EUROPEAN COMMISSION
Directorate General Health and Consumer Protection

SANCO/3029/99 rev.4
11/07/00

Residues:
Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414.

Working document

CONTENTS

1 Note: References in the text are given as bracketed numbers.

1 INTRODUCTION

In order to generate Annex II and III data for pre-registration purposes under Directive 91/414 EEC, robust, accurate and precise analytical methods are required.

Depending on proposed uses, methods are required (i) to generate residues data on which consumer dietary exposure assessments are based, and (ii) to support studies on the fate and behaviour of the active substance in foodstuffs, the environment, ecotoxicology and toxicology.

1.1 Scope

This document has been prepared to provide guidance to applicants on the pre-registration requirements for residue analytical methods for submissions under Directive 91/414/EEC. The guidance does not refer to methodology specifically supporting post-registration monitoring and control, which is covered by separate guidance (SANCO/825/00). In cases where the requirements of these guidelines cannot be fulfilled, full justification must be submitted. The requirements outlined in this guidance paper are applicable to a core data set for each method. It is not a requirement that the whole data set is generated every time a method is used.

Directive 96/46/EC, published in the Official Journal on 23.8.96 (1), amends section 4, Part A of Annex II and section 5, Part A of Annex III of Directive 91/414 EEC. This Directive addresses the development of analytical methods required for post-registration control and monitoring purposes. It is also broadly relevant to the methods of analysis required for registration. However, some of the requirements such as 'minimum cost' and 'commonly available' equipment do not apply to methods supporting pre-registration studies. It is recognised that there will be overlap between pre- and post-registration requirements for methods supporting both and requirements have been harmonised where possible.

1.2 Content

The document is divided into sections addressing the requirements for methods supporting the generation of data for registration. The following topics are discussed: method description; method validation; confirmatory techniques; derivatisation; non-specific and common moiety methods. In addition, immunological analysis is also considered. Annexes cover glossary/definitions and a summary of required method validation data.

1.3 GLP

Individual requirements for the validation of methods are described in each section. Full details of all validation studies must be reported. GLP compliance requirements for methods submitted in support of applications made under Directive 91/414 (including minor uses GLP requirements) are detailed in the Commission guideline documents 7109/VI/94 (2) and 7017/VI/95 (3). The development of a method is not subject to GLP, however where the method is used to generate data for safety purposes, for example residues data, data generation must be conducted to GLP.

1.4 Sampling and storage

This working document covers laboratory sample preparation, extraction and analysis only. For plant and animal product residue methods, information on the stability of residues on storage is covered under the amending Directive on residues requirements, 96/68/EC. FAO guidance on sampling methodology and initial sample preparation for the generation of residue trials data (for registration) is given in references (4) and (5). Directives 90/642/EEC

(6) and 79/700/EEC (7) and the FAO/WHO CODEX Alimentarius Commission (8) give limited guidance on sampling, sample sizes and initial preparation for post-registration purposes. Reference (9) gives sampling guidance for dislodgeable residues. For environmental sample residue methods, guidance on the stability of residues during storage, sampling methodology and initial sample preparation is given in reference (10).

1.5 Description and reporting of methods

Full descriptions of validated methods must be provided, including details of equipment, materials and conditions used. Where published methods are submitted, validation of the published method when applied to the relevant sample matrix and laboratory conditions is required. If a previously collaboratively tested method is used, this should be validated for new laboratory conditions. Full method details are required. In cases where the method significantly deviates from the standard method, validation data will be required.

The method description in the submitted method must include the following:

- principle of the method (including scope and method specificity)
- method summary; equipment/reagents (including details of any hazards or precautions required and reagent stability information)
- full details of standard compound purity where relevant.
- sample storage, where validation samples have been stored prior to analysis (conditions of storage, e.g. temperature and period of storage)
- general sample preparation techniques (including sample sizes and numbers of samples)
- analytical procedure (including extract preparation and instrumentation used)
- details of calibration
- interpretation of chromatograms (where appropriate)
- representative chromatograms (where appropriate) (control blank(s), analytical standard/matrix standard(s), lowest fortification(s), treated sample(s))
- calculations
- results of the determination of extraction efficiency
- references

Quantification procedures should be described, including detection system calibration, calculation of analyte concentration and any compliance with statistical parameters required. Supporting chromatograms/spectra or non-chromatographic data should be clearly labelled. Labelling should include sample description, scale, concentration and identification of all relevant components.

Validation data should also be submitted. A summary of method validation requirements is shown in Appendix 2.

This guideline document should be used in conjunction with Directive 96/46/EC.

For this paper the definitions discussed in Appendix 1 apply.

2 SCOPE AND APPLICABILITY OF RESIDUE METHODS SUPPORTING REGISTRATION

2.1 Requirements for methods of analysis

Residue analytical methods may be required to support many studies including the following:

(a) Residue studies:

- Plant residues - consumer risk assessment, setting MRLs
- Animal feeding studies - consumer risk assessment, setting MRLs
- Processing studies
- Stability of residues during storage

(b) Environmental fate:

- Field dissipation, accumulation, degradation or partition studies (non-radiolabelled) - parent and major environmental metabolites. Usual matrices of interest are soil, water and sediment. Air may also need to be considered.

(c) Efficacy:

- Soil - carry over of phytotoxic levels of the a.s. and/or biologically active metabolites.
- Water - assessing effectiveness of procedures for cleaning spray equipment.

(d) Ecotoxicology:

- To verify actual exposure - levels of a.s. and relevant/major metabolites in ecotoxicity tests. Usual matrices of interest are soil, water, sediments and feedstuffs.

(e) Toxicology

- Dietary and gavage non-radiolabelled studies
- Air - Inhalation studies

(f) Operator or Worker Exposure:

- Dosimetry samples
- Inhalation samples
- Biological samples

2.2 Sample extraction and purification techniques

Methods supporting registration may employ any suitable analytical techniques for sample preparation, providing the method validation criteria are adequately addressed.

2.3 Derivatisation

For the analysis of some compounds, such as those of high polarity or with poor chromatographic properties, derivatisation may be necessary. Derivatives may be prepared prior to chromatographic analysis or as part of the chromatographic procedure, either pre-or post-column.

- Where a derivatisation method is used, this must be fully reported and justified.
- The derivative must be stable and must be formed reproducibly.
- Where quantification is based on the determination of a derivative, the calibration should preferably be carried out using standard solutions of that derivative, unless the derivatisation step is an on-line part of the detection system.

- The mean yield and precision of the derivatisation step must be demonstrated, where possible.

The method is considered to remain specific to the analyte of interest if the derivatised species is specific to that analyte. However, where the derivative formed is a common derivative of two or more active substances or impurities or is classed as another active substance, the method should be considered non-specific. A consideration of non-specific methods is given in section 2.4.

2.4 Non-specific and common moiety methods

In the context of this guidance, common moiety methods are non-specific if the moiety is common to two or more active substances or significant, major or relevant impurities/metabolites, irrespective of how specific the determination is made towards the common moiety.

It is recognised that, for some analytes, a specific method may be unavailable or difficult to perform. Some common moieties are to be determined because all components containing that moiety are considered toxicologically important and because no single component is an adequate marker of residue concentration. However, where possible to do so, the use of non-specific methods is discouraged. Disadvantages of using non-specific or common moiety methods are:

- Where a non-specific method has been used, the identity of the source of the analyte is likely to be called into question. For example, the method may also detect impurities or breakdown products either containing a moiety common to the intended analyte, or which have been derivatised to a common species, or which cannot be resolved from the target analyte. Such methods may also be subject to interferences from other similarly structured compounds.

- when analysing a.s. content in a product that has undergone storage as part of a storage stability study, degradation may be impossible to determine with a method that is not specific to the a.s.

- Where the method determines a moiety common to two or more distinct active substances with differential toxicities, it is important to identify the origin of the residue, enabling the risk assessment to be carried out on the toxicologically significant residue components.

For the generation of plant and animal product residues trials data, in cases where it is likely that a multi-component residue definition will be required for risk assessment purposes, a common moiety method may be used. However, the choice of appropriate methods should take into consideration the needs of both risk assessment and MRL compliance. In practice this may mean generating the data in such a way as to give the regulatory authority flexibility to establish two separate residue definitions where appropriate, one for risk assessment and a second for MRL compliance monitoring. In such cases, where possible applicants should either:

(i) separately analyse for the individual components of the residue, rather than carrying out a total residue analysis; or

(ii) carry out a total residue analysis and a second series of analyses of the field trial samples for a suitable indicator molecule in parallel, if the total residue methodology is unsuitable for practical routine monitoring and enforcement of the MRL at reasonable cost. For

environmental sample residue methods, the use of common moiety methods in support of registration can be acceptable, however their use is not ideal. The availability of appropriate methods for monitoring purposes needs to be considered.

Non-specific and common moiety methods will only be acceptable in exceptional circumstances where there is no other practical means of determining the target analyte, and in these cases, full justification is required. This should include an explanation of why the compound cannot be determined by a specific analytical technique. When common moiety methods are proposed, validation data must be presented separately for all relevant components of the residue definition.

3 RESIDUES ANALYSIS IN PLANTS, PLANT PRODUCTS, FOODSTUFFS (OF PLANT AND ANIMAL ORIGIN) AND FEEDINGSTUFFS - (determination of the active substance and relevant metabolites) (*Annex IIA, point 4.2.1 and Annex IIIA , point 5.2.1 of Directive 91/414/EEC*)

3.1 Method Validation

Validation data should be submitted for all sample matrices to be analysed and should be carried out for all components of the residue definition. This is in contrast to validation requirements for post-registration control and monitoring using multi-residue methods (11). However, a case for matrix comparability and a reduced validation data set may be considered where two or more very similar matrices are to be analysed. For example, cereal grain. Reduced validation data for sample matrices within the same crop group (as defined in SANCO/825/00) are acceptable. In these cases data must include recovery at 2 fortification levels and associated precision (minimum 3 samples) together with an assessment of matrix interference.

For validation of methods for the determination of residues in products of animal origin, the following animal tissues should be used where appropriate:

- milk
- liver, kidney and muscle
- fat
- eggs

(i) *Specificity* - Methods of analysis of residues must be:

- appropriate to the residue definition used in the assessment of risk to consumers.

The method(s) must be capable of determining the active ingredient and/or relevant metabolites in the presence of the sample matrix. Where the sample contains more than one isomer, analogue, etc., of an active substance or relevant metabolite, the method(s) should distinguish between individual isomers/ analogues where this is necessary for carrying out risk assessments.

(ii) *Linearity* - The analytical calibration should extend over a range appropriate to the lowest and highest nominal concentration of the analyte in relevant analytical solutions $\pm$ at least 20%. Either duplicate determinations at three or more concentrations or single determinations at 5 or more concentrations must be made. The equation of the calibration line and a regression parameter, e.g. the correlation coefficient (r), must be reported and a typical calibration plot submitted. Details of the linear range should be given, e.g. µg/ml. Where a non-linear calibration is used, an explanation (including how calibration accuracy is to be maintained) must be provided.

Possible effects of sample components, e.g. co-extractives, on chromatographic transmission or detection system response must be addressed. Where appropriate, detection system calibration should be generated using standard solutions in a matrix similar to that of the samples to be analysed.

(iii) *Accuracy* - The accuracy of the method should be reported as mean recovery $\pm$ relative standard deviation (see iv below) for each component of the residue definition individually recovered from the sample matrix. Recovery data must be reported for 2 fortification levels appropriate to the proposed LOQ and likely residue levels or 10 x LOQ. Recoveries should

also be conducted during residue trial sample analysis and reported with residue trial results (Directive 96/68/EC) (12).

Samples to be utilised for recovery determinations should be of the untreated commodity, to which a known quantity of analyte is added and the whole sample analysed to reduce sampling error. The results should be compared to the known analyte "content" of the sample. Mean recoveries for each level should be in the range 70-110%, ideally with the mean in the range 80-100%. Lower recoveries may be acceptable for matrices which are difficult to analyse (e.g. hops, brassicas, bulb vegetables, herbs, tea) providing precision data are acceptable. Control (unfortified) samples should be analysed concurrently to determine any contamination by the analyte of interest or interferences.

In some methods the calibration requires the direct measurement of fortified test portions of blank samples without separate extraction and cleanup steps (e.g. headspace GC, SPME techniques). In these cases it is not possible to determine recovery and an estimate of the accuracy of the analytical technique may be made by an assessment of the linearity of matrix calibration or by comparison with other techniques.

(iv) *Precision - repeatability (r)* - The precision of the method must be reported as the RSD of repeatability at each fortification level (see iii above) and the overall relative standard deviation (RSD) must also be reported. Five determinations should be made at each fortification level. In general the RSD should be $\leq$ 20% per level. In certain justified cases higher variability may be accepted.

Where outliers have been identified using appropriate statistical methods (e.g. Grubbs (13) or Dixons (14) test), this should be justified. A maximum of 1 outlier may be discarded at each fortification level. Where more than one outlier has been identified at one fortification level, additional validation samples must be included and an explanation provided.

(v) *LOQ* - Methods must be validated at the proposed LOQ. Blank values (procedural blanks and untreated samples) should not exceed 30% of the LOQ. If this is exceeded, detailed justification is required.

(vi) *Precision - Reproducibility* (R) - Reproducibility, as defined in Appendix 1, is only required for method(s) designated as post-registration monitoring methods (see SANCO/825/00).

3.2 Confirmation of analyte identification

In general, additional confirmatory analysis will not be required where primary residue method(s) are shown to be specific to the analyte(s) of interest and the known source of the analyte(s). However, additional confirmation may be necessary (for example where the first method is an immunoassay or for confirmation of the identity of degradation products formed during sample storage), the confirmatory method should be appropriate to the matrix. Where relevant, standard solutions should be prepared in a matrix relevant to that containing the analyte to be measured (section 3.1 (ii)). Chromatographic confirmatory methods should be used, where practical, such as GC-MS, LC-MS and HPLC-DAD. Where mass spectrometric confirmatory analysis is carried out, a minimum of 3 ions (ideally with an m/z ratio of >100) should be used for identification/quantification. These ions should be reported and justified. Where MS is used in routine analysis of residue trial samples, 2 ions are sufficient for identification/quantification.

Where the primary method of determination cannot provide unequivocal identification and quantification of the analyte, confirmation may be possible using different separation systems. Examples are given in references 16 and 17.

3.3 Non-specific methods

See section 2.4. Where possible the method should be specific to the appropriate residue definition. In cases where analysis involves a non-specific method this may be acceptable for the analysis of residue trials samples where a history of previous applications to the trial site is known and reported. [2]

It should be noted that, where a non-specific method has been used, it is possible for the identity of the source of the analyte to be called into question. For example, the method may also detect impurities or breakdown products either containing a moiety common to the intended analyte, or which have been derivatised to a common species, or which cannot be resolved from the target analyte. Therefore, where the source of the analyte is unclear the method will not be acceptable. In cases where derivatisation to a common species is the only method available (for example dithiocarbamate compounds), justification for the use of the common moiety method may be based on the known source of the derivatised analyte and consideration of control samples.

3.4 Dislogeable residues

Information on dislodgeable residues is used for an estimation of dermal exposure of workers, who have contact with treated plants. For sampling and determination of dislodgeable residues, the method of Iwata et al. (9) applies generally. Methods should be fully described and validated as detailed in OECD guidelines (18). See also Appendix 2.

[2] In such cases, a monitoring method capable of determining all components of the MRL residue definition, or all relevant environmental residues to be monitored, must also be available.

4 RESIDUES ANALYSIS IN SOIL, WATER, SEDIMENT AND AIR SAMPLES - (determination of the active substance and relevant metabolites) (*Annex IIA, points 4.2.2 - 4.2.4 and Annex IIIA , points 5.2.2 - 5.2.4 of Directive 91/414/EEC*)

4.1 Method Validation

For methods supporting operator exposure studies, the method validation guidance detailed in the OECD guidance document for the conduct of occupational exposure studies (18) should be followed. Validation is summarised in Appendix 2.

(i) *Specificity* - Methods of analysis of residues must be:

- appropriate to the residue definitions (parent compound and relevant/major metabolites) used in the assessment of risk to non-target organisms and consumers / operators or bystanders (for drinking water and air)

Where the sample contains more than one isomer, analogue, etc., of an active substance or relevant/major metabolite, the method(s) should distinguish between individual isomers/ analogues where this is necessary for carrying out risk assessments.

(ii) *Linearity* - see section 3.1 (ii).

(iii) *Accuracy* - The accuracy of the method should be reported as mean recovery $\pm$ relative standard deviation (see iv below) for the active substance and/or relevant/major metabolites in the sample matrix. Recovery determinations should be made on representative samples containing a known quantity of each analyte. Recovery data must be reported for 2 fortification levels appropriate to the proposed LOQ and, where possible, likely residue levels. The fortification range should encompass the LOQ and 10 x LOQ (or highest residue level, if this is known). Mean recoveries for each level should be in the range 70-110%, ideally with the mean in the range 80-100%. Lower recoveries may be acceptable for difficult analytes, providing precision data are acceptable.

(a) *Soil, sediment and water*: Recoveries from soil, sediment and water should be conducted with untreated environmental samples to which a known quantity of analyte is added and the whole sample analysed to reduce sampling error. The results should be compared to the 'known' analyte content of the sample. Control (unfortified) samples should be analysed concurrently to determine any contamination by the analyte of interest or interferences. Procedural recoveries should also be conducted during environmental fate and ecotoxicology studies and reported with the results of these studies.

In some methods the calibration requires the direct measurement of fortified test portions of blank samples without separate extraction and cleanup steps (e.g. headspace GC, SPME techniques). In these cases it is not possible to determine recovery and an estimate of the accuracy of the analytical technique may be made by an assessment of the linearity of matrix calibration or by comparison with other techniques.

(b) *Air*[3]: The efficiency of extraction of the analyte from the adsorbent should first be determined. Following this, the retention efficiency of the sorbent material (i.e. the breakthrough volume or the maximum tested capacity (µg substance per tube) without breakthrough) must be determined and reported. This should be carried out by determining the recovery of the active ingredient and/or relevant degradation product, at defined air temperatures and relative humidities, after the flow of a defined air volume for at least 6

hours (19). Recovery from air should be reported as % of nominal concentration in air (mg/m^3).

The above tests should cover conditions comparable to those in studies where the methodology may be used. In optimising flow rate, consideration should be given to expected levels in air, retention of analyte and time period required for sampling replicates (18). Typical flow rates are 1-4 litres/minute. Further information may be found in references 18 and 19.

(iv) *Precision* - The precision of the method must be reported as repeatability of recovery at each fortification level (see iii above) and the overall relative standard deviation (RSD) must also be reported. Five determinations should be made at each fortification level. In general the RSD should be $\leq$ 20% per level. In certain justified cases higher variability may be accepted.

(v) *LOQ* - Methods must be validated at the LOQ. Blank values (procedural blanks and untreated samples) should not exceed 30% of the LOQ. If this is exceeded, detailed justification is required. For pre-registration methods, the required level of sensitivity should be determined on a case by case basis dependent on the required data to be generated. However consideration should be given to the required sensitivity criteria for a method designated for post-registration monitoring, as detailed below:

(a) *Soil and sediment*: The proposed LOQ should not exceed 0.05 mg/kg. If the phytotoxic concentration in soil for sensitive crops (ED_{10} or NOEL, Directive 93/71/EC, ref. 20) or the toxic concentration for non target organisms (NOEL or LC_{50}) is lower than 0.05 mg/kg, the LOQ must be < the ED_{10}, NOEL or LC_{50} concentration in soil/sediment.

(b) *Water*: The proposed LOQ should not exceed 0.1 µg/l for drinking water (Directive 96/46 EC, ref.1) and should take into account the lowest endpoint from aquatic toxicity studies (Directive 96/12/EC, ref. 21), or, where relevant, the lowest phytotoxic level**.** The LOQ for surface water must be less than the lowest chronic NOEC for either fish or Daphnia or the EC_{50} for algae. If no chronic data are available, the LOQ must be less than the lowest acute EC/LC_{50} for fish or Daphnia.

(c) *Air*[3]: The proposed LOQ should take into account relevant human and ecotoxicological-based limit values or exposure levels. It is recommended that the LOQ is sufficient to quantify exposures well below the NOEL divided by an appropriate safety factor (18).

4.2 Confirmation of analyte identification

See section 3.2.

4.3 Non-specific methods

See section 3.3.

[3] It should be noted that methods for air analysis must be available for determining both particle associated and gaseous residues. Consideration should be given to spray drift, dusts, vapour emission etc., potentially creating relevant exposures for aquatic systems, non-target plants, organisms, operators, workers and bystanders.

Analytical methods may also be required for active substances with low vapour pressures. Non-submission of methods must be justified.

5 RESIDUES ANALYSIS IN BODY FLUIDS AND TISSUES - (determination of the active substance and relevant metabolites) (*Annex IIA, point 4.2.5 and Annex IIIA , point 5.2.5 of Directive 91/414/EEC*)

Methods are required where the active substance and/or relevant metabolite is classed as toxic or highly toxic. Methods developed for animal tissues (section 3) may be appropriate. For validation of methods for the determination of residues in body fluids and tissues, the following should be used where appropriate:

- blood
- urine
- muscle and liver (if not investigated under section 3)

5.1 Method Validation

For methods supporting operator exposure studies, the method validation guidance detailed in the OECD guidance document for the conduct of occupational exposure studies (18) should be followed. Validation is summarised in Appendix 2.

(i) *Specificity* - Methods of analysis of residues must be:

- appropriate to the residue definition used in the assessment of risk to consumers / operators or bystanders.

The method must be capable of determining the a.s. and/or relevant metabolites in the presence of the sample matrix. Where the sample contains more than one isomer, analogue, etc., of an active substance or relevant metabolite, the method(s) should distinguish between individual isomers/ analogues where this is necessary for carrying out risk assessments.

(ii) *Linearity* - see section 3.1 (ii).

(iii) *Accuracy* - The accuracy of the method should be reported as mean recovery $\pm$ relative standard deviation (see iv below) for the active substance and/or relevant metabolites in the sample matrix. Recovery determinations should be made on representative samples containing a known quantity of each analyte. Recovery data must be reported at the proposed LOQ.

Samples should be control (untreated/unexposed) samples to which a known quantity of analyte is added and the whole sample analysed to reduce sampling error. The results should be compared to the 'known' analyte content of the sample. Mean recoveries for each level should be in the range 70-110%, ideally with the mean in the range 80-100%. Control (unfortified) samples should be analysed concurrently to determine any contamination by the analyte of interest or interferences. Lower recoveries may be acceptable for difficult analytes, providing precision data are acceptable. In some methods the calibration requires the direct measurement of fortified test portions of blank samples without separate extraction and cleanup steps (e.g. headspace GC, SPME techniques). In these cases it is not possible to determine recovery and an estimate of the accuracy of the analytical technique may be made by an assessment of the linearity of matrix calibration or by comparison with other techniques.

(iv) *Precision* - The precision of the method must be reported as repeatability of recovery at each fortification level (if more than one fortification level is used, see iii above) and the overall relative standard deviation (RSD) must also be reported. Five determinations should be

made at each fortification level. In general the RSD should be $\leq$ 20% per level. In certain justified cases higher variability may be accepted.

(v)Methods must be validated at the proposed LOQ. Blank values (procedural blanks and untreated samples) should not exceed 30% of the LOQ. The necessary LOQ will depend on the toxicological endpoint of interest. It is recommended that the LOQ is sufficient to quantify exposures well below the NOEL divided by an appropriate safety factor (18).

5.2 Confirmation of identification

See section 3.2.

5.3 Non-specific methods

See section 3.3.

6 IMMUNOLOGICAL METHODS OF ANALYSIS

In principle the use of immunoassay methodology for residue trial analysis is acceptable, providing the method has been adequately validated. However, the use of such methods in place of conventional analytical techniques must be fully justified. It is recognised that, whilst immunological methods may be highly selective and therefore useful screening tools, they are not very specific techniques. Their use in the context of residue trials is acceptable as, in principal, the source of the analyte(s) is known. There are some important points to be aware of in the use of immunological methods. Requirements are discussed below.

6.1 Method description

The guidelines described in section 1.5 should be followed. Presentation of chromatograms is not relevant, however examples of representative data, e.g. standard calibration curve, must be submitted. Full descriptions of the following are required:

- The principle of the test
- The commercial test kit ,or antibody source and production and hapten synthesis (see 6.2 (i) below)
- Details of all reaction steps in the method.

6.2 Method validation

Validation must be performed for each relevant sample matrix.

(i) *Specificity* - The specificity of the method must be addressed and reported. The method must be appropriate to the residue definition.

Prior to the process of antibody production, the antigen carrier (e.g. protein) is derivatised with a chemical similar to or derived from the analyte in such a way as to present characteristic parts of the analyte structure as antigens (hapten synthesis). The orientation of the antigen in respect to the carrier surface is important in determining the antibody's ability to differentiate between substances structurally related to the analyte. If the unbound part of the molecule is identical to that of other molecules present, such as other structurally-related pesticides, impurities or metabolites, the antibodies will bind to (cross-react with) these compounds or the target analyte.

Cross reactivities of multi-analytes (for a multi-compound residue definition) and potentially interfering compounds must be determined. Cross reactivities of the potential interfering compound should be expressed in comparison to that of the target analyte (e.g. the ratio of concentrations yielding a defined test response. For a multi-analyte determination (e.g. analysis of both parent and relevant metabolites together) the ratio of cross-reactivities should be such that an acceptable level of accuracy is obtained for the whole determination. For this, the cross reactivities must either be known or shown to be additive. Consideration must be given to potential cross-reactivities with all structurally-related compounds and a representative sample of other pesticides. For multi-analyte determinations a representative antigen conjugate should be used to coat the microtitre plates or equivalent in the test.

(ii) *Matrix effects* - For individual sample matrices, effects may be different for different pesticides or antibodies. Therefore the effect of the sample matrix on the performance of the test must be addressed.

Extraction solvents should be suitable and not cause significant interference (i.e. suppression of binding) at the concentrations used. Water miscible solvents are usually essential. The

matrix may have an effect on the LOQ, causing either a loss of sensitivity or in some cases higher sensitivity, where matrix components may shield solvent effects (22).

(iii) *Quantification procedures* - The general principles for quantification procedures described in section 1.5 should be followed. If matrix effects are experienced, calibration standards should be generated using fortified control sample matrix. For multi-analyte determinations the absorbance versus concentration standard curve should be generated using a similar analyte ratio as may be reasonably expected to occur in the samples, as the cross-reactivities of component analytes will differ.

All calculations and transformations of the data must be clearly reported and included in the validation of the method as a whole. Where a non-linear concentration-response curve is produced, a variety of data transformations may be carried out. However it is preferable to work within the linear part of the log-transformed calibration curve, as validation of the results becomes more difficult as the complexity of the mathematical transformation increases (23). Therefore there are distinct advantages in keeping the transformation simple. Some transformations may alter the data such that assay variability (precision) may be compressed or increased. Logit transformations, which linearly transform the response in the tail ends of the sigmoidal standard curve, do not necessarily take account of the inherent reduced precision at these tail ends (23).

(iv) *Accuracy* - The general principles for assessing method accuracy are as described in sections 3.1 (iii) and 4.1 (iii). Accuracy may be assessed using fortification experiments or by using samples containing incurred residues and comparing results with those from a conventional analytical technique (see also section 6.3) for which acceptable accuracy has been demonstrated. Statistical comparison of data generated with the immunological and conventional methods may be carried out using a paired T-test (24). Any discrepancies in accuracy should be justified. Mean recoveries for each level should be in the range 70-110%, ideally with the mean in the range 80-100%. For samples that may be analysed directly (e.g. milk, water, blood, wine) where it is not possible to determine recovery, an estimate of the accuracy of the analytical technique may be made by an assessment of the linearity of matrix calibration or by comparison with other techniques.

For multi-analyte determinations appropriate to the residue definition, the ratio of species in the fortification mixture should reflect that expected in samples and in the calibration mixture. The efficiency of any associated extraction procedures should be determined and reported.

(v) *Precision* - Precision should be reported as described in sections 3.1 (iv), 4.1 (iv) and 5.1 (iv). All standards and samples should be run under exactly the same conditions. Assessment of method precision should encompass the factors discussed below.

A number of factors may adversely affect the precision of an immunoassay, including drift in absorbance-measuring equipment, inhomogeneity of analyte distribution in sub-samples, reagent instability, unevenness of coating, inadequate temperature stability/distribution and operator error (22, 25). The enzyme reaction step of enzyme-linked immunoassays is normally conducted in non-equilibrium conditions, therefore the timing of the incubation step is important. The step-wise addition of reagents may lead to some samples being incubated for longer than others, causing assay drift.

(vi) *LOQ* - Methods must be validated at the proposed LOQ, see sections 3.1(v), 4.1(v) and 5.1(v). It is important with immunoassays to determine both the minimum and maximum LOQs. Limits of detection for immunoassays have been determined in a number of ways (23):

- by determining a minimum "inhibition" test value (the preferred method).
- by determining the standard deviation around the blank and applying a multiplication factor to this.
- by visual inspection.

Statistical methods for calculating the limit of detection are discussed in reference (23).

6.3 Confirmation of analyte identification

The principles discussed in section 3.2 apply. For method development purposes, a non-immunological confirmatory method is required for the unequivocal identification and quantification of analytes. This should be conducted on a statistically representative[4] number of both positive and negative samples of each type of sample analysed. For the analysis of residue trials samples where the application history is known, analyte confirmation is only required for the highest and lowest residues found in each type of sample. However, analyte identity and quantity should be confirmed in any samples containing toxicologically or environmentally significant levels. Correlation data (correlation coefficients), demonstrating comparable performance between the immunoassay and conventional methods must be reported.

Where immunochemical methods are used for biological pesticides which may not be amenable to analysis by a conventional technique such as LC or GC, confirmation may be attained by using a second non-chromatographic method where this is available.

4 5 validation samples and a minimum of 5 residue trial/ environmental samples.

7 REFERENCES

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[27] International Standard ISO 5725. *Precision of test methods - Repeatability and reproducibility*. Reference number ISO 5725 - 1986 (E).
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Appendix 1

1 GENERAL DEFINITIONS

For this paper the following definitions apply, as detailed in Directive 96/46/EC:

Impurities	Any component other than the pure active substance which is present in the active substance as manufactured (including non-active isomers) originating from the manufacturing process or from degradation during storage.
Relevant impurities	Impurities of toxicological and/or ecotoxicological or environmental concern which are known, or can be expected, to occur in the active substance as manufactured.
Significant impurities	Impurities with a content of $\geq$ 1 g/kg in the active substance as manufactured.
Metabolites	Metabolites are considered to be products resulting from biological or non-biological degradation or reaction of the active substance.
Major environmental metabolites	Metabolites representing >10% applied radioactivity at any time point in radiolabelled balance studies (Directive 95/36/EC, ref. 26; Working Guidance Document on Relevant metabolites, ref 28)
Relevant metabolites	Metabolites of toxicological and/or ecotoxicological or environmental concern (Working Guidance Document on Relevant metabolites, ref 28).
Specificity	The ability of a method to distinguish between the analyte being measured and other substances, based upon sufficient characteristics of the analyte as to make the results completely specific to the analyte, irrespective of the characteristics of other materials present.
Linearity	The ability of a detection system, within a defined range, to produce an acceptable linear correlation between the test results and the concentration of analyte in the sample. Allowing for any transformation of the data. The analyte concentration to be measured must be within the dynamic range of the analytical instrument.
Accuracy[5] (comprised of 'Trueness' and precision)	The degree to which the determined (observed) value of analyte in a sample corresponds to the accepted "reference" value.
Precision[5]	The closeness of agreement between independent test results obtained under prescribed conditions. A measure of random errors, which may be expressed as repeatability and reproducibility.
Repeatability[5]	The closeness of agreement between mutually independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short

	intervals of time. In the context of Directive 96/46/EC for residue analysis and for practical purposes, this is determined from recoveries.
Reproducibility[5,6]	In the context of Directive 96/46/EC for residue analysis, reliability of the method during routine use. Determination of repeatability of recovery by at least one laboratory which is independent from that which initially validated the method.
Limit of quantification (LOQ)	Defined as the lowest concentration tested, at which an acceptable mean recovery (70-110%), with an acceptable RSD (≤20%), is obtained (also referred to as the limit of determination (LOD) by CODEX and in EU MRL legislation). The proposed LOQ should not normally exceed 0.05 mg/kg (soil) and 0.1 µg/l (drinking water) and should only be reported to two decimal places on the mg/kg scale.
Recovery	The amount measured as a percentage of the amount of active substance or relevant metabolite originally added to a sample of the appropriate matrix which contains either no detectable level of the analyte or a known detectable level (not higher than 30% of the amount added).

[5] These terms are defined in ISO 5725 (27)

[6] The ISO 5725 definition is not considered practicable for residue analytical methods.

2 GLOSSARY OF TERMS

AOAC	AOAC International (formerly the Association of Official Analytical Chemists)
a.s.	active substance
C	concentration
C.V.	coefficient of variation
CODEX	
DAD	diode array detector
EC_{50}	effective concentration (50%)
ED_{50}	effective dose (50%)
FAO	Food and Agriculture Organisation of the United Nations
GC	gas chromatography
GLP	good laboratory practice
HPLC	high performance liquid chromatography
IR	infra-red (spectroscopy)
kg	kilogram
l	litre
LC	liquid chromatography
LOEC	lowest observable effect concentration
µg	microgram
m	metre
mg	milligram
MRL	maximum residue level
MS	mass spectrometry
NMR	nuclear magnetic resonance (spectroscopy)
NOEL	no observed effect level

RSD	relative standard deviation
UV	ultra-violet (spectroscopy or detection)

Appendix 2
SUMMARY OF REQUIRED VALIDATION DATA

Sample	**Analyte consideration**	**Accuracy**	**Precision (% RSD)**	**LOQ**	**Linearity**	**Interference**	**Specificity**	**Confirmatory analysis**
Plants, plant products, foodstuffs (of plant and animal origin) and feedingstuffs[1, 2]	Active substance Relevant metabolites	Determinations (mean ± % RSD) at 2 different fortification levels appropriate to the LOQ and MRL or likely residue levels. 2 controls. Procedural recoveries with residue trial samples.	5 determinations at each fortification level.		Calibration appropriate to nominal concentration range ± 20% in relevant analytical solutions. • duplicate determinations at 3 concentrations, *or* • single determinations at 5 concentrations. Matrix-matched standards where appropriate.	The control value must be < 30% of the LOQ.		Not required where primary method is shown to be specific to the analyte(s) of interest
Section reference		3.1 (iii)	3.1 (iv)	3.1 (v)	3.1 (ii)		3.1 (i)	3.2
Methods supporting operator/worker exposure studies[3]. E.g. dislogeable residues; environmental and biological samples.	Active substance Relevant metabolites	Determinations (mean ± % RSD) at 2 different fortification levels appropriate to the LOQ, 10x LOQ or likely residue levels. 1 control. Acceptable accuracy range 70-110%. Procedural recoveries with experimental samples.	7 determinations at each fortification level.		Calibration appropriate to expected values from field studies. • duplicate determinations at 3 concentrations, *or* • single determinations at 5 concentrations. Matrix-matched standards where appropriate.	The control value must be < 30% of the LOQ		Not required where primary method is shown to be specific to the analyte(s) of interest
Section reference		3.4, 4.1, 5.1	3.4, 4.1, 5.1	3.4, 3.1, 4.1, 5.1	3.1, 3.4, 4.1, 5.1	3.1 (v), 4.1 (v), 5.1 (v)	3.1 (i), 4.1 (i), 5.1 (i)	3.2, 4.2, 5.2

Continued

SUMMARY OF REQUIRED VALIDATION DATA continued

Soil	Active substance Relevant/major metabolites	Determinations (mean ± % RSD) at 2 different fortification levels appropriate to the LOQ and likely residue levels. Range to encompass LOQ - 10x LOQ. 2 controls. Procedural recoveries with experimental samples.	5 determinations at each fortification level.		Calibration appropriate to nominal concentration range ± 20% in relevant analytical solutions. • duplicate determinations at 3 concentrations, *or* • single determinations at 5 concentrations. Matrix-matched standards where appropriate.	The control value must be < 30% of the LOQ		Not required where primary method is shown to be specific to the analyte(s) of interest
Section reference		4.1 (iii) (a)	4.1 (iv)	4.1 (v) (a)	4.1 (ii)		4.1 (i)	4.2
Water	Active substance Relevant/major metabolites	Determinations (mean ± % RSD) at 2 different fortification levels appropriate to the LOQ and likely residue levels. Range to encompass LOQ - 10x LOQ. 2 controls. Procedural recoveries with experimental samples.	5 determinations at each fortification level.		Calibration appropriate to nominal concentration range ± 20% in relevant analytical solutions. • duplicate determinations at 3 concentrations, *or* • single determinations at 5 concentrations. Matrix-matched standards where appropriate.	-		Not required where primary method is shown to be specific to the analyte(s) of interest
Section reference		4.1 (iii) (a)	4.1 (iv)	4.1 (v) (b)	4.1 (ii)		4.1 (i)	4.2

continued

SUMMARY OF REQUIRED VALIDATION DATA continued

Air	Active substance Relevant/major metabolites	Determinations (mean ± % RSD) at 2 different fortification levels appropriate to the LOQ and likely residue levels. Range to encompass LOQ - 10x LOQ. 2 controls. Procedural recoveries with experimental samples.	5 determinations at each fortification level.		Calibration appropriate to nominal concentration range ± 20% in relevant analytical solutions. • duplicate determinations at 3 concentrations, *or* • single determinations at 5 concentrations. Matrix-matched standards where appropriate.	-		Not required where primary method is shown to be specific to the analyte(s) of interest
Section reference		4.1 (iii) (b)	4.1 (iv)	4.1 (v) (c)	4.1 (ii)		4.1 (i)	4.2
Body fluids and tissues	Active substance Relevant metabolites (when classed as toxic or highly toxic)	Determination (mean ± % RSD) at the LOQ. 2 controls. Procedural recoveries with experimental samples.	Minimum of 5 replicate sample determinations.		Calibration appropriate to nominal concentration range ± 20% in relevant analytical solutions. • duplicate determinations at 3 concentrations, *or* • single determinations at 5 concentrations. Matrix-matched standards where appropriate.	-		Not required where primary method is shown to be specific to the analyte(s) of interest
Section reference		5.1 (iii)	5.1 (iv)	5.1 (v)	5.1 (ii)		5.1 (i)	5.2

Must be addressed and reported

1 In addition, reproducibility (independent laboratory validation) must be addressed for the method(s) designated as suitable for post-registration monitoring purposes (see section 3.1 (vi)).

2 A case for matrix comparability and a reduced validation data set may be considered where two or more very similar matrices are to be analysed. For example, cereal grain. Reduced validation data for sample matrices within the same crop group (as defined in SANCO/825/00) are acceptable. In these cases data must include recovery at 2 fortification levels and associated precision (minimum 3 samples) together with an assessment of matrix interference.

3 In addition, refer to reference 18.