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Milk — Bacterial count — Protocol for the evaluation of alternative methods

*Lait — Dénombrement bactérien — Protocole pour l'évaluation des
méthodes alternatives*

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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.

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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 16297/IDF 161 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products* and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

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Foreword

IDF (the International Dairy Federation) is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented at the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

The main task of Standing Committees is to prepare International Standards. Draft International Standards adopted by the Standing Committees are circulated to the National Committees for endorsement prior to publication as an International Standard. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

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ISO 16297|IDF 161 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by Joint ISO-IDF Project Group (S07) of the Standing Committee on *Statistics and automation* under the aegis of its project leader, Mrs. I. Andersson (SE).

This first edition of ISO 16297|IDF 161 cancels and replaces IDF 161A:1995, which has been technically revised.

Introduction

Any quantitative measurement in microbiology should consider that there is a requirement for the microbiological state in a sample to be regarded as one point within the co-ordinates of a multidimensional system, which is to be projected on to the one-dimensional scale of the method applied, i.e. plate count, flow cytometry. Aspects such as flora (types and numbers of microorganisms and their distribution), growth phase, sub-lethal damage, metabolic activity, and history, influence to a greater or lesser extent any parameter that is measured. It is evident that any projection of an n -dimensional situation on to an one-dimensional scale is bound to provide a picture of the real situation that is rather restricted. In this respect one has to bow to the inevitable, regardless of which method of measurement is preferred.

The term reference (or official or anchor) method in this International Standard means a method internationally recognized by experts, used in legislation or by agreement between the parties. There are requirements for evaluation of an alternative method to refer to the reference method and to be based on the examination of suitable samples for its intended use.

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Milk — Bacterial count — Protocol for the evaluation of alternative methods

1 Scope

This International Standard gives guidelines for the evaluation of instrumental alternative methods for total bacterial count in raw milk from animals of different species.

NOTE The document is considered complementary to ISO 16140 and ISO 8196|IDF 128 (see Clause 2 and Reference [1]).

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

ISO 8196-1|IDF 128-1, *Milk — Definition and evaluation of the overall accuracy of alternative methods of milk analysis — Part 1: Analytical attributes of alternative methods*

ISO 8196-2|IDF 128-2, *Milk — Definition and evaluation of the overall accuracy of alternative methods of milk analysis — Part 2: Calibration and quality control in the dairy laboratory*

ISO 16140-1, *Microbiology of food and animal feed — Method validation — Part 1: Vocabulary*

ISO 16140-2, *Microbiology of food and animal feed — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*

ISO 21187|IDF 196:2004, *Milk — Quantitative determination of bacteriological quality — Guidance for establishing and verifying a conversion relationship between routine method results and anchor method results*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 8196-1|IDF 128-1 and ISO 8196-2|IDF 128-2 apply.

For the definitions of precision, repeatability and reproducibility, see ISO 5725-1, ISO 5725-2, ISO 8196-1|IDF 128-1, and ISO 16140-1.

4 Transformation of results

A prerequisite for statistics most common in the evaluation of measuring methods is the approximation of a normal distribution of the data. The exponential multiplication of microorganisms usually leads to a right-tailed distribution of quantitative microbiological parameters. Thus, in general, transformation of the raw data is necessary for approximation of normality. This is usually a common logarithmic transformation or a square root transformation for low bacteria levels. The most appropriate transformation can be checked by comparing histograms. All statistics are then computed from the

transformed data, unless specified otherwise, and only the final results are re-transformed to give a more expressive idea of the situation to the user (see also [Annex A](#)).

5 Attributes of the alternative method

NOTE The parameters outlined in this clause do not need to be evaluated completely for every alternative method. For example, the measuring range (see 5.2) of the plate loop method is determined by the loop(s) used.

5.1 Description of the method to be evaluated

5.1.1 Description

The description of the method under study shall be in line with the checklist in 5.1.2.

Most of the information is found in the specification of the method given by the responsible supplier or any other source (author) of the technique specified.

5.1.2 Checklist

- a) Principle of the method.
- b) Parameter or unit.
- c) Technical design of the measurement procedure.
- d) Field of application:
 - 1) purpose: e.g. research, screening, milk grading;
 - 2) matrix: e.g. raw milk from cows.
- e) Supplier(s) of instrument, reagents, standards.
- f) Specification of the method given by the producer or the author:
 - 1) prerequisites for sampling (often compared to the situation of fat analysis);
 - 2) possibilities for sample preservation [reagent(s), storage condition(s)];
 - 3) quantitative (units: method under study or reference method) and qualitative (the kind of microorganisms covered) spectrum;
 - 4) precision (in units of the method under study or in reference method units);
 - 5) accuracy of the estimate (in reference method units);
 - 6) samples per hour;
 - 7) list of references.

5.2 Measuring range

5.2.1 Lower limit of quantification

The lower limit of quantification is defined as the average of milk without bacteria plus the n -fold of its standard deviation; generally, $n = 10$. See also ISO 16140-2.

Analyse milk without bacteria or with a very low concentration of bacteria. Transform data by calculating square root from each result. Calculate the mean, $\bar{x}$, and the standard deviation, s , of the transformed results. Calculate the lower limit of quantification as $\bar{x} + ns$.

5.2.2 Upper limit of quantification

The upper limit of quantification is determined by the highest possible reading of the method or by methodological limitations, e.g. coincidence effects, inaccuracy in the upper measuring range, clogging of filters. Coincidence is when two or more elements of the measurand are detected simultaneously and identified as only one unit. For example, with flow cytometry, if two bacterial cells pass the detector simultaneously, they are detected as one. The coincidence effect is higher with higher concentrations of a measurand.

Upper limit of quantification is determined as the highest concentration where the instrument is still linear according to 5.2.3.

5.2.3 Linearity of the instrument signal

The relationship between the instrument readings and the expected values shall be linear within the concerned range of bacterial counts. Deviations from linearity may stem from non-specific signals and coincidence effects.

A linearity check is at first performed visually using appropriate graphs to obtain an impression of the shape of the relationship. Whenever deviation from linearity appears evident, a quantitative parameter is calculated to indicate whether the observed trend is acceptable or not.

To achieve this, use a high bacterial count milk diluted serially with low bacterial count milk, resulting in a set of at least 10 samples covering the concentration range of interest.

Measure all samples at least four times and calculate the average result for each sample. This gives the measured value per sample. Use the measured values for the high count milk and the low count milk to calculate values for the intermediate samples from the applied mixing ratios. This results in an expected value for each sample. Then apply linear regression with the expected values per sample, C_e , on the x -axis and the measured values per sample, C_{meas} , on the y -axis. Calculate the residuals $\Delta C_{1i} = C_{\text{meas}, i} - (a \times C_{e, i} + b)$ from the regression. Plot the residuals ΔC_{1i} on the y -axis versus the expected values, C_e , on the x -axis. A visual inspection of the data points usually yields sufficient information about the linearity of the signal. Any outlying residual should lead to deletion of the related result and to renewal of the calculation.

The curving can be expressed by the ratio, r_L , using Formula (1):

$$r_L = \frac{(\Delta C_{\text{max}} - \Delta C_{\text{min}})}{(C_{\text{meas, max}} - C_{\text{meas, min}})} \times 100 \quad (1)$$

where

ΔC_{max} is the value of the maximum residual from the regression;

ΔC_{min} is the value of the minimum residual from the regression;

$C_{\text{meas, max}}$ is the measured value for the high count milk;

$C_{\text{meas, min}}$ is the measured value for the low count milk.

The ratio, r_L , shall be less than 5 %.

NOTE To evaluate linearity, use the raw data expressed in units of the routine method without logarithmic or any other transformation.

5.3 Carry-over

Carry-over effects can occur in analytical systems that operate continuously. It derives from the transfer of a certain portion of sample material from one test sample to the next or further sample(s).

Due to the design of a mechanized process of analysis, not only the next sample, but also samples in a later position can be influenced due, for example, to incubation wells with a periodic circulation.

This effect can be tested by analysing consecutively milk with high bacterial count and blank samples. Carry-over causes an increase of blank sample values compared to normal blank sample value (value of blank sample analysed after another blank sample).

The carry-over can be expressed as percentage of the corresponding preceding milk sample.

For evaluation of carry-over, the number of samples and the bacterial count of the milk samples should be high enough to estimate the carry-over with sufficient certainty. The samples should be representative of the routine samples, especially regarding the storage time (longer storage time leading to higher milk viscosity and potentially higher carry-over). One way of setting up the test is described in the example below. For detailed and theoretical aspects and alternative setups of carry-over estimation, it is referred to ISO 8196-3|IDF 128-3.[1]

As an example, one way to estimate the carry-over effect is to analyse at least 10 sets of samples, each set containing one milk sample with very high bacterial count followed by two blank samples. Blank samples could be water or milk with negligible bacterial count.

(milk, blank₁, blank₂)₁, (milk, blank₁, blank₂)₂ ... (milk, blank₁, blank₂)_n

The relative carry-over, COR, expressed as a percentage, can be calculated for each sample set and then averaged:

$$COR_i = \frac{C_{b1i} - C_{b2i}}{C_{si}} \times 100 \quad (2)$$

$$COR = \frac{\sum_i COR_i}{n} \quad (3)$$

where

COR_i is the relative carry-over in the i th sample set;

C_{b1i} is the result of the first blank sample in the i th sample set;

C_{b2i} is the result of the second blank sample in the i th sample set;

C_{si} is the result of the milk sample in the i th sample set;

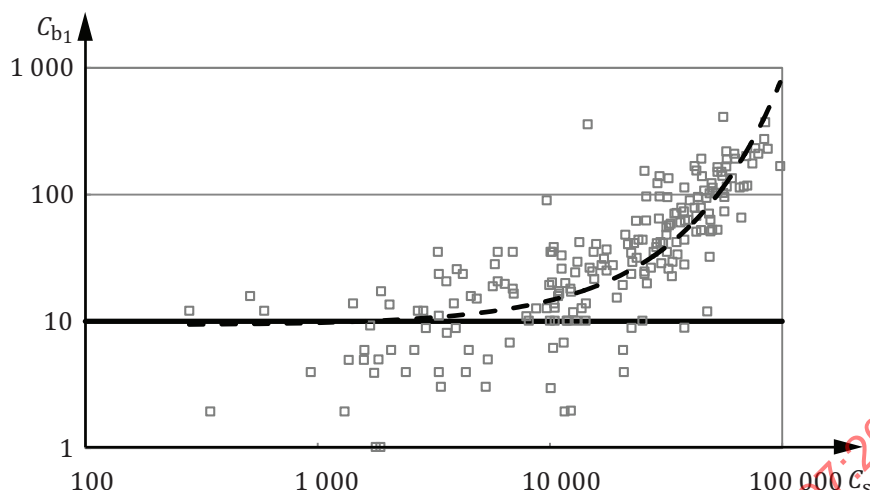
n is the number of sample sets.

Even a very low carry-over effect can be relevant if the corresponding preceding sample has a very high level in comparison to the next one. It can even cause the result of the next sample to exceed a given limit.

Carry-over shall be below 1 %.

An example of carry-over effect is given in Figure 1. The results of blank solutions analysed immediately after high count samples are plotted against the results of the corresponding preceding milk samples. From the graph, the measuring level of preceding milk samples which can lead to an increase of the blank values above the accepted level can be derived. The relation between sample and blank values can be approximated by a function, e.g. a polynomial.

NOTE To evaluate carry-over, use the raw data expressed in units of the routine method without logarithmic or any other transformation.



Key

- C_{b1} total bacterial count of blank solutions analysed immediately after milk sample in units/ml
- C_s total bacterial count of milk samples in units/ml
- results with individual sample sets
- trend line
- carry-over: 0 %

NOTE Carry-over in this example is 1 %.

Figure 1 — Example: Carry-over effect with regard to total bacterial count in raw milk

5.4 Stability

It is essential to check the stability of the instrument with suitable samples.

For many microbiological methods, reference materials are not available or their widespread application under field conditions is not possible due to short shelflife and thus restricted transportability.

Compensate for this deficiency by a reference material substitute or a ring test procedure. The relevant characteristics of a reference material substitute should be as similar as possible to the nature of the components and the matrix in which the measurement takes place.

When reference material substitutes with longer shelflife are available, the stability of instrumental methods shall be checked throughout the working day and also during the period between instrument standardization operations (quality control in the laboratory). Use a control chart according to ISO 8196-2|IDF 128-2.

Protocols for standardization and stability checks are described in ISO 8196-2|IDF 128-2.

5.5 Precision

5.5.1 General

For guidance on the determination of precision, repeatability and reproducibility, see ISO 5725-1, ISO 5725-2, ISO 8196-1|IDF 128-1, and ISO 16140-1.

5.5.2 Repeatability

The repeatability can be estimated from a large number ($n = 50 \dots 100$) of duplicate measurements made on samples covering the whole measuring range. If the repeatability is dependent on the level, it shall be specified as a function of the level, otherwise an average value can be used.

For total bacterial count in raw milk, the acceptability limits for the repeatability standard deviation, s_r , are:

- a) units of $0,09 \log_{10}$ units for contamination levels $\geq 2 \times 10^4$ cfu/ml;
- b) units of $0,12 \log_{10}$ units for contamination levels $< 2 \times 10^4$ cfu/ml.

5.5.3 Reproducibility

Estimate the reproducibility through an interlaboratory study according to ISO 5725-2 from duplicate measurements in representative samples at the lower, medium, and upper levels in the measuring range, preferably obtained from at least eight collaborators.

If no relationship exists between repeatability and the level, this can also be assumed to be true for the reproducibility. If there is a relationship between the reproducibility and the level, it shall be specified.

For total bacterial count in raw milk, the acceptability limit for the reproducibility standard deviation, s_R , is $0,16 \log_{10}$ units.

5.5.4 Evaluation of factors affecting the results

All non-bacteriological factors associated with the properties of the raw milk sample which could disturb the measurements by the alternative method shall be evaluated. Examples of factors are somatic cell count, composition of milk, history of milk, sampling of milk, preservation of milk, species and breed of animals.

Carefully consider which effects different factors may cause, and design experiments taking these into account.

EXAMPLE If linearity is expected to be affected by a certain factor (e.g. fat content), the linearity test should be repeated using samples with a low and high content of this affecting factor. If repeatability is expected to be affected, the repeatability test should be repeated using samples with high and low content. Certain preservatives can affect the level of the counts. To check for this, analyse a series of samples with and without addition of preservative.

6 The alternative method as an estimate of the reference method

This clause addresses the analysis of the interrelations of the results of the alternative method and the reference method. For the establishment and verification of a conversion relationship, see ISO 21187/IDF 196.

The analysis of the relation between two methods is based on the examination of test materials with both methods, covering the field of application and its spectrum of samples to be analysed with the method under study.

6.1 Evaluation of factors affecting the estimation

All factors associated with the properties of the raw milk sample that can affect the relation between reference and alternative method results shall be considered in order to make sure that samples chosen to evaluate the relationship are representative for the normal routine samples.

NOTE Factors influencing the relation can be bacteriological or non-bacteriological, e.g. type of bacteria, growth phase, storage condition, sample preservation, geographic differences, seasonal variations, species and breed of the animals from which the raw milk originates, method of milking, disinfection, feeding methods or individual supplier.

6.2 Measurement protocol

The evaluation of the alternative method as an estimate of the reference method requires a large amount of different samples (typically 100 per $\log_{10}$ step). A minimum number of samples can be calculated according to ISO 21187|IDF 196:2004, [Annex A](#).

Samples shall:

- a) be natural raw milk samples;
- b) uniformly cover the whole range of interest;
- c) be representative of the routine samples to be analysed especially taking into account the above mentioned factors.

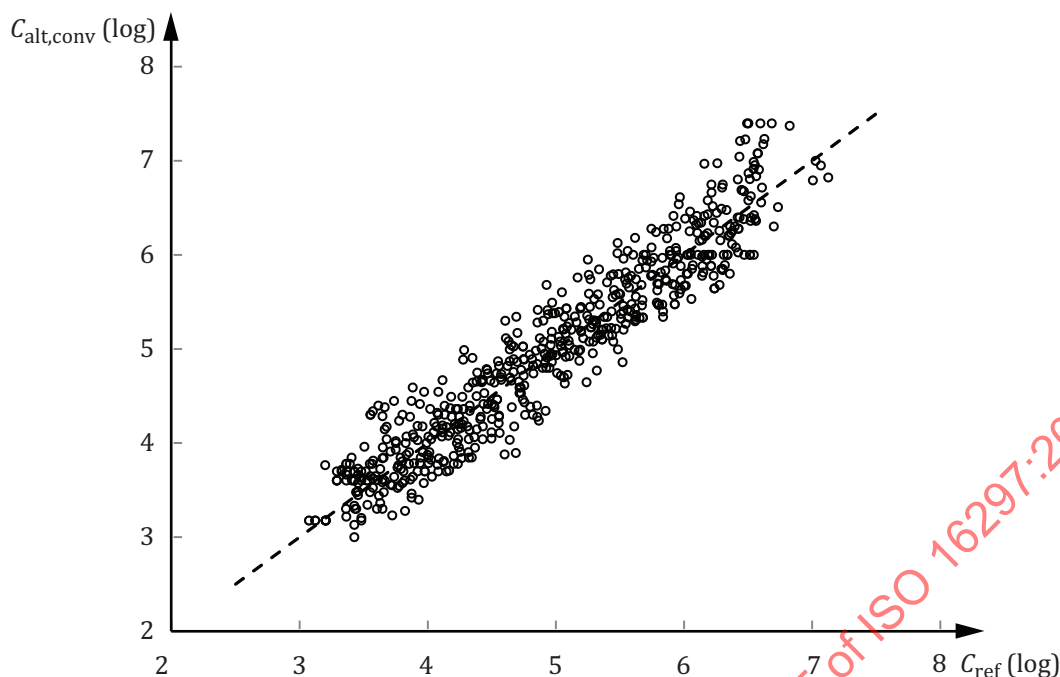
Samples shall be analysed with the reference method as well as with the alternative method at the same time or close to it (preferably within 2 h, whereby samples are kept at 0 °C to 4 °C during storage and transport).

6.3 Calculations

Before further evaluations, the alternative method results shall be converted into units of the reference method by the conversion function. Logarithmic transformation of reference method results as well as of alternative method results generally provides the required approximation of normality, see Clause 4.

6.3.1 Visual check of a scatter diagram

Before any calculation is made a scatter diagram shall be checked visually to obtain a first impression of the relationship and to determine whether the expected relationship between the methods is approximated. Plot reference results against results of alternative method (converted into units of the reference method). See Figure 2.



Key

- $C_{alt, conv} (\log)$ alternative method results after applying conversion function (log)
 $C_{ref} (\log)$ reference/anchor method results
 $\circ$ results of individual milk samples
 ----- $C_{ref} = C_{alt, conv}$

Figure 2 — Example: Relation between the results of an alternative method and of the corresponding reference method for total bacterial count in raw milk

6.3.2 Outliers

Outliers shall be carefully scrutinized. No data shall be discarded unless there is a sound microbiological reason to do so. For outlier evaluation, use ISO 21187/IDF 196.

6.3.3 Accuracy of the estimate, accuracy profile

The accuracy of the estimate is a measure of the reliability of the estimation of the value with one method from the measured value of another method. It can be described by the mean and standard deviation of differences between alternative method results and reference method results at different levels throughout the measuring range and illustrated by an accuracy profile.

For each sample, calculate the logarithmic difference between methods

$$\Delta C_{2i} = C_{alt, i} - C_{ref, i}$$

where

$C_{alt, i}$ is the result of the alternative method for the i th sample (logarithmic values);

$C_{ref, i}$ is the result of the reference method for the i th sample (logarithmic values).

Group results according to reference result in, for example, 0,5 log₁₀ unit intervals. Within each level:

- calculate the mean and standard deviation of reference results;
- calculate the average logarithmic difference, $\overline{\Delta C_{2i}}$, and standard deviation of logarithmic differences, $s_{\Delta C_{2i}}$.

Calculate the 95 % logarithmic confidence limits as $\overline{\Delta C_{2i}} \pm 1,96 s_{\Delta C_{2i}}$ (1,96 is the *t*-statistic for 95 % confidence limit).

Illustrate the result graphically as an accuracy profile. Plot results from each group on a graph with mean differences and 95 % confidence limits on the *y*-axis and mean reference results on the *x*-axis. See Figure 3.

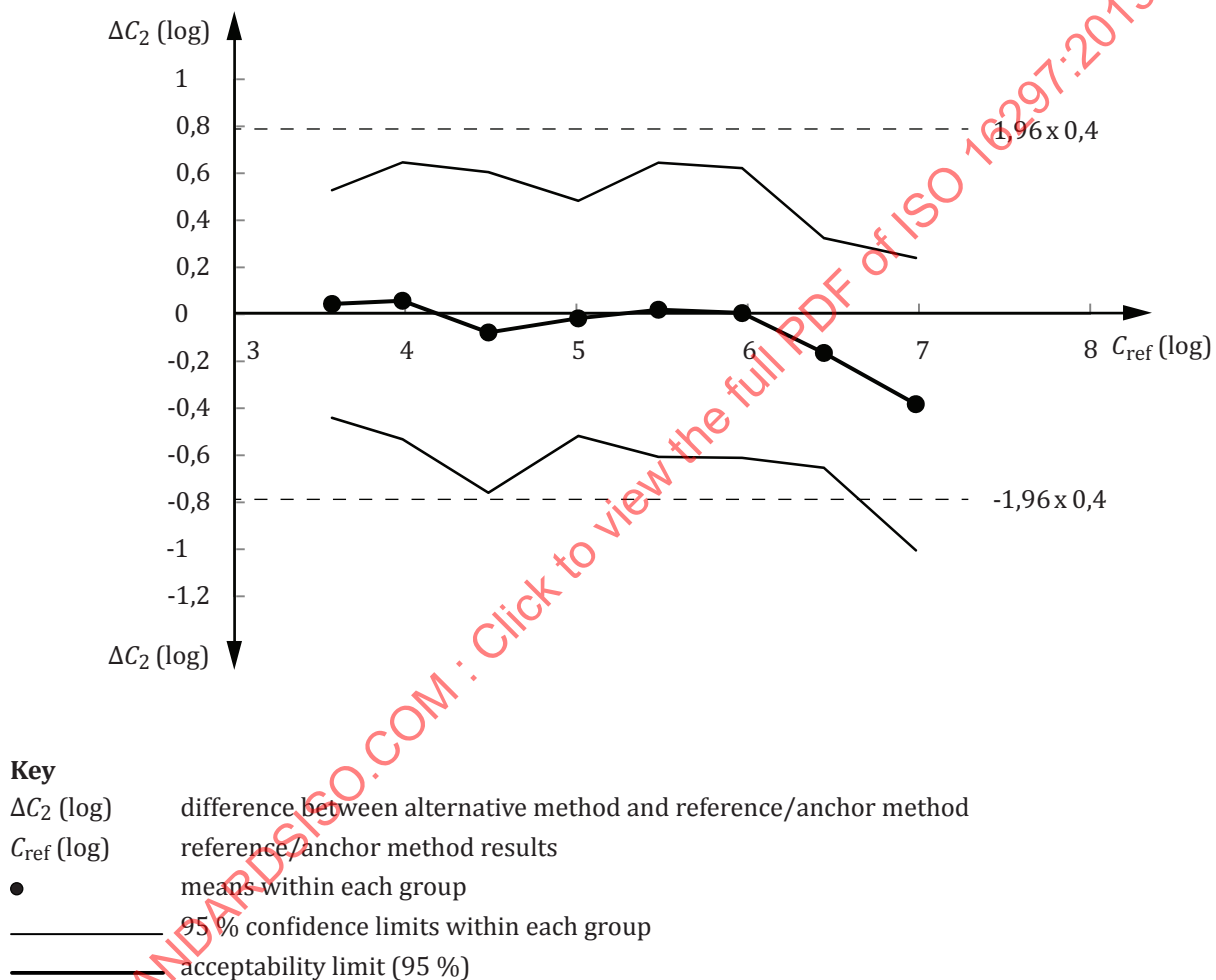


Figure 3 — Example: Accuracy profile for an alternative method for total bacterial count

6.3.4 Criterion of acceptability

The accuracy profile is compared to the criterion of acceptability. If the 95 % confidence limits fall within the acceptability limits, the alternative method fulfils the criterion. If the 95 % confidence limits fall outside the acceptability limits, the alternative method does not fulfil the criterion. If the criteria are fulfilled at some levels and not at other levels, the application of the method may be restricted by narrowing the measuring range.

For total bacterial count in raw milk, the overall accuracy expressed as standard deviation should not exceed 0,40 log₁₀ units (Reference [14]), that means 95 % confidence limit should be within $\pm 1,96 \times 0,4$ log₁₀ units, i.e. $\pm 0,8$ log₁₀ units.

6.4 Attributes of the alternative method expressed in units of the reference method

All attributes of the alternative method, which are expressed in the units of the alternative method, can be converted on to the reference scale by using the conversion relationship. The conversion function used shall be clearly stated.

7 Rating of the elaborated attributes

In general, a final report shall summarize the elaborated attributes of the alternative method under study. A rating of the results shall be given by expert opinion, especially in the light of the requirements set for the alternative method by its intended use.

Among others the following aspects might be of special importance.

- a) Range of the parameter of the alternative method to be expected under the conditions of its intended use. For example, with milk grading, the alternative method should have adequate discriminative power in the range of interest, thereby also having still a margin for situations where a future shift to stricter grading limits is anticipated.
- b) If a grading scheme is not based on single results, but on the averages of the results of several measurements (e.g. over a month), an appropriate measuring range of the alternative method with regard to the parameter's distribution in the population is of special importance: it is essential that low measurements not be disturbed by method background noise, otherwise the "buffer" to compensate for accidentally high measurements in a series is lost. This can lead to an unfair grading.
- c) The representativeness of sampling and preservation facilities plays an important role in the rating of a alternative method for the quantitative determination of the bacteriological quality of raw milk.