DRAFT GUIDANCE DOCUMENT ON SINGLE LABORATORY VALIDATION OF QUANTITATIVE ANALYTICAL METHODS IN SUPPORT OF PRE- AND POST-REGISTRATION DATA REQUIREMENTS FOR PLANT PROTECTION AND BIOCIDAL PRODUCTS

INTRODUCTION

1.   Accurate, precise and specific quantitative analytical methods are required for confirmation of identity, purity and stability of active substances (AS), and/or significant (*i.e.* $\geq 0.1\%$ relative to AS) or relevant (of toxicological, ecotoxicological or environmental significance) impurities (at any concentration) in technical materials and preparations containing active substances.

2.   This guideline is based on existing guidance documents and best practices from agencies and professional organizations pertinent to single laboratory validation of quantitative analytical methods.

- SANCO/3030/99 rev.4, 11 July 2000 (1) provides guidance for generating, validating (single laboratory validation) and reporting quantitative analytical methods in support of registration data requirements for the European Community.

- APVMA GL69 October 2004 (2) provides guidance for generating, validating (single laboratory validation) and reporting quantitative analytical methods in support of registration data requirements for Australia.

- AOAC, CIPAC, ISO and IUPAC (3) have published guidelines, in some cases harmonized guidelines, for single laboratory validation of quantitative analytical methods.

3.   Single laboratory validation is the logical conclusion to the method development process and provides assurance that the method has met specific requirements of performance. By its nature, a single laboratory validation does not provide data on the expectations for the method when used by other laboratories. A single laboratory validation may precede a more rigorous multi-laboratory collaborative validation or method transfer study. Neither of these is addressed in this guideline.

PARAMETERS FOR METHOD VALIDATION

4.   Method validation is a series of quality tests involving the quantitation of an analyte or analytes in a specific sample matrix using a specific laboratory procedure and measurement system. The validation data verifies that the laboratory procedure and measurement system are suitable to the purpose of the analysis. The parameters to be determined and the acceptance criteria differ slightly according to the analyte (active substance or impurity) and the sample (technical material or preparation). The validation process obtains performance data on the following parameters:

a) *Specificity (Selectivity)* – The ability of a method to distinguish between the analyte (active substance or impurity) being measured and other substances in the sample matrix.

b) *Linearity* – The ability of a method to produce an acceptable linear correlation between the measured response and the concentration of the analyte in the sample.

c) *Accuracy (Recovery)* – The degree to which the measured value for the analyte in a sample corresponds to the accepted, true or reference value.

d) *Precision* (*repeatability*) – The closeness of agreement independent test results with the same method, on identical test material, on the same equipment, by the same operator, in the same laboratory within short intervals of time.

e) *Limit of Quantification (LOQ)* – The lowest concentration of analyte in a sample that can be determined or quantitated with acceptable relative standard deviation.

f) *Limit of Detection* – The lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated as an exact value.

TECHNICAL MATERIALS

Determination of active substances and impurities

5.   The method validation parameters for methods to determine active substance(s) in technical materials are:

a) *Specificity (Selectivity)* – The degree of interference in the determination of impurities in the technical material is reported. Interferences from impurities should not contribute more than 3% to measured response of the target analyte. If the active substance is specified as being optically pure, the method must support this. Where an active substance consists of more than one isomer, analogue, etc., the method should be capable of determining the individual components present, with the exception that this requirement does not include determination of optical isomers in racemic mixtures, or isomers which are more or less equally active. For details of confirmatory techniques, see paragraph 7.

b) *Linearity* – The analytical calibration should extend over a range appropriate to the lowest and highest nominal concentration of the analyte in relevant analytical matrices + at least 20%. Either duplicate determinations at three or more concentrations or single determinations at five or more concentrations, is made. The equation of the calibration line and the correlation coefficient (r) must be reported and a typical calibration plot submitted. The limits of the linear range should be given, *e.g.* in % w/w. Where a linear correlation coefficient (r) is < 0.99, an explanation of how accurate calibration is to be maintained should be submitted. Where a non-linear calibration is used, an explanation (including how calibration accuracy is to be maintained) is provided.

c) *Accuracy (Recovery)* – The determination of accuracy for the active substance in the technical material is not required. An assessment of the accuracy of the method can be made through the assessment of interference and precision.

d) *Precision* (*repeatability*) – Details of the precision of the method are required for the active substance in the technical material. A minimum of five separate sample determinations is made and the mean, % RSD and number of determinations reported. The acceptability of the % RSD may be assessed using the modified Horwitz equation (details are given in Appendix). Where outliers have been identified using appropriate statistical methods (such as Dixon's or Grubbs Test) this should be made clear and justified. A maximum of one outlier may be discarded at each

fortification level. Where more than one outlier has been identified, additional determinations must be included.

6. The method validation parameters to determine significant and relevant impurities in technical materials are:

a) *Specificity (Selectivity)* – Where the technical material contains more than one significant and/or relevant impurity the method(s) must be capable of determining each in the presence of the other and in the presence of the active substance. Where more than one isomer, analogue, etc., of a relevant impurity is known to occur, the method(s) should distinguish between individual isomers/ analogues where this is relevant. Specificity for the analysis of impurities should be addressed to the extent that the technical material is properly characterised. For details of confirmatory techniques, see paragraph 7.

b) *Linearity* – as detailed in paragraph 5.b.

c) *Accuracy (Recovery)* – The accuracy of the method(s) for significant and/or relevant impurities should be reported as mean recovery and relative standard deviation (d)) in the technical material. At least two recovery determinations should be made on representative samples containing a known quantity of the analyte. Standard addition is an acceptable method of determining recoveries of impurities in the technical material. Recoveries should be determined at levels appropriate to the material specification. Where the process of recovery is identical to that used for calibration, for example, if there is no separation of the impurity from the active substance prior to the determinative step, there is no measure of recovery. In these cases, an estimate of the accuracy of the analytical technique may be made by an assessment of the linearity of matrix calibration by standard addition and by a comparison of accuracy with other techniques. Further discussion of the measurement of accuracy and statistical treatment of results is given in Appendix.

d) *Precision* (*Repeatability*) – Details of the precision of the method are required for significant and/or relevant impurities in technical materials, as detailed in paragraph 5.d.

LOQ – The LOQ must be reported for relevant impurities and must take into account the concentration of analyte which is of toxicological or environmental significance, or the concentration which is formed during storage of the product, where this is relevant.

The LOQ may be determined by preparing a standard solution at the estimated LOQ concentration (based on preliminary studies). The solution should be injected and analyzed several times (n = 6 to 10 times). The average measurement response (X) and standard deviation (SD) of the n results are calculated and the standard deviation should be less than 20% relative standard deviation (RSD). If the RSD exceeds 20%, a new standard solution of higher concentration is prepared and the above procedure repeated. The reported LOQ is calculated as:

$$LOQ = X + (10 \times SD).$$

The LOQ must be reported for significant impurities. In order to support the declared technical specification, the LOQ should be at or below the anticipated quantity of the significant impurity in the technical material.

7. The confirmation of analyte identification is done as follows:

a) The analytical method(s) used for the quantification of the active substance and impurities (significant and relevant) in the technical material may not establish the unequivocal identity of the analytes. As part of the validation and application of the method it may be a requirement of some regulatory authorities to confirm the identity of the active substance and impurities.

b) If the analysis has been performed using a highly specific/selective method then confirmation of analyte identity will have been established. Methods regarded as highly specific/selective are GC-MS and LC-MS, with three ions validated and LC-MS/MS, with two ion transitions validated.

c) Where the primary method of determination cannot provide unequivocal identification and quantification of the analyte, confirmation can be achieved using several approaches:

   - Analysis using a different analytical method, including using a different separation technique. The method should be fully validated.

   - Chromatographic peak (fraction) collection followed by off-line spectroscopic analysis (e.g. MS, IR, NMR). Full interpretation of the data to support the identity is required.

   - HPLC-DAD, but only where the UV spectrum of the analyte is characteristic. A retention time match to an authentic reference standard and a match to the corresponding UV spectrum of the analyte in the technical material must be established. The HPLC-UV method should be fully validated.

d) Where the primary method is not chromatographic, for example titration, a case justifying the specificity/selectivity of the method must be made.

e) Methods collaboratively tested by CIPAC may not require confirmation of identity in some jurisdictions.

PREPARATIONS

Determination of active substance(s) and relevant impurities

8.   The method validation parameters for methods to determine active substance(s) in preparations are:

a) *Selectivity (Specificity)* – Where the preparation contains more than one active substance the method(s) must be capable of determining each in the presence of the other. The degree of interference should be reported. Interferences from other substances present in the preparation, including other active substances, should not contribute more than 3% to the measured response for each active substance or the sum of the components of that active substance. If the active substance is specified as being optically pure, the method must support this. Where more than one isomer, analogue, etc., of an active substance is known to occur, the method(s) should distinguish between individual isomers/analogues where this is relevant, with the exception that this requirement does not include determination of optical isomers in racemic mixtures.

b) *Linearity* – as detailed in paragraph 5.b.

c) *Accuracy (Recovery)* – The accuracy of the method should be reported as mean recovery for the pure active substance in the preparation. The accuracy of the method may vary across the range of the method and therefore accuracy must be determined as different fortification levels. The accuracy should cover at least three concentrations (80, 100 and 120%) in the expected range.

Samples should ideally be laboratory-prepared co-formulant mixes to which a known quantity of analyte is added and the whole sample analysed to reduce sampling error. Where it is not possible to prepare a sample matrix without the presence of the analyte, or there are difficulties in replicating the sample to be analysed, the standard addition method may be used. Further discussion of the measurement of accuracy and statistical treatment of results is given in the Appendix.

d) *Precision* (*repeatability*) – Details of the precision of the method are required for the active substance in the formulation, as detailed in paragraph 5.d.

9. The method validation parameters to determine relevant impurities in preparations are:

a) *Specificity (Selectivity)* – Where the preparation contains more than one relevant impurity the method(s) must be capable of determining each in the presence of the other and in the presence of the active substance. Where more than one isomer, analogue, etc., of a relevant impurity is known to occur, the method(s) should distinguish between individual isomers/analogues where this is relevant.

Specificity for the analysis of relevant impurities should be addressed to the extent that the technical material is properly characterised. For details of confirmatory techniques, see paragraph 11.

b) *Linearity* – as detailed in paragraph 5.b.

c) *Accuracy (Recovery)* – The accuracy of the method(s) for relevant impurities should be reported as mean recovery and relative standard deviation (d) in the technical material. At least two recovery determinations should be made on representative samples containing a known quantity of the analyte. Standard addition is an acceptable method of determining recoveries of impurities in the technical material. Recoveries should be determined at levels appropriate to the material specification. Where the process of recovery is identical to that used for calibration, for example, if there is no separation of the impurity from the active substance prior to the determinative step, there is no measure of recovery. In these cases, an estimate of the accuracy of the analytical technique may be made by an assessment of the linearity of matrix calibration by standard addition and by a comparison of accuracy with other techniques.

Further discussion of the measurement of accuracy and statistical treatment of results is given in Appendix.

d) *Precision* (*Repeatability*) – Details of the precision of the method are required for relevant impurities in the preparation, as detailed in paragraph 5.d.

e) *LOQ* – The LOQ must be reported for relevant impurities and must take into account the concentration of analyte which is of toxicological or environmental significance, or the concentration which is formed during storage of the product, where this is relevant.

The LOQ may be determined by preparing a standard solution at the estimated LOQ concentration (based on preliminary studies). The solution should be injected and analyzed several times (n = 6 to 10 times). The average measurement response (X) and standard deviation (SD) of the n results should be calculated and the standard deviation should be less than 20% relative standard deviation (RSD). If the RSD exceeds 20%, a new standard solution of higher concentration should be prepared and the above procedure repeated. The reported LOQ is calculated as:

$$LOQ = X + (10 \text{ x SD})$$

The LOQ must also be reported for significant impurities. In order to support the declared technical specification, the LOQ should be at or below the anticipated quantity of the significant impurity in the technical material.

10.   The confirmation of analyte definition is done as follows:

a) The analytical methods used for the quantification of relevant impurities in the preparation may not establish the unequivocal identity of the relevant impurity. As part of the validation and application of the method it may be a requirement of some regulatory authorities to confirm the identity of relevant impurities in the preparation.

b) If the analysis has been performed using a highly specific/selective method then confirmation of analyte identity will have been established. Methods regarded as highly specific/selective are GC-MS and LC-MS, with three ions validated and LC-MS/MS, with two ion transitions validated.

c) Where the primary method of determination cannot provide unequivocal identification and quantification of the analyte, confirmation can be achieved using several approaches:

- Analysis using a different analytical method, including using a different separation technique. The method should be fully validated.

- Chromatographic peak (fraction) collection followed by off-line spectroscopic analysis (e.g. MS, IR, NMR). Full interpretation of the data to support the identity is required.

- HPLC-DAD, but only where the UV spectrum of the analyte is characteristic. A retention time match to an authentic reference standard and a match to the corresponding UV spectrum of the analyte in the preparation must be established. The HPLC-UV method should be fully validated.

d) Where the primary method is not chromatographic, for example titration, a case justifying the specificity/selectivity of the method must be made.

e) Methods collaboratively tested by CIPAC may not require confirmation of identity in some jurisdictions.

DATA REQUIREMENTS AND REPORTING METHODS

11.   A full description of a validated method shall be provided that includes details of equipment with associated operating parameters, materials, sample collection procedures, standard and/or sample preparation procedures, reagent preparation procedures, calculation procedures, pertinent references to ancillary documents and details related to hazards or necessary precautions. The applicability and limitations of the method should also be described. Example instrumental output like chromatograms, spectra, titration curves, etc. with applicable annotations identifying key features to be used in quantification. The example instrument output shall include analyses of control blank(s), analytical standard(s) or matrix-matched standard(s), lowest fortification(s) and nominal or expected concentration(s).

12.   The validation data may be amended to the method or provided as a separate report. All relevant data collected during validation should be provided. These data include the source and purity of reference substances, reagents and blank sample matrices. Annotated copies of all instrument output

(chromatograms, spectra, titration curves, etc.). The validation report shall list each method validation parameter and associated acceptance criterion and the validation data that demonstrates satisfactory performance of the method relative to the validation parameter.

LITERATURE

(1) SANCO/3030/99 rev. 4 (2000). European Commission Directorate General for Health and Consumer Affairs. *Technical Material and Preparations: Guidance for generating and reporting methods of analysis in support of pre- and post-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414.* Working document, 11th July 2000.

(2) APVMA GL69 (2004). Australian Pesticides & Veterinary Medicines Authority. *Guidelines for the validation of analytical methods for active constituent, agricultural and veterinary chemical products.* October 2004.

(3) Thompson M., Ellison S.L.R. and Wood R., "Harmonized Guidelines for Single-Laboratory Validation of Methods of Analisis," *Pure Appl. Chem.* 2002. **74**(5) 835-855.

(4) Brown S.D., Sum S.T. and Despagne F., *Anal. Chem.* 1996. **68** 21R-61R.

(5) Green J.J., *Analytical Chemistry News and Features.* 1996, May 1st. 305A-309A.

(6) Miller J.C. and Miller J.N., *Statistics for Analytical Chemistry (2nd edition).* 1988. Ellis Horwood. London.

(7) Grubbs F.E and Beck G., *Technometrics.* 1972. 14 847.

(8) Dixon W.J. *Ann. Math. Stat.* 1951. **22** 68.

(9) Albert R. and Horwitz W. *Anal. Chem.* 1997. **69** 789-790.

DEFINITIONS AND ABBREVIATIONS

AOAC – Association of Official Analytical Chemists

APVMA – Australian Pesticides & Veterinary Medicines Authority

AS – Active Substance

CIPAC – Collaborative International Pesticide Analytical Council

GC-MS – Gas Chromatography-Mass spectrometry

HPLC-DAD – High Performance Liquid Chromatography-Diode Array Detectors

LC-MS – Liquid Chromatography-Mass spectrometry

IR – Infrared Spectroscopy

ISO – International Organisation for Standadization

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IUPAC – International Union of Pure and Applied Chemistry

LOQ – Limit of Quantification

NMR – Nuclear Magnetic Resonance

RSD-SD – (Relative) Standard Deviation

SANCO (DG) – Directorate General for Health and consumer Affairs at the European Commission

APPENDIX

STATISTICAL CONSIDERATION OF VALIDATION RESULTS

General comments

The following guidelines are appropriate to the analysis of technical material and preparations and also reflect guidance given by CIPAC. It should be noted that the guidelines are not a prescriptive set of rules. Data must be considered in the light of appropriate scientific knowledge.

The statistical method used should be 'fit for purpose'. Therefore consideration should be given to the applicability of the statistical method chosen or indeed whether a statistical consideration of the results is necessary. A useful review of recent publications on the application of statistical methods to analytical methodology is given in (4).

Accuracy

Accuracy of a method may be measured in different ways (5) and the method should be appropriate to the matrix. Assessment may be made by analysing a sample of known concentration and comparing the measured value to the 'true' value. However a well characterised sample (*e.g.* a reference standard) must be used.

Alternatively, determination of accuracy may be based on the recovery of known amounts of analyte from a representative sample matrix. Samples should ideally be laboratory-prepared co-formulant mixes to which a known quantity of analyte is added (*e.g.* a 'spiked' placebo or sample matrix). Where it is not possible to prepare a sample matrix without the presence of the analyte, or there are difficulties in replicating the sample to be analysed (for example a pellet product form), the standard addition method may be used.

For example, when comparing the measured values with an expected or 'true' value using the Student's t-test (6), the choice of null hypothesis should be appropriate to the data set.

The precision of the data set will affect the interpretation of the statistical result in terms of significance. Data may be found to be precisely skewed in one direction, indicating a systematic difference between the measured and expected values, however if these data are skewed but with a large uncertainty, the result may be a non-significant difference. For example, if recovery data are precise and range between 95-96% in comparison with the 'expected' value of 100%, the t-test may yield a significant difference between measured and expected values, however the degree of accuracy would be acceptable. However if the data were less precise, for example 95-102%, the degree of accuracy would still be acceptable, however the data are less precise and the t-test would yield a non-statistical difference.

Confidence intervals for % mean recovery from preparations, based on consultation with industry, are as follows:

% Active Substance (nominal)	Mean % Recovery	% Impurity (nominal)	Mean % Recovery
>10%	98-102%	>1%	90-110%
1-10%	97-103%	0.1-1%	80-120%
<1%	95-105%	<0.1%	75-125%
0.01-0.1%	90-110%		
<0.01%	80-120%		

A discussion of the measured recovery of the method in relation to these guideline values is encouraged. The details of any statistical approach used must be reported.

Precision

A suitable test for outliers may be applied to the precision data, for example the Grubbs or Dixons Tests (7) (8). If outliers are discarded, justification must be given. Acceptability of the % RSD (coefficient of variation, CV) results for precision may be based on the Horwitz equation, an exponential relationship between the among-laboratory relative standard deviation (RSDR) and concentration (C):

$$\% \ RSD_R = 2^{(1-0.5 \ \log C)}$$

which, for estimation of repeatability (RSDr), is modified to:

$$\% \ RSD_r = \% \ RSD_R \ x \ 0.67$$

The Horwitz curve has been empirically derived and has been shown to be more or less independent of analyte, matrix and method of analysis over the concentration range C= (100%) to C = 10^{-9} by the analysis of vast numbers of method precision studies (9) The modified Horwitz values for repeatability CV given below may be used for guidance. If measured repeatability is outside these recommended values, a suggested explanation should be submitted for consideration.

% Analyte	Proposed acceptable RSD$_r$ (Horwitz value x 0.67)
100%	1.34% RSD
50%	1.49% RSD
20%	1.71% RSD
10%	1.90% RSD
5%	2.10% RSD
2%	2.41% RSD
1%	2.68% RSD
0.25%	3.30% RSD

The unmodified Horwitz equation is used as a criterion of acceptability for methods collaboratively tested by CIPAC.

Summary of Validation Characteristics and Requirements for Analytes

The validation parameters that need to be collected for a method depend on the application of the method. That is, the nature of the analyte and the nature of the sample matrix. The following table summarises the recommended characteristics for test methods described in this guideline:

Test characteristic	Technical Materials		Preparations	
	Assay of active substance	Measurement of significant and/or relevant impurities.	Assay of active substance	Measurement of relevant impurities
Specificity	Yes	Yes	Yes	Yes
Linearity	Yes	Yes	Yes	Yes
Accuracy	No	Yes	Yes	Yes
Precision	Yes	Yes	Yes	Yes
Range	*	Yes	Yes	Yes
Limit of Quantification	No	Yes	*	Yes

*May be required, depending on the nature or purpose of the specific test.