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Milk and milk products — Determination of antimicrobial residues — Tube diffusion test

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

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An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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

ISO/TS 26844 | IDFRM 215 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a Standing Committee may decide to publish an other type of normative document which is called by IDF: *Reviewed method*. Such a method represents an agreement between the members of a Standing Committee and is accepted for publication if it is approved by at least 50 % of the committee members casting a vote. A *Reviewed method* is equal to an ISO/PAS or ISO/TS and will, therefore, also be published jointly under ISO conditions.

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

ISO/TS 26844 | IDF/RM 215 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Action Team on *Veterinary residues*, of the Standing Committee on *Analytical methods for additives and contaminants*, under the aegis of its project leaders, Mr H. Stegeman (NL) and Mr J. Kerkhof (NL).

Milk and milk products — Determination of antimicrobial residues — Tube diffusion test

1 Scope

This Technical Specification (Reviewed Method) specifies a microbiological inhibitor test for the detection of a broad variety of antimicrobials in milk and milk products.

The method is applicable to raw milk, heat-treated milk and reconstituted dried milk.

2 Normative references

The following referenced documents are indispensable for the application 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.

ISO 4833, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of micro-organisms — Colony-count technique at 30 °C*

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

ISO 13969 | IDF 183, *Milk and milk products — Guidelines for a standardized description of microbial inhibitor tests*

ISO 18330 | IDF 188, *Milk and milk products — Guidelines for a standardized description of immunoassays or receptor assays for the detection of antimicrobial residues*

3 Terms and definitions

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

3.1
antimicrobial substances
substances that show an inhibition in the procedure specified in this document

3.2
limits of detection
concentration level at which a defined percentage of positive samples is detected

EXAMPLE 95 %.

4 Principle

A milk sample is added to two test tubes with agar media containing *Geobacillus stearothermophilus* ATCC 10149 (identical to NIZO strain C953). The test tubes differ from each other in pH, in added supplements and synergistic antibiotics. Incubation resulting in normal growth of the organism causes the pH

indicator in the agar to change colour from purple to yellow. When substances that are inhibitory to the growth of microorganisms are present in the milk, the colour of the pH indicator will remain purple.

Test tube A (pH 7,0; chloramphenicol) shows an improved sensitivity for tetracycline residues, and test tube B (pH 8,0; trimethoprim) for beta-lactams, macrolides, aminoglycosides, sulfonamides and trimethoprim residues.

5 Test organism, culture media, standard solutions and control samples

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or deionized water or water of equivalent purity.

5.1 Test organism

Use a suspension of *Geobacillus stearothermophilus* ATCC 10149 (identical to NIZO strain C953) [1)] adjusted to a viable count of approximately 5 000 000 colony-forming units/ml (see Annex B for preparation). Check the quality of each new batch of the test-organism suspension by determining the sensitivity for the standard solutions mentioned in Table 1.

Table 1 — Standard solutions for testing the sensitivity of the test-organism suspension

Standard solution	Content µg/kg milk
Benzylpenicillin (Penicillin-G)	2
Sulfadiazine	150
Neomycin	30
Erythromycin	10
Oxytetracycline	100

Perform the check with standard solutions and control milk in 5-fold, according to the procedure described in Clause 9. Determine the sensitivity of the test-organism suspension for benzylpenicillin and oxytetracycline with tube A (5.2.5) and the sensitivity for sulfadiazine, neomycin and erythromycin with tube B (5.2.6). A positive result should be obtained in all test tubes.

5.2 Culture media

In order to improve the reproducibility of the method, it is recommended to use dehydrated basic components or dehydrated complete media for the preparation of culture media. Follow the manufacturers' instructions.

5.2.1 Basic medium

5.2.1.1 Components

Casein trypton	5,0 g
Yeast extract	2,5 g
Glucose, anhydrous	1,0 g
Agar	10 g to 15 g
Water	1 000 ml

NOTE The basic dehydrated medium is commercially available as Plate Count Agar.

1) Suspension of *Geobacillus stearothermophilus* ATCC 10149 or NIZO strain C953 is an example of a product available commercially. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by ISO or IDF of this product.

5.2.1.2 Preparation

Dissolve the components in the water by heating. Adjust the pH so that after sterilization it is 7,0 ± 0,2.

Autoclave the medium at 121 °C ± 1 °C for 15 min.

The thus-prepared basic medium may be kept for a maximum of 3 months if stored in the dark at 0 °C to +5 °C.

5.2.2 Bromocresolpurple solution

5.2.2.1 Components

Bromocresolpurple	250 mg
Ethanol, 96%	5 ml
Water	100 ml

5.2.2.2 Preparation

Dissolve the bromocresolpurple in the ethanol. Dilute with water to 100 ml.

The bromocresolpurple solution may be kept for a maximum of 6 months if stored in the dark at 0 °C to +5 °C.

5.2.3 Chloramphenicol (CAP) solution

5.2.3.1 Components

Chloramphenicol	20,0 mg
Methanol	5 ml
Water	100 ml

5.2.3.2 Preparation

Dissolve the chloramphenicol in the methanol. Dilute with water to 100 ml.

The chloramphenicol solution may be kept for a maximum of 1 month if stored in the dark at 0 °C to +5 °C.

5.2.4 Trimethoprim (TMP) solution

5.2.4.1 Components

Trimethoprim	25,0 mg
Ethanol, 96 %	5 ml
Water	1 000 ml

5.2.4.2 Preparation

Dissolve the trimethoprim in the ethanol. Dilute with water to 1 000 ml.

The TMP solution may be kept for a maximum of 1 month if stored in the dark at 0 °C to +5 °C.

5.2.5 Test tubes A (pH 7)

Melt the basic medium (5.2.1). Cool the medium in a water bath (6.3) to 63 °C ± 1 °C. Add 1,5 ml of CAP solution (5.2.3) and 2 ml of bromocresolpurple solution (5.2.2) to 100 ml of the preheated basic medium, while keeping the medium in the water bath set at 63 °C. Mix the medium well.

Keeping the medium in the water bath set at 63 °C, adjust the pH (see 6.5) to 7,0 ± 0,1 at that temperature by using 1 mol/l NaOH or 1 mol/l HCl.

Subsequently, add per 100 ml of medium such an amount (approx. 2 ml) of test-organism suspension (5.1) that in the absence of antimicrobial substances the colour change does appear after incubation in the water bath at 63 °C for 4 h 15 min ± 30 min.

Mix and dispense the test medium in portions of 1 ml in tubes (6.4) and leave the medium to solidify.

The test tubes A may be stored at 0 °C to +5 °C for a maximum of 3 days, provided that the tubes are covered to avoid evaporation (e.g. with parafilm).

5.2.6 Test tubes B (pH 8)

Melt the basic medium (5.2.1). Cool the medium in a water bath (6.3) to 63 °C ± 1 °C. Add 0,6 ml of TMP solution (5.2.4) and 2 ml of bromocresolpurple solution (5.2.2) to 100 ml of the preheated basic medium, while keeping the medium in the water bath set at 63 °C. Mix the medium well.

Keeping the medium in the water bath set at 63 °C, adjust the pH (see 6.5) to 8,00 ± 0,02 at that temperature by using 1 mol/l NaOH or 1 mol/l HCl. Take care that while adjusting, the pH of the medium does not exceed a value of 8,05.

Subsequently, add an amount (approx. 2 ml) of test-organism suspension (5.1) per 100 ml of medium so that in the absence of antimicrobial substances the colour change appears after the incubation in the water bath at 63 °C for 4 h 15 min ± 30 min.

Mix and dispense the test medium in portions of 1 ml in tubes (6.4) and leave the medium to solidify.

The test tubes B may be stored at 0 °C to +5 °C for a maximum of 3 days, provided that the tubes are covered to avoid evaporation (e.g. with parafilm).

5.3 Standard solutions and control samples

Correct all weighing for purity and salt contents in accordance with ISO 13969 | IDF 183.

For the preparation of standard solutions and control samples, it may be assumed that 1 ml of solution is equal to 1 g of solution.

5.3.1 Benzylpenicillin standard solutions and control samples

Taking into account the limited stability of benzylpenicillin, it is advisable to prepare all the benzylpenicillin standard solutions freshly and to freeze the control milk samples on the same day at below −18 °C.

5.3.1.1 Benzylpenicillin standard stock solution

Dissolve 20,0 mg ± 0,1 mg of benzylpenicillin in 1 000 ml of water and mix. The thus-prepared benzylpenicillin standard stock solution contains 20 mg/l of benzylpenicillin.

The benzylpenicillin standard stock solution may be kept for a maximum of 2 days if stored at 0 °C to +5 °C.

5.3.1.2 Benzylpenicillin standard working solution

Dilute 10 ml of benzylpenicillin standard stock solution (5.3.1.1) with water to 1 000 ml and mix. The thus-prepared benzylpenicillin standard working solution contains 200 µg/l of benzylpenicillin.

5.3.1.3 Benzylpenicillin control milk sample

Dilute 1 ml of benzylpenicillin standard working solution (5.3.1.2) with negative milk (5.4) to 100 ml and mix. The thus-prepared control milk sample contains 2 µg/l of benzylpenicillin.

The benzylpenicllin control milk sample may be kept for a maximum of 2 months if stored in test tubes (6.4) at below −18 °C.

NOTE 1 mg of pure penicillin-G potassium salt is equivalent to 1 595 International Units of penicillin G. 1 mg of pure penicillin-G sodium salt is equivalent to 1 670 International Units of penicillin G.

5.3.2 Oxytetracycline standard solutions and control samples

5.3.2.1 Oxytetracycline standard stock solution

Dissolve 5,0 mg ± 0,1 mg of oxytetracycline in 10 ml of 0,1 mol/l HCl solution. Dilute to 100 ml with water and mix. The thus-prepared oxytetracycline standard stock solution contains 50 mg/l of oxytetracycline.

The oxytetracycline standard stock solution may be kept for a maximum of 1 week if stored in the dark at 0 °C to +5 °C.

5.3.2.2 Oxytetracycline standard working solution

Dilute 10 ml of oxytetracycline standard stock solution (5.3.2.1) with water to 100 ml and mix. The thus-prepared oxytetracycline standard working solution contains 5 000 µg/l of oxytetracycline.

5.3.2.3 Oxytetracycline control milk sample

Dilute 2 ml of oxytetracycline standard working solution (5.3.2.2) with negative milk (5.4) to 100 ml and mix. The thus-prepared control milk sample contains 100 µg/l of oxytetracycline.

The oxytetracycline control milk sample may be kept for a maximum of 3 months if stored in test tubes (6.4) at below −18 °C.

5.3.3 Sulfadiazine standard solutions and control samples

5.3.3.1 Sulfadiazine standard stock solution

Dissolve 15,0 mg ± 0,1 mg of sulfadiazine in 100 ml water and mix. The thus-prepared sulfadiazine standard stock solution contains 150 mg/l of sulfadiazine.

The sulfadiazine standard stock solution may be kept for a maximum of 2 weeks if stored at 0 °C to +5 °C.

5.3.3.2 Sulfadiazine standard working solution

Dilute 10 ml of sulfadiazine standard stock solution (5.3.3.1) with water to 100 ml and mix. The thus-prepared sulfadiazine standard working solution contains 15 000 µg/l of sulfadiazine.

5.3.3.3 Sulfadiazine control milk sample

Dilute 1 ml of sulfadiazine standard working solution (5.3.3.2) with negative milk (5.4) to 100 ml and mix. The thus-prepared control milk sample contains 150 µg/l of sulfadiazine.

The sulfadiazine control sample may be kept for a maximum of 2 months if stored in test tubes (6.4) at below −18 °C.