EUROPEAN
COMMISSION

Brussels, XXX
[…](2019) XXX draft

ANNEX

ANNEXES

to the

COMMISSION IMPLEMENTING REGULATION (EU) No… /.. on the performance of analytical methods for pharmacologically active substances, the interpretation of results and the methods to be used for sampling

[mandatory element]

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

Biological, biochemical or physico-chemical methods shall be used as screening methods. Qualitative, semi-quantitative or quantitative methods should be used as suitable screening methods.

1.1.2 Requirements of screening methods

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

For authorised pharmacologically active substances the CCβ shall be less 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, this result shall be confirmed by a confirmatory method.

Quantitative screening methods, used for both screening and confirmation shall meet the same requirements for accuracy, dynamic range, and precision as confirmatory methods.

1.2 Requirements of confirmatory methods

1.2.1 General requirements of confirmatory methods

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

For authorised pharmacologically active 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, 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 veterinary substances, other methods like e.g. HPLC-DAD and –FLD can be used or a combination of them.

When required according to the 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. 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 should be used.

1.2.2 General performance criteria for confirmatory methods

1.2.2.1 Trueness

For repeated analyses of a certified reference material, the deviation of the experimentally determined absolute recovery [EURLs please check entire document for correct use of 'recovery' and absolute 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

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 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 unacceptable high values. Therefore, the allowed maximum coefficient of variation shall not be greater than the values presented in Table 2.

Table 2. Acceptable coefficient of variation

Table 2 Acceptable coefficient of variation	
Mass fraction	Reproducibility CV (%)

> 1000 µg/kg	16 (according to Horwitz equation)
>120 µg/kg – 1000 µg/kg	22 (according to 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 Specific performance criteria for mass spectrometric detection

1.2.3.1 Chromatographic separation

For liquid or gas chromatography, 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 or a deviation of less than 5% of the retention time, in case the retention time is below 1 minute. In case an internal standard is used, the ratio of the chromatographic retention time of the analyte to that of the internal standard, i.e. 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 application of this Regulation onwards.

1.2.3.2. Mass spectrometric detection

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

- recording full scan (FS) mass spectra

- selected ion monitoring (SIM)

- sequential mass spectrometry (MS^n) techniques such as Selected Reaction Monitoring (SRM)

- 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.

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 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 should 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 diagnostic product or fragment ions should be diagnostic fragment ions/transitions. Non-selective transitions (e.g. the tropylium cation or loss of water) should 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 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 ±30 % 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).

1.2.3.3 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 non-authorized or prohibited substances, 5 identification points are required. One point can originate from the chromatographic separation. The table below 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.

(a) All mass spectrometric analyses are 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, electrophoresis, ion mobility spectrometry and super critical fluid chromatography.

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

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

Table 3. Identification points per technique

Table 3 Identification points per technique	
Technique	Identification Points

Separation (mode GC, LC, SFC, CE) and ion mobility)	1
LR-MS ion	1
Precursor ion selection at $<\pm0.5$ 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)

Table 4			
Examples of the number of identification points obtained for 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	$1 + 1 + 2\times1.5 = 5$
GC- or LC-MS/MS	GC or LC	2 precursor + 2 products	$1 + 2 + 2\times1.5 = 6$
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	$1 + n\times1.5$

GC- or LC-HRMS/MS	GC or LC	1 precursor ($<\pm0.5$ Da mass range) + 1 product	$1 + 1 + 2.5 = 4.5$
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.

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

For authorised pharmacologically active 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:

- full-scan diode array spectrophotometry (DAD) in case used with HPLC,

- fluorescence detection 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.

1.2.4.1. Performance criteria for Full-scan diode array spectrophotometry

The performance criteria for chromatographic separation included in chapter 1.2.3.1 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.

1.2.4.2. Performance criteria for fluorometric detection

The performance criteria for chromatographic separation included in chapter 1.2.3.1 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 occurrence of interfering components in blank sample extracts. There should be a minimum of 50 nanometres between the excitation and emission wavelength.

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	Substances	Identification in accordance with 1.2.2 and/or 1.2.3 and/or 1.2.4	CCα	CCβ	Trueness	Precision	Relative matrix effect/absolute recovery**	Selectivity	Stability[##]/ruggedness
			Table 5 Classification of analytical methods by the performance characteristics that have to be determined							
Confirmation	Qualitative	A	x	x	-					
	Quantitative	A, B	x	x		x	x	x	x	x
Screening	Qualitative	A, B			x				x	x
	Semi-quantitative	A, B			x		(x)		x	x
	Quantitative	A, B			x	x	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 as defined under SANTE 2017/11987

B: authorised substances as defined under SANTE 2017/11987

If stability data for analytes in 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.

2.2 Trueness, repeatability and within-laboratory reproducibility

This chapter provides examples and/or 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 under ruggedness below). 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 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[1].

An example is given below:

- analyse six replicates of the CRM in accordance with the test instructions for the method,

- determine the concentration of the analyte present in each sample of the replicates,

- calculate the mean, the standard deviation and the coefficient of variation (%) *for these six replicates*,

- 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.

---

[1] ISO 5725:1994 Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions; ISO 5725-2 Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method; Part 4: Basic methods for the determination of the trueness of a standard measurement method.

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:

- For methods validated from the date of application of this Regulation, select blank material and fortify at a concentration of

- 0.5, 1.0 and 1.5 times the RPA or,

- 0.1^2, 1.0 and 1.5 times the MRL or ML for authorised substances or,

- 1, 1.5 and 2 times the LCL for unauthorised substances (that do not have the RPA).

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

- Analyse the samples.

- Calculate the concentration detected in each sample.

- 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.

However, 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

For methods validated from the date of application of this Regulation, prepare a set of samples of identical blank matrices of the same species, fortified with the analyte to yield concentrations equivalent to

- 0.5, 1.0 and 1.5 times the RPA or,

- 0.1^2, 1.0 and 1.5 times the MRL or ML for authorised substances or,

- 1, 1.5 and 2 times the LCL for unauthorised or prohibited substances in case no RPA is applicable.

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

- Analyse the samples.

- Calculate the concentration detected in each sample.

---

[2] In case 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 a the lowest concentration between 0.1 times and 0.5 times the MRL, which is reasonable achievable.

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

- Repeat these steps on at least two other occasions, using different batches of blank matrices.

- Calculate the overall mean concentrations, standard deviation (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.

Alternatively, the calculation for repeatability can be performed according to ISO5725-2-3.

For methods for authorised substances validated before the date of application 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.

2.2.1.4 Within-laboratory reproducibility

For validations carried out after the date of application 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

- 0.5, 1.0 and 1.5 times the RPA

- 0.1^2, 1.0 and 1.5 times the MRL or ML for authorised substances

- 1, 1.5 and 2 times the LCL for unauthorised or prohibited substances in case no RPA is applicable.

- Perform the analysis at each concentration level with at least six different batches of blank material.

- Analyse the samples.

- Calculate the concentration detected in each sample.

- 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.

- 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 application 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 within-laboratory reproducibility can also be performed according to ISO5725-2-3[1], ISO 11843[3], Codex CAC/GL 59-2006[4], ISO WD TS 23471[5].

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 has to 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

---

[3] ISO 11843 – Capability of detection
[4] Guidelines on estimation of uncertainty of results (CAC/GL 59-2006)
[5] ISO WD TS 23471 – Experimental designs for evaluation of uncertainty – Use of factorial designs for determining uncertainty functions

sample populations that will be 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 non-authorised 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 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 eight factors (noise factors) can be examined. The study is designed in such a way that precision, trueness (based on spiked 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).

Table 6							
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 calculated as described by Jülicher et al.[6]

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

---

[6] 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

also be performed by conducting an inter laboratory study such as established by Codex Alimentarius, ISO or the IUPAC[7], or according to alternative methods such as single laboratory studies or in-house validation[8,9]. 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

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, depending on the recent analytical technique:

- 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 analytes.

- analyse an appropriate number of representative blank samples e.g. different lots or lots of different animal species (n >= 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;

- 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:

> - may lead to a false identification
>
> - hinders the identification of the target analyte
>
> - 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

---

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

[8] 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.

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

importance of these changes shall be evaluated, for instance, using the Youden approach[10,11]. 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. The ruggedness of the analytical method shall be determined using the following approach:

- Select factors of the sample pre-treatment, clean up and analysis, which may influence the measurement results. Such factors may include the analyst, the source and the age of reagents, solvents, standards and sample extracts, the rate of heating, the temperature, the pH-value as well as many other factors that may occur in the laboratory. These factors shall be modified in an order of magnitude that matches the deviations usually encountered among laboratories.

- Conduct a ruggedness test using the fractional factorial design approach of Youden[10] or an alternative method. Interactions between the different factors cannot be detected.

- Where a factor is found to influence the measurement results significantly, conduct further experiments to decide on the acceptability limits of this factor.

- Factors that significantly influence the results shall be identified clearly in the method protocol.

The Youden approach introduces several variations at once, instead of studying one alteration at a time. In the example below A, B, C, D, E, F, G denote the nominal values for seven different factors that could influence the results, if their nominal values are changed slightly.

Alternative values for the seven factors are denoted by the corresponding lower case letters a, b, c, d, e, f and g. This results in 27 to 128 different possible combinations.

It is possible to choose a subset of eight of these combinations that have a balance between capital and small letters (Table 7). Eight determinations have to be made, which will use a combination of the nominal or alternative values for the chosen factors A-G. An example of possible results of the determinations is shown in the bottom row of Table 7 values S-Z. These results are used for calculating the ruggedness of the method according to the calculations described by Youden[10].

Table 7: Experiment design for ruggedness studies (minor changes)

Table 7: Experiment design for ruggedness studies (minor changes)								
Factor value F	Combination of determinations number							
	1	2	3	4	5	6	7	8
A/a	A	A	A	A	a	a	a	a
B/b	B	B	b	b	B	B	b	b

---

[10] AOAC-I Peer Verified Methods, Policies and Procedures, 1993, AOAC International, 2200 Wilson Blvd., Suite 400, Arlington, Virginia 22201-3301, USA.
[11] W.J. Youden; Steiner, E.H.; 'Statistical Manual of the AOAC–Association of Official Analytical Chemists', AOAC-I, Washington DC: 1975, p. 35 ff.

C/c	C	c	C	c	C	c	C	c
D/d	D	D	d	d	d	d	D	D
E/e	E	e	E	e	e	E	e	E
F/f	F	f	f	F	F	f	f	F
G/g	G	g	g	G	g	G	G	g
Observed result R	S	T	U	V	W	X	Y	Z

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 outcome of the result of analysis. The mean value of five replicate solutions, which were stored, shall not differ by more than 10% from the mean value of 5 new replicate solutions. The mean value of the 5 new solutions shall be used as the basis for calculating the percentage difference.

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 available:

> - Calculate the concentration of the analyte(s) in each aliquot compared to the concentration of the analyte in the fresh solution, according to the formula included below for the determination of the stability of the analyte in solution.

In case the required stability data are not available, the following approaches shall be used:

Determination of the stability of the analyte in solution:

> - 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
>
> > - solutions of the analyte, which are used for fortification,
> >
> > - analyte solutions, used for the final analysis,
> >
> > - any other solution that is of interest (e.g. derivatised standards).

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

> - Dispense appropriate volumes into suitable containers, label and store according to the light and temperature conditions of the scheme included in Table 8. The storage time shall be two, three and four

14

weeks or longer, 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.

- Calculate the concentration of the analyte(s) in each aliquot compared to the concentration of the analyte in the fresh solution, following the formula below:

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

C_i = concentration at time point i

C_{fresh} = concentration of fresh solution

Determination of the stability of analyte(s) in matrix

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

- When incurred material is available, determine the concentration in the material, while the material is still fresh. Stored further aliquots of the homogenized incurred material 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.

- If no incurred material is available, take some blank material and homogenise it. Divide the material 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.

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

Table 8. Scheme for determination of analyte stability in solution

Table 8. Scheme for determination of analyte stability in solution			
	-20°C	+4°C	+20°C
Dark	10 aliquots	10 aliquots	10 aliquots
Light			10 aliquots

2.6 Decision limit (CCα for confirmation)

The CCα shall be determined for confirmatory methods. It 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 of this Annex.

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).

Therefore all concentrations at and above the CCα shall be considered non-compliant.

For <u>unauthorised or prohibited pharmacologically active substances,</u> a maximum α-error of 1% shall be ensured. The CCα shall be calculated as follows:

- Method 1: by the calibration curve procedure according to ISO 11843[12,13](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 concentration. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the decision limit. This is applicable to quantitative assays only, or,

- 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 should be verified by analyzing blank matrix fortified at the calculated decision limit.

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

For prohibited or unauthorised 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 is a good estimator for the measurement uncertainty, if determined by taking into account all relevant influencing factors.

For <u>authorised</u> pharmacologically active substances, a maximum α-error of 5% shall be ensured. The CCα shall be calculated as follows:

For authorised pharmacologically active substances for matrices or species for with an MRL has been set:

- Method 1: by the calibration curve procedure according to ISO 11843[3] (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. Analyse the samples. After identification, plot the signal 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 %), or

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

[12] ISO 11843:1997 Capability of detection — Part 1: Terms and definitions, Part 2: Methodology in the linear calibration case.

[13] E. Verdon, D. Hurtaud-Pessel, P. Sanders (2006), Evaluation of the limit of performance of an analytical method based on a statistical calculation of its critical concentrations according to ISO standard 11843: Application to routine control of banned veterinary drug residues in food according to European Decision 657/2002/EC; Accred Qual Assur, 11:58-62, DOI: 10.1007/s00769-005-0055-y & (2006) DOI: 10.1007/s00769-006-0218-5

For authorised 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.

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.

For authorised pharmacologically active substances for matrices or species for which no MRL has been set:

Method 1 or 2 of the paragraph above shall be applied but 'MRL or ML' shall be replaced by '1/10th of the cascade MRL, established under Regulation (EU) 2018/470 for the concerned substance, where analytically feasible'.

2.7 Detection capability (CCβ for screening)

The CCβ shall be determined for screening methods. The CCβ shall to 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) do not need to be applied for screening methods.

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

- Method 1: The calibration curve procedure according to ISO 11843[2] (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 below the lowest spike level shall be confirmed by experimental data at the validation step, or,

- 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.

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

For <u>authorised pharmacologically active substances,</u> a maximum β-error of 5% shall be ensured. The CCβ shall be calculated as follows:

- Method 1: by the calibration curve procedure according to ISO 11843[2] (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 value of the STC plus 1,64 times the standard deviation of the within-laboratory reproducibility equals the detection capability,

- Method 2: by investigation of fortified blank material at concentration levels at and 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 ≤ 5 % false compliant results remain, equals the detection capability of the method.

- Method 3: CCβ = STC + k(one sided, 95%) × measurement uncertainty at the MRL or ML

For authorised 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.

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.

The within-laboratory reproducibility is a good estimator for the measurement uncertainty, if determined by taking into account all relevant influencing factors.

2.8 Calibration curves

When calibration curves are used for quantification:

- equidistant of at least five levels (including zero) shall be used in the construction of the curve,

- the working range of the curve shall be described,

- the mathematical formula of the curve and the goodness-of-fit of the data to the curve shall be described,

- 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 is necessary, 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 procedure (see 3.5) or an internal standard shall be used instead of using a recovery correction factor.

The recovery shall be calculated for at least 6 representative lots of matrix.

An aliquot of blank matrix shall be spiked with the analyte before extraction and a second aliquot of blank matrix shall be spiked after sample preparation at a relevant concentration level and the concentration of the analyte shall be analysed:

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 should be determined when no internal standard or no matrix-fortified calibration is used. The calculation of the relative matrix effect should be done for at least 20 different blanks lots of matrix/species, according to the scope of the method e.g. different species to be covered.

The blank matrix should be spiked 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:

MF (standard) = (*area MMS* standard) /

  (*area solution* standard)

MF (IS) =   (*area MMS IS*) /

  (*area solution IS*)

MF (standard normalised for IS) = (*MF* (*standard*)) / (*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).

Chapter 3: quality control during routine analysis – ongoing method performance verification

The requirements for assuring the quality of analytical results of chapter 7.7 ISO 17025[14] 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 have ISO 17043 accreditation may be used as an alternative. As another alternative in-house reference materials, which are controlled regularly, may be used.

---

[14] ISO 17025: 2017 General requirement for the competence of testing and calibration laboratories.

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

- control sample for system suitability of the instrument, ideally method specific,
- quality control samples which are spiked before extraction (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),
- compliant control sample (blank samples), and,
- reagent blanks.

The following order is recommended for the quality control samples: control sample for system suitability of the instrument sample, 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 after and/or before 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 ISO 17025 chapter 7.7. If this requires a disproportionately large number of trueness determinations, the number of analytes may be reduced to a number of representative analytes.

Chapter 4: extension of the validated scope of a previously validated method via quality control samples during routing analysis

4.1 - Extensions of methods as regards to matrices/species

Additional matrices or species that are to be added to a previously comprehensively validated method can be integrated either by applying a reduced validation scheme or by integration in the on-going quality control procedure.

Simultaneous analyses of controlled samples of the original matrix with at least 5 different samples of the new matrix, with and without fortification at a relevant level (e.g. 0.5 RPA, 0.5 MRL or 0.5 ML) in duplicate on at least three different days. Repeatability, within-laboratory reproducibility and S/N ratios should be within an acceptable range from the values for the original matrix. If this condition is not fulfilled, additional validation will be necessary in order to determine the matrix specific parameters.

4.2 - Extensions of methods as regards to additional substances

Additional (structurally related) analytes that are to be added to a previously comprehensively validated method can be integrated either by applying a reduced validation scheme or by integration in the on-going quality control procedure.

Simultaneous analyses of controlled samples with the original analyte with at least 5 different batches, with and without fortification with the new substance at least at three different levels (e.g. 0.1, 1.0 and 1.5) RPA or MRL or ML in duplicate on at least three different days. Repeatability, within-laboratory reproducibility and S/N ratios should be within an acceptable range from the values for the original compound. If this condition is not fulfilled, additional validation will be necessary in order to determine the matrix specific parameters.

4.3 - Extensions of methods as regards to the range of concentrations

An extension of the stated concentration range for an analyte of a previously comprehensively validated method can be integrated either by applying a reduced validation scheme or by integration in the on-going quality control procedure.

Calibration curves for the modified range should be prepared according to the validated procedure. Repeatability and within-laboratory reproducibility should be within an acceptable range from those of the originally validated method. In case the range is extended to lower concentration, a recalculation of CCβ and, in case of non-authorized substances also CCα will be necessary.

ANNEX II

Sampling procedures and official sample treatment

1. Sample quantity

The minimum sample quantities shall be defined in the national residue control programme. It 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 should 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. In particular, for milk, according to the choice of the Member State, the samples can be taken

- either at the farm from the collection tank,

- 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:

— address of the competent authorities,

— name of the inspector or identification code,

— official code number of the sample,

— sampling date,

— name and address of the owner or the person having charge of the animals or the animal products,

— name and address of the animal's farm of origin (when sampling on farm),

— registration number of the establishment-slaughterhouse number,

— animal or product identification,

— animal species,

— sample matrix,

— medication within the last four weeks before sampling (when sampling on farm),

— substance or substance groups for examination,

— particular remarks.

Paper or electronic copies of the report are to be foreseen depending on the sampling procedure. The sampling report and its copies shall be signed at least 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.

5. Sampling report for the laboratory

The sampling report for the laboratory established by the competent authorities shall be in accordance with the requirements of ISO 17025 and shall contain at least the following information:

— address of the competent authorities or designated bodies,

— name of inspector or identification code,

— official code number of the sample,

— sampling date,

— animal species,

— sample matrix,

— substances or substance groups for examination

— particular remarks.

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

6. 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 should be as short as possible and the temperature during transport should 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.