codex alimentarius commission

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

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Agenda Items 11 a) and b) **CX/PR 04/7**
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JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON PESTICIDE RESIDUES
Thirty-sixth Session
New Delhi, India, 19 - 24 April 2004

Estimation of Uncertainty of Measurements and Confirmation of Results, Vienna 22-26 March 2004[1]

1. Introduction

The CCPR requested the FAO/IAEA Training and Reference Centre for Food and Pesticide Control to prepare 'Draft Guidelines on Estimation of Uncertainty of Results' and on the 'Use of Mass Spectrometry (MS) for Identification, Confirmation and Quantitative Determination of Residues' in cooperation with drafting partners from Australia, Belgium, Denmark, The Netherlands, and UK.

The Agrochemicals Unit of FAO/IAEA Agriculture and Biotechnology Laboratory prepared the working documents and circulated them among analysts who expressed interest in cooperation. Comments were received from Australia, Denmark, Germany, Hungary, The Netherlands, UK and USA.

The Joint FAO/IAEA Division called an expert consultation to:

- review the draft documents prepared by the Agrochemicals Unit and the comments received from the contributing analysts;
- discuss the pending issues and find solutions for contradicting opinions, and
- prepare the working document for CCPR with recommendations.

The Meeting was attended by 5 consultants and staff members of the Agrochemicals Unit.

The Meeting was of the opinion that the purpose of the documents is to provide guidance to analysts, accreditation bodies and decision-making risk managers in Member States for estimating and interpreting the uncertainty of measurements, taking into consideration the international requirements and technical possibilities of both least developed and most developed countries, and in particular to:

- assist analysts in identifying the sources of uncertainty to enable them to keep the procedures under better control;

[1] Document intends to serve as a background document for Agenda Items: 9a) Proposed Draft Guidelines on the Use of Mass Spectrometry (MS) for Identification, Confirmation and qualitative Determination of Residues and b) Proposed Draft Guidelines on the Estimation of Uncertainty of Results.

- facilitate satisfying the requirements of ISO 17025, export-import certification systems, and WTO;
- facilitate making correct decisions based on results of analysis;
- provide information for customers and accreditation bodies for realistic expectations for and assessment of the performance of pesticide residue laboratories.

The Meeting agreed that the working papers/recommendations should be:

- based on relevant ISO documents and related EURACHEM Guidance Documents;
- consistent with the Codex Standards, national guidelines, and recommendations made by CCMAS and CCFIC;
- practical and provide clear guidance how to act without being overly prescriptive (the analysts should be able to decide on appropriate actions taking into account the purpose of analysis, available facilities etc.);
- applicable in least developed countries having the minimum instrumentation necessary for performing reliable analyses as the results should meet the minimum quality standard regardless where and by what means they were produced.

3. WORK DONE

The Meeting reviewed the draft documents prepared by the Agrochemicals Unit and agreed with the written comments received stating that the complex topics were covered in a scientifically sound, systematic and comprehensive way. These documents will provide a very good source of background information for analysts who would like to study the specific aspects of the problems in detail.

Taking into consideration the objectives of the documents and the comments received from analysts of Member States, the meeting prepared two brief summary papers (Appendices 2 and 3), which can be incorporated in the Good Laboratory Practice document of CCPR, and recommendations for FAO/IAEA and for the CCPR.

4. RECOMMENDATIONS

The meeting recommends to FAO/IAEA Joint Division to:

(i) submit the Report to the Codex Secretariat for consideration at the next meeting of CCPR.

(ii) publish the referred working documents (Appendix 4) in scientific journals and/or place them on the Web site of TRC to make them readily available for persons interested in the detailed description of the procedures.

The meeting recommends to CCPR to:

I. incorporate the concept of a two-phase procedure for screening and confirmation of pesticide residues in the *revised guidelines on good laboratory practice in residue analysis*;

ii. review the present guidelines on reporting of the results to cover the combination of data from both confirmatory and screening experiments with their associated uncertainties;

iii. consider the concept of tentatively confirmed residues and their possible use in exposure assessment studies;

iv. note that there are two basic methodologies for estimating measurement uncertainty based either on component-by-component analysis [bottom-up] or method precision data [top-

down], and endorse for practical reasons application of the top-down approach in pesticide residue analysis;

v. incorporate the proposed *Draft Guidelines on the Estimation of Uncertainty of Results* and *Use of Mass Spectrometry) for Identification, Confirmation and Quantitative Determination of Residues* in the current *Revised Guidelines on Good Laboratory Practice in Residue Analysis,* and support the determination and documentation of measurement uncertainty by pesticide residue laboratories in Member States;

vi. initiate further work on elaboration of guidelines for consistent application of measurement uncertainty in decision making process in international trade.

DRAFT GUIDELINES ON ESTIMATION OF UNCERTAINTY OF RESULTS

1. INTRODUCTION

According to the (draft) CCMAS guidelines on measurement uncertainty, it is a requirement under ISO/IEC 17025 that laboratories determine and make available the uncertainty associated with each analytical method and result. To this end, food laboratories operating under Codex guidelines should have available considerable data derived from method validation /verification, inter-laboratory studies and in-house quality control activities, which can be applied to estimate the uncertainties particularly for the routine methods undertaken in the laboratory.

Since there is no common interpretation of analytical results among Codex Member States at the present time, different decisions are possible on the same analytical result. It is essential that interpretation of and action on analytical results is similar if there is to be equivalence according to the WTO TBT Agreement. This should be facilitated by taking into consideration the uncertainties associated with analytical results.

1.1 CONCEPT AND COMPONENTS OF UNCERTAINTY

Measurement uncertainty refers to the 'uncertainty' associated with data generated by a measurement process. In analytical chemistry, it generally defines the uncertainty associated with the laboratory process but may also include an uncertainty component associated with sampling and qualitative confirmation.

The uncertainty 'estimate' therefore describes the range around a reported or experimental result within which the true value can be expected to lie within a defined level of probability. This is a different concept to measurement error which can be defined as the difference between an individual result and the true value. The reporting of uncertainty is intended to provide a higher level of confidence in the validity of the reported result.

Contributions to data uncertainty are manifold and described in detail in Tables 1-3. The evaluation of uncertainty ideally requires an understanding and estimation of the contributions to the uncertainty of each of the activities involved in the measurement process.

2. IDENTIFICATION OF UNCERTAINTY SOURCES

In general, the uncertainty of measurements is comprised of many components, arising from activities involved with the sample. The uncertainty of an analytical result is influenced by three major phases of the determination:

- External operations: sampling (S_S), packing, shipping and storage of samples;
- Preparation of test portion: sample preparation and sample processing (S_{Sp});
- Analysis (S_A): extraction, cleanup, evaporation, derivatisation, instrumental determination

Packing, shipping, storage, and laboratory preparation of samples may have significant influence on the residues detected, but their contribution to the uncertainty cannot be quantified based on the current information.

The combined standard (S_{Res}) and relative (CV_L) uncertainty may be calculated according to the error propagation law:

$$S_{Res} = \sqrt{S_S^2 + S_{Sp}^2 + S_A^2} \; ; \; S_{Res} = \sqrt{S_S^2 + S_L^2} \qquad (1)$$

If the whole sample is analysed the mean residue remains the same and the equation can be written as:

$$CV_{Res} = \sqrt{CV_S^2 + CV_L^2} \text{ and } CV_L = \sqrt{CV_{Sp}^2 + CV_A^2} \qquad (2)$$

2.1 Errors in analytical measurements

In most measurements we can distinguish between three types of errors: gross, random and systematic errors.

Gross errors refer to unintentional/unpredictable errors while generating the analytical result. Errors of this type invalidate the measurement. Laboratory quality assurance procedures should minimize gross errors. It is not possible or desirable to statistically evaluate and include the gross errors in the estimation of uncertainty. They need no further discussion in this document.

Random errors are present in all measurements, and cause replicate results to fall on either side of the mean value. The random error of a measurement cannot be compensated for, but increasing the number of observations and training of the analyst may reduce the effects.

Systematic errors occur in most experiments, but their effects are quite different. The sum of all the systematic errors in an experiment is referred to as the bias. Since they do not sum to zero over a large number of measurements, individual systematic errors cannot be detected directly by replicate analyses. The problem with systematic errors is that they may go undetected unless appropriate precautions are taken. In practice, systematic errors in an analysis can only be identified if the analytical technique is applied to a reference material, the sample is analysed by another analyst or preferably in another laboratory, or by re-analysing the sample by another analytical method. However, only if the reference material matches identically in terms of analyte, matrix, and concentration does it meet the ideal conditions for determining the bias of the method. The bias of a method may also be investigated by recovery studies. However, recovery studies assess only the effects of analysis (S_A) and do not necessarily apply to naturally incurred samples, or components of the bias that may be introduced prior to the analytical step. In pesticide analysis, results are not normally corrected for the recovery, but should be corrected if the average recovery is significantly different from 100%. If the result has been corrected for recovery, the uncertainty associated with recovery should be incorporated in the uncertainty estimation of the measurement.

Some examples of sources of errors are illustrated in Tables 1, 2 and 3. It should be noted that not all sources mentioned have to be evaluated in the uncertainty estimation. Some sources are already incorporated in the overall uncertainty, while others are negligible and may be disregarded. However, it is important to recognise and assess all sources before elimination. Further information may be obtained from published documents[2],[3].

[2] EURACHEM Guide to Quantifying Uncertainty in Analytical Measurements, 2nd ed. 1999, http://www.measurementuncertainty.org

[3] Ambrus A. Reliability of residue data, Accred. Qual. Assur. 9, pp. xx. 2004

Table 1: Sources of errors in external operations

	Sources of systematic error	Sources of random error
Sampling (S_S)	Selection of sampling position, time of sampling.	Large variation of residue concentration in/on treated objects. Number of primary samples taken (Sample size)
	Incorrect labelling	
Packing, shipping and storage	Decomposition of analytes, contamination of the sample	Variation of storage temperature/condition

Table 2: Sources of error in preparation of the test portion

	Sources of systematic error	Sources of random error
Sample preparation	The portion of sample to be analysed (analytical sample) may be incorrectly selected	The analytical sample is in contact and contaminated by other portions of the sample
		Rinsing, brushing is performed to various extent, stalks and stones may be differentially removed
Sample processing (S_{Sp})	Decomposition of analyte during sample processing, cross contamination of the samples	Non homogeneity of the analyte in single units of the analytical sample
		Non homogeneity of the analyte in the ground/chopped analytical sample
		Variation of temperature during the homogenisation process
		Texture (maturity) of plant materials affecting the efficiency of homogenisation process

Table 3: Sources of error in analysis (S_A):

	Sources of systematic error	Sources of random error
Extraction/Clean up	Incomplete recovery of analyte	Variation in the composition (e.g. water, fat, and sugar content) of sample materials taken from a commodity
	Interference of co-extracted materials (load of the adsorbent)	Temperature and composition of sample/solvent matrix
Quantitative determination	Interference of co-extracted compounds	Variation of nominal volume of devices within the permitted tolerance intervals
	Unknown purity of analytical standard	Precision and linearity of balances
	Biased weight/volume measurements	Incomplete and variable derivatisation reactions
	Operator bias in reading analogue instruments, equipment	Changing of laboratory-environmental conditions during analysis

	Determination of substance which do not originate from the sample (e.g. contamination from the packing material)	Varying injection, chromatographic and detection conditions (matrix effect, system inertness, detector response, signal to noise variation etc.)
	Determination of substance differing from the residue definition	Operator effects (lack of attention)
	Biased calibration	Calibration

3. PROCEDURES FOR ESTIMATING MEASUREMENT UNCERTAINTY

Whilst there are a number of options available to laboratories for the estimation of measurement uncertainty, there are two preferred procedures described commonly as the 'bottom up' approach and the 'top down' approach[1,4]

The bottom-up method:

The bottom up or component-by-component approach incorporates an activity-based process whereby the analyst breaks down all the analytical operations into primary activities. These are then combined or grouped into common activities and an estimate made of the contribution of these activities to the combined uncertainty value of the measurement process. The bottom up approach can be very laborious and requires a detailed knowledge of the whole analytical process. The benefit to the analyst is that this approach provides a clear understanding of the analytical activities which contribute significantly to the measurement uncertainty and which therefore may be assigned as critical control points to reduce or manage measurement uncertainty in future applications of the method.

The top-down method:

The top down approach is based on method validation and long-term precision data derived from laboratory control samples, published literature data and/or inter-laboratory collaborative trials. Uncertainty estimates based on inter-laboratory studies may also take into account the between-laboratory variability of the data and is likely to provide the most reliable estimate of the method performance and the uncertainty associated with its application. It is important to acknowledge however that collaborative studies are designed to evaluate the performance of a specific method and participating laboratories. They normally do not evaluate imprecision due to sample preparation or processing as the samples generally tend to be highly homogenized.

Pesticide residue analytical laboratories normally look for over 200 residues in numerous commodities that lead to practically infinite number of combinations. Therefore it is recommended that, for estimating the uncertainty associated with multi residue procedures, laboratories use a properly selected range of analytes and sample matrices which represents the residues and commodities to be analysed in terms of physical chemical properties and composition according to the relevant parts of the *Revised Guidelines on Good Laboratory Practice* instead of establishing the uncertainty for each method/analyte/matrix combination.

In summary, laboratories should use either their own long-term precision data or the activity-based procedure (component by component calculation) to establish and refine the uncertainty data.

In certain situations it may also be appropriate to estimate the uncertainty contribution due to sample variability. This will require an understanding of the analyte variability within the sample lot and is not readily available to the laboratory or the analyst The values obtained from the statistical analysis of

[4] ISO, Guide to the Expression of Uncertainty in Measurement, ISO. Geneva, 1993

over 8500 residue data (Table 5) provide currently the best estimate[5]. These estimates can be incorporated into the combined uncertainty value.
Likewise it may be necessary to take into consideration the stability of analytes during sample storage and processing if these are likely to result in analyte variability between analysts and laboratories.

3.1 UNCERTAINTY ESTIMATES OF RESULTS INVOLVING ANALYSIS OF MULTI-COMPONENTS

The estimation of uncertainty of results for multi-component residues arising from the application of technical mixtures including structural and optical isomers, metabolites and other breakdown products may require a different approach particularly where the MRL has been established for the sum of all or some of the component residues. The assessment of the random and systematic errors of the results based on the measurements of multiple peaks is explained in detail in a recent publication[6] and should be consulted where necessary.

4. GUIDANCE VALUES FOR ACCEPTABLE UNCERTAINTIES

The estimation of the standard deviation, as a measure of standard uncertainty, requires the results of large number of tests which are not always available.

Depending on the number of observations (n), the relation of the true (σ) standard deviations, calculated (S) standard deviations, and the expected range of the mean value ($\bar{x}$) at 95% probability are illustrated in Table 4. The multiplying factor, ***f***, provides the link between the estimated and true values as the function of the number of measurements.

Table 4 The values of ***f*** for calculation of expected ranges of standard deviation and mean values

n	$S_{min}=f_1\sigma$	$S_{max}=f_2\sigma$	$\bar{x} = \pm f_3 S$
	f_1	f_2	f_3
5	0.35	1.67	1.24
7	0.45	1.55	0.92
15	0.63	1.37	0.55
31	0.75	1.25	0.37
61	0.82	1.18	0.26
121	0.87	1.13	0.18

The guidance values for standard uncertainty, given in Table 5, are based on a large number of data and can be used to assess the reality of the estimated uncertainty in a laboratory in order to avoid an unreasonable high or low value.

[5] Ambrus A and Soboleva E. Contribution of sampling to the variability of residue data; www.iaea.org/trc
[6] Soboleva E., Ambrus A., Jarju O., Estimation of uncertainty of analytical results based on multiple peaks, J. Chromatogr. A. 1029. 2004, 161-166

Table 5. Typical expected uncertainties of major steps of pesticide residue analysis

Procedure	Relative uncertainty	Comments
Sampling of commodities of plant origin. Reflects the variation of mean residues being in composite samples taken randomly from a lot. It does not incorporate the errors of follow-up procedures.	Medium and small commodities. (Sample size ≥10)[a]: 26-30%[b] Large commodities. (Sample size ≥5)[a]: 36-40%[b]	For testing compliance with MRLs in imported and domestic products, the sampling uncertainty is 0, as the MRLs refer to the average residues in bulk samples.
Sampling of animal products	The relation between the number of samples (n) to be taken for detection of a specified percentage of violation (β_p) with a given probability (β_t), is described by[a]: $1-\beta_t = (\beta_p)^n$	The primary samples should be selected randomly from the whole lot.
Sample processing Includes the physical operation performed for homogenizing the analytical sample, but excludes decomposition and evaporation of analytes.	Largely varying depending on sample matrix and equipment. No typical value can be given. The analysts should try to keep it[2] below 8-10%.	It may be influenced by the equipment used for chopping / homogenising the sample and the sample matrix, but it is independent from the analyte.
Analysis It includes all procedures performed from the point of spiking of test portions.	Within laboratory reproducibility: 16-53% for concentrations of 1µg/kg to 1 mg/kg[c]. Average between- laboratories reproducibility within 0.001-10 mg/kg: 25%[d]	The typical CV_A can be conveniently determined from the recovery studies performed with various pesticide-commodity combinations on different days and during the use of the method.

Notes:

(a) *Codex Secretariat. Recommended method of sampling for the determination of pesticide residues for compliance with MRLs, ftp://ftp.fao.org/codex/standard/en/cxg_033e.pdf .*

(b) *Ambrus A. Soboleva E. Contribution of sampling to the variability of residue data; www.iaea.org/trc*

(c) *Codex Secretariat, Revised Guidelines on Good Laboratory Practice in Residue Analysis ftp://ftp.fao.org/codex/alinorm03/al03_41e*

(d) *Alder L., Korth W., Patey A., van der Schee and Schoeneweis S., Estimation of Measurement Uncertainty in Pesticide Residue Analysis, J. AOAC International, 84, 1569-1578, 2001*

In addition to the estimated uncertainties made by the individual laboratories, regulatory authorities and other risk managers may decide on a default expanded uncertainty of measurements which can be used in judging compliance with MRLs (See section 5) based on between-laboratories reproducibility values. For instance, a 50% expanded uncertainty for CV_L is considered to be a reasonable default value.

5. Use of Uncertainty Information

If required, the result should be reported together with the expanded uncertainty, U, as follows

Result = x ± U (units)

The expanded uncertainty, U, may be calculated from the standard combined uncertainty (S_{Res}) with a coverage factor of 2 as recommended by EURACHEM or with the Student ***t*** value for the level of confidence required (normally 95%) where the effective degree of freedom is less than 20. The respective calculations for the expanded uncertainty are as follows

$U = 2S_{Res}$ or $U = t_{v,0.95}S_{Res}$

The numerical value of the reported results should follow the general rule that the last digit can be uncertain. Rounding the results should be done only when the final result is quoted since rounding at the initial stages of calculation may introduce unnecessary bias in the calculated values.

The interpretation of a residue value followed by the decision on the compliance of a lot with the MRL depends on how the number of reported significant figures, the uncertainty of the result and the recovery correction are used.

For the purpose of explication, it is assumed that the best estimate of the residue content is reported for a sample. How the results are interpreted depends upon the purpose of the testing. Typical reasons include testing compliance with the national MRL, certifying compliance with the Codex MRL of a commodity for export, and generating dietary intake estimates of residues. The first two purposes are routinely encountered in residue testing environments and are examined further.

5.1 Testing compliance with an MRL at national level

The expanded uncertainty should be calculated using S_L from equation 1 as $U = kS_L$.where $S_L = CV_L$ x residue.

Figure 1 shows how the testing results can be displayed in terms of the measured value of the residue, the corresponding uncertainty interval, and the MRL.

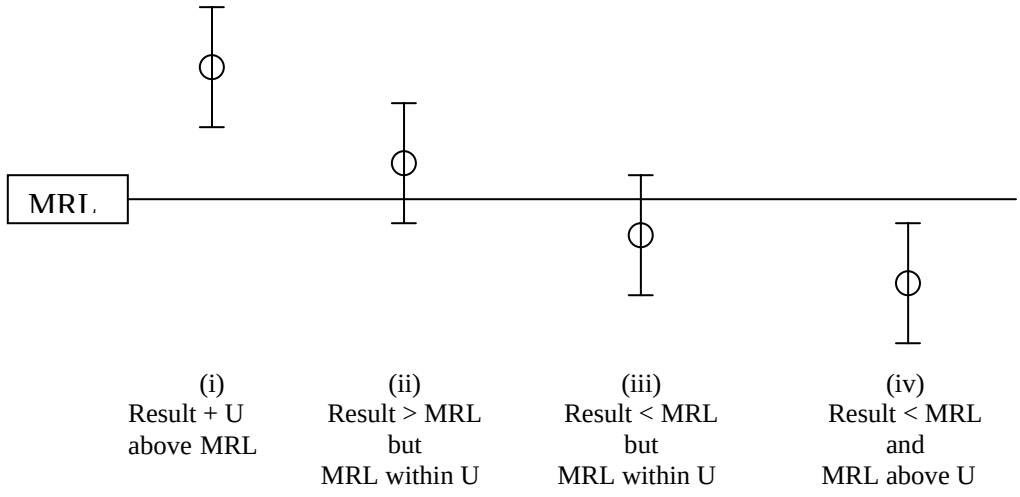

Figure 1. Illustration of the relationship of measured value, ○ , expanded uncertainty, ⊤ , and MRL

Situation (i)

The analytical result bounded by the measurement uncertainty endpoints is greater than the MRL. The result indicates that the residue in the sampled lot is above the MRL.

Situation (ii)

The analytical result is greater than the MRL with the lower endpoint of the measurement uncertainty less than the MRL

Situation (iii)

The analytical result is less than the MRL with the upper endpoint of the measurement uncertainty being greater than the MRL.

Situation (iv)

The analytical result bounded by the expanded measurement uncertainty endpoints is less than the MRL.

5.1.1 Decision Environment

The decision-making in Situation (i) is clear. In order to avoid lengthy explanation of the uncertainty in a court case involving the performance of the analysis for testing compliance with the MRL at the national level in locally produced or imported commodities, the laboratory may report the results as the sample contains "not less than 'x – U' residues." Hence, any enforcement action is only taken after the analyst is certain that the specification has been significantly exceeded. This satisfies the requirement to prove beyond reasonable doubt that a limit has been exceeded if the case should come to court.

The same clarity is observed in Situation (iv). The sample would be considered compliant by all Enforcement Authorities**.**

The middle situations are problematic for decision-makers. If the uncertainty of the result is not used in Situation (ii), the lot would be declared noncompliant which is an incorrect decision. Since the deviation from the MRL is within the uncertainty of the measurement, the sampled lot should be declared as being compliant with the MRL. In Situation (iii), the sampled lot would be considered as being compliant with the MRL by Enforcement Authorities in general, but some Enforcement Authorities could incorrectly decide otherwise.

5.2 Certifying compliance of a lot to be exported

The certification of any composite sample of a specified size complying with the MRL by the laboratory requires that the uncertainty of sampling is specified and the compliance is stated at a specified probability level with a given confidence level.

There is a basic problem in that as there is no internationally agreed or nationally declared value for the acceptable violation rate other than the USA where β_p = 99% compliance is required at β_t = 99% confidence level.

The coverage factors required for the calculation of the expanded uncertainty depend on the number of effective degrees of freedom of the estimated standard uncertainty. They are given in Table 6.

Table 6 Coverage factors for the calculation of expanded uncertainty $U= kS_{Res}$ [a]

Degree of freedom	t at 95%[b]	k at β_p=0.95,β_t=0.95[c]	k at β_p=0.99,β_t=0.99[c]
5	2.6	3.7	7.3
15	2.1	2.6	4.3
20	2.1	2.4	3.9
∞	2	1.65	2.3

Notes: (a) The expanded uncertainty uses S_{Res} from equation 1.
(b) This is recommended by EURACHEM.
(c) The coverage is important on the upper end of the distribution: one sided tolerance factors are included in the table.

The tested lot is compliant if the analytical result, X, plus the upper bound of the measurement uncertainty limit is less than the MRL. That is,

$X+kS_{Res} < MRL$

For a commodity to be exported to the USA, the upper endpoint of measurement uncertainty must be less than the MRL. This criterion implies that the measured residue must be significantly lower.

For instance, let the MRL be 1 mg/kg, the combined relative standard uncertainty of the pesticide result be 0.33 based on 21 observations, and the measured residue be 0.55 mg/kg.

(i) It follows that residues in 95% of a samples taken from the lot may be expected to be lower than the MRL (0.55 + 2.4 * 0.33* 0.55 = 0.99 mg/kg). Where a 95% compliance is acceptable the sampled lot would satisfy the requirements of the importing country.

(ii) However, when the commodity is intended for export to the USA, the residue must be less than 1 mg/kg in 99% of the samples. Based on the 0.55 mg/kg measured residue it may be expected that residues up to 1.3 mg/kg can occur in 99% of the samples (0.55 + 3.9*0.33*0.55 = 1.258). Therefore, the residue measured in one sample must be ≤0.43 mg/kg to certify compliance (0.43 + 3.9*0.43*0.33 = 0.983; 0.44+3.9*0.33*0.44=1.006 mg/kg).

Use of Mass Spectrometry (MS) for Identification, Confirmation and Quantitative Determination of Residues

Confirmatory Tests

When analyses are performed for monitoring or enforcement purposes, it is particularly important that confirmatory data are generated before reporting on samples containing residues of pesticides that are not normally associated with that commodity, or where MRLs appear to have been exceeded. Samples may contain interfering chemicals that may be misidentified as pesticides. Examples in gas chromatography include the responses of electron-capture detectors to phthalate esters and of phosphorus-selective detectors to compounds containing sulphur and nitrogen.

Analysis of pesticide residues with multi-residue methods generally consists of two phases: screening and confirmation. The process is schematically depicted in Fig. 2. The first phase comprises establishment of those pesticide residues that are likely to be present from interpreting the raw data, avoiding false negatives as much as possible. The second phase is the confirmation, which focuses on the pesticides found in phase 1. The importance of the results to be reported, and consequent management decision determines the efforts put in the confirmatory process. The choice of the technique used for confirmation depends on their availability, time and cost. They are based on, either further interpretation of chromatographic and mass spectrometric data, or alternative methods using different physico-chemical properties of the compound, the combination of various separation and detection methods. Some alternative procedures for confirmation are given in Table 6.

Whenever chromatographic techniques are used in screening or confirmation proper settings of the retention time windows is pivotal. Care should be taken that the instrument is adjusted correctly before starting the analysis, a system suitability test should be performed prior to each batch of analysis[7]. Retention times data base should be adjusted for the current conditions[8]. In phase 1 tolerance intervals of 1.5 to 3% of the absolute retention time may be applied for capillary GC depending on the peak shape. For confirmation of the retention time the absolute tolerance intervals will increase at higher retention time. The tolerance interval should be less than 1 sec for an RT less than 500 sec. For retention times between 500 and 5000 sec. an interval of 0.2% RRT is recommended. For higher retention times 6 sec. is an suitable interval.

Confirmatory tests may be quantitative and/or qualitative but, in most cases, both types of information will be required. Particular problems occur when residues must be confirmed at or about the limit of determination but, although it is difficult to quantify residues at this level, it is essential to provide adequate confirmation of both level and identity.

The need for confirmatory tests may depend upon the type of sample or its known history. In some crops or commodities, certain residues are frequently found. For a series of samples of similar origin, which contain residues of the same pesticide, it may be sufficient to confirm the identity of residues in a small proportion of the samples selected randomly. Similarly, when it is known that a particular pesticide has been applied to the sample material there may be little need for confirmation of identity, although a number of randomly selected results should be confirmed. Where "blank" samples are available, these should be used to check the occurrence of possible interfering substances.

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[7] Soboleva E. Ambrus A., Application of system suitability test for quality assurance and performance optimization of a gas chromatographic system for pesticide residue analysis, J. Chromatogr. A. 1027. 2004. 55-65.

[8] Lantos J., Kadenczki L., Zakar F., Ambrus A. Validation of gas chromatographic Databases for qualitative identification of active ingredients of pesticide residues in Fajgelj A. Ambrus A. (eds) Principles of Method Validation, Royal Society of Chemistry, Cambridge, 2000, pp 128-137.

The necessary steps to positive identification are a matter of judgement on the analyst's part and particular attention should be paid to the choice of a method that would minimise the effect of interfering compounds. The technique(s) chosen depend(s) upon the availability of suitable apparatus and expertise within the testing laboratory.

Gas Chromatography/Mass spectrometry (GC/MS)

Residue data obtained using mass spectrometry can represent the most definitive evidence and, where suitable equipment is available, it is the confirmatory technique of choice. The technique is also used commonly for residue screening purposes (phase 1).

Tolerance intervals of ion ratios and retention times based on injection of pesticide standard in pure solvent at the concentration close to critical level should have been established at this point. The tolerance intervals for the ion ratios should be within the limits of ± 30 % of absolute ion abundances ratios. When 2 (or 3) selected ion ratios are within the established tolerance intervals the residue is confirmed[9].

When the ions detected still indicate the possible presence of a residue the result may be reported as tentatively identified. However, when the result would lead to regulatory action, further confirmation of analyte identity shall be sought. This can be achieved with the same GC-MS equipment, by injecting matrix-matched standards of the suspected analyte, in order to compensate for matrix influence on ion ratios. In this case subsequent injections of matrix matched standard and suspected sample has to be made. The deviation of RRT of analyte in standard and suspected peak in sample should typically be less than 0.1 %. Two ion ratios measured in a sample should be within the tolerance interval calculated based on the ion ratios in matrix-matched standard. The residue is considered to be confirmed if it complies with the general rule stated above. If the ion rations are not within the tolerance intervals, additional confirmation of identity may be obtained by the use of alternative analytical techniques, examples are listed in Table 6.

HPLC and HPLC-MS

Confirmation of residues detected following separation by HPLC is generally more problematic than where gas chromatography is used. If detection is by UV absorption, production of a complete spectrum can provide good evidence of identity. However, UV spectra of some pesticides are poorly diagnostic, being similar to those produced by many other compounds possessing similar functional groups or structures, and co-elution of interfering compounds can create additional problems. UV absorption data produced at multiple wavelengths may support or refute identification but, in general, they are not sufficiently characteristic on their own. Fluorescence data may be used to support those obtained by UV absorption. LC-MS can provide good supporting evidence but, because the spectra generated are generally very simple, showing little characteristic fragmentation, results produced from LC-MS are unlikely to be definitive. LC-MS/MS is a more powerful technique, combining selectivity with specificity, and often provides good evidence of identity. LC-MS techniques tend to be subject to matrix effects, especially suppression, and therefore confirmation of quantity may require the use of standard addition or isotopically-labelled standards. Derivatisation may also be used for confirmation of residues detected by HPLC (Table 6).

Thin Layer Chromatography (TLC)

In some instances, confirmation of gas chromatographic findings is most conveniently achieved by TLC. Identification is based on two criteria, Rf value and visualisation reaction. Detection methods based on bioassays (e.g. enzyme -, fungal growth or chloroplast inhibition) are especially suitable for qualitative confirmation as they are specific to certain type of compounds, sensitive and normally very

[9] Soboleva E. Ahad K. Ambrus A. Applicability of some MS criteria for the confirmation of pesticide residues, http://www.iaea.org/trc

little affected by the co-extracts[10,11]. The scientific literature contains numerous references to the technique[12]. The quantitative aspects of thin-layer chromatography are, however, limited. A further extension of this technique involves the removal of the area on the plate corresponding to the Rf of the compound of interest followed by elution from the layer material and further chemical or physical confirmatory analysis. A solution of the standard pesticide should always be spotted on the plate alongside the sample extract to obviate any problems of non-repeatability of Rf. Over-spotting of extract with standard pesticide can also give useful information. The advantages of thin layer chromatography are speed, low cost and applicability to heat sensitive materials; disadvantages include (usually) lower sensitivity and separation power than instrumental chromatographic detection techniques and need for more efficient cleanup in case of detections based on chemicals colour reactions.

Derivatisation

This area of confirmation may be considered under three broad headings.

(a) Chemical reactions

Small-scale chemical reactions resulting in degradation, addition or condensation products of pesticides, followed by re-examination of the products by chromatographic techniques, have frequently been used. The reactions result in products possessing different retention times and/or detector response from those of the parent compound. A sample of standard pesticide should be treated alongside the suspected residue so that the results from each maybe directly compared. A fortified extract should also be included to prove that the reaction has proceeded in the presence of sample material. Interference may occur where derivatives are detected by means of properties of the derivatising reagent. A review of chemical reactions which have been used for confirmatory purposes has been published by Cochrane, W.P. (Chemical derivatisation in pesticide analysis, Plenum Press, NY (1981)). Chemical reactions have the advantages of being fast and easy to carry out, but specialised reagents may need to be purchased and/or purified.

(b) Physical reactions

A useful technique is the photochemical alteration of a pesticide residue to give one or more products with a reproducible chromatographic pattern. A sample of standard pesticide and fortified extract should always be treated in a similar manner. Samples containing more than one pesticide residue may give problems in the interpretation of results. In such cases pre-separation of specific residues may be carried out using TLC, HPLC or column fractionation prior to reaction.

(c) Other methods

Many pesticides are susceptible to degradation/transformation by enzymes. In contrast to normal chemical reactions, these processes are very specific and generally consist of oxidation, hydrolysis or de-alkylation. The conversion products possess different chromatographic characteristics from the parent pesticide and may be used for confirmatory purposes if compared with reaction products using standard pesticides.

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[10] Ambrus[1]* Á.,. Füzesi[2] I.; Susán[2] M.; Dobi[3] D., Lantos[4] J., Zakar[5] F., Korsós[4] I., Oláh[3] J., Beke[3] B.B., and L. Katavics[5] A cost effective screening methods for pesticide residue analysis in fruits, vegetables and cereal grains, J. Environ Sci. Health B39 **2004** *accepted for publication.*

[11] Ambrus Á.; Füzesi I.; Lantos J.; Korsos I.; Hatfaludi T. Repeatability and Reproducibility of Rf and MDQ Values with Different TLC Elution and Detection Systems. J. Environ Sci. Health B39 **2004** *accepted for publication.*

[12] IUPAC Report on Pesticides (13) (Bátora, V., Vitorovic, S.Y., Thier, H.-P. and Klisenko, M.A.; Pure & Appl. Chem., 53, 1981, 1039-1049

Table 6. Detection methods suitable for screening (Phase 1) and confirmation (Phase 2) of residues.

		Phase 1 - Screening							
		GC with capillary column – ECD, NPD, FPD, PFPD	GC-MS	LC-MS	LC-DAD or scanning UV	LC-UV/VIS (single wavelength)	LC-fluorescence	GC with packed column – ECD, NPD, FPD	TLC – enzyme -, fungal growth or chloroplast inhibition
Phase 2, confirmation	GC – capillary column – ECD, NPD, FPD, PFPD	x[1]	x[1]	x	x	x	x	x	x
	GC-MS	x	x[2]	x	x	x	x	x	x
	LC-MS	x	x		x	x	x	x	x
	Full scan techniques	x	x	x	x	x	x	x	x
	$(MS)^n$, HRMS, alternative ionisation techniques	x	x	x	x	x	x	x	x
	LC-DAD or scanning UV	x	x	x		x	x	x	x
	LC-UV/VIS (single wavelength)	x	x				x	x	x
	LC-fluorescence	x	x		x	x		x	x
	TLC – enzyme, fungal growth or chloroplast inhibition	x	x	x	x	x	x	x	x[3]
	Derivatisation	x	x	x	x	x	x	x	x
	Specific isomers profile	x	x	x	x	x	x	x	
	GC with packed column – ECD, NPD, FPD	x	x	x	x	x	x	x[1]	x

1 – Either the column of different polarity, which results in different elution order of the residues and contaminants eluting in the vicinity to the peak of interest, or another specific detector shell be used.

2- The same GC-MS technique can be used for the phase 2 (confirmation) if different ions are selected or tolerance intervals are established based on matrix matched solutions.

3 – Mobile or stationary phase of different polarity shall be used.

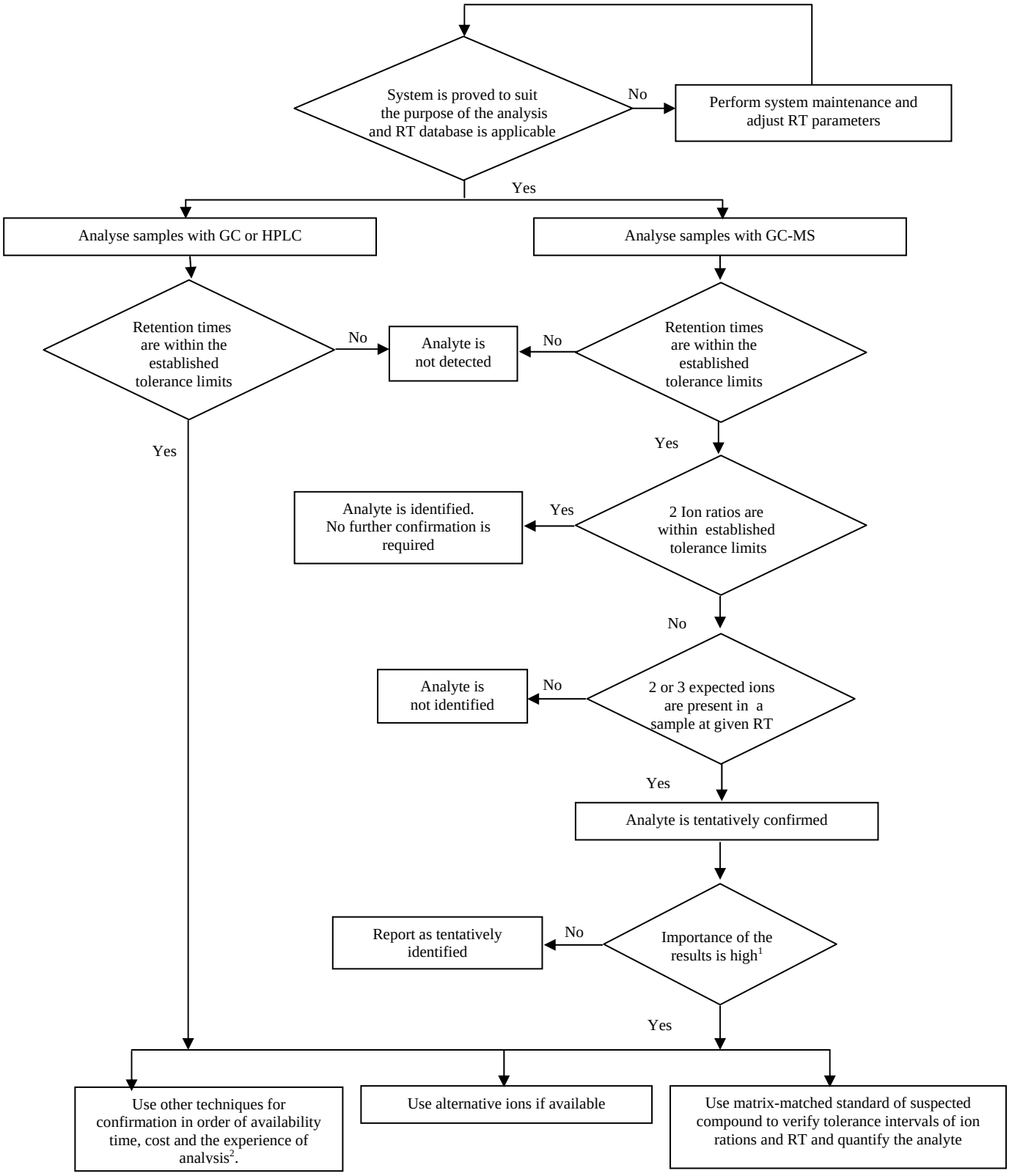

Figure 2. Schematic Representation of Screening and Confirmation (Phase 1 and Phase 2) for Pesticide Residues

1 - Unusual values including banned substances, MRL violation or study requirements as in e.g. exposure assessment

2 – Refer to table 6 for other means of confirmation

LIST OF WORKING DOCUMENTS

Ambrus A. Soboleva E. Contribution of sampling to the variability of residue data; Submitted for publication in JAOAC 2004 or Lecture presented at the 4th European, Pesticide Residue Workshop, Rome 2002. www.iaea.org/trc

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