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COMMISSION IMPLEMENTING REGULATION (EU) 2021/808

of 22 March 2021

on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC

(Text with EEA relevance)

(OJ L 180, 21.5.2021, p. 84)

Amended by:

		Official Journal		
		No	page	date
►**M1**	Commission Implementing Regulation (EU) 2021/810 of 20 May 2021	L 180	112	21.5.2021

Corrected by:

►**C1** Corrigendum, OJ L 186, 27.5.2021, p. 33 (2021/810)

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(Text with EEA relevance)

Article 1

Subject matter and scope

This Regulation lays down rules concerning the methods of analysis used for sampling and for laboratory analyses in relation to residues of pharmacologically active substances in live food-producing animals, their body parts and fluids, excrements, tissues, products of animal origin, animal by-products, feed and water. It also lays down rules for the interpretation of analytical results of these laboratory analyses.

This Regulation applies to official controls aimed at verifying compliance with the requirements on the presence of residues of pharmacologically active substances.

Article 2

Definitions

For the purposes of this Regulation, the definitions in Article 2 of Commission Delegated Regulation (EU) 2019/2090 ([1]), in Commission Regulation (EU) 2019/1871 ([2]), in Article 2 of Regulation (EC) No 470/2009 of the European Parliament and of the Council ([3]) and in Council Regulation (EEC) No 315/93 ([4]) shall apply.

([1]) Commission Delegated Regulation (EU) 2019/2090 of 19 June 2019 supplementing Regulation (EU) 2017/625 of the European Parliament and Council regarding cases of suspected or established non-compliance with Union rules applicable to the use or residues of pharmacologically active substances authorised in veterinary medicinal products or as feed additives or with Union rules applicable to the use or residues of prohibited or unauthorised pharmacologically active substances (OJ L 317, 9.12.2019, p. 28).

([2]) Commission Regulation (EU) 2019/1871 of 7 November 2019 on reference points for action for non-allowed pharmacologically active substances present in food of animal origin and repealing Decision 2005/34/EC (OJ L 289, 8.11.2019, p. 41).

([3]) Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council (OJ L 152, 16.6.2009, p. 11).

([4]) Council Regulation (EEC) No 315/93 of 8 February 1993 laying down Community procedures for contaminants in food (OJ L 37, 13.2.1993, p. 1).

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The following definitions shall also apply:

(1) ‘absolute recovery’ means the yield of the final stage of an analytical process for an analyte divided by the amount of the analyte in the original sample, expressed as a percentage;

(2) ‘accuracy’ means the closeness of agreement between a test result and the accepted true reference value, determined by estimating trueness and precision ([5]);

(3) ‘alpha (α) error’ means the probability that the tested sample is compliant, even though a non-compliant measurement result has been obtained;

(4) ‘analyte’ means the component of a system to be analysed;

(5) ‘authorised substance’ means a pharmacologically active substance authorised for use in food-producing animals in accordance with Directive 2001/82/EC of the European Parliament and of the Council ([6]);

(6) ‘beta (β) error’ means the probability that the tested sample is truly non-compliant, even though a compliant measurement result has been obtained;

(7) ‘bias’ means the difference between the estimated value of the test result and an accepted reference value;

(8) ‘calibration standard’ means a traceable reference for measurements that represents the quantity of substance of interest in a way that ties its value to a reference base;

(9) ‘certified reference material’ (CRM) means a reference material, accompanied by documentation issued by a delegated body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures ([7]);

(10) ‘co-chromatography’ means a technique in which an unknown substance is applied to a chromatographic support together with one or more known compounds, in the expectation that the relative behaviour of the unknown and known substances will assist in the identification of the unknown one;

(11) ‘collaborative study’ means analysing the same sample(s) by using the same method to determine performance characteristics of the method in different laboratories, where the study allows to calculate the random measurement error and laboratory bias for the method used;

(12) ‘confirmatory method’ means a method that provides full or complementary information enabling the substance to be unequivocally identified and if necessary quantified in one of the following manners:

(a) at the maximum residue level or maximum level for authorised substances;

([5]) ISO 3534-1: 2006 Statistics – Vocabulary and symbols – Part 1: General statistical terms and terms used in probability (Chapter 1).

([6]) Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products (OJ L 311, 28.11.2001, p. 1).

([7]) JCGM 200:2008, International vocabulary of metrology – Basic and general concepts and associated terms (VIM), Third Edition 2008: https://www.iso.org/sites/JCGM/VIM-JCGM200.htm (Chapter 5 Measurement standards (Etalons)).

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(b) at the reference points for action (RPA) for prohibited or unauthorised substances, for which a reference point for action is established;

(c) at a concentration as low as reasonably achievable for prohibited or unauthorised substance, for which no reference point for action is established;

(13) ‘coverage factor (k)’ means a number which expresses the desired level of confidence and which is associated with the expanded measurement uncertainty;

(14) ‘decision limit for confirmation (CCα)’ means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant and the value 1 – α means statistical certainty in percentage that the permitted limit has been exceeded;

(15) ‘detection capability for screening (CCβ)’ means the smallest content of the analyte that may be detected or quantified in a sample with an error probability of β:

(a) in the case of prohibited or unauthorised pharmacologically active substances, the CCβ is the lowest concentration at which a method is able to detect or quantify, with a statistical certainty of 1 – β, samples containing residues of prohibited or unauthorised substances;

(b) in the case of authorised substances, the CCβ is the concentration at which the method is able to detect concentrations below the permitted limit with a statistical certainty of 1 – β;

(16) ‘fortified sample material’ means a sample enriched with a known amount of the analyte to be detected or quantified;

(17) ‘inter-laboratory study’ means the organisation, performance and evaluation of tests on the same sample(s) by two or more laboratories in accordance with predetermined conditions to evaluate testing performance, either as a collaborative study or a proficiency test;

(18) ‘internal standard (IS)’ means a substance not contained in the sample and having physico-chemical properties as similar as possible to those of the analyte to be identified or quantified;

(19) ‘level of interest’ means the concentration of a substance or analyte in a sample that is significant to determine its compliance with the legislation as regards:

(a) the maximum residue level or maximum level for authorised substances in accordance with Commission Regulation (EC) No 124/2009 ([8]) and Commission Regulation (EU) No 37/2010 ([9]);

([8]) Commission Regulation (EC) No 124/2009 of 10 February 2009 setting maximum levels for the presence of coccidiostats or histomonostats in food resulting from the unavoidable carry-over of these substances in non-target feed (OJ L 40, 11.2.2009, p. 7).

([9]) Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin (OJ L 15, 20.1.2010, p. 1).

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(b) reference points for action for prohibited or unauthorised substances, for which a reference point for action is established in accordance with Regulation (EU) 2019/1871;

(c) a concentration as low as analytically achievable for prohibited or unauthorised substance, for which no reference point for action is established;

(20) ‘lowest calibrated level’ (LCL) means the lowest concentration on which the measuring system has been calibrated;

(21) ‘matrix’ means the material from which a sample is taken;

(22) ‘matrix effect’ means the difference in analytical response between a standard dissolved in the solvent and a matrix-matched standard either without a correction using an internal standard or with correction using an internal standard;

(23) ‘matrix-matched standard’ means a blank (i.e. analyte-free) matrix to which the analyte is added at a range of concentrations after sample processing;

(24) ‘matrix-fortified standard’ means a blank (i.e. analyte-free) matrix, which prior to solvent extraction and sample processing, is spiked with the analyte at a range of concentrations;

(25) ‘measurand’ means the particular quantity subject to measurement;

(26) ‘measurement uncertainty’ means a non-negative parameter associated with the result of measurement, which characterises the dispersion of values that could reasonably be attributed to the measurand, based on the information used;

(27) ‘performance criteria’ means requirements for a performance characteristic according to which it can be judged that the analytical method is fit for the intended use and generates reliable results;

(28) ‘precision’ means the closeness of agreement between independent test results obtained under stipulated conditions and is expressed as the standard deviation or coefficient of variation of the test results;

(29) ‘qualitative method’ means an analytical method, which detects or identifies a substance or a group of substances on the basis of its chemical, biological or physical properties;

(30) ‘quantitative method’ means an analytical method, which determines the amount or mass fraction of a substance so that it may be expressed as a numerical value of appropriate units;

(31) ‘recovery’ means the recovery corrected amount of an analyte divided by the fortified amount of the analyte in the matrix sample, expressed as a percentage;

(32) ‘recovery correction’ means the use of internal standards, the use of a matrix calibration curve as well as the use of a recovery correction factor and also a combination of these approaches;

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(33) ‘reference material’ means a material sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process or in examination of nominal properties ([10]);

(34) ‘relative matrix effect’ means the difference in analytical response between a standard dissolved in the solvent and a matrix-matched standard with a correction using an internal standard;

(35) ‘repeatability’ means precision under conditions, where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time;

(36) ‘reproducibility’ means precision under conditions, where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment ([11]);

(37) ‘ruggedness’ means the susceptibility of an analytical method to changes in experimental conditions under which the method can be applied as presented or with specified minor modifications;

(38) ‘screening method’ means a method that is used for screening of a substance or class of substances at the level of interest;

(39) ‘screening target concentration’ (STC) means the concentration lower than or equal to the CCβ at which a screening measurement categorises the sample as potentially non-compliant ‘Screen Positive’ and triggers a confirmatory testing;

(40) ‘selectivity’ means the ability of a method to distinguish between the analyte being measured and other substances;

(41) ‘single laboratory study’ or ‘in-house validation’ means an analytical study involving a single laboratory using one method to analyse the same or different test materials under different conditions over justified long time intervals;

(42) ‘standard addition’ means a procedure in which one part of the sample is analysed as such and known amounts of the standard analyte are added to the other test portions before analysis;

(43) ‘standard analyte’ means an analyte of known and certified content and purity to be used as a reference in the analysis;

(44) ‘substance’ means matter of constant composition characterised by the entities which compose it and by certain physical properties;

(45) ‘test portion’ means the quantity of material drawn from the sample on which the test or observation is carried out;

([10]) Codex Alimentarius Commission, Food and Agriculture Organization of the United Nations/World Health Organization, Guidelines on analytical terminology (CAC/GL 72-2009).

([11]) ISO 5725-1:1994 Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions (Chapter 3).

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(46) ‘trueness’ means the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value;

(47) ‘units’ means those units described in ISO 80000 ([12]) and Council Directive 80/181/EEC ([13]);

(48) ‘validation’ means the demonstration by examination and the provision of effective evidence that the particular requirements of a specific intended use are fulfilled ([14]), through a single laboratory study or a collaborative study;

(49) ‘within-laboratory reproducibility’ or ‘intermediate precision/in-house reproducibility’ means measurement precision under a set of within-laboratory conditions in a specific laboratory.

Article 3

Methods of analysis

Member States shall ensure that the samples taken in accordance with Article 34 of Regulation (EU) 2017/625 are analysed using methods that comply with the following requirements:

(1) they are documented in test instructions, preferably according to Annexes of ISO 78-2:1999 Chemistry-Layouts for standards – Part 2: Methods of chemical analysis ([15]);

(2) they comply with the performance criteria and other requirements for analytical methods laid down in Chapter 1 of Annex I to this Regulation;

(3) they have been validated in accordance with the requirements laid down in Chapters 2 and 4 of Annex I to this Regulation;

(4) they allow enforcement of the reference points for action laid down in Regulation (EU) 2019/1871, the identification of the presence of prohibited and unauthorised substances and the enforcement of maximum levels (MLs), which have been set on the basis of Regulation (EEC) No 315/93 and Regulation (EC) No 124/2009 and maximum residue limits (MRLs), which have been set on the basis of Regulations (EC) No 1831/2003 and (EC) No 470/2009.

Article 4

Quality control

Member States shall ensure the quality of the results of analyses performed pursuant to Regulation (EU) 2017/625, in particular by monitoring tests or calibration results in accordance with ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories and with the requirements for quality control during routine analysis as laid down in Chapter 3 of Annex I to this Regulation.

([12]) ISO 80000-1:2009 Quantities and units – Part 1: General (Introduction).
([13]) Council Directive 80/181/EEC of 20 December 1979 on the approximation of the laws of the Member States relating to units of measurement and on the repeal of Directive 71/354/EEC (OJ L 39, 15.2.1980, p. 40).
([14]) ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories (Chapter 3).
([15]) ISO 78-2: 1999 Chemistry – Layouts for standards – Part 2: Methods of chemical analysis (Annexes).

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Article 5

Interpretation of results

(1) The result of an analysis shall be considered non-compliant where it is equal to or above the decision limit for confirmation (CCα).

(2) For authorised substances for which an MRL or ML has been established, the decision limit for confirmation (CCα) shall be the concentration at and above which it can be decided with a statistical certainty of numerical value 1 – α that the permitted limit has been exceeded.

(3) For unauthorised or prohibited substances or for authorised substances for which no MRL or ML has been established in a specific species or product, the decision limit for confirmation (CCα) shall be the lowest concentration level at which it can be decided with a statistical certainty of numerical value 1 – α that the particular analyte is present.

(4) For unauthorised or prohibited pharmacologically active substances the α error shall be 1 % or lower. For all other substances, the α error shall be 5 % or lower.

Article 6

Methods for sampling

Member States shall ensure that samples are taken, handled and labelled in accordance with the detailed methods for sampling laid down in Annex II to this Regulation.

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Article 7

Repeals and transitional measures

Decisions 2002/657/EC and 98/179/EC are repealed from the date of entry into force of this Regulation.

However, until 10 June 2026, the requirements laid down in points 2 and 3 of Annex I to Decision 2002/657/EC shall continue to apply to methods, which have been validated before the date of entry into force of this Regulation.

For the purposes referred to in the second paragraph of Article 8 of Regulation (EU) 2019/1871, Annex II to Decision 2002/657/EC shall continue to apply until 27 November 2022.

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Article 8

Entry into force

This Regulation shall enter into force on the twentieth day following that of its publication in the *Official Journal of the European Union*.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

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ANNEX I

CHAPTER 1

PERFORMANCE CRITERIA AND OTHER REQUIREMENTS FOR ANALYTICAL METHODS

1.1. **Requirements of screening methods**

1.1.1. *Categories of suitable screening methods*

Qualitative, semi-quantitative or quantitative methods shall be used as suitable screening methods.

1.1.2. *Requirements for biological, biochemical or physico-chemical screening* methods

For prohibited or unauthorised substances, the CCβ shall be as low as reasonably achievable and in any case lower than the reference point of action (RPA) for substances for which RPAs are established under Regulation (EU) 2019/1871.

For authorised pharmacologically active substances, the CCβ shall be lower than the MRL or ML.

Only those analytical methods, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate lower than or equal to 5 % (β error), shall be used for screening purposes. In the case of a suspected non-compliant result, that result shall be confirmed by a confirmatory method.

Quantitative screening methods, used for both screening and confirmation shall meet the same requirements for accuracy, range, and precision as described in 1.2.2.1 and 1.2.2.2.

1.2. **Requirements of confirmatory methods**

1.2.1. *General requirements for confirmatory methods*

For prohibited or unauthorised substances, the CCα shall be as low as reasonably achievable. For prohibited or unauthorised substances, for which an RPA is established under Regulation (EU) 2019/1871 the CCα shall be lower than or equal to the reference point for action.

For authorised substances, the CCα shall be higher than but as close as possible to the MRL or ML.

For confirmation purposes, only analytical methods for which it can be demonstrated in a documented traceable manner that they are validated and have a false non-compliant rate (α error) which is less or equal to 1 % for prohibited or unauthorised substances or which is less or equal to 5 % for authorised substances shall be used.

Confirmatory methods shall provide information on the structural chemical composition of the analyte. Consequently, confirmatory methods based only on chromatographic analysis without the use of mass spectrometric detection are not suitable on their own for use as confirmatory methods for prohibited or unauthorised pharmacologically active substances. In the case of mass spectrometry not being suitable for authorised substances, other methods such as HPLC-DAD and -FLD, or a combination of them, can be used.

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When required according to the confirmatory method, a suitable internal standard shall be added to the test portion at the beginning of the extraction procedure. Depending on availability, either stable isotope-labelled forms of the analyte, which are particularly suited for mass spectrometric detection, or analogue compounds that are structurally closely related to the analyte, shall be used. When no suitable internal standard can be used, the identification of the analyte shall preferably be confirmed by co-chromatography ([1]). In this case only one peak shall be obtained, the enhanced peak height (or area) being equivalent to the amount of added analyte. If this is not practicable, matrix-matched or matrix-fortified standards shall be used.

1.2.2. *General performance criteria for confirmatory methods*

1.2.2.1. Trueness by recovery

For repeated analyses of a certified reference material, the deviation of the experimentally determined recovery corrected mean mass fraction from the certified value shall comply with the minimum trueness ranges listed in Table 1.

Table 1

Minimum trueness of quantitative methods

Mass Fraction	Range
≤ 1 µg/kg	–50 % to +20 %
> 1 µg/kg to 10 µg/kg	–30 % to +20 %
≥ 10 µg/kg	–20 % to +20 %

When no certified reference materials are available, it is acceptable that trueness of measurements is assessed in other ways, such as using materials with assigned values from inter-laboratory studies or through additions of known amounts of the analyte(s) to a blank matrix.

1.2.2.2. Precision

The coefficient of variation (CV) for the repeated analysis of a reference or fortified material, under within-laboratory reproducibility conditions, shall not exceed the level calculated by the Horwitz Equation. The equation is:

$$CV = 2^{(1 - 0,5 \log C)}$$

where C is the mass fraction expressed as a power (exponent) of 10 (e.g. 1 mg/g = 10^{-3}). For mass fractions below 120 µg/kg the application of the Horwitz equation yields unacceptably high values. Therefore, the allowed maximum coefficient of variation shall not be greater than the values presented in Table 2.

([1]) Co-chromatography is a procedure in which the sample extract prior to the chromatographic step(s) is divided into two parts. Part one is chromatographed as such. Part two is mixed with the standard analyte that is to be measured. Then this mixture is also chromatographed. The amount of added standard analyte has to be similar to the estimated amount of the analyte in the extract. Co-chromatography is used to improve the identification of an analyte when chromatographic methods are used, especially when no suitable internal standard can be used.

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Table 2

Acceptable coefficient of variation

Mass fraction	Reproducibility CV (%)
> 1 000 µg/kg	16 (adapted from Horwitz equation)
> 120 µg/kg – 1 000 µg/kg	22 (adapted from Horwitz equation)
10 – 120 µg/kg	25 (*)
< 10 µg/kg	30 (*)

(*) The CV (%) presented is a guideline and should be as low as reasonably possible.

For analyses carried out under repeatability conditions, the coefficient of variation under repeatability conditions shall be equal or below two thirds of the values listed in Table 2.

1.2.3. *Requirements for chromatographic separation*

For liquid (LC) or gas chromatography (GC), the minimum acceptable retention time for the analyte(s) under examination shall be twice the retention time corresponding to the void volume of the column. The retention time of the analyte in the extract shall correspond to that of the calibration standard, a matrix-matched standard or a matrix-fortified standard with a tolerance of ± 0,1 minute. For fast chromatography, where the retention time is below 2 minutes, a deviation of less than 5 % of the retention time is acceptable. In case an internal standard is used, the ratio of the chromatographic retention time of the analyte to that of the internal standard, that means the relative retention time of the analyte, shall correspond to that of the calibration standard, matrix-matched standard or matrix-fortified standard with a maximum deviation 0,5 % for gas chromatography and 1 % for liquid chromatography for methods validated from the date of entry into force of this Regulation.

1.2.4. *Specific performance criteria for mass spectrometry*

1.2.4.1. Mass spectrometric detection

Mass spectrometric detection shall be carried out by using some of the following options:

1. recording full scan (FS) mass spectra;

2. selected ion monitoring (SIM);

3. sequential mass spectrometry (MS^n) techniques such as Selected Reaction Monitoring (SRM);

4. a combination of mass spectrometry (MS) or sequential mass spectrometry (MS^n) techniques with appropriate ionisation modes.

Both low-resolution mass spectrometry (LRMS, at unit mass resolution) and high-resolution mass spectrometry (HRMS), including e.g. double focusing sectors, Time of Flight (TOF) and Orbitrap instruments are appropriate.

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For confirmation of the identity of an analyte in high-resolution mass spectrometry (HRMS) the mass deviation of all diagnostic ions shall be below 5 ppm (or in case of m/z < 200 below 1 mDa). On basis of this the effective resolution should be selected fit for purpose and the resolution shall typically be greater than 10 000 for the entire mass range at 10 % valley or 20 000 at full width at half maximum (FWHM).

When mass spectrometric determination is performed by the recording of full scan spectra (both LRMS and HRMS), only diagnostic ions with a relative intensity of more than 10 % in the reference spectrum of the calibration standard, matrix-matched standard or matrix-fortified standards are suitable. Diagnostic ions shall include the molecular ion (if present at ≥ 10 % intensity of the base peak) and characteristic fragment or product ions.

Precursor ion selection: When mass spectrometric determination is performed by fragmentation after precursor ion selection, precursor ion selection is carried out at unit mass resolution or better. The selected precursor ion shall be the molecular ion, characteristic adducts of the molecular ion, characteristic product ions or one of their isotope ions. In case the precursor selection has a mass selection window of more than one Dalton (e.g. in case of Data Independent Acquisition) the technique is considered as full-scan confirmatory analysis.

Fragment and product ions: The selected fragment or product ions shall be diagnostic fragment for the analyte/product measured. Non-selective transitions (e.g. the tropylium cation or loss of water) shall be omitted whenever possible. The abundance of diagnostic ions shall be determined from the peak area or height of integrated extracted ion chromatograms. This is also applicable when full-scan measurements are used for identification. The signal-to-noise (S/N) ratio of all diagnostic ions shall be greater or equal than three to one (3:1).

Relative intensities: The relative intensities of the diagnostic ions (ion ratio) are expressed as a percentage of the intensity of the most abundant ion or transition. The ion ratio has to be determined by comparing spectra or by integrating the signals of the extracted ion mass traces. The ion ratio of the analyte to be confirmed shall correspond to those of the matrix-matched standards, matrix-fortified standards or standard solutions at comparable concentrations, measured under the same conditions, within ± 40 % relative deviation.

For all mass spectrometric analyses, at least one ion ratio shall be determined. These are preferably ions obtained within a single scan, but the ions can also originate from different scans in the same injection (i.e. full scan and fragmentation scan).

1.2.4.2. Identification

A system of identification points shall be used to select adequate acquisition modes and evaluation criteria. For confirmation of the identity of substances in a matrix for which an MRL is established (authorised use), a minimum of 4 identification points is required. For unauthorised or prohibited substances, 5 identification points are required. One point can originate from the chromatographic separation. Table 3 shows the number of identification points that each of the techniques yields. To qualify for the identification points required for confirmation, identification points obtained from different techniques can be added.

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1. All mass spectrometric analyses shall be combined with a separation technique that shows sufficient separation power and selectivity for the specific application. Suitable separation techniques are amongst others liquid and gas chromatography, capillary electrophoresis (CE) and supercritical fluid chromatography (SFC). In the case of analyte which presents any isobar or isomer compound, the acceptability of the retention time (i.e. ± 0,5 % in GC and ± 1 % in LC and SFC) is mandatory to confirm its identity.

2. A maximum of three separate techniques can be combined to achieve the minimum number of identification points.

3. Different ionisation modes (e.g. electron ionisation and chemical ionisation) are considered as different techniques.

Table 3

Identification points per technique

Technique	Identification Points
Separation (mode GC, LC, SFC, CE)	1
LR-MS ion	1
Precursor ion selection at <±0,5 Da mass range	1 (indirect)
LR-MS^n product ion	1,5
HR-MS ion	1,5
HR-MS^n product ion	2,5

Table 4

Examples of the number of identification points specific techniques and combinations of techniques (n = an integer)

Technique(s)	Separation	Number of ions	Identification points
GC-MS (EI or CI)	GC	n	1 + n
GC-MS (EI and CI)	GC	2 (EI) + 2 (CI)	1 + 4 = 5
GC-MS (EI or CI) 2 derivates	GC	2 (Derivate A) + 2 (Derivate B)	1 + 4 = 5
LC-MS	LC	n (MS)	1 + n
GC- or LC-MS/MS	GC or LC	1 precursor + 2 products	1 + 1 + 2 × 1,5 = 5
GC- or LC-MS/MS	GC or LC	2 precursor + 2 products	1 + 2 + 2 × 1,5 = 6
GC- or LC-MS^3	GC or LC	1 precursor + 1 MS^2 product + 1 MS^3 product	1 + 1 + 1,5 + 1,5 = 5
GC- or LC-HRMS	GC or LC	n	1 + n × 1,5
GC- or LC-HRMS/MS	GC or LC	1 precursor (<±0,5 Da mass range) + 1 product	1 + 1 + 2,5 = 4,5

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Technique(s)	Separation	Number of ions	Identification points
GC- or LC-HRMS and HRMS/MS	GC or LC	1 full scan ion + 1 HRMS product ion ([a])	1 + 1,5 + 2,5 = 5
GC- and LC-MS	GC and LC	2 ions (GCMS) + 1 ion (LCMS)	1 + 1 + 2 + 1 + 1 = 6

([a]) No additional identification point is obtained for the precursor ion selection, if this precursor ion is the same ion (or an adduct or isotope) as the HRMS ion monitored in full scan.

1.2.5. *Specific performance criteria for the determination of an analyte using liquid chromatography with detection techniques other than mass spectrometry*

For authorised substances only, the following techniques can be used as alternative for mass spectrometry based methods, provided that the relevant criteria for these techniques are fulfilled:

1. full-scan diode array detection spectrophotometry (DAD) in case used with HPLC;

2. fluorescence detection spectrophotometry (FLD) in case used with HPLC.

Liquid chromatography with UV/VIS detection (single wavelength) is not suitable on its own for use as a confirmatory method.

1.2.5.1. Performance criteria for full-scan diode array spectrophotometry

The performance criteria for chromatographic separation included in Chapter 1.2.3 shall be fulfilled.

The absorption maxima in the UV spectrum of the analyte shall be at the same wavelengths as those of the calibration standard in matrix within a maximum margin, which is determined by the resolution of the detection system. For diode array detection, this maximum margin is typically within ± 2 nm. The spectrum of the analyte above 220 nm shall, for those parts of the two spectra with a relative absorbance greater than or equal to 10 %, not be visibly different from the spectrum of the calibration standard. This criterion is met when firstly the same maxima are present and secondly when the difference between the two spectra is at no point greater than 10 % of the absorbance of the calibration standard. In the case computer-aided library, searching and matching are used, the comparison of the spectral data in the official samples to that of the calibration solution has to exceed a critical match factor. This factor shall be determined during the validation process for every analyte on the basis of spectra for which the criteria described above are fulfilled. Variability in the spectra caused by the sample matrix and the detector performance shall be checked.

1.2.5.2. Performance criteria for fluorescence detection spectrophotometry

The performance criteria for chromatographic separation included in Chapter 1.2.3 shall be fulfilled.

The selection of the excitation and emission wavelengths in combination with the chromatographic conditions shall be done in such a way to minimise the effects of interfering components in blank sample extracts. There should be a minimum of 50 nanometres between the excitation and emission wavelength.

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The nearest peak maximum in the chromatogram shall be separated from the designated analyte peak by at least one full peak width at 10 % of the maximum height of the analyte peak.

This applies to molecules that exhibit native fluorescence and to molecules that exhibit fluorescence after either transformation or derivatisation.

CHAPTER 2

VALIDATION

2.1. **Performance characteristics to be determined for analytical methods**

By means of the validation of the method, it shall be demonstrated that the analytical method complies with the criteria applicable for the relevant performance characteristics. Different control purposes require different categories of methods. Table 5 determines which performance characteristic shall be verified for which type of method, further explanation of each parameter is given in this chapter.

Table 5

Classification of analytical methods by the performance characteristics that have to be determined

Method	Confirmation		Screening		
	Qualitative	Quantitative	Qualitative	Semi-quantitative	Quantitative
Substances	A	A, B	A, B	A, B	A, B
Identification in accordance with 1.2	x	x			
CCα	x	x			
CCβ	-		x	x	x
Trueness		x			x
Precision		x		(x)	x
Relative matrix effect/absolute recovery (*)		x			x
Selectivity/Specificity		x	x	x	x
Stability (#)		x	x	x	x
Ruggedness		x	x	x	x

x: It is required to prove by means of the validation that the requirements for the performance characteristic are met.
(x) The precision requirements of Chapter 1.2.2.2 do not need to be met for semi-quantitative screening methods. However, the precision shall be determined to prove the suitability of the method for avoiding false compliant analytical results.
A: prohibited or unauthorised substances
B: authorised substances
(#) If stability data for analytes in a matrix are available from scientific literature or from another laboratory, these data do not need to be determined again by the concerned laboratory. However, a reference to available stability data of analytes in solution is only acceptable if identical conditions are applied.
(*) Relevant for MS methods to prove by means of the validation that the requirements for the performance characteristics are met. The relative matrix effect of the method shall be determined when this effect was not assessed during the validation procedure. The absolute recovery of the method shall be determined when no internal standard or no matrix-fortified calibration is used.

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2.2. **Trueness, repeatability and within-laboratory reproducibility**

This chapter provides examples and references for validation procedures. Other approaches to demonstrate that the method complies with performance criteria may be used, provided that they achieve the same level and quality of information.

2.2.1. *Conventional validation*

The calculation of the parameters in accordance with conventional methods requires the performance of several individual experiments. Each performance characteristic has to be determined for each major change (see Section 2.4). For multi-analyte methods, several analytes can be analysed simultaneously, as long as possibly relevant interferences have been ruled out. Several performance characteristics can be determined in a similar way. Therefore, in order to minimise the workload, it is advised to combine experiments as much as possible (e.g., repeatability and within-laboratory reproducibility with specificity, analysis of blank samples to determine the decision limit for confirmation and testing for specificity).

2.2.1.1. Trueness on the basis of a certified reference material

It is preferred to determine the trueness of an analytical method by means of certified reference material (CRM). The procedure for this is described in ISO 5725-4:1994 ([2]).

An example is given below:

1. Analyse six replicates of the CRM in accordance with the test instructions for the method;

2. Determine the concentration of the analyte present in each sample of the replicates;

3. Calculate the mean, the standard deviation and the coefficient of variation (%) *for these six replicates*;

4. Calculate the trueness by dividing the detected mean concentration by the certified value (measured as concentration) and multiply by 100, to express the result as a percentage.

Trueness (%) = (mean recovery-corrected concentration detected) × 100/certified value

2.2.1.2. Trueness on basis of fortified samples

If no certified reference material is available, the trueness of the method shall be determined by experiments using a fortified blank matrix, as a minimum in accordance with the following scheme:

1. For methods validated from the date of entry into force of this Regulation, select blank material and fortify at a concentration of:

([2]) ISO 5725-4:2020 Accuracy (trueness and precision) of measurement methods and results – Part 4: Basic methods for the determination of the trueness of a standard measurement method (Clause 3).

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(a) 0,5 ([3]), 1,0 and 1,5 times the RPA; or

(b) 0,1 ([4]), 1,0 and 1,5 times the MRL or ML for authorised substances; or

(c) 1,0, 2,0 and 3,0 times the LCL for unauthorised substances (for which no RPA has been established).

2. At each level, the analysis shall be performed with six replicates.

3. Analyse the samples.

4. Calculate the concentration detected in each sample.

5. Calculate the trueness for each sample using the equation below and subsequently calculate the mean trueness and coefficient of variation for the six results at each concentration level.

Trueness (%) = (mean recovery-corrected concentration detected) × 100/fortification level

For methods for authorised substances validated before the date of application of this Regulation, a determination of the trueness of the method using 6 fortified aliquots at 0,5, 1,0 and 1,5 times the MRL or ML is sufficient.

2.2.1.3. Repeatability

1. For methods validated from the date of entry into force of this Regulation a set of samples of identical blank matrices of the same species shall be prepared. They shall be fortified with the analyte to yield concentrations equivalent to:

(a) 0,5 ([5]), 1,0 and 1,5 times the RPA, or

(b) 0,1 ([6]), 1,0 and 1,5 times the MRL or ML for authorised substances, or

(c) 1,0, 2,0 and 3,0 times the LCL for unauthorised or prohibited substances in case no RPA is applicable.

2. At each level, the analysis shall be performed with at least six replicates.

3. Analyse the samples.

4. Calculate the concentration detected in each sample.

5. Calculate the mean concentration, standard deviation and the coefficient of variation (%) of the fortified samples.

6. Repeat these steps on at least two other occasions.

7. Calculate the overall mean concentrations, standard deviations (by averaging the standard deviation squared of the individual occasions and taking the square root of that) and coefficients of variation for the fortified samples.

([3]) Where, for a non-allowed pharmacologically active substance validation of a concentration of 0,5 times the RPA is not reasonably achievable, the concentration of 0,5 times the RPA can be replaced by the lowest concentration between 0,5 times and 1,0 times the RPA, which is reasonably achievable.

([4]) Where, for a specific pharmacologically active substance validation of a concentration of 0,1 times the MRL is not reasonably achievable, the concentration of 0,1 times the MRL can be replaced by the lowest concentration between 0,1 times and 0,5 times the MRL, which is reasonably achievable.

([5]) Where, for a non-allowed pharmacologically active substance validation of a concentration of 0,5 times the RPA is not reasonably achievable, the concentration of 0,5 times the RPA can be replaced by the lowest concentration between 0,5 times and 1,0 times the RPA, which is reasonably achievable.

([6]) Where, for a specific pharmacologically active substance validation of a concentration of 0,1 times the MRL is not reasonably achievable, the concentration of 0,1 times the MRL can be replaced by the lowest concentration between 0,1 times and 0,5 times the MRL, which is reasonably achievable.

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For methods for authorised substances validated before the date of entry into force of this Regulation, a determination of the repeatability with fortified matrices in concentrations at 0,5, 1,0 and 1,5 times the MRL or ML is sufficient.

Alternatively, the calculation for repeatability can be performed according to ISO 5725-2:2019 ([7]).

2.2.1.4. Within-laboratory reproducibility

1. For validations carried out after the date of entry into force of this Regulation, prepare a set of samples of specified test material (identical or different matrices), fortified with the analyte(s) to yield concentrations equivalent to:

   (a) 0,5([5]), 1,0 and 1,5 times the RPA, or

   (b) 0,1([6]), 1,0 and 1,5 times the MRL or ML for authorised substances, or

   (c) 1,0, 2,0 and 3,0 times the LCL for unauthorised or prohibited substances in case no RPA is applicable.

2. Perform the analysis at each concentration level with at least six replicates of blank material.

3. Analyse the samples.

4. Calculate the concentration detected in each sample.

5. Repeat these steps on at least two other occasions with different batches of blank material, different operators and as many different environmental conditions as possible, e.g. different batches of reagents, solvents, different room temperatures, different instruments or a variation of other parameters.

6. Determine the mean concentration, standard deviation and the coefficient of variation (%) of the fortified samples.

   For methods for authorised substances validated before the date of entry into force of this Regulation, a determination of the within-laboratory reproducibility with fortified matrices in concentrations at 0,5, 1,0 and 1,5 times the MRL or ML is sufficient.

   Alternatively, the calculation for within-laboratory reproducibility/intermediate precision can also be performed according to ISO 5725-2:2019, ISO 11843-1:1997 ([8]), Codex CAC/GL 59-2006 ([9]).

2.2.2. *Validation according to alternative models*

The calculation of the parameters in accordance with alternative models requires the performance of an experimental plan. The experimental plan shall be designed depending on the number of different species and different factors under investigation. Hence, the first step of the entire validation procedure is to consider the sample populations that will be

([7]) ISO 5725-2:2019 Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method (Clause 3).

([8]) ISO 11843-1:1997 Capability of detection – Part 1: Terms and definitions.

([9]) Codex Alimentarius Commission, Food and Agriculture Organization of the United Nations, World Health Organization, Guidelines on estimation of uncertainty of results (CAC/GL 59-2006).

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analysed in the laboratory in the future, in order to determine the most important species and the factors, which may influence the measurement results. The factorial approach allows the assessment of the measurement uncertainty of the test results, obtained under a variety of test conditions in a given laboratory, such as different analysts, different instruments, different lots of reagents, different matrices, different elapsed assay times and different assay temperatures. Subsequently, the concentration range has to be chosen in a purpose-adapted way according to the MRL or ML for authorised substances or the RPA or LCL for prohibited or unauthorised substances.

The factorial approach aims at establishing reliable precision data and measurement data by simultaneous controlled variation of the selected factors. It allows the evaluation of the combined impact of factorial effects and random effects. The experimental design allows also the investigation of the ruggedness ([10]) of the analytical method and the determination of the in-house reproducibility standard deviation across matrices.

In the following an example for an alternative approach using an orthogonal experimental design plan is given.

Up to seven factors (noise factors) can be examined. The study is designed in such a way that precision, trueness (based on fortified samples), sensitivity, measurement uncertainty and critical concentrations can be determined simultaneously by implementation of the experimental plan.

Table 6

Example of an orthogonal experimental design plan with 7 factors (I – VII) varied at two levels (A/B) in a validation study with eight runs (factor level combination)

Factor	I	II	III	IV	V	VI	VII
Run 01	A	A	A	A	A	A	A
Run 02	A	A	B	A	B	B	B
Run 03	A	B	A	B	A	B	B
Run 04	A	B	B	B	B	A	A
Run 05	B	A	A	B	B	A	B
Run 06	B	A	B	B	A	B	A
Run 07	B	B	A	A	B	B	A
Run 08	B	B	B	A	A	A	B

The calculation of the method characteristics shall be performed as described by Jülicher et al. ([11]).

([10]) The changes in experimental conditions referred to therein can consist of the sample materials, analytes, storage conditions, environmental and/or sample preparation conditions. For all experimental conditions, which could in practice be subject to fluctuation (e.g. stability of reagents, composition of the sample, pH, temperature) any variations which could affect the analytical result shall be indicated.

([11]) Jülicher, B., Gowik, P. and Uhlig, S. (1998) Assessment of detection methods in trace analysis by means of a statistically based in-house validation concept. Analyst, 120, 173.

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2.2.3. *Other validation approaches*

Other approaches to demonstrate that the method complies with performance criteria for the performance characteristics may be used, provided that they achieve the same level and quality of information. Validation can also be performed by conducting an inter-laboratory study such as established by Codex Alimentarius, ISO or the IUPAC ([12]), or according to alternative methods such as single laboratory studies or in-house validation ([13]). When alternative validation procedures are applied, the underlying model and strategy with the respective prerequisites, assumptions and formulae shall be laid down in the validation protocol or at least references shall be given to their availability.

2.3. **Selectivity/Specificity**

The power of discrimination between the analyte and closely related substances shall be determined to the best possible extent. Interference of homologues, isomers, degradation products, endogenous substances, analogues, metabolic products of the residue of interest, of matrix compounds or of any other possibly interfering substance shall be determined and if needed the method shall be amended to avoid the identified interferences. For determining the specificity of the method, the following approach shall be used:

1. Select a range of chemically related compounds or other substances likely to be encountered with the compound of interest that may be present in the samples and verify whether they could interfere with the analysis of the target analyte(s).

2. Analyse an appropriate number of representative blank samples e.g. different lots or lots of different animal species ($n \geq 20$) and check for any interferences of signals, peaks or ion traces in the region of interest where the target analyte is expected to elute.

3. Fortify representative blank samples at a relevant concentration with substances that could possibly interfere with the identification and/or quantification of the analyte and investigate whether the added substance:

   (a) may lead to a false identification;

   (b) hinders the identification of the target analyte;

   (c) influences the quantification notably.

2.4. **Ruggedness**

The analytical method shall be tested for its continued performance under different experimental conditions, which include for example different sampling conditions and minor changes that can occur in routine testing. For testing the ruggedness of the method, the changes introduced in the experimental conditions should be minor. The importance of these changes shall be evaluated. Each performance characteristic shall be determined for all minor changes that have been shown to have a significant effect on the performance of the assay.

([12]) IUPAC (1995), Protocol for the design, conduct and interpretation of method-performance studies, Pure & Applied Chem, 67, 331.

([13]) Gowik, P., Jülicher, B. and Uhlig, S. (1998) Multi-residue method for non-steroidal anti-inflammatory drugs in plasma using high performance liquid chromatography-photo-diode-array detection. Method description and comprehensive in-house validation. J. Chromatogr., 716, 221.

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2.5. **Stability**

The stability of the calibration standard, matrix-matched standard and/or matrix-fortified standards and of analyte or matrix constituents in the sample during storage or analysis shall be determined, as instabilities might influence the test results.

Usually the analyte stability is well characterised under various storage conditions. The experiments carried out for monitoring the storage conditions of standards and samples, which are carried out as part of the normal laboratory accreditation and quality control system, can provide the required information. If stability data for analytes in the matrix are available (e.g. on the basis of information from the EURLs, published data, etc.), these data do not need to be determined by each laboratory. However, referring to available stability data of analytes in solution and in matrix is only acceptable if identical conditions are applied.

In case the required stability data are not available, the following approaches should be used.

2.5.1. *Determination of the stability of the analyte in solution*

1. Prepare fresh stock solutions of the analyte(s) and dilute as specified in the test instructions to yield sufficient aliquots (e.g. 40) of each selected concentration. Samples shall be prepared of:

   (a) Solutions of the analyte, which are used for fortification;

   (b) Analyte solutions, used for the final analysis;

   (c) Any other solution that is of interest (e.g. derivatised standards).

2. Measure the analyte content in the freshly prepared solution according to the test instructions.

3. Dispense appropriate volumes into suitable containers, label and store according to the light and temperature conditions of the scheme included in Table 7. The storage time shall be chosen taking into account the applied analytical practice, ideally until the first degradation phenomena are observable during identification and/or quantification. If no degradation is observed during the stability study, the storage duration of the stability study shall be equal to the duration of the maximum storage period of the solution.

4. Calculate the concentration of the analyte(s) in each aliquot compared to the concentration of the analyte in the freshly prepared solution, following the formula below:

$$\text{Analyte Remaining (\%)} = C_i \times 100/C_{fresh}$$

C_i = concentration at time point i

C_{fresh} = concentration of fresh solution

The mean value of five replicate solutions, which were stored, shall not differ by more than 15 % from the mean value of five freshly prepared replicate solutions. The mean value of the five freshly prepared solutions shall be used as the basis for calculating the percentage difference.

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Table 7

Scheme for determination of analyte stability in solution

	–20 °C	+4 °C	+20 °C
Dark	10 aliquots	10 aliquots	10 aliquots
Light			10 aliquots

2.5.2. *Determination of the stability of analyte(s) in matrix*

1. Use where possible incurred samples. When no incurred matrix is available, a blank matrix fortified with the analyte shall be used.

2. When incurred matrix is available, determine the concentration in the matrix, while the matrix is still fresh. Store further aliquots of the homogenised incurred matrix at minus 20 °C or lower if required, and determine the concentrations of the analyte as long as the sample is retained in the laboratory.

3. If no incurred matrix is available, take some blank matrix and homogenise it. Divide the matrix into five aliquots. Fortify each aliquot with the analyte, which should preferably be prepared in a small quantity of aqueous solution. Analyse one aliquot immediately. Store the remaining aliquots at least minus 20 °C or lower if required and analyse them after short term, mid-long term and long term storage taken into account the applied analytical methods.

4. Record the maximum acceptable storage time and the optimum storage conditions.

The mean value of five replicate solutions, which were stored, shall not differ by more than the within-lab reproducibility of the method from the mean value of five freshly prepared replicate solutions. The mean value of the five freshly prepared solutions shall be used as the basis for calculating the percentage difference.

2.6. **Decision limit for confirmation (CCα)**

The CCα shall be determined for confirmatory methods. The CCα shall be established under conditions complying with the requirements for identification or identification plus quantification as defined under 'Performance criteria and other requirements for analytical methods' as laid down in Chapter 1.

For the control of the compliance of samples, the combined standard measurement uncertainty has already been taken into account in the CCα value (decision limit for confirmation).

1. For unauthorised or prohibited pharmacologically active substances, the CCα shall be calculated as follows:

   (a) Method 1: by the calibration curve procedure according to ISO 11843-1:1997 ([14]) (here referred to as critical value of the net state variable). In this case, blank material shall be used, which is fortified at and above the RPA or LCL in equidistant steps. Analyse the samples. After identification, plot the signal where possible, or the recalculated concentration against the added

([14]) ISO 11843-1:1997 Capability of detection – Part 1: Terms and definitions.

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concentration. The corresponding concentration at the y-intercept plus 2,33 times the standard deviation of the within-laboratory reproducibility at the intercept equals the decision limit. This method is applicable to quantitative assays only. Decision limits obtained with this approach shall be verified by analysing blank matrix fortified at the calculated decision limit.

(b) Method 2: by analysing at least 20 representative blank materials per matrix to be able to calculate the signal to noise ratio at the time window in which the analyte is expected. Three times the signal-to-noise ratio can be used as the decision limit. This is applicable to quantitative and qualitative assays. Decision limits obtained with this approach shall be verified by analysing blank matrix fortified at the calculated decision limit.

(c) Method 3: CCα = LCL + k(one-sided, 99 %) × (combined) standard measurement uncertainty at LCL

For unauthorised or prohibited pharmacologically active substances, depending on the validation experiment (and its respective degrees of freedom) the t-distribution might be reasonably applied, or – if the Gaussian distribution (one-sided, n=∞) is taken as a basis – a k-factor of 2,33 shall be used.

The within-laboratory reproducibility and the trueness are suitable to define the (combined) standard measurement uncertainty, if determined by taking into account all relevant influencing factors.

Method 2 for the calculation of CCα can only be used until 1 January 2026 in case of methods validated before the date of entry into force of this Regulation. For the methods validated after the entry into force of this Regulation, only Methods 1 or 3 shall be used.

2. For authorised substances, the CCα shall be calculated as follows:

(a) For authorised substances in matrix/species combinations for which an MRL or ML has been set:

(i) Method 1: by the calibration curve procedure according to ISO 11843-1:1997 (here referred to as critical value of the net state variable). In this case, blank material shall be used, which is fortified at and above the MRL or ML in equidistant steps. Analyse the samples. After identification, plot the signal, where possible, or the recalculated concentration, against the added concentration. The corresponding concentration at the MRL or ML plus 1,64 times the standard deviation of the within-laboratory reproducibility at the permitted limit equals the decision limit (α = 5 %).

(ii) Method 2: CCα = MRL (or ML) + k(one-sided, 95 %) × (combined) standard measurement uncertainty at the MRL or ML.

For authorised substances, depending on the validation experiment (and its respective degrees of freedom) the t-distribution might be reasonably applied, or – if the Gaussian distribution (one-sided, n=∞) is taken as a basis, a k-factor of 1,64 shall be used.

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The within-laboratory reproducibility and the trueness are suitable to define the (combined) standard measurement uncertainty, if determined by taking into account all relevant influencing factors.

For pharmacologically active substances for which the MRL is established for the sum of different substances, the CCα of the substance with the highest concentration in the sample shall be used as the CCα to assess the sum of substances in the measured sample.

(b) For authorised substances in matrix/species combinations for which no MRL has been set, no residues shall be present unless an authorised treatment in accordance with Article 11 of Directive 2001/82/EC took place. For authorised substances, for which no MRL has been set, the cascade MRL, established under Commission Implementing Regulation (EU) 2018/470 ([15]), shall be used for the calculation of the CCα. Method 1 or 2 of the paragraph above shall be applied but 'MRL' refers to the '0,5 times cascade MRL, with the target 0,1 times cascade MRL, where reasonably feasible'.

2.7. **Detection capability for screening (CCβ)**

The CCβ shall be determined for screening methods. The CCβ shall be established as defined under 'Performance criteria and other requirements for analytical methods' as laid down in Chapter 1 of this Annex and according to the requirements laid down in Table 5. However, the full requirements for identification (cf. 1.2.3, 1.2.4, 1.2.5) do not need to be applied for screening methods.

1. For unauthorised or prohibited pharmacologically active substances, a maximum β error of 5 % shall be ensured. The CCβ shall be calculated as follows:

(a) Method 1: The calibration curve procedure according to ISO 11843-1:1997 (here referred to as minimum detectable value of the net state variable). In this case, representative blank material shall be used, which is fortified at and below the RPA, or if no RPA has been established, around the STC in equidistant steps. Analyse the samples. Plot the signal against the added concentration. The corresponding concentration at the STC plus 1,64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the STC equals the detection capability. Extrapolation far below the lowest fortification level (< 50 % of lowest fortification level) shall be confirmed by experimental data at the validation step.

(b) Method 2: Investigation of fortified blank material at concentration levels at and above the STC. For each concentration level 20 fortified blanks shall be analysed in order to ensure a reliable basis for this determination. The concentration level, where only ≤ 5 % false compliant results remain, equals the detection capability of the method.

(c) Method 3: CCβ = STC + k(one-sided, 95 %) × (combined) standard measurement uncertainty at or above the STC.

For unauthorised or prohibited pharmacologically active substances, depending on the validation experiment (and its respective degrees of freedom) the t-distribution might be reasonably applied, or if the Gaussian distribution (one-sided, n=∞) is taken as a basis, a k-factor of 1,64 shall be used.

([15]) Commission Implementing Regulation (EU) 2018/470 of 21 March 2018 on detailed rules on the maximum residue limit to be considered for control purposes for foodstuffs derived from animals which have been treated in the EU under Article 11 of Directive 2001/82/EC (OJ L 79, 22.3.2018, p. 16).

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The within-laboratory reproducibility and the trueness are suitable to define the (combined) standard measurement uncertainty, if determined by taking into account all relevant influencing factors.

2. For authorised substances, a maximum β error of 5 % shall be ensured. The CCβ shall be calculated as follows:

(a) Method 1: by the calibration curve procedure according to ISO 11843-1:1997 (here referred to as a minimum detectable value of the net state variable). In this case, representative blank material shall be used, which is fortified at and below the permitted limit, starting from the STC in equidistant steps. Analyse the samples and identify the analyte(s). Calculate the standard deviation of the mean measured content at the STC.

The corresponding concentration at the STC plus 1,64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the STC equals the detection capability,

(b) Method 2: by investigation of fortified blank material at concentration levels below the permitted limit. For each concentration level 20 fortified blanks shall be analysed in order to ensure a reliable basis for this determination. The concentration level, where only $\leq$ 5 % false compliant results remain, equals the detection capability of the method.

(c) Method 3: CCβ = STC + k(one-sided, 95 %) × (combined) standard measurement uncertainty at or above STC.

For authorised substances, depending on the validation experiment (and its respective degrees of freedom) the t-distribution might be reasonably applied, or if the Gaussian distribution (one-sided, n=∞) is taken as a basis, a k-factor of 1,64 shall be used (whatever under cascade use or under regular MRL use).

The within-laboratory reproducibility and the trueness are suitable to define the (combined) standard measurement uncertainty, if determined by taking into account all relevant influencing factors.

For pharmacologically active substances for which the MRL is established for the sum of different substances, the CCβ of the substance with the highest concentration in the sample shall be used as the CCβ to assess the sum of substances in the measured sample.

2.8. **Calibration curves**

When calibration curves are used for quantification:

(1) at least five preferably equidistant levels (including zero level) should be used in the construction of the curve;

(2) the working range of the curve shall be described;

(3) the mathematical formula of the curve and the goodness-of-fit of the data (coefficient of determination R^2) to the curve shall be described;

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(4) acceptability ranges for the parameters of the curve shall be described.

For calibration curves based on a standard solution, matrix-matched standards or matrix-fortified standards acceptable ranges shall be indicated for the parameters of the calibration curve, which may vary from series to series.

2.9. **Absolute recovery**

The absolute recovery of the method shall be determined when no internal standard or no matrix-fortified calibration is used.

When requirements for trueness, as set out in Table 1, are fulfilled, a fixed correction factor may be used. Otherwise, the recovery factor obtained for that specific batch shall be used. Alternatively, the standard addition ([16]) procedure or an internal standard shall be used instead of using a recovery correction factor.

The absolute recovery shall be calculated for at least six representative lots of matrix.

An aliquot of blank matrix shall be fortified with the analyte before extraction, and a second aliquot of blank matrix shall be fortified after sample preparation at a relevant concentration level and the concentration of the analyte shall be determined.

The recovery shall be calculated as:

Rec (analyte) = (*area* matrix-fortified standard)/(*area* matrix-matched standard) × 100

2.10. **Relative matrix effects**

The relative matrix effect shall be determined in all cases. This can be done either as part of the validation or in separate experiments. The calculation of the relative matrix effect shall be done for at least 20 different blanks lots (matrix/species), according to the scope of the method e.g. different species to be covered.

The blank matrix should be fortified after extraction with the analyte at the RPA, MRL or ML and should be analysed together with a pure solution of the analyte.

The relative matrix effect or matrix factor (MF) is calculated as:

$$\text{MF (standard)} = \frac{\text{peak area of MMS standard}}{\text{peak area of solution standard}}$$

$$\text{MF (IS)} = \frac{\text{peak area of MMS IS}}{\text{peak area of solution IS}}$$

$$\text{MF (standard normalised for IS)} = \frac{\text{MF (standard)}}{\text{MF (IS)}}$$

IS: internal standard

MMS: matrix-matched standard

The coefficient of variation shall not be greater than 20 % for the MF (standard normalised for IS).

([16]) The amount of the standard analyte added, can be, for example, between two and five times the estimated amount of the analyte in the sample. This procedure is designed to determine the content of an analyte in a sample, taking account of the recovery of the analytical procedure.

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CHAPTER 3

QUALITY CONTROL DURING ROUTINE ANALYSIS – ONGOING METHOD PERFORMANCE VERIFICATION

The requirements for assuring the quality of analytical results of Chapter 7.7 of ISO/IEC 17025:2017 ([17]) shall be complied with.

During routine analysis, the analysis of certified reference materials (CRMs) is the preferable option to provide evidence of method performance. Since CRMs that contain the relevant analytes at the required concentration levels are seldom available, also reference materials provided and characterised by the EURLs or laboratories that hold an ISO/IEC 17043:2010 ([18]) accreditation may be used as an alternative. As another alternative in-house reference materials, which are controlled regularly, may be used.

The ongoing method performance verification during routine analysis should be carried out at the screening step and the confirmatory step.

1. For the screening step:

   For each series (batch) of analyses performed, a set of the following quality control samples shall be simultaneously analysed:

   (a) control sample for system suitability of the instrument, ideally method specific;

   (b) quality control samples which are fortified at a concentration close to the STC and ideally at the CCβ of screening for authorised pharmacologically active substances as well as for the prohibited or unauthorised substances);

   (c) compliant control sample (blank samples), and when relevant, reagent blanks.

2. For the confirmatory step:

   For each series (batch) of analyses performed, a set of the following quality control samples shall be simultaneously analysed:

   (a) control sample for system suitability of the instrument, ideally method specific;

   (b) quality control samples which are fortified at a concentration close to the MRL or ML for authorised pharmacologically active substances or close to the RPA or LCL for prohibited or unauthorised substances (non-compliant control samples);

   (c) compliant control sample (blank samples), and when relevant, reagent blanks.

The following order is recommended for the quality control samples: control sample for system suitability of the instrument, compliant control sample, sample(s) to be confirmed, compliant control sample again and fortified quality control sample (non-compliant control samples).

For quantitative methods with each batch of official samples, a calibration curve shall be analysed and measured before or after the above listed samples.

Where practicable, trueness (on basis of fortified samples) of all target analytes in the non-compliant control samples shall be evaluated, by means of quality control charts in accordance with Chapter 7.7 of ISO/IEC 17025:2017. If this requires a disproportionately large number of trueness determinations, the number of analytes may be reduced to a number of representative analytes.

([17]) ISO/IEC 17025: 2017 General requirements for the competence of testing and calibration laboratories (Chapter 7.7).

([18]) ISO/IEC 17043:2010 Conformity assessment – General requirements for proficiency testing.

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CHAPTER 4

EXTENSION OF THE VALIDATED SCOPE OF A PREVIOUSLY VALIDATED METHOD

Sometimes it is necessary to extend the scope of a previously comprehensively validated method. In these cases an extension of the scope should be accomplished in an efficient and analytically sound way. This can be achieved by carrying out a validation on a reduced number of samples (e.g. the half number of samples) compared to a full validation.

Nevertheless, the type and number of modifications to be validated in a single reduced validation scheme shall always be based on expert knowledge and previous experiences, e.g. a change in detection technique would require a complete validation in any case.

In general, to assure the ongoing validity of the method, its performance shall be monitored continuously and compared to the initially obtained validation parameters. Ideally, this ongoing method performance control is designed in a way that the missing data for a complete validation can be collected over time (e. g. with a few data points from QC samples in each analytical series).

4.1. Extensions of methods as regards to the range of concentrations

Due to changes of MRLs, MLs, and RPAs it may become necessary to adjust the concentration range for which a method is validated. For such a case, the application of a reduced validation scheme is acceptable.

Calibration curves for the modified range should be prepared according to the validated procedure. Different batches fortified at different concentration levels (cf. 2.2.1, 2.2.2) should be analysed. Trueness, repeatability and within-laboratory reproducibility/intermediate precision should be within an acceptable range compared to those of the originally validated method. A recalculation of CCβ (screening methods) and CCα (confirmation methods) should be performed, where relevant.

4.2. Extensions of methods as regards to additional substances

Generally, the method extension to additional compounds is only possible for analytes, which are similar structure and characteristic-wise compared to those already included in the analytical method. For such a case, the application of a reduced validation scheme is acceptable. Likewise, no divergence from the method description is allowed.

Calibration curves for the additional substances should be prepared according to the validated procedure. Different batches of matrix materials fortified at different concentration levels (cf. 2.2.1, 2.2.2) should be analysed. Trueness, repeatability and within-laboratory reproducibility/intermediate precision should be within a comparable range to those of the other analytes of the originally validated method and in line with the requirements set in 1.2.2. A calculation of CCβ (screening methods) and CCα (confirmation methods) for the new analytes has to be done.

4.3. Extensions of methods as regards to matrices/species

The inclusion of new matrices or species in an already validated analytical method shall always be a case-by-case decision based on the knowledge and experiences gained so far with the method and preliminary experiments assessing potential matrix effects and interferences. Generally, this will only be possible for matrices that exhibit similar properties and for non-critical analytes (stability, detectability).

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Calibration curves (standard or matrix) should be prepared according to the validated procedure. Different batches of matrix material fortified at different concentration levels (cf. 2.2.1, 2.2.2) should be analysed. Trueness, repeatability and within-laboratory reproducibility/intermediate precision should be within an acceptable range to those of the originally validated method and in line with the requirements set in 1.2.2. Depending on the validation approach, a recalculation of CCβ (screening methods) or CCα (confirmation methods) might be necessary.

If the results are not within an acceptable range compared to the values for the original matrix, an additional full validation will be necessary, in order to determine the matrix/species specific performance parameters.

In cases where MRLs for a specific substance differ for certain matrices, it will most likely be difficult to adapt the method scope to the additional matrix/species and concentration, since in this case two modifications have to be considered. In such cases a full validation is recommended.

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ANNEX II

SAMPLING PROCEDURES AND OFFICIAL SAMPLE TREATMENT

1. **Sample quantity**

The minimum sample quantities shall be defined in the national residue control programme. The minimum sample quantities shall be sufficient to enable the approved laboratories to carry out the analytical procedures necessary to complete the screening and the confirmatory analyses. Specifically for poultry, aquaculture, rabbits, farmed game, reptiles and insects a sample consists out of one or more animals, depending the requirements of the analytical methods. For eggs, the sample size is at least 12 eggs or more, according to the analytical methods used. In case several substance categories need to be analysed in one sample with different analytical methods, the sample size shall be increased accordingly.

2. **Division into sub-samples**

Unless technically impossible or not required by national legislation, each sample shall be divided into at least two equivalent sub-samples each allowing the complete analytical procedure. The subdivision can take place at the sampling location or in the laboratory.

3. **Traceability**

Each sample shall be taken in such way that it is always possible to trace it back to the farm of origin and the batch of animals or the individual animal, where relevant. In particular, for milk, according to the choice of the Member State, the samples can be taken, in either of the following places:

1. at the farm from the collection tank;

2. at the level of the dairy industry, before the milk has been discharged.

4. **Sample containers**

Samples shall be collected in suitable containers to maintain sample integrity and traceability. In particular, containers shall prevent substitution, cross-contamination and degradation. The containers shall be officially sealed.

5. **Sampling report**

A report shall be produced after each sampling procedure.

The inspector collects at least the following data in the sampling report:

1. address of the competent authorities;

2. name of the inspector or identification code;

3. official code number of the sample;

4. sampling date;

5. name and address of the owner or the person having charge of the animals or the animal products;

6. name and address of the animal's farm of origin (when sampling on farm);

7. registration number of the establishment-slaughterhouse number;

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8. animal or product identification;

9. animal species;

10. sample matrix;

11. where relevant, medication within the last four weeks before sampling (when sampling on farm);

12. substance or substance groups for examination;

13. particular remarks.

Paper or electronic copies of the report are to be provided depending on the sampling procedure. The sampling report and its copies shall be completed in a way that ensures their authenticity and legal validity, which may require that these documents are signed by the inspector. In case of on-farm sampling, the farmer or his deputy may be invited to sign the original sampling report.

The original of the sampling report remains at the competent authority, which has to guarantee that unauthorised persons cannot access this original report.

If necessary, the farmer or the owner of the establishment may be informed of the sampling undertaken.

6. **Sampling report for the laboratory**

The sampling report for the laboratory established by the competent authorities shall be in accordance with the requirements set in Chapter 7 of ISO/IEC 17025:2017 ([1]) and shall contain at least the following information:

1. address of the competent authorities or designated bodies;

2. name of inspector or identification code;

3. official code number of the sample;

4. sampling date;

5. animal species;

6. sample matrix;

7. substances or substance groups for examination;

8. particular remarks.

The sampling report for the laboratory shall accompany the sample when sent to the laboratory.

7. **Transport and storage**

Residue control programmes shall specify the suitable storage and transport conditions for each analyte/matrix combination to ensure analyte stability and sample integrity. The transport time shall be as short as possible and the temperature during transport shall be adequate to ensure analyte stability.

Specific attention shall be paid to transport boxes, temperature and delivery times to the responsible laboratory.

In case of any non-compliance with the requirements of the control programme, the laboratory shall inform the competent authority without delay.

([1]) ISO/IEC 17025: 2017 General requirements for the competence of testing and calibration laboratories (Chapter 7.7).