BRITISH STANDARD

BS ISO 8196-3:2009

Milk — Definition and evaluation of the overall accuracy of alternative methods of milk analysis

Part 3: Protocol for the evaluation and validation of alternative quantitative methods of milk analysis

ICS 67.100.10

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British Standards

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National foreword

This British Standard is the UK implementation of ISO 8196-3:2009.

The UK participation in its preparation was entrusted to Technical Committee AW/5, Chemical analysis of milk and milk products.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

Compliance with a British Standard cannot confer immunity from legal obligations.

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Part 3: Protocol for the evaluation and validation of alternative quantitative methods of milk analysis

Lait — Définition et évaluation de la précision globale des méthodes alternatives d'analyse du lait —

Partie 3: Protocole pour l'évaluation et la validation des méthodes quantitatives alternatives d'analyse du lait

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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.

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 8196-3|IDF 128-3 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.

ISO 8196|IDF 128 consists of the following parts, under the general title *Milk — Definition and evaluation of the overall accuracy of alternative methods of milk analysis*:

— *Part 1: Analytical attributes of alternative methods*

— *Part 2: Calibration and quality control in the dairy laboratory*

— *Part 3: Protocol for the evaluation and validation of alternative quantitative methods of milk analysis*

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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 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 document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

ISO 8196-3|IDF 128-3 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 ISO and IDF.

All work was carried out by the Joint ISO-IDF Action Team on *Automated methods* of the Standing Committee on *Quality assurance, statistics of analytical data and sampling* under the aegis of its project leader, Mr. O. Leray (FR).

This edition of ISO 8196-3|IDF 128-3, together with ISO 8196-1|IDF 128-1 and ISO 8196-2|IDF 128-2, cancels and replaces IDF 128:1985, which has been technically revised.

ISO 8196|IDF 128 consists of the following parts, under the general title *Milk — Definition and evaluation of the overall accuracy of alternative methods of milk analysis*:

— *Part 1: Analytical attributes of alternative methods*

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Introduction

This part of ISO 8196|IDF 128 is complementary to ISO 8196-1|IDF 128-1. It describes a protocol for the evaluation of new alternative methods for which ISO 8196-1|IDF 128-1 cannot apply, e.g. when the organization of interlaboratory studies is hampered by too small a number of new instruments available for study.

The latter is generally the case with dedicated instrumental methods (e.g. milk payment analysis, milk recording analysis) of which the commercialization depends on official approvals for use. An application for such an official approval is to be accompanied by one or more assessments of the relevant performance characteristics.

This part of ISO 8196|IDF 128 specifies a harmonized protocol for such a method validation by an expert laboratory. It lists the evaluation steps, provides a criteria-based approach for the assessment of the performance characteristics, including guidance for checking statistical compliance.

On the basis of such a harmonized protocol, only a limited number of evaluations should suffice for a decision on approval either by national bodies or by an international organization for the application of the methods and/or equipment in their area. An example is given for the evaluation of a method for the determination of fat, protein, lactose, urea and somatic cell count in milk.

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Milk — Definition and evaluation of the overall accuracy of alternative methods of milk analysis —

Part 3:
Protocol for the evaluation and validation of alternative quantitative methods of milk analysis

1 Scope

This part of ISO 8196|IDF 128 specifies a protocol for the evaluation and validation of alternative quantitative methods of milk analysis.

The protocol is applicable to all milk components including somatic cells. For microbiological parameters other standards, such as ISO 16140[5], apply. This part of ISO 8196|IDF 128 is also applicable to the validation of new alternative methods where a limited number of analysts does not allow the organization of an interlaboratory study and ISO 8196-1|IDF 128-1, therefore, does not apply.

This part of ISO 8196|IDF 128 also establishes general principles of a procedure for granting international approvals of these alternative methods. These principles are based on the validation protocol defined in this part of ISO 8196|IDF 128.

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 3534-1, *Statistics — Vocabulary and symbols — Part 1: General statistical terms and terms used in probability*

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

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 9622, *Whole milk — Determination of milkfat, protein and lactose content — Guidance on the operation of mid-infrared instruments*[1)]

ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

1) Equivalent to IDF 141.

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3 Terms and definitions

For the purpose of this document, the terms and definitions given in ISO 8196-1|IDF 128-1, ISO 8196-2|IDF 128-2, ISO 3534-1 and ISO 5725-1 apply, together with the following.

3.1
validation of an alternative method
demonstration that results obtained with an alternative method are comparable to those obtained with the reference method, thereby showing compliance of accuracy with defined requirements and fitness for purpose

3.2
measurand
component
analyte
criterion
particular quantity or characteristic subject to measurement

EXAMPLES A measurand may be a milk component, a physical characteristic or a biological element.

NOTE Adapted from ISO/IEC Guide 99:2007[8], 2.6.

3.3
quantitative method
method of analysis whereby the result is an amount of a quantity, a concentration or a value of a **measurand** (3.2) determined either directly or on a test portion

3.4
methods comparison study
study, performed by an organizing laboratory of an alternative method against the reference method under test bed conditions

3.5
method confirmation study
study performed in routine laboratories, of an alternative method to confirm results of a previous **methods comparison study** (3.4)

3.6
interlaboratory study
study of performance of an alternative method on one or more “identical” laboratory samples of homogeneous, stable materials under documented conditions in several laboratories and under the control of an **organizing laboratory** (3.7)

3.7
organizing laboratory
laboratory having qualified staff and equipment to perform a **methods comparison study** (3.4)

3.8
national approval
authorization of use of a method for defined purposes in a country — generally for reasons of collective interest and/or having an official character — delivered by an official body

3.9
international approval
authorization of use of a method for defined purposes at the international level — generally for reasons of collective interest and/or having an official character — delivered by an international organization for the benefit of stakeholders

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4 General principles for the validation of alternative methods

4.1 Validation protocol

4.1.1 General

The validation protocol comprises two phases as specified in 4.1.2 and 4.1.3 respectively.

4.1.2 Phase I

A methods comparison study includes the assessment of the analytical attributes and a comparison of the alternative method against the reference method under test bed conditions. This part of the evaluation has to be carried out by an organizing laboratory specialized in analytical evaluations as well as being experienced in the application of the relevant reference method. The laboratory shall conform to ISO/IEC 17025 for this activity.

4.1.3 Phase II

A method confirmation study under routine testing conditions is initiated after a successful Phase I. The examination is recommended of at least two instruments located in different routine laboratories under routine testing conditions for a minimum period of two months. Care should be taken that each instrument is exposed to the level of sample variation normally expected during that period. Each instrument should fulfil the day-to-day quality control demands specified in ISO 8196-2|IDF 128-2 by checking compliance of results with figures of overall accuracy obtained in Phase I. The alternative method should also be assessed for general convenience aspects such as speed, consumables, user-friendliness, security, and robustness.

4.1.4 National approval

Based on the content of submitted reports, national bodies can authorize the use of an alternative method for defined purposes. Compliance with requirements stated in this protocol provides assurance of a sufficient quality in measurement results and comparability with other methods and/or instruments of a similar type validated elsewhere according to the same protocol.

4.1.5 International approval

International organizations can grant an international approval, e.g. for international milk recording, or to respond to a criteria approach. A number of successful individual (i.e. national) validations, reported in a standardized way, can provide sufficient confidence in the new alternative method performance and replace interlaboratory studies. The overall evaluation should be renewed successfully in a minimum number of distinct countries. Three independent validations are recommended.

4.2 Field of validity of the approval

4.2.1 An approval is given only under the circumstances specified in 4.2.2 and 4.2.3.

4.2.2 The field of application in which the instruments are used has been evaluated (component, concentration range, animal species, etc.). For instance, if milk of different animal species is to be analysed, specific evaluations for each species have to be carried out to assess that the instrument is appropriate for the expected use. If milk from breeds with unusual contents (e.g. Jersey breed with high fat and protein) is to be analysed, the evaluation should be carried out over the whole range of occurrence of the relevant component.

4.2.3 The specific method and/or instrument configuration used has been evaluated. If the configuration changes, proof should be obtained that it does not affect the precision and the accuracy beyond acceptable limits.

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4.2.4 Carefully note and report all characteristics of both the milk products analysed and the configuration(s) of the alternative method assessed.

5 Technical protocol for the validation

5.1 Course of operations

Whatever the alternative method, a standard measurement process can be represented schematically as in Figure A.1. Each step corresponds to a source of error that may contribute to the overall uncertainty of the method (element in the breakdown of the overall accuracy). The evaluation protocol and experimental designs are constructed to fit the sequence of signal treatment and to permit verification that they are set up in such a way that precision and accuracy of the method can respond to the limits required in practice.

It is necessary for each step of the evaluation described in the following paragraphs to fulfil the appropriate limits for each analytical criterion before starting the next step.

The first part of the protocol (5.2.2) is compulsory as it defines the minimum assessment sequence to be carried out.

A second part (5.2.3) is recommended to provide complementary information for future use.

5.2 Methods comparison study

5.2.1 General

This part specifies the elements of the evaluation which are compulsory.

The evaluation is to be carried out from test results expressed in standardized units of the reference method. For methods covering large ranges of measurand values (i.e. wider than 1 log unit), it is recommended to split the range into segments, each of maximum width one log unit, so as to obtain a minimum of three segments and to perform statistical calculations separately on each segment.

NOTE For instance, for fat in commercial milk, distinction can be made between skim milk, half-skim milk and whole milk; for raw milk, natural fat and protein ranges are often related to the species, which are then to be assessed by separate evaluations (4.2); somatic cells in raw milk typically cover a range of several log units.

For methods where precision and accuracy are found to be proportional to the measurand value, apply an appropriate correction to the raw values.

Evaluation results should comply with specifications stated in the following paragraphs. For general dairy industry purposes, limits for the different analytical characteristics mentioned have been extracted or derived from existing International Standards.

Annex B summarizes these limits for fat, protein (crude protein, true protein and casein), lactose, urea and somatic cells.

NOTE For liquid milk during milking or processing, there may be different assessment criteria for in-line and on-line analysis systems and at-line systems.

5.2.2 Compulsory assessments for the validation

5.2.2.1 Assessment of preliminary instrumental fittings

Before starting any further assessment, basic criteria indicating a proper functioning of the method or the instrument require verification. These criteria are daily precision (including repeatability and short-term stability), carry-over, and linearity.

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5.2.2.1.1 Daily precision (repeatability and short-term stability)

Basically, the method used should present a measurement signal stability which complies with the precision requirements. If not, the analyser is either not functioning correctly (and should not be used) or its precision is not suitable for the objective of the analysis. Hence, the instantaneous stability (repeatability) and the signal level stability have to be assessed prior to any other characteristics.

EXAMPLE 1

The precision should be evaluated at three different concentration levels of the component measured: low, medium, and high. To achieve this, three different milk samples should be split into as many identical test portions as necessary for the analyses.

During the day, for each level, analyse the same milk sample in triplicate ($n = 3$) using the instrument every 15 min to 20 min without any change in the calibration in order to obtain a minimum of 20 check test series ($q \geqslant 20$). Preferably, it should be operated under conditions as close as possible to routine circumstances. Sufficient numbers of samples should be processed to keep the instrument running between the periodic checks.

Using a one-way analysis of variance (ANOVA), estimate the standard deviation of repeatability, s_r, the standard deviation between check series, s_c, and the standard deviation of daily reproducibility, s_R, or, equivalently, according to the following:

For every check, j ($j = 1 \ldots q$), calculate the mean,

$$\bar{x}_j = \sum x_{ij}/n$$

and the standard deviation,

$$s_{rj} = \left[\sum (x_{ij} - \bar{x}_j)^2 /(n-1)\right]^{1/2}$$

of replicates.

For the whole check sequence, calculate:

a) the standard deviation of repeatability: $s_r = \left(\sum s_{rj}^2 / q\right)^{1/2}$

b) the standard deviation of means:

$$s_{\bar{x}} = \left[\sum (\bar{x}_j - \bar{x})^2 /(n-1)\right]^{1/2} = \left\{\left[\sum \bar{x}_j^2 - \left(\sum \bar{x}_j\right)^2 / q\right] /(q-1)\right\}^{1/2}$$

with

$$\bar{x} = \sum \bar{x}_j / q$$

c) the standard deviation between checks: $s_c = (s_{\bar{x}}^2 - s_r^2 / n)^{1/2}$ with $s_c = 0$ if $s_c < 0$

d) the standard deviation of daily reproducibility: $s_R = (s_c^2 + s_r^2)^{1/2}$

EXAMPLE 2 The values obtained for s_r and s_R should comply with the limits stated in Annex B.

The stability of the method response during the sequence of check tests can be visualized by plotting measurement results and means, y, versus the check sequence numbers, x.

The significance of a possible observed deviation or fluctuation can be verified with the F-test of a one-way ANOVA or, equivalently, by calculating the observed value of F, F_{obs}:

$$F_{obs} = n s_{\bar{x}}^2 / s_r^2$$

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The test is significant if $F_{obs} > F_{1-\alpha}$ with $k_1 = q - 1$, $k_2 = q(n - 1)$, and $\alpha = 0{,}05$.

5.2.2.1.2 Carry-over effect

5.2.2.1.2.1 Strong differences in component concentrations between two successively analysed samples may influence the result of the second.

Differences can be caused by incomplete rinsing of the flow system and the measuring cell by liquid circulation and contamination by the stirring device. Automatic correction of results is acceptable within certain limits, provided it can be proven that there is a systematic transfer of a small quantity of material from one measurement to the next.

Automated analysers for liquids often allow automatic correction to compensate for the overall carry-over effect when necessary. Carry-over has to be clearly distinguished from rinsing efficiency.

5.2.2.1.2.2 The overall carry-over effect should be assessed including the correction factors either set in the instrument or obtained using the method supplied by the manufacturer. It should not exceed the values stated per component.

NOTE Limits are defined from the prerequisite that carry-over effect should not produce an error higher than the repeatability of the method. Hence, limits for the carry-over ratio (COR), L_C, should fulfil the condition $L_C \leqslant (r/\Delta L_{range}) \times 100$ where r is the repeatability limit at the level of the bias measured and ΔL_{range} is the difference between the maximum and the minimum concentration in the range of interest. For components where repeatability is not constant over the measuring range, the COR limits are set based on the levels of best repeatability (e.g. somatic cell counting). Common limits for COR are in the range 1 % to 2 %.

5.2.2.1.2.3 The rinsing efficiency of the flow system has to be assessed separately by running tests without any correction (correction factor set to zero) in manual mode that bypasses the automated stirrer. Rinsing efficiency should not be less than 99 % or the internal carry-over should not exceed 1 %.

5.2.2.1.2.4 Analyse two samples, with high and low concentrations, respectively, of prior distribution in series of test portions. Repeat, as many times, N_C, as necessary (see below) the analytical sequence in terms of component concentration, low, low, high, high, in order to obtain N_C sets of results, L_{L_1}, L_{L_2}, L_{H_1}, L_{H_2}. The minimum number of sequence replications, N_C, should be 20.

A sufficient number is recommended to reduce the relative uncertainty of the COR estimate, δ_{rel}, and to enable a clear differentiation from zero. A relative uncertainty of 20 % or less is sought. The relevant number of sequences can be obtained by $N_C \geqslant (100/\delta_{rel})^2$. Increasing the number of sequences is especially to be considered in case of estimating COR for adjustment of a correction factor.

NOTE For components where repeatability is not constant over the measuring range and for levels with high repeatability, more numerous sequences can be required. Alternative numbers of sequences can be calculated by $N_C \geqslant [r \times 100/(L_C \Delta L_{test})]^2$ where ΔL_{test} is the range between high and low concentration samples (equal to or greater than ΔL_{range}).

5.2.2.1.2.5 Method requirements for samples: Prepare a sufficient number of test portions from each low and high concentration laboratory sample prior to analysis in order to analyse each test portion only once. The low and high concentration laboratory samples should preferably be milks or liquid products with similar viscosity to those routinely analysed.

Individual component concentrations have to differ considerably. For milk, this can, for instance, be achieved by using natural separation (creaming for fat), artificial separation (ultrafiltration for protein, microfiltration for somatic cells), or addition (lactose and urea).

For biochemical component determinations, the low and high concentrations of the laboratory samples should, preferably, be extreme values in the measuring range.

NOTE Sufficiently large ranges are recommended to easily differentiate carry-over effects from random error. The minimum range needed, $\Delta L_{test} = L_H - L_L$, can be calculated according to $\Delta L_{test} \geqslant r \times 100/(L_C \sqrt{N_C})$ where r and L_C are the stated limits and N_C is the number of sequences applied (see Annex B).

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For milk components or criteria covering large ranges of concentration, e.g. a 3 $\log_{10}$ scale or more, the ratio of carry-over error may not be constant over the whole range. This should be verified by assessing the carry-over at different concentrations.

In such case, it is recommended to choose a level $L_{\mathrm{H}i}$ at the median of each part, i, previously defined in the whole range. A minimum number of two levels in the medium and high concentration range are needed that can be extended to three for particularly wide ranges.

EXAMPLE For somatic cell counting in individual animal milk, the definition of three levels, at about 500×10^3 cells/ml, $1\,000 \times 10^3$ cells/ml, and $1\,500 \times 10^3$ cells/ml, is recommended.

5.2.2.1.2.6 Calculation: Calculate the mean and the standard deviations of the differences, $d_{L_{\mathrm{L}}i} = L_{\mathrm{L}_1 i} - L_{\mathrm{L}_2 i}$ and $d_{L_{\mathrm{H}}i} = L_{\mathrm{H}_2 i} - L_{\mathrm{H}_1 i}$, respectively, $\bar{d}_{L_{\mathrm{L}}}$, $s_{L_{\mathrm{L}}}$, $\bar{d}_{L_{\mathrm{H}}}$, $s_{L_{\mathrm{H}}}$ and the mean difference of concentration, $\bar{d}_{\rho} = \bar{L}_{\mathrm{H}_2} - \bar{L}_{\mathrm{L}_2}$.

Then calculate the CORs, C, and their standard deviations, s_C, by using the following equations:

$$C_{\mathrm{H/L}} = \bar{d}_{L_{\mathrm{L}}} \times 100 / \bar{d}_{\rho} \text{ and } s_{C_{\mathrm{H/L}}} = sd_{L_{\mathrm{L}}} \times 100 / \bar{d}_{\rho} \sqrt{N_C}$$

$$C_{\mathrm{L/H}} = \bar{d}_{L_{\mathrm{H}}} \times 100 / \bar{d}_{\rho} \text{ and } s_{C_{\mathrm{L/H}})} = sd_{L_{\mathrm{H}}} \times 100 / \bar{d}_{\rho} \sqrt{N_C}$$

The COR can also be obtained by using the following equivalent formulas:

$$C_{\mathrm{H/L}} = (\sum L_{\mathrm{L}_1} - \sum L_{\mathrm{L}_2}) \times 100 / (\sum L_{\mathrm{H}_2} - \sum L_{\mathrm{L}_2}) = (\bar{L}_{\mathrm{L}_1} - \bar{L}_{\mathrm{L}_2}) \times 100 / (\bar{L}_{\mathrm{H}_2} - \bar{L}_{\mathrm{L}_2})$$

$$C_{\mathrm{L/H}} = (\sum L_{\mathrm{H}_2} - \sum L_{\mathrm{H}_1}) \times 100 / (\sum L_{\mathrm{H}_2} - \sum L_{\mathrm{L}_2}) = (\bar{L}_{\mathrm{H}_2} - \bar{L}_{\mathrm{H}_1}) \times 100 / (\bar{L}_{\mathrm{H}_2} - \bar{L}_{\mathrm{L}_2})$$

The two COR values obtained should not significantly differ from each other and should not exceed the limit, L_C, in the test condition stated for the component in Annex B.

Verify this by checking whether the following conditions are fulfilled:

$$C_{\mathrm{H/L}} - C_{\mathrm{L/H}} \geqslant t_{1-\alpha/2} \left[s^2_{C_{\mathrm{H/L}}} + s^2_{C_{\mathrm{L/H}}} \right]^{1/2}$$

$$C_{\mathrm{H/L}} \leqslant L_C - t_{1-\alpha}\, s_{C_{\mathrm{H/L}}}$$

$$C_{\mathrm{L/H}} \leqslant L_C - t_{1-\alpha}\, s_{C_{\mathrm{L/H}}}$$

with $\alpha = 0{,}05$.

5.2.2.1.3 Linearity

5.2.2.1.3.1 **General**. According to the classical definition of an indirect method, the instrument signal should result from a characteristic of the component measured and thereby allow the definition of a simple relationship to the component concentration.

Linearity expresses the constancy of the ratio between the increase in the concentration of a milk component and the corresponding increase of the alternative method result. Therefore, linearity of the measurement signal is in most cases essential to maintain a constant sensitivity over the measuring range and to allow easy handling of calibration and fittings. Moreover, it allows in routine (to some extent) measurements beyond the calibration range through linear extrapolation.

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NOTE Current alternative methods are frequently based on multiple signals using a multivariate approach. For these methods, in particular for examples involving small relative changes in the sample matrix and signals with low specificity, linearity assessment can be difficult due to large random error (low signal to noise ratio). In these cases, as the linearity error is contained in the overall accuracy component, linearity assessment can be omitted provided it is covered in the further step of accuracy evaluation.

The method is specified in 5.2.2.1.3.2 to 5.2.2.1.3.4.

5.2.2.1.3.2 Samples. Linearity can be assessed using sets of 8 to 15 samples with component concentrations evenly distributed over the measuring range.

a) Samples should preferably be milks or liquids of similar physical characteristics (i.e. density, viscosity), e.g. by combining (weighing) a high content sample, L_H, and a low content sample, L_L.

b) Concentrations should vary in regular intervals. Depending on the component, that can for instance be achieved by natural separation (creaming for milk fat), artificial separation (ultrafiltration for protein, microfiltration for somatic cells) and recombination, or by using pure solutions (lactose and urea).

c) The linearity assessment range should be congruent with the concentration range for the validation study (Annex B).

d) Reference values for linearity samples can be established from either the mixing ratio or the theoretical concentrations as calculated from the concentrations of the initial samples. Depending on the alternative method, they should be obtained from volume by volume mixing ratios where analysis is performed on a milk volume (volumetric intake measurement) and mass by mass mixing ratios where analysis is applied to a weighed milk portion (see Annex E).

5.2.2.1.3.3 Analyses. Analyse each sample, firstly in order of increasing concentrations in $N_L/2$ replicates, secondly in order of decreasing concentrations in $N_L/2$ replicates, so as to obtain the total replicate number relevant for the measurand (see Annex B).

5.2.2.1.3.4 Calculation and assessment. Calculate the linear regression equation $y = bx + a$ (y = instrument, x = reference) and the residuals e_i ($e_i = y_i - bx_i - a$) from the means of replicates and the theoretical reference.

Plot the residuals, e_i, on the ordinate against theoretical concentrations on the abscissa. Visual inspection of the data points usually yields sufficient information about the linearity of the signal.

Any deviation from linearity or obvious trend in the data in this plot indicates a potential problem and should lead to further investigation of the method, as detailed below.

Any residual obviously being out of the current distribution (outlier) should lead to deletion of that result and repetition of the calculation before applying further tests.

Calculate the relative linearity bias by the ratio of the residual range to the signal values range:

$$\frac{\Delta e}{\Delta \rho} = \frac{e_{\max} - e_{\min}}{\rho_{\max} - \rho_{\min}}$$

where

$e_{\max}$ is numerical value of the upper residual;

$e_{\min}$ is the numerical value of the lower residual;

$\rho_{\max}$ is the numerical upper value measured with the instrument;

$\rho_{\min}$ is the numerical lower value measured with the instrument.

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NOTE 1 Limits are defined from the prerequisite that deviation from linearity should not produce a larger error than the repeatability of the method over the usual measuring range. Hence, limits of the relative linearity bias, $L_{\Delta e/\Delta L}$, are meant to fulfil the condition $L_{\Delta e/\Delta L} \leqslant r/\Delta L_{\text{range}}$ for the upper acceptable repeatability, with r being the repeatability limit and ΔL_{range} being the difference between the maximum and the minimum concentration in the concentration range of interest. For components where repeatability is not constant over the measuring range, the relative linearity bias limits are set based on the levels of largest repeatability (e.g. somatic cell counting). Common limits for $\Delta e/\Delta L_{\text{range}}$ are in the range 0,01 to 0,02.

NOTE 2 The number of replicates needed to ensure significance of the $\Delta e/\Delta L$ test can be estimated by the conditions: $N_L \geqslant 8\,\sigma_r^2/(L_{\Delta e/\Delta L}^2\,\Delta L_{\text{test}}^2)$ or $N_L \geqslant r^2/(L_{\Delta e/\Delta L}^2\,\Delta L_{\text{test}}^2)$.

NOTE 3 Concentration ranges, ΔL_{test}, larger than ΔL_{range} allow the measurement of larger linearity bias, Δe, with a similar relative linearity bias and increased significance for the same maximum repeatability value. The minimum concentration range can be estimated by the conditions: $\Delta L_{\text{test}} \geqslant 2\sqrt{2}\,\sigma_r/(L_{\Delta e/\Delta L}\sqrt{N_L})$ or $\Delta L_{\text{test}} \geqslant r/(L_{\Delta e/\Delta L}\sqrt{N_L})$.

A one-way ANOVA can be carried out to confirm the statistical significance of non-linearity. Statistical tests for comparison of variances can be applied to confirm the significance of difference between residual variances.

Furthermore, if needed, non-linear trends can be approached by second and third degree polynomial and statistical tests, $\Delta e/\Delta L$ and F-tests used to select and assess the equation that allows the best linear fit.

Examples are given in Annex D.

5.2.2.1.4 Measurement limits

Limits of a measurement with an instrumental method exist at both extremities of the analytical range, e.g. a lower limit and an upper limit.

It is not required to determine these limits when natural concentration ranges for the respective components and species are normally located far from zero (which is generally the case for biochemical components, i.e. fat, protein, lactose, urea), and within the linearity range of the method.

The assessment of the measurement limits can be carried out in combination with the evaluation of the linearity. If linearity is not achieved throughout the whole concentration range, determine the actual range of application for the method concerned.

5.2.2.1.5 Lower limits

5.2.2.1.5.1 General. Lower limits are defined, as multiples of the standard deviation, σ, of random error observed near zero (blank), in three ways depending on the risk of error accepted and the precision requirements, as specified in 5.2.2.1.5.2 to 5.2.2.1.5.4.

5.2.2.1.5.2 Critical level, which is the smallest amount that can be detected (non-null) but not quantified as an exact value (risk $\beta = 50$ %). Below it cannot be assumed that the value is non-null:

$$L_{\text{crit}} = u_{1-\alpha}\,\sigma$$

EXAMPLES $L_{\text{crit}} = 1{,}645\sigma$ with $\alpha = 5$ %; $L_{\text{crit}} = 3\sigma$ with $\alpha = 0{,}13$ %.

5.2.2.1.5.3 Detection limit, for which the second type of error is minimized up to a defined level, generally equal to the level of risk, $\beta = 5$ %. It defines the lowest result, which differs significantly from zero (first type error, α), that can be produced with a sufficiently low probability (second type error, β) of including the blank value (zero) and with a sufficient confidence interval:

$$L_{\text{det}} = (u_{1-\alpha} + u_{1-\beta})\,\sigma$$

EXAMPLES $L_{\text{det}} = 3{,}29\sigma$ with $\alpha = \beta = 5$ %; $L_{\text{det}} = 6\sigma$ with $\alpha = \beta = 0{,}13$ %.

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