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Validation of alternative analysis methods

Application to the food industry

Validation protocol for methods of detecting and quantifying traces of animal medicines in food products

Requirements regarding the preliminary and inter-laboratory studies carried out by an expert laboratory

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Concerns

This document defines:

1. The experimental conditions to be applied by the Expert Laboratory for validation of methods for the detection and quantification of antibiotic residues in food, pursuant to the Brand Certification Rules in the NF Validation (NF102).
2. The preliminary study report and inter-laboratory study report forms with which the Expert Laboratory must comply.
3. The arrangements for dealing with validation modifications/extensions should the manufacturer request this, and revalidations.

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A. Introduction

This document describes the methods for determining the performance characteristics which must be verified to validate a screening method (qualitative, quantitative).
The objective is to characterise the screening method and to compare its performances with the performance criteria expected to determine the validity of the method.
This criteria approach is based on European regulation CE/2021/808 (2021) (which abrogated European decision 2002/657 (2002)) and the European validation guide for screening methods (EURL September 2023).

B. Scope

This document sets out the general principle as well as the technical protocol for validating screening methods in the field of detection and quantification of veterinary drugs in animal foods.
The validation of a method relates simultaneously to the test procedure recommended by the manufacturer, the products and equipment required for implementation of the method, and to a specified scope.

C. Compliance with these requirements

The Expert Laboratory must present in its draft studies all discrepancies (if any) compared with the screening test protocol proposed for validation by the manufacturer and/or compared with the requirements of this document. If no discrepancy is mentioned, compliance with these elements is implicit and under its responsibility.

D. Definitions

- **Analyte:** the substance which must be detected, identified and/or measured, or the derivatives produced during its analysis.
- **Applicability:** potential use of the same method for different matrices.
- **Detection capability (CCβ):** the smallest content in substance which can be detected, identified and/or measured in a sample with an error probability β.
- **Performance characteristic:** functional quality which can be attributed to a method of analysis. These are in particular the specifics, detection capability and precision.
- **Performance criterion:** requirements in performance characteristic terms from which it is possible to judge that a method of analysis is suitable for the objective pursued and gives reliable results.
- **Target concentration or Level of interest:** the concentration which will give a positive result (potentially non-compliant) with the screening test. For authorised substances, the target concentration should be less than or equal to the Maximum Residues Limit (MRL). For a banned substance, it should be less than or equal to the Minimum Performance Level Required (MPLR). The more the target concentration is below the regulatory limit, the less are the risks of obtaining a false-compliant result for samples containing a residue at the regulatory limit.

- **Accuracy:** narrowness of the agreement between the test result and the accepted benchmark (certified reference material value or supplementation value). It is determined by combining trueness and precision.
- **False-compliant:** sample which contains a molecule targeted by the test, at a concentration above the regulatory limit chosen in the performance criterion (MRL, MPLR) and which gives a negative result with the method to be validated.
- **False non-compliant:** Blank sample or one which contains a molecule targeted by the test, at a concentration below the regulatory limit chosen in the performance criterion (MRL, MPLR) or which contains a molecule that should not be detected by the test and which gives a positive result with the method to be validated.
- **False negative (FN):** sample which contains a molecule targeted by the test, at a concentration greater than CCβ and which gives a negative result with the method to be validated.
- **False positive (FP):** Blank sample or one which contains a molecule targeted by the test, at a concentration below CCβ or which contains a molecule that should not be detected by the test and which gives a positive result with the method to be validated..
- **Precision**: Closeness of the agreement between the results of independent tests obtained under conditions of repeatability and reproducibility.
- **Trueness:** closeness of the agreement between the mean value obtained from a broad series of test results and an accepted benchmark.
- **Independent laboratory**: Laboratory different from that of the manufacturer and likely to supply data feeding the preliminary and inter-laboratory studies.
- **Expert laboratory**: Test body independent of the manufacturer for the screening test to be validated, qualified by AFNOR Certification for the field in question, pursuant to the NF102 certification rules in force. Chosen directly by the manufacturer in the list of qualified laboratories supplied by AFNOR Certification, it is responsible for conducting and/or supervising the validation study in compliance with the requirements of this document.
- **Authorised limit:** Maximum Residue Limit (MRL) (for example in Europe, Regulation No 470/2009/EC (2009) or other maximum tolerance applicable to substances and drawn up in the reference legislation, such as the Reference Value (RV; reference point for action (RPA)) (Regulation (EU) 2019/1871)
- **Maximum Residue Limit (MRL)**: The MRL is the maximum concentration of the residue of a pharmacologically active substance which can be authorised in food of animal origin. To protect public health, maximum residue limits are set, taking into account toxicological risks, environmental contamination and the microbiological and pharmacological effects of the residues.
- **Matrix:** all the constituents of the sample for testing, except the analyte.
- **Qualitative method:** analytical method which detects a substance on the basis of its chemical, biological or physical properties.
- **Quantitative method:** analytical method which determines the quantity or fraction of weight of a substance in order to be able to express it as a numerical value with appropriate units.
- **Supplemented material:** sample enriched with a known quantity of analyte to be detected.

- **Screening method:** method used to detect the presence of a substance or of a class of substances at the levels in question. These methods are applied to sifting a number of samples in order to detect potential non-compliant results.
- **Cross-reactions:** Analytical response of the method with analogues of the target analyte, its metabolites, or other components that may be present in the matrix.
- **Repeatability:** degree of concordance between the results of independent analyses, using the same method, with an identical fraction for analysis under the same conditions.
- **Reproducibility:** degree of concordance between the results of independent analyses, using the same method, with an identical fraction for analysis, but under different conditions (different laboratories, operators and equipment).
- **Analytical response:** The phenomenon observed at the end of the analytical process in relation with the analytes present in the matrix, as the case may be.
- **Reference value (RV; reference point for action (RPA)):** Reference values are established under Regulation (EU) 2019/1871 and take into account both analytical considerations and the toxic potential of these substances. Foodstuffs of animal origin containing residues of a pharmacologically active substance at or above the reference value are considered to be non-compliant with EU legislation.
- **Robustness:** sensitivity of a method of analysis to variations of experimental conditions, which may be expressed by the list of samples, analytes, storage conditions, the conditions of the environment and/or preparation of the sample for which the method may be applied as is or with certain minor modifications. For all experimental conditions which, in practice, are subject to variations (e.g. stability of reagents, composition of the sample, pH, temperature), all variations which could affect the analytical result must be shown.
- **Specific feature:** ability of a method to distinguish the measured analyte from other substances (e.g. isomers, metabolites, breakdown products, endogenic substances, matrix constituents, etc.).
- **Broad spectrum test:** this is a test which detects several families of antibiotics, without identification of the detected family of antibiotics.
- **Specific action spectrum test**: this is a test with identification of the family of antibiotics or of a specific antibiotic.

E. Framework of the certification application

The certification documents must be filed by the applicant with AFNOR Certification, under the conditions and within the deadlines set by the NF102 certification rules (see 4.2).

The applicant must describe the application precisely. The information items below are to be provided on the test for validation in order to specify the scope submitted for certification. They are to appear in the draft preliminary study presented by the Expert Laboratory before organising the actual preliminary study.

- Principle of the test, principle of reading and interpretation of the test
- Type of method (qualitative or quantitative)
- Test formats (if there is more than one) (e.g. ampoules/microplates)
- Matrices: animal species, types of milk (blended milk, individual, large blend, untreated milk, reconstituted milk, UHT, etc.), with or without preservative, etc.
- Range of action for the test: list of antibiotics and expected detection limits, broad or specific spectrum
- Detailed protocol(s): if minor modifications must be made to the method, depending on the matrix, they must be announced in the protocol (Supplier's kit instructions)
- Analyses in single, double or other repetitions. If the protocol mandates analyses in duplicate, the protocol must define how to move forward if the results are inconsistent.
- Robustness: To provide parameters influencing the test protocol, as well as the tolerances applicable to these parameters.

In a single application, there may be more than one format for the same product and several matrices, provided that the protocol is described precisely for each case.

- If the formats/matrices are declared by the manufacturer as non-equivalent in terms of detection capabilities, they will undergo a complete validation study.
- If the formats/matrices are declared by the manufacturer as equivalent in terms of detection capabilities:
    - Either the performance characteristics (CCβ/specific feature) will be determined for a mix of formats/matrices.
    - Or one of the formats/matrices will be validated in full and the applicability to the other formats/matrices will be studied.
        - If applicability is proven for other formats/matrices, they will be considered as equivalent.
        - If the results show that other formats/matrices are not equivalent, then it will be necessary to have a complete validation of the other formats/matrices.

Note: Acceptance of external results.

It is possible to accept external results obtained earlier as part of another validation study by an independent laboratory. It will be for the Technical Board to judge, on the basis of the items provided by the Expert Laboratory, the admissibility of the validation protocols used and to accept all or part of the data.

F. Experimental conditions

I. Organising the study

The validation study is conducted by the Expert Laboratory and includes:

- A preliminary study which supplies the characterisation of the method,
- An inter-laboratory analysis which determines the precision of the method under the given conditions of repeatability and reproducibility,
- Use and interpretation of the results compared with expected performance criteria.

The validation study (drafts and results) is investigated by the Technical Board in accordance with the NF102 certification rules (section 4.3).

II. Preparation of master antibiotic solutions

The active principle content contained in the standard, from the standard analysis certificate (attached by the supplier), taking into account the different parameters (e.g. purity, water content, salts etc.).
Master antibiotic solutions may have different stabilities, depending on the antibiotic molecules, preparation mode (solvent) and method of storage. The Expert Laboratory will have to refer to the data from the table in Appendix 2. Table for preparing and keeping parent antibiotic solutions (at 0.5 mg/ml). to prepare and keep its master solutions. In addition, the Expert Laboratory will be able to refer to studies of stability in solution and in the matrix (milk and muscle) carried out by a liquid chromatography method paired in tandem with mass spectrometry (CL-SM/SM) (data publishes in an international scientific journal) (Gaugain et al. 2013). The data from this table was obtained for master solutions at 0.5 mg/ml, in other words, for example, by weighing 25 mg of active substance in a 50 ml phial.

III. Preliminary study

1. Characterisation of the performance of the method

1.1 Blank matrices

1.1.1 Liquid matrices

Special case of untreated milk:

Specification for the milk producer (for selecting a standard milk to be used in the validation study):

- The validation study will have to be done only on fresh untreated milk (to be used within 36 hours after it is taken and to be kept chilled at between 0 and 6°C).
- Blend of milk from at least 10 animals.
- No treatment for at least 8 weeks before treating it.
- Ask the milk producer for its history covering 2 months for the composition and quality and over 6 months for antibiotics. See whether the producer's results are stable and compare with the ranges in table 1 (standard milk selection criteria). The values obtained for this milk must remain within acceptable ranges.

- Ask the producer to alert the Expert Laboratory if a treatment was used during the period when it was supplying milk for the validation study.
- Normal standard fresh milk for its composition, according to table 1 (cells, bacteria, fatty matter (MG), protein materials (MP), pH).

Table 1. Selection criteria for a standard milk.

		Cells	Germs	MG	MP	pH	Antibiotics	Lactation period
Cow	Target value	< 200000	<30000	40	33	6.7-6.8	None	Between 30 and 270 days after calving
	Range acceptable	< 400000	< 100000	35-45	30-36	6.6-6.9		
Goat	Target value	< 2000000	<60000	38	34	6.7-6.8	None	Between 20 and 150 days after calving
	Range acceptable			30-50	28-40	6.6-6.9		
Ewe	Target value	< 2000000	<60000	70	55	6.7-6.8	None	Between 20 and 150 days after calving
	Range acceptable			50-90	40-70	6.6-6.9		

Target value: The target value is used as an indicator for selecting producer(s).
Acceptable range: The acceptable range is an interval outside which the results obtained with this milk will fail.

The composition and quality of the milk (cells, bacteria, fatty matter, protein materials, pH) will be analysed at the start of the study and again at the end of the study in order to check the milk supplied by the producer(s).
Supplementations will have to made on a day by day basis on the fresh milk.
Each validation day, a milk sample (blank matrix) must be kept and stored at -25°C ± 5°C. If there are dubious results during the validation study (false positive results, CCβ lower than the CCβ declared by the manufacturer), the Expert Laboratory will defreeze the sample for a screening analysis, or even a confirmation analysis.

1.1.2 Solid matrices

Specification for solid matrices:

- Solid matrices will be available from trade (e.g. supermarkets, farm shops, others) or from a sub-contractor guaranteeing the absence of antibiotic residues in these matrices, or even be sourced from animals bred at the Expert Laboratory.
- The validation study could be performed on solid matrices frozen earlier for practical validation study reasons, unless the kit protocol expressly demands analysis of non-frozen matrices.
- Supplementation should therefore be on a day to day basis.

Each validation day, a sample from each blank matrix used must be kept and stored at -25°C ± 5°C. If there are dubious results during the validation study (false positive results, CCβ lower than the CCβ declared by the manufacturer), the Expert Laboratory will defreeze the sample for a screening analysis, or even a confirmation analysis.

1.2 Qualitative methods

A qualitative method is characterised by its detection capability/capabilities, its applicability and its robustness.

1.2.1 Detection capabilities (CCβ)

1.2.1.1 Choice of antibiotics to validate

There are two cases:

1. The test specifically targets one or more antibiotics separately: The kit detection capability will be determined only for the antibiotic(s) targeted specifically.
2. The test targets one or more families of antibiotics in a differentiated manner (test with a specific range of action) or, as the case may be (broad spectrum test): The detection capability will then be determined by referring, each time this is relevant, to the tables in Appendix 1. List of antibiotics to be validated, based on the type of test and the antibiotics targeted by the test., which give the lists of antibiotics to use, according at least to the matrices of interest, the type of test and the molecular family.

***Note*:**

- *These lists will be revised regularly by the Technical Board (e.g. to take account of changes in the use of veterinary drugs).*
- *The Expert Laboratory will have to use for the validation study at least antibiotics from the lists in* Appendix 1. List of antibiotics to be validated, based on the type of test and the antibiotics targeted by the test. *and, if need be, propose supplementary antibiotics based on the manufacturer's requests.*
- For food for which no list is available in Appendix 1. List of antibiotics to be validated, based on the type of test and the antibiotics targeted by the test., the Technical Board will study on a case by case basis proposals to be validated by the Expert Laboratory.

1.2.1.2 Choice of target concentrations

Each chosen antibiotic will be tested at least at one target concentration for each matrix, in order to determine the CCβ. The target concentration choice will be based on the authorised limit for the antibiotic concerned in the interest matrix, on the detection capability/capabilities declared by the kit supplier and/or on previous studies carried out in other laboratories, if these exist.

The target concentration choice must approach as nearly as possible the actual performances of the screening method.

The target concentration will be determined as follows:

1. If the manufacturer declares a CCβ, then the target concentration to test will be equal to the CCβ declared by the supplier. For a declared CCβ > 20% of the MRL, it will be necessary to test only the CCβ declared by the supplier (**diagram 1**).
2. If the manufacturer does not declare a CCβ for the antibiotic of interest, it will necessary to carry out preliminary assessment tests (**diagram 2**). The target concentration to test will then be equal to the authorised limited and will have to be tested 5 times. If the CCβ proves > the authorised limit, it will be necessary to assess the CCβ up to 5 times the authorised limit only, unless the supplier asks to assess the CCβ precisely, even above 5 times the authorised limit.

In both cases of, for the target concentration tested, the percentage of positive results is > 95%, the CCβ is at least equal to this concentration. This means that a lower concentration can be tested in order to approach the actual CCβ as nearly as possible.
Conversely, a percentage of results < 95% obtained at this concentration means that a higher concentration must be tested.

The 2 approaches are explained step by step in each of diagrams 1 and 2 set out below.

Summary diagrams of the approach.

Diagram 1. Case where the CCβ is declared by the manufacturer.

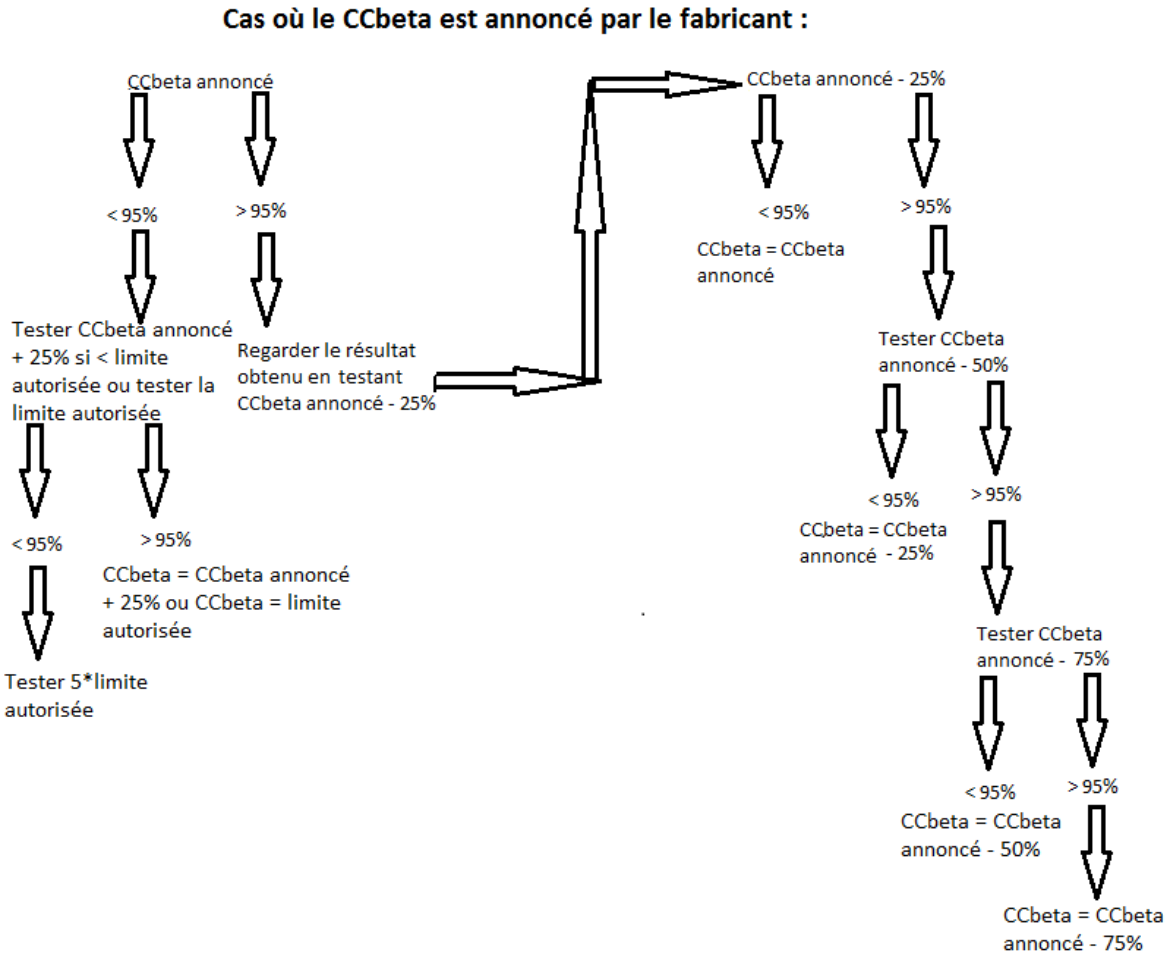
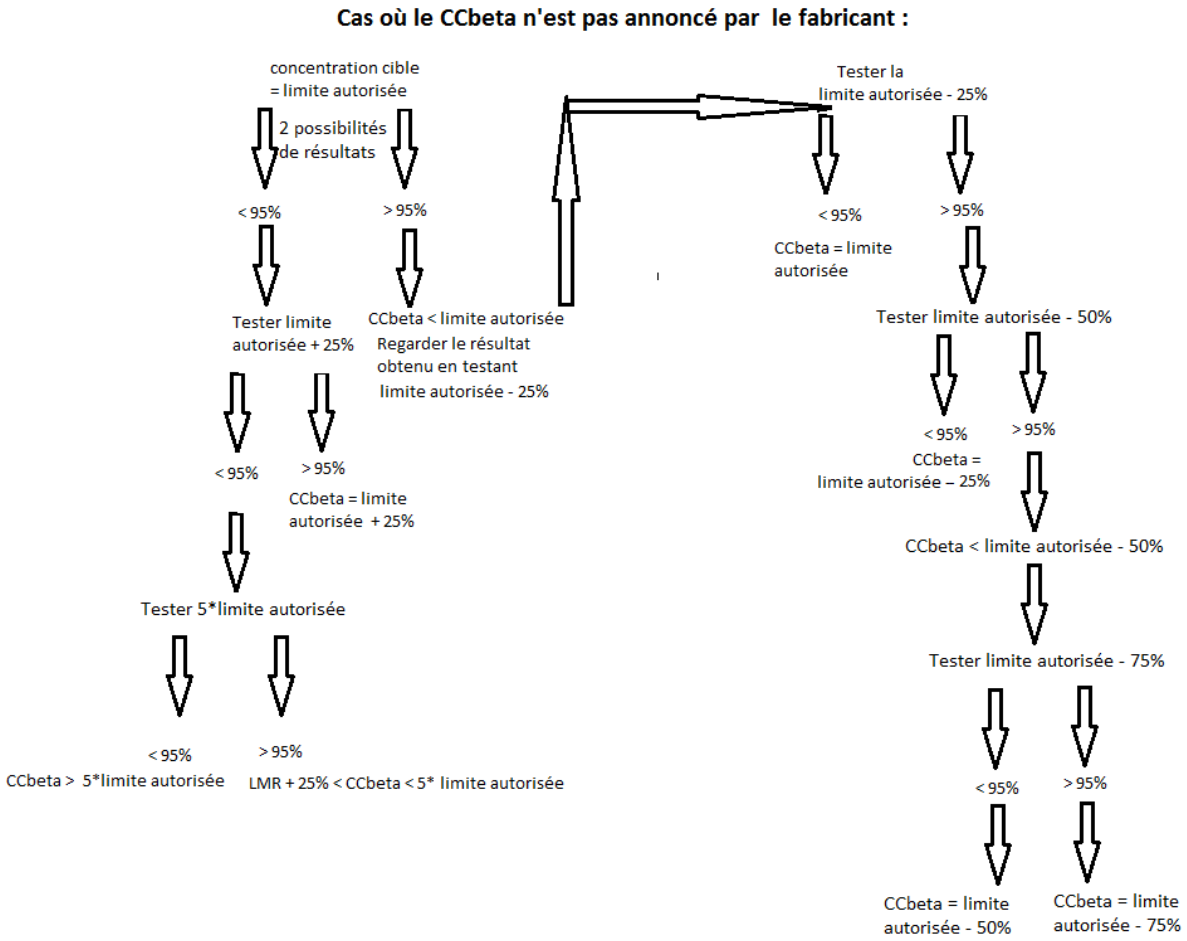


Diagram 2. Case where the CCβ is not declared by the manufacturer.



1.2.1.3 Number of samples to analyse to determine a CCβ

According to regulation CE/2021/808 (2021), at least 20 tests must be carried out for at least one concentration level to obtain a reliable basis for this determination. In this case, the method's detection capability is equal to the concentration level where only 5% or fewer negative results remain, in other words at most 1 negative sample out of 20 loaded.
The number of samples for the validation study depends on the degree of statistical confidence required in the result, and the relationship between the target screening concentration and the regulatory limit. The lower the target concentration compared with the authorised limit, the more the number of samples to analyse can be reduced. Indeed, the same statistical confidence that the test will be capable of detecting the residues at the authorised limit will be obtained with fewer samples. The different cases are presented in table 2.

Table 2. Number of samples to test for the validation study, depending on the cases.

Target concentration value	Number of samples to test	Performance criterion Maximum authorised number of negative results
> Authorised limit	20	1
Close to the authorised limit (10% below the authorised limit)	60	3
Between 50% and 90% of the authorised limit	40	2
≤ half of the authorised limit	20	1

Among the n samples to analyse, these are either n samples from the same matrix, or n samples consisting of a blend from different matrices (see 2nd applicability approach).
These studies can be undertaken by sequential steps: the first 10 supplemented samples are tested and, if more than one supplemented sample gives a negative result, the validation can be abandoned for this concentration. The screening target concentration must then be increased and the exercise repeated.

1.2.1.4 CCβ determination protocol

It is mandatory to use at least 3 lots of reagents during the detection capability test, if possible one of which is used shortly after production and one just before the expiry date. In this case, there is no longer any requirement for supplementary tests to evaluate the differences between lots and the age of lots in the robustness section.
The detection capability must be determined from the analysis of doped materials (artificially or not) at the target concentrations, with at least one molecule with an antibiotic activity. The mixture of several molecules is valid only for specific tests and only if the specific feature has been demonstrated beforehand.

- For liquid matrices (milk, etc.), supplementation of the matrix with antibiotic solutions is well suited. Supplementation of the matrix must be done with antibiotic solutions, finally with a maximum of 10% of the working solution in the matrix.

➢ For solid matrices (meat, etc.), 2 cases must be considered, based on the type of kit:

- If the method for preparing the sample before analysis needs crushing and homogenisation of the sample, the same procedure for supplementation as for liquid matrices is applicable.
- If the sample is analysed without any preparation (raw matrix), supplementation is unsuitable. In this case, production or collection of samples loaded naturally must then be contemplated. However, in the first instance a study based on aqueous solutions of antibiotics may be planned in order to cover a broad spectrum of antibiotics in order to determine the sensitivity and specific features of the kit. A study of materials loaded naturally would then confirm or invalidate the results of this first study. Exemptions from this protocol could be granted by the Technical Board if they are justified by the Expert Laboratory. For example, it is possible in certain cases to use meats that are crushed and then supplemented in antibiotics.

➢ The target antibiotic concentration will be prepared **at least as 20 copies with at least 3 different origins for the corresponding milk, i.e. on 3 different days, from 3 different animal groups or 3 different breeding herds).**

➢ The samples must be coded at random to carry out all the analyses **blind**, by a person who is not the same as the person who will do the analysis. Each sample is analysed by the screening method, by following the supplier's protocol.

For example, the samples can be coded from 1 to 20. Several antibiotics will be tested on Day 1, as well as the blank samples.

The following table represents a coding table for blind analyses on Day 1.

Coding	1	2	3	4	5	6	7	8	9	10
Content	AB3	B	AB1	AB2	AB3	AB2	AB2	AB1	AB3	AB2
Coding	11	12	13	14	15	16	17	18	19	20
Content	AB1	AB3	AB2	AB3	AB2	AB1	B	AB3	AB1	AB1

B: Blank; AB1: antibiotic 1; AB2: antibiotic 2; AB3: antibiotic 3.

The same antibiotics will be tested on Day 2, but with a different coding. As many coding tables must be prepared as necessary, to obtain the number of samples required for determination of the CCβ and the specific feature (rate of false positives).

1.2.1.5 Use and interpretation of the results

After analysing n supplemented (or loaded naturally) samples, **the smallest target concentration for which fewer than 5% of negative results remain is equal to the method's CCβ detection capability.**

A table (see example below) will summarise for all validated antibiotics:

- The family of antibiotics to which it belongs,
- The name of the antibiotic,
- The authorised limit in the interest matrix,

- The number of positive results obtained over the total number of samples tested for the concentration corresponding to the CCβ (or the percentage of positives and the total number of samples tested),
- The CCβ,
- The comparison with the regulatory limit (performance criteria).

Table 3. Summary of the results obtained to be incorporated in the validation study report.

Family	Antibiotic	MRL	Number of positive results over the total number of samples tested	CCβ	≤ or ≥ the authorised limit

1.2.1.6 Conclusions

If the CCβ obtained is different from the CCβ declared by the manufacturer in the technical instructions, then the technical instructions must be amended.

1.2.2 Rate of false positives/Cross reactions

The rate of false positives reflects the capability of the method to guarantee that negative samples are genuinely negative. This determination relates to all types of test (specific and broad spectrum).
The analysis is used to highlight matrix interferences (endogenic substances, constituents of the matrix, etc.). The Expert Laboratory must check potential interference.

1.2.2.1 False-positives rate determination protocol

At least 20 blank matrix samples (see § 1.1, chapter III), **if possible from different sources** (devoid of antibiotic residues or other substances with antibacterial activity) will be analysed according to **the manufacturer's protocol** with the screening method to be validated.

1.2.2.2 Cross-reaction determination protocol

For tests with a specific action range only, the Expert Laboratory will study the cross-reactions of the test with antibiotics from the same family or from other families. This will establish the power of discrimination between the analyte and associated substances (isomers, metabolites, breakdown products, etc.).
For this, blank matrices containing substances with antibiotic activity (at least one per large family of antibiotics (penicillins, cephalosporins, tetracyclins, macrolides, aminosids, sulfamids, quinolones and sundry others)) will be analysed. It will be necessary to test substances associated chemically (e.g. metabolites) when this is relevant. Other substances likely to be found in the presence of the component

of interest on the samples and likely to interfere with detection of the component of interest (e.g. preservatives) could also be tested.
In this case:

- 3 blank samples will be supplemented to 100 times the authorised limit with these substances on a type of milk, meat, etc.
- Each sample will be analysed.

1.2.2.3 Use and interpretation of the results

For blank matrix samples (§ 1.2.2.1, chapter III) then for supplemented samples (§ 1.2.2.2, chapter III), the rate of false positives is determined as follows: Rate of false positives = number of positive results/number of samples analysed x 100.

1.2.3 Applicability

Matrices can have an impact on the results of a test. Most methods are designed for a principal matrix. Owing to the large number of possible analyte/matrix combinations, it is necessary to define the scope of the method in matrix terms. The validation study is performed for a defined "matrix/analyte" pair.

In general, the parameters to test during applicability may be:

- Matrices: Matrices can be different tissues (muscle, kidney, liver, etc.), of different animal origin (eggs, honey, shrimps, etc.) or coming from different animal species (bovine, ovine, poultry, rabbits, etc.). For milk, there is also a distinction between blended milk, individual milk, powdered milk, etc.
- Format of the test,
- Preservatives or not.

1.2.3.1 Prerequisite

The applicant will need to define the scope of the applicability of the method in its initial certification application, before writing the draft preliminary study (see E. Framework of the certification application). The different criteria to be tested in applicability will be chosen based on this application.

Applicability tests are included only if the protocol used for the two matrices is identical. If the protocol used is different, a complete validation study must be done.

Two approaches are possible and are described below.

1.2.3.2 First approach

A first approach consists in determining the rate of false positives and the CCβ for the new matrix, then to compare the new CCβ with the CCβ from the initial validation. ***This approach will be mandatory for milk and dairy products.***

In general, the authorised limits do not differ between species in the same type of matrix (e.g. milk). Nonetheless, if the CCβ was determined for a matrix (e.g. cow's milk) during the initial validation and if the test must be applied to the same matrix resulting from another species (e.g. ewe's milk), a matrix

effect (interferences) must be studied. It can only be presumed that the same CCβ will be applied to this new matrix. **Consequently,** the CCβ in this new matrix will have to be determined for the target substance in the test (targeted test) or, at least on a certain number of representative substances (when the method has a broad detection spectrum).

➢ **Experimental protocol:**

For each new matrix (if possible at least matrices from 3 different sources for milk, in other words different days, different animal groups or different breeds),

- To determine the rate of false positives, it will be necessary to analyse at least 10 different blank materials.
- To determine the detection capability/capabilities (CCβ), it will be necessary to analyse at least 10 different materials supplemented at the level of interest, for 1 to 2 representative molecules per antibiotic family according to the tables in Appendix 3. Selection of one to 2 representative molecules per antibiotics family..

All these analyses will have to be performed blind (coded but unknown samples), on different days with different qualified operators, if possible.

These will be tested:

- 10 different blank raw milk samples with normal composition, quality and pH,
- 10 different blank raw milk samples supplemented with a substance for the family in question (maximum 4 families), by choosing the most relevant substance for the matrix tested. The supplementation is set at or just below the detection capability level (CCβ) (maximum +20%).
- For the beta-lactamines family, test at least one penicillin and one cephalosporin, for milk and muscle matrices.

➢ **Interpreting the results:**

- No criterion is set on the rate of false positives compared with the initial validation. If the rate of false positives is higher than during the initial validation, the difference will be logged clearly in the study report.
- Concerning the detection capability:
  - If the 10 supplemented samples are all detected positive, the method is applicable to new matrices (or species), with the same CCβ as the original matrix.
  - If one supplemented sample alone is found negative, it will be necessary to retest 10 supplemented samples:
    - If no negative result is found in the next 10 samples, the method is applicable with the same CCβ.
    - If at least 1 negative result is found again, it must be deduced from this that the CCβ for this new matrix is greater than that estimated for the original matrix. The method is therefore not applicable with the same CCβ. In this case, the screening method must be revalidated entirely for the new matrix (the target concentration must be increased).

➢ **Specimen application:**

If the manufacturer wishes its test to be validated for raw cow's milk, as well as for UHT cow's milk, it will be necessary to validate raw cow's milk completely and test UHT cow's milk in applicability.
Each new type of milk will have to be tested against raw milk on each occasion.
Examples of different milks (list not closed according to the manufacturer's application):

- UHT milk,
- Pasteurised milk,
- Defrosted milk,
- Reconstituted milk powder,
- Milks from species other than the cow.

1.2.3.3 Second approach

*The second approach consists in validating (specific feature determination of CCβ) by combining several species or several matrices (**excluding milk or dairy products**).*

Example: If the manufacturer wishes its test to be validated for bovine muscle, as well as for pork, ovine and poultry muscle, it must be validated by combining all these species. The same matrix (example: muscle), originating from four different animal species, will be used.

The rate of false positives will be determined by analysing at least 20 blank samples (5 samples per species). The rate of false positives determined in this way will be global rate of false positives, all species combined.

The CCβ will be determined by analysing the same 20 blank samples supplemented at the target screening concentration (5 samples per animal species).

- If the 20 supplemented samples are all detected positive or there is a maximum of 1 negative result, the method is applicable to all matrices (or species), with a single CCβ.
- If 2 or more than 2 supplemented samples are detected negative, the CCβ for this combination of matrices is greater than that expected. In this case, the target concentration must be increased to determine the common CCβ.

Whatever the approach chosen, if the method is not applicable, it must be validated by following the complete protocol for the new matrix.

1.2.3.4 Reporting the results

The results of the applicability study are reported either in the form of a comparative table between the CCβs from the first validated matrix and the CCβs for the same antibiotics for the new validated matrix for **approach no. 1** (see table 4), or in the form of CCβ table 3, presented in § 1.2.1.5 of chapter III for **approach no. 2**.

Table 4. Approach no. 1: Example of summary table of the results obtained to be incorporated in the validation study report.

			1st matrix		nth matrix	
Family	Antibiotic	MRL	CCβ	≤ or ≥ MRL	Number of positive results over the total number of samples tested	Applicability (Yes/No)

1.2.4 Robustness

1.2.4.1 Objectives of the robustness study

The objectives of the robustness study are to observe the consequences of the deliberate introduction by the laboratory of reasonable minor variations. Minor variations are those of an order of magnitude that may be encountered during the everyday routine in a laboratory (10 to 20% of variations).
The first step is to choose sample pre-processing factors and analyse them, which could influence measured results. These factor can include the analyst, the source and age of reagents, solvents, standards and extracts from the samples, temperature, pH, etc.

1.2.4.2 Choice of factors which can be tested (incomplete list)

On the basis of the information supplied by the manufacturer and according to the type of test, the Expert Laboratory will determine the relevant factors to be checked. The factors that it is mandatory to test are those which constitute critical steps of the method, according to the planned conditions of use. If critical factors (identified by the Expert Laboratory) are not documented by the manufacturer, it will be mandatory for the Expert Laboratory to check these during the study.

- **Influences of the test protocol:**
  - Incubation temperature: the temperature required (= benchmark) compared with a lower temperature and a higher temperature.
  - Comment: if a test is performed at ambient temperature (without incubator), the test will be performed at 20°C (= benchmark) and at extreme temperatures to be defined.
  - The incubation period (for each incubation step): the incubation period required (= benchmark) compared with a longer period and with a shorter period.
    Comment: for a test with several incubation steps, test each step independently.

Comment: for a test with several incubation steps, several combinations can also be tested (shorter-longer, longer – shorter, etc.).

- Advance or retard reading: reading as recommended in the protocol (= benchmark) compared with advanced or retarded reading (e.g. 30 sec., 1 min, 5 min, 15 min).
- Test portion volume: correct volume stated in the protocol (= benchmark) compared with a smaller (-10%) and a larger volume (+ 10%).
- Matrix/reagent contact time before starting incubation. To be determined according to the test by the Expert Laboratory.

- **Influence of age on test lots:** If this was not tested previously when determining the detection capability, the effect of lot ageing will have to be tested:
  - The test is performed on the same milk samples, on the same day, with two lots, one used shortly after production and one just before the expiry date.

- **Quality of the matrix/Influences of the composition:**
  - pH value: Against an extreme pH value - high and low.
  - Factors which apply to milk alone:
    - A high count of somatic cells: Milk with normal composition/quality (= benchmark) *against* milk with a count > 10^6 per ml.
    - High total flora count: Milk with normal composition/quality (= benchmark) *against* milk with a count > 5×10^5 per ml.
    - Low fatty matter rate: Milk with normal composition/quality (= benchmark) *against* milk with a low fatty matter rate (e.g. MG < 2 g par 100 g).
    - High fatty matter rate: Milk with normal composition/quality (= benchmark) *against* milk with a high fatty matter rate (e.g. MG > 6 g par 100 g).
    - Low protein rate: Milk with normal composition/quality (= benchmark) *against* milk with a low protein rate (e.g. MP < 2.5 g par 100 g).
    - High protein rate: Milk with normal composition/quality (= benchmark) *against* milk with a high protein rate (e.g. MP > 4 g par 100 g).
    - Frozen milk or not, storage temperature, sample temperature before analysis.

  The set thresholds (low and high) for fatty matter and protein rates could be based on an interval of 50% around current milk samples encountered locally.

  - Low pH: Milk with "normal composition/quality" (= benchmark) *against* milk with 6.0 < pH < 6.3.
  - High pH: Milk with "normal composition/quality" (= benchmark) *against* milk with 7.10 < pH < 7.50.
  - Frozen milk/non-frozen milk.
  - Milk temperature: Cold milk (3 ± 2°C) (= benchmark) against milk at 20 ± 2°C.

1.2.4.3 Choosing tolerances for testing

1) When the manufacturer specifies the intervals, the declared tolerances will be tested.
2) When the manufacturer does not specify the intervals, the factors to test will have to be modified to an order of magnitude which corresponds to the usual deviations.

1.2.4.4 Choosing substances for testing

Substances for supplementation are chosen to be representative of the series of analytes in question.

- For a test with a specific action range for an analyte or a family of analytes, only one substance (A) is incorporated into the robustness test (see Appendix 3. Selection of one to 2 representative molecules per antibiotics family.).
- For a broad spectrum test, two substances A and B from different families among those most representative (in the opinion of the Expert Laboratory) will be incorporated into the robustness test (e.g. for a βlactamines test: a penicillin and a cephalosporin) (see Appendix 3. Selection of one to 2 representative molecules per antibiotics family.).

1.2.4.5 Number of samples for analysis

➢ Conventional approach to a parameter:
  - 3 different blank matrix samples,
  - 3 different blank matrix samples, supplemented with a substance (A) at or just above the detection capability level (CCβ) (maximum +20%).
  - 3 different blank matrix samples, supplemented with a substance (B) at or just above the detection capability level (CCβ) (maximum +20%).

➢ Approach by experimental plan:
  - 10 different blank matrix samples,
  - 10 different blank matrix samples, supplemented with a substance (A) at or just above the detection capability level (CCβ) (maximum +20%).
  - 10 different blank matrix samples, supplemented with a substance (B) at or just above the detection capability level (CCβ) (maximum +20%).

Note*: *10 samples (10 blank + 10 substance A + 10 substance B) are necessary in this approach to be able to calculate a statistically valid false negatives rate and a false positives rate, which is not the case with 3 samples only. However, the total number of total analyses reduces, as at least 4 factors can be tested simultaneously by an experimental plan.

1.2.4.6 Robustness study protocol

It is recommended that the robustness study be performed on different days with different operators. A robustness study can be conducted in two ways.

➢ Conventional approach to a parameter:

Each parameter will have to be varied individually and tested for each of the parameters and for each of the target values of the parameter (low, benchmark and high) with the number of samples

recommended above. The operation of this type of study is simple, but the study requires more analytical work.

➢ Approach by experimental plan:

Feedback of various factors must be used and this will reduce the analytical loading. However, it must be noted that the operation of experimental plans is more complex. Their use demands sound statistical databases (Goupy 2001, 2005).

Experimental plans and their interpretation are presented in brief in Appendix 4. Implementation and interpretation of an experimental plan for the robustness study..

If the Expert Laboratory chooses this approach, it will have to describe in the draft preliminary study the feedback used, the calculations to be performed and how to interpret the results.

1.2.4.7 Use and interpretation of the results

➢ Conventional approach to a parameter:

The analysis will be done factor by factor. A factor will have an impact on the result if its variation causes 1 or more false positive results (for 3 blank matrix samples) or 1 or more false negative results (for 3 samples supplemented for one and/or other of the substances tested).

The impact of variations of each factor (plus or minus) will be determined by comparison with the benchmark (standard matrix) results. The results will be reported in the form of a table (see below).

Table 5. Results of the robustness study.

	Impact on blank samples (yes/no)	Impact on supplemented samples (yes/no)	Conclusion
Factor 1	N	N	Robust
Factor 2	N	N	Robust
Factor 3	N	N	Robust
Factor 4	N	Y	Not robust
Factor 5	Y	N	Not robust

The unprocessed results will be reported by the Expert Laboratory in an appendix to the study report.

➢ Approach by experimental plan:

The analysis of the experimental plans is described, for example, in "(Goupy 2001, 2005)".

1.2.4.8 Conclusions

An analytical method is robust if the results are not sensitive to variations of the experimental conditions.

If the method is not robust for a factor in the range tested, the range tested will be reduced. The analyses will be repeated with this reduced range (2nd robustness study).

The robustness study will serve to highlight critical points. These critical points, once identified, will have to be highlighted in the manufacturer's technical instructions and in the preliminary study report. If a declared tolerance is not achieved, the manufacturer's technical instructions will have to be edited to include the new tolerance.

1.3 Quantitative methods

The parameters to be determined for quantitative methods are the same as those for a qualitative method, plus the parameters described below.

1.3.1 Cross-reactions

The percentage of cross-reactions between the component of interest and the interfering substances (from the same family or from other families) will have to be determined. The cross-reaction results will be reported in a table (example below).

Table 6. Examples from a cross-reactions results table.

	Cross-reaction percentages (%)*
Component of interest	100
Interfering substance 1	10
Interfering substance 2	52
Interfering substance 3	34
Interfering substance 4	17

*Values given by way of example.

1.3.2 Trueness and precision

Trueness and precision (and/or accuracy) must be determined for a quantitative method. Trueness, precision and accuracy are defined and described in European regulation CE/2021/808 (2021). Precision can be determined in conditions of repeatability or reproducibility. During the preliminary study (intra-laboratory), only the repeatability (inter-days, same method, fraction for analysis identical and under the same conditions) will be determined.

1.3.2.1 Choosing substances to test for trueness and precision

The principle for choosing substances to test (number of molecules to test, based on the type of test) is the same as for the robustness study (see § 1.2.4.4, chapter III).
Two approaches are proposed in this baseline to determine trueness and precision, an **individual approach** (characteristic by characteristic) and a **global approach** (combination of several characteristics into just one: accuracy profile). The choice of approach will be made by the Expert Laboratory.

1.3.2.2 Individual approach

- **Choosing concentrations for testing:**

Trueness:

A blank matrix sample will be supplemented at a single level, concentration of interest. The concentration of interest is most often the authorised limit.

Repeatability:

Identical matrix samples will be supplemented with the substance for testing at 3 levels, in order to obtain concentrations equivalent to 1, 1.5 and 2 times the regulatory limit for banned substances or 0.5, 1 and 1.5 times the authorised limit.

Note*: *The unprocessed repeatability results, if the authorised limit was chosen as the concentration of interest, can be used to determine trueness.

- **Protocols for determining trueness and precision:**

Trueness:

The procedure is described in detail in the ISO 5725-2 standard (5725-2 1994).

In accordance with the supplier's protocol, six replicas of the supplemented sample must be analysed.

Repeatability:

- At each level of concentration, the analysis must be performed with six replicas.
- Analyse each replica three times.

- **Use and interpretation of the results:**

Trueness:

- the analyte concentration present in each replica is determined,
- the mean, standard deviation and variation coefficient (%) are calculated for these concentration values,
- trueness is calculated by dividing the mean concentration detected by the concentration of interest and multiplying by 100 to express the result as a percentage.

Trueness (%) = mean concentration detected × 100/concentration of interest.

European regulation CE/2021/808 (2021) sets the criteria to achieve for trueness of the quantitative methods. The difference between the mean concentration determined experimentally and the supplementation value must lie within the following limits.

Justesse minimale des méthodes quantitatives

Fraction massique	Fourchette
≤ 1 µg/kg	−50 % à +20 %
> 1 µg/kg à 10 µg/kg	−30 % à +20 %
≥ 10 µg/kg	−20 % à +20 %

Repeatability:

- the concentration detected in each sample is calculated.
- the mean concentration, repeatability standard deviation and variation coefficient (CV) (%) of the supplemented samples are calculated for each level.

$$s = \sqrt{\frac{\sum_1^n (x_i - \bar{x})^2}{(n-1)}}$$

where: xi: i[th] value, obtained over a series of n measurements of a sample

$\bar{x}$: mean value over the series of n measurements

n: number of measurements

and $CV = \frac{s}{\bar{x}} * 100$

the smaller the standard deviation, the better the precision of the method.

The following table sets out the repeatability criteria to be met so that the repeatability of the method is satisfactory, based on the supplementation concentration.

Table 7. Repeatability criteria.

Supplementation concentration (µg/kg)	Repeatability variation coefficient (%)
1	≤ 20%
10	≤ 20%
100	≤ 15%
1000	≤ 12%

1.3.2.3 Global approach: Accuracy profile

The accuracy profile is a global approach, which can replace the individual approach proposed above to determine trueness and precision individually.

Accuracy is a combination of trueness (systematic error) and precision (random errors). It can be determined using the accuracy profile approach.

By using measurements of repeatability and intermediate precision, the accuracy profile calculates an interval in which a known proportion of measurements will lie. If this interval is compared with an acceptability interval defined by the user or by the regulations, it is possible to decide simply whether a method is valid or not(Feinberg 2007).

The experimental protocol, as well as the interpretation of the accuracy profile, are reported in Appendix 5. Global accuracy profile approach.. In addition, the implementation and interpretation of the accuracy profiles are described well in the articles by M. Feinberg (Feinberg 2010a, b).

2. Practicability of the kit

The practicability of the method is not a performance criterion, but provides important information for a future user, such as the speed of analysis, for example.

Practicability is the ease of use test associated with the necessary hardware, reagents, instruments and environmental conditions. The objective will be to verify whether the methodology is appropriate or not for routine analyses

Practicability and adaptability cover several aspects. These are listed in 12 criteria. The method of communication of this criterion to the user and the method for controlling it have been defined for each of them.

Indeed, certain criteria need a communication on the packaging or instructions, whereas others need a communication on the NF VALIDATION certificate.

Practicability criteria are set out in the following table.

Table 8. Alternative method practicability criteria.

	Criteria for checking	Communication on the criterion to the user	Method of controlling the criterion
1	Reagent packaging method	Packaging or instructions	Verification by the Expert Laboratory
2	Volume of the reagents	Packaging or instructions	Verification by the Expert Laboratory
3	Conditions for storing reagents (+ expiry date of unopened products)	Packaging or instructions	Verification by the Expert Laboratory that the conditions exist
4	Method of use after the 1st use (in particular, existence of best before dates)	Packaging or instructions	Verification by the Expert Laboratory that the arrangements exist
5	Special equipment or premises needed	Note	Verification by the Expert Laboratory
6	Reagents ready for use or for reconstitution (in this case, existence of an operating mode)	Packaging or instructions	Verification by the Expert Laboratory of the veracity of the written instructions
7	Training time for an operator unfamiliar with the method	Report	Measured by the Expert Laboratory (possibility to use periods taken by collaborating laboratories) and split into one of the following 3 categories: less than one day, between 1 day and 1 week, more than 1 week
8	Actual handling time/Flexibility of the technique, depending on the number of samples to analyse, etc.	Report	Handling time measured for the analysis/time to obtain a result verified by the Expert Laboratory
9	Time to obtain the results	Report	Verification by the Expert Laboratory
10	Type of operator qualification	Report	Defined by the Expert Laboratory compared with the minimum skills required to perform the test
11	If stipulated by the method, conditions for traceability of the results	Note	Example: visual or optical reading Verification by the Expert Laboratory
12	Maintenance by the laboratory	Report	Duration and frequency verified by the Laboratory

The data resulting from this practicability study will be incorporated:

- in the preliminary study report for criteria 1, 2, 3, 4, 5, 6, 8, 9, 11 and 12,
- in the inter-laboratory study report for criteria 7 and 10.

3. Conclusions on the preliminary study data

No maximum number of false positives is laid down in the regulations, as samples declared positive (including false-positive results) must be confirmed by a physico-chemical method for identification and quantification as part of the official inspection. A screening method should have as low a rate of false-positives as possible.

A valid screening method (qualitative or quantitative) should have a detection capability (CCβ) equal to or less than the level of interest (authorised limit, regulatory limit) or as low as possible when no limit is set.

- When a method has a broad detection spectrum (substances with a defined authorised limit), for each analyte detected (corresponding to a minimum list of substances), the detection capability CCβ, as well as its delta compared with the MRL should be defined.
- For banned substances, the method must be very specific for the analyte of interest. Furthermore, the components of the matrix must not have any influence on the test results.

The applicability of the method to different matrices must also be proven.
Finally, the robustness of the method must be proven.

Table 9. Summary of the results of the preliminary study.

Performance characteristics		Conclusions of the validation study
	Rate of false-positives (%)	
	Cross-reactions (%)	
Detection capability	CCβ AB1 (µg/kg)	
	..... (µg/kg)	
	..... (µg/kg)	
	CCβ ABn (µg/kg)	
Applicability	List of matrices	
Robustness	Critical factors identified	
Accuracy	Trueness	
	Precision	

***Note*:**

- *The table above will have to be repeated in the NF VALIDATION certificate.*
- *In parallel, the manufacturer will have to publish the performances of the alternative method in the technical instructions for the method:*
  - *The rate of false-positives,*
  - *All determined CCβs,*
  - *List of applicable matrices,*
  - *Critical factors emerging from the robustness study,*
  - *For quantitative methods, trueness and precision.*

IV. Interlaboratory study

The objective of the interlaboratory study is to determine the variability of the results obtained in different laboratories using identical samples (repeatability, reproducibility).

1. Organisation of the interlaboratory study

1.1 Choosing collaborating laboratories

The choice of collaborating laboratories is made jointly by the manufacturer and the Expert Laboratory. However, the Expert Laboratory is responsible for proposing and supervising the collaborating laboratories.

The Expert Laboratory must propose to the Technical Board when presenting the draft interlaboratory study, a list of competent testing laboratories, French and/or foreign, public or private, which have not participated in developing the method and are independent of the manufacturer. The Technical Board will validate the list of participants.

These laboratories will be at least 10 in number, in order to obtain at least 8 series of interpretable results. The Expert Laboratory will participate in the tests but its results will not be incorporated into the global data analysis.

The Expert Laboratory will forward to participants the analysis protocol to be used for the screening method. The manufacturer must equip the laboratories with all the equipment and documentation necessary to use the alternative method. Prior training of collaborators by the manufacturer is recommended.

1.2 Choosing matrices for testing

If the preliminary study covered several matrices, the interlaboratory study could also cover these matrices, depending on the expert report by the Expert Laboratory and the opinion of the Technical Board. The Expert Laboratory will rely on the data produced by the preliminary study.

1.3 Choosing antibiotics

There are 3 cases for choosing antibiotics:

- The action range of the kit is limited to one antibiotic: the interlaboratory study will cover this antibiotic alone.
- The action range of the kit is limited to one family of antibiotics: the interlaboratory study will cover at least 4 antibiotics from this family, if there are at least 4 of them in the tables at Appendix 1. List of antibiotics to be validated, based on the type of test and the antibiotics targeted by the test.. For beta-lactamines, at least 2 penicillins and 2 cephalosporins should be studied.
- The action spectrum of the kit is broad: the interlaboratory study will cover a broader variety of antibiotics: at least one antibiotic per large family (2 beta-lactamines + 4 other large families) (6 antibiotics). ***Note:*** *Provided that the manufacturer has asked that the test be validated for these families of antibiotics.*

For the choice of antibiotics, their frequency of use in veterinary medicine or the occurrence in the form of residues in the matrix in question, if this data is known, may also be taken into account.

1.4 Choosing concentrations

Four different concentration levels must be used. These 4 levels will be defined in the draft interlaboratory study.

The 1st material L_0 is a blank material (devoid of substances with an antibiotic activity), the 1st concentration level L_1 corresponds to a concentration leading potentially to a negative result (concentration less than CCβ determined during the preliminary study = 1/2 CCβ), the 2nd level L_2 to a concentration slightly greater than CCβ (CCβ + 20%), and finally the 3rd level L_3 to a concentration leading to a positive result (CCβ + 50%). The concentration L_1 could give a certain percentage of positive results with certain participants, as the concentration is close to the detection limit.

1.5 Number of samples to be sent to each laboratory

There are 3 cases:

- the action range of the kit is limited to one antibiotic: one antibiotic * 4 levels (1 blank + 3 concentrations) in double blind, i.e. a total of 8 samples.
- the action range of the kit is limited to one family of antibiotics: at least 4 antibiotics * 4 levels (1 blank + 3 concentrations) in double blind, (i.e. a total of at least 32 samples),
- the action spectrum of the kit is broad: at least 6 antibiotics * 4 levels (1 blank + 3 concentrations) in double blind, (i.e. a total of at least 48 samples).

1.6 Preparation and shipping of samples

1.6.1 Source of the materials

The materials are prepared from a blank matrix (free of antibiotic residues). The characteristics of blank matrices for the interlaboratory study are identical to those described in § 1.1 of chapter III.

Immediately on arrival at the Expert Laboratory, an aliquot fraction will be tested with a suitable antibiotics test. If the result is positive, the blank matrix will be discarded and the test deferred as soon as possible. If not, the test will move to the next step.

1.6.2 Preparation of materials

The Expert Laboratory will prepare samples for the collaborating laboratories.

In most cases, the prepared materials will be supplemented with antibiotics at concentrations of interest. When this is possible, naturally charged materials taken from treated animals, will be prepared.

During preparation, every precaution will be taken to avoid cross-contamination between the different materials:

☞ clear identification of the glassware,

- ☞ the first material treated will be the blank material,
- ☞ contaminated materials will be treated one after the other. No material will be prepared and packed while the handling of the preceding material is unfinished,
- ☞ for liquid matrices, magnetic agitation (average speed) for at least 10 minutes should guarantee satisfactory homogeneity of the materials. This magnetic agitation will have to be maintained throughout the aliquot phase,
- ☞ each contaminated material will be homogenised in a container once only, with the container being different for each material.

1.6.2.1 Preparation of samples, coding

Materials will be distributed in pots labelled in advance codes).
Each material is prepared, coded and sent in double blind.
The material storage conditions at the Expert Laboratory depend on the matrix, the stability of the antibiotics and the robustness results.

1.6.2.2 Verification of the materials

Each material will be inspected before the packages leave, in order to verify the consistency of the results achieved with those projected. These tests may be performed at the same time as the homogeneity tests or at the null stability point. Supernumerary samples are prepared in order to verify stability and homogeneity.
The Expert Laboratory must verify the stability and homogeneity of the materials before their departure. For homogeneity analyses, 10 samples with 2 test portions must be taken and analysed for each antibiotic at the concentration L_3 (see § 1.4, chapter IV). For stability analyses, 3 samples must be analysed, at 3 time intervals (at the time of preparing the materials, at an intermediate point and at the final point after the date of analysis by the participants), for each antibiotic at the concentration L_3, by the method which is the subject of the validation study. The homogeneity analysis can replace the null stability point.

1.6.3 Preparation of negative and positive markers

Markers are samples whose quality is known by the participant and which are used purely to validate the test (or not). Their results will not be incorporated into the data analysis.
A positive marker is a blank matrix supplemented to the concentration L_3.
Negative markers and positive markers (supplemented in antibiotics) are sent to participating laboratories with the coded materials.

- If a negative marker is supplied by the manufacturer, it is this marker that must be used with the protocol by the participant and it could be used to exclude a laboratory in the event of a positive result.
- If a negative marker is not supplied by the manufacturer, the Expert Laboratory will send a negative marker (all participants will have the same); it could be used to exclude a laboratory in the event of a positive result.

1.6.4 Contractors

Suitable packaging for transport (including dry ice) and transport of the parcels will be provided by a contractor.
The blank matrix supplier (e.g. cow's milk (raw milk from the tank, free from antibiotic residues)) could be a contractor.

1.6.5 Sending materials

The transport conditions (timings, temperature, etc.) and storage of the materials (before departure and on arrival) depend on the matrix, the stability of the antibiotics and the robustness results. It is recommended that a temperature monitoring device be added to the parcel (e.g. a thermobutton).

1.6.6 Communication with participants

Each participant will be identified by a code which will be known only by it and by the Expert Laboratory. The Expert Laboratory will send instructions to the participants. It will also set an analysis date to be observed for all participants (this is an exclusion condition). The Expert Laboratory must in particular set and communicate very clearly for collaborating laboratories the conditions for eliminating a laboratory's results at the time of sending the samples. This will achieve clear rules, not for discussion, for elimination of the results and will also avoid a collaborating laboratory carrying out useless tests (see § 2.1 cases for laboratory exclusion below).

1.6.7 Analyses by the participants

The collaborating laboratories, as well as the Expert Laboratory will carry out analyses with the screening method, while applying strictly the protocol that was sent for the test by the Expert Laboratory. All analyses will be performed **in double** (2 separate series of analyses) and **in blind** in each of the collaborating laboratories and on the stipulated date.

2. Calculations and interpretation of the results of the interlaboratory study

2.1 Laboratory exclusions

Results from excluded laboratories will not be incorporated into the global results analysis. The cases quoted below are exclusion cases:

- If negative markers give a positive result.
- If positive markers give a negative result.
- Storage and transport: If the temperature and duration of transport of the samples does not correspond with the limits set by the Expert Laboratory.
- If the analysis date is not compliant compared with the date set by the Expert Laboratory.

The Technical Board may reverse an exclusion declared by the Expert Laboratory, if it finds it unjustified. Similarly, the Technical Board may demand other exclusions, for reasons that it will justify.

A search for the causes must be made at the laboratory which has obtained aberrant results. The Expert Laboratory will have to state in the report why this laboratory was eliminated and why it obtained aberrant results.

2.2 Analysis of the interlaboratory study for a qualitative method

2.2.1 Specific features and sensitivity

The positive results obtained by the screening method for each antibiotic must be reported in the form of the table which follows.

Table 10. Number of positive results from the screening method by contamination level.

Laboratories	Contamination level			
	L_0	L_1	L_2	L_3
Laboratory 1	/4	/4	/4	/4
Laboratory 2	/4	/4	/4	/4
Laboratory 3	/4	/4	/4	/4
etc.	/4	/4	/4	/4
Number of positive results				

The Expert Laboratory must then calculate:

- the % of the specific feature SP **for the level L_0** using the following equation:

$SP = (1-(P_0/N_0))*100\%$

where: N_0: total number of results at level L_0

P_0: number of positive [results] at level L_0.

- the % of positive results **at level L_1**: $P_1/N_1*100\%$

where: N_1: total number of results at level L_1

P_1: number of positive [result] at level L_1.

- the % sensitivity SE **for each level** of positive contamination (L_2 and L_3) using the following equation:

$SE = (P/N+))*100\%$

where: N: total number of results L_2 (N_2+) or L_3 (N_3+) respectively

P_2: number of positive [results] at level L_2.

P_3: number of positive [results] at level L_3.

P: number of true positives at level L_2 (P_2) or at level L_3 (P_3).

- the % sensitivity SE **global** (L_2+L_3) using the following equation:

$SE = (P/N+))*100\%$

where: N_{global}: total number of results $L_2 + L_3$ ($N_2+ + N_3+$)

P_{global}: number of positives at level L_2 and at level L_3

P: number of true positives at level $L_2 + L_3$ (P_2+P_3).

2.2.2 Repeatability

The repeatability in each laboratory is estimated by comparing:

- The results of the 2 analyses performed on each sample (2 different series of analyses), given that knowledge of the first result may influence reading during the second analysis.
- The results obtained with the 2 samples from each pair.

The repeatability, expressed as a percentage, is the ratio of the number of identical results per pairs of analyses over the total number of pairs.

The results can be reported in a table (see the example below), combining the results from all the samples tested (negative and supplemented).

Table 11. Repeatability study.

		(Number of identical analyses for **a single sample** / N)*100 (%)	(Number of identical analyses for **2 identical samples** (pair)/ N)*100 (%)
Participants	1		
	2		
	etc.		
	i		
	Total		

N: total number of samples

2.2.3 Interlaboratory reproducibility

The interlaboratory study aims to compare performances (accuracy and precision) of the method with preset performance criteria. It is performed by different participants by using identical samples examined in reproducibility conditions.

Reproducibility, expressed as a percentage, is the ratio of the number of identical results, of the most frequent type (e.g. negative results for samples free from molecules with antibiotic activity or a positive result for samples contaminated with a concentration greater than the detection limit) over the total number of analyses.

The results can be reported in the form of the following table:

Table 12. Interlaboratory reproducibility study

Antibiotic (or blank milk)	Contamination level	Concentration (µg/kg)	Reproducibility (%)
blank milk	L_0	/	
	L_0	/	
	L_0	/	
Antibiotic 1	L_1		
	L_2		
	L_3		
Antibiotic 2	L_1		
	L_2		
	L_3		
etc.	etc.	etc.	etc.
Antibiotic n	L_1		
	L_2		
	L_3		
Total			

2.3 Analysis of the interlaboratory study for a quantitative method

2.3.1 Specific features and sensitivity

The same calculations must be performed for a quantitative method as for a qualitative method.

2.3.2 Repeatability

The repeatability in each laboratory at non-quantifiable levels L_0 and L_1 is estimated as for a qualitative method, i.e. by taking into consideration the positive and/or negative results and not the quantitative results. Indeed, these two types of materials should give negative results in the majority of cases.

The repeatability in each laboratory at quantifiable levels L_2 and L_3 is estimated by calculating for each antibiotic and each concentration level: the mean of 2 measurements (2 analyses/pair), the standard deviation and the variation coefficient, as well as the mean of 4 measurements (2 pairs in double blind, 2 analyses/pair), the standard deviation and the variation coefficient.

The results must be reported in the following form, for each antibiotic.

Table 13. Results of the repeatability study for the levels L_2 and L_3 per antibiotic.

Participant no.		L2			L3		
		Mean	SD*	VC (%)	Mean	SD*	VC (%)
1	Pair 1 (n=2)						
	Pair 2 (n=2)						
	2 pairs (n=4)						
2	Pair 1 (n=2)						
	Pair 2 (n=2)						
	2 pairs (n=4)						
3	Pair 1 (n=2)						
	Pair 2 (n=2)						
	2 pairs (n=4)						
etc.	Pair 1 (n=2)						
	Pair 2 (n=2)						
	2 pairs (n=4)						
i	Pair 1 (n=2)						
	Pair 2 (n=2)						
	2 pairs (n=4)						
Total participants Interlaboratories	**Pair 1 (n=2)**						
	Pair 2 (n=2)						
	2 pairs (n=4)						

*SD = standard deviation.

Acceptability criteria were set to determine whether the method is repeatable or not.

Table 14. Acceptability criteria for repeatability.

Supplementation concentration (µg/kg)	Repeatability variation coefficient (%)	
1	≤ 20%	
10	≤ 20%	
100	≤ 15%	
1000	≤ 12%	

2.3.3 Interlaboratory reproducibility

The interlaboratory reproducibility at non-quantifiable levels L_0 and L_1 is estimated as for a qualitative method, i.e. by taking into consideration the positive and/or negative results and not the quantitative results. Indeed, these two types of materials should give negative results in the majority of cases.

The interlaboratory reproducibility at quantifiable levels L_2 and L_3 is estimated by calculating for each antibiotic and for each concentration level. The reproducibility in each laboratory is estimated by calculating for each antibiotic and for each concentration level.

The results must be reported in the following form.

Table 15. Results of the reproducibility study for the levels L_2 and L_3 per antibiotic.

Antibiotic (or blank milk)	Contamination level	Mean	SD*	VC (%)
Antibiotic 1	L_2			
	L_3			
Antibiotic 2	L_2			
	L_3			
etc.	etc.			
Antibiotic n	L_2			
	L_3			
Total				

Acceptability criteria were set to determine whether the method is reproducible or not.

Table 16. Acceptability criteria for interlaboratory reproducibility.

Supplementation concentration (µg/kg)	Reproducibility VC (%)
1	≤ 30%
10	≤ 30%
100	≤ 25%
1000	≤ 20%

2.3.4 Trueness

The trueness in each laboratory is estimated by calculating, for each antibiotic and each quantifiable concentration level L_2 and L_3, the closeness of agreement between the result obtained by the laboratory on 4 measurements (mean of 4 measurements) and the supplementation value of the sample:
Trueness (%) = mean concentration detected × 100/supplementation value.

2.3.5 Accuracy profile

The accuracy profile is a global approach, which can replace the individual approach proposed above to determine trueness and precision.
The accuracy profile can also be used to exploit the results of an interlaboratory study. The protocol proposed here is inspired by the EN ISO 16140-2 (2016) standard.

➢ **Interlaboratory study protocol:**

- At least 3 contamination levels, 2 pairs per level and per antibiotic.
- Contamination levels: L_1 less than, L_2 intermediate and L_3 greater than the quantification range + a blank matrix level (L_0).
- Analyses in duplicate by each laboratory.

➢ **Reporting the results:**

		Results	
Participants (i)	Level (k)	Duplicate 1	Duplicate 2
1	L_0		
2	L_0		
i	L_0		
1	L_1		
2	L_1		
i	L_1		
1	L_2		
2	L_2		
i	L_2		
1	L_3		
2	L_3		
i	L_3		

➢ **Calculating the accuracy profile and interpreting the results:**

The lack of trueness is estimated by calculating the absolute bias, which is equal to the difference between the mean value for each contamination level i and the "so-called true" supplementation value. The calculations are the same as those proposed in the EN ISO 16140-2 (2016) standard, as well as the interpretation of the accuracy profile, outside the bias calculation (which is based on the reference method in the EN ISO 16140-2 standard). The acceptability criteria which are set for reproducibility (§ 2.3.3, chapter IV) are applicable here (see European regulations).

G. Study and summary reports template

The preliminary study and interlaboratory study report templates (frames) are shown in Appendix 6. Template for the preliminary study report and for the interlaboratory study report..

Initially, the Expert Laboratory will write a preliminary study report then, at a later date, an interlaboratory study report, at the time of the first validation request.

Following the decision for initial certification, rework or extension of the validation of a method, the Expert Laboratory must then draft a summary document of the studies (preliminary and interlaboratory) on the basis of the study report templates set out in Appendix 6. Template for the preliminary study report and for the interlaboratory study report., with the instructions below:

- It must repeat the important elements of these studies, and only those validated.
- Its objective is to be distributable to any person requesting it. The manufacturer must also validate its content, as regards the confidentiality of the items appearing in it.
- It must be sent by the Expert Laboratory to AFNOR Certification no later than 2 months after a positive vote by the Technical Board.

Summary reports published are made available to the public, by AFNOR Certification, on the website http://nf-validation.afnor.org.

H. Processing modifications/extensions

The general conditions for dealing with extensions/modifications are defined in section 5.4 of the NF102 certification rules. The examples which are specified here do not apply to alternative methods for screening antibiotic residues. This is why special provisions have been defined and are repeated below.

If there is an extension request (for example new matrix), after the first validation study, presented on the basis of an identical test protocol, an additional preliminary study must be conducted by the Expert Laboratory. An applicability study will be required to validate extensions/modifications.

Examples of modifications to the alternative method that must generate an additional study by an Expert Laboratory are set out below:

- Instrumental reading instead of visual reading,
- Matrices,
- Format of the test,
- Preservatives or not.

If the applicability of the alternative method following this modification is proven during this additional study, the need to repeat an interlaboratory study will be assessed by the Technical Board.

If the results of the applicability study during this additional study are unsatisfactory (specific features and/or CCβ different from those obtained during the initial validation with the first matrix), a complete validation study will have to be performed. Therefore, a new certification application will have to be filed by the applicant.

In the case of a new family of antibiotics added to the scope (strips, etc.), this involves a new certification application (the alternative method cannot be considered as the same product validated initially).

The initial summary report will be complemented by the summary of the study addenda produced.

I. Rework

The conditions for dealing with rework studies are defined in section 5.3 of the NF102 certification rules.

The summary report will comprise an abstract of the principal results obtained during the first validation study and, as the case may be, during extension studies, as well as the summary of the rework study addenda produced.

J. References

2009. Commission Regulation (EC) N° 470/2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin Official Journal of the European Union L152: 11-22.

NF EN FDIS 16140-2 Microbiologie des aliments – Validation des méthodes – Partie 2 : protocole pour la validation de méthodes alternatives (commerciales) par rapport à une méthode de référence. In 2016.

5725-2 NI, Exactitude (justesse et fidélité) des résultats et méthodes de mesure. In Partie 1: Principes généraux et définitions; ISO 5725-2 Partie 2: Méthode de base pour la détermination de la répétabilité et de la reproductibilité d'une méthode de mesure normalisée; Partie 4: Méthodes de base pour la détermination de la justesse d'une méthode de mesure normalisée., 1994.

Bourdat-Deschamps M. 2010. Validation de l'analyse des hydrocarbures aromatiques polycycliques dans les eaux de pluie par la méthode du profil d'exactitude. Le Cahier des Techniques de l'Inra. 85-98.

EURL Guidance Document on Screening Method Validation version 1.1., 21 September 2023
https://eurl-residues.eu/wp-content/uploads/2023/10/Guidance_screening_20230921_v1_0.pdf
Feinberg M. 2007. Validation of analytical methods based on accuracy profiles. J. Chromatogr. A. 1158:174-183.

Feinberg M. 2010a. Interprétation du profil d'exactitude. Le Cahier des Techniques de l'Inra. 45-60.

Feinberg M. 2010b. Mise en oeuvre du profil d'exactitude. Le Cahier des Techniques de l'Inra. 27-44.

Gaugain M, Chotard M-P, Verdon E. 2013. Stability Study for 53 Antibiotics in Solution and in Fortified Biological Matrixes by LC/MS/MS. J. AOAC Int. 96:471-480.

Goupy J, Introduction aux plans d'expériences. 2001; p 293.

Goupy J. 2005. What kind of experimental design for finding and checking robustness of analytical methods? Analytica Chimica Acta
Papers Presented at the 9th International Conference on Chemometrics in Analytical Chemistry. 544:184-190.

Hubert P, Nguyen-Huu JJ, Boulanger B, Chapuzet E, Cohen N, Compagnon PA, Dewé W, Feinberg M, Laurentie M, Mercier N, Muzard G, Valat L, Rozet E. 2008. Harmonization of strategies for the validation of quantitative analytical procedures: A SFSTP proposal: Part IV. Examples of application. J. Pharm. Biomed. Anal. 48:760-771.

Règlement (UE) 2019/1871 de la Commission du 7 novembre 2019 relatif aux valeurs de référence pour les substances pharmacologiquement actives non autorisées présentes dans les denrées alimentaires d'origine animale et abrogeant la décision 2005/34/CE. Journal officiel de l'Union européenne L 289 du 8.11.2019, p. 41.

Règlement d'exécution (UE) 2021/808 de la Commission du 22 mars 2021 concernant les performances des méthodes d'analyse des résidus de substances pharmacologiquement actives utilisées chez les animaux producteurs d'aliments et l'interprétation des résultats ainsi que les méthodes à employer pour l'échantillonnage et abrogeant les décisions 2002/657/CE et 98/179/CE. Journal officiel de l'Union européenne L180 du 21.05.2021, p. 84-109.

Appendix 1. List of antibiotics to be validated, based on the type of test and the antibiotics targeted by the test.

MILK

Broad spectrum test: 20 antibiotics to be validated in the milk from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
BETA-LACTAMINES	Penicillin G	4
	Amoxicillin	4
	Cloxacillin	30
	Cefalexin	100
	Cefalonium	20
	Sum of cefapirin and deacetylcefapirin (metabolite)	60
	Cefquinome	20
	Sum of all residues maintaining the betalactam structure, expressed as defuroylceftiofur (metabolite)	100
TETRACYCLINS	Chlortetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Oxytetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Tetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
SULPHONAMIDES	Sulfadimethoxin	100
	Sulfadiazin	100
MACROLIDES	Tylosin	50
	Erythromycin A	40
AMINOSIDES	Dihydrostreptomycin	200
	Neomycin B	1500
	Sum of gentamicin C1, gentamicin C1a, gentamicin C2 and gentamicin C2a	100
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	100
LINCOSAMIDES	Lincomycin	150

Specific beta-lactamines test: 8 antibiotics to be validated in the milk from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
BETA-LACTAMINES	Penicillin G	4
	Amoxicillin	4
	Cefalexin	100
	Cefalonium	20
	Sum of cefapirin and deacetylcefapirin (metabolite)	60
	Cefquinome	20
	Sum of all residues maintaining the betalactam structure, expressed as defuroylceftiofur (metabolite)	100
	Cloxacillin	30

Special tetracyclins test: 3 antibiotics to be validated in the milk from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
TETRACYCLINS	Chlortetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Oxytetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Tetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100

Special sulphonamides test: 6 antibiotics to be validated in the milk from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
SULPHONAMIDES	Sulfadimethoxin	100
	Sulfadiazin	100
	Sulfadoxin	100
	Sulfadimidin (sulfamethazin)	100
	Sulfamethoxazol	100
	Sulfachlorpyridazin	100

Special macrolides test: 4 antibiotics to be validated in the milk from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
MACROLIDES	Erythromycin A	40
	Tylosin	50
	Tilmicosin	50
	Sum of spiramycin and neospiramycin	200

Special aminosides test: 4 antibiotics to be validated in the milk from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
AMINOSIDES	Dihydrostreptomycin	200
	Streptomycin	200
	Neomycin B	1500
	Sum of gentamicin C1, gentamicin C1a, gentamicin C2 and gentamicin C2a	100

Special quinolones test: 4 antibiotics to be validated in the milk from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	100
	Marbofloxacin	75
	Danofloxacin	30
	Flumequin	50

MUSCLE

Broad spectrum test: 15 antibiotics to be validated in the muscle from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
BETA-LACTAMINES	Penicillin G	50
	Amoxicillin	50
	Cloxacillin	300
	Cefalexin	200
	Sum of all residues maintaining the betalactam structure, expressed as defuroylceftiofur	1000
TETRACYCLINS	Chlortetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Oxytetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Doxycyclin	100
SULPHONAMIDES	Sulfadimethoxin	100
	Sulfadiazin	100
MACROLIDES	Erythromycin A	200
	Tylosin	100
AMINOSIDES	Dihydrostreptomycin	500
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	100
LINCOSAMIDES	Lincomycin	100
POLYPEPTIDES	Sum of metabolites capable of being hydrolysed into 8-a-hydroxymutilin	100 (pork, poultry, rabbit)

Specific beta-lactamines test: 6 antibiotics to be validated in the muscle from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
BETA-LACTAMINES	Penicillin G	50
	Amoxicillin	50
	Cloxacillin	300
	Cefalexin	200
	Sum of all residues maintaining the betalactam structure, expressed as defuroylceftiofur	1000
	Cefquinome	50

Special tetracyclins test: 7 antibiotics to be validated in the muscle from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
TETRACYCLINS	Chlortetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Oxytetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Tetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Doxycyclin	100

Special sulphonamides test: 6 antibiotics to be validated in the muscle from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
SULPHONAMIDES	Sulfadimethoxin	100
	Sulfadiazin	100
	Sulfadoxin	100
	Sulfadimidin (sulfamethazin)	100
	Sulfamethoxazol	100
	Sulfachlorpyridazin	100

Special macrolides test: 6 antibiotics to be validated in the muscle from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
MACROLIDES	Erythromycin A	200
	Tylosin A	100
	Tilmicosin	50 (all species)/75 (poultry)
	Sum of spiramycin and neospiramycin	200 (bovine, poultry)
	Spiramycin 1	250 (pork)
	Sum of tylvalosin and 3-O-acetyltylosin and 3-O-acetyltylosin	50 (pork)

Special aminosides test: 6 antibiotics to be validated in the muscle from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
AMINOSIDES	Dihydrostreptomycin	500
	Streptomycin	500
	Neomycin B	500
	Sum of gentamicin C1, gentamicin C1a, gentamicin C2 and gentamicin C2a	50 (bovine, porcine)
	Spectinomycin	300
	Paromomycin	500

Special quinolones test: 6 antibiotics to be validated in the muscle from different species.

FAMILY	Antibiotics	MRL (µg kg^{-1})
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	100
	Marbofloxacin	150
	Danofloxacin	200 (bovine, poultry)/100 (pork)
	Difloxacin	400/300 (poultry)
	Flumequin	50/400 (turkey)

AQUACULTURE PRODUCTS

Broad spectrum test: 11 to 18 antibiotics to be validated in different aquaculture products.

FAMILY	Antibiotics	MRL (µg kg^{-1})
BETA-LACTAMINES	*Amoxicillin*	*50*
TETRACYCLINS	**Oxytetracyclin**	**100**
	Chlortetracyclin *Sum of the parent substance and its epimers in 4 (metabolite)*	*100*
SULPHONAMIDES	Sulfadimethoxin	100
	Sulfadiazin	**100**
MACROLIDES	*Erythromycin A*	*200*
	Tylosin	100
AMINOSIDES	*Neomycin B*	*500*
	Spectinomycin	300
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	100
	Flumequin	**600**
	Oxolinic acid	**100**
	Sarafloxacin	*30*
LINCOSAMIDES	Lincomycin	100
	Colistin	150
PHENICOLS	**Sum of florfenicol and its metabolites measured as florfenicolamin**	**1000**
	Thiamphenicol	50
	Trimethoprim	**50**

In boldface type, the priority antibiotics in Europe.

In italics, antibiotics of interest as supplement.

Other antibiotics have a MRL but have less interest related to usages.

Reference: Joint FAO/WHO/OIE Expert Consultation on Antimicrobial Use in Aquaculture and Antimicrobial Resistance (Seoul, South Korea, June 13-16, 2006). Towards a risk analysis of antimicrobial use in aquaculture

Victoria Alday, Benjamin Guichard, Peter Smith, Carl Uhland.

Specific beta-lactamines test: 6 antibiotics to be validated in different aquaculture products.

FAMILY	Antibiotics	MRL (µg kg^{-1})
BETA-LACTAMINES	Penicillin G	50
	Amoxicillin	50
	Penicillin	50
	Cloxacillin	300
	Dicloxacillin	300
	Oxacillin	300

Special tetracyclins test: 7 antibiotics to be validated in different aquaculture products.

FAMILY	Antibiotics	MRL (µg kg^{-1})
TETRACYCLINS	Chlortetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Oxytetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Tetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Doxycyclin	100

Special sulphonamides test: 6 antibiotics to be validated in different aquaculture products.

FAMILY	Antibiotics	MRL (µg kg^{-1})
SULPHONAMIDES	Sulfadimethoxin	100
	Sulfadiazin	100
	Sulfadoxin	100
	Sulfadimidin (sulfamethazin)	100
	Sulfamethoxazol	100
	Sulfachlorpyridazin	100

Special macrolides test: 6 antibiotics to be validated in different aquaculture products.

FAMILY	Antibiotics	MRL (µg kg^{-1})
MACROLIDES	Erythromycin A	200
	Tylosin A	100
	Tilmicosin	50
	Sum of spiramycin and neospiramycin	/
	Sum of tylvalosin and 3-O-acetyltylosin and 3-O-acetyltylosin	/

Special aminosides test: 6 antibiotics to be validated in different aquaculture products.

FAMILY	Antibiotics	MRL (µg kg^{-1})
AMINOSIDES	Neomycin B	500
	Spectinomycin	300
	Paromomycin	500
	Dihydrostreptomycin	/
	Streptomycin	/
	Sum of gentamicin C1, gentamicin C1a, gentamicin C2 and gentamicin C2a	/

Special quinolones test: 7 antibiotics to be validated in different aquaculture products.

FAMILY	Antibiotics	MRL (µg kg^{-1})
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	100
	Danofloxacin	100
	Difloxacin	300
	Flumequin	600
	Oxolinic acid	100
	Sarafloxacin	30

EGGS

Broad spectrum test: 13 antibiotics to be validated in eggs.

FAMILY	Antibiotics	MRL (µg kg^{-1})
BETA-LACTAMINES	Amoxicillin	/
TETRACYCLINS	Chlortetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	200
	Oxytetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	200
	Tetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	200
SULPHONAMIDES	Sulfadimethoxin	/
	Sulfadiazin	/
MACROLIDES	Erythromycin A	150
	Tylosin A	200
AMINOSIDES	Neomycin B	500
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	/
LINCOSAMIDES	Lincomycin	50
POLYPEPTIDES	Colistin	300
MISCELLANEOUS	Sum of metabolites capable of being hydrolysed into 8-a-hydroxymutilin	1000

Specific beta-lactamines test: 7 antibiotics to be validated in eggs.

FAMILY	Antibiotics	MRL (µg kg^{-1})
BETA-LACTAMINES	Penicillin G	/
	Amoxicillin	/
	Penicillin	/
	Cloxacillin	/
	Dicloxacillin	/
	Oxacillin	/
	Sum of cefapirin and deacetylcefapirin (metabolite)	/

Special tetracyclins test: 7 antibiotics to be validated in eggs.

FAMILY	Antibiotics	MRL (µg kg^{-1})
TETRACYCLINS	Chlortetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Oxytetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Tetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	100
	Doxycyclin	100

Special sulphonamides test: 6 antibiotics to be validated in eggs.

FAMILY	Antibiotics	MRL (µg kg^{-1})
SULPHONAMIDES	Sulfadimethoxin	/
	Sulfadiazin	/
	Sulfadoxin	/
	Sulfadimidin (sulfamethazin)	/
	Sulfamethoxazol	/
	Sulfachlorpyridazin	/

Special macrolides test: 6 antibiotics to be validated in eggs.

FAMILY	Antibiotics	MRL (µg kg^{-1})
MACROLIDES	Erythromycin A	150
	Tylosin A	200
	Tilmicosin	/
	Sum of spiramycin and neospiramycin I	/
	Josamycin	/

Special aminosides test: 6 antibiotics to be validated in eggs.

FAMILY	Antibiotics	MRL (µg kg^{-1})
AMINOSIDES	Neomycin B	500
	Spectinomycin	/
	Paromomycin	/
	Dihydrostreptomycin	/
	Streptomycin	/
	Sum of gentamicin C1, gentamicin C1a, gentamicin C2 and gentamicin C2a	/

Special quinolones test: 6 antibiotics to be validated in eggs.

FAMILY	Antibiotics	MRL (µg kg^{-1})
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	/
	Danofloxacin	/
	Difloxacin	/
	Flumequin	/
	Sarafloxacin	/

HONEY

Broad spectrum test: 15 antibiotics to be validated in different types of honey.

FAMILY	Antibiotics	RC (µg kg^{-1})
BETA-LACTAMINES	Amoxicillin	/
TETRACYCLINS	Oxytetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	20
	Tetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	20
SULPHONAMIDES	Sulfadimidin (sulfamethazin)	50
	Sulfathiazol	50
	Sulfamethoxazol	50
MACROLIDES	Erythromycin A	20
	Tylosin	20
AMINOSIDES	Streptomycin	40
	Dihydrostreptomycin	40
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	/
	Norfloxacin	/
LINCOSAMIDES	Lincomycin	/
	Trimethoprim	/

No MRL in honey.

RC: Recommended Concentration (LRUE 2007 document).

Special tetracyclins test: 7 antibiotics to be validated in different types of honey.

FAMILY	Antibiotics	RC (µg kg^{-1})
TETRACYCLINS	Chlortetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	20
	Oxytetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	20
	Tetracyclin Sum of the parent substance and its epimers in 4 (metabolite)	20
	Doxycyclin	20

Special sulphonamides test: 8 antibiotics to be validated in different types of honey.

FAMILY	Antibiotics	RC (µg kg^{-1})
SULPHONAMIDES	Sulfadimethoxin	50
	Sulfadiazin	50
	Sulfathiazol	50
	Sulfadimidin (sulfamethazin)	50
	Sulfamethoxazol	50
	Sulfachlorpyridazin	50
	Sulfapyridin	50
	Sulfaquinoxalin	50

Special macrolides test: 6 antibiotics to be validated in different types of honey.

FAMILY	Antibiotics	RC (µg kg^{-1})
MACROLIDES	Erythromycin A	20
	Tylosin A	20
	Tylosin B = desmycosin	20
	Tilmicosin	/
	Sum of spiramycin and neospiramycin	/
	Josamycin	/

Special aminosides test: 6 antibiotics to be validated in different types of honey.

FAMILY	Antibiotics	RC (µg kg^{-1})
AMINOSIDES	Dihydrostreptomycin	40
	Streptomycin	40
	Neomycin B	/
	Sum of gentamicin C1, gentamicin C1a, gentamicin C2 and gentamicin C2a	/
	Spectinomycin	/
	Paromomycin	/

Special quinolones test: 7 antibiotics to be validated in different types of honey.

FAMILY	Antibiotics	MRL (µg kg^{-1})
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	/
	Ciprofloxacine	/
	Norfloxacin	/
	Marbofloxacin	/
	Danofloxacin	/
	Difloxacin	/
	Flumequin	/

Appendix 2. Table for preparing and keeping parent antibiotic solutions (at 0.5 mg/ml).

Family	Antibiotic	Dilution solvent(s)	Storage temperature (°C)	Maximum storage period (days)
PENICILLINS	Amoxillin	Water-methyl alcohol 50:50, v/v	-18	>358
	Penicillin	Water-methyl alcohol 50:50, v/v	-18	31
	Penicillin G	Water-methyl alcohol 50:50, v/v	-18	218
	Penicillin V	Water-methyl alcohol 50:50, v/v	-18	299
	Oxacillin	Water-methyl alcohol 50:50, v/v	-18	>358
	Cloxacillin	Water-methyl alcohol 50:50, v/v	-18	>358
	Nafcillin	Water-methyl alcohol 50:50, v/v	-18	74
	Dicloxacillin	Water-methyl alcohol 50:50, v/v	-18	74
CEPHALOSPORINS	Cephapirin	Water-methyl alcohol 50:50, v/v	-18	>353
	Cafquinome	Water-methyl alcohol 50:50, v/v	-18	49
	Cafalonium	Water-methyl alcohol 50:50, v/v	-18	ND
	Cefazolin	Water-methyl alcohol 50:50, v/v	-18	>353
	Cefalexin	Water-methyl alcohol 50:50, v/v	-18	>353
	Ceftiofur	Water-methyl alcohol 50:50, v/v	-18	219
	Cefoperazon	Water-methyl alcohol 50:50, v/v	-18	219
SULPHONAMIDES	Sulfaguanidin	Methanol	-18	>348
	Sulfadiazin	Methanol	-18	>348
	Sulfathiazol	Methanol	-18	>348
	Sulfadimerazin	Methanol	-18	>348
	Sulfamethoxypyridazin	Methanol	-18	>348
	Sulfamonomethoxin	Methanol	-18	>348
	Sulfadoxin	Methanol	-18	>348
	Sulfaquinoxalin	Methanol	-18	>348
	Sulfadimethoxin	Methanol	-18	>348
MACROLIDES	Tulathromycin	Methanol	-18	>350
	Neospiramycin	Methanol	-18	168
	Spiramycin	Methanol	-18	>350
	Tilmicosin	Methanol	-18	>350
	Tylosin	Methanol	-18	ND
	Tylvalosin	Methanol	-18	72
	o-acetyltylvalosin	Methanol	-18	>350
	Josamycin	Methanol	-18	72
AMINOSIDES	Spectinomycin	Water-methyl alcohol 50:50, v/v	-18	204
	Streptomycin	Water-methyl alcohol 50:50, v/v	-18	204
	Dihydrostreptomycin	Water-methyl alcohol 50:50, v/v	-18	204
	Kanamycin	Water-methyl alcohol 50:50, v/v	-18	105
	Paromomycin	Water-methyl alcohol 50:50, v/v	-18	ND
	Gentamicin C1	Water-methyl alcohol 50:50, v/v	-18	36
	Neomycin	Water-methyl alcohol 50:50, v/v	+4	105
	Lincomycin	Methanol	-18	204

Family	Antibiotic	Dilution solvent(s)	Storage temperature (°C)	Maximum storage period (days)
TETRACYCLINS	Tetracyclin	Methanol	-18	203
	Doxycyclin	Methanol	-18	203
	Oxytetracyclin	Methanol	-18	203
QUINOLONES	Nalidixic acid	Methanol + 1 ml NaOH 1M	-18	203
	Oxolinic acid	Methanol + 1 ml NaOH 1M	-18	203
	Flumequin	Methanol + 1 ml NaOH 1M	-18	203
	Norfloxacin	Methanol + 1 ml NaOH 1M	-18	203
	Ciprofloxacin	Methanol + 1 ml NaOH 1M	-18	>349
	Danofloxacin	Methanol + 1 ml NaOH 1M	-18	105
	Enrofloxacin	Methanol + 1 ml NaOH 1M	-18	>349
	Marbofloxacin	Methanol + 1 ml NaOH 1M	-18	>350
	Sarafloxacin	Methanol + 1 ml NaOH 1M	-18	>351
	Difloxacin	Methanol + 1 ml NaOH 1M	-18	203

ND: not determined

Appendix 3. Selection of one to 2 representative molecules per antibiotics family.

MILK

FAMILY	Antibiotics	MRL (µg kg^{-1})
PENICILLINS	Amoxicillin	4
	Cloxacillin	30
CEPHALOSPORINS	Cefalexin	100
TETRACYCLINS	Oxytetracyclin	100
	Tetracyclin	100
SULPHONAMIDES	Sulfadimethoxin	100
	Sulfadiazin	100
MACROLIDES	Tylosin A	
	Erythromycin A	
AMINOSIDES	Dihydrostreptomycin	
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	
LINCOSAMIDES	Lincomycin	

MUSCLE

FAMILY	Antibiotics	MRL (µg kg^{-1})
PENICILLINS	Amoxicillin	50
	Cloxacillin	300
CEPHALOSPORINS	Cefalexin	200
TETRACYCLINS	Chlortetracyclin	100
	Oxytetracyclin	100
SULPHONAMIDES	Sulfadimethoxin	100
	Sulfadiazin	100
MACROLIDES	Erythromycin A	200
	Tylosin A	100
AMINOSIDES	Dihydrostreptomycin	500
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	100
LINCOSAMIDES	Lincomycin	100
POLYPEPTIDES	Sum of metabolites capable of being hydrolysed into 8-a-hydroxymutilin	100 (pork, poultry, rabbit)

AQUACULTURE PRODUCTS

FAMILY	Antibiotics	MRL (µg kg^{-1})
BETA-LACTAMINES	Amoxicillin	50
TETRACYCLINS	Oxytetracyclin	100
	Chlortetracyclin	100
SULPHONAMIDES	Sulfadiazin	100
MACROLIDES	Erythromycin A	200
AMINOSIDES	Neomycin B	500
	Spectinomycin	300
QUINOLONES	Flumequin	600
	Oxolinic acid	100
LINCOSAMIDES	Lincomycin	100
PHENICOLS	Florfenicol	1000
	Trimethoprim	50

EGGS

FAMILY	Antibiotics	MRL (µg kg^{-1})
BETA-LACTAMINES	Amoxicillin	/
TETRACYCLINS	Chlortetracyclin	200
SULPHONAMIDES	Sulfadimethoxin	/
MACROLIDES	Erythromycin A	150
	Tylosin A	200
AMINOSIDES	Neomycin B	500
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	/
LINCOSAMIDES	Lincomycin	50
POLYPEPTIDES	Colistin	300
MISCELLANEOUS	Sum of metabolites capable of being hydrolysed into 8-a-hydroxymutilin	1000

HONEY

FAMILY	Antibiotics	RC (µg kg^{-1})
BETA-LACTAMINES	Amoxicillin	/
TETRACYCLINS	Oxytetracyclin	20
	Tetracyclin	20
SULPHONAMIDES	Sulfathiazol	50
MACROLIDES	Tylosin A	20
AMINOSIDES	Streptomycin	40
QUINOLONES	Sum of enrofloxacin and ciprofloxacin	/
LINCOSAMIDES	Lincomycin	/

Appendix 4. Implementation and interpretation of an experimental plan for the robustness study.

An example of an experimental plan matrix is presented in table A4.1 below.

The basic concept is not to study one variation at a time, but to introduce a number of variations simultaneously. For example, we mean by A, B, C and D 4 different factors likely to influence the results if their nominal values are modified slightly. The nominal values of these parameters are + (high value) or – (low value) in the table below. This gives 2^4, i.e. 32 potential different combinations.

It is possible to select a sub-set of eight of these combinations having a balance between + and –. Eight determinations (e.g. 8 series of different tests over 8 days) using a combination of the selected factors A-D) must be performed. The results of the determinations will be shown in the final column of the table. The result observed and measured may be, for example, a rate of false-negative results or a rate of false-positive results, an incubation period, etc.

Table A4.1. Example of an experimental plan matrix.

	Levels								
Day	**1**	**A**	**B**	**C**	**D=ABC**	**AB+CD**	**AC+BD**	**BC+AD**	**Observed result**
1	+	-	-	-	-	+	+	+	
2	+	+	-	-	+	-	-	+	
3	+	-	+	-	+	-	+	-	
4	+	+	+	-	-	+	-	-	
5	+	-	-	+	+	+	-	-	
6	+	+	-	+	-	-	+	-	
7	+	-	+	+	-	-	-	+	
8	+	+	+	+	+	+	+	+	

Two variation levels are studied for each factor (in this case 4 factors A, B, C and D): a minimum level (-) and a maximum level (+). In addition, the interactions between the different factors can also be studied.

Use and interpretation of the results:

The table A4.2 below summarises the results obtained after the robustness study. In this example, 4 factors had been chosen and they had been varied from a minimum value (-) to a maximum value (+), around the reference value. The impact on the response was observed (rate of false-negatives, rate of false-positives) compared with the calculated mean for each type of response (I). In this case, for example, the mean of the rate of false-negatives is 0.63. The factor D which shows an influence of -0.63 is therefore a factor which influences the result; in this case, it therefore reduces the rate of false-negatives, which is favourable for the test. In contrast, the factor A, which has an influence of +0.63, increases the rate of false-negatives. The factor A therefore has an adverse impact on the result of the test. This factor A could prejudice the robustness of the test if its impact is very large.

In this example, only the factor C has no influence on the results in terms of false-positives and false-negatives.

Table A4.2. Example of the results of a robustness study.

	Factor				**Interactions**			**Mean**
	A	**B**	**C**	**D=ABC**	**E**	**F**	**LH**	
Response	**A**	**B**	**C**	**D=ABC**	**AB+CD**	**AC+BD**	**BC+AD**	**1**
Rate of false +	**0.63**	**0.63**	-0.05	**-0.63**	**0.63**	-0.63	-0.63	0.63
Rate of false -	**0.63**	**0.63**	-0.05	**-0.63**	**0.63**	-0.63	-0.63	0.63

Appendix 5. Global accuracy profile approach.

Experimental protocol:

The choice of substances for testing will be same as for the trueness and precision approach determined individually (§ 1.3.2.1 of chapter III.).

Two experience plans must be built:

- A calibration plan (standards in solution and/or in matrix), which is used to estimate the response function of the method.
- A validation plan (standards supplemented in the matrix), which is used to determine the validation characteristics.

Table A5.1.Construction of experience plans (calibration and validation).

	Calibration plan	Validation plan
Concentration levels	k' levels, number k' ≥ 1	k levels, k ≥ 3
Number of series	I series, spread over I days, number I ≥ 3	I series, spread over I days, number I ≥ 3
Number of repetitions	J' repetitions, recommended number J' ≥ 2, discretionary for each calibration and for each series	J repetitions, number J ≥ 2, constant for each series
Total number of tests	I*J'*k' tests	I*J*k tests

In summary, the unprocessed result are used to choose the ideal calibration model and build the accuracy profile.

Choice of calibration model:

From the results of the calibration plan, it is possible to test several calibration models and thus to build a number of accuracy profiles for the same data (Bourdat-Deschamps 2010).

From the validation plan data in the matrix, the concentrations found must be calculated, for each series I, from the corresponding calibration model. For this, the inverse function of the calibration model is used.

Building the accuracy profile:

To build the accuracy profile, the objectives of the method must first be set:

- The tolerances (%) are related to the proportion β. This proportion is set arbitrarily, generally between 80 and 95%, depending on the scope of the method. It corresponds to the percentage of results that will be included on average in the calculated tolerance interval.
- The acceptability limits (± λ) correspond to the performances demanded for the method. They are set according to the use stipulated as routine (e.g. regulatory limits (e.g. trueness criteria in the regulation CE/2021/808 (2021)), client request), sometimes dependent on the concentration level. They are expressed in the same unit as the variable one wishes to measure and encompass the reference value; they delineate the dosage interval.

The trueness (bias or recovery) and precision calculations are then performed from the recovered concentrations z, per concentration level k, for each calibration model that will be tested.

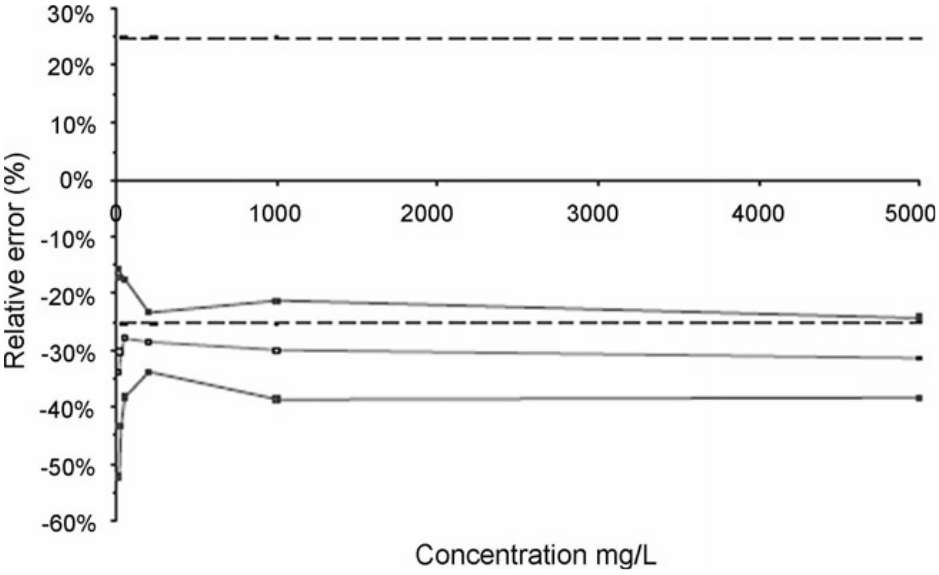
Interpreting the accuracy profile:

The graphic representation of the profile enables an initial visual interpretation.

The validity of the method will be determined based on the tolerances and acceptability limits.

To conclude that the method is valid, the major part of the tolerance intervals must be included in the acceptability interval. The validity domain of the method corresponds to the domain in which the proportion of acceptable results is at least equal to β. The part which is included in the acceptability interval corresponds to the dosage interval. Furthermore, a low quantification limit (LOQ) and a high quantification limit will be derivable from the accuracy profile. The low LOQ corresponds to the lower end of the validity domain and the high LOQ to the upper end of the validity domain.

Table A5.2. Accuracy profile extracted from the publication by Hubert *et al.* (Hubert et al. 2008). Accuracy profile with a bias.



Appendix 6. Template for the preliminary study report and for the interlaboratory study report.

I. PRELIMINARY STUDY REPORT

It must contain in the introduction the following items:

- the name of the Expert Laboratory and of its sub-contractors, if any (with, in this case, the scope of the sub-contracting),
- the name of the applicant,
- the heading for the study screening method and its scope,
- the study start and end dates,
- a summary of the principal results,
- a brief bibliographical analysis if possible and/or if necessary.

Each of the following criteria must be populated:

- the measurement protocol and, in particular:
  - the method for preparing the samples,
  - number and type of samples tested (e.g. by matrix types, natural or artificial contaminations, etc.),
  - an analysis flow chart.
- The statement of results: The unprocessed data must be appended.
- Interpretation of the results: Summary tables may summarise all the results.

The general conclusion of the preliminary study must be shown:

- a conclusion for each of the characteristics studied, based on the acceptability criteria,
- A general conclusion (the Expert Laboratory must not give an opinion on the way forward for the case).

II. INTERLABORATORIES' STUDY REPORT

In the first place, it must contain in the introduction the following items:

- the name of the Expert Laboratory and of its sub-contractors, if any (with, in this case, the scope of the sub-contracting),
- the name of the applicant,
- the heading for the study screening method and its scope,
- the number of laboratories that have participated in the study, as well as the details for all of these laboratories (name of the entity, town, country),
- the study start and end dates,
- a summary of the principal results.

In addition, each of the following criteria must be populated:

- the measurement protocol and, in particular:
  - method for preparing the samples, their storage and despatch,
  - condition of the samples on arrival in the collaborating laboratories (stability) (for example, samples remain frozen until arrival),
  - number and type of samples tested (e.g. matrices, natural or artificial contaminations, etc.),
  - choice of molecules and concentration levels tested, number of samples free from substances with antibiotic activity, etc.,
  - an analysis flow chart.
- The statement of results: The results from the Expert Laboratory will be reported. The results obtained by the collaborating laboratories, with the screening method, must be expressed **anonymously**. A general table will report the individual results from each collaborating laboratory. A 2nd table will summarise for each material and for all the laboratories the positive result percentages. The statement of the results must be made in compliance with the applicable standards (ISO 5725 (5725-2 1994) for quantitative methods). The unprocessed data must be appended.
- Interpretation of the results: An interpretation must be made in light of the unprocessed results. In particular, it must explore any causes of disagreement between the expected results and those achieved, and in each case explain the elimination, if any, of the results and/or laboratories.

The general conclusion of the interlaboratory study must be shown:

- A conclusion for each of the characteristics studied, based on the acceptability criteria,
- A general conclusion (the Expert Laboratory must not give an opinion on the way forward for the case).