Guidance Document #10
Analytical Methods for Feed Ingredients
January 2026
At Step 7: Steering Committee Endorsement

ANALYTICAL METHODS FOR FEED INGREDIENTS

January 2026

Endorsed by the Steering Committee
January 2026

It is recommended for the companies planning to submit applications/dossiers for pre-market authorization, to contact the jurisdictions of the countries to confirm their acceptance of the current guidance document.

The International Cooperation for Convergence of Technical Requirements for the Assessment of Feed Ingredients (ICCF) was launched in 2017 and aims to develop and establish common guidance documents to provide technical recommendations for the assessment of feed ingredients, including new uses of existing feed ingredients.

This guidance document has been developed by the appropriate ICCF Experts Working Group and was subject to consultation by the Parties, in accordance with the ICCF Process.

The founding members of the ICCF include the Canadian Food Inspection Agency (CFIA), the European Commission (DG SANTE), the U.S. Food and Drug Administration (FDA), as well as the American Feed Industry Association (AFIA), the Animal Nutrition Association of Canada (ANAC), the EU Association of Specialty Feed Ingredients and their Mixtures (FEFANA) and the International Feed Industry Federation (IFIF).

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ANALYTICAL METHODS FOR FEED INGREDIENTS

1. INTRODUCTION

1.1 Objective of the guidance

This document provides guidance on the information and data to be included in an application for pre-market approval or authorization of feed ingredients, with regards to analytical methods used to establish identity, specification, characterization to support stability and homogeneity, to support the nutritional or technical effect and to support target animal safety and human food safety. The analytical methods are intended for identifying, detecting and/or determining the concentration/level of analytes, contaminants, and constituent entities in feed ingredients, premixes(premixtures), feeds, edible products, target organs, blood/plasma, urine, feces, and manure. This document supports the requirements provided in the other ICCF Guidance Documents.

This guidance document has been developed with an international team of experts and is considered the best practice for the development and implementation of suitable methods of analysis, used in the assessment of feed ingredients.

While the guidance document provides recommendations for the various parameters to be included when providing a method of analysis for the purpose of an assessment of a feed ingredient, applicants are advised to consult the appropriate regulatory authorities and their guidelines, during the development phase of new feed ingredients or for new uses of an authorized feed ingredient. This will ensure that the methods of analysis provided to support the market approval or authorization of the feed ingredient are acceptable.

1.2 Definitions

The definitions applicable in the context of this guidance document are provided in the ICCF Glossary of Terms.

1.3 Scope of the Guidance

This guidance document is applicable for all types of feed ingredients subject to an assessment in the context of their pre-market approval or authorization. It covers the identification and the evaluation of the concentration/amount of active substance(s) and/or their metabolites in different matrices, such as:

- Feed ingredient,
- Ingredient market formulation,
- Premixtures containing the feed ingredient,
- Feeds containing the feed ingredient,
- Edible products, produced by animals consuming the feed ingredient,
- Target organs, considered for the evaluation of the metabolism of the active substance(s) and/or its (their) metabolites, in the context of safety assessment,
- The urine/feces or the manure from animals consuming the feed ingredient.

It may not be necessary for each feed ingredient to provide methods in each of the above-mentioned matrices. Methods of analysis should be provided, when relevant, for supporting the overall assessment of the feed ingredient and/or its ingredient market formulation(s). Therefore, this guidance document should be read in conjunction with the intended conditions of use of the feed ingredient and other guidance documents, providing further details on the information to be submitted to support this assessment.

This guidance document is also applicable for the evaluation of contaminants, relevant for the safety assessment of the feed ingredient and/or its ingredient market formulation(s), as applicable.

Methods of analysis may be developed for various purposes. The following purposes are considered in this guidance document:

- Official control and monitoring on the marketplace,
- In-process controls,
- Internal controls (e.g., product release),
- Use in studies supporting the proposal for pre-market approval or authorization of the feed ingredient.

This guidance document does not provide recommendations for specific methods of analysis. However, methods of analysis such as, but not limited to, High Performance Liquid Chromatography (HPLC), High Performance Thin Layer Chromatography (HPTLC), Mass Spectrometry (MS), Inductively Coupled Plasma (ICP) Spectroscopy, electrochemistry, pH

measurements, titrations, gravimetry, combustion, spectrometry, immunoassay, microscopy, and cell enumeration are considered in this guidance document.

The methods of analysis proposed may either be official methods, standardized methods or developed by a single laboratory.

This guidance document is composed of three parts:

- The general recommendations covering all types of methods of analysis (Section 2),
- The specific recommendations to measure chemically defined analytes, in any matrix(ces) (Section 3),
- The specific recommendations to measure the presence of microorganisms in ingredient market formulations, premixtures and feeds (Section 4).

2. GENERAL RECOMMENDATIONS

2.1 Methods of analysis of the active substance and its metabolites

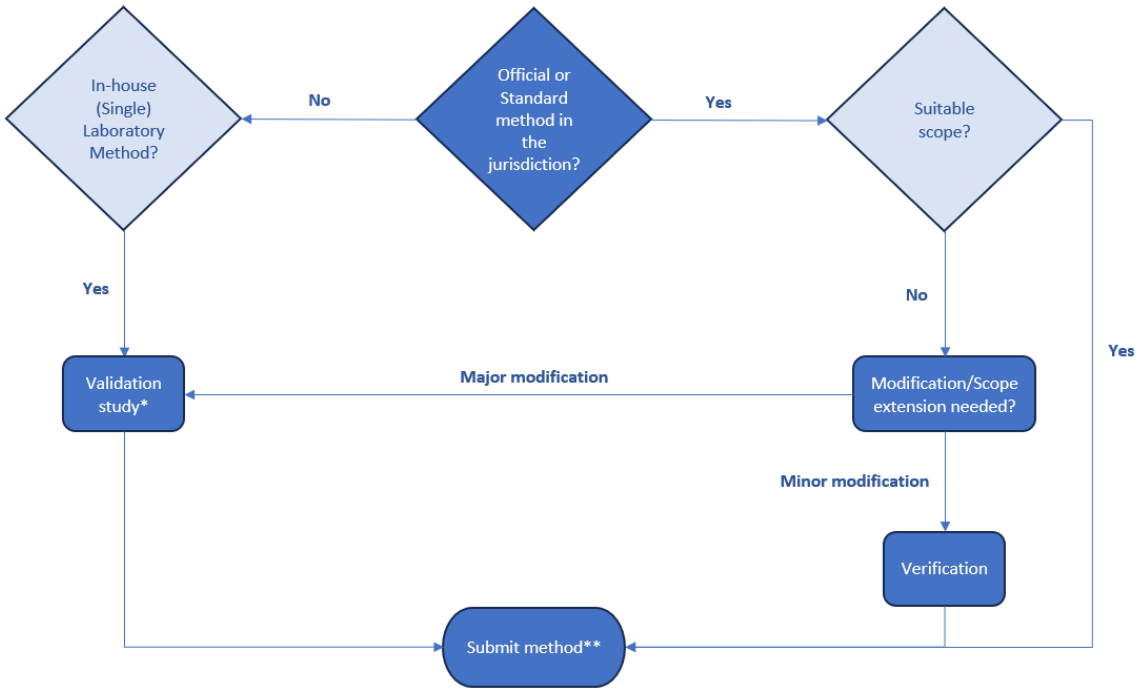
The analytical procedure of each method of analysis of the active substance(s) and/or the relevant constituent entity(ies) and/or the metabolites of the feed ingredient should be provided, including the description of equipment used and its parameters, and contain at least the following details to enable replication of the analysis:

- The matrix(ces) covered by the scope of the method of analysis,
- The analyte(s) analyzed (in the case of multi-analyte methods, the list of analytes measured should be provided),
- The description of the processing of the sample, including, where appropriate, grinding and sieving procedures (e.g., for feed and premixture),
- The description of the incubation phase (i.e., for enzymes, microorganisms, and immunoassay), if relevant,
- The equipment (apparatus/instruments) used for conducting the analysis and the relevant conditions of use,
- The description of the reagents, and preparation, if appropriate (e.g., the titer of antibody used in the immunoassays, buffers, extractants),
- Reference standards used and description of preparations of standards and dilutions,
- The description of the various steps to follow, including the relevant time if appropriate,
- The relevant mathematical method/equation(s) for calculating the concentration of the analyte in the sample tested.

When the proposed method of analysis is either an official or a standardized method, the applicant shall evaluate whether the scope of the methods of analysis appropriately covers the intended analysis in the proposed pre-market approval or authorization application, especially the matrices covered and the range of the method of analysis. If the intended use of the feed ingredient is not covered by the scope of the method of analysis, the applicant should provide a method's verification assessment to modify the scope of the method, by introducing new matrices, including a different range or introducing a new analyte in case of multi-analyte methods.

If the applicant needs to modify or amend the official or standardized method, then a method validation study shall be organized. This method validation study shall cover the modifications introduced in the method. Figure 1 provides a decision tree to determine the information that should be provided for method of analysis, for example, information on the official standardized method, validation study, and verification.

Figure 1 – Decision tree for the evaluation of the method of analysis.



* with a transferability study , if required
** including the operating procedure applied

When the applicant submits a method of analysis developed for the measurement of the relevant analyte in the intended matrix(ces), the following information should be provided, depending on the objective of further use of the method of analysis:

- If the method of analysis is aimed at being used for official controls and monitoring on the marketplace, the description, and the results of the method validation study (as described in Section 2.1) should be provided. Additionally, some jurisdictions may request the submission of the results of a method transferability study (as described in the Section 2.2), using an independent laboratory, other than the one which developed and validated the method of analysis.
- If the method of analysis is only used for internal control, it may not be provided in the submission package for a pre-market approval or authorization application. However, the companies using methods of analysis for internal control should ensure that the results obtained by these methods of analysis are comparable to the results obtained with the method(s) of analysis used for official controls and monitoring on the marketplace, as appropriate. It is recommended to conduct a method validation study of these methods (as described in the Section 2.1). A transferability study may not be necessary if the method is not used by multiple laboratories, but exclusively by the applicant for specific purposes.
- If the method is used in the context of stability and homogeneity studies (Stability Testing of Feed Ingredients, Homogeneity Testing of Feed Ingredients), or for setting the feed ingredient specification (Manufacturing Process and Specification), the description of the method of analysis and the results of the validation study should be provided. The method may be different from the methods proposed for official control purposes.
- If the method is used in the context of the safety studies (Sub-chronic Oral In vivo studies on Laboratory Animals, ADME evaluation in the context of risk assessment of feed ingredient) and studies demonstrating the intended effect of the feed ingredient (Effectiveness assessment), the description of the method of analysis and the results of the method validation study should be submitted. It is recommended that the results obtained by these methods of analysis are representative of the results obtained with the method(s) of analysis for official controls.

Method transferability studies may be provided, for the method of analysis to be used for official control and monitoring (e.g., setting Maximum Residue Limits).

Table 1 provides the relevant recommendations for the provision of method validation and transferability studies.

Table 1 – Recommendations for the validation and transferability studies depending on the scope of method of analysis (except the official and standardized methods)

	Feed[1]	Edible products	Target organs[2]	Excreta[3]	Contaminants
Validation study	X	X	X	X	X
Transferability study**	X	X*			

[1] analyte in the feed ingredient, ingredient market formulation, premixture, and feed

[2] target organs, blood, and plasma

[3] urine, feces, and manure

* if for safety reasons, Maximum Residue Limits in edible products are required

** if required by the jurisdiction, where the information is submitted

Depending on the scope of the method of analysis, the following information could be required:

- Methods of analysis, describing the analytical procedure and information on equipment used and its parameters,
- Result of the method verification assessment if the proposed method of analysis deviates from an official method or a standardized method (Figure 1),
- Result of the method validation study (Table 1),
- Result of the method transferability study, if requested by the jurisdiction and if the method is planned to be used for official controls and monitoring. In certain jurisdictions, the method transferability study may be organized by the authorities with their reference laboratory(ies),
- Result of an inter-laboratory collaborative study, if available (Table 1).

The types of analytical methods vary depending on the feed ingredient and/or its active substance(s), which may be either a chemical substance or a microorganism. Further details for each type of analyte are provided in Sections 3 and 4.

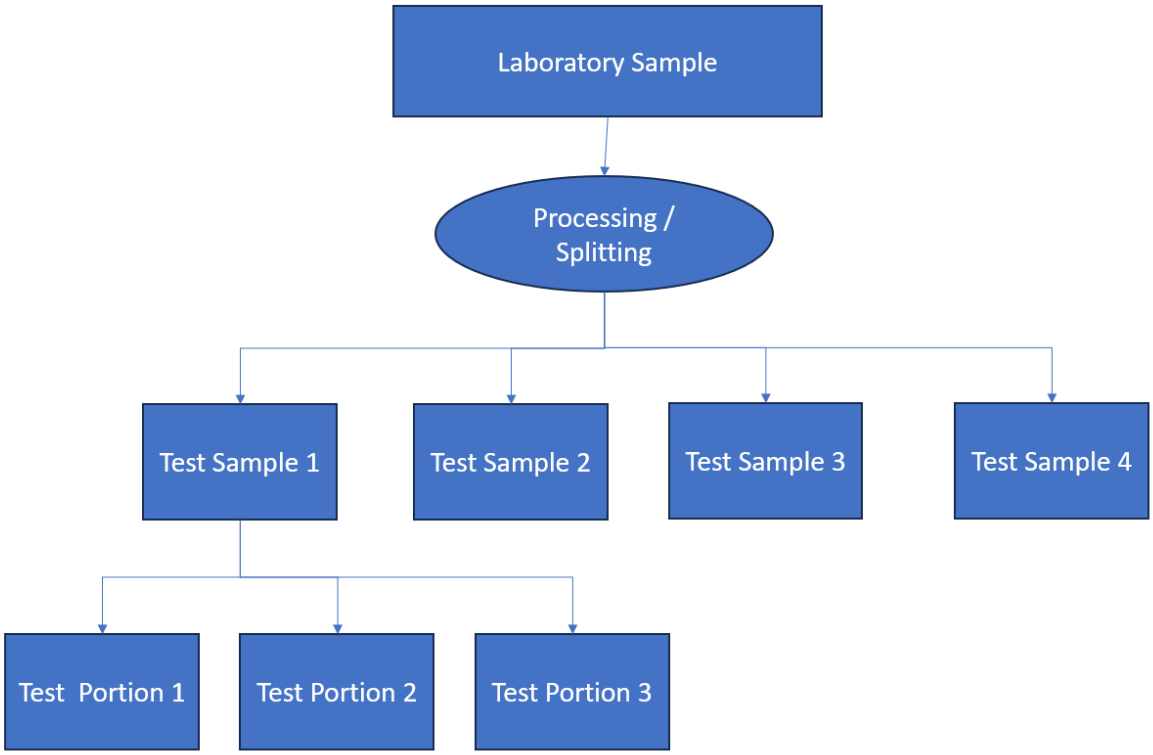
2.2 Sampling approaches

It is essential that the samples are representative of the feed ingredient and of the matrix(ces) covered by the scope of the method of analysis, with regards to homogenization of the analyte and particle size of the test sample, which typically ranges from 0.5 to 2 mm.

Some factors may also influence the test results obtained, such as stability of the analyte under given conditions (e.g., oxidation, light degradation), which should be considered when developing a method of analysis.

The description of the analytical methods, to be provided in support of the application for approval or authorization, should include information on the samples' preparation (i.e., homogenization, stabilization). The proper management of the samples favors the reliability and repeatability of the test results. Figure 2 provides a schematic description of the sample preparation.

Figure 2 – Schematic description of sample's preparation



The laboratory sample corresponds to the sample of the matrix(ces) arriving in the laboratory that should be analyzed. It may be supplied by the manufacturers (e.g., feed ingredient, premixture, feed) or collected during the relevant studies for the edible products, the target organs, blood/plasma, urine/feces, and manure. It is further processed and split to prepare the test samples for analysis. These test samples should be sufficiently homogenized and should be of the relevant particle size for the analysis. The test samples may need to undergo further preparation (e.g., extraction, solubilization, cleaning up or concentration) and potential incubation (see the case of enzymes) under well controlled conditions. These preparation steps

of the test portion should be clearly described in the method of analysis (see Section 3.4). After preparation of the test samples, the laboratory prepares the test portion, which will be used in the equipment relevant for the method of analysis.

If the method of analysis is aimed at evaluating multiple analytes (e.g., evaluation of contaminants, or amino acids or trace elements), the preparation of the test samples should be properly assessed to ensure that the preparation steps do not influence the concentration of certain analytes.

The appropriate laboratory sample quantity, depending on the objective of the test, should be provided to enable the analytical process to be conducted, considering the number of replicates indicated in the method of analysis. The minimum number of replicates to be considered depends on the statistical analysis of the precision and reproducibility limits of the method of analysis. In general, the number of replicates to be considered is between 3 and 5, depending on the method of analysis.

2.3 Method validation study

The main objective of the method validation study is to demonstrate that the analytical procedure described and applied under the described conditions is suitable for its intended purpose, based on validation parameters and pre-determined criteria. The validation parameters will vary depending on the type of analytical method (chemical, microbiological). The Sections 3 and 4 provide the relevant parameters for the various methods of analysis and their scope(s).

For methods of analysis in feed matrices, the analytical range of the methods should cover the concentration range of the analyte in the feed ingredient and/or its ingredient market formulation(s), as well as in premixture and feeds, as proposed in the conditions of use provided by the applicant.

For methods of analysis in edible products, target organs, blood/plasma, urine, feces and manure, the concentration range should cover the expected concentration of the constituent entities and/or their metabolites in these matrices.

Table 2 provides typical performance parameters to be considered in the method validation study, depending on the scope of the analytical method.

Table 2 – Typical performance parameters

Parameters	Qualitative		Quantitative				
			Feeds	Edible Products	Tissues[1]	Excreta	Contaminants
	Identification	Detection (presence /absence)	Constituent entities /active substance(s)	Active substance(s) /metabolites			Process /other contamination
Trueness	-[2]	-	X	X	X	X	X
Precision: - Repeatability - Intermediate precision	-	-	X	X	X	X	X
Specificity / Selectivity	X	X	X	X	X	X	X
System Suitability	X	X	X	X	X	X	X
Ruggedness (Robustness)	-	-	X	X	X	X	X
Working Range	-	X	X	X	X	X	X
- Including LOD	-	X	X	X	X	X	X
- Including LOQ	-	-	X	X	X	X	X
Linearity (Calibration curve)	-	-	X	X	X	X	X

[1] target tissues/organs, blood, and plasma

[2] Not Applicable

Note: if properly justified, some of the listed parameters may be omitted

Before starting a method validation study, a protocol containing the below-mentioned information should be established:

- The intended purpose of the analytical method,
- The performance parameters to be validated (see Section 3 and 4 for more details),
- The pre-determined criteria.

After the method validation study, the results should be summarized in a validation report, for submission to the relevant jurisdictions.

2.4 Method transferability study

In some jurisdictions, the method of analysis shall be tested by at least one independent laboratory, if it is proposed to be used for market controls or monitoring. The objective of the method transferability study is to evaluate whether the proposed methods of analysis can be transferred reliably for application in other laboratories.

For evaluating the capacity of the method of analysis to be used by other laboratories than the one having developed and validated it, the following information should be considered:

- The analyte/matrix combination(s) relevant to the scope,
- The concentration ranges of the analytes in the relevant matrix(ces) corresponding to the validated range,
- The implementation of the analytical procedures by the independent laboratory with no deviations.

The laboratory, having developed and validated the method of analysis, should provide all samples for analysis to the independent laboratory(ies). The concentrations of the characterized analyte should be:

- provided for ‘known’ samples, used for training,
- kept undisclosed for the other samples.

The various samples should be prepared from the same batch of the feed ingredient, and ideally, the same samples should be used for the validation and transferability studies.

3. SPECIFIC RECOMMENDATIONS FOR THE ANALYSIS OF CONSTITUENT ENTITIES

3.1 Generalities

Depending on the methods of analysis submitted by the applicant, various parameters may be needed. However, for each of the parameters chosen, it is important that appropriate data and statistical analysis are provided.

In addition to the method of analysis of the feed ingredient and/or its active substance and/or its constituent entity(ies) in the feed ingredient, ingredient market formulation, premixture and feed containing it, methods of analysis for the quantification and/or proof of presence of the constituent entity(ies) and/or its metabolite(s) in edible products, target organs, blood/plasma, and urine/feces or manure shall be considered. These latter methods of analysis may be developed in the context of the evaluation of Absorption, Distribution, Metabolism, and Excretion (ADME evaluation in the context of risk assessment of feed ingredients), target animal safety, intended nutritional and technical effects, human food safety, and in the context of the environmental risk assessment (Feed Ingredients Environmental Risk Assessment (Phase 2)). It should be noted that in some cases, the use of radiolabeled materials is required. In that case, the method of analysis for determining the level of radioactivity should be provided, including its method validation study, as appropriate.

Further to the information described under Section 2.1, the following information should be provided for methods of analysis of constituent entity(ies) and metabolites:

- The analyte stability,
- The linearity (calibration curve).

3.2 Consideration of the matrix

The description of the methods of analysis should provide a description of the matrix(ces), included in its scope, such as feed ingredient, ingredient market formulation, premixture, feed, edible products, target organs, blood/plasma, urine/feces, or manure.

For methods of analysis involving the quantitative determination of a feed ingredient and/or its constituent entities in feed, appropriate consideration must be given to the extraction of the active substance from the ingredient's market formulation (e.g., coatings). The method of analysis should include a detailed description of the extraction procedure for the constituent entities from the relevant matrix or matrices. This should encompass all relevant parameters, including the potential use of solvents.

In certain occasions, it is possible that some or all components of the matrix (e.g., presence of substance adsorbing the constituent entities of the feed ingredient) or degradation of the matrix (e.g., hemolysis) influence the results of the measurement, leading to bias. These interactions should be considered when developing the method of analysis and included, as necessary, in its description.

In the case of edible products, target organs, blood/plasma, the storage conditions of the matrices and the potential decay of the concentration of the metabolites during storage are essential parameters to control. These matrices are prone to modifications (e.g., through oxidation), and the metabolites are potentially further degraded. Furthermore, these matrices are usually analyzed sometime after sampling. The applicant should therefore clearly describe the storage conditions of the samples and the stability of the analyte in the relevant matrix(ces) during the storage period.

3.3 Definition of the analyte

The analyte(s) of the method of analysis should be clearly indicated (e.g., chemical formula), supported by identification criteria such as NMR, mass spectrometry or spectrophotometry, and indicate their unit (e.g., IU, mg, µg) in the analytical procedure.

3.4 Enzyme activity measurements specific approach

The measurement of enzyme activities in each relevant matrix (feed ingredient, ingredient market formulation, premixture, and feed) is based on two phases:

- An incubation phase, with defined conditions (i.e., pH, temperature, time, substrate, buffer used),
- A measurement phase, where the effect of the enzyme on the substrate during the incubation phase (e.g., reduction of the viscosity of the substrate, production of specific metabolites (e.g., reducing sugars) is quantified, using an analytical procedure appropriate to the measured effect (e.g., measurement of viscosity, quantification of the metabolite(s) of the incubation phase).

The information describing the two above-mentioned phases shall be provided when a method of analysis for enzyme activities is proposed.

For enzymes, the activity under specific incubation conditions is measured. Hence, the enzyme activity is defined based on the conditions of the incubation phase. The description of the incubation conditions should include:

- the substrate and its concentration used,
- the buffer used,
- the end results of the incubation phase (e.g., specific sugar or viscosity measurement),
- the pH and the temperature at which the incubation is run,
- the time of the incubation phase,
- the rate of the enzymatic reaction, and
- any other parameters that could have an influence on the result of the incubation phase.

The following description of an enzyme activity may serve as an example:

EA (Enzyme Activity) (unit per kg[1]): the amount of enzyme which liberates xx µmol[1] of reducing sugar (expressed in xx equivalent) per minute from a [substrate description] at pH xx and xx °C.

3.5 Method validation study

The objective of the analytical method should be clear enough to enable proper understanding, as this will govern the validation parameters that should be considered.

The method validation study shall cover the scope of the method of analysis, including the matrix(ces) and the defined range. If the scope of the method of analysis covers multiple matrices, the validation shall be run on one specific matrix. Other matrices, covered by the scope of the method of analysis, could be evaluated through a verification study, if technical information is provided, demonstrating that the results in one matrix can be extrapolated to others.

If the feed ingredient is used in various ingredient market formulations, a representative ingredient market formulation should be tested in the method validation study.

[1] Other units may be used such as mg or µg, if appropriate

3.5.1 Parameters

The typical validation parameters[2] (described in more detail in Sections 3.5.1.1 to 3.5.1.8) are listed[3] below:

- Accuracy
  - Trueness, including recovery,
  - Precision, including repeatability and intermediate precision,
- Specificity/Selectivity,
- System suitability,
- Ruggedness (Robustness),
- LOD,
- LOQ,
- Linearity (calibration curve), and
- Range.

3.5.1.1 Trueness

The trueness is demonstrated through comparison of the measured test results and the expected value in the sample tested. This is particularly important if the method of analysis is aimed at quantifying the measurand.

The trueness may be measured either in comparison with a reference standard containing a known amount of the analyte, or by spiking the matrix (not containing the feed ingredient) with a known amount of the analyte.

For validating the trueness, an appropriate number of determinations with various concentrations, covering the defined range of the method of analysis must be performed. In general, a minimum of three (3) concentrations and of three (3) replicates of each concentration (i.e., nine (9) determinations) are recommended.

Trueness results shall be reported as the mean percent recovery by the analysis of a known added amount of analyte in the sample or in comparison with the reference standard.

A confidence interval of 95% for the mean percent recovery should be determined and compared with the acceptability criterion. This allows the evaluation of the measurement bias.

[2] Aligned with other guidelines, e.g., VICH
[3] The order of the list has no specific relevance, i.e., does not relate to any priority

Note that the concentration used in the trueness testing should be compatible with the specification of the feed ingredient and/or the expected concentrations in ingredient market formulation(s) and with the intended use level of the feed ingredient in feed.

3.5.1.2 Precision

The precision of the analytical method should be evaluated on homogeneous samples or spiked samples. The precision evaluation should cover repeatability and intermediate precision.

For the repeatability test, a minimum of nine (9) values should be determined covering three (3) concentrations.

For the intermediate precision, possible variations that could affect the performance of the analytical method, such as days, environmental conditions, equipment, analysts, should be studied.

The standard deviation (SD) and relative standard deviation (RSD) should be calculated and reported. The results should be compliant with pre-determined criteria.

The repeatability and the trueness evaluation may be covered by the same test.

3.5.1.3 Selectivity/Specificity

The specificity can be demonstrated by the absence of interference, that might be inherent to the analytical procedure. This evaluation may be combined with the accuracy tests (i.e., trueness and precision), and the results should be compared with pre-determined criteria.

If the specificity does not meet the pre-determined criteria, the potential interferences should be minimized, to prove the appropriateness of the analytical method.

3.5.1.4 Limit of Detection (LOD)

For evaluating the LOD, two (2) samples should be produced, a blank sample (containing the matrix without the analyte) and a sample containing a known and low amount of the analyte. The amount to be tested should correspond to the limit set in the analytical procedure provided by the laboratory having developed the analytical method.

Several procedures may be applied to measure LOD, such as:

- based on visual evaluation,
- based on signal-to-noise ratio through comparing the signal obtained with samples containing known low concentration of the analyte with blank samples. In that case, the LOD is calculated as the signal to noise ratio multiplied by three (3), or
- based on the standard deviation of the response and the slope of the calibration curve (see Section 3.5.1.6)

3.5.1.5 Limit of Quantification (LOQ)

For evaluating the LOQ, two (2) samples should be produced:

- blank sample(s) (containing the matrix without the analyte), and
- samples containing known and low amounts of the analyte.

The amount to be tested should correspond to the limit set in the analytical procedure provided by the laboratory having developed the analytical method.

Several procedures may be applied to measure the LOQ such as:

- based on signal-to-noise ratio through comparing the signal obtained with samples containing known low concentration(s) of the analyte with blank samples. In general, the LOQ represents about ten (10) times the signal to noise ratio, or
- based on the standard deviation of the response and the slope of the calibration curve (see Section 3.5.1.6).

For further details on the calculation of the LOQ, you may refer to the VICH Guidelines (VICH GL2) or the EURL guide on the determination of LOD/LOQ.

3.5.1.6 Linearity (calibration curve)

The linearity of the test results is evaluated by testing various samples across the range of the analytical procedure. The linearity should be first established by visual examination of a plot of signals, as a function of the analyte concentration. Test results should be established by appropriate statistical methods (e.g., by calibration of a regression line by the method of the least squares), using a minimum of five (5) different concentrations. In certain cases, the linearity is obtained after mathematical transformation[4]. For an acceptable linear relationship, a minimum correlation coefficient (r) of 0.995 or coefficient of determination (R^2) of 0.990 is recommended.

[4] For further details on the mathematical transformation is available in the VICH guidelines 6

The minimum accepted values for the correlation coefficient and coefficient of determination may not be achieved. In this case, attempts to increase those values (e.g., by reducing the width of the range of the method of analysis) should be considered.

In certain cases, a curve pattern results from the plot of data, suggesting a lack of fit due to nonlinear calibration function. In the case of enzymes, it may be recommended to use a polynomial evaluation. In this case, a test of significance, combined with a residual plot analysis, should be conducted and the coefficient of determination (R^2) shall not be used.

If the linearity of the results is confirmed, it is recommended to evaluate the y axis intercept, by using at least five (5) calibration standards, evenly spaced over the range of the method of analysis, covering 0-150% or 50-150% of the concentration likely to be encountered in practice. The samples should be analyzed in duplicate and in a random order, unless there is a risk of carry-over. In that case, the samples may be analyzed from the least concentrated to the most concentrated.

The general matrix effect should be considered by adding the analyte to a test solution derived from the blank matrix, if appropriate, or use the standard addition method. If the calibration is linear, the slopes of the function from a usual calibration curve prepared from analytes in pure solvents, and the analyte additions plot can be compared for significant difference. A lack of significance means that there is no general matrix effect.

3.5.1.7 Working Range

The working range of the method of analysis is provided in the scope of the method of analysis. The working range should specify the lower limit (i.e., LOQ or lowest concentration investigated) and the upper limit (i.e., the highest concentration of the calibration or another defined criterion). It is validated by testing that the analytical method provides acceptable precision, accuracy, and linearity when applied to samples containing the analyte at the extremes of the range and within the range.

3.5.1.8 Ruggedness (Robustness)

The ruggedness (robustness) of the method of analysis aims at evaluating the resistance to change in the results produced by the analytical method, when minor deviations are made from the experimental conditions described in the procedure. It is tested to deliberately introduce small changes to the procedure (e.g., reagent lots, column supplier and batch, extraction solvent composition) and examine the effect on the results.

3.5.2 Report

The method validation study should be reported, including the protocol and the results of analysis of each of the parameters of the method validation study, such as those originating from the analysis of the parameters mentioned under Sections 3.5.1.1 to 3.5.1.8.

3.6 Method transferability study

When the method transferability study is recommended (Section 2.4), the following conditions should be considered:

- The analyte/matrix combination(s) to be tested is covered by the scope of the method of analysis, however, the transferability study may cover fewer combinations than the method validation study,
- The concentrations of the analyte in the relevant matrix(ces) are within the range of the method of analysis,
- The analytical procedure used by the independent laboratory is applied with no deviations. However, if deviations need to be applied, these should be discussed with and agreed upon by the laboratory that developed and validated the method.
- All samples (blank samples with no analyte, reference samples with known concentration of the analyte covering the range of the method of analysis, and samples with undisclosed concentration of the analyte) should be prepared and characterized by the laboratory that developed and validated the method, if possible, using the same batch of feed ingredient or relevant matrix(ces). The independent laboratory(ies) should then use the provided samples for the method transferability study.

The reference samples are used for training, while the other samples are used for the method transferability study.

3.6.1 Parameters

The following parameters should be verified:

- Trueness, generally expressed as recovery,
- Precision, including repeatability and intermediate precision,
- LOD determined during the method validation study, and
- LOQ determined during the method validation study.

The determination of the standard deviations for repeatability and recovery rate should be based on six (6) samples containing a known amount of the analyte covering the range of the methods for two (2) consecutive days.

The determination of the LOQ and LOD should be based on the analysis of at least three (3) subsamples of the blank sample (matrix with no feed ingredient) and three (3) samples containing low levels of the analyte on two (2) different days.

3.6.2 Report

The results obtained should be reported in the method transferability study report.

In addition, the results of the analysis of three (3) undisclosed samples on a single day should be provided in the method transferability study report and any information concerning the analytical procedure or deviation should be provided.

4. SPECIFIC REQUIREMENTS FOR THE MICROBIOLOGICAL ANALYSIS

The methods of microbiological analysis may cover the intentional use of a living microorganism in a feed (direct fed microorganisms) or the potential microbial contamination of the feed ingredient, including the presence of the production microbial strain in fermentation products. The methods to be used for each purpose have different characteristics, which are described in the following sections (Section 4.1 and 4.2, respectively).

4.1 Method of analysis for intentional (intended) use

The level of microorganisms intentionally incorporated in feed matrices is usually very high (order of magnitude 10^6 colonies forming units (cfu)/g or above) and its presence is known.

4.1.1 Enumeration methods

The enumeration methods of analysis aim at quantifying the number of microorganisms provided by the feed ingredient and contained in the relevant feed matrix(ces) (i.e., ingredient market formulation, premixture, feed). The enumeration methods of analysis should be combined with the identification of the microorganism under evaluation. When the feed ingredient contains living microorganisms, the enumeration should also provide indications on the viability of the microorganisms.

In the case of ingredient market formulation(s) containing more than one microorganism, the incubation period may lead to the over-development of one strain compared to another. In that case, it is recommended to grow the relevant microorganisms on different substrates/media, if feasible.

The enumeration shall be accompanied by information relevant to the identification of the microorganisms being enumerated (either at the level of the family, the genus, the species, or the strain, depending on the submission). As a first step for the identification of the relevant microorganisms, a phenotypic identification may be enough to confirm the microorganism identity. If the phenotypic identification would not provide the relevant information, then a genotypic identification of the microorganism (e.g., using Polymerase Chain Reaction (PCR) analysis) should be considered. It is also possible to use the Whole Genome Sequencing (WGS) approach.

When the feed ingredient contains a specific microorganism (claimed on the label), it is recommended to maintain the strain in a culture collection, following the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure[5].

In a more complex matrix than the feed ingredient and the ingredient market formulation(s), the presence of 'native' microorganisms may influence the results of the enumeration. However, it will be necessary to properly evaluate the specificity of the incubation substrate/medium, to facilitate the enumeration of the relevant microorganism.

The method of analysis should clearly indicate the minimum number of replicates to be considered in the quantitative measurement of viable microorganism.

4.1.1.1 Method analysis types

4.1.1.1.1 Existing methods of analysis

The most common method of analysis used for enumerating viable microorganisms in a sample is the plate count method. It consists of growing the microorganism on a defined substrate/medium, allowing the specific growth of the microorganism under study, while limiting/excluding the growth of other microorganism under defined conditions (e.g., temperature, time). The number of colonies formed by the microorganisms are counted after the period of incubation on the Petri dishes. The results are expressed in cfu per xx gram(s).

Other methods have been developed such as:

- Flow cytometry, measuring the total fluorescent unit, the non-active fluorescent unit and the active fluorescent unit.
- Digital PCR

[5] World Intellectual Property Organization (2024) Budapest treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure. 6 pages

- Real time PCR

If choosing such types of methods of analysis, the applicant should provide the results of a method validation study and a comparison with the results of a plate count method using the same microorganism(s).

4.1.1.1.2 New methods

If other methods are proposed by the applicant, they should be validated and a comparison with the results of a plate count method using the same microorganism(s) should be provided.

4.1.1.2 Method validation Study

The objective of the analytical method should be clear enough to enable proper understanding, as this will govern the validation parameters that need to be considered.

The method validation study shall cover the scope of the method of analysis, including the matrix(ces) and the defined range. If the scope of the method of analysis covers multiple matrices, the validation shall be run on one specific matrix. Other matrices, covered by the scope of the method of analysis, could be evaluated through a verification study, if technical information is provided, demonstrating that the results in one matrix can be extrapolated to others.

If the feed ingredient is used in various ingredient market formulations, a representative ingredient market formulation should be tested in the method validation study.

4.1.1.2.1 Parameters

The method validation study should enable the validation of the method of analysis for the specific strain(s) to be evaluated and should consider the following parameters together with acceptance criteria:

- LOQ,
- Specificity, the level of specificity (e.g., at the family or at the species level) will depend on the objective of the enumeration (e.g., labeling),
- Sensitivity,
- Inclusivity,
- Linearity,
- Reproducibility,
- Repeatability, and
- Ruggedness (Robustness).

In addition, the equations required for calculating the results should be included in the method of analysis, with an example calculation. It should be strictly followed with no deviation, during the method validation study.

If the feed ingredient contains more than one claimed microorganism, it may not be possible to differentiate the various microorganisms, especially when the microorganisms are from the same genus. In that case, the enumeration will measure the total number of microorganisms colonies.

4.1.1.2.1.1 Limit of Quantification (LOQ)

The evaluation of the LOQ for microbial analysis is only necessary for instrumentally based methods. Hence, when the method of analysis is based on the counting of individual colonies, a LOQ study is not necessary.

If an LOQ study is recommended, the method validation study evaluates the results of the method in matrix samples that contain a known amount of the microorganism using replicates. Alternatively, the theoretical LOQ should then be provided.

4.1.1.2.1.2 Specificity

The specificity can be demonstrated by the fact that non-target microorganisms are not identified/quantified by the proposed method of analysis. Blank samples, i.e., not containing the target microorganisms, shall be analyzed. If more than one of these blank samples yield a positive result (i.e., presence of the target microorganism), the method cannot be validated. A minimum of 20 blank samples is recommended for this study.

4.1.1.2.1.3 Sensitivity

The sensitivity study aims at demonstrating the limit that the method of analysis can discriminate, with an acceptable repeatability. Various samples should be tested at different concentrations from the lowest and the highest limit of the range for the matrix(ces), where the microorganisms have been incorporated in a known amount.

The evaluation of the sensitivity of the methods of analysis for microorganisms in the feed ingredient or its ingredient market formulation(s) is not recommended for the method using the plate count, but may be necessary when using an alternative method to the plate count (see Sections 4.1.1.1.1 and 4.1.1.1.2)

4.1.1.2.1.4 Inclusivity

Various strains of well characterized microorganisms, including the microorganisms contained in the feed ingredient shall be considered for the inclusivity study. At least fifty (50) pure cultures of target microorganisms shall be tested. For some microorganisms, it will be difficult to obtain the required number of strains. In these cases, an agreed set of test strains should be selected by the parties involved in the method validation study. The concentration of microorganisms, after growth on a non-selective broth, shall be ten (10) times to one hundred (100) times greater than the minimum detection level of the method of analysis.

4.1.1.2.1.5 Linearity

Plate count procedures do not require a calibration curve for quantification, but some methods do. The following requirements apply to these methods.

The linearity of the results of the method of analysis is evaluated by testing five (5) samples with different concentrations evenly spaced across the range of the method of analysis, covering 0-150% or 50-150% of the concentrations to be encountered in practice. The samples should be analyzed in duplicate and in a random order. Linearity should first be established by visual examination of a plot of signals, as a function of the target microorganism concentration, after mathematical transformation (log values). A minimum correlation coefficient (r) of 0.995 or coefficient of determination (R^2) of 0.990 is proposed. If the above-mentioned minimums are not reached, attempts to increase the obtained values (e.g., by reducing the width of the range of the method of analysis) could be proposed.

If the linearity cannot be demonstrated, a test of significance, combined with a residual plot analysis, should be conducted and the coefficient of variation shall not be used.

The general matrix effect should be considered, by applying the method of microorganism additions to a test solution derived from a typical test material. For more details, reference is made to Annex B of the ISO guidelines 16140-3.

4.1.1.2.1.6 Reproducibility

If the evaluation of reproducibility is necessary, a minimum of two (2) laboratory samples of the same matrix should be tested, by multiple laboratories. The laboratory samples should be prepared from different batches of the same matrix and potentially tested by various technicians and on different days.

The concentration of microorganisms in the sample shall be pre-determined and representative of the expected concentration in the matrix tested.

4.1.1.2.1.7 Repeatability

Repeatability is measured by testing the analytical procedure on the same sample, in the same laboratory on the same date. A minimum of nine (9) values should be considered covering three (3) concentrations.

4.1.1.2.1.8 Ruggedness (Robustness)

The ruggedness (robustness) of the method of analysis shall be verified during the method validation study, to evaluate the capability of the method of analysis to work under variable conditions in the same laboratory, such as operators, days of analysis, and equipment used.

4.1.1.2.2 Report

The method validation study should be reported, including the protocol of the study and the test results of each of the parameters of the method validation study, such as those originating from the analysis of the parameters mentioned under Sections 4.1.1.2.1.1 to 4.1.1.2.1.8.

4.1.1.3 Method transferability Study

When the method transferability study is recommended (Section 2.4), the following conditions should be considered:

- The target microorganism/matrix combination(s) to be tested is covered by the scope of the method of analysis. However, the method transferability study may cover fewer combinations than the method validation study.
- The concentration ranges of the microorganism count in the relevant matrix(ces) are within the range of the method of analysis.
- The analytical procedures used by the independent laboratory are applied with no deviations. However, if deviations need to be applied, these should be discussed with and agreed upon by the laboratory that validated the method of analysis.
- All samples (blank samples with no target microorganism, references samples with known concentration of the target microorganism, and samples with undisclosed concentration of the target microorganism) should be prepared and characterized by the laboratory that developed and validated the method of analysis, if possible, with the same batch of feed ingredient and sent under appropriate conditions, to ensure the

stability of the target microorganism in the relevant matrix. The independent laboratory(ies) should then use the provided samples for the method transferability study.

The reference samples are used for training, while the other samples are used for the method transferability study.

4.1.1.3.1 Parameters

For the method transferability study, the following parameters should be specifically reviewed, if relevant:

- LOQ,
- Specificity, the level of specificity (e.g., at the genus or at the species level) will depend on the objective of the enumeration (e.g., labeling),
- Sensitivity,
- Inclusivity,
- Repeatability, and
- Ruggedness (Robustness).

4.1.1.3.2 Report

The test results obtained should be reported in the transferability study report.

In addition, the results of the analysis of the three (3) undisclosed samples on a single day may need to be provided in the transferability study report and any information concerning the analytical procedure or deviations should be provided.

4.1.2 Identification methods

The identification method of analysis aims at verifying the identity of the microorganism present in the feed ingredient or in the matrix(ces) containing it. It usually follows the enumeration and identifies the microorganism that has grown during the incubation phase. The phenotypic evaluation can be established (using Analytical Profile Index [API] galleries) either at the cell or colony level, as well as sequencing with functional biology. The evaluation is based on reference to databases for evaluation or with culture collection, for feed ingredients containing living microorganisms.

Pulsed Field Gel Electrophoresis (PFGE) is a genotyping technique, used to separate large DNA molecules after digestion with unique restriction enzymes and application to a gel matrix under an electric field with periodically alternating direction of charge.

Quantitative PCR can amplify the part of the genome of interest. However, 16S and 15S sequencing would not be sufficiently reliable for accurate identification. It is to be noted that interactions (such as inhibition of the reaction by a matrix component or amplification of a closely related strain, when using a strain—specific primer, leading to false-positive results) should be considered when implementing PCR methodologies. Furthermore, PCR requires the use of strain specific primers.

The Random Amplified Polymorphic DNA (RAPD) method does not require the DNA sequence of the target microorganism, but allows proper identification, based on the amplification of random segments of genomic DNA with a single primer of an arbitrary nucleotide sequence. The RAPD method could be applicable for the identification of intentionally used microorganisms.

Whole Genome Sequencing (WGS) is a valid technology for accurate identification.

The Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry method can be used as a method for identifying the microorganism at the species level.

When identifying the microorganism strain, the applicant should apply the relevant name and provide a culture collection number (Manufacturing Process and Specification).

As the methods for identification described above have been validated and are transferable, it is neither recommended to conduct a method validation study nor a method transferability study for this purpose.

4.2 Method of analysis for microbial contamination

4.2.1 General case

The methods of analysis for microbial contamination are usually official methods, whose scope should be evaluated for application in the relevant matrix(ces) tested. It is recommended that the applicant uses these methods when evaluating the potential contamination of the feed ingredient and/or its ingredient market formulation.

The objective of these methods of analysis is not to define the strain of the microorganisms contaminating the feed ingredient or its ingredient market formulation; hence methods of analysis based on proteomics and presence of 16S would be considered as sufficient for identification.

4.2.2 Case of fermentation products

For fermentation products, where the absence of the production microorganisms should be demonstrated (especially for genetically modified microorganisms), specific methods of analysis should be developed and submitted by the applicants. The scope of the method should at least cover the following matrices: the feed ingredient and/or its ingredient market formulation(s). These methods of analysis usually consist of the analysis of the production microorganism in the feed ingredient sample and after spiking with known amount of the viable production strain.

The methods of analysis shall be validated considering the following parameters:

- Sensitivity
- LOD

The sensitivity study aims at demonstrating the limit that the method of analysis can discriminate with good accuracy. Various samples should be tested at different concentrations from the lowest and the highest limit of the range for the matrices, where the microorganisms have been incorporated at a known amount.

The evaluation of the sensitivity of the methods of analysis for microorganisms in the feed ingredient and/or its ingredient market formulation(s) is not recommended for the method using the plate count but may be necessary when using an alternative method to the plate count, while the LOD of the method should be determined.

The result of the sensitivity study, if appropriate, shall be provided in the validation study report.

The limit of detection of the method of analysis is based on the lowest concentration of the microorganism spiked in the feed ingredient.

The result of the sensitivity study, if appropriate, and the limit of detection shall be reported in the validation study report.

5. ACRONYMS

AOAC	AOAC International
API	Analytical Profile Index
cfu	colony forming unit
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
ICP	Inductively Coupled Plasma
ISO	International Standardization Organization
LOD	Limit of Detection
LOQ	Limit of Quantification
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization-Time of Flight
MS	Mass Spectrometry
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
r	Correlation coefficient
R^2	Coefficient of determination
RAPD	Random Amplified Polymorphic DNA
RSD	Relative Standard Deviation
SD	Standard Deviation
WGS	Whole Genome Sequencing

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