1 pH unit from 20 °C to 25 °C.

6.9 Sterile tubes, **bottles**, or **flasks** with caps, of appropriate capacity.

6.10 Sterile filter, with a pore size of 0,2 µm.

6.11 Sterile Petri dishes, with a diameter of approximately 90 mm and (optional) a diameter of approximately 140 mm.

6.12 Sterile graduated pipettes or automatic pipettes, of appropriate nominal capacities

7 Sampling

Sampling is not included in this document. Sampling should be performed carefully, following the principles described in EN 1482 (all parts) with the appropriate adaptations to account for the specificities of organic, organo-mineral and mineral fertilisers, and for the microbiological quality of the samples

8 Preparation of test sample

The preparation of the test sample is not included in this document. The test sample shall be prepared following prEN 17803.

9 Procedure

9.1 General

The procedure as given in Annex A shall be followed.

9.2 Preparation of the initial suspension

Preparation of the initial suspension shall be performed according to prEN 17803.

9.3 Non-selective pre-enrichment

The initial suspension (9.2) shall be incubated at 36 °C ± 2 °C (6.3) for 18 h ± 2 h. It is allowed to keep the pre-enrichment culture at 5 °C ± 3 °C (6.5) for 72 h maximum after the incubation.

9.4 Selective enrichment

After the incubation, the RVS broth (B.2) shall be inoculated with 0,1 ml of the pre-enriched initial suspension (9.3). If the RVS broth has been stored at a low temperature, it shall be brought to room temperature before inoculation.

The inoculated RVS broth shall be incubated at 41,5°C ± 1 °C (6.3) for 24 h ± 3 h. It is allowed to keep the selective enrichment at 5 °C ± 3 °C (6.5) for 72 h maximum after the incubation [7, 8, 9, 10].

9.5 Isolation

From the selective enrichment (9.4), the surface of two selective media shall be inoculated by means of a 10 µl loop (6.7) or comparable, so that well-isolated colonies are obtained after incubation. The two selective media based on different biochemical reactions characteristic for *Salmonella* spp. (Annex D) shall be chosen by the testing laboratory.

The plates with the two selective media shall be allowed to reach room temperature if they were stored at a lower temperature. If necessary, dry the surface of the plates (6.2) before use, as indicated in EN ISO 11133:2014 [3].

To obtain well-isolated colonies, large-size Petri dishes (diameter approximately 140 mm) or two normal size plates (diameter approximately 90 mm) may be used (6.11).

The plates with the two selective media shall be incubated at 36 °C ± 2 °C (6.3) for 24 h ± 3 h, or according to the manufacturer's instructions if explicitly recommended.

After incubation, both selective media shall be checked for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella* spp.

9.6 Confirmation

9.6.1 General

Biochemical or serological testing can be used to determine whether a presumptive colony belongs to the genus *Salmonella* or not.

For serological testing, it is recommended to subculture this colony on a non-selective agar (B.5) to get a pure culture with enough material to work with. For biochemical testing, it is also possible to use a selective medium (Annex D) for subculturing, to ensure that there is a pure culture to work with.

NOTE Any other method capable of unambiguously identifying *Salmonella* spp. than the one described in this document [e.g. polymerase chain reaction (PCR) analyses or Matrix-Assisted Laser Desorption Ionization - Time-of-Flight Mass Spectrometry (MALDI-TOF)] may be used for confirmation of *Salmonella* spp. if giving the same results as the method specified in this document.

In cases where identification of *Salmonella* spp. is unclear, after choosing either biochemical testing (9.6.3) or serological testing (9.6.4), the testing option that had not been chosen initially shall be tried. After that, if the identification of *Salmonella* spp. is still unclear, other tests shall be carried out (e.g., PCR).

For the characterization of *Salmonella* spp. strains (optional), full serotyping is needed. Guidance for serotyping is given in e.g. CEN ISO/TR 6579-3 [6].

9.6.2 Selection of colonies for confirmation

Mark presumptive colonies on each plate (9.5). If well-isolated colonies are available on the selective media (9.5), the biochemical or serological confirmation can be performed directly on a presumptive colony. It is recommended to subculture this colony on a non-selective agar (B.5) to get a pure culture with enough material to work with.

Select one presumptive colony for confirmation. If the result is negative, select a combination of up to four presumptive colonies from both selective media (testing up to five colonies in total).

9.6.3 Biochemical testing

9.6.3.1 General

Inoculate the biochemical confirmation media with each of the cultures obtained from the colonies selected in 9.6.2. For biochemical confirmation of *Salmonella* spp*.,* at least the tests specified in 9.6.3.2 to 9.6.3.4 shall be performed. The tests specified in 9.6.3.5 and 9.6.3.6 may also be performed when the results of the other confirmation tests do not give a clear identification. The use of biochemical strips (9.6.3.7) replace the tests specified in 9.6.3.2 to 9.6.3.4.

9.6.3.2 Triple sugar/iron (TSI) agar

Streak the TSI agar (B.6) slant surface and stab the butt. Incubate at 36 °C ± 2 °C (6.3) for 24 h ± 3 h.

Interpret the changes in the medium as follows:

a) butt

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

b) slant surface

- yellow: lactose and/or sucrose positive (lactose and/or sucrose fermentation);
- red or unchanged: lactose and sucrose negative (no fermentation of lactose or sucrose).

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

When a lactose-positive *Salmonella* is isolated, 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 9.6.3.1).

NOTE Kligler-Hajna medium gives similar results as TSI agar.

9.6.3.3 Urea agar

Streak the urea agar (B.7) slant surface. Incubate at 36 °C ± 2 °C (6.3) for up to 24 h.

If the reaction is positive, urea is hydrolysed, liberating ammonia. This 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.

Typical *Salmonella* cultures do not hydrolyse urea, so that the colour of the urea agar will remain unchanged.

9.6.3.4 L-Lysine decarboxylation medium (LDC)

Inoculate just below the surface of the liquid LDC medium (B.8). Incubate at 36 °C ± 2 °C (6.3) for 24 h ± 3 h.

Turbidity and a purple colour after incubation indicate a positive reaction. A yellow colour indicates a negative reaction.

The majority of the typical *Salmonella* cultures show a positive reaction in LDC.

9.6.3.5 Detection of *β*-galactosidase (optional)

The β-galactosidase test can be used to distinguish *Salmonella enterica* subspecies *arizonae* and *diarizonae* and other members of the *Enterobacteriaceae* (all give a positive reaction) from other subspecies of *Salmonella enterica* (in general these give a negative reaction).

Several procedures to perform the β-galactosidase test exist. An example is given below.

Suspend a loopful of the suspected colony in a tube containing 0,25 ml of the saline solution (B.10).

Add one drop of toluene and shake the tube. Place the tube in a water bath set at 36 °C ± 2 °C (6.4) and leave for several minutes (approximately 5 min). Add 0,25 ml of the reagent for detection of β-galactosidase (B.8) and mix.

Replace the tube in the water bath set at 36 °C ± 2 °C (6.3) and leave for up to 24 h.

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

If prepared paper discs are used for the detection of β-galactosidase, follow the manufacturer's instructions.

9.6.3.6 Medium for indole reaction (optional)

The indole test can be used when there is a need to differentiate *Salmonella* (generally indole negative, see Table 1) from *Escherichia coli* and *Citrobacter* (both indole positive), as these organisms can give typical reactions on some of the *Salmonella* isolation media.

Inoculate a tube containing 5 ml of the tryptone/tryptophan medium (B.9.1) with the suspected colony.

Incubate at 36 °C ± 2 °C (6.3) for 24 h ± 3 h. After incubation, add 1 ml of the Kovacs reagent (B.9.2).

The formation of a red ring (surface layer) indicates a positive reaction. A yellow-brown ring (surface layer) indicates a negative reaction.

9.6.3.7 Biochemical strips (optional)

Biochemical strips can also be used to confirm presumptive Salmonella *spp*. colonies. Use the biochemical strips according to the manufacturer´s instructions.

9.6.4 Serological testing

9.6.4.1 General

Presumptive colonies (9.6.2) can be tested for the presence of *Salmonella* spp. O-and H-antigens by slide agglutination using polyvalent antisera (Annex B.11). Use the antisera according to the manufacturer's instructions if different from the method described below. The following tests (9.6.4.2 to 9.6.4.5) are the minimum required for serological testing of *Salmonella* spp. Strains that are auto-agglutinable cannot be tested for the presence of *Salmonella* spp. Antigens and shall be confirmed by biochemical tests (9.6.3).

NOTE Guidance on serological confirmation and on serotyping is given in e.g. CEN ISO/TR 6579-3 [6].

9.6.4.2 Elimination of auto-agglutinable strains

Place one drop of saline solution (Annex B.10) on a clean glass slide. Using a loop (6.7), disperse part of the colony to be tested in the saline solution in order to obtain a homogeneous and turbid suspension.

Rock the slide gently for 5 s to 60 s (depending on the manufacturer's instructions). Observe the suspension, preferably against a dark background. If the bacteria have formed granules in the suspension, this indicates auto-agglutination and serological confirmation will not be possible.

NOTE Additional information on the treatment of auto-agglutinating strains is given in e.g. CEN ISO/TR 6579-3 [6].

9.6.4.3 Examination for O-antigens

Using one non-auto-agglutinating pure colony, proceed according to 9.6.4.2 using one drop of polyvalent anti-O sera (Annex B.11) in place of the saline solution.

If agglutination occurs, this is considered a positive reaction.

9.6.4.4 Examination for H-antigens

Using one non-auto-agglutinating pure colony, proceed according to 9.6.4.2 using one drop of polyvalent anti-H sera (Annex B.11) in place of the saline solution.

If agglutination occurs, this is considered a positive reaction.

9.6.4.5 Interpretation of serological reactions

Table 1 gives the interpretation of the confirmatory serological tests (9.6.4.2, 9.6.4.3 and 9.6.4.4) carried out on the colonies used (9.6.2).

Table 1 – Interpretation of results of confirmatory serological tests

Culture morphology	Auto-agglutination	Serological reactions	Interpretation
Typical	No	O- and H-antigens positive	Detection
Typical	No	O- or H-antigens negative	Presumptive *Salmonella* spp., biochemical tests (9.6.3) necessary
Typical	Yes	Not tested because of auto-agglutination (see 9.6.4.2)	Presumptive *Salmonella* spp., biochemical tests (9.6.3) necessary

Atypical	No	O- and/or H-antigens positive	Presumptive *Salmonella* spp., biochemical tests (9.6.3) necessary
Atypical	No	O- and/or H-antigens negative	Not considered *Salmonella* spp.

9.6.5 Serotyping

If required, confirmed strains can be sent to a recognized *Salmonella* reference centre for definitive typing (serotyping, phage typing, molecular typing). If the strain is sent to a reference centre, it should be accompanied by all relevant information, such as confirmation results and source from which the strain was isolated.

10 Expression of results

The results shall indicate if confirmed *Salmonella* spp. have been detected or not in the test portion of 25 g or 25 ml of product according to the procedure (9) specified in this document.

11 Performance assays

The content of this clause will be added after the interlaboratory study has been completed.

12 Test report

The test report shall contain at least the following information:

— all information necessary for the complete identification of the sample;

— the sampling method used (EN 1482), with an indication of all deviations from the sampling method, if known;

— the size of the test portion and/or the nature of the object examined;

— the test method used, with reference to this document;

— any deviation in the media or the incubation conditions or confirmation used;

— any unusual features observed;

— all operating conditions not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the result(s);

— the results obtained, indicating clearly the method of expression used;

— if repeatability has been checked, the final result obtained; and

— the date(s) of start and end of the test.

Annex A
(normative)

Diagram of the procedure

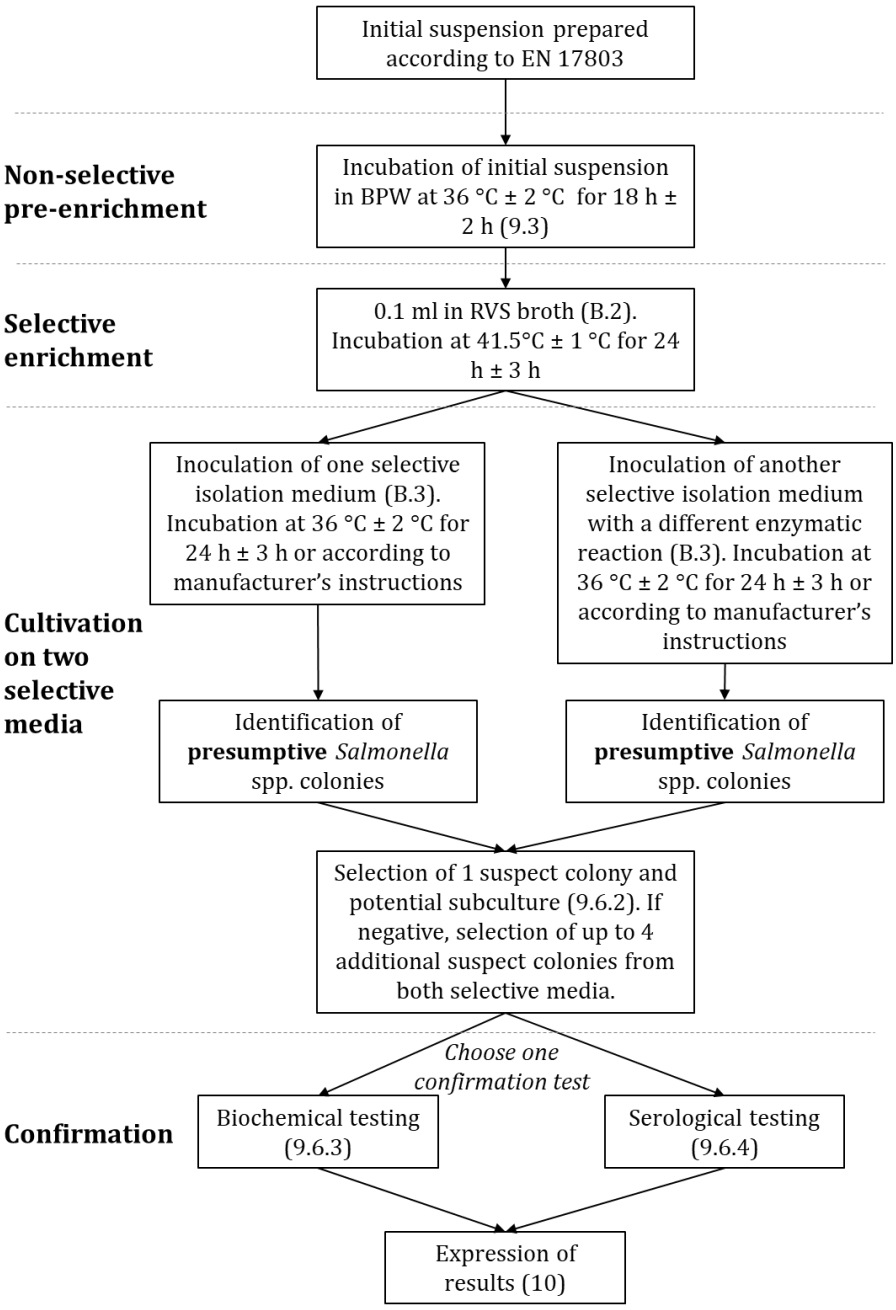

Figure A.1 — Diagram of procedure for the detection of *Salmonella* spp. in organic, organo-mineral and inorganic fertilisers

Annex B
(normative)

Culture media and reagents

B.1 General

The preparation and performance of culture media is a fundamental step to ensure the integrity of microbiological examination.

When ready-to-use media are used, the manufacturers of these media should have a quality programme that ensures the quality of the media they supply, according to e.g. EN ISO 11133:2014 [3]. Under these conditions, the user/laboratory does not need to run additional testing on such media, but shall ensure the storage conditions according to the manufacturers' recommendations.

For diluents and media prepared by the user/laboratory directly from commercially available dehydrated formulations and/or from basic individual components, the performance of these diluents/media should be evaluated according to e.g. EN ISO 11133:2014 [3].

B.2 Rappaport-Vassiliadis Soya (RVS) broth

B.2.1 Solution A

B.2.1.1 Composition

Enzymatic digest of soya	4,5 g
Sodium chloride	7,2 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,26 g
Dipotassium hydrogen phosphate (K_2HPO_4)	0,18 g
Water	1 000 ml

B.2.1.2 Preparation

Dissolve the components in water by heating to about 70 °C, if necessary.

The solution shall be prepared on the day of preparation of the complete RVS broth.

B.2.2 Solution B

B.2.2.1 Composition

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	400 g
Water	1000 ml

B.2.2.2 Preparation

Dissolve the magnesium chloride in water.

As this salt is very hygroscopic, it is advisable to dissolve the entire contents of $MgCl_2 \cdot 6H_2O$ from a newly opened container according to the formula. For instance, 250 g of $MgCl_2 \cdot 6H_2O$ is added to 625 ml of water, giving a solution of total volume of 788 ml and a mass concentration of about 31.7 g per 100 ml of $MgCl_2 \cdot 6H_2O$.
The solution may be kept in a dark glass bottle with tight stopper at room temperature for at least two years.

B.2.3 Solution C

B.2.3.1 Composition

Malachite green oxalate	0,4 g
Water	100 ml

B.2.3.2 Preparation

Dissolve the malachite green oxalate in water.

The solution may be kept in a dark glass bottle at room temperature for at least eight months.

B.2.4 Complete medium

B.2.4.1 Composition

Solution A	1000 ml
Solution B	100 ml
Solution C	10 ml

B.2.4.2 Preparation

Add to 1000 ml of solution A, 100 ml of solution B, and 10 ml of solution C.

Dispense the medium into closed flasks (6.9) with a volume of 10 ml.

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

If necessary, adjust the pH (6.8) to 5,2 ± 0,2 at 25 °C after sterilization.

Store the medium at 5 °C ± 3 °C (6.5) for up to three months.

NOTE: Commercially available RVS broth can be used.

B.3 Selective media

At least two different selective media based on two different biochemical reactions characteristic for *Salmonella* spp. (Annex D, Table D.1) shall be used in this method. For examples of such media, see Annex D and Annex E.

B.4 Nutrient agar (example of non-selective medium)

B.4.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Sodium chloride (NaCl) (optional)	5,0 g
Agar	9 g to 18 g
Water	1 000 ml

B.4.2 Preparation

Dissolve the components or the dehydrated complete medium in water by heating, with frequent agitation.

Transfer the culture medium into tubes or flasks (6.9) of appropriate capacity. Sterilize for 15 min in the autoclave (6.1) set at 121 °C. If necessary, adjust the pH (6.8) to 7,0 ± 0,2 at 25 °C after sterilization.

B.4.3 Preparation of nutrient agar plates

Cool the medium to 47 °C to 50 °C in a water bath (6.4), mix, and pour into sterile Petri dishes (6.11). Allow to solidify.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven (6.2) set between 25 °C and 50 °C until the surface of the agar is dry.

Store the poured plates protected from drying, at 5 °C ± 3 °C (6.5) for up to four weeks.

B.5 Triple sugar/iron agar (TSI agar; example for H_2S production agar)

B.5.1 Composition

Meat extract	3,0 g
Yeast extract	3,0 g
Peptone	20,0 g
Sodium chloride (NaCl)	5,0 g
Lactose	10,0 g
Sucrose	10,0 g
Glucose	1,0 g
Iron(III) citrate	0,3 g
Sodium thiosulfate	0,3 g
Phenol red	0,024 g

Agar	9 g to 18 g[1]
Water	1 000 ml

B.5.2 Preparation

Dissolve the components or the dehydrated complete medium in water by heating with frequent agitation.

Dispense the medium into tubes or bottles (**Erreur ! Source du renvoi introuvable.**) in aliquots of 10 ml.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C. If necessary, adjust the pH (6.8) to 7,4 ± 0,2 at 25 °C after sterilization.

Allow to set in a sloping position to give a butt of depth 2,5 cm to about 5 cm. Store the poured tubes protected from drying, at 5 °C ± 3 °C (6.5) for up to four weeks.

B.6 Urea agar (Christensen)

B.6.1 Base medium

B.6.1.1 Composition

Peptone[a]	1,0 g
Glucose	1,0 g
Sodium chloride (NaCl)	5,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	2,0 g
Phenol red	0,012 g
Agar	9 g to 18 g[b]
Water	1000 ml

[a] For example, enzymatic digest of gelatine.

[b] Depending on the gel strength of the agar.

B.6.1.2 Preparation

Dissolve the components or the dehydrated complete base in water by heating with frequent agitation.

If necessary, adjust the pH (6.8) to 6,8 ± 0,2 at 25 °C after sterilization.

Pour the base medium into tubes or flasks (6.9) of appropriate capacity.

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

The base medium may be stored in closed tubes or flasks at 5 °C ± 3 °C (6.5) for up to three months.

[1] Depending on the gel strength of the agar.

B.6.2 Urea solution

B.6.2.1 Composition

Urea	400 g
Water, to a final volume of	1 000 ml

B.6.2.2 Preparation

Dissolve the urea in water. Sterilize by filtration through a filter with a pore size of 0,22 µm (6.10).

B.6.3 Complete medium

B.6.3.1 Composition

Base medium (B.6.1)	950 ml
Urea solution (B.6.2)	50 ml

B.6.3.2 Preparation

Under aseptic conditions, add the urea solution to the base previously melted and then cooled to 47 °C to 50 °C.

Dispense the complete medium into sterile tubes (6.9) in quantities of 10 ml.

Allow the medium to set in a sloping position.

Store the poured tubes protected from drying, at 5 °C ± 3 °C (6.5) for up to four weeks.

B.7 L-Lysine decarboxylation medium (LDC)

B.7.1 Composition

L-Lysine monohydrochloride	5,0 g
Yeast extract	3,0 g
Glucose	1,0 g
Bromocresol purple	0,015 g
Water	1000 ml

B.7.2 Preparation

Dissolve the components in water by heating, if necessary.

If necessary, adjust the pH (6.8) to 6,8 ± 0,2 at 25 °C after sterilization.

Transfer the medium in quantities of 2 ml to 5 ml to narrow tubes (6.9) with screw caps.

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

Store the poured tubes at 5 °C ± 3 °C (6.5) for up to three months.

B.8 β-galactosidase reagent (optional)

Additional to the reagent described below, toluene is needed for the β-galactosidase test.

B.8.1 Buffer solution

B.8.1.1 Composition

Sodium dihydrogen phosphate (NaH_2PO_4)	6,9 g
Sodium hydroxide, 10 mol/l solution	approx. 3 ml
Water, to a final volume of	50 ml

B.8.1.2 Preparation

Dissolve the sodium dihydrogen phosphate in approximately 45 ml of water in a volumetric flask.

Adjust the pH (6.8) to 7,0 ± 0,2 at 25 °C with the sodium hydroxide solution.

Add water to a final volume of 50 ml.

The buffer solution may be stored in closed flasks at 5 °C ± 3 °C (6.5) for up to six months.

B.8.2 ONPG solution

B.8.2.1 Composition

o-Nitrophenyl β-D-galactopyranoside (ONPG)	0,08 g
Water	15 ml

B.8.2.2 Preparation

Dissolve the ONPG in water at approximately 50 °C.

Cool the solution.

B.8.3 Complete reagent

B.8.3.1 Composition

Buffer solution (B.8.1)	5 ml
ONPG solution (B.8.2)	15 ml

B.8.3.2 Preparation

Add the buffer solution to the ONPG solution.

Store the complete reagent in closed flasks (6.9) at 5 °C ± 3 °C (6.5) for up to three months. Discard the complete reagent as soon as the colour changes to yellow.

B.9 Medium and reagent for indole reaction

B.9.1 Tryptone/tryptophan medium

B.9.1.1 Composition

Tryptone	10 g
Sodium chloride (NaCl)	5 g
DL-Tryptophan	1 g
Water	1 000 ml

B.9.1.2 Preparation

Dissolve the components in boiling water.

If necessary, adjust the pH to (6.8) 7,5 ± 0,2 at 25 °C after sterilization.

Dispense the medium into tubes (6.9) in aliquots of 5 ml.

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

Store the poured tubes at 5 °C ± 3 °C (6.5) for up to three months.

B.9.2 Kovacs reagent (optional)

B.9.2.1 Composition

4-Dimethylaminobenzaldehyde	5 g
Hydrochloric acid, ρ = 1,18 g/ml to 1,19 g/ml	25 ml
2-Methyl-2-butanol	75 ml

B.9.2.2 Preparation

Mix the components.

Store the complete reagent in closed flasks (6.9) in the dark at 5 °C ± 3 °C (6.5) for up to six months.

B.10 Saline solution

B.10.1 Composition

Sodium chloride (NaCl)	8,5 g
Water	1 000 ml

B.10.2 Preparation

Dissolve the sodium chloride in water.

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

If necessary, adjust the pH (6.8) to 7,0 ± 0,2 at 25 °C after sterilization.

Dispense the solution into flasks or tubes (6.9) of suitable capacity to obtain the portions necessary for the test.

Store the solution in closed flasks/tubes at 5 °C ± 3 °C (6.5) for up to six months.

B.11 Antisera

Several types of agglutinating sera containing antibodies for one or several O-antigens are available commercially, i.e. antisera containing one or more "O" groups (called monovalent or polyvalent anti-O sera), and antisera containing antibodies for one or several H-factors (called monovalent or polyvalent anti-H sera). Commercially available *Salmonella* latex kits can be used/are preferred in order to carry out the serological testing. Commercially available latex kits that do not allow to differentiate between O- and H- antigens shall not be used.

B.12 Performance testing for the quality assurance of the culture media

The definition of selectivity and productivity is specified in e.g. EN ISO 11133:2014 [3]. In general, it is recommended to follow the procedures for performance testing described in e.g. EN ISO 11133:2014 [3]. For the other media, the inoculum levels for the target and the non-target organisms are specified in e.g. EN ISO 11133:2014 [3].

Annex C
(informative)

Method validation studies and performance characteristics

[Comment from the project leader: *Please note that Annex C will be developed after the interlaboratory studies have been performed (prior to the CEN Enquiry).****]***.

NOTE This method has been validated in an interlaboratory study for the following materials:

Annex D
(informative)

Examples of selective media with different biochemical reactions characteristic for *Salmonella* spp.

NOTE 1 Derived from EN ISO 6579-1 [4]

NOTE 2 The list of selective media in Table D.1 is not exhaustive.

NOTE 3 Two different selective media based on different indicator systems (with "X" in different columns of Table D.1) shall be chosen for isolation of presumptive *Salmonella* spp. Colonies (9.4).

Table D.1 – Indicator systems used in some selective media for isolation of *Salmonella* spp. Indicated are reactions as shown by the majority of the *Salmonella* spp. strains. Between brackets the concentration of the relevant agent is given in g/l

Medium[a]	*α*-galactosidase positive	*β*-galactosidase negative	*β*-glucosidase negative	Esterase positive	Cellobiose neg.	H_2S pos.	Lactose negative	Lysine decarboxylase positive[b]	Mannitol positive	Propylene glycol positive	Salicin negative	Sucrose negative	Trehalose pos.	Xylose positive
ABC[c]	X	X												
BGA							X (10)					X (10)		
BS						X								
BSA™ (OSCM II)[d]			X	X										
CHROMagar™ Salmonella[i]		X	X	X										
CHROMagar™ Salmonella Plus[i]			X	X										
CSE				X			X (14,6)							
DCLS							X (5,0)					X (5,0)		
DCA						X	X (10,0)							
HE						X	X (12,0)				X (2,0)	X (12,0)		
MLCB						X		X (5,0)	X (3,0)					
MM		X			X (5,0)	X	X (10,0)		X (1,2)				X (1,33)	
Önöz[e]						X	X (11,5)					X (13,0)		
Rambach™ Agar[i]		X								X (10,5)				
chromID® *Salmonella* (SMID2)[f]		X	X	X			X (6,0)							
SS						X	X (10,0)							
XLD						X	X (7,5)	X (5,0)				X (7,5)		X (3,75)
XLT4						X	X (7,5)	X (5,0)				X (7,5)		X (3,75)
ASAP®[f]			X	X										
IBISA®[f]			X	X										

Table D.1 *(continued)*

Medium[a]	*α*-galac-tosidase positive	*β*-galac-tosidase negative	*β*-glu-cosidase negative	Esterase positive	Cello-biose neg.	H_2S pos.	Lactose negative	Lysine decar-boxylase positive[b]	Mannitol positive	Propylene glycol positive	Salicin negative	Sucrose negative	Tre-halose pos.	Xylose positive
IRIS *Salmonella*®[g]			X	X										
Rapid'*Salmonella*[h]			X	X										

[a] ABC: *αβ*-Chromogenic medium (www.labm.com); BGA: Brilliant Green Agar; BS: Bismuth sulphite agar; BSA: Brilliance™ *Salmonella* Agar (formerly called OSCM II: Oxoid *Salmonella* Chromogenic medium II; www.oxoid.com); CSE: Chromogenic *Salmonella* Esterase; DCLS: Desoxycholate Citrate Lactose agar; DCA: Desoxycholate Citrate agar (Leifson agar); HE: Hektoen Enteric agar; MLCB: Mannitol Lysine Crystal Violet Brilliant green agar; MM: Miller Mallinson agar; SMID2: *Salmonella* Identification medium II (also known as chromID *Salmonella*;www.biomerieux.com); SS: *Salmonella Shigella* agar; XLD: Xylose Lysine Deoxycholate agar; XLT4: Xylose Lysine Tergitol 4 agar.

[b] The agent is L-lysine hydrochloride.

The following information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results:

[c] LABM is an example of a suitable supplier of ABC medium.

[d] BSA™ is a trade name of a product available commercially and supplied by Oxoid.

[e] Merck is an example of a suitable supplier of Önöz medium.

[f] chromID® *Salmonella*, ASAP®, IBISA® are trade names of products available commercially and supplied by bioMerieux.

[g] IRIS *Salmonella*® is a trade name of a product available commercially and supplied by SOLABIA S.A.S. and BIOKAR Diagnostics.

[h] Biorad is an example of a suitable supplier of Rapid'*Salmonella.*

[i] CHROMagar™ Salmonella, CHROMagar™ Salmonella Plus, Rambach™ Agar are trade names of products available commercially and supplied by CHROMagar.

Annex E
(informative)

Additional examples and manufacturers of selective media with two different biochemical reactions characteristic for *Salmonella* spp.

NOTE The lists of selective media showing C-8 esterase activity (Table E.1) and H_2S production (Table E.2) are not exhaustive.

Table E.1 – Examples of selective chromogenic media for the expression of the C8-esterase activity

Medium	Examples of manufacturers
ASAP® agar	Biomérieux
CHROMagar® Salmonella	Chromagar
ChromID® Salmonella	Biomérieux
CASE[a]	Neogen
Compass® Salmonella	Solabia
IRISa Salmonella®	Solabia
Rapid'Salmo	Bio-rad
Salmonella Chromogenic agar	Oxoid
[a] CASE : Chromogenic Agar for Salmonella Esterase ; ABC : α β Chromogenic	

Table E.2 – Examples of confirmation selective media highlighting the production of H_2S

Medium	Examples of manufacturers
Bismuth Sulfite agar	Oxoid, Sigma-Aldrich, Merck millipore
Hektoen	Bio-rad
Kligler-Hajna	Bio-rad, Oxoid
Miller-Mallinson	Sigma-Aldrich, BD, VWR
Önöz	Merck
Shigella Salmonella	BD, Neogen, Oxoid, Solabia
TSI	BD, Oxoid, Bio-rad
XLD	Solabia

Preparation and storage of the media shall be carried out in accordance with the manufacturer's instructions.

Bibliography

[1] Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003

[2] EN ISO 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination (ISO 6887)*

[3] EN ISO 11133:2014[2], *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media (ISO 11133)*

[4] EN ISO 6579-1, *Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella — Part 1: Detection of Salmonella spp. (ISO 6579-1)*

[5] EN ISO 6579-1:2017/A1:2020, Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 1: Detection of *Salmonella* spp. AMENDMENT 1: Broader range of incubation temperatures, amendment to the status of Annex D, and correction of the composition of MSRV and SC (ISO 6579-1:2017/Amd 1:2020)

[6] CEN ISO/TR 6579-3:2014, *Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella — Part 3: Guidelines for serotyping of Salmonella spp. (ISO/TR 6579-3:2014)*

[7] D'AOUST J.Y. MAISHMENT C. BRUGENER D.M. CONLEY D.R., LOIT A., MILLING M. AND PURVIS U. *Detection of Salmonella in refrigerated preenrichment and enrichment broth cultures. J. Food Prot*. 1980, 43 (5) pp. 343–345

[8] D'AOUST J.Y. BECKERS H.J. BOOTHROYD M. MATES A., MCKEE C.R., MORAN A.B., SADO P., SPAIN G.E., SPERBER W.H., VASSILIADIS P., WAGNER D.E. AND WIBERG C. ICMSF methods studies. XIV. *Comparative study on recovery of Salmonella from refrigerated preenrichment and enrichment broth cultures. J. Food Prot.* 1983, 46 (5) pp. 391–399

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[10] DAVIES R.H. BEDFORD S. AND SHANKSTER S. *Enhanced culture techniques for the detection of Salmonella*. *Vet. Rec.* 2001, pp. 539–540

[11] Community reference laboratory for salmonella. *Analytical methods — Detection of Salmonella*: https://www.eurlsalmonella.eu/publications/eurl-manual (site visited on 2020-12-08)

[2] As impacted by EN ISO 11133:2014/A1:2018 and EN ISO 11133:2014/A2:2020.