INTERNATIONAL STANDARD 4831

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION•МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ•ORGANISATION INTERNATIONALE DE NORMALISATION

Microbiology — General guidance for the enumeration of coliforms — Most probable number technique at 30 °C

Microbiologie — Directives générales pour le dénombrement des coliformes — Technique du nombre le plus probable après incubation à 30 °C

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First edition — 1978-08-15

ISO 4831:1978
https://standards.iteh.ai/catalog/standards/sist/4b5cec50-67ae-4e65-bc22-232e7bc2f817/iso-4831-1978

UDC 663.1

Ref. No. ISO 4831-1978 (E)

Descriptors : food products, animal products, microbiological analysis, counting, coliform bacteria.

Price based on 8 pages

FOREWORD

ISO (the International Organization for Standardization) is a worldwide federation of national standards institutes (ISO member bodies). The work of developing International Standards is carried out through ISO technical committees. Every member body interested in a subject for which a technical committee has been set up 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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council.

International Standard ISO 4831 was developed by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the member bodies in July 1976.

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It has been approved by the member bodies of the following countries :

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Chile
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Printed in Switzerland

TC 34

INTERNATIONAL STANDARD ISO 4831-1978 (E)/ERRATUM

Published 1979-03-01

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ERRATUM

Page 2

Sub-clause 6.4, line 2 : Substitute "20 mm x 200 mm" for "200 mm x 200 mm".

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Microbiology – General guidance for the enumeration of coliforms – Most probable number technique at 30 °C

0 INTRODUCTION

0.1 This International Standard is intended to provide general guidance for the examination of products not dealt with by existing International Standards and for the consideration of bodies preparing reference 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, these guidelines may not be appropriate for some products in every detail, and for some 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 guidelines provided as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviation from them in the case of particular products.

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

0.2 The technique described in this International Standard is less precise than that described in ISO 4832, but allows a microbiological examination to be carried out on a larger test portion, thus permitting a lower number of coliforms per gram or per millilitre of product to be detected. Moreover, since the definition of "coliforms" adopted in the two documents is different, the micro-organisms enumerated are not necessarily the same.

For any particular product, the method to be chosen will be specified in the International Standard dealing with that product.

0.3 For the purposes of a practicable test method, the definition of "coliforms" given in clause 3 and used as the basis for the procedure is not necessarily identical with corresponding definitions given in other published texts. A proportion of strains of the micro-organisms described in other published texts as "coliforms" (including *Escherichia coli*) fail to produce enough gas to be detectable by use of a Durham tube (i.e. "anaerogenic strains"). Therefore, the method described by this International Standard will not detect all strains of the micro-organisms referred to in other publications as "(presumptive) coliforms" (for example *Citrobacter, Enterobacter, Klebsiella*)[1].

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1 SCOPE AND FIELD OF APPLICATION

This International Standard gives general guidelines for the enumeration of coliforms present in products intended for human consumption or feeding of animals, by means of the culture technique involving a liquid medium, and calculation of the most probable number (MPN) after incubation at 30 °C.

A limitation on the applicability of this International Standard is imposed by the method's susceptibility to a large degree of variability. The method should be applied and the results interpreted in the light of the information given in 10.4.

2 REFERENCES

ISO 2293, *Meat and meat products – Aerobic count at 30 °C (Reference method).*

ISO 3565, *Meat and meat products – Detection of* salmonellae *(Reference method).*

ISO 4832, *Microbiology – General guidance for enumeration of coliforms – Colony count technique at* 30 °C.

ISO . . ., *Microbiology – General guidance for preparation of dilutions.*[2]

1) See Edwards, P. R., and Ewing, W. H. (1972) : "*Identification of* Enterobacteriaceae", 3rd edition, Burgess Publishing Company, Minneapolis, Minnesota, U.S.A.

2) In preparation.

3 DEFINITION

For the purpose of this International Standard, the following definition applies :

"coliforms" : Bacteria which, at 30 °C, cause fermentation of lactose with the production of gas under the operational conditions described.

4 PRINCIPLE

4.1 Inoculation of three tubes of double-strength liquid selective culture medium (a)[1] with a specified quantity of the test sample if the initial product is liquid, or with a specified quantity of an initial suspension in the case of other products.

4.2 Inoculation of three tubes of single-strength liquid selective culture medium (b)[1] with a specified quantity of the test sample if the initial product is liquid, or with a specified quantity of an initial suspension in the case of other products; then, under the same conditions, inoculation of further tubes of medium (b) with decimal dilutions of the test sample or of the initial suspension.

4.3 Incubation of the tubes containing medium (a) at 30 °C for 24 h.

4.4 Incubation of the tubes containing medium (b) at 30 °C for 24 h or 48 h, and examination of these tubes.

4.5 Inoculation of a new series of tubes of medium (b), with the cultures from the tubes of medium (a) and with those cultures from the first series of tubes of medium (b) in which opacity or gas formation has been noted.

4.6 Incubation at 30 °C for 24 h or 48 h, and examination of the new series of tubes.

4.7 Calculation of the number of coliforms per millilitre or per gram of sample (i.e. the MPN), from the number of tubes in the new series in which gas formation has been noted, using a table for determination of most probable numbers.

5 SAMPLING

Carry out sampling in conformity with the International Standard dealing with the product concerned.

6 APPARATUS AND GLASSWARE

Usual microbiological laboratory equipment, and in particular :

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

Apparatus which will enter into contact with the culture medium, the dilution fluid or the sample, except for apparatus that is supplied sterile (particularly plastics apparatus), shall be sterilized either

— by being kept at 170 to 175 °C for not less than 1 h in an oven, or

— by being kept at 121 ± 1 °C for not less than 20 min in an autoclave.

6.2 Incubator, capable of being controlled at 30 ± 1 °C.

6.3 Loop, of platinum-iridium or nickel-chromium, diameter approximately 3 mm.

6.4 Test tubes, approximately 16 mm × 160 mm and 200 mm × 200 mm, or **bottles** of equivalent capacity.

6.5 Durham tubes, of a size suitable for use in 16 mm × 160 mm test tubes.

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6.6 Total delivery pipettes (blow-out pipettes), having nominal capacities of 1 ml and 10 ml.

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6.7 pH meter.

7 CULTURE MEDIUM AND DILUTION FLUID

7.1 Basic materials

In order to improve the precision of the results, it is recommended that, for the preparation of the culture medium, dehydrated basic components or a complete dehydrated medium should be used. The manufacturer's instructions shall be rigorously followed.

The chemicals used shall be of analytical quality.

The water used shall be distilled or deionized, and shall be free from substances that might inhibit growth of coliforms under the test conditions.

If the medium and dilution fluid are not used immediately, they shall be kept in the dark at a temperature between 0 and + 5 °C, and in conditions that prevent any change in their composition. They shall not be kept for longer than 1 month.

7.2 Dilution fluid

Use a peptone-based dilution fluid containing sodium chloride, buffered or not; for example, peptone-saline dilution fluid (see 5.3 of ISO 2293) or buffered peptone water (see 6.2.1 of ISO 3565).

1) Where appropriate, a liquid enrichment medium may be used prior to the inoculation of the selective medium.

7.3 Lactose bile brilliant green broth (selective medium)

Composition

	a) Double-strength medium	b) Single-strength medium
peptone	20 g	10 g
lactose	20 g	10 g
dehydrated ox bile	40 g	20 g
brilliant green corresponding to the specifications in the annex of ISO 3565	0,026 6 g	0,013 3 g
water	1 000 ml	1 000 ml

Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling.

If necessary, adjust the pH (checking with the pH meter) so that after sterilization it is 7,2 ± 0,1 at 25 °C.

Dispense the medium, in quantities of 10 ml, in 16 mm × 160 mm test tubes (6.4) containing Durham tubes (6.5) in the case of the single-strength medium and in 20 mm × 200 mm test tubes (6.4) (without Durham tubes) in the case of the double-strength medium.

Sterilize in an autoclave at 121 ± 1 °C for 15 ± 1 min.

The Durham tubes shall not contain air bubbles after sterilization.

NOTE — Because the complete medium may not always produce the expected result, its performance should be checked before use. (A method for the purpose will be developed and a standard published.)

8 PREPARATION OF THE TEST SAMPLE

Refer to the particular International Standard dealing with the product under examination. If an International Standard is not available, it is recommended that agreement be reached on this subject by the parties concerned.

9 PROCEDURE (see the diagram in annex A)

Where several samples taken from the same batch are to be examined, carry out the following operations for each sample.

9.1 Test portion, initial suspension and dilutions

Refer to ISO . . . (see clause 2) and to the International Standard dealing with the product under examination.

Prepare the initial suspension and the dilutions using a dilution fluid meeting the requirements given in 7.2.

Make a sufficient number of dilutions to ensure that all the tubes corresponding to the final dilution will yield a negative result.

9.2 MPN technique

9.2.1 *Inoculation*

9.2.1.1 Take three tubes of double-strength selective medium [7.3 a)]. Transfer to each of these tubes, using a pipette (6.6), 10 ml of the test sample, if liquid, or 10 ml of the initial suspension.

9.2.1.2 Then take three tubes of single-strength selective medium [7.3 b)]. Transfer to each of these tubes, using a pipette (6.6), 1 ml of the test sample, if liquid, or 1 ml of the initial suspension.

9.2.1.3 For each of the following dilutions (from 1/10 or 1/100, according to the circumstances), take three tubes of single-strength selective medium [7.3. b)]. Transfer 1 ml of the respective dilution into each of these tubes. Change the pipette for each dilution. Carefully mix the inoculum and the medium.

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9.2.2 *Incubation*

9.2.2.1 Leave the tubes of double-strength medium (9.2.1.1) in the incubator (6.2) at 30 ± 1 °C for 24 ± 2 h.

9.2.2.2 Leave the tubes of single-strength medium (9.2.1.2 and 9.2.1.3) in the incubator (6.2) at 30 ± 1 °C for 24 ± 2 h or, if neither gas formation nor opacity preventing the observation of gas formation is observed at this stage, for 48 ± 2 h.

9.2.3 *Inoculation from the incubated tubes*

9.2.3.1 From each of the incubated tubes from 9.2.2.1, inoculate with a loop (6.3) a tube of single-strength selective medium [7.3 b)]. Incubate at 30 ± 1 °C for 24 ± 2 h or, if gas formation is not observed at this stage, for 48 ± 2 h.

9.2.3.2 Carry out the same procedure for the incubated tubes from 9.2.2.2 showing gas formation or opacity, when either of these features is first observed (i.e. after 24 ± 2 h or after 48 ± 2 h).

9.2.4 *Interpretation*

For each dilution, count the total number of tubes in which gas formation is observed in 9.2.3 (positive tubes) after 24 ± 2 h and (if used) 48 ± 2 h.

10 EXPRESSION OF RESULTS

10.1 Selection of dilutions[1)]

For each sample examined, select three consecutive dilutions in accordance with one of the three following rules, whichever is appropriate :

a) *When at least one dilution yielding three positive tubes exists*

Select the highest dilution (i.e. that having the lowest sample concentration) yielding three positive tubes, together with the next two higher dilutions (i.e. those having sample concentrations of 1/10 and 1/100 of that of the first dilution selected) (see example 1).

See also rule c).

If insufficient further dilutions were made beyond the highest dilution yielding three positive tubes, select instead the three highest dilutions in the series (i.e. those having the lowest sample concentration) (see example 2).

b) *When no dilution yielding three positive tubes exists*

If rule a) cannot be applied, select the three highest dilutions in the series (i.e. those having the lowest sample concentration) (see example 3).

See also rule c).

c) *Special case*

In all cases where more than one of the three dilutions selected in accordance with rules a) and b) does not yield positive tubes, select from these dilutions the lowest one not yielding positive tubes (i.e. that having the highest sample concentration) and the two next lower dilutions in the series (i.e. those having sample concentrations of ten times and one hundred times that of the first dilution selected) (see examples 4 and 5), except when positive tubes are only found at the level of the first dilution prepared from the sample. In this last case, it is necessary to select the first three dilutions for calculation of the MPN even though this series includes two dilutions yielding no positive tube.

10.2 Determination of MPN index

10.2.1 According to the number of samples examined per batch, check, using the tables in annexes B and C, whether the sequences of numbers of positive tubes corresponding to the dilutions selected in accordance with 10.1 are statistically acceptable. Acceptability depends both on the number of samples examined and on the decision as to whether or not to accept category 2 results.

Thus, for example, if only category 1 results are accepted, the sequence 221 is acceptable only when 10 samples (of the batch concerned) have been examined. On the other hand, if the less likely category 2 results are also accepted, the sequence 221 is also acceptable when only 2, 3 or 5 samples have been examined. However, when the sequence 221 is the result of a single examination, it is never acceptable.

10.2.2 For each sequence found to be acceptable in accordance with 10.2.1, obtain the MPN index from the table in annex B or C.

10.3 Calculation of most probable number (MPN)

Obtain the number of coliforms per millilitre or per gram by multiplying the MPN index (see 10.2) by the reciprocal of the lowest dilution selected (i.e. that having the highest sample concentration).

When the lowest dilution selected corresponds to the tubes prepared with double-strength medium (inoculation with 10 ml), first divide the MPN index by 10.

Express the result as a number between 1,0 and 9,9 multiplied by 10^n, n being the appropriate power of 10.

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Example 1 : Case of a **solid** sample

Initial suspension 1/10 (10 ml) : 3 tubes +
Initial suspension 1/10 (1 ml) : [3] tubes +
Dilution 1/100 (1 ml) : [2] tubes +
Dilution 1/1 000 (1 ml) : [1] tube +
Dilution 1/10 000 (1 ml) : 0 tube +

Retain 321.

The table in annex B or C gives an MPN index of 15 and the calculation gives an MPN of 15 × 10, i.e.

$1,5 \times 10^2$ coliforms per gram

Example 2 : Case of a **solid** sample

Initial suspension 1/10 (10 ml) : 3 tubes +
Initial suspension 1/10 (1 ml) : [3] tubes +
Dilution 1/100 (1 ml) : [3] tubes +
Dilution 1/1 000 (1 ml) : [0] tube +

Retain 330.

The table in annex B or C gives an MPN index of 20 and the calculation gives an MPN of 20 × 10, i.e.

2×10^2 coliforms per gram

1) In this sub-clause, the initial suspension and, if necessary, the test sample are considered as dilutions.

Example 3 : Case of a **liquid** sample

Test sample (dilution 1/1)	(10 ml)	: 2 tubes +
Test sample (dilution 1/1)	(1 ml)	: 2 tubes +
Dilution 1/10	(1 ml)	: **1** tube +
Dilution 1/100	(1 ml)	: **1** tube +
Dilution 1/1 000	(1 ml)	: **0** tube +

Retain 110.

The table in annex B or C gives an MPN index of 0,7 and the calculation gives an MPN of 0,7 × 10, i.e.

7×10^0 coliforms per millilitre

Example 4 : Case of a **solid** sample

Initial suspension 1/10	(10 ml)	: **3** tubes +
Initial suspension 1/10	(1 ml)	: **3** tubes +
Dilution 1/100	(1 ml)	: **0** tube +
Dilution 1/1 000	(1 ml)	: 0 tube +

Retain 330.

The table in annex B or C gives an MPN index of 20 and the calculation gives an MPN of $\frac{20}{10} \times 10$, i.e.

$2{,}0 \times 10^1$ coliforms per gram

Example 5 : Case of a **solid** sample

Initial suspension 1/10	(10 ml)	: 2 tubes +
Initial suspension 1/10	(1 ml)	: **2** tubes +
Dilution 1/100	(1 ml)	: **1** tube +
Dilution 1/1 000	(1 ml)	: **0** tube +
Dilution 1/10 000	(1 ml)	: 0 tube +

Retain 210.

The table in annex B or C gives an MPN index of 1,5 and the calculation gives an MPN of 1,5 × 10, i.e.

$1{,}5 \times 10^1$ coliforms per gram

10.4 Precision of the method

It is recognized that wide variations in results may occur with the MPN technique. Results obtained from this method should therefore be used with caution.

Confidence limits are given in the tables in annexes B and C.

11 TEST REPORT

The test report shall show the method used and the result obtained; it shall also mention all operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the result.

The report shall include all details for complete identification of the sample.

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