METHOD VALIDATION AND QUALITY CONTROL PROCEDURES FOR PESTICIDE RESIDUES ANALYSIS IN FOOD AND FEED

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METHOD VALIDATION AND QUALITY CONTROL PROCEDURES FOR PESTICIDE RESIDUES ANALYSIS IN FOOD AND FEED

Introduction

1. The guidance in this document is intended for laboratories control or in the monitoring of pesticide residues in food involved in official and feed in the European Union. The document describes the method validation and analytical quality control (AQC) requirements to support the validity of data used for checking compliance with maximum residue limits (MRLs), enforcement actions, or assessment of consumer exposure to pesticides

The key objectives are:

(ii) to provide a harmonized cost-effective quality assurance system in the EU

(iii) to ensure the quality and comparability of analytical results

(iv) to ensure that acceptable accuracy is achieved

(v) to ensure that false positives or false negatives are not reported

(vi) to support compliance with ISO/IEC 17025 (accreditation standard)

2. This document is complementary and integral to the requirements in ISO/IEC 17025.

3. This document supersedes Document No. **SANCO/3131/2007**.

4. The glossary (Appendix C) should be consulted for explanation of terms used in the text.

Accreditation and legal background

5. In accordance with Article 12 of Regulation 882/2004, laboratories designated for official control of pesticide residues must be accredited to ISO/IEC 17025. According to article 11 of Regulation 882/2004, analysis methods used in the context of official controls shall comply with relevant Community rules or with internationally recognised rules or protocols or, in the absence of the above, with other methods fit for the intended purpose or developed in accordance with scientific protocols. Where the above does not apply, validation of methods of analysis may further take place within a single laboratory according to an internationally accepted protocol. According to Article 28 of Regulation 396/2005, technical guidelines dealing with the specific validation criteria and quality control procedures in relation to methods of analysis for the determination of pesticide residues may be adopted in accordance with the procedure referred to in Article 45(2) of this regulation. The present document entails mutually acceptable scientific rules for official pesticide residue analysis within the EU as agreed by all Member States of the European Union and constitutes a technical guideline in the sense of article 28 of Regulation 396/2005. It should thus be consulted in audits and accreditations of official pesticide residue laboratories according to ISO/IEC 17025.

Sampling, transport, processing and storage of samples

Sampling

6. Laboratory samples should be taken in accordance with Directive 2002/63/EC or superseding legislation. Where it is impractical to take primary samples randomly within a lot, the method of sampling must be recorded.

Laboratory sample transportation

7. Samples must be transported under appropriate conditions to the laboratory in clean containers and robust packaging. Polythene bags, ventilated if appropriate, are acceptable for most samples but low-permeability bags (e.g. nylon film) must be used for samples to be analysed for residues of fumigants. Samples of commodities pre-packed for retail sale should not be removed from their packaging before transport. Very fragile or perishable products (e.g. ripe raspberries) may have to be frozen to avoid spoilage and then transported in "dry ice" or similar, to avoid thawing in transit. Samples that are frozen at the time of collection must be transported without thawing. Samples that may be damaged by chilling (e.g. bananas) must be protected from both high and low temperatures.

8. Rapid transportation to the laboratory, preferably within one day, is essential for samples of most fresh products. The condition of samples delivered to the laboratory should approximate to that acceptable to a discerning purchaser, otherwise samples should normally be considered unfit for analysis.

9. Samples must be identified clearly and indelibly, in a way that prevents inadvertent loss or confusion of labelling. The use of marker pens containing organic solvents should be avoided for labelling bags containing samples to be analysed for fumigant residues, especially if an electron capture detector is to be used.

Sample preparation and processing prior to analysis

10. On receipt, each laboratory sample must be allocated a unique reference code by the laboratory.

11. Sample preparation, sample processing and sub-sampling to obtain analytical portions should take place before visible deterioration occurs. This is particularly important when the analytical result is to be used to assess consumer intake. Canned, dried or similarly processed samples should be analysed within the stated shelf life.

12. Sample preparation must be in accordance with the definition of the commodity and the part(s) to be analysed, see Regulation 396/2005 Annex1.

13. Sample processing and storage procedures should be demonstrated to have no significant effect on the residues present in the analytical sample (see Directive 2002/63/EC). Where there is evidence that comminution (cutting and homogenisation) at ambient temperature has a significant influence on the degradation of certain pesticide residues, it is recommended that samples are homogenised at low temperature (e.g. frozen and/or in the presence of "dry ice"). Where comminution is known to affect residues (e.g. dithiocarbamates or fumigants) and practical alternative procedures are not available, the test portion should consist of whole units of the commodity, or segments removed from large units. For all other analyses, the whole laboratory sample (in most cases 1-2 kg) needs to be comminuted. All analyses should be undertaken within the shortest time practicable, to minimise sample storage. Analyses for residues of very labile or volatile pesticides should be started, and the

procedures involved in potential loss of analyte completed, on the day of sample receipt. In any case, sample comminution should ensure that the sample is homogeneous enough so that sub-sampling variability is acceptable. If this is not achievable, the use of larger test portions should be considered.

14. If a single analytical portion is unlikely to be representative of the analytical sample, replicate portions must be analysed, to provide a better estimate of the true value.

Pesticide standards, calibration solutions, etc.

Identity, purity, and storage of standards

15. "Pure" standards of analytes should be of known purity and each must be uniquely identified and the date of receipt recorded. They should be stored at low temperature, preferably in a freezer, with light and moisture excluded, i.e. under conditions that minimise the rate of degradation. Under such conditions, the supplier's expiry date, which is often based on less stringent storage conditions, may be replaced, as appropriate for each standard, by a date allowing for storage up to 10 years. The pure standard may be retained if its purity is shown to remain acceptable. The purity should be checked by the allocated time after which a "pure" standard may be retained if its purity is shown to remain acceptable and a new expiry date is allocated. Ideally, the identity of freshly acquired "pure" standards should be checked if the analytes are new to the laboratory.

Preparation and storage of stock standards

16. When preparing stock standards (solutions, dispersions or gaseous dilutions) of "pure" standards of analytes and internal standards, the identity and mass (or volume, for highly volatile compounds) of the "pure" standard and the identity and amount of the solvent (or other diluents) must be recorded. The solvent(s) must be appropriate to the analyte (solubility, no reaction) and method of analysis. Moisture must be excluded during equilibration of the "pure" standard to room temperature before use and concentrations must be corrected for the purity of the "pure" standard.

17. Not less than 10 mg of the "pure" standard should be weighed using a 5 decimal place balance. The ambient temperature should be that at which the glassware is calibrated, otherwise preparation of the standard should be based on mass measurement. Volatile liquid analytes should be dispensed by weight or volume (if the density is known) directly into solvent. Gaseous (fumigant) analytes may be dispensed by bubbling into solvent and weighing the mass transferred, or by preparing gaseous dilutions (e.g. with a gas-tight syringe, avoiding contact with reactive metals).

18. Stock standards must be labelled indelibly, allocated an expiry date and stored at low temperature in the dark in containers that prevent any loss of solvent and entry of water. Currently available data show that stock standards of the large majority of pesticides in toluene and acetone are stable for at least 5 years in the freezer when stored in tightly closed glass containers.

19. For suspensions (e.g. dithiocarbamates) and solutions (or gaseous dilutions) of highly volatile fumigants that should be prepared freshly, the accuracy of the solution should be compared with a second solution made independently at the same time.

Preparation, use and storage of working standards

20. When preparing working standards, a record must be kept of the identity and amount of all solutions and solvents employed. The solvent(s) must be appropriate to the analyte (solubility, no reaction) and method of analysis. The standards must be labelled indelibly, allocated an expiry date and stored at low temperature in the dark in containers that prevent any loss of solvent and entry of water. Septum closures are particularly prone to evaporation losses (in addition to being a source of contamination) and should be replaced as soon as practicable after piercing, if solutions are to be retained. Following equilibration to room temperature, solutions must be re-mixed and a check made to ensure that no analyte remains undissolved, especially where solubility at low temperatures is limited.

21. At method development or validation, or for analytes new to the laboratory, the response detected should be shown to be due to the analyte, rather than to an impurity or artefact. If the techniques used can lead to degradation of the analyte during extraction, clean-up or separation, and they generate a product that is commonly found in samples but which is excluded from the residue definition, positive results must be confirmed using techniques that avoid this problem.

Testing and replacement of standards

22. Whenever any standard is used beyond its expiry date its stability should be verified. Existing stock and working solutions may be tested against newly prepared solutions by comparing the detector responses obtained from appropriate dilutions of individual standards or mixtures of standards. The purity of an old "pure" standard may be checked by preparing a new stock standard and comparing the detector responses obtained from freshly prepared dilutions of old and new stock standards. Inexplicable differences in apparent concentration between old and new standards must be investigated.

23. The means from at least three replicate measurements for each of two solutions (old and new) should not normally differ by more than ±10%[1]. The mean from the new solution is taken to be 100%. If the mean response of the old standard differs by more than ±10% from the new, storage time or conditions must be adjusted as necessary on the basis of the results and should be checked against a second solution independently prepared from the first one. The use of an internal standard may reduce the number of replicate injections required to achieve a ±10% difference.

Extraction and concentration

Extraction conditions and efficiency

24. Test portions should be disintegrated thoroughly during extraction to maximise extraction efficiency, except where this is known to be unnecessary or inappropriate (e.g. for determination of fumigants or surface residues). Temperature, pH, etc., must be controlled if these parameters affect extraction efficiency, analyte stability or solvent volume. To improve the extraction efficiency of low moisture containing commodities (cereals, dried fruits), it is recommended to add water to the samples before extraction is carried out. However, the time between addition of water and extraction should be controlled in order to avoid any significant losses of pesticides.

[1] Alternatively, a t-test of the means should not show a significant difference at the 5% level

Extract concentration and dilution to volume

25. Great care must be exercised when extracts are evaporated to dryness, as trace quantities of many analytes can be lost in this way. A small volume of high boiling point solvent may be used as a "keeper" and the evaporation temperature should be as low as practicable. Frothing and vigorous boiling of extracts, or dispersion of droplets, must be avoided. A stream of dry nitrogen or vacuum centrifugal evaporation is generally preferable to the use of an air stream for small-scale evaporation, as air is more likely to lead to oxidation or to introduce water and other contaminants.

26. Where extracts are diluted to a fixed volume, accurately calibrated vessels of not less than 1 ml capacity should be used and further evaporation avoided.

27. Analyte stability in extracts should be investigated during method validation. Storage of extracts in a refrigerator or freezer will minimise degradation but potential losses at the higher temperatures of an autosampler rack should not be ignored.

Contamination and interference

Contamination

28. Samples must be separated from each other, and from other sources of potential contamination, during transit to, and storage at, the laboratory. This is particularly important with surface or dusty residues, or with volatile analytes. Samples known, or thought, to bear such residues should be doubly sealed in polythene or nylon bags and transported and processed separately.

29. Pest control in, or near, the laboratory must be restricted to pesticides that will not be sought as residues.

30. Volumetric equipment, such as flasks, pipettes and syringes must be cleaned scrupulously, especially for re-use. As far as practicable, separate glassware, etc., should be allocated to standards and sample extracts, in order to avoid cross-contamination. Avoid using excessively scratched or etched glassware. Solvents used for fumigant residues analysis should be checked to ensure that they do not contain the analyte.

31. Where an internal standard is used, unintended contamination of extracts or analyte solutions with the internal standard, or *vice versa*, must be avoided.

32. Where the analyte occurs naturally in, or is produced from, samples (e.g. inorganic bromide in all commodities; sulphur in soil; or carbon disulfide produced from the *Brassicaceae)*, low-level residues from pesticide use cannot be distinguished from natural levels. Natural occurrence of these analytes must be considered in the interpretation of results. Dithiocarbamates, ethylenethiourea or diphenylamine can occur in certain types of rubber articles and this source of contamination must be avoided.

Interference

33. Equipment, containers, solvents (including water), reagents, filter aids, etc., should be checked as sources of possible interference. Rubber and plastic items (e.g. seals, protective gloves, wash bottles), polishes and lubricants are frequent sources. Vial seals should be PTFE-lined. Extracts should be kept out of contact with seals, especially after piercing, by keeping vials upright. Vial seals may have to be replaced quickly after piercing, if re-analysis of the extracts is necessary. Analysis of reagent blanks should identify sources of interference in the equipment or materials used.

34. Interference from natural constituents of samples is frequent. The interference may be peculiar to the determination system used, variable in occurrence and intensity, and may be subtle in nature. If the interference takes the form of a response overlapping that of the analyte, a different clean-up or determination system may be required. Interference in the form of suppression or enhancement of detection system response is dealt with in paragraph 45. If it is not practicable to eliminate interference, or to compensate for it by matrix-matched calibration, the overall accuracy (bias) and precision of analysis should nonetheless comply with the criteria in paragraphs 64 and 65.

Analytical calibration, representative analytes, matrix effects and chromatographic integration

General requirements

35. Correct calibration is dependent upon correct identification of the analyte (see paragraphs 69-82) Bracketing calibration should be used unless the determination system has been shown to be free from significant drift in its absolute (external standardisation) or relative (internal standardisation) response. In a batch of parallel determinations (e.g. ELISA with 96-well plates), the calibration standards should be distributed to detect differences in response due to position. Responses used to quantify residues must be within the dynamic range of the detector.

36. Batch sizes for determination should be adjusted so that detector response to a single injection of bracketing calibration standards does not drift >20% at ≥2 x LCL (lowest calibrated level), or >30% at 1–2 x LCL (if the LCL is close to the LOQ). If the drift exceeds these values, repeat of determinations is not necessary where the samples clearly contain no analyte, providing that the response at the calibration level corresponding with the reporting level (RL) remains measurable throughout the batch.

37. Extracts containing high-level residues may be diluted to bring them within the calibrated range. Where calibration solutions are matrix-matched (paragraph 44) the concentration of matrix extract may also have to be adjusted.

Calibration

38. Residues below the LCL, if corresponding with the RL, should be considered uncalibrated, and therefore reported as <RL, whether or not a response is evident. If it is desirable to report measurable residues below the original RL and corresponding LCL, determinations must be repeated with a lower LCL. If the signal to noise ratio produced by the target LCL is inadequate (less than 6:1), a higher level must be adopted as the LCL. An additional calibration point, for example at two times the target LCL, provides a back-up LCL if there is a risk that the target LCL will not be measurable. Validation of analytical methods should include determination of recovery at the proposed RL.

39. Calibration by interpolation between two levels is acceptable providing the difference between the 2 levels is not greater than a factor of 4, and where the mean response factors, derived from replicate determinations at each level, indicate acceptable linearity of response with the higher being not more than 120% of the lower response factor (110% in cases where the MRL is approached or exceeded).

40. Where three or more levels are utilised, an appropriate calibration function may be calculated and used between the lowest and highest calibrated levels. The calibration curve (which may or may not appear to be linear) should, in

general, not be forced through the origin. The fit of the calibration function must be plotted and inspected visually and/or by calculation of the residuals, avoiding unjustified reliance on correlation coefficients, to ensure that the fit is satisfactory in the region relevant to the residues detected. If individual residuals deviate by more than ±20% (±10% in cases where the MRL is approached or exceeded) from the calibration curve in the relevant region, an alternative calibration function must be used. In general, the use of weighted linear regression (1/x) is recommended, compared to linear regression.

41. Single-level calibration may provide more accurate results than multi-level calibration if the detector response is variable with time. When single-level calibration is employed, the sample response should be within ±20% of the calibration standard response if the MRL is exceeded. If the MRL is not exceeded, the sample response should be within ±50% of the calibration response, unless further extrapolation is supported by evidence of acceptable linearity of response. Where analyte is added for recovery determination at a level corresponding to the LCL, recovery values <100% may be calculated using a single point calibration at the LCL. This particular calculation is intended only to indicate analytical performance achieved at the LCL and does not imply that residues <LCL should be determined in this way.

Representative analytes

42. Where practicable, each determination system should be calibrated with all the targeted analytes for every batch of analyses. If this requires a disproportionately large number of calibrations, the determination system must be calibrated with a minimum number of representative analytes. Reliance on representative analytes is associated with an increased risk of incorrect results, especially false negatives. Therefore representative analytes must be chosen very carefully, to provide enough evidence that acceptable screening is achieved for all other analytes. The choice should be made according to the probability of finding residues in the sample and the physico-chemical characteristics of the analytes i.e. analytes likely to give the poorest and most variable response. The representative analytes to be calibrated in each batch must be at least 15 analytes plus 25% of the total number of analytes included in the analytical scope of each determination system. For example, if the analytical scope of an instrument method covers 40 analytes, the determination system must be calibrated with at least 25 representative analytes. If the scope of analysis in determination system is 20 or less, then all analytes should be calibrated. The minimum frequency for calibration of representative and all other analytes is given in Table 1.

Table 1. Minimum frequencies for calibration

	Representative analytes	All other analytes
Minimum frequency of calibration	In each batch of analyses.	Within a rolling programme at least every third month*
	At least one calibration point corresponding to the reporting limit.	At least one calibration point corresponding to the reporting limit See also paragraph 43.

*The minimum requirements are :

(i) at the beginning and end of a survey or programme and

(ii) when potentially significant changes are made to the method.

43. Where an analyte that is not a representative analyte is detected in a sample, the result must be considered tentative until calibrated (see paragraphs 36–41). When the screening result indicates that an MRL might be exceeded, or in the case of other violative residues, the sample must be reanalysed and accompanied by acceptable recovery (see paragraph 66) of the detected analyte. The recovery test may be omitted when the standard addition approach as described in paragraph 47 is employed or when employing the isotope-dilution approach with the isotope-labelled internal standard being added to the analytical portion prior to extraction, provided that the reporting level (RL) still can be achieved.

Matrix effects and matrix-matched calibration

44. The potential for matrix effects to occur should be assessed at method validation. They are notoriously variable in occurrence and intensity but some techniques are particularly prone to them. If the techniques used are not inherently free from such effects, calibration should be matrix-matched routinely, unless an alternative approach can be shown to provide equivalent or superior accuracy. Extracts (or samples, for calibration of headspace and SPME analysis) of blank matrix preferably of the same type as the sample may be used for calibration purposes. An alternative practical approach to minimise matrix effects in GC-analyses is the use of "analyte protectants" (e.g. sorbitol, γ-gulonolactone, δ-gluconolactone, 3-ethoxy-1,2-propanediol (ethylglycerol)) that are added to both the sample extracts and the calibration solutions (in pure solvent or in matrix) in order to produce equivalent matrix effects. The most effective ways to negate matrix effects are calibrations by standard addition (see paragraphs 47 and 4849 and isotope dilutions with the isotope-labelled internal standard being added at any stage of the procedure prior to measurement.

45. A potential problem is that different samples, different types of extract, different commodities and different "concentrations" of matrix may exhibit matrix effects of variable magnitude. Where a slight risk of erroneous calibration is acceptable, a representative matrix (see glossary) may be used to calibrate a wide range of sample types.

46. If required in GC analysis, priming should be performed immediately prior to the first series of calibration determinations in a batch of analyses.

Standard addition

47. Standard addition may be used as an alternative approach to the use of matrix-matched calibration standards. In particular, it is recommended that standard addition is used for quantification of confirmatory analyses in cases of MRL exceedances and/or when no suitable blank commodity is available for the preparation of matrix-matched standard solutions. Standard addition means a procedure in which the test sample is divided in three (or more) test portions. One portion is analysed as such, and known amounts of the standard analyte are added to the other test portions immediately prior to extraction. The amount of the standard analyte added should be between one 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, inherently taking into account the recovery of the analytical procedure and also compensating for any matrix effect. The quantity of analyte present in the "unspiked" sample extract is calculated by simple proportion. This technique assumes some knowledge of the likely concentration of the analyte in the sample, so that the amount of added analyte is similar to that already present in the sample. If the concentration of the analyte is completely unknown then it may be necessary

to "spike" a number of replicate samples with increasing quantities of analyte, so that a calibration curve can be constructed in a similar way to normal standard calibration. This technique automatically adjusts for both recovery and calibration. Standard addition will not, of course, overcome chromatographic interferences caused by overlapping/unresolved peaks from co-extracted compounds. In the standard addition approach the unknown concentration of the analyte in the sample is derived by extrapolation, thus a linear response in the appropriate concentration range is essential for achieving accurate results.

48. Addition of a known quantity of analyte to an aliquot of sample extract, etc prior to injection is another form of standard addition, but in this case adjustment is only for calibration including matrix effects.

Effects of pesticide mixtures on calibration

49. Calibration using mixed analyte solutions made up in pure solvent, etc. should be checked at method validation (paragraphs 55–57) for similarity of detector response to that obtained from the separate analytes. If the responses differ significantly, or in cases of doubt, residues must be quantified using individual calibration standards in matrix, or better still, by standard addition.

Calibration for pesticides that are mixtures of isomers

50. Where a calibration standard is a mixture of isomers, etc., of the analyte, detector response generally may be assumed to be similar, on a molar basis, for each component. However, enzyme assays, immuno-assays and other assays with a biological basis may give calibration errors if the component ratio of the standard differs significantly from that of the measured residue. An alternative detection system should be used to quantify such residues. In those cases where the response of a "selective" detector to isomers differs (e.g. the electron-capture efficiency of HCH isomers), separate calibration standards must be used. If separate standards are not available for this purpose, an alternative detection system should be used to quantify residues.

Calibration using derivatives or degradation products

51. Where the pesticide is determined as a degradation product or derivative, the calibration solutions should be prepared from a "pure" standard of that degradation product or derivative, if available. Procedural standards should only be used if they are the only practical option.

Chromatographic integration

52. Chromatograms must be examined by the analyst and the baseline fitting checked and adjusted, as required. Where interfering or tailing peaks are present, a consistent approach must be adopted for the positioning of the baseline. Peak height or peak area data may be used; whichever yields the more accurate and repeatable results.

53. Unless biosensor detection is employed, calibration by mixed isomer (or similar) standards may utilise summed peak areas, summed peak heights, or measurement of a single component, whichever is the more accurate.

Analytical method validation and performance criteria

Qualitative screening methods

54. Qualitative screening methods (e.g. bioassays, chemical methods using automated MS-based detection) can be useful for detection of pesticides, which potentially have low probability to be present in the samples.

For qualitative screening methods the confidence of detection or identification of an analyte at a certain concentration level should be established. Validation in case of qualitative screening methods is focused on detectability. The detection is the lowest spiking level for which has been demonstrated that a certain analyte can be detected (not necessarily identified) in at least 95% of the samples (i.e. a false-negative rate of 5% is accepted) at the concentration of interest (e.g. at the RL of the method used for confirmation). The samples included in the validation should be representative for the matrix scope of the screening method. When strictly used as qualitative method, there are no requirements with regard to linearity and recovery. With respect to selectivity, the presence of false positives should be excluded using unspiked samples (preferably "blank" samples). However, as long as analytes found during screening are identified and confirmed by a second sample analysis using an appropriate confirmatory method, there is no strict need for a criterion for the number of false positives from a QC point of view.

A basic validation of a screening method would typically involve analysis of 10 different samples in duplicate from each group of commodities (see Annex 1.) spiked with analytes at the anticipated screening reporting level (SRL). Upon application in routine analysis, on-going QC data should be acquired and the validity of the method should be periodically reassessed. For analytes that have not been included in the (on-going) method validation, the confidence level of detection at a certain concentration of analyte(s) is not known. Consequently, although analytes outside the scope of validation can be detected using the method, no screening reporting level can be specified or guaranteed.

Initial method validation

55. Within-laboratory method validation should be performed to provide evidence that a method is fit for the purpose for which it is to be used. Method validation is a requirement of accreditation bodies, and must be supported and extended by method performance verification during routine analysis (analytical quality control and on-going method validation). All procedures (steps) that are undertaken in a method should be validated, if practicable.

56. For both multi- and selective residue methods, representative matrices may be used. As a minimum, one representative commodity from each commodity group as described in Annex I must be validated, depending on the intended scope of the method. When the method applied in routine for a wider variety of matrices, complementary, on-going QC- and validation data should be acquired during the routine analyses. A practical approach to the validation procedure is presented in Appendix A.

57. The method must be tested to assess for sensitivity, mean recovery (as a measure of trueness or bias), precision, and limit of quantification (LOQ). This effectively means that spiked recovery experiments to check the accuracy of the method should be undertaken. A minimum of 5 replicates is required (to check the precision) at both the reporting limit (to check the sensitivity of the method), and at least another higher level, perhaps an action level, for example the MRL. The (method) LOQ is defined as the lowest validated spike level meeting the method performance acceptability criteria (mean recoveries for each representative commodity in the range 70-120%, with a $RSD_r \leq 20\%$). Other approaches to demonstrate that the analytical method complies with

performance criteria may be used, provided that they achieve the same level and quality of information. Where the residue definition incorporates two or more analytes, if possible, the method should be validated for all analytes included in the residue definition.

58. If the analytical method does not permit determination of recovery (for example, direct analysis of liquid samples, SPME, or headspace analysis), the precision is determined from repeat analyses of calibration standards. The bias is usually assumed to be zero, although this is not necessarily so. In SPME and headspace analysis, the trueness and precision of calibration may depend on the extent to which the analyte has equilibrated, particularly with respect to the sample matrix. If these methods depend upon equilibrium, this must be demonstrated during method development.

Acceptability of analytical method performance–extended method validation

59. A quantitative analytical method should be demonstrated at initial and extended validation as being capable of providing mean recovery values at each spiking level and for at least one representative commodity from each relevant group within the range 70–120%, repeatability RSD_r and within laboratory reproducibility $RSD_{wR} \leq 20\%$, for all compounds to be sought using the method In certain justified cases, typically with multiresidue methods, recoveries outside this range may be accepted. Where the method does not permit this, and there is no satisfactory alternative, the relatively poor mean recovery must be considered before taking enforcement action. Exceptionally, where recovery is low but consistent (i.e. demonstrating good precision) and the basis for this is well established (e.g. due to pesticide distribution in partition), a mean recovery below 70% may be acceptable. However, a more accurate method should be used, if practicable. Within- laboratory reproducibility (RSD_{wR}) should be ≤ 20%, excluding any contribution due to sample heterogeneity.

On-going performance verification (routine recovery determination)

60. Where practicable, recovery of analytes determined should be measured with each batch of analyses. If this requires a disproportionately large number of recovery determinations, the minimum acceptable frequency of recovery may be as given in Table 2. The choice must include at least 10 % of the representative analytes per detection system. However, the number of representative analytes in each batch must not be less than 5 per detection system. Analysis of reference materials is e a preferable option to use, though rarely practical due to the lack of CRMs providing that the materials contain the relevant analytes at appropriate levels.

Table 2. Frequency for routine recovery (performance verification)

	Representative analytes	All other analytes
Minimum frequency of recovery	10% of representative analytes (at least 5 per detection system) in each batch of analyses	Within a rolling programme to include all other analytes at least every 12 months, but preferably every 6 months
	Within a rolling program covering all representative analytes as well as different types of commodities, at least at the level corresponding to the reporting limit.	At least at the level corresponding to the reporting limit.

61. If the rolling programme (Table 1 and 2) for calibration or recovery of a representative analyte produces unacceptable results, all results produced after the previous successful calibration or recovery of that analyte must be considered to be potentially erroneous.

62. Analyte recovery should normally be determined by spiking within a range corresponding to 1–10 times the RL, or at the MRL, or at a level of particular relevance to the samples being analysed. The level of addition may be changed intermittently or regularly, to provide information on analytical performance over a range of concentrations. Recovery at levels corresponding to the RL and MRL is particularly important. In cases where blank material is not available (e.g. where inorganic bromide is to be determined at low levels) or where the only available blank material contains an interfering compound, the spiking level for recovery should be ≥3 times the level present in the blank material. The analyte (or apparent analyte) concentration in such a blank matrix should be determined from multiple test portions. If necessary, recoveries should be corrected by blank values. Blank values and uncorrected recoveries must also be reported. They must be determined from the matrix used in spiking experiments and the blank values should not be higher than 30% of the residue level corresponding to the RL.

63. As far as practicable, the recovery of all components defined by the MRL should be determined routinely. Where a residue is determined as a common moiety, routine recovery may be determined using the component that either normally predominates in residues or is likely to provide the lowest recovery.

Methods for determination of fat or dry weight content

64. Where results are expressed on the basis of dry weight or fat content, the method used to determine the dry weight or fat content must be consistent. Ideally it should be validated against a widely recognised method.

Acceptability of analytical performance for routine recoveries

65. The mean recovery is calculated from (different matrices) one commodity group. Acceptable limits for a single recovery result should normally be within the range of the mean recovery +/- 2x RSD and may be adjusted using within laboratory reproducibility (routine on going recovery) data or repeatability (initial validation) However, a generalized range of 60-140 % may be used in routine multi residue analysis. Recoveries outside the above mentioned range require re-analysis of the batch but may be acceptable in

certain justified cases. Where the individual recovery is unacceptably high and no residues are detected, it is not necessary to re-analyse the samples to prove the absence of residues. However, consistently high recovery should be investigated. If a significant trend occurs in recovery, or potentially unacceptable (RSD beyond ± 20 %) results are obtained, the cause(s) must be investigated.

In order to assure the correct execution of the whole procedure for each individual sample and the correct injection of each final sample extract in the GC- or LC-system, the use of one or more quality control (QC-) standards, is recommended. These compounds, which are added at different stages of the procedure e.g. to the samples prior to extraction (surrogate standards) or to the final sample extract just before injection (instrument internal standards) , should be chosen to be outside of the target pesticide scope and should preferably represent the entire scope of pesticides in terms of polarity and susceptibility to degradation.

66. Data on numerical exceedences of the MRL residues must be supported by individual recovery results in the same batch within the range of the mean recovery (70-120 %) ± 2 x RSD, at least for the confirmatory analyses. If recovery within this range cannot be achieved, enforcement action is not necessarily precluded, but the risk of relatively poor accuracy must be taken into account. It is recommended to correct for recovery preferably by using standard addition according to paragraph 47 or isotopically labelled standards in all cases of violation

Proficiency testing and analysis of reference materials

67. The laboratory must participate regularly in relevant proficiency tests. When a low number of compounds (e.g.: <90%) are analysed with respect to the pesticides present in the test sample, false positive(s) or negative(s) are reported or the accuracy achieved in any of the tests is questionable or unacceptable, the problem(s) should be investigated. Particularly for false positive(s), negative(s) and, or unacceptable performance, have to be rectified before proceeding with further determinations of the analyte/matrices combinations involved.

68. In-house reference materials may be analysed regularly to help provide evidence of analytical performance. Where practicable, exchange of such materials between laboratories provides an additional, independent check of accuracy.

Confirmation of results

69. Negative results (residues below the RL) can be considered confirmed if the recovery and LCL measurement for the batch are acceptable (paragraphs 38 and 64). Negative results for represented analytes are supported only indirectly by the recovery and LCL data for representative analytes and must be interpreted with caution.

70. Positive results (residues at or above the RL) usually require additional confirmation to that given in paragraph 69. In addition to the general requirements of paragraphs 71-80, confirmation of positive results for represented analytes (i.e. those with no concurrent calibration and recovery) should be supported by the appropriate concurrent calibration and recovery determinations. Confirmation is not mandatory for all positive results given that the requirements for the recovery in the batch comply with the requirements in paragraph 65, and must be decided by the laboratory on a case-by-case basis.

71. Suspected MRL exceedances or unusual residues must be identified. The criteria for identification are given in 71-79. The use of a highly specific detection system, such as mass spectrometry, is recommended.

Identification

72. Selective detectors employed with GC or LC such as ECD, FPD, NPD, DAD and fluorescence, offer only limited specificity. Their use, even in combination with different polarity columns, does not provide unambiguous identification. These limitations may be acceptable for frequently found residues, especially if some results are also confirmed using a more specific detection technique. Such limitations in the degree of identification should be acknowledged when reporting the results.

Mass spectrometry coupled to chromatography

73. Mass spectrometry in conjunction with chromatographic separation is a very powerful combination for identification of an analyte in the extract. It simultaneously provides:

i. retention time
ii. ion mass/charge ratio; and
iii. abundance data

Requirements for chromatography

74. For GC-MS procedures, the chromatographic separation should be carried out using capillary columns. For LC-MS procedures, the chromatographic separation can be performed using any suitable LC column. In either case, the minimum acceptable retention time for the analyte(s) under examination should be at least twice the retention time corresponding to the void volume of the column. The retention time (or relative retention time) of the analyte in the sample extract must match that of the calibration standard (may need to be matrix matched) within a specified window after taking into consideration the resolving power of the chromatographic system. The ratio of the chromatographic retention time of the analyte to that of a suitable internal standard, i.e. the relative retention time of the analyte, should correspond to that of the calibration solution with a tolerance of ±0.5% for GC and ±2.5% for LC[2].

Requirements for mass spectrometry (MS)

75. Reference spectra for the analyte should be generated using the instruments and techniques employed for analysis of the samples. If major differences are evident between a published spectrum and that generated within the laboratory, the latter must be shown to be valid. To avoid distortion of ion ratios, the response of the analyte ions must not overload the detector. The reference spectrum in the instrument software can originate from a previous injection without matrix present, but preferably from the same batch.

[2] Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002657/EC

76. Identification relies on proper selection of diagnostic ions. The (quasi) molecular ion is a diagnostic ion that should be included in the measurement and identification procedure whenever possible. In general, and especially in single MS, high m/z ions are more diagnostic than low m/z ions (e.g. m/z < 100). However, high m/z ions arising from loss of water or from common moieties may be of little use. Although characteristic isotopic ions, especially Cl or Br clusters, may be of particular utility, the selected diagnostic ions should not exclusively originate from the same part of the parent molecule. Choice of diagnostic ions may change depending on background interferences.

77. Diagnostic ion chromatograms should have peaks (exceeding S/N 3:1) of similar retention time, peak shape and response ratio to those obtained from a calibration standard analysed at comparable concentration in the same batch. Chromatographic peaks from different diagnostic ions for the same analyte must overlap with each other. Where an ion chromatogram shows evidence of significant chromatographic interference, it must not be relied upon to quantify or identify residues. The ion that shows the best signal-to-noise ratio and no evidence of significant chromatographic interference should be used for quantification.

78. In case of full scan measurement, careful subtraction of background spectra, either manual or automatically by deconvolution or other algorithms may be required to ensure that the resultant spectrum of the chromatographic peak is representative. Whenever background correction is applied, this must be applied uniformly throughout the batch and should be clearly indicated.

79. Different types and modes of mass spectrometric detectors provide different degrees of selectivity, which relates to the confidence in identification. The requirements for identification are given in Table 3. They should be regarded as guidance criteria for identification, not as absolute criteria to prove presence or absence of a compound.

Table 3 Identification requirements for different types of mass spectrometers

MS mode:	Single MS (standard mass resolution)	Single MS (high resolution/high mass accuracy)	MS/MS
Typical systems (examples)	quadrupole, ion trap, time-of-flight (TOF)	TOF, Orbitrap, FTMS, magnetic sector	Triple quadrupole ion trap, hybride MS (e.g. Q-TOF, Q-trap)
Acquisition:	Full scan, Limited m/z range, Selected ion monitoring (SIM)	Full scan, Limited m/z range, Selected ion monitoring (SIM)	Selected/multiple reaction monitoring (SRM/MRM), full scan product-ion spectra
Requirements for identification:	≥ 3 diagnostic ions, (preferably including quasi molecular ion)	≥ 2 diagnostic ions (preferably including the quasi molecular ion). Mass accuracy < 5 ppm. At least one fragment ion.	≥ 2 product ions
Ion ratio(s):	according to Table 4		

The intensities of diagnostic ions or product ions can be determined through spectra or by integrating the signals of the single mass traces (extracted ion chromatograms). The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense (abundant) ion or product ion, should correspond to those of the calibration standard at comparable concentrations and measured under the same conditions. Matrix-matched calibration solutions may need to be employed. Table 4 below indicates the maximum tolerances.

It should be noted that some analytes and instruments achieve better performance, and others worse, which is also a function of concentration and matrix. Actual measurement of the variability of the ion ratios can be conducted experimentally over time using calibration standards to devise performance-based criteria rather than the fixed generic criteria given in Table 4.

Table 4. Default recommended maximum permitted tolerances for relative ion intensities using a range of spectrometric techniques[2].

Relative intensity (% of base peak)	EI-GC-MS (relative)	CI-GC-MS, GC-MSn, LC-MS, LC-MSn (relative)
> 50 %	± 10 %	± 20 %
> 20 % to 50 %	± 15 %	± 25 %
> 10 % to 20 %	± 20 %	± 30 %
≤ 10%	± 50 %	± 50 %

Larger tolerances may lead to a larger percentage of false positive results. Likewise, if the tolerances are decreased, then the likelihood of false negatives increases[3]. As for Table 4, the tolerances should not be taken as absolute limits and automated data interpretation based on the criteria without complementary interpretation by an experienced analyst is not recommended.

For a higher degree of confidence in identification, further evidence may be required. This can be achieved through additional mass spectrometric information, for example evaluation of full scan spectra, additional accurate mass (fragment) ions, additional product ions (in MS/MS), or accurate mass product ions. If the isotope ratio of the ion(s), or the chromatographic profile of isomers of the analyte, is highly characteristic it may provide sufficient evidence. Otherwise, additional evidence may be sought using a different chromatographic separation system and/or a different ionisation technique, or any other means providing supporting information.

Confirmation by an independent laboratory

80. Where practicable, confirmation of results in an independent expert laboratory provides strong supporting evidence of quantity. If different determination techniques are used, the evidence will also support identification.

[3] Eugenia Soboleva, Karam Ahad and Árpád Ambrus, Applicability of some mass spectrometric criteria for the confirmation of pesticide residues, Analyst, 2004, 129, 1123-1129

Reporting of results

Expression of results

81. Results for individual analytes should be expressed as the chemical name defined by the MRL residue definition and in mg/kg. Where the MRL is a sum of metabolites, degradates or transformation products, the concentrations of these products should be expressed according to the residue definition and then added to the total residue concentration, see Appendix B. Residues below the Reporting Limit should be reported as <RL mg/kg.

Calculation of results

82. In general, residues data do not have to be adjusted for recovery, when the mean recovery is in the range of 70-120%. If residues data are adjusted for recovery, then this must be stated.

83. Where confirmed data are derived from a single test portion (i.e. the residue does not exeed the MRL), the reported result should be that derived from the detection technique considered to be the most accurate. Where results are obtained by two or more equally accurate techniques, the mean value may be reported.

84. Where two or more test portions have been analysed, the arithmetic mean of the most accurate results obtained from each portion should be reported. Where good comminution and/or mixing of samples has been undertaken, the RSD of results between test portions should not exceed 30% for residues significantly above the LOQ. Close to the LOQ, the variation may be higher and additional caution is required in deciding whether or not a limit has been exceeded. Alternatively, the limits for repeatability, or reproducibility, given in Annex VI to Directive 91/414/EEC, may be applied, although these do not incorporate sub-sampling error (which is particularly important when undertaking dithiocarbamate or fumigant analyses).

Rounding of data

85. It is essential to maintain uniformity in reporting results. In general, results ≥0.001 and <0.01 should be rounded to one significant figure; results ≥0.01 and <10 mg/kg should be rounded to two significant figures; results ≥10 mg/kg may be rounded to three significant figures or to a whole number. Reporting limits should be rounded to 1 significant figure at <10 mg/kg and two significant figures at ≥10 mg/kg. These requirements do not necessarily reflect the uncertainty associated with the data. Additional significant figures may be recorded for the purpose of statistical analysis. In some cases the rounding may be specified by, or agreed with the customer/stakeholder of the monitoring. In any case, the rounding of results should never lead to a different decision as regards the exceedance or of a limit of legal relevance (e.g. MRL). Thus, rounding to significant figures shall be done after the final calculation of the result.

Qualifying results with uncertainty data

86. It is a requirement under ISO/IEC 17025 that laboratories determine and make available the uncertainty associated with analytical results. To this end, laboratories should have available sufficient data derived from method valida-

tion/verification, inter-laboratory studies (e.g. proficiency tests) and in-house quality control tests, which are applied to estimate the uncertainties[4].

Measurement uncertainty is a quantitative indicator of the confidence in the analytical data and describes the range around a reported or experimental result within which the true value can be expected to lie within a defined probability (confidence level). Uncertainty ranges must take into consideration all sources of error.

87. Uncertainty data[5] should be applied cautiously to avoid creating a false sense of certainty about the true value. Estimates of typical uncertainty are based on previous data and may not reflect the uncertainty associated with analysis of a current sample. Typical uncertainty may be estimated using an ISO (Anonymous 1995,'Guide to the expression of uncertainty in measurement' ISBN 92-67-10188-9) or Eurachem (EURACHEM/CITAC Guide, Quantifying Uncertainty in Analytical Measurement, 2nd edition, (http://www.measurementuncertainty.org/mu/guide/index.html) approach. The values used may be derived from in-house validation data, the analysis of reference materials, from collaborative method development data, or estimated based on judgment. Reproducibility RSD (or repeatability RSD if reproducibility data are not available) may be used as the basis, but the contribution of additional uncertainty sources (e.g. heterogeneity of the sample from which the analytical test portion should be taken [due to differences in the procedures used for sample preparation, sample processing and sub-sampling], extraction efficiency, differences in standard concentrations) should be included. These RSD values may be derived from recovery data or the analysis of reference materials. Uncertainty data relate primarily to the analyte and matrix used to generate them and should be extrapolated to other analytes and matrices with caution. Uncertainty tends to be greater at lower levels, especially as the LOQ is approached. It may therefore be necessary to generate uncertainty data for a range of concentrations if typical uncertainty is to be provided for a wide range of residues data.

Another practical alternative for a laboratory to estimate its measurement uncertainty and to verify its estimation based on own within-laboratory data is by evaluating its performance during proficiency tests. Proficiency test results can provide an important indication about the contribution of inter laboratory bias to the measurement uncertainty of an individual laboratory as well as indirectly justifying the measurement uncertainty value reported.

88. Replicate analyses of a specific sample combined with concurrent recovery determinations, can improve the accuracy of the single-laboratory result and justify the use of a refined figure for the measurement uncertainty. In that case, care should still be taken with the influence of inter-laboratory bias. These uncertainty data will embrace the repeatability of sub-sampling and analysis. This practice will be typically applied when the analytical results are extremely important (e.g. doubt about MRL compliance and associated economical implications).

89. The use of reporting limits based on the LCL eliminates the need to consider uncertainty associated with residue levels found <reporting limits.

[4] Codex Alimentarius Commission Guideline CAC/GL 59-2006 (Guidelines on estimation of uncertainty of results)

[5] Lutz Alder et al. Estimation of Measurement Uncertainty in Pesticide Residue Analysis. Journal of AOAC International. Vol 84, No 5, 2001, 1569-1577

Interpretation of results for enforcement purposes

90. Assessment of whether or not a sample contains a violative residue is generally only a problem in cases where the level is relatively close to the MRL. The decision should take account of concurrent AQC data and the results obtained from replicate test portions, together with any assessment of typical uncertainty. The possibility of residue loss or cross-contamination having occurred before, during or after sampling must also be considered[4].

91. Considering the results obtained from EU proficiency tests, a default expanded uncertainty figure of 50% (corresponding to a 95% confidence level and a coverage factor of 2), in general covers the inter-laboratory variability between the European laboratories and is recommended to be used by regulatory authorities in cases of enforcement decisions (MRL-exceedences). A prerequisite to be allowed to use a 50% default expanded uncertainty by regulatory authorities, is that the laboratory proves its own calculated expanded uncertainty to be less than 50%. In cases where exceedences of an MRL at the same time cause an exceedence of the acute reference dose, an expanded uncertainty with a lower confidence level can be applied as a precautionary measure.

92. If laboratories experience, in individual cases, unacceptably high repeatability- or within-laboratory reproducibility-RSD_{wR} (e.g. at very low concentration levels), or unsatisfactory z-scores during proficiency tests, the use of a correspondingly higher uncertainty figure must be considered, on a case-by-case basis [5]. For results obtained with single-residue methods (in particular, if stable isotopically labelled internal standards are used), lower expanded uncertainties can be justified, if supported by correspondingly better between-reproducibility RSD_R (≤ 25%)

93. It is common practice that pesticide analysis results are not corrected for recovery, but may be corrected if the average recovery is significantly different from 100% (typically if outside of the range 70-120%, with good precision). In those cases, the uncertainty associated with recovery correction should also be taken into account (see 66)..

94. If required, the result should be reported together with the expanded uncertainty (U), as follows: Result = x ± U (units), with x representing the measured value. In case of official food control by regulatory authorities, compliance with the MRL must be checked by assuming the lower limit of the uncertainty interval (x - U) to be the highest confirmed analyte concentration in the sample. Thus, the MRL is exceeded if x-U > MRL. E.g., in case the MRL = 1 and x = 2.2, then x-U = 2.2 – 1.1 (= 50% of 2.2), which is > MRL.

Additional recommended guidance

Report of the forty-first session of the Codex Committee on Pesticide Residues, Beijing, China, 20-25 April 2009, ALINORM 09/32/24**,** Appendix X**.**

Annex 1.

Selection of representative matrices[6]

Vegetables, fruits and cereals

Commodity groups	Commodity categories	Typical representative commodities included in the category
High water content	Pome fruit	Apples, pears
	Stone fruit	Apricots, cherries, peaches
	Bulb vegetables	Bulb onion
	Fruiting vegetables/cucurbits	Tomatoes, peppers, cucumber, melon
	Brassica vegetables	Cauliflower, Brussels sprout, cabbage, broccoli
	Leafy vegetables and fresh herbs	Lettuce, spinach, basil
	Stem and stalk vegetables	Leek, celery, asparagus
	Forage/fodder crops	Fresh alfalfa, fodder vetch, fresh sugar beets
	Fresh legume vegetables	Fresh peas with pods, petit pois, mange tout, broad bean, runner bean, dwarf French bean
	Leaves of root and tuber vegetables	Sugar beet and fodder beet tops
	Fresh Fungi	Champignons, chanterelles
	Root and tuber vegetables or feed	Sugar beet and fodder beet roots, carrot, potato, sweet potato

[6] OECD Environment, Health and safety Publications, Series on Testing and Assessment , No72 and Series on Pesticides No. 39

Commodity groups	Commodity categories	Typical representative commodities included in the category
High oil content	Tree nuts	Walnut, hazelnut, chestnut
	Oil seeds and products thereof	Oilseed rape, sunflower, cotton-seed, soybeans, peanuts, sesame etc. Oils and pastes (e.g. peanut butter, tahina) thereof,
	Oily fruits and products	Olives, Avocados and oils and pastes thereof
High starch and/or protein content and low water and fat content	Dry legume vegetables/pulses	Field bean, dried broad bean, dried haricot bean (yellow, white/navy, brown, speckled)
	Cereal grain and products thereof	Wheat, rye, barley and oat grain; maize, rice,wholemeal bread, white bread, crackers, breakfast cereals, pasta
High acid content and high water content	Citrus fruit	Lemons, mandarins, tangerines, oranges
	Small fruit and berries	Strawberry, blueberry, raspberry, Black currant, red currant, white currant, grapes
	Other	kiwifruit, pineapple, rhubarb
High sugar and low water content	Dried fruit	Raisins, dried apricots, dried plums, fruit jams
"Difficult or unique commodities"*		Hops Cocoa beans and products thereof, Coffee, Tea Spices

*"Difficult commodities" should only be fully validated if they are frequently analysed. If they are only analysed occasionally, validation may be reduced to just checking the reporting levels using spiked blank extracts.

Products of animal origin

Commodity groups	Commodity categories	Typical representative commodities included in the category
Meat	Red meat	Beef, pork, lamb, game, horse
	White meat	Chicken, duck, turkey
	Fish	Cod, haddock, salmon, trout,
	Offal *	Liver, kidney
	fat from meat	
Milk and milk products	Milk	Cow, goat and buffalo milk
	Cheese	Cow, goat cheese
	Yogurt	
	Cream	
	Butter	
Eggs	Eggs	Chicken, duck, quail, goose eggs
Honey	Honey	

* Offal (liver, kidney) should be validated separately, if necessary

Appendix A.

The validation procedure: outline and example approaches

Validation is undertaken following the completion of the method development or before a method that has not been previously used is to be introduced for routine analysis. We distinguish between initial validation of a quantitative analysis method to be applied in the laboratory for the first time and to extension of the scope of an existing validated method for new analytes and matrices.

Quantitative analysis

1. Initial full validation

Validation needs to be performed

- for all analytes within the scope of the method

- for at least 1 commodity from each of the commodity groups (as far as they are within the claimed scope of the method or as far as applicable to samples analysed in the laboratory)

Experimental:

A typical example of the experimental set up of a validation is:

Sample set (sub samples from 1 homogenised sample)

Reagent blank

1 unspiked sample

5 spiked samples at LOQ

5 spiked samples at 2-10x LOQ or MRL

Instrumental sequence:

Calibration standards in solvent at LOQ level

Calibration standards in matrix at LOQ level

Reagent blank

Unspiked sample

5 spiked samples at LOQ

Calibration standards in matrix at LOQ level

5 spiked samples at 2-10x LOQ or MRL

Calibration standards in matrix at 2-10x LOQ or MRL

Data evaluation:

Calibrate and inject the sequence and quantify as is anticipated in the AQC document.

From the data determine at least the parameters from Table 1 and verify them against the criteria.

Table 1: Validation parameters and criteria.

Parameter	What/how	Criterion	Cross reference to AQC document
Linearity	Through calibration curve	Residuals < ±20%	35-41
Matrix effect	Comparison of response from solvent standards and matrix-matched standards	-	44-48
LOQ	By definition: lowest level for which it has been demonstrated that criteria for accuracy and precision have been met	≤ MRL	56
Specificity	Response in reagent blank and control samples	< 30% of LOQ	63
Accuracy	Determine average recovery for both spike levels	70-120%	58
Precision (RSD_r)	Determine repeatability RSD_r, determine for both spike levels	≤ 20%	58
Precision* (RSD_{wR})	Determine within-laboratory reproducibility*	≤ 20%	58
Robustness	Can be derived from on-going method validation / verification through establishing average recovery and RSD_{wR}?	See above	

* Within-lab reproducibility is to be derived from on-going QC (see below)

2. Extension of the scope of the method: new analytes

New analytes that are added to a previously validated method need to be validated using the same procedure as outlined above for initial validation.

Alternatively, the validation of new analytes can be integrated in the on-going quality control procedure. As an example: with each batch of routine samples one or more commodities from the applicable commodity category are fortified at LOQ and one higher level. Determine recovery and occurrence of any interference in the corresponding unfortified sample. When for both levels 5 recovery values have been collected, the average recovery and within - laboratory reproducibility (RSD_{wR}) can be determined and tested against the criteria from table 1.

3. Extension of the scope of the method: new matrices

A pragmatic way of validation of the applicability of the method to other matrices from the same commodity category is to do this during the on-going quality control performed concurrently with analysis of the samples. See below.

4. On going performance validation / verification

The purpose of on-going method validation is to:

- demonstrate robustness through evaluation of mean recovery and within -laboratory reproducibility (RSD_{wR})
- demonstrate that minor adjustments made to the method over time do not unacceptably affect method performance
- demonstrate applicability to other commodities from the same commodity category (see also above)
- determine acceptable limits for individual recovery results during routine analysis
- collect information for estimation of the within-laboratory measurement uncertainty

Experimental:

Typically, with each batch of samples routinely analysed, one or more samples of different commodities from the applicable commodity category are fortified with the analytes and analysed concurrently with the samples.

Data evaluation:

Determine for each analyte the recovery from the fortified sample and occurrence of any interference in the corresponding unfortified sample. Periodically (e.g. annually) determine average recovery and reproducibility (RSD_{wR}) and verify data obtained against the criteria from Table 1. These data can also be used to set or update limits for acceptability of individual recovery determinations as outlined in paragraph 65 of the AQC document and for estimation of the measurement uncertainty.

Appendix B.

Examples of conversion factors

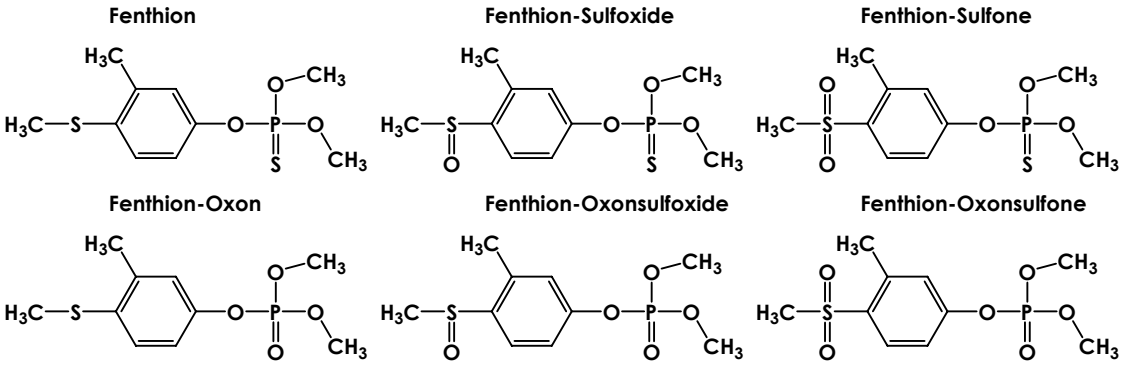
The MRL residue definitions for a number of pesticides include not only the parent pesticide, but also its metabolites or other transformation products.

In Example 1, the sum of the components is expressed as fenthion following adjustment for the different molecular weights (conversion factors), in Example 2 the sum is expressed as arithmetic sum and Example 3 of Thiodicarb and Methomyl .

The following examples illustrate the three types of additions that are required in order to meet the requirements of the residue definition.

Example 1.

Fenthion, its sulfoxides and sulfones, and their oxygen analogues (oxons), all appear in the residue definition and all should be included in the analysis.



Example of calculating the conversion factor (Cf)

$$C_{FenthionSO\ to\ Fenthion} = \frac{MW_{Fenthion}}{MW_{FenthionSO}} \times C_{FenthionSO} = \frac{278.3}{294.3} \times C_{FenthionSO} = 0.946 \times C_{FenthionSO}$$

Compound			Mw	Cf
Fenthion	RR´S	P=S	278.3	1.00
Fenthion sulfoxide	RR´SO	P=S	294.3	0.946
Fenthion sulfone	RR´SO2	P=S	310.3	0.897
Fenthion oxon	RR´S	P=O	262.3	1.06
Fenthion oxonsulfoxide	RR´SO	P=O	278.3	1.00
Fenthion oxonsulfone	R´SO2	P=O	294.3	0.946

Residue Definition:

Fenthion (fenthion and its oxygen analogue, their sulfoxides and sulfones expressed as fenthion

Where the residue is defined as the sum of the parent and transformation products, the concentrations of the transformation products should be adjusted according to their molecular weight being added to the total residue concentration.

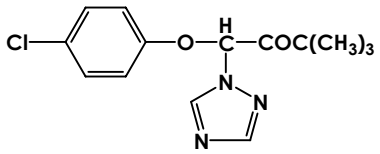
$$C_{Fenthion\ Sum} = 1.00 \times C_{Fenthion} + 0.946 \times C_{FenthionSO} + 0.897 \times C_{FenthionSO_2} + 1.06 \times C_{Fenthion\ oxon} + 1.00 \times C_{Fenthion\ oxonSO} + 0.946 \times C_{Fenthion\ oxonSO_2}$$

Example 2.

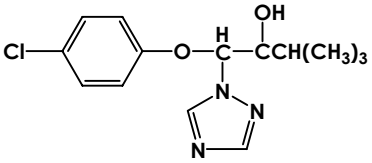
Residue Definition:

Triadimefon and triadimenol (sum of triadimefon and triadimenol)

Triadimefon



Triadimenol



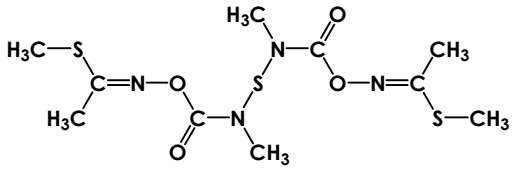
$C_{\text{Triadimefon and triadimenolSum}} = 1.00 \times C_{\text{Triadimefon}} + 1.00 \times C_{\text{Triadimenol}}$

Example 3.

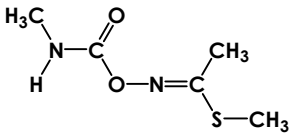
Residue Definition:

Methomyl and Thiodicarb (sum of methomyl and thiodicarb expressed as methomyl)

Thiodicarb



Methomyl



$$C_{Methomyl\ Sum} = C_{Methomyl} + C_{Thiodicarb} \times \left(\frac{2 \times MW_{\text{Methomyl}}}{MW_{\text{Thiodicarb}}} \right) = C_{Methomyl} + C_{Thiodicarb} \left(\frac{2 \times 162.2}{354.5} \right)$$

$$C_{Methomyl\ Sum} = C_{Methomyl} + 0.915 \times C_{Thiodicarb}$$

Appendix C.

Glossary

accuracy	Closeness of agreement between a test result and the true, or the accepted reference value. When applied to a set of test results, it involves a combination of random error (estimated as precision) and a common systematic error (trueness or bias) (ISO 5725-1).
analyte	The chemical species of which the concentration (or mass) is to be determined. For the purposes of these procedures: a pesticide or a metabolite, breakdown product or derivative of a pesticide or an internal standard.
analytical sample	See test sample
analytical portion	See test portion.
API	Atmospheric pressure ionisation (for LC-MS). A generic term including electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI).
AQC	Analytical quality control. Measurement and recording requirements intended to demonstrate the performance of the analytical method in routine practice. The data supplement those generated at method validation. AQC data may be used to validate the extension of methods to new analytes, new matrices and new levels. Synonymous with the terms internal quality control (IQC) and performance verification. Concurrent AQC data are those generated during analysis of the batch in which the particular sample is included.
batch (analysis)	For extraction, clean-up and similar processes, a batch is a series of samples dealt with by an analyst (or team of analysts) in parallel, usually in one day, and should incorporate at least one recovery determination. For the determination system, a batch is a series undertaken without a significant time break and which incorporates all relevant calibration determinations (also referred to as an "analysis sequence", a "chromatography sequence", etc.). With formats such as 96-well plates, a plate or group of plates may form a batch. A determination batch may incorporate more than one extraction batch. This document does not refer to "batch" in the IUPAC or Codex sense, which relates to manufacturing or agricultural production batches.
bias	Also referred to as "accuracy". The difference between the mean measured value and the true value, i.e. the total systematic error.

blank	(i) Material (a sample, or a portion or extract of a sample) known not to contain detectable levels of the analyte(s) sought. Also known as a matrix blank. (ii) A complete analysis conducted using the solvents and reagents only; in the absence of any sample (water may be substituted for the sample, to make the analysis realistic). Also known as a reagent blank or procedural blank.
bracketing calibration	Organisation of a batch of determinations such that the detection system is calibrated immediately before and after the analysis of the samples. For example, calibrant 1, calibrant 2, sample 1, sample *n*, calibrant 1, calibrant 2.
calibration	Determination of the relationship between the observed signal (response produced by the detection system) from the target analyte in the sample extract and known quantities of the analyte prepared as standard solutions. In the present document, calibration does not refer to calibration of weighing and volumetric equipment, mass calibration of mass spectrometers, and so on.
calibration standard	A solution (or other dilution) of the analyte (and internal standard, if used) used for calibration of the determination system. May be prepared from a working standard and may be matrix-matched.
certified reference material (CRM)	See reference material.
CI	Chemical ionisation (for GC-MS).
comminution	The process of reducing a solid sample to small fragments.
confirmation	Confirmation is the combination of two or more analyses that are in agreement with each other (ideally, using methods of orthogonal selectivity), at least one of which meets identification criteria)[7]. It is impossible to confirm the complete absence of residues. Adoption of a "reporting limit" at the LCL avoids the unjustifiably high cost of confirming the presence, or absence, of residues at unnecessarily low levels. The nature and extent of confirmation required for a positive result depends upon importance of the result and the frequency with which similar residues are found. Assays based on ELISA or ECD tend to demand confirmation, because of their lack of specificity. Mass spectrometric techniques are often the most practical and least equivocal approach to confirmation. AQC procedures for confirmation should be rigorous.

[7] S.J. Lehotay, K. Mastovska, A. Amirav, A.B. Fialkov, T. Alon, P.A. Martos, A. de Kok, A.R. Fernandez-Alba, Trends in Anal. Chem. 27 (2008) 1070-1090

contamination	Unintended introduction of the analyte into a sample, extract, internal standard solution etc., by any route and at any stage during sampling or analysis.
determination/detection system	Any system used to detect and determine the concentration or mass of the analyte. For example, GC-FPD, LC-MS/MS, LC with post-column derivatisation, ELISA.
Diagnostic ion	Mass spectrometric term for ions that are highly characteristic for the compound measured.
ECD	Electron-capture detector.
EI	Electron ionisation.
ELISA	Enzyme-linked immuno-sorbent assay.
EU	European Union.
False negative	A result wrongly indicating that the analyte concentration does not exceed a specified value.
False positive	A result wrongly indicating that the analyte concentration exceeds a specified value.
FPD	Flame-photometric detector (may be specific to sulphur or phosphorus detection).
FWHM	Full-width at half maximum
GC	Gas chromatography (gas-liquid chromatography).
Identification	Is a qualitative result from a method capable of providing structural information (e.g., using mass spectrometric (MS) detection) that meets acceptable criteria for the purpose of the analysis. The process of generating of sufficient evidence to ensure that a result for a specific sample is valid. Analytes must be identified correctly in order to be quantified. AQC procedures for identification should be rigorous.
interference	A positive or negative response produced by a compound(s) other than the analyte, contributing to the response measured for the analyte, or making integration of the analyte response less certain or accurate. Interference is also loosely referred to as "chemical noise" (as distinct from electronic noise, "flame noise", etc.). Matrix effects are a subtle form of interference. Some forms of interference may be minimised by greater selectivity of the detector. If interference cannot be eliminated or compensated, its effects may be acceptable if there is no significant impact on accuracy (bias) or precision.
High resolution MS	Detection using mass spectrometers with high resolving power, typically > 10,000 FWHM
internal quality control (IQC)	see AQC
Within-laboratory reproducibility	see reproducibility

internal standard	An analyte 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. The analyte concentration is deduced from its response relative to that produced by the internal standard. In case of using MS-detection isotopically labelled analytes form ideal internal standards, where available.
laboratory sample	The sample sent to and received by the laboratory.
LC	Liquid chromatography (primarily high performance liquid chromatography, HPLC).
LCL	Lowest calibrated level. The lowest concentration (or mass) of analyte with which the determination system is successfully calibrated, throughout the analysis batch. See also "reporting level".
LC-MS	Liquid chromatographic separation coupled with mass spectrometric detection.
Level	In this document, refers to concentration (e.g. mg/kg, µg/ml) or quantity (e.g. ng, pg).
LOD	Limit of determination (see LOQ below).
LOQ	Limit of quantitation (quantification) (also known as limit of determination, LOD). The minimum concentration or mass of the analyte that can be quantified with acceptable accuracy and precision. Should apply to the complete analytical method. Variously defined but must be a value greater than the limit of detection. With most methods and determination systems, the LOQ has no fixed value. LOQ is preferable to LOD because it avoids possible confusion with "limit of detection". However, in legislation MRLs that are set at the limit of quantification/determination are referred to as "LOD MRLs", not "LOQ MRLs".

Mass accuracy:	Mass accuracy is the deviation of the measured *accurate* mass from the calculated *exact* mass of an ion. It can be expressed as an absolute value in milliDaltons (mDa) or as a relative value in parts-per-million (ppm) error and is calculated as follows: (accurate mass – exact mass) Example: the experimentally measured mass = 239.15098, the theoretical exact mass of the ion m/z = 239.15028. The mass accuracy = (239.15098 – 239.15028) = 7.0 mDa or (accurate mass – exact mass) / exact mass * 10^6 Example: the experimentally measured mass = 239.15098, the theoretical exact mass of the ion m/z = 239.15028 The mass accuracy = (239.15098 – 239.15028) / 239.15028 * 10^6 = 2.9 ppm
Mass resolution	The resolution of a mass spectrometry instrument is the ability to distinguish between two ions with similar m/z values (IUPAC definition[8]: the smallest mass difference between two equal magnitude peaks so that the valley between them is a specified fraction of the peak height).
Mass resolving power	The resolving power, defined at full-width half maximum (FWHM), is m/Δm, where m is the m/z being measured and Δm the width of the mass peak at half peak height. Note 1: for magnetic sector instruments another definition is used ("10% valley"). Roughly the difference between the two definitions is a factor of 2 (i.e. 10,000 resolving power by the 10% valley method equals 20,000 resolving power by FWHM). Note 2: mass resolving power is often confused or interchangeably used with mass resolution (see definition above).
matrix blank	See blank.

[8] http://www.iupac.org/web/ins/2003-056-2-500 and http://old.iupac.org/reports/provisonal/abstarct06/murray_prs.pdf

matrix effect	An influence of one or more undetected components from the sample on the measurement of the analyte concentration or mass. The response of some determination systems (e.g. GC, LC-MS, ELISA) to certain analytes may be affected by the presence of co-extractives from the sample (matrix). Partition in headspace analyses and SPME is also frequently affected by components present in the samples. These matrix effects derive from various physical and chemical processes and may be difficult or impossible to eliminate. They may be observed as increased or decreased detector responses, compared with those produced by simple solvent solutions of the analyte. The presence, or absence, of such effects may be demonstrated by comparing the response produced from the analyte in a simple solvent solution with that obtained from the same quantity of analyte in the presence of the sample or sample extract. Matrix effects tend to be variable and unpredictable in occurrence, although certain techniques and systems (e.g. HPLC-UV, isotope dilution) are inherently less likely to be influenced. More reliable calibration may be obtained with matrix-matched calibration when it is necessary to use techniques or equipment that are potentially prone to the effects. Matrix-matched calibration may compensate for matrix effects but does not eliminate the underlying cause. Because the underlying cause remains, the intensity of effect may differ from one matrix or sample to another, and also according to the "concentration" of matrix. Isotope dilution or standard addition may be used where matrix effects are sample dependent.
matrix-matched calibration	Calibration intended to compensate for matrix effects and acceptable interference, if present. The matrix blank (see "blank") should be prepared as for analysis of samples. In practice, the pesticide is added to a blank extract (or a blank sample for headspace analysis) of a matrix similar to that analysed. The blank matrix used may differ from that of the samples if it is shown to compensate for the effects. However, for determination of residues approaching or exceeding the MRL, the same matrix (or standard addition) should be used.
method	A sequence of analytical procedures, from receipt of a sample through to the calculation of results.
method development	The process of design and preliminary assessment of the characteristics of a method, including ruggedness.
method validation	The process of characterising the performance to be expected of a method in terms of its scope, specificity, accuracy (bias), sensitivity, repeatability and within laboratory reproducibility. Some information on all characteristics, except within laboratory reproducibility, should be established prior to the analysis of samples, whereas data on reproducibility and extensions of scope may be produced from AQC, during the analysis of samples. Wherever possible, the assessment of accuracy (bias) should involve analysis of certified reference materials, participation in proficiency tests, or other inter-laboratory comparisons.

MRL	Maximum residue level. In Regulation 396/2005 list MRLs for pesticide/commodity combinations, an asterisk indicates that the MRL* is set at or about the LOQ, with the LOQ being here a consensus figure rather than a measured value.
MS	Mass spectrometry.
MS/MS	Tandem mass spectrometry, here taken to include MS^n. An MS procedure in which ions of a selected mass to charge ratio (*m*/z) from the primary ionisation process are isolated, fragmented usually by collision, and the product ions separated (MS/MS or MS^2). In ion-trap mass spectrometers, the procedure may be carried out repetitively on a sequence of product ions (MS^n), although this is not usually practical with low-level residues.
May	**MAY** within this document means perhaps or possibly an option (the action is optional).
Must	**MUST or SHALL** within this document means an absolute requirement (the action is mandatory). **MUST/SHALL NOT** means an absolute no.
NPD	Nitrogen-phosphorus detector.
Non-compliance	See violative residue
performance verification	see analytical quality control (AQC)
priming (of GC injectors and columns)	Priming effects resemble long-lasting matrix effects and are typically observed in gas chromatography. Typically, an aliquot of sample extract that has not been subjected to clean-up may be injected after a new column or injector liner is fitted, or at the beginning of a batch of determinations. The objective is to "deactivate" the GC system and maximise transmission of the analyte to the detector. In some cases, large quantities of analyte may be injected with the same objective. In such cases it is critically important that injections of solvent or blank extracts are made before samples are analysed, to ensure the absence of carryover of the analyte. Priming effects are rarely permanent and may not eliminate matrix effects.
procedural blank	See blank.
(Quasi)-molecular ion	A molecular ion (M^+ or M^-) or a protonated ($M+H^+$) or deprotonated molecule ($M-H^+$).
reagent blank	See blank.
recovery (of analyte through an analytical method)	The proportion of analyte remaining at the point of the final determination, following its addition (usually to a blank sample) immediately prior to extraction. Usually expressed as a percentage. Routine recovery refers to the determination(s) performed with the analysis of each batch of samples.

reference material	Material characterised with respect to its notionally homogeneous content of analyte. Certified reference materials (CRMs) are normally characterised in a number of laboratories, for concentration and homogeneity of distribution of analyte. In-house reference materials are characterised in the owner's laboratory and the measurement accuracy (bias) may be unknown.
reference spectrum	A spectrum of absorption (e.g. UV, IR), fluorescence, ionisation products (MS), etc., derived from the analyte and which may be characteristic of it. The reference mass spectrum preferably should be produced from the "pure" standard (or a solution of the "pure" standard) by the instrument used for analysis of the samples, and similar ionisation conditions must be used.
"pure" standard	A relatively pure sample of the solid/liquid analyte (or internal standard), of known purity. Usually >90% purity, except for certain technical pesticides.
Repeatability (r)	The precision (standard deviation) of measurement of an analyte (usually obtained from recovery or analysis of reference materials), obtained using the same method on the same sample(s) in a single laboratory over a short period of time, during which differences in the materials and equipment used and/or the analysts involved will not occur. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the test result. May also be defined as the value below which the absolute difference between two single test results on identical material, obtained under the above conditions, may be expected to lie with a specified probability (e.g. 95%).
reporting level	The lowest level at which residues will be reported as absolute numbers. It may represent the practical LOQ, or it may be above that level to limit costs. It must not be lower than the corresponding LCL. For EU monitoring purposes where samples for surveys are analysed over a 12-month period, the same reporting limit should be achievable throughout the whole year.
representative analyte	An analyte used to assess probable analytical performance in respect of other analytes notionally sought in the analysis. Acceptable data for a representative analyte are assumed to show that performance is satisfactory for the represented analytes. Representative analytes must include those for which the worst performance is expected.

Reproducibility (R)	The precision (standard deviation) of measurement of an analyte (usually by means of recovery or analysis of reference materials), obtained using the same method in a number of laboratories, by different analysts, or over a period in which differences in the materials and equipment will occur. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the test result. Within-reproducibility (w_R) is that produced in a single laboratory under these conditions. May also be defined as the value below which the absolute difference between two single test results on identical material, obtained under the above conditions, may be expected to lie with a specified probability (e.g. 95%).
response	The absolute or relative signal output from the detector when presented with the analyte.
RSD	Relative standard deviation (coefficient of variation).
sample	A general term with many meanings but, in these guidelines, refers to laboratory sample, test sample, test portion, or an aliquot of extract.
sample preparation	The first of two processes which may be required to convert the laboratory sample into the test sample. The removal of parts that are not to be analysed, if required.
sample processing	The second of two processes which may be required to convert the laboratory sample into the test sample. The process of homogenization, comminution, mixing, etc., if required.
Screening Reporting Level (SRL)	Lowest spiking level for which has been demonstrated that a certain analyte can be detected (not necessarily identified) in at least 95% of the samples
SD	Standard deviation.
selectivity	The ability of the extraction, the clean-up, the derivatisation, the separation system and (especially) the detector to discriminate between the analyte and other compounds. GC-ECD is a selective determination system providing no specificity.
Shall	**See must**
Should	**SHOULD** within this document means a recommendation that may be ignored but only in particular circumstances (because of valid reasons) and the full implications of ignoring the recommendation must be understood and carefully assessed before choosing a different course of action. **SHOULD NOT** means not recommended, although it may be acceptable in particular circumstances, but the full implications of ignoring the recommendation must be understood and carefully assessed.

Significant figures	Those digits in a number that are known with certainty, plus the first uncertain digit. Ex. 3 significant figures 0.104, 1.04, 104, 1.04×10^4 The 1 and the middle 0 are certain, and the 4 is uncertain, but significant. Note: Initial zeroes are never significant. Exponential number has no effect on the number of significant figures.
SIM	Selected ion monitoring. Operation of a mass spectrometer in which the abundance of several ions of specific m/z values are recorded rather than the entire mass spectrum
SRM	Selected reaction monitoring. Measurement of specific product ions corresponding to m/z selected precursor ions recorded via two or more stages of mass spectrometry (MS^n).
solid phase dilution	Dilution of a pesticide by distribution within a finely divided solid, such as starch powder. Normally used only for insoluble analytes such as the complex dithiocarbamates.
S/N	Signal-to-noise ratio.
specificity	The ability of the detector (supported by the selectivity of the extraction, clean-up, derivatisation or separation, if necessary) to provide signals that effectively identify the analyte. GC-MS with EI is a fairly non-selective determination system capable of high specificity. High resolution mass MS and MS^n can be both highly selective and highly specific.
spike or spiking	Addition of analyte for the purposes of recovery determination or standard addition.
SPME	Solid phase micro-extraction.
standard	A general term which may refer to a "pure" standard, stock standard, working standard, or calibration standard.
stock standard	The most concentrated solution (or solid dilution, etc.) of the "pure" standard or internal standard, from which aliquots are used to prepare working standards or calibration standards.
surrogate standard,	A substance of known concentration added to the samples for QC-purposes. This substance should be unlikely to be found in samples and should have properties similar to the pesticides targeted (more than one surrogate standard may be required to represent a broad analyte spectrum). Surrogate standards are intended to monitor for recovery difference as well as problems during extraction and determinative analysis. Adding surrogate standards at different stages of the analytical procedure may help to localize the sources of errors.

test portion	Also referred to as the "analytical portion". A representative sub-sample of the test sample, i.e. the portion which is to be analysed.
test sample	Also referred to as the "analytical sample". The laboratory sample after removal of any parts that are not to be analysed, e.g. bones, adhering soil. It may or may not be comminuted and mixed before withdrawing test portions. See also Directive 2002/63/EC.
trueness	The measure of trueness is normally expressed as 'bias'. The closeness of agreement between the average value obtained from a series of test results (i.e. the mean recovery) an accepted reference or true value (ISO 5725-1).
uncertainty (of measurement)	A range around the reported result within which the true value can be expected to lie with a specified probability (confidence level, usually 95%). Uncertainty data should encompass trueness (bias) and reproducibility
unit (sample)	A single fruit, vegetable, animal, cereal grain, can, etc. For example, an apple, a T-bone steak, a grain of wheat, a can of tomato soup.
validation	see method validation
violative residue	A residue which exceeds the MRL or is unlawful for any other reason.
working standard	A general term used to describe dilutions produced from the stock standard, which are used, for example, to spike for recovery determination or to prepare calibration standards.

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