

Guide

GUIDE FOR VALIDATION OF ANALYTICAL METHODS

Version 5.0

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**GUIDE FOR THE VALIDATION OF ANALYTICAL METHODS
FOR THE ANALYSIS OF RESIDUES AND CONTAMINANTS IN BIOLOGICAL
MATRICES AND FOODSTUFFS BY MASS SPECTROMETRY**

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1. SCOPE

The purpose of the following guide is to define the parameters for the validation of the analytical methods developed in LABERCA regarding the analysis of residues and contaminants in biological matrices and food. The guide also intends to precise the criteria fixed at the European level (when they exist) for these parameters. The guide applicability is expected to cover the analysis of forbidden substances or maximal residue level substances (MRL), screening and confirmatory methods, but is limited to a mass spectrometric (MS) measurement. Practical modalities for the calculation of critical limits such as decision limit ($CC\alpha$) and detection capability ($CC\beta$) are especially detailed. The present guideline intends to establish a common working plan for any validation project of the newly developed methods in LABERCA. However, some modifications of the procedure may become necessary according either to the specificity of the detection technique (GC-MS, GC-MS/MS, LC-MS/MS, GC-HRMS), or to schedule requirements, or co-operation with other laboratories...

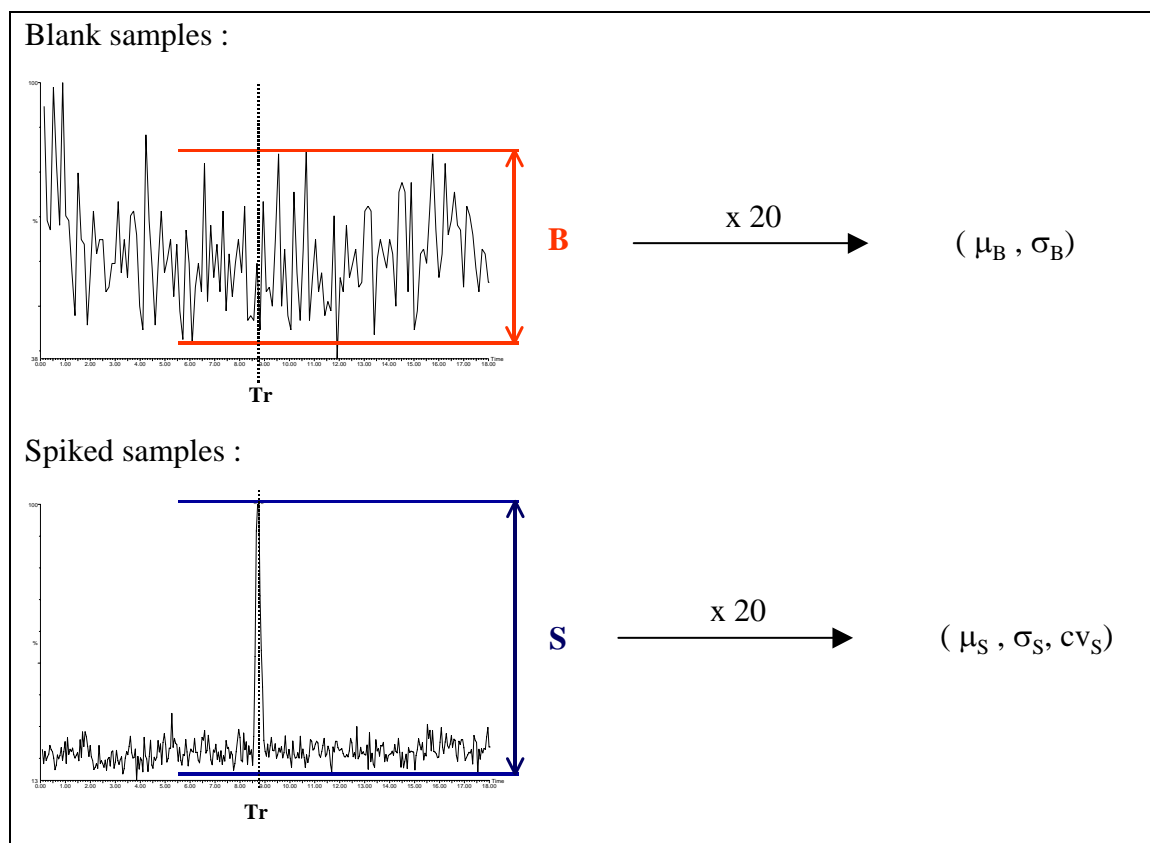
2. RECORDED DATA

The raw analytical data in use in LABERCA are exclusively ion chromatograms obtained by mass spectrometry. According to the 2002/657/EC decision, the only ions to be considered as diagnostic ones in mass spectrometry are: molecular or pseudomolecular ion, specific adducts or fragment ions, as well as isotopic ions. For screening purposes, a single signal may be used. For confirmatory purposes, at least 3 (for the compounds belonging to group B in annex one of 96/23/EC) or 4 (for the compounds belonging to group A in the 96/23/EC annex) identification points have to be achieved, each of them being defined by a specific number of ions according to the technique. The minimum number of diagnostic ions is for example:

- 4 ions for GC-MS or LC-MS
- 1 precursor ion and 2 product ions for GC-MS/MS or LC-MS/MS
- 2 ions for HRMS

The recorded parameters for the analyte during an analytical process are the following:

- the relative retention time,
- the intensities of the analyte signal (signal or noise amplitude) reported to the signal of the internal standard (Figure 1).
- the relative abundances between the various analyte signals (ion ratio).



3. PARAMETERS TO BE CONSIDERED IN THE VALIDATION PROCESS

3.1. Specificity

Definition

Ability of the method to distinguish between the analyte of interest from other interfering substances.

Method of estimation

Checking the ions chromatograms of blank samples for potential co-eluted interfering compounds which may disturb the interpretation.

Objective (fixed by the 2002/657/EC decision)

Absence of interferences leading to interpretation disturbances at the retention time of the analyte.

3.2. Linearity**Definition**

Aptitude of the method to give an analytical response proportional to the analyte concentration in a given concentration range.

Method of estimation

Calculation of the coefficient of determination (R^2) of the regression curve (relative intensity versus concentration), obtained by spiking a mixture of various blank samples ($n=20$) at a minimum of five fortification levels.

Objective (LABERCA own requirement)

$$R^2 > 0,98$$

3.3. Trueness**Definition**

Defined usually as bias: difference between the mean value measured for the analyte in a certified reference material and its certified value. If no certified reference material is available, a fortified sample should be used.

Method of estimation

Analyse 20 replicates of a reference sample, calculate the observed mean (C_{Obs}) and the difference with the theoretical content (C_{Theo}), estimate the relative difference to the real content (Figure 2).

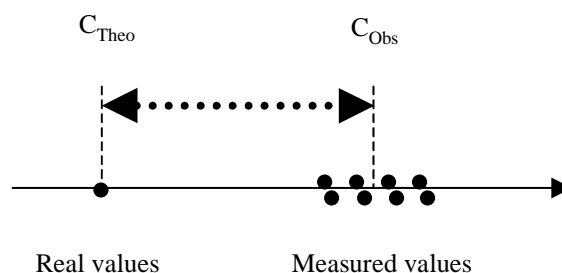


Figure 2: graphical representation of the trueness.

Objective (fixed by the 2002/657/EC decision)

$$\begin{array}{llll}
 100 \times (C_{\text{obs}} - C_{\text{theo}}) / C_{\text{theo}} & \in & [-50, +20 \%] & \text{for } C < 1 \text{ ppb} \\
 100 \times (C_{\text{obs}} - C_{\text{theo}}) / C_{\text{theo}} & \in & [-30, +10 \%] & \text{for } 1 \text{ ppb} < C < 10 \text{ ppb} \\
 100 \times (C_{\text{obs}} - C_{\text{theo}}) / C_{\text{theo}} & \in & [-20, +10 \%] & \text{for } C > 10 \text{ ppb}
 \end{array}$$

3.4. Precision**Definition**

The measure of precision is usually expressed in terms of imprecision and is calculated as a standard deviation of the results obtained by applying the experimental procedure several times under prescribed conditions or as a relative standard deviation to the mean (CV%). The conditions may be repeatability, reproducibility and within-laboratory reproducibility.

Method of estimation

Calculation of the signal relative standard deviation for 20 various spiked samples at a concentration close to the detection capability or the MRL (from a factor 0.5; 1; 1.5 or 2). Calculate the standard deviation of the results and the coefficient of variation (CV%) (Figure 3).

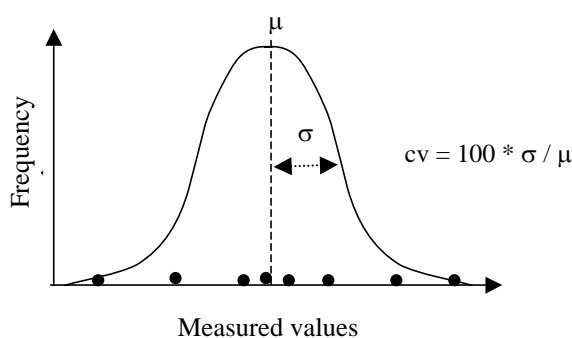


Figure 3: graphical representation of the precision.

Objective (fixed by the 2002/657/EC decision)

For relative retention times : CV < 2,5 % en LC et CV < 0,5 % en GC

For relative signals : CV < $2^{0,5 - (\log C)/2}$ for 1 ppb < C < 100 ppb (Horwitz model)

CV < 23 % si 10 ppb < C < 100 ppb

CV < 32 % si 1 ppb < C < 10 ppb

CV < X pour C < 1 ppb

X should be fixed according to the objectives

For ion ratios

: $R = \text{less intense signal} / \text{most intense signal}$

R	GC-MS ⁿ	GC-MS
	LC-MS ⁿ	CV %
	CV %	CV %
> 50 %	20 %	10 %
]20-50] %	25 %	15 %
]10-20] %	30 %	20 %
≤ 10 %	50 %	50 %

3.5. Decision limit (CC α) and detection capability (CC β)

3.5.1. Introduction

An analytical method will be characterised by a decision limit (CC α) and a detection capability (CC β). For screening methods, these parameters will be calculated using the most intense signal (signal permitting to detect the analyte). For confirmatory methods and in the case of MRL substances, these parameters will be calculated using the less intense signal (“critical” signal permitting the unambiguous identification of the analyte). The ratio between the detection capabilities of the screening and confirmation methods may be used as complementary criteria for the characterisation of the confirmation method performances. The concepts of decision limit and detection capability have been introduced in the ISO 11843 standard in order to propose a method to determine the limit from which a system can be declared different than its basic state (Figure 4). In the present case, the system is a diagnostic ion chromatogram of the target analyte, and the basic state correspond to this chromatogram for a blank sample (forbidden substances) or for a sample containing the analyte at a concentration equal to the MRL (MRL substances). In practice, CC α and CC β allow to characterise the two main sources of signal variability, i.e. coming from the background noise (mainly depending on the method specificity) and from the measure (mainly depending on the method repeatability).

3.5.2. Decision limit (CC α)

Definition

“Limit from which a sample can be declared non-compliant with an error probability equal to α ”. In practice, CC α corresponds to the maximal amplitude of the noise (for a blank sample, forbidden substances) or of the analyte signal (sample containing the analyte at a concentration equal to the MRL, MRL substances), with a confidence level equal to $1-\alpha$.

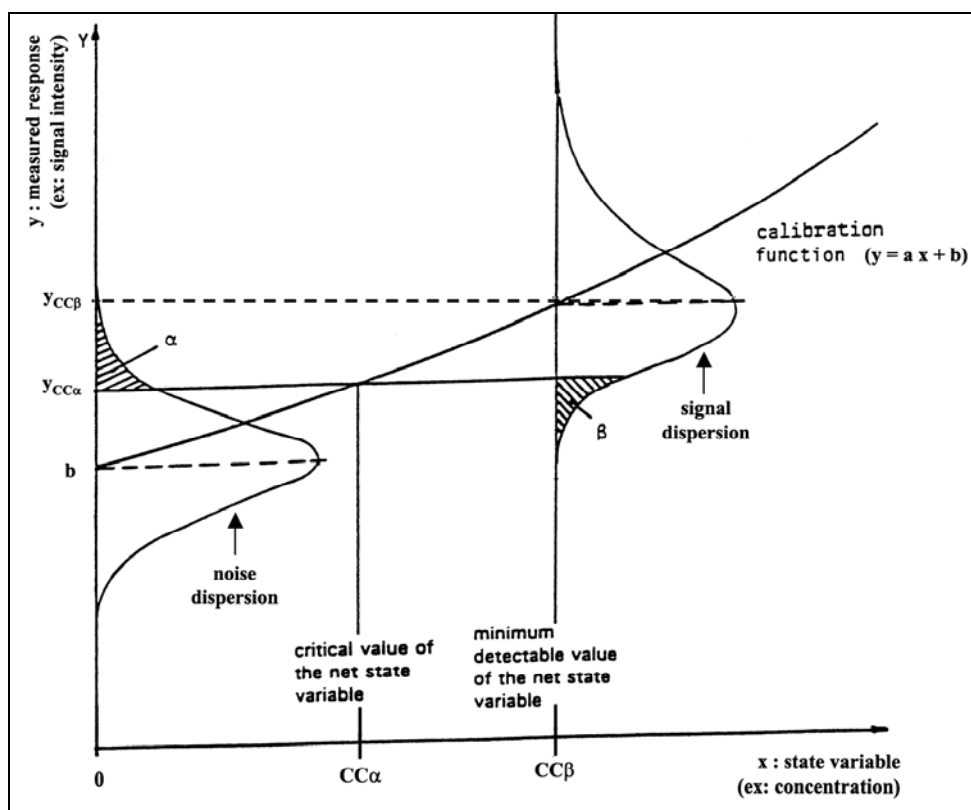


Figure 4 : Definition of the decision limit and detection capability according to the ISO 11843.

Method of estimation

Forbidden substances

In the case of forbidden substances, $CC\alpha$ is the concentration corresponding to an analytical response intensity $I_{CC\alpha}$, defined by the relation:

$$I_{CC\alpha} = \mu_B + 2.33 \sigma_B$$

where μ_B and σ_B are respectively the mean and standard deviation of the noise amplitude, estimated on the basis of various blank samples ($n=20$). The relation signal intensity/concentration is established by using a calibration curve elaborated on the basis of a mixture of various blank samples, spiked at least at 5 concentration levels ($n=1$ replicate per level). The signal μ_B , obtained after the analysis of the 20 blank samples, is used as forced intercept of the calibration curve. According to this procedure, $I_{CC\alpha}$ can be defined as:

$$I_{CC\alpha} = \mu_B + a \cdot CC\alpha$$

Finally, according to these two relations :

$$CC\alpha = 2.33 \sigma_B / a \quad (\text{Eq. I})$$

MRL substances

In the case of MRL substances, $CC\alpha$ is the concentration corresponding to an analytical response intensity $I_{CC\alpha}$, defined by the relation:

$$I_{CC\alpha} = \mu_{MRL} + 1.64 \sigma_{MRL}$$

where μ_{MRL} and σ_{MRL} are respectively the mean and standard deviation of the analyte signal amplitude for samples containing the analyte at a concentration equal to the MRL ($n=20$). The relation signal intensity/concentration is established by using a calibration curve elaborated on the basis of a mixture of various blank samples, spiked at least at 6 fortification levels including: 0 ($n=5$); $x.MRL$ ($n=5$); 0.5 MRL ($n=20$); 1.5.MRL ($n=10$) and $y.MRL$ ($n=5$), where x and y are fixed according to the objectives. The signal μ_B , obtained for the 20 blank samples, is used as forced intercept of the calibration curve. According to this procedure, $I_{CC\alpha}$ can be defined as:

$$I_{CC\alpha} = \mu_B + a \cdot CC\alpha$$

Finally, according to these two relations :

$CC\alpha = (\mu_{MRL} - \mu_B + 1.64 \sigma_{MRL}) / a$	(Eq. II)
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Objective (LABERCA own requirement)

No EC indication for the moment.

3.5.3. Detection capability ($CC\beta$)***Definition***

“Limit from which the analyte can be detected, identified, and/or quantified (according to the needs) with an error probability of β ”. In practice, $CC\beta$ corresponds to the lowest measurement result from which the sample can be declared non-compliant, i.e. containing the analyte (forbidden substances) or containing the analyte at a concentration higher than the MRL (MRL substances).

Method of estimationForbidden substances

In the case of forbidden substances, $CC\beta$ corresponds to the concentration leading to a signal intensity $I_{CC\beta}$ defined as:

$$I_{CC\beta} = \mu_B + 2.33 \sigma_B + 1.64 \sigma_{CC\beta}$$

where μ_B and σ_B are defined as previously and where $\sigma_{CC\beta}$ is the standard deviation of the analyte signal intensity at the concentration $CC\beta$. According to the difference between the real value of

$CC\beta$ and the concentration C at which the assay is performed in order to estimate this signal variability (concentration estimated during the method development, globally inducing a signal to noise ratio around 6), this last equation should be modified in order to do an approximation on the relative standard deviation rather than on the standard deviation. According to this remark, the relation is now:

$$I_{CC\beta} = \mu_B + 2.33 \sigma_B + 1.64 CV_C CC\beta$$

The relation signal intensity/concentration is established by using a calibration curve elaborated on the basis of a mixture of various blank samples, spiked at least at 5 concentration levels ($n=1$ replicate per level). The signal μ_B obtained after the analysis of the 20 blank samples, is used as forced intercept of the calibration curve. According to this procedure, $I_{CC\beta}$ can be defined as:

$$I_{CC\beta} = \mu_B + a \cdot CC\beta$$

Finally, according to these two relations :

$$CC\beta = (2.33 \sigma_B + 1.64 \mu_B CV_C) / a (1 - 1.64 CV_C) \quad (\text{Eq. III})$$

MRL substances

In the case of MRL substances, $CC\beta$ is the concentration corresponding to an analytical response intensity $I_{CC\beta}$, defined by the relation:

$$I_{CC\beta} = \mu_{MRL} + 1.64 \sigma_{MRL} + 1.64 \sigma_{CC\beta}$$

where μ_{MRL} and σ_{MRL} and $\sigma_{CC\beta}$ are defined as previously. According to the difference between the real value of $CC\beta$ and the concentration C at which the assay is performed in order to estimate this signal variability (concentration estimated during the method development, globally inducing a signal to noise ratio around 6), this last equation should be modified in order to do an approximation on the relative standard deviation rather than on the standard deviation. According to this remark, the relation is now:

$$I_{CC\beta} = \mu_{MRL} + 1.64 \sigma_{MRL} + 1.64 CV_C I_{CC\beta}$$

The relation signal intensity/concentration is established by using a calibration curve elaborated on the basis of a mixture of various blank samples, spiked at least at 6 concentration levels including: 0 ($n=5$) ; $x.MRL$ ($n=5$) ; 0.5 MRL ($n=20$) ; 1.5.MRL ($n=10$) and $y.MRL$ ($n=5$), where x and y are fixed according to the objectives. The signal μ_B , obtained for the 20 blank samples, is used as forced intercept of the calibration curve. According to this procedure, $I_{CC\beta}$ can be defined as:

$$I_{CC\beta} = \mu_B + a \cdot CC\beta$$

Finally, according to these two relations :

$$CC\beta = (\mu_{MRL} - \mu_B + 1.64 \sigma_{MRL} + 1.64 \mu_B CV_C) / a (1 - 1.64 CV_C) \quad (\text{Eq. IV})$$

Objective (LABERCA own requirement)

No EC indication for the moment.

3.6. Inclusion criteria for the analytes in the validated method

Additional criteria have been defined in order to decide, after the validation, the inclusion or non inclusion of each analyte in the validated method, regarding the obtained validation results. Two different approaches are proposed ; the first systematical approach is described in § 3.6.1. and the second approach (§ 3.6.2) may be used if the introduction criteria are not fulfilled.

3.6.1. CC α and MRPL

One compound will be included in the method if **the detection capability (CCb) calculated for confirmation analysis (i.e. on the less intense diagnostic signal) and for this analyte is below or equal to the minimum required performance limit (MRPL)**. This criteria was adopted during the 15th July 2004 meeting of the European commission regarding the interpretation and practical application of the 2002/657/EC decision.

3.6.2. Identification criteria

A compound can be included on the list of the target analytes covered by a method if this compound was successfully identified in at least 19 samples among the 20 different samples (representative of the various matrices covered by the method application range) fortified at a concentration below or equal to the MRPL and that have been analysed for the calculation of the detection capability.

3.7. Extraction recovery***Definition***

Fraction of the analyte initially present in the sample, which is retrieved in the final extract after application of the sample preparation method.

Estimation mean

$$\text{Rdt} = 100 \times \frac{\left(\frac{\text{analyte}}{\text{EE}} \right)_{\text{Ajout}}}{\left(\frac{\text{analyte}}{\text{EE}} \right)_{\text{Standard}}} \quad (\text{Eq. III})$$

With :

- EE = external standard
- Spike = sample fortified with the analyte at C level (before extraction).
- Standard = extracted sample fortified with the analyte at C level (after extraction).

Objective (LABERCA own requirement)

None. The specificity of the method in term of absence of interfering compounds, as well as the signal to noise ratio of the monitored signal are considered as more relevant parameters to characterise the method performances. This value may be considered as informative.

4. CONCLUSION

The purpose of the present report was to extract some essential elements from the 2002/657/EC decision, that are required for its practical application in the field of residue and contaminant analysis by mass spectrometry. The determination of the critical limits such as decision limit $CC\alpha$ and detection capability $CC\beta$ were particularly detailed. Following this guideline, the minimal number of experimental assays necessary for such validation procedure appears to be 45 in the case of forbidden substances, and 55 in the case of MRL compounds. Some additional assays may be performed in order to evaluate other parameters (stability, robustness,...). Moreover, a continuous monitoring of the method performances should be suggested, for example using quality control charts.