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Milk — Enumeration of colony-forming units of psychrotrophic micro-organisms — Colony-count technique at 6,5 °C

Lait — Dénombrement des unités formant colonie de micro-organismes psychrotrophes — Technique par comptage des colonies à 6,5 °C

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Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 6730 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 5, *Milk and milk products*, in collaboration with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC), and will also be published by these organizations.

Annex A of this International Standard is for information only.

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Milk — Enumeration of colony-forming units of psychrotrophic micro-organisms — Colony-count technique at 6,5 °C

1 Scope

This International Standard specifies a method for the enumeration of colony-forming units (CFU) of psychrotrophic micro-organisms in raw and heat-treated milk by means of the colony-count technique at 6,5°C.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

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

ISO 8261:1989, *Milk and milk products — Preparation of test samples and dilutions for microbiological examination.*

3 Definition

For the purposes of this International Standard, the following definition applies.

3.1 psychrotrophic micro-organisms: Bacteria, yeasts and moulds forming countable colonies under the conditions specified in this International Standard.

4 Principle

4.1 Preparation of poured plates using a specified culture medium and a specified quantity of the test sample. Preparation of other plates, under the same conditions, using decimal dilutions of the test sample.

4.2 Aerobic incubation of the plates at 6,5 °C for 10 days.

4.3 Calculation of the number of colony-forming units (CFU) of micro-organisms per millilitre of sample from the number of colonies obtained on plates chosen at dilution levels so as to give a significant result.

5 Diluent and culture medium

5.1 General

For general guidance, see ISO 7218.

5.2 Basic materials

See ISO 8261.

5.3 Diluents for general use

See ISO 8261.

5.4 Distribution, sterilization and storage

See ISO 8261.

5.5 Culture medium

5.5.1 Components

Tryptone	5,0 g
Yeast extract	2,5 g
Glucose monohydrate ($C_6H_{12}O_6 \cdot H_2O$)	1,0 g
Skimmed milk powder[1)]	1,0 g
Agar	10 g to 15 g [2)]
Water	1 000 ml

1) The skimmed milk powder shall be free from inhibitory substances. This should be proved by comparative tests using skimmed milk powder known to be free from such substances.

2) According to the gel strength of the agar.

5.5.2 Preparation

5.5.2.1 Preparation from commercial dehydrated complete medium

Follow the manufacturer's instructions but, in all cases, add the skimmed milk powder, even if the manufacturer considers such an addition unnecessary.

Adjust the pH, if necessary, so that after sterilization it is 7,0 at 25 °C.

5.5.2.2 Preparation from dehydrated basic components

Dissolve and disperse in the water, in the following order, the yeast extract, the tryptone, the glucose and, finally, the skimmed milk powder.

NOTE 1 Heating the water will assist in this procedure.

Add the agar and heat to boiling, stirring frequently until the agar is completely dissolved, or steam for about 30 min.

NOTE 2 If the solution is not clear, filter through filter paper.

Adjust the pH, if necessary, so that after sterilization it is 7,0 at 25 °C.

5.5.2.3 Distribution, sterilization and storage

Dispense the medium into test tubes (6.9), in quantities of 12 ml to 15 ml per tube, or into flasks or bottles (6.9), in quantities of 100 ml to 150 ml.

Sterilize in an autoclave (6.1) at 121 °C ± 1 °C for 15 min. If the medium is to be used immediately, cool it to 45 °C in the water-bath (6.5). If not, before beginning the microbiological examination, in order to avoid any delay when pouring the medium, completely melt the medium in a hot water-bath (6.6) then cool it to 45 °C in the water-bath (6.5). (See also 8.4.4.)

Store the medium in the dark at a temperature between 0 °C and + 5 °C for no longer than 3 months after preparation.

NOTE 3 It is recommended, in order to check the temperature of the agar, to place a thermometer into a portion of 15 g/l agar solution in a separate container identical to that used for the medium. This temperature control solution should be exposed to the same heating and cooling operations as the medium itself.

6 Apparatus and glassware

CAUTION — Sterilize all apparatus that will come into contact with the test sample, the diluent, the dilutions or the culture medium in accordance with ISO 8261:1989, 6.1.

NOTE 4 Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment, the apparatus required for the preparation of test samples and dilutions as specified in ISO 8261 and, in particular, the following.

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

See ISO 7218.

6.2 Incubator, capable of operating at 6,5 °C ± 0,5 °C.

6.3 Petri dishes, made of glass or plastic, of 90 mm to 100 mm diameter.

6.4 Graduated pipettes, plugged with cotton wool, calibrated to deliver 1 ml ± 0,02 ml or 10 ml ± 0,2 ml or 11 ml ± 0,2 ml.

6.5 Water-bath, capable of operating at 45 °C ± 1 °C.

6.6 Water-bath, capable of operating at more than 100 °C.

6.7 Colony-counting equipment, consisting of an illuminated base with a dark background, fitted with a magnifying lens to be used at a magnification of × 1,5 and a mechanical or electronic digital counter.

6.8 Temperature-compensated pH-meter, accurate to ± 0,1 pH units at 25 °C.

6.9 Test tubes, of approximately 20 ml capacity (or flasks or bottles of suitable capacity), and **flasks** or **bottles** of 150 ml to 250 ml capacity, for sterilization and storage of the culture medium.

NOTE 5 Bottles or flasks with non-toxic metal screw-caps may be used.

7 Sampling

Sampling should have been carried out in accordance with ISO 707.

8 Procedure

NOTE 6 In order to improve the precision of the method, the preparation of dilutions should be carefully standardized. Factors that affect precision are as follows:

— type of blending equipment;

— blending time;

— diluent;

— time allowed for large particles to settle;

— mixing time allowed in the preparation of decimal dilutions.

CAUTION — Usual aseptic precautions shall be taken. The operations described in 8.1 and 8.2 shall not be carried out in sunlight.

8.1 Preparation of the test sample and primary dilution

See ISO 8261:1989, 8.1.1.

8.2 Further decimal dilutions

See ISO 8261:1989, 8.2.

NOTE 7 Other dilution series may be used, e.g. a primary dilution of 10 ml of test sample in 90 ml of diluent, or 11 ml of test sample in 99 ml of diluent. The accuracy and precision of the method are greater when the larger quantities of sample and diluent are used.

8.3 Duration of the procedure

See ISO 8261:1989, 8.3.

8.4 Inoculation and incubation

8.4.1 Take two sterile Petri dishes (6.3). Transfer to each dish, by means of a sterile pipette (6.4), 1 ml of the test sample.

8.4.2 Take two further sterile Petri dishes. Transfer to each dish, by means of another sterile pipette, 1 ml of the 10^{-1} dilution of the test sample.

8.4.3 If necessary, repeat this operation using further decimal dilutions.

8.4.4 Check that the temperature of the culture medium (5.5) does not exceed 46 °C.

NOTE 8 If the culture medium is at a temperature greater than 46 °C, it may damage or kill the psychrotrophic microflora of the sample. If any damage is expected, spread plating plus a low incubation temperature should be used.

Pour 12 ml to 15 ml of the culture medium (5.5) into each Petri dish.

NOTE 9 If 15 ml is insufficient to obtain a homogeneous distribution of the organisms, a volume of 20 ml should be used.

8.4.5 Carefully mix the inoculum with the medium by rotating the Petri dishes, and allow the mixture to solidify by leaving the Petri dishes to stand on a cool horizontal surface.

8.4.6 The time taken between the preparation of the first dilution and the mixing of inoculum with medium shall not exceed 15 min.

8.4.7 Prepare a sufficient number of control plates to check the sterility.

8.4.8 Invert the prepared dishes and place them in the incubator (6.2) set at 6,5 °C for 10 days.

NOTE 10 To prevent spreading, some precautions should be taken, such as

— the addition of an overlayer of culture medium after solidifying, or

— the addition of a drop of glycerol on filter paper in the lid of the dish.

8.4.9 Do not stack the dishes more than six high. Separate the stacks of dishes from one another and from the walls and top of the incubator.

8.5 Interpretation

8.5.1 Count the colonies on each plate (see 9.1), using the colony-counting equipment (6.7).

Examine the plates in subdued light. It is important that pinpoint colonies be included in the count but it is essential that the operator avoid mistaking particles of undissolved or precipitated matter in dishes for pinpoint colonies. Examine doubtful objects carefully, using higher magnification where required, to distinguish colonies from foreign matter.

8.5.2 Spreading colonies shall be considered as single colonies. If less than one-quarter of the surface is overgrown by spreading colonies, count the colonies on the unaffected part of the plate and calculate the corresponding number for the entire plate. If more than one-quarter of the surface is overgrown by spreading colonies, discard the count.

9 Expression of results

9.1 Retain dishes containing at least 10 colonies and not more than 300 colonies.

Calculate the number of CFU of micro-organisms, N, per millilitre of milk using the following formula:

$$N = \frac{\sum C}{(n_1 + 0{,}1n_2)d}$$

where

$\sum C$ is the sum of colonies counted on all the dishes retained;

n_1 is the number of dishes retained in the first dilution resulting in 10 to 300 colonies;

n_2 is the number of dishes retained in the second dilution resulting in 10 to 300 colonies;

d is the dilution factor corresponding to the first dilution.

NOTE 11 If there are more than two countable dilutions resulting in 10 to 300 colonies, the formula should be modified to take into account the further dilutions. For three dilutions, the formula becomes

$$N = \frac{\sum C}{(n_1 + 0{,}1n_2 + 0{,}01n_3)d}$$

where n_3 is the number of dishes retained in the third dilution resulting in 10 to 300 colonies.

Round the result obtained to two significant figures. When the number to be rounded is 5, with no further significant figures, round the number immediately to the left of the 5 to give an even figure. For example, 28 500 is rounded to 28 000, and 11 500 is rounded to 12 000.

Take as the result the number of CFU of psychrotrophic micro-organisms per millilitre of milk, expressed as a number between 1,0 and 9,9 multiplied by 10^x, where x is the appropriate power of 10.

EXAMPLE

A count of the CFU of micro-organisms gave the following results (two Petri dishes per dilution were incubated):

— at the first dilution retained (10^{-2}), 168 and 215 colonies

— at the second dilution retained (10^{-3}), 14 and 25 colonies

$$N = \frac{\sum C}{(n_1 + 0{,}1n_2)d}$$

$$= \frac{168 + 215 + 14 + 25}{[2 + (0{,}1 \times 2)] \times 10^{-2}} = \frac{422}{0{,}022} = 19\ 182$$

Rounding the result as specified above gives 19 000 or $1{,}9 \times 10^4$ CFU psychrotrophic micro-organisms per millilitre of milk.

9.2 If the two dishes corresponding to the test sample contain fewer than 10 colonies, report the result as "less than $10 \times 1/d$ CFU of psychrotrophic micro-organisms per millilitre of milk", where d is the dilution factor of the lowest dilution.

9.3 If there are only dishes containing more than 300 colonies, calculate an estimated count from dishes having a count nearest to 300 colonies and multiply this number by the reciprocal of the value corresponding to the highest dilution. Report the result as the "estimated number of colony-forming units of psychrotrophic micro-organisms per millilitre of milk".

10 Repeatability

The absolute difference between two single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, shall not be greater than 30 % of the lower result.

NOTES

12 If the repeatability requirements are not met in 5 % or more of the cases, an investigation into possible sources of error should be considered.

13 For repeatability definitions, see ISO 5725.

11 Test report

The test report shall specify the method used and the results obtained, indicating clearly the method of expression used. It shall also mention all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the results.

The test report shall include all information necessary for the complete identification of the sample.

Annex A
(informative)

Bibliography

[1] ISO 707:1985, *Milk and milk products — Methods of sampling.*

[2] ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests.*

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