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Microbiology of food and animal feeding stuffs — Protocol for the validation of alternative methods

Microbiologie des aliments — Protocole pour la validation des méthodes alternatives

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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 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 International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 16140 was prepared by the European Committee for Standardization (CEN) in collaboration with Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Throughout the text of this document, read "...this European Standard..." to mean "...this International Standard...".

Contents

Foreword

This document (EN ISO 16140:2003) has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN, in collaboration with Technical Committee ISO/TC 34 "Agricultural food products".

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by November 2003, and conflicting national standards shall be withdrawn at the latest by November 2003.

The annexes A, C to K and M to T are normative. The annexes B, L and U are informative.

This document contains also a Bibliography.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Slovakia, Spain, Sweden, Switzerland and the United Kingdom.

Introduction

The need for the food industry to rapidly assess the microbiological quality of raw materials and finished products and the microbiological status of manufacturing procedures, has led to the development and refinement of alternative microbiological methods of analysis that are quicker and/or easier to perform than the corresponding reference method; some can also be automated.

Among these alternative methods, some can yield results that are equivalent to those provided by the reference method, while others can lead to results that differ appreciably.

The suppliers/producers of the alternative methods, the food and drink industry, the public health services and other authorities need a reliable common protocol for the validation of such alternative methods. The data generated can also be the basis for the certification of a method by an independent organisation.

Because of the extent of the methods comparative study described in this standard for use by the organising laboratory, the procedure is sometimes not appropriate for use as an "in house" method for the validation of an alternative method by an individual laboratory.

1 Scope

This document establishes the general principle and the technical protocol for the validation of alternative methods in the field of microbiological analysis of food, animal feeding stuff and environmental and veterinary samples (see 5.1.1.2.1) for:

— the validation of alternative methods which can be used in particular in the framework of the official control;

— the international acceptance of the results obtained by the alternative method.

It also establishes the general principles of certification of these alternative methods, based on the validation protocol defined in this document (see 4.2).

Where an alternative method is used on a routine basis for internal laboratory use without the requirement to meet (higher) external criteria of quality assurance, a less stringent comparative validation of the alternative method than that set in this standard may be appropriate.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text, and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

ISO 3534-1, *Statistics – Vocabulary and symbols – Part 1: Probability and general statistical terms.*

ISO 5725, *Accuracy (trueness and precision) of measurement methods and results.*

3 Terms and definitions

For the purposes of this European Standard, the following terms and definitions apply:

3.1
alternative method
method of analysis that demonstrates or estimates, for a given category of products, the same analyte (3.4) as is measured using the corresponding reference method (3.2).

NOTE 1 The method can be proprietary or non commercial, and does not need to cover an entire analysis procedure, that is from the preparation of samples to the test report.

NOTE 2 The alternative method exhibits attributes appropriate to the users' needs, for example:

— speed of analysis and/or response;

— ease of execution and/or automation;

— analytical properties (precision, accuracy, limit of detection, etc.);

— miniaturisation;

— reduction of cost.

NOTE 3 The term "alternative" is used to refer to the entire "test procedure and reaction system". This term includes all ingredients whether material or otherwise, required for implementing the method.

3.2
reference method
internationally recognised method and widely accepted.

NOTE For the purpose of this standard, these are International and European Standards and if not existing, certain national standards of equivalent standing.

3.3
validation of an alternative method
demonstration that adequate confidence is provided that the results obtained by the alternative method are comparable to those obtained using the reference method

NOTE The word "comparable" is defined in this EN ISO 16140 by a technical protocol adapted to each type of method (see clauses 5 and 6).

3.4
analyte
component measured by the method of analysis. It may be the microorganism

3.5
qualitative method
method of analysis whose response is either the presence or absence of the analyte (3.4) detected either directly or indirectly in a certain amount of sample

3.6
quantitative method
method of analysis whose response is the amount of the analyte (3.4) measured either directly (enumeration in a mass or a volume), or indirectly (colour absorbance, impedance, etc.) in a certain amount of sample

3.7
methods comparison study
study, performed by the organising laboratory of the alternative method against the reference method

3.8
inter-laboratory study
study of the method's performance using common samples in several laboratories and under the control of the organising laboratory

3.9
organising laboratory
laboratory having the qualified staff and skills to perform the method comparison study and organise the interlaboratory study.

NOTE The availability of an experienced statistician is essential for the analysis of the results.

4 General principles for the validation and the certification of alternative methods

4.1 Validation protocol

The validation protocol comprises two phases:

— a methods comparison study (3.7) of the alternative method (3.1) against the reference method (3.2) carried out in the organizing laboratory;

— an interlaboratory study (3.8) of each of the two methods.

If appropriate, the two phases may be undertaken in parallel.

The technical rules for performing the methods comparison study and the interlaboratory study are given in clauses 5 and 6, depending upon whether the alternative method is qualitative or quantitative in nature.

If the alternative method has already been validated and meets the requirements set by another organisation, specific rules are defined in annex A for accepting the results of this prior validation.

4.2 Principles of the certification

4.2.1 If a subsequent certification of the alternative method is required, the two following principles shall also be applied (in addition to 4.1):

Details on the organisation of the certification (management of the method comparison study and the interlaboratory study, all the different bodies involved including the expert laboratory – designated in this standard as the "organising laboratory"- the reviewers, the certification body, etc) are provided [8] by the certification body.

4.2.2 The manufacturer shall apply a **quality system** covering the production line of the product for which the certification is sought and based on the appropriate European Standard relative to quality systems or other equivalent international standard (for example EN ISO 9001).

In granting the certification, the certification organisation shall take into account the existence of any quality system certificate issued by a certification body accredited for quality systems.

4.2.3 A **regular verification** of the quality of the certified method shall be undertaken after the certification is granted. An audit is to be performed regularly to verify that the following are still met:

— the quality assurance requirements, (see 4.2.1);

— the product's production control requirements, (see 4.2.1).

In addition to the general requirements of the appropriate European Standard relative to the quality system, the manufacturer presents regularly to the certification organisation updated documentation that take into account any modification made to the product or production process which may affect the instructions for using the method and/or the method's performance. The certification organisation then decides whether these modifications affect the certification.

5 Qualitative methods - Technical protocol for their validation

5.1 Methods comparison study

5.1.1 Relative accuracy, relative specificity and relative sensitivity

5.1.1.1 Terms and definitions

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

5.1.1.1.1 relative accuracy (*AC*)

degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples[1)] (see 5.1.1.3.1.).

NOTE The term "relative accuracy" used here is complementary to the "accuracy" and "trueness" as defined in ISO 5725-1 and ISO 3534-1. These state that accuracy is "the closeness of agreement between a test result and the accepted reference value", and that the trueness is "the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value". For the purpose of this standard, the accepted reference value is chosen as the value obtained by the reference method. Thus, the term "relative" implies that the reference method does not automatically provide the accepted reference value.

5.1.1.1.2 positive deviation (PD)

The alternative method becomes a false positive when it presents a positive deviation if it gives a positive result when the reference method gives a negative result.

A positive deviation becomes a false positive result when the true result can be proven as being negative.

A positive deviation is considered as a true positive when the true result can be proven as being positive.

5.1.1.1.3 negative deviation (ND)

The alternative method presents a negative deviation if it gives a negative result when the reference method gives a positive result.

A negative deviation becomes a false negative result when the true result can be proved as being positive.

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1) Difficult to achieve if the pre-enrichment steps are different.

5.1.1.1.4 relative sensitivity (*SE*)

ability of the alternative method to detect the analyte when it is detected by the reference method (see 5.1.1.3.1.).

5.1.1.1.5 relative specificity (*SP*)

ability of the alternative method to not detect the analyte when it is not detected by the reference method (see 5.1.1.3.1.).

5.1.1.2 Measurement protocol

5.1.1.2.1 Food samples

It is of the highest priority to find food samples naturally contaminated with the analyte to be detected for the validation.

If it is sought to validate the method for all foods, study five categories of food. This number may be reduced to 1, 2, 3 or 4 categories if the validation of alternative method is restricted to these stated categories, at the producer's request. The recommended categories are listed in annex B.

Appropriate environmental samples may be included as one category. Veterinary samples may be treated as another category (see annex B).

It is desirable that food samples come from as wide a distribution as possible in order to reduce any bias from local food specialities and broaden the range of validation.

When analysing naturally contaminated samples, the range and distribution of contamination of the samples should be representative of the levels usually found in that product but with emphasis on smaller numbers.

If it is not possible to acquire a sufficient number of naturally contaminated foods for each of the categories, artificial contamination of food samples is permissible. The method and levels of contamination should result in samples behaving similarly to naturally contaminated ones. See methods of inoculation and restrictions in annex C.

5.1.1.2.2 Number of samples

The total number of test portions to be analysed is 60 for each food category chosen from the categories stated in annex B. Within each category, select representative food types and analyse 20 test portions of each food type by the proposed method and the reference method to produce at least 60 total results for each category by each method. For naturally contaminated food types prepare the sample as described in annex D. For artificially contaminated food type adjust the inoculation levels to achieve fractional positive recovery of the test portions analysed by at least one of the methods. Fractional recovery is achieved when some number, but not all, of the test portions are determined to be positive by one or both methods, alternative method or reference method.

It is desirable to produce approximately 50 % of the results that are positive and 50 % that are negative. This is, however, a recommendation, not an absolute percentage, provided that some number of the test portions are positive and some number are negative for the same food type.

5.1.1.2.3 Test sample preparation

The reference and alternative methods shall be performed with, as far as possible, exactly the same sample.

Thus, if the first stage of the two methods is the same (for example the same pre-enrichment broth), perform the replication at the second step (case 1, annex D).

If this is not the situation, that is the first culture media, methodology or dilutions are different, prepare paired test portions for analysis. There are two primary methodologies for such preparations.

In the first instance, mix a double weight of sample with an equal weight/volume of sterile water or other suitable diluents and homogenize very thoroughly. Then divide into two portions taking particular care to increase the concentration of the primary enrichment by (approximately 10 %) to compensate for the dilution effect of the diluted, homogenised sample (case 2, annex D).

In the second instance, directly inoculate the food type with a starting inoculum sufficient to allow a fractional recovery of the micro-organisms in the test portions analysed by at least one of the methods after the microorganisms have equilibrated in the food type. Then weigh 25 g test portions and proceed as described in annex D. This may be preferred for liquid products but is acceptable for any food type provided that the food is properly homogenised.

5.1.1.3 Calculation and interpretation

5.1.1.3.1 Treatment of data

Tabulate the data of the paired results of the reference and alternative methods and calculate the following parameters for each food category (60 samples) according to the Table 1.

Table 1 - Paired results of the reference and alternative method

Responses	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	+/+ positive agreement (PA)	-/+ positive deviation (PD) (R-/A+)
Alternative method negative (A-)	+/- negative deviation (ND) (A-/R+)	-/- negative agreement (NA)

The calculations shall be performed on a number of negative results obtained by the reference method which for the results in Table 1 cannot exceed twice the number of positive results; the negative results being selected if necessary as immediately following a positive result, in the order of analysis of the samples.

Express the three criteria as follows:

— **Relative accuracy:** $AC = \frac{(PA + NA)}{N} \times 100\,\%$;

— **Relative specificity:** $SP = \frac{NA}{N_-} \times 100\,\%$;

— **Relative sensitivity:** $SE = \frac{PA}{N_+} \times 100\%$

where

N is the total number of samples (NA + PA + PD +ND);

N_- is the total number of negative results with the reference method (NA + PD);

N_+ is the total number of positive results with the reference method (PA + ND).

5.1.1.3.2 Confidence intervals

The calculation of confidence intervals associated with the number of samples tested is given in annex E.

5.1.1.3.3 Discordant results

Examine the discordant results as described in annex F (The McNemar test), by using the count of PD and ND (see 5.1.1.3.1).

When the values for PD and ND are high and almost equal, no statistical difference between the methods can be detected using the McNemar test. In this case, the organising laboratory shall pay further attention to explain the reasons for the high values of PD and ND. Moreover, it shows that the relative accuracy of a method shall never be interpreted by taking into account only the McNemar test.

5.1.1.3.4 Summary of calculation

All the calculations shall be summarised in Table 2:

Table 2 - Calculation of the relative accuracy, the relative sensitivity and the relative specificity

Matrices	PA	NA	ND	PD	Sum	Relative Accuracy *AC* (%)	N_+	Relative sensitivity *SE* (%)	N_-	Relative specificity *SP* (%)
					N	$\frac{100\times(PA+NA)}{N}$	PA + ND	$\frac{100\times PA}{N+}$	NA + PD	$\frac{100\times NA}{N-}$
Food cat. 1										
Food cat. 2										
Food cat. 3										
Food cat. 4										
Food cat. 5										
TOTAL										

5.1.1.3.5 Interpretation

A table giving the raw results (that is **all** the positive and negative results, Table 1) shall be provided.

Taking into account the number of positive deviations and the number of negative deviations, the capability of the alternative method to give more or fewer true positive results than the reference method is evaluated.

The report of the study shall distinguish the results obtained with naturally contaminated and artificially contaminated samples.

The procedure for the artificial contamination of test samples shall be described in the report of the study.

Data published elsewhere and meeting the conditions defined in annex A may be used for evaluating the relative accuracy.

5.1.2 Relative detection level

5.1.2.1 Definition

For the purpose of this standard, the relative detection level is the smallest number of culturable microorganisms (3.4) that can be detected in the sample in 50 % of occasions by the alternative and reference methods.

5.1.2.2 Measurement protocol

Test the following:

— use one food product within each food category chosen from 5.1.1.2.1, depending of the scope of the validation (see annex B);

— use five different target microorganisms (or less, depending on the scope of the validation) each one associated with one food category, if possible. (See annex G.1 for the definition of the target microorganism);

— preferably test five levels (but a minimum of three levels) of one target microorganism per food, including the negative control, etc. The first level shall be the negative control. The second level shall be the theoretical detection level. The third level shall be just above the theoretical detection threshold and any further levels shall be higher than the previous one. A factor of about three between each concentration in the upper levels could be applied;

— replicate each combination (food product, level of contamination) six times by both the alternative and reference methods. Perform the division at the level where the two methods differ as illustrated in annex D. Thus, if the 1st stage of each method is the same (for example the same pre-enrichment broth), perform the division at the 2nd step (case 1, annex D). If this is not the case, i.e. the first culture media, methodology or dilutions being different, mix a double weight of sample with an equal w/v of sterile water or other suitable diluent and then divide into two portions;

— apply the complete procedure of the alternative method and the reference method, including the preparation of the sample. Inoculation of each food sample may be prior to its addition to the culture medium or afterwards.

If necessary, for assuring a better precision of the lowest inoculum level, increase the amount of food sample or the number of replicate samples. For example, 75 g of food sample contaminated with three cells instead of 25 g contaminated with one cell.

The greater the number of inoculum levels used the more precise is the determination of the detection threshold.

5.1.2.3 Calculation

For each level L_i (i = 0 to 3) and each food/strain combination (j = 1 to 5), compare both methods as stated in Table 3:

Table 3 - Calculation of relative detection level

		Results		
		Negatives (-)	Positives (+)	Total
Method	Reference	a	$n - a$	n=6
	Alternative	b	$n - b$	n=6
	Total	$a+b$	$2n - (a + b)$	$2n$=12

For small 2 by 2 tables, perform exact Fisher tests [8].

Comparisons

Instead of only comparing both methods at each level and each food/strain, the same test to compare two food/strains at the same level can be used.

If food/strains seem to be comparable, the same test is available with $n > 6$ in pooling food/strains for each level L_i.

The levels can also be pooled to do checks, but using the ranking order: $L_0 + L_1$, $L_0 + L_1 + L_2$, $L_1 + L_2$, $L_0 + L_1 + L_2 + L_3$, $L_1 + L_2 + L_3$, $L_2 + L_3$… with or without pooling the food/strains.

Report all the significant differences between methods, food/strains and/or levels.

5.1.2.4 Interpretation

The interpretation shall be done by the organising laboratory in charge of the methods comparison study.

The relative detection level lies between the two contamination levels giving respectively less and more than 50 % detection level. The relative detection level is therefore expressed as a range.

5.1.3 Inclusivity and exclusivity

5.1.3.1 Definition

Inclusivity is the ability of an alternative method to detect the target analyte from a wide range of strains.

Exclusivity is the lack of interference from a relevant range of non-target strains of the alternative method.

5.1.3.2 Measurement protocol

5.1.3.2.1 Selection of test strains

5.1.3.2.1.1 General

For microorganisms a range of strains is chosen to avoid any local bias.

Criteria for selecting test strains are given in annex G.

Each strain shall be characterised biochemically, serologically and if relevant genetically, in sufficient detail for its identity to be established and should be preferentially isolated from food. Also the food material from which it was originally isolated shall be known and recorded.

5.1.3.2.1.2 Target microorganisms

Select at least 50 pure cultures of microorganisms relevant to the alternative method and the food product being used (see G.3), except for *Salmonella*.

For *Salmonella* methods, select at least 30 pure cultures of microorganisms.