

## II

(Acts whose publication is not obligatory)

## COMMISSION

## COMMISSION DECISION

of 12 August 2002

**implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results**

(notified under document number C(2002) 3044)

(Text with EEA relevance)

(2002/657/EC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC <sup>(1)</sup>, and in particular the second subparagraph of Article 15(1) thereof,

Whereas:

- (1) The presence of residues in products of animal origin is a matter of concern for public health.
- (2) Commission Decision 98/179/EC of 23 February 1998 laying down detailed rules on official sampling for the monitoring of certain substances and residues thereof in live animals and animal products <sup>(2)</sup> provides that the analysis of samples is to be carried out exclusively by laboratories approved for official residue control by the competent national authority.
- (3) It is necessary to ensure the quality and comparability of the analytical results generated by laboratories approved for official residue control. This should be achieved by using quality assurance systems and specifically by applying of methods validated according to common procedures and performance criteria and by ensuring traceability to common standards or standards commonly agreed upon.
- (4) Council Directive 93/99/EEC of 29 October 1993 on the subject of additional measures concerning the official control of foodstuffs and Decision 98/179/EC <sup>(3)</sup> require

official control laboratories to be accredited according to ISO 17025 (1) from January 2002 onwards. Pursuant to Decision 98/179/EC, participation in an internationally recognised external quality control assessment and accreditation scheme is required for approved laboratories. Moreover, approved laboratories must prove their competence by regular and successful participation in adequate proficiency testing schemes recognised or organised by the national or Community reference laboratories.

- (5) A network of Community reference laboratories, national reference laboratories and national control laboratories operates under Directive 96/23/EC to enhance coordination.
- (6) As a result of advances in analytical chemistry since the adoption of Directive 96/23/EC the concept of routine methods and reference methods has been superseded by criteria approach, in which performance criteria and procedures for the validation of screening and confirmatory methods are established.
- (7) It is necessary to determine common criteria for the interpretation of test results of official control laboratories in order to ensure a harmonised implementation of Directive 96/23/EC.
- (8) It is necessary to provide for the progressive establishment of minimum required performance limits (MRPL) of analytical method for substances for which no permitted limit has been established and in particular for those substances whose use is not authorised, or is specifically prohibited in the Community, in order to ensure harmonised implementation of Directive 96/23/EC.

<sup>(1)</sup> OJ L 125, 23.5.1996, p. 10.

<sup>(2)</sup> OJ L 65, 5.3.1998, p. 31.

<sup>(3)</sup> OJ L 290, 24.11.1993, p. 14.

- (9) Commission Decision 90/515/EEC of 26 September 1990 laying down the reference methods for detecting residues of heavy metals and arsenic <sup>(1)</sup>, Commission Decision 93/256/EEC of 14 May 1993 laying down the methods to be used for detecting residues of substances having a hormonal or a thyrostatic action <sup>(2)</sup>, and of Commission Decision 93/257/EEC of 15 April 1993 laying down the reference methods and the list of the national reference laboratories for detecting residues <sup>(3)</sup>, as last amended by Decision 98/536/EC <sup>(4)</sup> have been re-examined before in order to take account of developments in scientific and technical knowledge, have been found outdated in their scope and provisions and should accordingly be repealed with this Decision.
- (10) In order to allow methods for the analysis of official samples to be adapted to the provisions of this Decision, a transitional period should be laid down.
- (11) The measures provided for in this Decision are in accordance with the opinion of the Standing Committee on the Food Chain and Animal Health,

HAS ADOPTED THIS DECISION:

#### Article 1

##### Subject matter and scope

This Decision provides rules for the analytical methods to be used in the testing of official samples taken pursuant to Article 15(1), second sentence, of Directive 96/23/EC and specifies common criteria for the interpretation of analytical results of official control laboratories for such samples.

This Decision shall not apply to substances for which more specific rules have been laid down in other Community legislation.

#### Article 2

##### Definitions

For the purpose of this Decision the definitions in Directive 96/23/EC and in the Annex to this decision shall apply.

#### Article 3

##### Analytical methods

The Member States shall ensure that official samples taken pursuant to Directive 96/23/EC are analysed using methods that:

- (a) are documented in test instructions, preferably according to ISO 78-2 (6);
- (b) comply with Part 2 of the Annex to this Decision;
- (c) have been validated according to the procedures described in Part 3 of the Annex;

<sup>(1)</sup> OJ L 286, 18.10.1990, p. 33.

<sup>(2)</sup> OJ L 118, 14.5.1993, p. 64.

<sup>(3)</sup> OJ L 118, 14.5.1993, p. 75.

<sup>(4)</sup> OJ L 251, 11.9.1998, p. 39.

- (d) comply with the relevant minimum required performance limits (MRPL) to be established in accordance with Article 4.

#### Article 4

##### Minimum required performance limits

The present Decision shall be reviewed to progressively establish the minimum required performance limits (MRPL) of analytical methods to be used for substances for which no permitted limit has been established.

#### Article 5

##### Quality control

The Member States shall ensure the quality of the results of the analysis of samples taken pursuant to Directive 96/23/EC, in particular by monitoring tests and/or calibration results according to Chapter 5.9 of ISO 17025 (1).

#### Article 6

##### Interpretation of results

1. The result of an analysis shall be considered non-compliant if the decision limit of the confirmatory method for the analyte is exceeded.

2. If a permitted limit has been established for a substance, the decision limit is the concentration above which it can be decided with a statistical certainty of  $1 - \alpha$  that the permitted limit has been truly exceeded.

3. If no permitted limit has been established for a substance, the decision limit is the lowest concentration level at which a method can discriminate with a statistical certainty of  $1 - \alpha$  that the particular analyte is present.

4. For substances listed in Group A of Annex I to Directive 96/23/EC, the  $\alpha$  error shall be 1 % or lower. For all other substances, the  $\alpha$  error shall be 5 % or lower.

#### Article 7

##### Repeal

Decisions 90/515/EEC, 93/256/EEC and 93/257/EEC are repealed.

#### Article 8

##### Transitional provisions

The methods for the analysis of official samples of substances listed in Group A of Annex I to Directive 96/23/EC, which satisfy the criteria set out in Decisions 90/515/EEC, 93/256/EEC and 93/257/EEC may be used for up to two years after this Decision enters into force. Methods currently applied for substances listed in Group B of Annex I to Directive 96/23/EC shall comply with this Decision at the latest five years after the date of application of this Decision.

*Article 9***Date of application**

This Decision shall apply from 1 September 2002.

*Article 10***Addressees**

This Decision is addressed to the Member States.

Done at Brussels, 12 August 2002.

*For the Commission*  
David BYRNE  
*Member of the Commission*

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## ANNEX

**PERFORMANCE CRITERIA, OTHER REQUIREMENTS AND PROCEDURES FOR ANALYTICAL METHODS****1. DEFINITIONS**

- 1.1. Accuracy means the closeness of agreement between a test result and the accepted reference value (2). It is determined by determining trueness and precision.
- 1.2. Alpha ( $\alpha$ ) error means the probability that the tested sample is compliant, even though a non-compliant measurement has been obtained (false non-compliant decision).
- 1.3. Analyte means the substance that has to be detected, identified and/or quantified and derivatives emerging during its analysis.
- 1.4. Beta ( $\beta$ ) error means the probability that the tested sample is truly non-compliant, even though a compliant measurement has been obtained (false compliant decision).
- 1.5. Bias means the difference between the expectation of the test result and an accepted reference value (2).
- 1.6. Calibration standard means a device for measurements that represents the quantity of substance of interest in a way that ties its value to a reference base.
- 1.7. Certified reference material (CRM) means a material that has had a specified analyte content assigned to it.
- 1.8. Co-chromatography means a procedure in which the extract prior to the chromatographic step(s) is divided into two parts. Part one is chromatographed as such. Part two is mixed with the standard analyte that is to be measured. Then this mixture is also chromatographed. The amount of added standard analyte has to be similar to the estimated amount of the analyte in the extract. This method is designed to improve the identification of an analyte when chromatographic methods are used, especially when no suitable internal standard can be used.
- 1.9. Collaborative study means analysing the same sample by the same method to determine the performance characteristics of the method. The study covers random measurement error and laboratory bias.
- 1.10. Confirmatory method means methods that provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest.
- 1.11. Decision limit ( $CC\alpha$ ) means the limit at and above which it can be concluded with an error probability of  $\alpha$  that a sample is non-compliant.
- 1.12. Detection capability ( $CC\beta$ ) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of  $\beta$ . In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of  $1 - \beta$ . In the case of substances with an established permitted limit, this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of  $1 - \beta$ .
- 1.13. Fortified sample material means a sample enriched with a known amount of the analyte to be detected.
- 1.14. Interlaboratory study (comparison) means organisation, performance and evaluation of tests on the same sample by two or more laboratories in accordance with predetermined conditions to determine testing performance. According to the purpose the study can be classified as collaborative study or proficiency study.
- 1.15. Internal Standard (IS) means a substance not contained in the sample with physical-chemical properties as similar as possible to those of the analyte that has to be identified and which is added to each sample as well as to each calibration standard.
- 1.16. Laboratory sample means a sample prepared for sending to a laboratory and intended for inspection or testing.
- 1.17. Level of interest means the concentration of substance or analyte in a sample that is significant to determine its compliance with legislation.
- 1.18. Minimum required performance limit (MRPL) means minimum content of an analyte in a sample, which at least has to be detected and confirmed. It is intended to harmonise the analytical performance of methods for substances for which no permitted limit has been established.

- 1.19. Performance characteristic means functional quality that can be attributed to an analytical method. This may be for instance specificity, accuracy, trueness, precision, repeatability, reproducibility, recovery, detection capability and ruggedness.
- 1.20. Performance criteria means requirements for a performance characteristic according to which it can be judged that the analytical method is fit for the purpose and generates reliable results.
- 1.21. Permitted limit means maximum residue limit, maximum level or other maximum tolerance for substances established elsewhere in Community legislation.
- 1.22. Precision means the closeness of agreement between independent test results obtained under stipulated (predetermined) conditions. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the test result. Less precision is determined by a larger standard deviation (2).
- 1.23. Proficiency study means analysing the same sample allowing laboratories to choose their own methods, provided these methods are used under routine conditions. The study has to be performed according to ISO guide 43-1 (3) and 43-2 (4) and can be used to assess the reproducibility of methods.
- 1.24. Qualitative method means an analytical method which identifies a substance on the basis of its chemical, biological or physical properties.
- 1.25. Quantitative method means an analytical method which determines the amount or mass fraction of a substance so that it may be expressed as a numerical value of appropriate units.
- 1.26. Reagent blank determination means the complete analytical procedure applied without the test portion or using an equivalent amount of suitable solvent in place of the test portion.
- 1.27. Recovery means the percentage of the true concentration of a substance recovered during the analytical procedure. It is determined during validation, if no certified reference material is available.
- 1.28. Reference material means a material of which one or several properties have been confirmed by a validated method, so that it can be used to calibrate an apparatus or to verify a method of measurement.
- 1.29. Repeatability means precision under repeatability conditions (2).
- 1.30. Repeatability conditions means conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment (2).
- 1.31. Reproducibility means precision under reproducibility conditions (2)(4).
- 1.32. Reproducibility conditions means conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment (2)(4).
- 1.33. Ruggedness means the susceptibility of an analytical method to changes in experimental conditions which can be expressed as a list of the sample materials, analytes, storage conditions, environmental and/or sample preparation conditions under which the method can be applied as presented or with specified minor modifications. For all experimental conditions which could in practice be subject to fluctuation (e.g. stability of reagents, composition of the sample, pH, temperature) any variations which could affect the analytical result should be indicated.
- 1.34. Sample blank determination means the complete analytical procedure applied to a test portion taken from a sample from which the analyte is absent.
- 1.35. Screening method means methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are specifically designed to avoid false compliant results.
- 1.36. Single laboratory study (in-house validation) means an analytical study involving a single laboratory using one method to analyse the same or different test materials under different conditions over justified long time intervals.
- 1.37. Specificity means the ability of a method to distinguish between the analyte being measured and other substances. This characteristic is predominantly a function of the measuring technique described, but can vary according to class of compound or matrix.

- 1.38. Standard addition means a procedure in which the test sample is divided in two (or more) test portions. One portion is analysed as such and known amounts of the standard analyte are added to the other test portions before analysis. The amount of the standard analyte added has to be between two and five times the estimated amount of the analyte in the sample. This procedure is designed to determine the content of an analyte in a sample, taking account of the recovery of the analytical procedure.
- 1.39. Standard analyte means an analyte of known and certified content and purity to be used as a reference in the analysis.
- 1.40. Substance means matter of particular or definite chemical constitution and its metabolites.
- 1.41. Test portion means the quantity of material drawn from the test sample on which the test or observation is carried out.
- 1.42. Test sample means a sample prepared from a laboratory sample and from which test portions will be taken.
- 1.43. Trueness means the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. Trueness is usually expressed as bias (2).
- 1.44. Units means those units described in ISO 31 (20) and Directive 71/354/EC (19).
- 1.45. Validation means the confirmation by examination and the provision of effective evidence that the particular requirements of a specific intended use are fulfilled (1).
- 1.46. Within-laboratory reproducibility means precision obtained in the same laboratory under stipulated (predetermined) conditions (concerning e.g. method, test materials, operators, environment) over justified long time intervals.

## 2. PERFORMANCE CRITERIA AND OTHER REQUIREMENTS FOR ANALYTICAL METHODS

Analytical methods or combinations of methods other than those described below may only be used for screening or confirmatory purposes if it can be proven that they fulfil the relevant requirements established in this Decision.

### 2.1. GENERAL REQUIREMENTS

#### 2.1.1. Handling of samples

Samples shall be obtained, handled and processed in such a way that there is a maximum chance of detecting the substance. Sample handling procedures shall prevent the possibility of accidental contamination or loss of analytes.

#### 2.1.2. Performance of tests

##### 2.1.2.1. Recovery

During the analysis of samples the recovery shall be determined in each batch of samples, if a fixed recovery correction factor is used. If the recovery is within limits, the fixed correction factor may then be used. Otherwise the recovery factor obtained for that specific batch shall be used, unless the specific recovery factor of the analyte in the sample is to be applied in which case the standard addition procedure (see 3.5) or an internal standard shall be used for the quantitative determination of an analyte in a sample.

##### 2.1.2.2. Specificity

A method shall be able to distinguish between the analyte and the other substances under the experimental conditions. An estimate to which extent this is possible has to be provided. Strategies to overcome any foreseeable interference with substances when the described measuring technique is used, e.g. homologues, analogues, metabolic products of the residue of interest have to be employed. It is of prime importance that interference, which might arise from matrix components, is investigated.

### 2.2. SCREENING METHODS

Only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate of < 5 % ( $\beta$ -error) at the level of interest shall be used for screening purposes in conformity with Directive 96/23/EC. In the case of a suspected non-compliant result, this result shall be confirmed by a confirmatory method.

## 2.3. CONFIRMATORY METHODS FOR ORGANIC RESIDUES AND CONTAMINANTS

Confirmatory methods for organic residues or contaminants shall provide information on the chemical structure of the analyte. Consequently methods based only on chromatographic analysis without the use of spectrometric detection are not suitable on their own for use as confirmatory methods. However, if a single technique lacks sufficient specificity, the desired specificity shall be achieved by analytical procedures consisting of suitable combinations of clean-up, chromatographic separation(s) and spectrometric detection.

The following methods or method combinations are considered suitable for the identification of organic residues or contaminants for the substance groups indicated:

Table 1

## Suitable confirmatory methods for organic residues or contaminants

Measuring technique	Substances Annex 1 96/23/EC	Limitations
LC or GC with mass-spectrometric detection	Groups A and B	Only if following either an on-line or an off-line chromatographic separation Only if full scan techniques are used or using at least 3 (group B) or 4 (group A) identification points for techniques that do not record the full mass spectra
LC or GC with IR spectrometric detection	Groups A and B	Specific requirements for absorption in IR spectrometry have to be met
LC-full-scan DAD	Group B	Specific requirements for absorption in UV spectrometry have to be met
LC -fluorescence	Group B	Only for molecules that exhibit native fluorescence and to molecules that exhibit fluorescence after either transformation or derivatisation
2-D TLC - full-scan UV/VIS	Group B	Two-dimensional HPTLC and co-chromatography are mandatory
GC-Elektron capture detection	Group B	Only if two columns of different polarity are used
LC-immunogram	Group B	Only if at least two different chromatographic systems or a second, independent detection method are used
LC-UV/VIS (single wavelength)	Group B	Only if at least two different chromatographic systems or second, independent detection method are used.

## 2.3.1. Common performance criteria and requirements

Confirmatory methods shall provide information on the chemical structure of the analyte. When more than one compound gives the same response, then the method cannot discriminate between these compounds. Methods based only on chromatographic analysis without the use of spectrometric detection are not suitable on their own for use as confirmatory methods.

Where used in 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 compounds that are structurally related to the analyte shall be used.

When no suitable internal standard can be used, the identification of the analyte shall 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. With gas chromatography (GC) or liquid chromatography (LC), the peak width at half-maximum height shall be within the 90-110 % range of the original width, and the retention times shall be identical within a margin of 5 %. For thin layer chromatography (TLC) methods, only the spot presumed to be due to the analyte shall be intensified; a new spot shall not appear and the visual appearance shall not change.

Reference or fortified material containing known amounts of analyte, at or near either the permitted limit or the decision limit (non-compliant control sample) as well as compliant control materials and reagent blanks should preferably be carried through the entire procedure simultaneously with each batch of test samples analysed. The order for injecting the extracts into the analytical instrument is as follows: reagent blank, compliant control sample, sample(s) to be confirmed, compliant control sample again and finally non-compliant control sample. Any variation from this sequence shall be justified.

### 2.3.2. Additional performance criteria and other requirements for quantitative methods of analysis

#### 2.3.2.1. Trueness of quantitative methods

In the case of repeated analyses of a certified reference material, the guideline ranges for the deviation of the experimentally determined recovery corrected mean mass fraction from the certified value are as follows:

**Table 2**

#### Minimum trueness of quantitative methods

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

When no such CRMs are available, it is acceptable that trueness of measurements is assessed through recovery of additions of known amounts of the analyte(s) to a blank matrix. Data corrected with the mean recovery are only acceptable when they fall within the ranges shown in Table 2.

#### 2.3.2.2. Precision of quantitative methods

The inter-laboratory coefficient of variation (CV) for the repeated analysis of a reference or fortified material, under 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<sup>-3</sup>). Examples are shown in the table 3.

**Table 3**

#### Examples for reproducibility CVs for quantitative methods at a range of analyte mass fractions

Mass fraction	Reproducibility CV(%)
1 µg/kg	(*)
10 µg/kg	(*)
100 µg/kg	23
1 000 µg/kg (1 mg/kg)	16

(\*) For mass fractions lower than 100 µg/kg the application of the Horwitz Equation gives unacceptable high values. Therefore, the CVs for concentrations lower than 100 µg/kg shall be as low as possible.

For analyses carried out under repeatability conditions, the intra-laboratory CV would typically be between one half and two thirds of the above values. For analyses carried out under within-laboratory reproducibility conditions, the within-laboratory CV shall not be greater than the reproducibility CV.

In the case of substances with an established permitted limit, the method shall achieve within-laboratory reproducibility not greater than the corresponding reproducibility CV at a concentration of 0,5 × the permitted limit.



### 2.3.3. Performance criteria and other requirements for mass spectrometric detection

Mass spectrometric methods are suitable for consideration as confirmatory methods only following either an on-line or an off-line chromatographic separation.

#### 2.3.3.1. Chromatographic separation

For GC-MS procedures, the gas chromatographic separation shall be carried out using capillary columns. For LC-MS procedures, the chromatographic separation shall be carried out using suitable LC columns. In any case, the minimum acceptable retention time for the analyte under examination is twice the retention time corresponding to the void volume of the column. The retention time (or relative retention time) of the analyte in the test portion shall match that of the calibration standard within a specified retention time window. The retention time window shall be commensurate with the resolving power of the chromatographic system. 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 solution at a tolerance of  $\pm 0,5\%$  for GC and  $\pm 2,5\%$  for LC.

#### 2.3.3.2. Mass spectrometric detection

Mass-spectrometric detection shall be carried out by employing MS-techniques such as recording of full mass spectra (full scans) or selected ion monitoring (SIM), as well as MS-MS<sup>n</sup> techniques such as Selected Reaction Monitoring (SRM), or other suitable MS or MS-MS<sup>n</sup> techniques in combination with appropriate ionisation modes. In high-resolution mass spectrometry (HRMS), the resolution shall typically be greater than 10 000 for the entire mass range at 10 % valley.

**Full scan:** When mass spectrometric determination is performed by the recording of full scan spectra, the presence of all measured diagnostic ions (the molecular ion, characteristic adducts of the molecular ion, characteristic fragment ions and isotope ions) with a relative intensity of more than 10 % in the reference spectrum of the calibration standard is obligatory.

**SIM:** When mass spectrometric determination is performed by fragmentography, the molecular ion shall preferably be one of the selected diagnostic ions (the molecular ion, characteristic adducts of the molecular ion, characteristic fragment ions and all their isotope ions). The selected diagnostic ions should not exclusively originate from the same part of the molecule. The signal-to-noise ratio for each diagnostic ion shall be  $\geq 3:1$ .

**Full scan and SIM:** The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion or transition, shall correspond to those of the calibration standard, either from calibration standard solutions or from spiked samples, at comparable concentrations, measured under the same conditions, within the following tolerances:

**Table 4**

#### Maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques

Relative intensity (% of base peak)	EI-GC-MS (relative)	CI-GC-MS, GC-MS <sup>n</sup> LC-MS, LC-MS <sup>n</sup> (relative)
> 50 %	$\pm 10\%$	$\pm 20\%$
> 20 % to 50 %	$\pm 15\%$	$\pm 25\%$
> 10 % to 20 %	$\pm 20\%$	$\pm 30\%$
$\leq 10\%$	$\pm 50\%$	$\pm 50\%$

**Interpretation of mass spectral data:** The relative intensities of the diagnostic ions and/or precursor/product ion pairs have to be identified by comparing spectra or by integrating the signals of the single mass traces. Whenever background correction is applied, this shall be applied uniformly throughout the batch (see 2.3.1, paragraph 4) and shall be clearly indicated.

**Full scan:** When full scan spectra are recorded in single mass spectrometry, a minimum of four ions shall be present with a relative intensity of  $\geq 10\%$  of the base peak. The molecular ion shall be included if it is present in the reference spectrum with a relative intensity of  $\geq 10\%$ . At least four ions shall lie within the maximum permitted tolerances for relative ion intensities (Table 5). Computer-aided library searching may be used. In this case, the comparison of mass spectral data in the test 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 below are fulfilled. Variability in the spectra caused by the sample matrix and the detector performance shall be checked.

SIM: When mass fragments are measured using other than full-scan techniques, a system of identification points shall be used to interpret the data. For the confirmation of substances listed in Group A of Annex I of Directive 96/23/EC, a minimum of 4 identification points shall be required. For the confirmation of substances listed in Group B of Annex I of Directive 96/23/EC, a minimum of 3 identification points are required. The table below shows the number of identification points that each of the basic mass spectrometric techniques can earn. However, in order to qualify for the identification points required for confirmation and the sum of identification points to be calculated:

- (a) a minimum of at least one ion ratio shall be measured, and
- (b) all relevant measured ion ratios shall meet the criteria described above, and
- (c) a maximum of three separate techniques can be combined to achieve the minimum number of identification points.

Table 5

**The relationship between a range of classes of mass fragment and identification points earned**

MS technique	Identification points earned per ion
Low resolution mass spectrometry (LR)	1,0
LR-MS <sup>n</sup> precursor ion	1,0
LR-MS <sup>n</sup> transition products	1,5
HRMS	2,0
HR- MS <sup>n</sup> precursor ion	2,0
HR-MS <sup>n</sup> transition products	2,5

## Footnotes:

- (1) Each ion may only be counted once.
- (2) GC-MS using electron impact ionisation is regarded as being a different technique to GC-MS using chemical ionisation.
- (3) Different analytes can be used to increase the number of identification points only if the derivatives employ different reaction chemistries.
- (4) For substances in Group A of Annex 1 to Directive 96/23/EC, if one of the following techniques are used in the analytical procedure: HPLC coupled with full-scan diode array spectrophotometry (DAD); HPLC coupled with fluorescence detection; HPLC coupled to an immunogram; two-dimensional TLC coupled to spectrometric detection; a maximum of one identification point may be contributed, providing that the relevant criteria for these techniques are fulfilled.
- (5) Transition products include both daughter and granddaughter products.

Table 6

**Examples of the number of identification points earned for a range of techniques and combinations thereof (n = an integer)**

Technique(s)	Number of ions	Identification points
GC-MS (EI or CI)	N	n
GC-MS (EI and CI)	2 (EI) + 2 (CI)	4
GC-MS (EI or CI) 2 derivatives	2 (Derivative A) + 2 (Derivative B)	4
LC-MS	N	n
GC-MS-MS	1 precursor and 2 daughters	4
LC-MS-MS	1 precursor and 2 daughters	4
GC-MS-MS	2 precursor ions, each with 1 daughter	5
LC-MS-MS	2 precursor ions, each with 1 daughter	5
LC-MS-MS-MS	1 precursor, 1 daughter and 2 granddaughters	5,5
HRMS	N	2 n
GC-MS and LC-MS	2 + 2	4
GC-MS and HRMS	2 + 1	4

### 2.3.4. Performance criteria and other requirements for chromatography coupled to infrared detection

Adequate peaks: Adequate peaks are absorption maxima in the infrared spectrum of a calibration standard fulfilling the following requirements.

#### 2.3.4.1. Infra-red detection

Absorption maximum: This shall be in the wavenumber range 4 000-500  $\text{cm}^{-1}$ .

Intensity of absorption: This shall not be less than either:

- (a) a specific molar absorbance of 40 with respect to peak base line; or
- (b) a relative absorbance of 12,5 % of the absorbance of the most intense peak in the region 4 000-500  $\text{cm}^{-1}$

when both are measured with respect to zero absorbance, and 5 % of the absorbance of the most intense peak in the region 4 000-500  $\text{cm}^{-1}$  when both are measured with respect to their peak base line.

Note: Although adequate peaks according to (a) may be preferred from a theoretical point of view, those according to (b) are easier to determine in practice.

The number of peaks in the infrared spectrum of the analyte whose frequencies correspond with an adequate peak in the spectrum of the calibration standard, within a margin of  $\pm 1 \text{ cm}^{-1}$  is determined.

#### 2.3.4.2. Interpretation of infra-red spectral data

Absorption shall be present in all regions of the analyte spectrum which correspond with an adequate peak in the reference spectrum of the calibration standard. A minimum of six adequate peaks is required in the infrared spectrum of the calibration standard. If there are less than six adequate peaks (7), the spectrum at issue cannot be used as a reference spectrum. The 'score', i.e. the percentage of the adequate peaks found in the infrared spectrum of the analyte, shall be at least 50. Where there is no exact match for an adequate peak, the relevant region of the analyte spectrum shall be consistent with the presence of a matching peak. The procedure is only applicable to absorption peaks in the sample spectrum with an intensity of a least three times the peak to peak noise.

### 2.3.5. Performance criteria and other requirements for the determination of an analyte using LC with other detection techniques

#### 2.3.5.1. Chromatographic separation

An internal standard shall be used if a material suitable for this purpose is available. It shall preferably be a related standard with a retention time close to that of the analyte. The analyte shall elute at the retention time that is typical for the corresponding calibration standard under the same experimental conditions. The minimum acceptable retention time for an analyte shall be two times the retention time corresponding to the void volume of the column. The ratio of the retention time of the analyte to that of the internal standard, i.e. the relative retention time of the analyte, shall be the same as that of the calibration standard in the appropriate matrix, within a margin of  $\pm 2,5 \%$ .

#### 2.3.5.2. Full-scan UV/VIS detection

The performance criteria for LC methods have to be fulfilled.

The absorption maxima in the spectrum of the analyte shall be at the same wavelengths as those of the calibration standard within a margin determined by the resolution of the detection system. For diode array detection, this is typically within  $\pm 2 \text{ nm}$ . The spectrum of the analyte above 220  $\text{nm}$  shall, for those parts of the two spectra with a relative absorbance  $\geq 10 \%$ , 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 observed 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 test 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.

#### 2.3.5.3. *Performance criteria for fluorimetric detection*

The performance criteria for LC methods have to be fulfilled.

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

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.

#### 2.3.5.4. *Performance criteria for the determination of an analyte by an LC-immunogram*

A LC immunogram is not suitable on its own for use as a confirmatory method.

Relevant criteria for LC methods have to be fulfilled.

The pre-defined quality control parameters, e.g. non-specific binding, the relative binding of the control samples, the absorbance value of the blank have to be within the limits obtained during validation of the assay.

The immunogram has to be constructed of at least five fractions.

Each fraction shall be less than half of the peak width.

The fraction with the maximum content of the analyte has to be the same for the suspect sample, the non-compliant control sample and the standard.

#### 2.3.5.5. *Determination of an analyte using LC with UV/VIS detection (single wavelength)*

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

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.

#### 2.3.6. **Performance criteria and other requirements for the determination of an analyte by 2-D TLC coupled to full-scan UV/VIS spectrometric detection**

Two-dimensional HPTLC and co-chromatography are mandatory.

The RF values of the analyte shall agree with the RF values of the standards within  $\pm 5\%$ .

The visual appearance of the analyte shall be indistinguishable from that of the standard.

For spots of the same colour the centre of the nearest spot should be separated from the centre of the spot of the analyte by at least half the sum of the spot diameters.

The spectrum of the analyte shall not be visually different from the spectrum of the standard, as described for full-scan UV/VIS detection.

In the case computer-aided library searching and matching are used, the comparison of the spectral data in the test 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.

#### 2.3.7. **Performance criteria and requirements for the determination of an analyte by GC in combination with electron capture detection (ECD)**

An internal standard shall be used if a material suitable for this purpose is available. It shall preferably be a related substance with a retention time close to that of the analyte. The analyte shall elute at a retention time which is typical for the corresponding calibration standard under the same experimental conditions. The minimum acceptable retention time for an analyte shall be two times the retention time corresponding to the void volume of the column. The ratio of the retention time of the analyte to that of the internal standard, i.e. the relative retention time of the analyte, shall be the same as that of the calibration standard in the appropriate matrix, within a margin of  $\pm 0,5\%$ . 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. For additional information, co-chromatography may be used.

## 2.4. CONFIRMATORY METHODS FOR ELEMENTS

Confirmatory analyses for chemical elements shall be based on the concept of unequivocal identification and accurate as well as precise quantification by means of physical-chemical properties unique to the chemical element at hand (e.g. element characteristic wavelength of emitted or absorbed radiation, atomic mass) at the level of interest.

The following methods or combinations of methods are considered suitable for the identification of chemical elements:

Table 7

**Suitable confirmatory methods for chemical elements**

Technique	Measured parameter
Differential pulse anodic stripping voltametry	Electric signal
Atomic absorption spectrometry	
Flame	Absorption wavelength
Hydride generation	Absorption wavelength
Cold vapour	Absorption wavelength
Electrothermal atomisation (graphite furnace)	Absorption wavelength
Atomic emission spectrometry	
Inductively coupled plasma	Emission wavelength
Mass spectrometry	
Inductively coupled plasma	Mass-to-charge-ratio

2.4.1. **Common performance criteria and other requirements for confirmatory methods**

Reference or fortified material containing known amounts of analyte, at or near either the maximum permitted limit or the decision limit (non-compliant control sample) as well as compliant control materials and reagent blanks should preferably be carried through the entire procedure simultaneously with each batch of test samples analysed. The recommended order for injecting the extracts into the analytical instrument is as follows: reagent blank, compliant control sample, sample to be confirmed, compliant control sample and finally non-compliant control sample. Any variation from this shall be justified.

In general, most analytical techniques require complete digestion of the organic matrix to obtain solutions prior to determination of the analyte. This can be achieved by using microwave mineralisation procedures, which minimise the risk of loss and/or contamination of the analytes of interest. Decontaminated Teflon vessels of good quality shall be used. If other wet or dry digestion methods are resorted to, documented evidence shall be available to exclude potential loss or contamination phenomena. As an alternative to digestion, separation procedures (e.g. extraction) may under certain circumstances be chosen to separate analytes from matrix components and/or to concentrate analytes in order to introduce them into the analytical equipment.

As regards calibration, be it external or based on the standard addition method, care shall be taken not to exceed the working range established for the analysis. In the case of external calibration, it is mandatory that calibration standards are prepared in a solution that matches as closely as possible the composition of the sample solution. Background correction shall be also applied if required by specific analytical circumstances.

2.4.2. **Additional performance criteria and other requirements for quantitative methods of analysis**2.4.2.1. *Trueness of quantitative methods*

In the case of repeated analyses of a certified reference material for elements, the deviation of the experimentally determined mean content from the certified value shall not lie outside the limit  $\pm 10\%$ . When no such CRMs are available, it is acceptable that trueness of measurements is assessed through recovery of additions of known amounts of the element to the unknown samples. Attention is drawn to the fact that, unlike the analyte, the added element is not chemically bound in the real matrix and that therefore results obtained by this approach have lesser validity than those achieved through the use of CRMs. Recovery data are only acceptable when they are within  $\pm 10\%$  of the target value.

#### 2.4.2.2. Precision of quantitative methods

In the case of repeated analysis of a sample carried out under within-laboratory reproducibility conditions, the intra-laboratory coefficient of variation (CV) of the mean shall not exceed the following values:

**Table 8**

**CVs for quantitative methods at a range of element mass fractions**

Mass fraction	CV (%)
≥ 10 µg/kg to 100 µg/kg	20
> 100 µg/kg to 1 000 µg/kg	15
≥ 1 000 µg/kg	10

#### 2.4.3. Specific requirements for differential pulse anodic stripping voltametry (DPASV)

Complete destruction of organic matter in samples prior to DPASV determinations is of the greatest importance. No broad signals due to the presence of organic materials shall be seen in the voltamograms. Inorganic matrix constituents may influence peak heights in DPASV. Therefore, quantification has to be done by the method of standard additions. Specimens of typical voltamograms of a sample solution shall be supplied with the method.

#### 2.4.4. Specific requirements for atomic absorption spectrometry (AAS)

This technique is basically mono-elemental and requires therefore optimisation of the experimental settings depending on the particular element to be quantified. Wherever possible, results shall be checked qualitatively and quantitatively by resorting to alternative absorption lines (ideally, two different lines shall be selected). Calibration standards shall be prepared in a solution matrix that matches as closely as possible that of the sample measurement solution (e.g. acid concentration or modifier composition). To minimise blank values, all reagents shall be of the highest available purity. Depending on the mode chosen to vaporise and/or atomise the sample, various types of AAS can be distinguished.

##### 2.4.4.1. Specific requirements for flame AAS

The instrument settings shall be optimised for each element. Especially the gas composition and flow rates have to be checked. A continuum source corrector shall be used to avoid interferences caused by background absorption. In the case of unknown matrices, a check shall be made as to whether or not background correction is required.

##### 2.4.4.2. Specific requirements for graphite furnace AAS

Contamination in the laboratory often affects accuracy when working at ultra-trace levels in the graphite furnace. Therefore high purity reagents, deionised water and inert plastic ware for sample and standard handling should be used. The instrument settings for each element shall be optimised. Especially the pre-treatment- and atomisation-conditions (temperature, time) and the matrix modification have to be checked.

Working under isothermal atomisation conditions (e.g. transverse heated graphite tube with integrated L'vov platform (8)) will reduce the influence of the matrix concerning the atomisation of the analyte. In combination with matrix modification and Zeeman-background correction (9), quantification by means of a calibration curve based upon measuring of aqueous standard solutions will be allowed.

#### 2.4.5. Specific requirements for hydride generation atomic absorption spectrometry

Organic compounds containing elements such as arsenic, bismuth, germanium, lead, antimony, selenium, tin and tellurium can be very stable and require oxidative decomposition to obtain correct results for total element content. Therefore, microwave digestion or high-pressure ashing under strong oxidative conditions is recommended. The greatest care shall be devoted to the complete and reproducible conversion of the elements into their corresponding hydrides.

The formation of arsenic hydride in hydrochloric acid solution with NaBH<sub>4</sub> depends on the oxidation state of arsenic (As III: fast formation, As V: longer formation period). To avoid a loss of sensitivity for the determination of As V with flow injection technique, caused by the short reaction time in this system, As V has to be reduced to As III after the oxidative decomposition. Potassium iodide/ascorbic acid or cysteine are suitable for this purpose. Blanks, calibration solutions and sample solutions shall be treated in the same way. Working with a batch system allows determining both arsenic species without affecting accuracy. Due to the delayed formation of As V-hydride, calibration shall be performed by peak area integration. The instrument settings shall be optimised. The gas flow, which transfers the hydride to the atomiser, is especially important and shall be checked.

#### 2.4.6. Specific requirements for cold vapour atomic absorption spectrometry

Cold vapour is used only in the case of mercury. Due to volatilisation and adsorption losses of elemental mercury, special care is necessary during the whole analysis. Contamination by reagents or the environment has to be avoided carefully.

Organic compounds containing mercury require oxidative decomposition to obtain correct results for total mercury content. For decomposition, sealed systems with microwave digestion or high pressure asher are to be used. Special care is required for cleaning the equipment that had contact with mercury.

Working with the flow injection technique is advantageous. For lower decision limits, adsorption of elemental mercury on gold/platinum adsorber followed by thermal desorption is recommended. Contact of the adsorber or the cell with moisture will disturb the measurement and shall be avoided.

#### 2.4.7. Specific requirements for inductively coupled plasma atomic emission spectrometry (ICP-AES)

Inductively coupled plasma atomic emission spectrometry (10) is a multi-element method, which allows a simultaneous measurement of various elements. To use the ICP-AES, the samples first have to be digested to decompose organic matrices. Sealed systems with microwave digestion or high pressure ashing shall be used. For a meaningful ICP-AES analysis, the instrument calibration and element or wavelength selection play an essential role. For instrument calibration, in case of linear calibration curves, it is usually necessary to measure calibration solutions of only four concentrations, because ICP-AES calibration curves are generally linear over four to six orders of magnitude of concentration. Calibration of the ICP-AES system should normally be performed with a multi-element standard, which shall be prepared in a solution that contains the same acid concentration as the measurement solution. For the linear curve, the element concentrations shall be checked.

The selection of wavelengths for measurement of the emission from the analytes is appropriate for the concentrations of the elements to be determined. When the analyte concentration falls outside of the working range of an emission line, a different emission line shall be used. At first, the most sensitive emission line (not interfered) shall be chosen, then a less sensitive line. When working at or near the detection limit, the most sensitive line for the respective analyte is usually the best choice. Spectral and background interferences are causing the major difficulties in ICP-AES. Possible interferences are e.g. simple background shift, sloping background shift, direct spectral overlap and complex background shift. Each of these interferences has its own causes and remedies. Depending on the matrices, interference corrections and optimisation of operating parameters shall be applied. Some interferences can be avoided by dilution or by adaptation of the matrices. With each batch of test samples analysed, reference and fortified material containing known amounts of the analyte(s) as well as blank material shall be treated in the same way as the test samples. For testing for a drift, the standard shall be checked e.g. after 10 samples. All reagents and the plasma gas shall be of the highest available purity.

#### 2.4.8. Specific requirements for inductively coupled mass spectrometry (ICP-MS)(11)

The determination of trace elements of average atomic mass, such as chromium, copper and nickel may be subject to strong interference from other isobaric and polyatomic ions. This can be circumvented only when a resolution power of at least 7 000-8 000 is available. Difficulties associated with the MS techniques include instrumental drift, matrix effects and molecular ion interference ( $m/z < 80$ ). Multiple internal standardisation covering the same mass range as the elements to be determined is required to correct instrumental drift and matrix effects.

Complete decomposition of organic matter in samples prior to ICP-MS measurements is required. As in the AAS, after digestion in sealed vessels, volatile elements e.g. iodine are to be transferred to a stable oxidation state. Most severe interference results from molecular ion combinations of argon (plasma gas), hydrogen, carbon, nitrogen and oxygen (dissolution acids, plasma gas impurities and entrained atmospheric gases) and the sample matrix. Complete digestion, background measurements, appropriate choice of analytical masses sometimes associated with a lower abundance (poorer detection limit) and of dissolution acids, e.g. nitric acid, are required to avoid interferences.

For the elements to be determined, interferences are to be excluded by the appropriate choice of specific analytical masses including confirmation of isotope ratios. Instrument response considering Fano-factors shall be checked for each measurement by the use of internal standards.

## 3. VALIDATION

Validation shall demonstrate that the analytical method complies with the criteria applicable for the relevant performance characteristics.

Different control purposes require different categories of methods. The following table determines which performance characteristic shall be verified for which type of method.

Table 9

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

		Detection limit CCB	Decision limit CCa	Trueness/recovery	Precision	Selectivity/ specificity	Applicability/ ruggedness/ stability
Qualitative methods	S	+	-	-	-	+	+
	C	+	+	-	-	+	+
Quantitative methods	S	+	-	-	+	+	+
	C	+	+	+	+	+	+

S = screening methods; C = confirmatory methods; + = determination is mandatory.

## 3.1. VALIDATION PROCEDURES

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

Validation can also be performed by conducting an interlaboratory study such as established by Codex Alimentarius, ISO or the IUPAC (12), or according to alternative methods such as single laboratory studies or in-house validation (13)(14). This part focuses on single laboratory studies (on in-house validation) using a modular approach. This approach consists of:

1. a set of common performance characteristics independent of the validation model used and
2. more specific model-dependent procedures as described in Table 10.

Table 10

## Model-independent and model-dependent performance parameters

Validation		
Model-independent performance parameters	Model-dependent performance parameters	
Common performance characteristics (3.1.1)	Conventional validation approach (3.1.2)	In-house validation approach (3.1.3)
Specificity Trueness Ruggedness: minor changes Stability	Recovery Repeatability Within-laboratory reproducibility Reproducibility Decision limit (CCa) Detection capability (CCβ) Calibration curves Ruggedness: major changes	Recovery Repeatability Within-laboratory reproducibility Reproducibility Decision limit (CCa) Detection capability (CCβ) Calibration curve Ruggedness



### 3.1.1. Model-independent performance characteristics

Irrespective of the validation approach chosen, the following performance characteristics have to be determined. To minimise the workload, a carefully designed and statistically sound approach can be used to combine experiments performed to determine different parameters.

#### 3.1.1.1. Specificity

For analytical methods, the power of discrimination between the analyte and closely related substances (isomers, metabolites, degradation products, endogenous substances, matrix constituents, etc) is important. Two approaches are necessary to check for interferences.

Therefore, potentially interfering substances shall be chosen and relevant blank samples shall be analysed to detect the presence of possible interferences and to estimate the effect of the interferences:

- select a range of chemically related compounds (metabolites, derivatives, etc.) or other substances likely to be encountered with the compound of interest that may be present in the samples;
- analyse an appropriate number of representative blank samples ( $n \geq 20$ ) and check for any interferences (signals, peaks, ion traces) in the region of interest where the target analyte is expected to elute;
- additionally, representative blank samples shall be fortified at a relevant concentration with substances that are likely to interfere with the identification and/or quantification of the analyte;
- after analysis, investigate whether:
  - the presence may lead to a false identification,
  - the identification of the target analyte is hindered by the presence of one or more of the interferences, or
  - the quantification is influenced notably.

#### 3.1.1.2. Trueness

In this paragraph, the determination of trueness (one component of accuracy) is described. Trueness can only be established by means of certified reference material (CRM). A CRM be used whenever available. The procedure is described in detail in ISO 5725-4 (5). 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 concentrations,
- calculate the trueness by dividing the detected mean concentration by the certified value (measured as concentration) and multiply by 100, to express the result as a percentage.

Trueness (%) = mean recovery-corrected concentration detected  $\times$  100/certified value.

If no CRM is available, instead of trueness, the recovery can be determined as described under 4.1.2.1 below.

#### 3.1.1.3. Applicability/ruggedness (minor changes)

Such studies use the deliberate introduction of minor reasonable variations by the laboratory and the observation of their consequences.

The pre-investigative studies have to be carried out by selecting 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 should be modified in an order of magnitude that matches the deviations usually encountered among laboratories.

- Identify possible factors that could influence the results.
- Vary each factor slightly.

- Conduct a ruggedness test using the approach of Youden (15)(16). (Other approved methods may be used at this point. The Youden approach, however, keeps the required time and effort to a minimum). The Youden approach is a fractional factorial design. 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 should be identified clearly in the method protocol.

The basic idea is not to study one alteration at a time but to introduce several variations at once. As an example, let 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. Let their alternative values be denoted by the corresponding lower case letters a, b, c, d, e, f and g. This results in  $2^7$  or 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 11). Eight determinations have to be made, which will use a combination of the chosen factors (A-G). The results of the determinations are shown in Table 11 below as S-Z.

Table 11

**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
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

For calculations see examples for ruggedness testing in 3.3.

#### 3.1.1.4. Stability

It has been observed that insufficient stability of the analyte or matrix constituents in the sample during storage or analysis may give rise to significant deviations in the outcome of the result of analysis. Furthermore, the stability of the calibration standard in solution should be checked. Usually analyte stability is well characterised under various storage conditions. Monitoring of the storage condition will form part of the normal laboratory accreditation system. When this is not known, examples are given below on how the stability can be determined.

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 (around the minimum required performance limit for substances for which no permitted limit has been established or around the permitted limit for other substances. Prepare both solutions of the analyte used for fortification and used in the final analysis solution, and 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 scheme:

Table 12

## Scheme for the determination of analyte stability in solution

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

- The storing time could be selected as one, two, three and four weeks or longer if necessary, e.g. until the first degradation phenomena are observable during identification and/or quantification. The maximum storing time and the optimum storing conditions have to be recorded.
- The calculation of the concentration of the analyte(s) in each aliquot should be carried out by using the solution of the analyte freshly prepared at the time of analysis as 100 %.

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

$C_i$  = concentration at time point

$C_{\text{fresh}}$  = concentration of fresh solution

## Stability of analyte(s) in matrix

- Whenever possible, incurred samples should be used. When no incurred material is available, matrix fortified with the analyte should be used.
- When incurred material is available, the concentration in the material should be determined while the material is still fresh. Further aliquots of material could be taken after one, two, four and 20 weeks and the concentrations should be determined. The tissue should be stored at least minus 20 °C or lower if required.
- 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 one, two, four and 20 weeks.

## 3.1.1.5. Calibration curves

When calibration curves are used for quantification:

- at least five levels (including zero) should be used in the construction of the curve,
- the working range of the curve should be described,
- the mathematical formula of the curve and the goodness-of-fit of the data to the curve should be described,
- acceptability ranges for the parameters of the curve should be described.

When serial calibration based on a standard solution is necessary, acceptable ranges shall be indicated for the parameters of the calibration curve, which may vary from series to series.

## 3.1.2. Conventional validation procedures

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 applicability/ruggedness above). For multi-analyte methods, several analytes can be analysed simultaneously, as long as possibly relevant interferences are ruled out previously. Several performance characteristics can be determined in a similar way. So, to minimise 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).

## 3.1.2.1. Recovery

If there is no CRM available, the recovery has to be determined by experiments using fortified blank matrix using, for example, the following scheme:

- select 18 aliquots of a blank material and fortify six aliquots at each of 1, 1,5 and 2 times the minimum required performance limit or 0,5, 1 and 1,5 times the permitted limit,
- analyse the samples and calculate the concentration present in each sample,

- using the equation below, calculate the recovery for each sample,
- calculate the mean recovery and CV from the six results at each level,
- % Recovery =  $100 \times \text{measured content}/\text{fortification level}$ .

This conventional method for the determination of recovery is a variant of the standard addition method described in 3.5, when:

- the sample is considered as a blank sample instead of a sample to be analysed,
- it is considered that yield <sup>(1)</sup> and recovery <sup>(2)</sup> are similar for the two test portions,
- the test samples have the same masses and the test portion extracts the same volumes,
- the amount of the calibration standard that is added to the second (spiked) test portion is noted  $x_{\text{ADD}}$  ( $x_{\text{ADD}} = \rho_A \cdot V_A$ ),
- $x_1$  is the measured value for the blank and  $x_2$  the measured value for the second (spiked) test portion,
- then, % Recovery =  $100 (x_2 - x_1)/x_{\text{ADD}}$ .

When any of the above conditions is (or is supposed) not to be achieved, then the complete procedure for determination of the recovery by mean of the standard addition method as described in 3.5 has to be performed.

#### 3.1.2.2. Repeatability

- Prepare a set of samples of identical matrices, fortified with the analyte to yield concentrations equivalent to 1, 1,5 and 2 times the minimum required performance limit or 0,5, 1 and 1,5 times the permitted limit.
- At each level the analysis should be performed with at least six replicates.
- Analyse the samples.
- Calculate the concentration detected in each sample.
- Find the mean concentration, standard deviation and the coefficient of variation (%) of the fortified samples.
- Repeat these steps on at least two other occasions.
- Calculate the overall mean concentrations and CVs for the fortified samples.

#### 3.1.2.3. Within-laboratory reproducibility

- Prepare a set of samples of specified test material (identical or different matrices), fortified with the analyte(s) to yield concentrations equivalent to 1, 1,5 and 2 times the minimum required performance limit or 0,5, 1 and 1,5 times the permitted limit.
- At each level the analysis should be performed with at least six replicates.
- Repeat these steps on at least two other occasions with different operators and different environmental conditions, e.g. different batches of reagents, solvents etc., different room temperatures, different instruments, etc. if possible.
- Analyse the samples.
- Calculate the concentration detected in each sample.
- Find the mean concentration, standard deviation and the coefficient of variation (%) of the fortified samples.

#### 3.1.2.4. Reproducibility

When reproducibility has to be verified, laboratories should participate in collaborative studies according to ISO 5725-2 (5).

#### 3.1.2.5. Decision Limit (CC<sub>α</sub>)

The decision limit has to be established according to the requirements for identification or identification plus quantification as defined under 'Performance criteria and other requirements for analytical methods' (part 2).

<sup>(1)</sup> Yield: that fraction of mass of the analyte contained in the sample, which is present in the final extract.

<sup>(2)</sup> Recovery (here): that fraction of mass of the analyte added to the sample, which is present in the final extract. Throughout the rest of the document it is assumed that yield and recovery are equal and therefore only the term 'recovery' is used.

In the case of substances for which no permitted limit has been established, CC $\alpha$  can be established:

- either by the calibration curve procedure according to ISO 11843 (17) (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 minimum required performance level in equidistant steps. Analyse the samples. After identification, plot the signal 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 ( $\alpha = 1\%$ ),
- or by analysing at least 20 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 decision limit. This is applicable to quantitative and qualitative assays.

In the case of substances with an established permitted limit, CC $\alpha$  can be established:

- either by the calibration curve procedure according to ISO 11843 (17) (here referred to as critical value of the net state variable). In this case blank material shall be used, which is fortified around the permitted limit in equidistant steps. Analyse the samples. After identification, plot the signal against the added concentration. The corresponding concentration at the permitted limit plus 1,64 times the standard deviation of the within-laboratory reproducibility equals the decision limit ( $\alpha = 5\%$ ),
- or by analysing at least 20 blank materials per matrix fortified with the analyte(s) at the permitted limit. The concentration at the permitted limit plus 1,64 times the corresponding standard deviation equal the decision limit ( $\alpha = 5\%$ ).

See also Article 5 and point 3.2.

#### 3.1.2.6. Detection capability (CC $\beta$ )

The detection capability should be determined according to the requirements for screening, identification or identification plus quantification as defined (see part 2).

In the case of substances for which no permitted limit has been established, CC $\beta$  can be established by:

- the calibration curve procedure according to ISO 11843 (17) (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 minimum required performance level in equidistant steps. Analyse the samples. After identification, plot the signal against the added concentration. The corresponding concentration at the decision limit plus 1,64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability ( $\beta = 5\%$ ),
- analysing at least 20 blank materials per matrix fortified with the analyte(s) at the decision limit. Analyse the samples and identify the analytes. The value of the decision limit plus 1,64 times the standard deviation of the within-laboratory reproducibility of the measured content equals the detection capability ( $\beta = 5\%$ ),
- where no quantitative results are available, the detection capability can be determined by the investigation of fortified blank material at and above the decision limit. In this case the concentration level, where only  $\leq 5\%$  false compliant results remain, equals the detection capability of the method. Therefore, at least 20 investigations for at least one concentration level have to be carried out in order to ensure a reliable basis for this determination.

In the case of substances for which a permitted limit has been established, CC $\beta$  can be established:

- either by the calibration curve procedure according to ISO 11843 (17) (here referred to as minimum detectable value of the net state variable). In this case representative blank material shall be used, which is fortified around the permitted limit in equidistant steps. Analyse the samples and identify the analyte(s). Calculate the standard deviation of the mean measured content at the decision limit. The corresponding concentration at the value of the decision limit plus 1,64 times the standard deviation of the within-laboratory reproducibility equals the detection capability ( $\beta = 5\%$ ),
- or by analysing at least 20 blank materials per matrix fortified with the analyte(s) at the decision limit. The value of the decision limit plus 1,64 times the corresponding standard deviation equals the detection capability ( $\beta = 5\%$ ).

See also section 3.2.

### 3.1.2.7. Ruggedness (major changes)

The analytical method should be tested under different experimental conditions, which include for example, different species, different matrices or different sampling conditions. The changes introduced should be major. The importance of these changes can be evaluated, for instance, using the Youden approach (15)(16). Each performance characteristic should be determined for all major changes that have been shown to have a significant effect on the performance of the assay.

### 3.1.3. Validation according to alternative models

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. In the following an example for an alternative approach is given. When applying e.g. the in-house validation model, the performance characteristics are determined in a manner that permits validation for major changes within the same validation procedure. This requires design of an experimental plan for validation.

#### 3.1.3.1. Experimental plan

An 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 shall consider the sample populations that will be analysed in the laboratory in the future in order to select the most important species and those factors which may influence the measurement results. Subsequently, the concentration range shall be chosen in a purpose-adapted way according to the level of interest.

Example:

- several analytes can be investigated simultaneously with the analytical method being validated,
- two variations of the leading factor have been identified (A and B). Leading factors form the basis on which the factor levels are combined. These leading factors may include factors such as species or matrix. In this example the leading factor was varied on two levels, i.e. two different species (species A and B) were considered. In general, it is possible to vary the leading factors on more than two levels, which only increase the number of analyses to be performed,
- the selected factors are to be varied on two levels (indicated as either + or -).

**Table 13**

#### Examples for factors considered important for a validation procedure

Gender of the animal	(factor 1)
Breed	(factor 2)
Transport conditions	(factor 3)
Storing conditions	(factor 4)
Freshness of the sample	(factor 5)
Fattening conditions	(factor 6)
Different operators with different experience	(factor 7).

**Table 14**

#### Possible experimental plan for the above example

Species	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Sample No
A	+	+	+	+	-	+	-	1
A	+	+	-	-	+	-	-	2
A	+	-	+	-	-	-	+	3
A	+	-	-	+	+	+	+	4
A	-	+	+	-	+	+	+	5
A	-	+	-	+	-	-	+	6
A	-	-	+	+	+	-	-	7
A	-	-	-	-	-	+	-	8

Species	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Sample No
B	+	+	+	+	+	-	+	9
B	+	+	-	-	-	+	+	10
B	+	-	+	-	+	+	-	11
B	+	-	-	+	-	-	-	12
B	-	+	+	-	-	-	-	13
B	-	+	-	+	+	+	-	14
B	-	-	+	+	-	+	+	15
B	-	-	-	-	+	-	+	16

As each sample (each factor level combination) has to be spiked with four different concentrations around the level of interest, and one blank sample shall be analysed for each level,  $5 \times 16 = 80$  analyses have to be performed for the entire validation experiment.

From these 80 measurement results it is possible to compute (13)(14).

#### Recovery

- repeatability per concentration level ( $s_{ir}$ ),
- within-laboratory reproducibility per concentration level ( $s_{lr}$ ),
- decision limit ( $CC\alpha$ ),
- detection capability ( $CC\beta$ ),
- power curve ( $\beta$ -error rate versus concentration (see 3.1.3.2),
- ruggedness of major changes; ruggedness to minor changes can be determined according to paragraph 3.1.1.3,
- 16 sample-related calibration curves,
- one overall calibration curve,
- prediction interval of the overall calibration curve,
- matrix-induced deviations ( $s_{mat}$ ),
- run-induced deviations ( $s_{run}$ ),
- effect of the individual factors on the measurement results.

These performance characteristics allow the comprehensive evaluation of the performance level of the method, since not only the influence of the individual factors is investigated, but also the relevant combinations of these factors. With the help of this experiment design it is possible to decide if one or the other of the selected factors shall be excluded from the overall calibration curve, because it significantly deviates from the standard deviations of the other factors.

#### 3.1.3.2. Power curve

The power curve provides information about the detection capability of the method within the chosen concentration range. It refers to the  $\beta$ -error risk when applying the investigated method. The power curve allows to calculate the detection capabilities for the respective categories (screening, confirmation) or types (qualitative or quantitative) of methods for a certain  $\beta$ -error (e.g. 5 %).

Figure 1  
Power curve

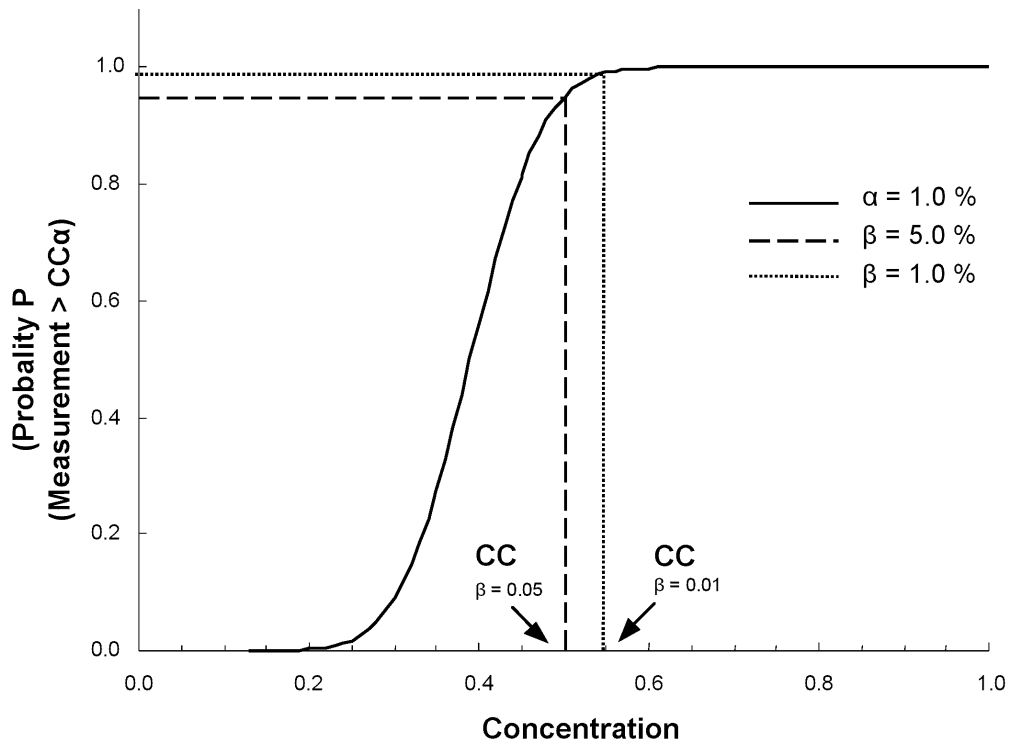


Figure 1 shows an example of the graphical establishment of detection capability ( $CC\beta$ ) of an analytical method. This particular method has a remaining risk of taking a false decision of 5 % at a concentration of 0,50  $\mu\text{g}/\text{kg}$ . At a concentration of 0,55  $\mu\text{g}/\text{kg}$  the risk of taking a false compliant decision decreases to 1 %.

3.1.3.3. Reproducibility

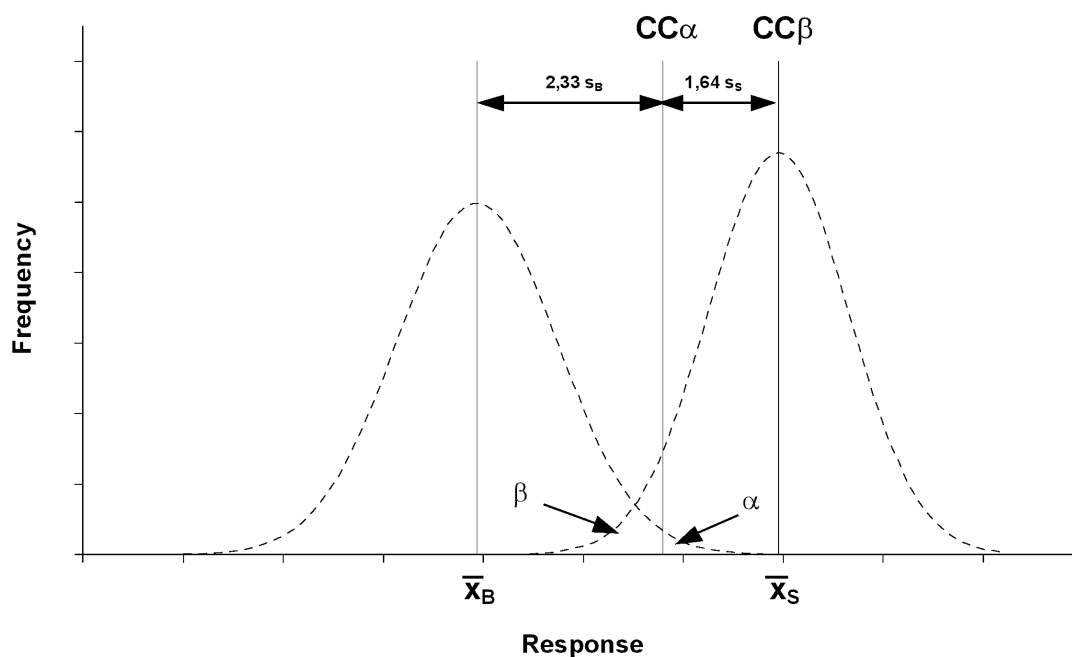
The determination of a method's reproducibility by the single laboratory studies (in-house validation) concept requires repeated participation in proficiency studies in accordance with ISO guide 43-1 (3) and 43-2 (4). The laboratories are allowed to choose their own methods, provided these methods are used under routine conditions. The standard deviation of the laboratory can be used to assess the reproducibility of the method.



## 3.2. GRAPHICAL REPRESENTATION OF THE DIFFERENT ANALYTICAL LIMITS

Figure 2

Substances for which no permitted limit has been established



$\bar{x}_S$  Mean response value of the contaminated sample

$s_B$  Standard deviation of the blank sample (determined under within-laboratory-reproducibility conditions)

$s_S$  Standard deviation of the contaminated sample (determined under within-laboratory-reproducibility conditions)

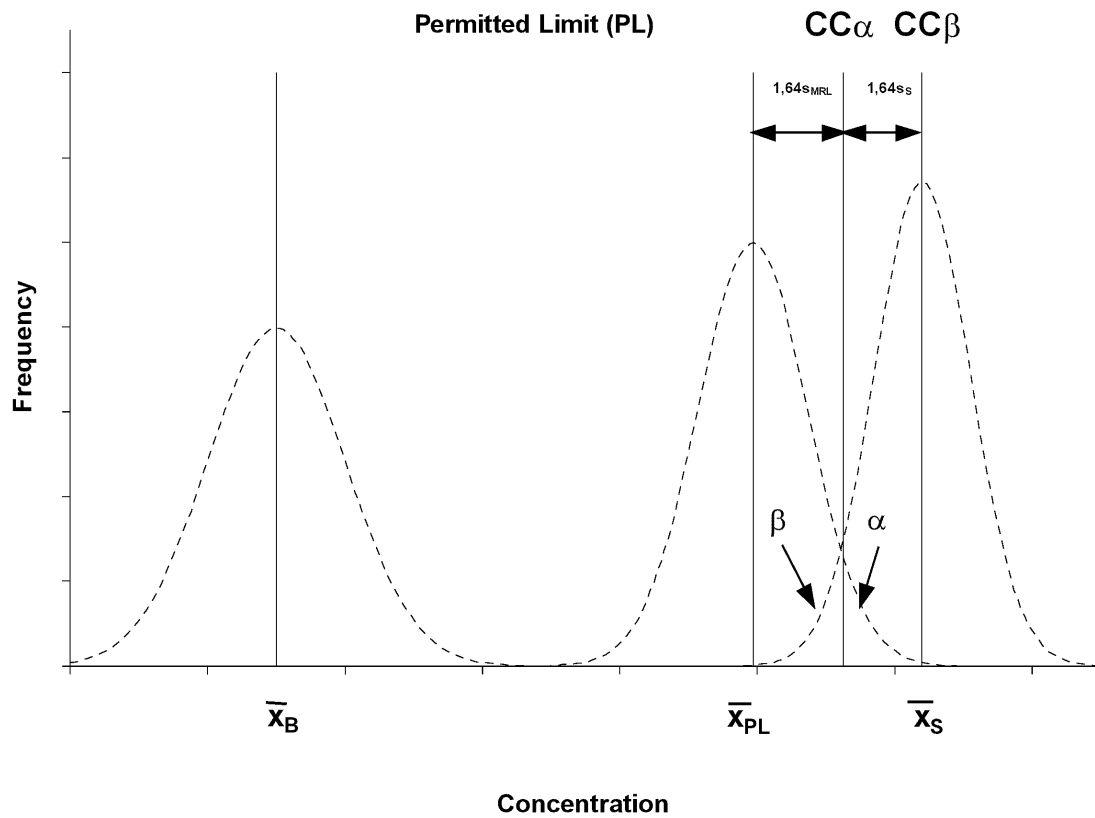
$\alpha$  Rate of false non-compliant results

$\beta$  Rate of false compliant results

$CC\alpha$  Response with a given  $\alpha$ -error and 50 %  $\beta$ -error

$CC\beta$  Response with a very small  $\alpha$ -error and  $\beta$ -error

Figure 3  
Substances with an established permitted limit



- $\bar{X}_B$  mean 'concentration' of the blank sample
- $\bar{X}_{PL}$  mean concentration of the sample containing the analyte at the permitted limit
- $\bar{X}_S$  mean concentration of the contaminated sample
- $S_{PL}$  Standard deviation of the sample containing the analyte at the permitted limit (determined under within-laboratory-reproducibility conditions)
- $S_S$  Standard deviation of the containing sample (determined under within-laboratory-reproducibility conditions)
- $\alpha$  rate of false non-compliant results
- $\beta$  rate of false compliant results
- $CC_\alpha$  Response with a given  $\alpha$ -error and 50 %  $\beta$ -error
- $CC_\beta$  Response with a very small  $\alpha$ -error and a given  $\beta$ -error

### 3.3. CALCULATION EXAMPLE FOR RUGGEDNESS TESTING OF MINOR CHANGES ACCORDING TO THE YOUTDEN APPROACH (16)

#### Comparison of averages (A)

$A_A = \Sigma(A_i)/4$	Compare the averages of the capital letters ( $A_A$ to $A_G$ ) with the averages of their corresponding small letters ( $A_a$ to $A_g$ ). If a factor have an effect, the difference will be significant larger than the differences of the other factors.
$A_B = \Sigma(B_i)/4$	
$A_C = \Sigma(C_i)/4$	A robust method should not be affected by changes encountered almost certainly between laboratories.
$A_D = \Sigma(D_i)/4$	
$A_E = \Sigma(E_i)/4$	If there is no outstanding difference, the most realistic measure of the random error is given by the seven differences.
$A_F = \Sigma(F_i)/4$	
$A_G = \Sigma(G_i)/4$	
$A_a = \Sigma(a_i)/4$	
$A_b = \Sigma(b_i)/4$	
$A_c = \Sigma(c_i)/4$	
$A_d = \Sigma(d_i)/4$	
$A_e = \Sigma(e_i)/4$	
$A_f = \Sigma(f_i)/4$	
$A_g = \Sigma(g_i)/4$	

#### Differences (D)

$$\begin{aligned} D_a &= A - a = \Sigma(A_i) - \Sigma(a_i) \\ D_b &= B - b = \Sigma(B_i) - \Sigma(b_i) \\ D_c &= C - c = \Sigma(C_i) - \Sigma(c_i) \\ D_d &= D - d = \Sigma(D_i) - \Sigma(d_i) \\ D_e &= E - e = \Sigma(E_i) - \Sigma(e_i) \\ D_f &= F - f = \Sigma(F_i) - \Sigma(f_i) \\ D_g &= G - g = \Sigma(G_i) - \Sigma(g_i) \end{aligned}$$

#### Squares of differences ( $D_i^2$ )

$$\begin{aligned} D_a^2 &= \text{value a} \\ D_b^2 &= \text{value b} \\ D_c^2 &= \text{value c} \\ D_d^2 &= \text{value d} \\ D_e^2 &= \text{value e} \\ D_f^2 &= \text{value f} \\ D_g^2 &= \text{value g} \end{aligned}$$

Standard deviation of the differences  $D_i$  ( $S_{D_i}$ ):

$$S_{D_i} = \sqrt{2 * \Sigma(D_i^2 / 7)}$$

When  $S_{D_i}$  is significantly larger than the standard deviation of the method carried out under within-laboratory reproducibility conditions according (see above) it is a foregone conclusion that all factors together have an effect on the result even if every single factor does not show a significant influence and that the method is not sufficiently robust against the chosen modifications.

### 3.4. CALCULATION EXAMPLES FOR THE IN-HOUSE VALIDATION PROCEDURE

Examples and calculations for the in-house validation protocol as described under validation according to alternative models (3.1.3) (13) (14).

### 3.5. EXAMPLES FOR THE STANDARD ADDITION METHOD

A test sample with a content T of the analyte is divided in two test portions 1 and 2 of respective masses  $m_1$  and  $m_2$ . The test portion 2 is spiked with a volume  $V_A$  of a solution of concentration  $\rho_A$  of the analyte. Two extracts of the test portions of respective volumes  $V_1$  and  $V_2$  are obtained after extraction and purification steps of the method. The recovery of the analyte is supposed to be rc. Both extracts are assayed with a measurement method of sensitivity b and give an analytical response of  $x_1$  and  $x_2$  respectively.

If assumed that rc and b are the same for the analyte in the native sample and in the spiked sample, then the content T can be calculated as:

$$T = x_1 \cdot V_1 \cdot \rho_A \cdot V_A / (x_2 \cdot V_2 \cdot m_1 - x_1 \cdot V_1 \cdot m_2)$$

The method will allow the determination of the recovery rc. Then, in addition with the assay described above, part of the extract of the test portion 1 (volume  $V_3$ ) is spiked with a known amount  $\rho_B \cdot V_B$  of the analyte and assayed. The analytical response is  $x_3$  and the recovery is:

$$rc = x_2 \cdot V_1 \cdot V_2 \cdot \rho_B \cdot V_B / [x_3 \cdot V_1 \cdot V_3 (T \cdot m_2 + \rho_A \cdot V_A) - x_2 \cdot V_2 \cdot T \cdot m_1 (V_3 - V_B)]$$

Moreover, it is possible to calculate the sensitivity b, as:

$$b = x_1 \cdot V_1 / rc \cdot T \cdot m_1$$

All conditions of application and details have been described (18).

#### 4. ABBREVIATIONS USED

AAS	Atomic absorption spectrometry
AES	Atomic emission spectrometry
AOAC-I	Association of Official Analytical Chemists INTERNATIONAL
B	bound fraction (immunoassays)
CI	chemical ionisation
CRM	Certified reference material
CV	coefficient of variation
2 D	two dimensional
DAD	diode array detection
DPASV	differential pulse anodic stripping voltametry
ECD	electron capture detection
EI	electronic impact ionisation
GC	gas chromatography
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HRMS	high resolution (mass spectrometry)
ICP-AES	inductively coupled plasma-atomic emission spectrometry
ICP-MS	inductively coupled plasma-mass spectrometry
IR	infrared
ISO	International Standard Organisation
LC	liquid chromatography
LR(MS)	low resolution (mass spectrometry)
MRPL	Minimum required performance limit
MS	mass spectrometry
m/z	mass/charge ratio
RF	relative migration to the solvent front (TLC)
RSDL	relative standard deviations of the laboratory
SIM	selected ion monitoring
TLC	thin layer chromatography
UV	ultra violet light
VIS	visible light

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