EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate E – Safety of the Food Chain

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GUIDELINES FOR THE IMPLEMENTATION OF DECISION 2002/657/EC [2]

1. RE. CHAPTER 2: PERFORMANCE CRITERIA AND OTHER REQUIREMENTS FOR ANALYTICAL METHODS

2.1.2.1. Recovery.

It can be taken from the text (cf. 2.1.2.1 and 2.3.2.1) that analytical recovery must be applied to all results – both during the validation phase and application of the method in practice. These prescriptions do not apply to qualitative methods.

2.3.1. Common performance criteria and requirements.

As a general point, it is recommended that for each series (batch) of analyses performed, quality control samples which are spiked (fortified) at a concentration close to MRPL or MRL should be included. For banned and unauthorised compounds where no MRPL has yet been established, the CCβ should be used. The ISO 11843 provides the international recommendation to applied and efficient quality control.

2.3.3.2. Mass spectrometric detection.

Calculation of relative intensities of diagnostic ions must be made on the basis of assigning 100% to the most intense signal originating from the analyte and which, among those, is used for the identification of the analyte.

General point: When a sample has been concluded suspect after a screening process, the same attention has to be paid to the compliant/non-compliant test result generated by the confirmatory method. This means that *all* the information must be given either to prove the presence of the analyte (see Table 5-6 of the Decision) or, where presence can not be proven, the reasons why should be identified and *documented*.

Measurement uncertainty

Measurement uncertainty was not explicitly mentioned in CD 2002/657/EC. However, when determined correctly by systematically taking into account all relevant influencing factors possibly affecting the measurement results, the within-laboratory reproducibility can be regarded as a good estimator for the combined measurement uncertainty of the individual methods. Further prerequisites are the use of recovery-corrected data and the fact that the uncertainty of the recovery was taken into account the one or the other way.

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[1] These guidelines have been drafted by the Community Reference Laboratories for residue control.

[2] References to Commission Decision 2002/657/EC cited in the text relate to the English translation.

For the calculation of the extended uncertainty a certain safety factor is needed. When determining the CCα the factor of the Gaussian distribution for 99% (group A substances) or 95% (group B substances) certainty was used respectively, i. e. a factor of 2.33 in the first and of 1.64 in the latter case. Consequently these factors are also to be used for the calculation of the extended uncertainty at any concentration under validation.

For the control of compliance the measurement uncertainty is already taken into account by applying the CCα as decision limit.

2. RE. CHAPTER 3: VALIDATION

In the Decision the second paragraph of this Chapter (page 23) states that "Validation can also be performed by conducting an inter-laboratory study…." Strictly speaking this is not true as only some performance characteristics of a method can be assessed by means of an inter-laboratory collaborative study which can not *per se* be (solely) used for validation purposes. N.B. inter-laboratory collaborative studies should not be confused with proficiency tests.

3.1.1.1. Specificity.

An example of a practical tool to deal with this issue is given in Annex I.

3.1.1.4. Stability.

It is recommended that stability of analyte in matrix does not need to be calculated by each laboratory provided that they can justify the reasons why they have not done so (on the basis of published data, information from the CRLs etc).

3.1.2.1., 3.1.2.2. and 3.1.2.3. **Recovery, Repeatability and within-laboratory reproducibility**.

In addition to the experimental models described in the Decision (which deal with substances for which MRLs or MRPLs have been established), it is recommended that for those substances for which *no* MRPL has been established, the spiking concentrations should be 1, 1.5 and 2 times the CCβ. These prescriptions do not apply to qualitative methods.

3.1.2.5. and 3.1.2.6. CCα and CCβ .

For compounds for which an MRPL has been established, the value for CCβ must be less than or equal to the MRPL. (Consequently CCα must always be less than the MRPL).

For banned and unauthorised compounds where no MRPL has yet been established, CCβ should be as low as reasonably achievable (ALARA).

Protocols for the determination of CCα and CCβ are given on page 28 of the Decision. Regardless of the protocol used, it is recommended that in all cases the figures generated for both CCα and CCβ must be verified by blank samples which are fortified with the respective concentrations of CCα and CCβ. At the CCβ this must be performed with at least 20 replicates for the verification of the β-error of ≤ 5%. This requirement has to be fulfilled. Methods for screening should be able to detect the analyte at the CCβ in 95% of the cases. For confirmation, the method should also be able to identify the analyte with the same percentage at the CCβ and in 50% of all cases at the CCα. Where the

percentages obtained are significantly lower than these theoretical ones, it can be concluded that the calculated values for CCα and CCβ are too low, necessitating further investigation of these performance characteristics.

Determination of CCα for substances for which there is no permitted limit. Indent 1 page 28 states that "CCα can be established by the calibration curve procedure according to ISO 11843 (here referred to as critical value of the net state variable). In this case blank material must be used, which is fortified at and above the *minimum required performance level* in equidistant steps…"

[It should be noted that the *minimum required performance level* is not the MRPL. The *minimum required performance level* is the lowest concentration of analyte which is expected to be detected (screening methods) or its identity unequivocally confirmed (confirmatory methods). It follows that for substances for which an MRPL has been proposed, the *minimum required performance level* should be less than the MRPL].

It is therefore recommended that representative blank material must be used, which is fortified at and above the *minimum required performance level*. The lowest calibration point is that at which the method starts to detect or identify the analyte. (This does not mean that the method should detect or identify the analyte in 100 % of the cases).

It should be noted that the two approaches are only valid for a linear curve and therefore linearity has to be proven in order to use these approaches.

It is recognised that in some instances where ISO 11843 has been used the extrapolated theoretical values for CCα may be too low to be confirmed experimentally. In such cases parallel extrapolation to x axis at the lowest calibration concentration in spiked samples is recommended.

It should also be noted that utilisation of the ISO 11843 approach will only provide validation data for individual matrix/analyte combinations. The use of a multi-matrix multi-analyte approach (see section 3.1.3. of the Decision) can usefully address this limitation.

Indent 2 page 28 states that "CCα can be established…by analysing at least 20 blank samples per matrix…." When this approach is used, it should be stressed that such blank samples are representative.

3.1.2.6. Detection capability (CCβ)

Determination of CCβ for substances for which there is no permitted limit. Similar clarification to that described for the calculation of CCα above is required for determination of CCβ using the ISO 11843 approach. Again it is recommended that representative blank material must be used, which is fortified at and above the lowest concentration of analyte which is expected to be detected (screening methods) or its identity unequivocally confirmed (confirmatory methods)."

With regard to screening methods, validation procedures for which are not comprehensively described in the Decision, it is recommended that for the generation of CCβ, 20 representative blank samples should be spiked with at least 2 concentration levels spanning the level of interest which is also covered by the corresponding confirmatory method.

3. ASSESSMENT OF NON-COMPLIANT RESULTS OF SUBSTANCES FOR WHICH A "SUM MAXIMUM RESIDUE LIMIT (MRL)" IS ESTABLISHED

For some pharmacologically active substances the MRLs laid down in Community legislation are expressed in form of sum-MRLs. Examples include sulfonamides, tetracyclines, benzimidazoles, and azaperone. In these cases the question of how to validate the respective methods arose. Theoretically an infinite number of possibilities exist as the number of concentration level combinations approximates infinity. It has been agreed to validate these substances as described in chapter 3.1.

There are two different cases to be distinguished

1.) independent substances like sulfonamides,

2.) substances and their metabolites which occur in particular ratios and these ratios are not known for all species and matrix-combinations.

For the assessment the following technical procedure can be applied for both cases:

In real samples it cannot be predicted which combination of substances will be present. Consequently for the assessment of non-compliant results in case of sum-MRLs it is not possible to validate and calculate the precise decision limit (CCα) for each combination of analytes that might occur in practice. Therefore, as a pragmatic approach, it is recommended to calculate the 'sum CCα' for the confirmatory method by using the corresponding uncertainties of the within-laboratory reproducibility, determined during the validation study. In this calculation the uncertainties of the closest concentrations (measured during the validation study) to the detected concentrations (in the real sample) are used. If a concentration is found exactly in the middle between two validated concentrations, the within-lab reproducibility of the higher concentration validated should be used.

This approach adds the individual uncertainties of the detected substances by means of error propagation law and weighing the uncertainties in accordance with their detected concentration levels. A safety factor of 1.64 is used for substances with an MRL to determine CCα (see section 3.1.2.5. of the Annex to Commission Decision 2002/657/EC). If the sum of the found concentrations is higher than the sum CCα calculated according to this approach, the result is non-compliant (i.e. sum of individual substance concentrations > sum CCα).

This approach is demonstrated in the following example where residues of three different sulfonamides have been detected in the same sample.

Theoretical example

sum MRL = 100 µg/kg
Sum of concentrations found = c1 + c2 + c3 = 115 µg/kg

substance 1(sulfamethazine): c1 = 15 µg/kg; w1 = 0.13 (SD1 = 3 µg/kg; CCα for sulfamethazine = 133 µg/kg)
substance 2 (sulfadiazine): c2 = 30 µg/kg; w2 = 0.26 (SD2 = 3.6 µg/kg; CCα for sulfadiazine = 120 µg/kg)
substance 3 (sulfaquinoxaline): c3 = 70 µg/kg; w3 = 0.61 (SD3 = 5.6 µg/kg; CCα for sulfaquinoxaline = 113 µg/kg)

Square root (w1*SD1 ^{2} + w2*SD2 ^{2} + w3*SD3 ^{2}) =

Square root ($0.13*3^2 + 0.26*3.6^2 + 0.61*5{,}6^2$) = 4.86 µg/kg

Sum CCα = 100 µg/kg + 1.64*4.86 µg/kg = 108 µg/kg

In this case the sum of found concentrations exceeds the sum CCα. The result in this case is considered NON-compliant.

ci : found concentration of substance i
wi : weighing factor of substance i
SDi: standard deviation derived from the within-laboratory reproducibility of substance i, closest to the found concentration ci
Safety factor: 1.64 for substances with an MRL.
Calculation of the weighing factors:
e.g. w1 = c1/(c1 + c2 + c3) = 15/(15+30+70) = 15/115 = 0.13

Real life example:

	Found concentration [µg/kg]	Weighing factor	Standard deviation derived from the within laboratory reproducibility **[µg/kg]**
Sulfamerazine	33	0.28	14.7
Sulfadimethoxine	36	0.31	13.2
Sulfaquinoxaline	49	0.42	7.2
Sum of found concentrations	119		
Sum MRL (muscle) [µg/kg]	100		
Safety factor	1.64		
Square root of sum of squared uncertainties	11.7		
CCα at sum-MRL	118		
Result:	Sample is considered compliant		

In this case the sum of found concentrations does not exceed the calculated CCα at the sum MRL. The result is considered compliant.

3.1. Validation of substances for which a sum "Maximum Residue Limit (MRL)" is established.

In these cases the question of how to validate the respective methods arose. Theoretically an infinite number of possibilities exist as the number of concentration level combinations approximates infinity. It has been agreed that each individual substance has to be validated around the MRL concentration, i.e. at 0.5, 1 and 1.5 times the MRL. In addition it is recommended to determine, or at least to have some knowledge of, the lowest concentration level detectable with the method in question and of the accuracy (precision and recovery) at that concentration.

For substances for which MRLs are laid down for the sum of parent drugs and their metabolites, provided that the same metabolites and the same relative rates of metabolites are always produced, in addition to the assessment of positive results by adding the weighted variances, a second procedure can be applied during the validation. It is the use of a CCα which has been determined during the validation in such a way that the concentrations of the parent drug and its metabolites are summed up always before using the values for further calculation of the validation parameters. Applying this procedure the calculated CCα is already the decision limit for the sum of all relevant substances. It cannot be used for the assessment of the individual substances (parent drug or individual metabolites).

3.2. The particular case for malachite green and leuco-malachite green

Currently there is one substance for which a sum Minimum Required Performance Limit (MRPL) has been established – malachite green and its metabolite leuco-malachite green have a sum MRPL of 2 µg/kg.

In contrast to substances with an MRL, **CCα and CCβ** for substances with an **MRPL** must always be ***below*** **the MRPL**.

Provided the full identification criteria have passed for the analytes, any result in excess of CCα should be investigated. To that end, for those methods which distinguish between malachite green and its leuco-metabolite (and measure both substances separately), it is recommended that each individual CCα should always be less than ½ MRPL.

N.B.: Leuco-malachite green very often accounts for more than 80% of the total malachite green residues found in fish (for those methods which distinguish between malachite green and its leuco metabolite and measure both substances separately).

4. GUIDELINES FOR THE IMPLEMENTATION OF DECISION 2002/657/EC REGARDING SOME CONTAMINANTS (MYCOTOXINS, DIOXINS AN DIOXIN-LIKE PCBS AND HEAVY METALS

Article 1 paragraph 2 of Commission Decision 2002/657/EC states that "this Decision shall not apply to substances for which more specific rules have been laid down in other Community legislation"

At the moment, the substances concerned are the following:

(1) Mycotoxins in foodstuffs (see Commission Regulation (EC) No 401/2006[3] laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs establishes rules on sampling methods and performance criteria for the methods of analysis to be used for official control of mycotoxins in food.

(2) Dioxins and dioxin-like PCBs in foodstuffs (see Commission Regulation (EC) No 1883/2006[4] laying down methods of sampling and analysis for the official control of levels of dioxins and dioxin-like PCBs in certain foodstuffs, contains provisions on sampling, sample preparation and requirements for methods of analysis used in official controls of dioxins (PCDD/PCDF) and dioxin-like PCBs in certain foodstuffs.

(3) Lead, Cadmium, Mercury, Inorganic tin, 3-MCPD and benzopyrene in foodstuffs (see Commission Regulation (EC) No 333/2007[5] laying down the methods of sampling and analysis for the official control of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs establishes provisions for the sampling and analysis for the official controls for these substances

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[3] OJ L 70, 9.3.2006, p. 12
[4] OJ L 364, 20.12.2006, p. 32
[5] OJ L 88, 29.3.2007, p.29.

Annex I

SPECLOG - the Specificity Log

An underpinning documentation tool to transparently demonstrate the reliability of a specificity, confirmation and/or identification claim for an analytical method used in regulatory testing for residues of veterinary drugs in food and food related materials.

According to laboratory accreditation standard ISO 17025 results of a test for residues of veterinary drugs have to be reported inclusive the uncertainty in the result.

To fulfil this requirement the overwhelming majority of official protocols with instructions how to validate a method for official testing for residues of veterinary drugs in food, however, is nearly exclusively dedicated to the validation aspects of the *quantitative* part of the analyses.

The validation of the *qualitative* part, namely the proper identification or confirmation of the detected drug residue or its metabolite (the so-called analyte), is largely neglected and / or explicit validation requirements are missing.

The most important characteristic for a proper identification of the analyte in a sample of an edible tissue of animal origin is the specificity of the testing method, in other testing areas also sometimes called the selectivity of a method.

In case the nature of the analyte is unknown the structure of the analyte has to be *elucidated* by combination of all data derived from the test on the bases of a *working hypothesis* about the molecular structure.

In case the nature of the analyte is known and a standard of the analyte is available and/or relevant data of such a standard (e.g. a reference mass spectrum) are available the identity of the analyte is only *confirmed* against such *standard information*.

In cases of (legal) dispute this absence of explicit and transparent proof of the specificity claim of the method “beyond (any) reasonable doubt” is a lethal shortcoming. This is most evident in dispute cases related to non-authorised veterinary drugs or unauthorised use of approved drugs.

In arbitration cases with challenged non-compliant (“positive”) residue findings the so-called “Specificity log” *, in short “SPECLOG” successfully could be used.

The SPECLOG is nothing more or less than a structured logging document of all the information that leads to the conclusion that a method is (examples given) “highly specific”, “very specific” or “specific”.

To each of these qualifications of the specificity of a method an explicit numerical “error probability” (or its *complementary* “correctness probability”) has to be linked. Such a numerical probability has to be fit-for-the-purpose and has to be set as a validation parameter in beforehand by a competent authority, e.g. CODEX CCRVDF, Reference Laboratories or Accrediting Bodies.

How to demonstrate that a test method meets this error probability (and in consequence is “fit-for-the-purpose”) is a big challenge and needs a lot of effort of all kind of scientists.

However, in the EU network of reference laboratories (CRL-NRL network) analytical chemists and chemometrists are working on this topic for quite some years.

Recently also the American Society for Mass Spectroscopy (ASMS)and some Forensic Laboratories are involved in such studies.

In anyway the SPECLOG must be an underpinning and transparent database for the validation of any specificity claim.

Up to the present for the analyte we discriminated in the SPECLOG the following different sub-databases.

1. data from the chemistry used in the extraction and clean-up procedure
2. data from the subsequent chromatography
3. data from the detecting spectroscopy or electrochemistry
4. data from the “blank” reagents
5. data from the “blank” samples
6. data from library searches for potential interferences or matches
7. data and arguments why potential interferences in practice do not or likely will not interfere
8. whatever other data of interest or importance, like choice of sample matrix, sampling and transport / storage history and other explicit quality control data.

The data under point 1. and 2. supply indirect information (from material - material interaction). Data under point 3. supply direct information (from material – radiation interaction or from material – force field interaction). In general direct information is more reliable than indirect information.

All data under point 5. for the “blanks” have to be continuously and cumulatively documented and archived as these data sets are growing and are getting more and more impact the more the test method has been applied in time and number of (quality control) samples. At the end it is the most important dataset to demonstrate explicitly that any “ad random, non predictable” interferences within the population of samples for which the

method has been validated are “under control”. Also explicit error probabilities for *ad random* errors can be derived from this data set.

As “blanks” have to be taken into account *true blanks* (from animals never been treated with the drug under consideration) as well as *virtual blanks*. Virtual blanks are samples of unknown origin which samples show in the test method no detectable signals for the analyte.

Unfortunately such data sets in practice are largely wasted by the laboratories or at best are not archived in a structured and retrievable way.

It is strongly suggested to include the SPECLOG approach in the discussions of the CCRVDF about method validation as laid down in the appropriate CODEX documents.

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* for the same documentation tool we also used in the past the name “Selectivity budget”. This name, however, has been abandoned because in practice it created confusion with the “Uncertainty budget” as officially used in some protocols (like ISO 17025) to estimate the “uncertainty” of quantitative test.