INTERNATIONAL STANDARD

ISO 13969

IDF 183

First edition
2003-10-01

Milk and milk products — Guidelines for a standardized description of microbial inhibitor tests

Lait et produits laitiers — Lignes directrices pour une description normalisée des méthodes microbiologiques de dépistage d'inhibiteurs microbiens

Reference numbers
ISO 13969:2003(E)
IDF 183:2003(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

International Dairy Federation
Diamant Building • Boulevard Auguste Reyers 80 • B-1030 Brussels
Tel. + 32 2 733 98 88
Fax + 32 2 733 04 13
E-mail info@fil-idf.org
Web www.fil-idf.org

Published in Switzerland

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.

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 13969 | IDF 183 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

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 and AOAC International in the development of standard methods of analysis and sampling for milk and milk products.

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 IDF National Committees 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. IDF shall not be held responsible for identifying any or all such patent rights.

ISO 13969 | IDF 183 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

All work was carried out by the Joint ISO/IDF/AOAC Action Team *Antimicrobials and other veterinary medical residues*, of the Standing Committee on *Analytical methods for additives and contaminants*, under the aegis of its project leader, Mrs G. Suhren (DE).

Introduction

The parameters outlined in this International Standard may not need to be evaluated completely for every test, depending on

a) the field of application of the test under study (e.g. screening or reference method, type of milk, i.e. animal species or raw/heat treated milk),

b) the information needed [e.g. the introduction of a new substance with fixed maximum residue limit (MRL)], and

c) the detection pattern (e.g. sensitivity of the test microorganism to a broad or narrow variety of antimicrobial compounds).

Thus “the terms of reference” between the producer and user of a certain test should be agreed upon in the context of these guidelines omitting, for example, those aspects that are not relevant to the intended field of application.

A general disadvantage related to the interpretation of microbial inhibitor tests is that they are usually evaluated in a subjective way and in very few steps, i.e. negative, questionable, and positive by comparison with positive and/or negative control samples.

In cases where the medium contains an indicator, the type of the resultant colour change can depend upon the type of antimicrobial present. This sometimes makes it difficult to obtain a clear distinction between positive and negative results. Test interpretation in few steps also means that small alterations or minor colour developments, which may be of importance in a validation programme, need major experimental effort.

Milk and milk products — Guidelines for a standardized description of microbial inhibitor tests

1 Scope

This International Standard gives guidance for a standardized description of microbial inhibitor tests for milk and milk products. It is intended to give a framework and basis for the evaluation/validation of microbial inhibitor tests, allowing the comparison of data obtained from different tests and experimental studies.

2 Terms and definitions

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

2.1
false positives
percentage of positive results when testing negative samples

2.2
false negatives
percentage of negative results at the claimed detection level(s)

2.3
limit of detection
concentration level at which a defined percentage of samples is detected

EXAMPLE 95 % together with the respective confidence level.

3 Information needed from the developer/manufacturer

3.1 Methodology

The developer or manufacturer of the test should provide information regarding methodology by mentioning the following:

a) description of the method;

b) principle of the method;

c) technical design of the procedure (e.g. degree of automation, data processing);

d) evaluation of test results (e.g. scores, scale and definition of what to consider “positive” or “negative”);

e) capacity (e.g. sample throughput);

f) special requirements for sampling, preservation and testing;

g) procedure for the purpose of quality assurance by the developer/manufacturer;

h) field of application concerning

   1) the intended test use (e.g. screening or confirmation), and

   2) the substrate or matrix (e.g. milk from cows or other animals, milk powder or other foods).

3.2 Operating requirements

The following information should be given regarding operating requirements:

a) requirements for user experience and training;

b) requirements for safety and special laboratory service (e.g. electric power, S1-lab, waste disposal and maximum concentration level);

c) requirements for quality control by the manufacturer and/or user.

3.3 Test specifications

The following information should be given regarding test definitions:

— limit of detection: see 2.3.

3.4 Documentation

The following information should be given regarding documentation:

a) user manual, including a trouble-shooting guide;

b) suppliers of instruments, reagents, standards, technical services and customer support;

c) status of official recognition/general introduction in specified countries;

d) availability of reference material;

e) availability of internationally recognized/validated reference from ISO, IDF and AOAC International, or others;

f) availability of, for example, literature and practical experiences.

4 Evaluation of the attributes of the microbiological inhibitor test

4.1 General

The validation of a method should always be carried out under controlled conditions, i.e. based on defined test samples. The influence of particular conditions is described in 4.3.6.

4.2 Prerequisites

4.2.1 Milk free from antimicrobials (“negative milk”)

The cows from which milk is collected in order to serve as “negative milk” shall meet the following requirements. If, however, a test is applied for milk of an animal species other than cows, the requirements with respect to the status of the animal should be adjusted accordingly.

a) The clinical and sub-clinical health status shall be good, with special emphasis on udder health (less than 150 000 somatic cells per millilitre).

b) The treatment or feeding with antimicrobial substances shall be prohibited for at least 8 weeks before milk collection. In the case of dry cow treatment, milk should not be collected earlier than 60 days after calving provided the dry cow period has been at least 4 weeks.

c) The cows shall be mid-lactation: more than 60 days and less than 200 days after calving, producing more than 5 kg of milk per day.

d) The milking of at least five to seven cows shall be combined to overcome individual variations in milk composition.

e) The total viable count shall be less than 10^4 CFU (colony-forming units) per millilitre before the preservation process (deep-freezing, lyophilization). The possible presence of β-lactamase-producing microorganisms shall be kept in mind in the case of β-lactam antibiotic testing.

4.2.2 Test substances

The test substances that are used in the testing procedure should be obtained from a recognized developer/manufacturer, preferably with an analytical certificate with a guaranteed specification. The concentration required should be calculated based on the free acid or base forms of the drug, corrected for purity. Special considerations should be given to those substances with stability/potency problems.

Unless otherwise stated, it is preferable that the evaluation of detection limits (4.3.2) should be undertaken using those antimicrobials and/or concentrations that the developer/manufacturer claims the test will detect.

4.2.3 Solvents

If special solvents or other chemicals are required to dissolve the substances, it should be ensured that these solvents or chemicals in the test samples have no influence on the test result. The use of solvents other than water should be restricted.

4.2.4 Preparation of test samples

4.2.4.1 General

The preparation of test samples can cause problems and is a very laborious task for the test evaluation laboratories. Therefore, it might be appropriate to employ a centralized test sample preparation system, which agrees to supply interested laboratories with test samples in stable form (e.g. lyophilized).

For large-scale evaluations (e.g. to obtain the data basis for a generalized description), all dilutions required should be prepared in one batch to avoid day-to-day variations of weighing, diluting and differences in the status of the “negative milk” (4.2.1).

4.2.4.2 Selection of concentrations

The selection of concentrations for the determination of the detection limits is described in 4.3.2. For estimated purposes, if not otherwise stated, the concentration found to represent the detection limit should be tested, together with one concentration step higher and two or three concentration steps lower than the claimed detection limit and the corresponding negative milk. As an approximate guideline, it is recommended to divide the concentration range giving 50 % to 100 % positives into three to four equal distant levels (linear and logarithmic scales respectively) as demonstrated in Figure 1.

4.2.4.3 Dilution

The following precautions should be met when preparing a dilution series of test substances.

a) The preparation of the dilution series should be carried out in such a way that only the final dilution is prepared with milk in order to avoid protein binding.

b) The proportion of the added aqueous standard solution in the final milk dilution step should be the same for all dilutions and less than 1 %.

4.2.4.4 Preservation

The preservation of test samples should preferably be carried out by lyophilization, if this is not deprecated by the developer/manufacturer of the test under study, or the test principle. The following procedure for preservation has proved to be feasible.

a) Immediately after the preparation of the various test samples, all dilutions should be dispensed into test tubes with the desired volume and be frozen at −18 °C ± 2 °C in a sloping position.

b) Lyophilization should be carried out as soon as possible and not later than one week after deep freezing. During the lyophilization process, the temperature should not exceed 25 °C.

c) Test tubes should be hermetically sealed immediately after lyophilization and stored in the dark at ⩽ 6 °C.

d) Test samples should be reconstituted with distilled water. The added volume of water should be 10 % less than the volume of the sample that was lyophilized in order to compensate for the dry matter of milk.

e) Reconstituted test samples may be used on the day of reconstitution only. They should be kept in a refrigerator between uses and discarded at the end of the day.

4.2.4.5 Reduction of mistakes during preparation

In most cases, it is not possible to confirm the concentration of antimicrobial in the test samples by an independent quantitative method. To cope with this problem pragmatically, the following procedure regarding a reduction in the number of mistakes during preparation is proposed.

a) Each of three to five persons should prepare their own and independent dilution series.

b) Dilution series should be tested with appropriate microbiological inhibitor tests or other suitable methods.

c) Only those dilution series that give similar test results should be chosen for the mixture of the final test samples.

4.2.5 Experimental design

4.2.5.1 Number of replicates

4.2.5.1.1 Test with subjective reading

For microbial inhibitor tests with subjective reading and few possible outcomes, all experiments should be performed as blind coded studies. Participating person(s), preferably more than one, responsible for the analysis receive(s) coded samples without any knowledge of its substance/concentration combination. The results should be expressed as the positive results, in percent, out of the total number of replicates within each evaluated concentration step. Calculation of percentages generally requires at least 10 to 20 replicates at each selected concentration. The reading resolution at each tested concentration step equates to ± 5 %, when testing 20 replicates. The resolution equates to ± 10 %, when testing 10 replicates.

4.2.5.1.2 Test with objective reading

For microbial inhibitor tests with objective reading (e.g. ELISA reader and a measuring scale), the experimental design is not necessarily a blind coded study. The number of replicates depends on the repeatability of the method, but should be at least three to five replicates for each substance/concentration combination. If a defined statistical confidence is required, the appropriate number of replicates should be calculated.

For example, to determine a 90 % negative rate with 95 % confidence, 60 samples are needed for subjective readings and 30 samples for tests with instrumental interpretation. If not stated otherwise, at least two different test kit batches should be used for the evaluation of the different attributes.

4.2.5.2 Evaluation of experimental data

For data analysis, a graphical presentation is recommended, whereby the x-axis represents the concentration of the substance under study and the y-axis the percent of positive results or the scaled values ("dose-response curves").

The choice of whether the x-axis is scaled linear or logarithmic depends on the range of concentrations tested. If the range covers more than 100-fold, a logarithmic scale is more appropriate than a linear one. Using such coordinates, the different test conditions (e.g. test kit batch) provide dose-response curves that allow a visual comparison with respect to the effect of the parameter under study.

For a statistical evaluation, the definition of the confidence level for the method under study is imperative. For example, such a definition might be that the detection limit equals that concentration at which 95 % of the test results are interpreted as positive.

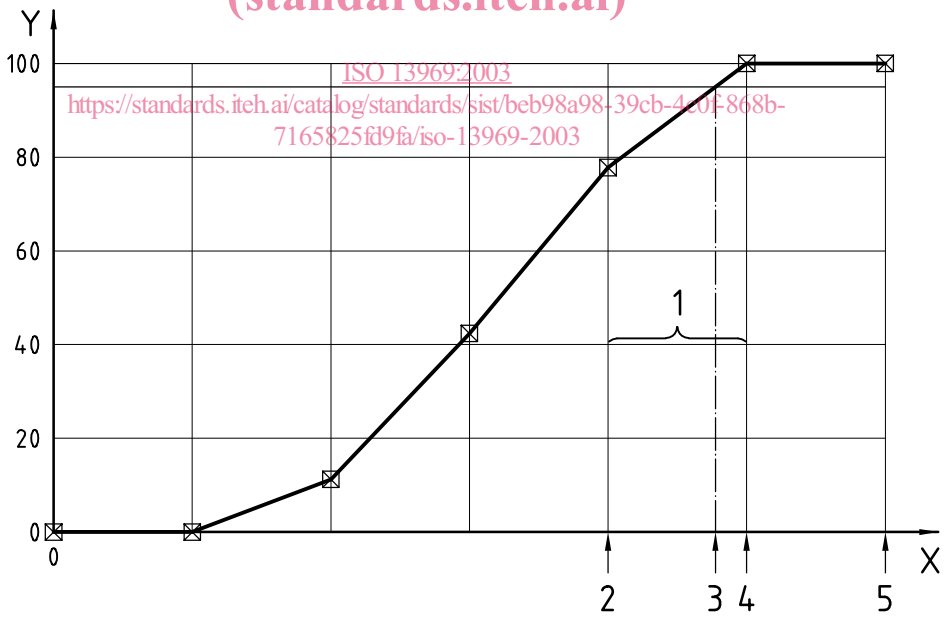
The detection limits may be expressed in two ways (see Figure 1)

a) either the two tested concentrations between which the intersection of the dose-response curve and the line representing "95 % positive results" value lies (as a range in µg/kg), or

b) the concentration corresponding to the intersection of the dose-response curve with the line representing "95 % positive results".

For methods with continuous scales, the mean values ($\bar{X}$) and standard deviations (s) for each tested concentration should be calculated, i.e. the intersection of the mean value minus $2s$ ($\bar{X} - 2s$) or the mean value plus $2s$ ($\bar{X} + 2s$), depending on whether the response is inversely related to the concentration or not. The value on the y-axis, which is to be interpreted as positive, corresponds to the detection limit.

It should be noted that the estimated detection limits depend to a certain extent on the concentrations tested.



Key

X antimicrobial content, µg/kg
Y positive results, %
1 range of detection limit
2 expected positive
3 detection limit
4 MRL
5 1,5 × expected positive

Figure 1 — Model of dose-response curve for the determination of detection limits