INTERNATIONAL STANDARD

ISO 10718

Second edition
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Cork stoppers — Enumeration of colony-forming units of yeasts, moulds and bacteria capable of growth in an alcoholic medium

Bouchons en liège — Dénombrement des unités formant colonie de levures, de moisissures et de bactéries capables de se développer dans un milieu alcoolique

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Foreword

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

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

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

International Standard ISO 10718 was prepared by Technical Committee ISO/TC 87, *Cork*.

This second edition cancels and replaces the first edition (ISO 10718:1993), which has been technically revised.

Cork stoppers — Enumeration of colony-forming units of yeasts, moulds and bacteria capable of growth in an alcoholic medium

1 Scope

This International Standard specifies a method to enumerate the colony-forming units of yeasts, moulds and bacteria which can exist on cork stoppers and can grow in an alcoholic solution under certain conditions.

This International Standard applies to cork stoppers which were submitted to the usual sanitizing procedures.

2 Normative reference

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

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

3 Principle

Direct counting of colonies of living micro-organisms (yeasts, moulds and bacteria) by incubation in a cultural medium after extraction in an alcoholic solution charged with tartaric acid and followed by a membrane filtration procedure.

4 Reagents and cultural media

4.1 Physiological solution (0,85 % NaCl)[1)] or **Ringer solution** (1/4 X)[1)] with the following composition:

Sodium chloride	2,25 g/l
Potassium chloride	0,105 g/l
Calcium chloride $6H_2O$	12 g/l
Sodium bicarbonate	0,05 g/l
Final pH (obtained from the mixture)	7,0 ± 0,2

1) This product is commercially available.

4.2 WLD (for counting bacteria) with the following composition.

Yeast extract	4,0 g/l
Casein hydrolysate	5,0 g/l
Dextrose	50,0 g/l
Potassium dihydrogen phosphate	0,55 g/l
Magnesium chloride	0,425 g/l
Calcium chloride	0,125 g/l
Magnesium sulfate	0,125 g/l
Manganese sulfate	0,002 5 g/l
Ferric chloride	0,002 5 g/l
Bromocresol green	0,022 g/l
Cycloheximide (actidione)	0,004 g/l
Final pH (obtained from the mixture)	5,5 ± 0,2

4.3 M-Green (for counting yeasts and moulds) with the following composition.

Yeast extract	9,0 g/l
Dextrose (cerelose)	50,0 g/l
Peptone	10,0 g/l
Magnesium sulfate	2,10 g/l
Potassium phosphate	2,0 g/l
Diastase	0,05 g/l
Thiamine	0,05 g/l
Bromocresol	0,026 g/l
Final pH (obtained from the mixture)	4,6 ± 0,2

4.4 Tartaric acid.

4.5 Ethanol, 96 %.

4.6 Surface-active agent.

4.7 Triptone gel.

4.8 Diphenyl.

Reagents and cultural media should be stored according to the manufacturer's instructions

5 Apparatus

Usual microbiological laboratory apparatus and, in particular, the following.

5.1 Membrane filtration system.

One of the membrane filtration systems described at 5.1.1 or 5.1.2 may be used.

5.1.1 Sterile filtration system, ready to use, including a polypropylene funnel with a capacity of at least 100 ml, a sterile membrane (porosity 0,45 μm), a sterile dish and a vacuum pump with a three-way cock to turn off the vacuum.

NOTE This system is commercially available.

5.1.2 Traditional filtration system including a funnel with a minimum capacity of 100 ml (of stainless steel, glass or polycarbonate which can be sterilized in an autoclave or an oven), a sterile membrane (porosity 0,45 μm), a sterile Petri dish with a blotting pad and a vacuum pump.

5.2 **Refrigerated incubator**, capable of being controlled at 30 °C ± 2 °C.

5.3 **Refrigerator**, capable of being controlled at a temperature between 2 °C and 8 °C.

5.4 **Orbital shaker**, with a plate or rocking-motion shaker that can be set at a speed between 140 r/min and 160 r/min or a **reciprocating shaker** that can be set at a speed of 140 to 60 back-and-forth sloshing motions.

5.5 **pH-meter**, with a temperature compensation, accurate to ± 0,1 at 25 °C.

5.6 **Glass flasks**, with screw caps and an appropriate capacity to allow the four stoppers to be immersed in a 100 ml solution.

6 Sampling

Sampling shall be carried out aseptically.

Use sterile containers to preserve the sample at a temperature between 2 °C and 8 °C up to the time of testing.

7 Test conditions

The preparation of the material and the test procedure shall be carried out aseptically and following the rules specified in ISO 7218.

8 Extraction

8.1 Prepare the physiological solution or the Ringer solution (4.1). While stirring, add the surface-active agent (4.6) to obtain a 10 g/l concentration and than add triptone gel (4.7) to obtain a 1 g/l concentration. Afterwards, adjust to a pH value between 3 and 3,5 using tartaric acid (4.4). Dispense about 90 ml of the solution to each flask (5.6) and sterilize.

8.2 After cooling add, to each flask, 10 ml of ethanol (4.5) aseptically.

8.3 Put four cork stoppers into each flask, checking that the cork stoppers are completely immersed. Shake the flasks for 1 h at a speed between 140 r/min to 160 r/min, and a temperature between 20 °C and 25 °C.

The number of flasks depends on the sampling plan that has been chosen. Half of the flasks are to be used for seeding on WLD and the remaining for seeding on M-Green. For each cultural media, prepare an additional flask for the blank test.

9 Procedures

9.1 General

Follow procedure 9.2 when using a sterile filtration system and a sterile cultural media that is ready to use.

Follow procedure 9.3 when using a filtration system that has to be sterilized and a dehydrated cultural media.

9.2 Fast determination using a filtration system and a sterile culture media ready to use

9.2.1 Preparation

Prepare the filtration system (5.1.1).

9.2.2 Seeding on WLD

Place the complete funnel with the sterile membrane on the vacuum-pump filtration head. Aseptically filter the extraction solution prepared in accordance with clause 8. At the end of the filtration, turn off the vacuum from the suction circuit to re-equilibrate the atmospheric pressure.

Just before the seeding, add diphenyl (4.8) dissolved in a 10 % ethanol solution to the WLD media (4.2) in order to obtain a 30 ppm diphenyl concentration. Add the WLD media contained in the ampoule, suck it lightly and turn off the vacuum. Remove the filtration set and put the stopper on the base of the filtration set to avoid retro-infection Take away the cylindrical part of the funnel. Lift the funnel cap and fit it on the filter/Petri dish set.

Repeat this procedure for each flask.

9.2.3 Seeding on M-Green

Place the complete funnel with the sterile membrane on the vacuum-pump filtration head. Aseptically filter the extraction solution prepared in accordance with clause 8. At the end of the filtration, turn off the vacuum from the suction circuit to re-equilibrate the atmospheric pressure.

Add the M-Green cultural media (4.3) contained in the ampoule, suck it lightly and turn off the vacuum. Remove the filtration set and put the stopper on the base of the filtration set to avoid retro-infection. Take away the cylindrical part of the funnel. Lift the funnel cap and fit it on the filter/Petri dish set.

Repeat this procedure for each flask.

Dehydrated cultural media on the membrane shall be rehydrated using sterilized and demineralized water.

9.3 Determination using a filtration system to be sterilized and a dehydrated cultural media

9.3.1 Preparation of media

Prepare and sterilize the WLD media (4.2) and the M-Green media (4.3), following the manufacturer's instructions.

Add diphenyl (4.8), dissolved in a 10 % ethanolic solution, to the WLD media to obtain a 30 ppm concentration in diphenyl.

Prepare the Petri dishes.

9.3.2 Preparation of filtration system

Sterilise and prepare the filtration system (5.1.2).

9.3.3 Seeding on WLD

Aseptically filter the extraction solution prepared in accordance with clause 8 using a sterile membrane.

Place the membrane on a Petri dish containing WLD.

Repeat this procedure for each flask.

9.3.4 Seeding on M-Green

Aseptically filter the extraction solution prepared in accordance with clause 8, using a sterile membrane.

Place the membrane on a Petri dish containing M-Green.

Repeat this procedure for each flask.

10 Blank test

Prepare a blank test for each media.

11 Incubation

Invert the WLD and the M-Green dishes and incubate in a refrigerated incubator (5.2) at 30 °C ± 2 °C for 3 days.

Observe and count the colonies on each plate at least every 24 h.

12 Expression of results

12.1 Determination of the CFU number of bacteria per cork stopper

After the specified incubation period, count the colonies of bacteria on each WLD dish, always referring to the last valid counting.

For each dish, the number of colony-forming units (CFU) per cork stopper is given by the following formula:

$$\frac{N_b}{4}$$

where

N_b is the total number of colonies of bacteria counted;

4 is the number of cork stoppers tested.

The test result is the arithmetical mean of the values obtained for each dish, rounded upwards to the nearest unit.

12.2 Determination of the CFU number of yeasts and moulds per cork stopper

After the specified incubation period count the colonies of yeasts and moulds on each M-Green dish always referring the last valid counting.

For each dish the number of colony-forming units (CFU) per cork stopper is given by the following formula:

$$\frac{N_{y,m}}{4}$$

where

$N_{y,m}$ is the total number of colonies of yeasts and moulds;

4 is the number of cork stoppers tested.

The test result is the arithmetical mean of the values obtained for each dish, rounded upwards to the nearest unit.