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

INTRODUCTION

1. The International Organization for Standardization (ISO) has defined method validation as “the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled” (1). In other words, method validation is required in order to demonstrate that a particular method of analysis is fit for its intended purpose.

2. Accurate, precise and specific quantitative analytical methods are required for confirmation of the identity, purity and stability of target analytes in technical materials and preparations/formulations of pesticidal products. Plant protection products and biocidal products come under the umbrella of pesticidal products for the purpose of this guidance document.

3. Analytes of interest for method validation purposes may include active substances (AS), significant impurities and relevant impurities. Significant impurities are impurities that are present in the technical active substance as manufactured at concentrations of ≥ 0.1% w/w. Relevant impurities are impurities that are also present in the technical material but are considered to be significant from a toxicological, ecotoxicological or environmental point of view. Relevant impurities may be present at concentration levels ≥ or ≤ 0.1% w/w in the technical active substance as manufactured. Validated methods of analysis are required for the active substance, significant impurities and relevant impurities in the technical material as manufactured. In contrast to technical materials validation requirements, validated method of analysis are only required for the active substance(s) and relevant impurities in preparations/formulations. Validated methods of analysis are not required for significant impurities in preparations/formulations.

4. It should be noted that the terms “active ingredients” and “active substances” are used in North America and Europe respectively. The guidance document wants to highlight the subsequent use of these two terms in order to avoid confusion and wants to state that both terms are equivalent and valid for purposes of this guidance document.

5. The guidance document is based on existing guidance documents and best practices from agencies and professional organizations pertinent to single laboratory validation of quantitative analytical methods (2) (3) (4).

6. 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

7. 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 the suitability of a specific laboratory procedure and measurement system for the target analyte(s) in particular matrices.

8. Commercially certified reference standards should be used for method validation. In the event that such reference standards are not available for method validation, a thorough explanation should be provided with regards to the choice of reference standards that are used for the method validation process. Reference standards which are not fully certified should be fully characterised (for example by NMR and Mass Spectral data) before being considered acceptable for method validation purposes.

9. The validation parameters to be determined and the acceptance criteria may differ 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)**

10. Specificity (selectivity) can be defined as the ability of a method to distinguish between the analyte (active substance or impurity) being measured and other substances in the sample matrix.

11. The specificity/selectivity of a method refers to the extent to which it can determine particular analyte(s) in a complex mixture without interference from other components.

12. The degree of interference in the determination of target analytes in the technical material and preparation/formulation must be reported. Interferences from non-target analytes in the technical material or preparation/formulation should not contribute more than 3% to the measured response of the target analyte. If the active substance is specified as being optically pure, the method for the technical material and preparation/formulation must support this. Where an active substance or relevant impurity consists of more than one isomer, analogue, etc., the methods should be capable of determining the individual components present. However, some regulatory authorities provide an exemption to the requirement of determining individual isomers such as when optical isomers are present in racemic mixtures, or when the optical isomers have approximately the same efficacy and toxicity profile.

13. Where a preparation/formulation contains more than one active substance the method(s) must be capable of determining each in the presence of the other, and where a technical material or preparation/formulation contains more than one impurity the method(s) must be capable of determining each in the presence of the other and in the presence of the active substance(s). Specificity for the analysis of active substances and impurities should be addressed to the extent that the technical material and preparation/formulation is properly characterised (see confirmatory validation criteria for details).

   b) **Linearity**

14. Linearity can be defined as the ability of a method to produce an acceptable linear correlation between the measured response and the concentration of the analyte in the sample.

15. 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.

16. Linearity data is required for the active substance, significant and relevant impurities in technical materials. Linearity data is also required for the active substance(s) and relevant impurities in preparations/formulations. Linearity data is not required for significant impurities in preparations/formulations.

c) **Accuracy (Recovery)**

17. Accuracy (recovery) can be defined as the degree to which the measured value for the analyte in a sample corresponds to the accepted, true or reference value.

18. The experimental determination of accuracy for the active substance in the technical material is not required in some jurisdictions on the basis that it may be possible to make an assessment of the accuracy of the method through the available interference and precision validation data.

19. In contrast to the technical material, the experimental determination of the accuracy of the active ingredient in the preparation/formulation is required in all regulatory jurisdictions. The accuracy of the method should be reported as mean recovery for the pure active substance in the preparation/formulation. 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.

20. The accuracy of the method(s) for significant and/or relevant impurities should be reported as mean recovery and relative standard deviation in the technical material. At least two independent 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.

21. The accuracy of the method(s) for relevant impurities should be reported as mean recovery and relative standard deviation in the preparation. The same accuracy criterion as described for relevant impurities in technical materials also applies to relevant impurities in preparations/formulations.

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

d) **Precision (repeatability)**

23. Precision (repeatability) can be defined as the closeness of agreement of 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.

24. Details of the precision of the method are required for the active substance, significant impurities and relevant impurities in the technical material as manufactured. A minimum of five separate sample
determinations is made and the mean, % RSD and number of determinations are 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.

25. The same criteria as outlined above apply for the active substance(s) and relevant impurities in the preparation/formulation. Precision (repeatability) validation data is not required for significant impurities in the preparation/formulation.

e) Limit of Quantification (LOQ)

26. The LOQ can be defined as the lowest concentration of analyte in a sample that can be determined or quantitated with acceptable relative standard deviation.

27. The LOQ can also be described as the lowest concentration level for which acceptable recoveries are obtained. The LOQ is sometimes described as being equal to 10 times the signal to noise ratio. Scientifically accepted procedures for determining the LOQ are encouraged. However it should be noted that there are several ways of determining the LOQ and that specific ways of determining the LOQ should be checked with specific regulatory authorities.

28. The LOQ does not have to be reported for the active substance in the technical material as manufactured or in the preparation/formulation.

29. The LOQ must be reported for significant and relevant impurities in the technical material as manufactured. In order to support the declared technical specification, the LOQ for significant impurities should be at or below the anticipated quantity of the significant impurity in the technical material. Some regulatory authorities may have specific limits that must be obtained for significant impurities. The LOQ for relevant impurities in the technical material should be based on the concentration of analyte which is considered to be of toxicological, eco-toxicological or environmental significance.

30. The LOQ must also be reported for relevant impurities in the preparation/formulation and must take into account the concentration of analyte which is considered to be of toxicological or environmental significance, or the concentration which is formed during storage of the preparation/formulation, where this is relevant. The LOQ does not have to be reported for significant impurities in the preparation/formulation.

f) Limit of Detection (LOD)

31. The LOD can be defined as the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated as an exact value.

32. The LOD is not required for the active substance in technical materials or preparations/formulations.

33. Information in relation to the LOD for impurities in the technical material and preparation/formulations is only required in some regulatory jurisdictions. The LOD is sometimes described as being equal to 5 times the signal to noise ratio. Scientifically accepted procedures for determining the LOD are encouraged. It should be noted that there are several ways of determining the LOD and that suggested approaches should be checked with specific regulatory authorities.
g) **Confirmation of analyte identification**

34. Confirmation of identity can be defined as the unequivocal establishment of the chemical identity of the analyte in a particular matrix. It should be noted that the confirmation of analyte identity during analysis is not required by all regulatory authorities.

35. However if required, the confirmation of analyte identification during analysis can be carried out by the procedures outlined below:

   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.

36. It should be noted that the confirmation of relevant impurities in preparations/formulations is also carried out in the same manner as described above. However it is also important to note that it is not necessary to confirm the identity of significant impurities in the preparation/formulation as part of the analysis.

37. It needs to be highlighted that the procedures outlined above are only acceptable for the purposes of establishing the identity as part of the chemical analysis. Some regulatory authorities have an additional data requirement of confirming/establishing the full chemical structure of impurities in the technical material. In such cases full NMR, Mass Spectral data are likely to be requested.
DATA REPORT

38. 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. Matrix or solvent effects that result in signal enhancement, masking, or suppression should be described. Methods which allow little variation in the described procedure should be highlighted. 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).

39. 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


DEFINITIONS AND ABBREVIATIONS

AOAC – Association of Official Analytical Chemists

APVMA – Australian Pesticides & Veterinary Medicines Authority

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 Standardization

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 (5).

Accuracy

Accuracy of a method may be measured in different ways (6) 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 (7), 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:
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.

**Preciseion**

A suitable test for outliers may be applied to the precision data, for example the Grubbs or Dixons Tests (8) (9). 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):

\[
\% \text{ RSD}_R = 2^{(1 - 0.5 \log C)}
\]

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

\[
\% \text{ RSD}_r = \% \text{ RSD}_R \times 0.67
\]

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 (10) 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.

<table>
<thead>
<tr>
<th>% Analyte</th>
<th>Proposed acceptable RSD_r (Horwitz value x 0.67)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>1.34%</td>
</tr>
<tr>
<td>50%</td>
<td>1.49%</td>
</tr>
<tr>
<td>20%</td>
<td>1.71%</td>
</tr>
<tr>
<td>10%</td>
<td>1.90%</td>
</tr>
<tr>
<td>5%</td>
<td>2.10%</td>
</tr>
<tr>
<td>2%</td>
<td>2.41%</td>
</tr>
<tr>
<td>1%</td>
<td>2.68%</td>
</tr>
<tr>
<td>0.25%</td>
<td>3.30%</td>
</tr>
</tbody>
</table>
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:

<table>
<thead>
<tr>
<th>Test characteristic</th>
<th>Technical Materials</th>
<th>Preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Linearity</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Accuracy</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Precision</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Range</td>
<td>*</td>
<td>Yes</td>
</tr>
<tr>
<td>Limit of Quantification</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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