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Technical Guide for Pesticide Residues Analysis on Tobacco and Tobacco Products

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0. INTRODUCTION

The CORESTA Sub-Group Agrochemicals Analysis has a long history of promoting improved methodologies for the determination of pesticide residues in tobacco and tobacco products. On occasions in the past these efforts resulted in the desired goal of ISO approved methods. However, in the past most improvements were to single pesticide methods or small groups of pesticides with similar characteristics.

The advent of mass spectrometry in pesticide determinations and in particular triple quadrupole technology has revolutionised the approach to residue determinations. Now a large diverse group of pesticides, sometimes in the hundreds, are determined by a single sample preparation and a single instrumental determination. These multi-residue methodologies are now the norm for most pesticide residue laboratories. Unfortunately, these multi-residue methods are highly individualised to suit the data requirements and technology available to each laboratory. In addition, as scientific interest in certain residues increases, it also wanes in others. The result is an ever changing list of residues of current interest. And finally, multi-residues are being measured to ever lowering detection levels.

Several standardised methods are available in the field of pesticide multi-residue methods: Luke method, Swedish Ethyl Acetate method, DFG S 19, sorbent-assisted liquid-liquid extraction and QuEChERS method. There is a strong request on standardized methods for laboratories accredited according ISO/IEC 17025 in order to comply with requirements of national accreditation bodies.

However, it has been recognised that within this context, it may not be appropriate to work towards a single ISO standardised multi-residue method but would be more useful to provide a technical guideline, which would allow for a diverse group of methodologies but still achieve the group's ultimate goal of high quality residue data for use by the tobacco industry.

The European Union document SANTE/11813/2017 [Ref. 12.1] sets out best practices for the general quality control procedures for pesticide residue analysis in foodstuffs.

This guideline, presented herein and similarly structured as the SANTE document, is intended to address issues more specific to tobacco analysis and may provide some minimum criteria for successful method implementation and also information on tobacco-specific aspects that should be considered.

The document may be particularly relevant in giving guidance not only to laboratories that are newly introducing multi-residue methods for tobacco samples but also to laboratories that need improvements in their performance.

Although this Technical Guideline has been written to support the development and routine use of multi-residue methods, a number of targeted or single residue methods continue to be utilised and this document applies equally to them.

1. SAMPLE RECEIPT AND SAMPLING

1.1 The objective of sampling should be to obtain a composite tobacco sub-sample that is representative of the original sample.

1.2 CORESTA Recommended Methods (N° 24, 43 and 47) are available for guidance on suitable sampling regimes for processed tobacco in blends of cigarettes, fine-cut tobacco and cigars respectively. The ISO 4874:2000 standard is also available as a method of sampling batches of raw tobacco.

1.3 Each tobacco sample should ideally be supplied in a clean, clear plastic zip-lock bag, which is appropriately labelled and enclosed in a second zip-lock bag to prevent accidental contamination during transport.

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

1.5 Each tobacco sample should be processed to ensure that it is homogeneous prior to any sub-samples being taken.

1.6 Grinding or milling a sample is an effective tool to ensure a sample is homogeneous.

1.7 It is advisable to keep a portion of non-homogenised tobacco in reserve in case of possible retests.

1.8 If a single extract is unlikely to be representative of the sample provided for analysis, replicate analyses must be performed, to provide a better estimate of the true value.

1.9 Where it is known that particular residues would be adversely affected by the processing procedure, a sub-sample should be taken prior to the sample being homogenised.

1.10 There are several possible approaches for grinding/milling tobacco. However, those grinding processes that generate significant heat may adversely affect the tobacco and should be avoided, especially where subsequent methods involve the analysis of thermally labile or volatile residues.

1.11 Cryogenic milling is a simple technique which can be applied to those residues that may be lost when tobacco is ground at ambient temperature (e.g. DTCs). It involves the sample being frozen at - 20°C in the presence of dry ice before being disintegrated into a fine powder. By reducing the temperature at which samples are processed potential reactions can be slowed and loss of pesticides can be minimised.

1.12 The ideal particle size is around one millimetre in diameter or less, although up to 4 mm can be used, as this will maximise the surface area and subsequent extraction efficiency. Sieves are often incorporated into the grinding equipment which can be used to gauge the particle size of the tobacco.

1.13 The analysis of thermally labile or volatile residues should be the first to be undertaken.

1.14 Sample processing, sub-sampling and storage can profoundly influence the results of analysis and should be investigated as part of the method validation procedure.

2. STORAGE

2.1 All tobacco samples should be extracted and analysed within the shortest timeframe possible, thereby minimizing sample storage.

2.2 Tobacco samples should be kept in a cool, dry, dark place until analysis.

2.3 If long term storage is required, it is advisable that in order to maintain integrity samples should be kept in a freezer (approx. - 18°C). Long term frozen samples may not necessarily represent the tobacco they originated from as the tobaccos will have been stored under different climatic conditions. Consequently, residue content may vary considerably.

3. STANDARDS

3.1 Standards must be purchased from a reputable source.

3.2 Certified standards must be used.

3.3 Standards must be relatively pure and of known purity.

3.4 The use of a 'pure' standard material is preferable to that of a standard in solution (e.g. 10 µg/mL in acetonitrile) as this allows the laboratory greater flexibility in terms of standard preparation as well as method validation at higher fortification levels.

3.5 Each 'pure' standard material used in the laboratory must be uniquely identified and its date of receipt and its expiry date recorded.

3.6 'Pure' standard materials must be stored appropriately, preferably in a freezer, with light and moisture excluded, i.e. under conditions that minimise the rate of degradation.

3.7 No 'pure' standard material should be used after ten years of storage.

3.8 A 'pure' standard material may be retained and used for longer than the supplier's expiration date if it can be demonstrated that the purity of the standard remains acceptable.

3.9 The purity of a 'pure' standard material may be evaluated using a freshly acquired 'pure' standard material, preferably from an alternative supplier.

3.10 Great care must be used in applying the 'pure' standards where several isomers are included, because the proportion of isomers could vary among standard suppliers.

Preparation of stock standards

3.11 Prior to preparing a stock standard the 'pure' standard material must be allowed to equilibrate to room temperature - at least 30 minutes to 2 hours depending on storage condition temperature.

3.12 Stocks standards should be prepared with not less than 10 mg 'pure' standard material using a 5 decimal place analytical balance.

3.13 Appropriate solvents for residue analysis should be used to prepare the standards. Acetone or acetonitrile are universal solvents and can be used in the preparation for the majority of residues. The criteria for solvent selection should be based on the following:

- Residue is soluble in solvent at the required concentration.
- Residue is not easily degraded in the solvent.
- Solvent is appropriate for the method of analysis.

Note: Pyrethroids, for example, are known to degrade in acetone and should be prepared with a more suitable solvent, such as ethyl acetate.

3.14 Preparation of stock standards using protic solvents (e.g. methanol and water) should only be used when other solvents are not appropriate. If methanol and or water are necessary, expect higher degradation rates in these solvents.

3.15 When a stock standard is prepared the accuracy of the solution should be compared with a second independently prepared solution – ideally, the previous stock standard if still stable and available.

3.16 To minimise any transcription errors it is advisable that the calculation of the stock solution concentration from the weight and purity of the ‘pure’ standard material be double-checked and recorded by a second person.

3.17 To reduce the possibility of degradation, the ‘pure’ standard material should not be exposed to light. Use amber coloured glassware when preparing stock solutions or wrap the container used with aluminium foil.

3.18 Stock standard must be allocated a unique ID and appropriate expiry date. Analyte name, date of preparation, type of solvent and concentration should also be included on the stock solution container.

3.19 Stock standards must be stored in containers that prevent any loss of solvent and entry of water.

3.20 Standards must be stored appropriately; ideally, in a freezer or refrigerator.

3.21 The stability of standard may be checked by preparing a new stock solution and comparing the detector responses. The comparison should be undertaken using appropriate dilutions of individual standards or mixtures of standards. Inexplicable differences between old and new standards must be investigated. Discrepancies between the concentrations of new and old solutions may be due to a number of factors other than simply analyte degradation (e.g. analyte precipitation, solvent evaporation, differences in the purities between the old and new reference standards, errors in weighing, or errors in the instrumental analysis).

4. EXTRACTION AND SAMPLE PREPARATION

4.1 The objective when extracting a tobacco sample is to maximise the extraction efficiency. Certain factors can affect this efficiency and since the extraction is being applied within a multi-residue method, a pragmatic approach is required and a balance must be struck between different factors.

4.2 Parameters such as temperature, time, pH, etc. may affect extraction efficiency, analyte stability and required solvent volume of different compounds in the MRM analysis suite in different ways. Usually, these factors are not controlled since their effects will be reflected in the various MRM recovery rates.

4.3 Extracting a tobacco sample by shaking it with a solvent is a time-tested and simple approach that is fast, convenient and inexpensive. The organic solvents most commonly used include acetone, acetonitrile, ethyl acetate and methanol. With the exception of the more polar pesticides (e.g. methamidophos) these solvents are equivalent in their extraction efficiency.

4.4 The choice of solvent should be made so that both polar and non-polar compounds encompassed in the MRM analysis suite are extracted with adequate recoveries. Solvents with different polarities will extract residues with different polarities to different extents.

Polar friendly solvents like acetone or acetonitrile will best extract analytes with high polarity, and less polar solvents like n-hexane or cyclohexane will best extract analytes with low polarity. When extractions with polar friendly solvents are done, greater clean-up procedures may be necessary. For these analyses solvents such as acetone or acetonitrile have been shown to give sufficiently good overall recoveries for most compounds in the MRM suite. However, for certain highly polar pesticides such as methamidophos, chlorothalonil, and endosulfan sulfate, even acetonitrile may not be polar enough to achieve complete extraction. In these cases, 10 % to 20 % water in acetonitrile will improve the extraction efficiency at the cost of increased sample clean-up.

4.5 Where extracts are diluted to a fixed volume, accurately calibrated volumetric vessels should be used.

4.6 Further and/or complete evaporation of the solvent of extracts should be avoided, because analytes in extracts could be degraded or become hard to be re-dissolved.

4.7 Great care must be exercised when tobacco extracts are evaporated to dryness, as trace quantities of many residues can be lost in this way. A small volume of high boiling point solvent may be used as a 'keeper' and the evaporation temperature should be kept as low as practical. When evaporation to dryness is necessary, usually rotary evaporation at reduced pressure is less detrimental to the sample than nitrogen purging. This is especially true when higher boiling solvents are used such as acetonitrile or toluene.

4.8 The stability of analytes in tobacco extracts must be investigated during method validation. Storage of extracts in a fridge or freezer will minimise degradation but potential losses at the higher temperatures of an autosampler tray should not be ignored.

4.9 In order to protect against potential losses pesticide residue analysis should be performed in the shortest time-frame possible.

4.10 Appropriate Solid Phase Extraction (SPE) columns or relevant adsorbent materials can be used to clean up extracts. Careful attention should be paid to type, manufacturer and lot of these columns and materials, because clean-up efficiency could depend significantly on these parameters.

Note: Once exposed to the atmosphere, SPE columns should be kept in dry conditions, e.g. desiccator, as humidity could otherwise damage the sorbent.

4.11 As far as practical, appropriate solvents should be prepared freshly for conditioning and/or eluting from SPE and other columns. When old solutions are used (e.g. older than one week), there might be problems in clean-up procedures, because the concentration or property of the solutions could be changed during long term storage.

5. RECOVERY

5.1 Recovery is the procedure in which a known amount of one or more analytes is added to a 'blank' tobacco sample prior to extraction. The result is typically expressed as a percentage.

5.2 The analytical method should be demonstrated at validation as being capable of providing a mean recovery in the range (70 % - 120 %) and relative standard deviation ≤ 20 %. In certain justified cases, recoveries outside of this range may be accepted, for example, if the recovery was low but demonstrated good precision.

5.3 Recoveries are generally fortified onto the tobacco prior to extraction. It is highly recommended to perform recovery experiments on the four major types of cured tobacco (Burley, Virginia and Oriental and dark tobaccos), especially during method validation.

5.4 If practical, the stock solutions used to fortify the tobacco for recovery should originate from a separate source compared to those stock solutions used to prepare the instrument calibration solutions.

5.5 Although recoveries may not be reflective of the extractability of incurred residues in tobacco, which may have penetrated the tobacco or become bound to it, they do indicate whether subsequent steps of the analytical procedure are performed with sufficient accuracy and precision.

5.6 Once a recovery sample has been fortified allow at least 30 minutes for the residues to penetrate the tobacco prior to adding extraction solution.

5.7 Furthermore, recoveries should be performed at different fortification levels in order to validate method (e.g. LOQ, 10 × LOQ, when possible GRL) and at one fortification level on routine basis.

5.8 Recoveries performed at the LOQ are the most important. Most data reported will be less than the LOQ. Validating the pesticides could be detected and quantified at this level is crucial to protect the validity of a database.

5.9 For targeted or single residue methods (e.g. DTCs and MH) recoveries should be performed with every batch of samples.

5.10 Ideally, recoveries for all residues incorporated in a multi-residue method should be undertaken with every batch of tobacco samples analysed. Where this would involve performing a disproportionately large number of recovery determinations recoveries should be performed on a rolling basis.

5.11 If a residue is found on a frequent basis it would be prudent to incorporate it as a recovery with every batch of samples analysed.

Table 1. Frequency of residues included in recovery samples

Targeted or Single Residues	Frequently Observed Residues	All other residues
Included with every batch of samples	Included with every batch of samples	Regularly reviewed

5.12 It is important that a recovery sample does not contain residues which may interact resulting in poor recoveries.

5.13 Separate recoveries should be performed when analysing precursors and metabolites to prevent erroneous results.

5.14 At least one recovery must be performed with each batch of samples extracted and analysed.

6. CONTAMINATION AND INTERFERENCE

6.1 A reagent blank should be analysed in conjunction with every batch of samples analysed.

6.2 Samples must be separated from each other, and from other potential sources of contamination, during transport to, and storage at laboratory.

6.3 Volumetric equipment, such as flasks, pipettes and syringes must be meticulously cleaned with an appropriate high purity solvent (e.g. pesticide or environmental grade acetone), before use.

6.4 To avoid the possibility of cross-contamination, glassware should be separately allocated to standards and sample extracts.

6.5 Great care around the use of detergent should be exercised in cleaning glassware. Glassware must be rinsed and dried thoroughly. Alkaline detergents must be avoided because most analytes would be degraded if alkaline components remain in glassware.

6.6 Pest control in, or near the laboratory should be avoided if possible. When pesticides are used, these must be restricted to those that will not be analysed as residues in the laboratory.

6.7 Equipment, containers, solvents including water, reagents, filter aids, etc. should be checked as sources of possible interference. Rubber and plastic items (e.g. seals, protective gloves, wash bottles, connecting devices), polishes and lubricants are frequent sources.

6.8 Interference from natural constituents of samples often occurs. The interference may be peculiar to determination system used, variable in frequency and intensity, and the cause should be understood and checked by the analyst.

6.9 Instruments detectors such as ECD, FPD, NPD, PDA and fluorescence should be regularly checked for contamination and/or interference in order to maintain their performance.

6.10 Instrument consumable, including: syringe, liner, septum, guard column, and analytical column, should be replaced frequently.

Matrix effects

6.11 Matrix effects arise from the presence of co-extractives in a tobacco extract and may be difficult or impossible to eliminate. They are observed as a decreased (suppression) or increased (enhancement) detector response for the analyte compared with that produced by the analyte in a simple solvent solution.

6.12 Matrix effects cannot be predicted. Therefore, it may be necessary to know the tobacco types being analysed so that appropriate calibration standards can be prepared. If the tobacco type is not known then standard addition may provide more accurate quantification.

6.13 Matrix effects can seriously affect the accuracy of an analytical method if not addressed and must not be overlooked. Potential matrix effects from the various types of tobacco analysed on a routine basis should be evaluated during method validation.

6.14 Matrix effects can be minimised through a variety of means:

- Improving the sample clean-up
- Diluting the sample (if the residue level is sufficient to permit dilution)
- Using labelled internal standards
- Using matrix matched standards
- Using standard addition
- Using procedural standards

7. CALIBRATION

7.1 There are several different approaches for quantification:

- Calibration using solvent standards
- Calibration using matrix matched standards
- Calibration using procedural standards
- Standard addition

7.2 The choice of calibration technique employed is dependent on several factors and can differ for different analytical methods.

Solvent standards

7.3 Solvent standards may be used for quantitation provided that it has been demonstrated that no matrix effects occur from the analysis of different tobacco types.

7.4 When using solvent standards samples and standards must be in the same solvent.

Matrix matched standards

7.5 Matrix-matched standards are the preferred choice for quantitation.

7.6 Matrix-matched standard solutions are prepared by extracting a ‘blank’ tobacco and adding an appropriate aliquot of the standard solution.

7.7 The ‘blank’ tobacco should be a relevant tobacco previously shown to contain none or minimal levels of residues.

7.8 To prevent errors in quantitation the matrix concentration in the matrix-matched standards must be identical to that of the sample extracts being analysed. For example, when sample extracts are found to contain high-level residues beyond the calibration range they are usually diluted to bring it within the range; the matrix concentration in the matrix-matched standards must be also be adjusted so it is identical to that of the diluted sample extracts being analysed.

7.9 Great care must be exercised when matrix-matched solutions are used, as certain isomers and/or metabolites may react in the matrix. For example, fenamiphos has been found to be easily oxidised in matrix-matched standards.

Procedural standards

7.10 This approach can compensate for matrix effects and low extraction recoveries associated with certain pesticide/commodity combinations, especially where isotopically labelled standards are not available or are too costly.

7.11 Procedural standards are prepared by spiking a series of blank tobacco with different amounts of analyte, prior to extraction, and are analysed in exactly the same way as the samples.

Standard addition

7.12 Standard addition is a viable alternative to the use of matrix-matched standards. This technique assumes some knowledge of the concentration of the analyte in the sample, so that the amount of added analyte is similar to the level present in the sample.

7.13 This technique automatically adjusts for both recovery and matrix effects.

7.14 Standard addition does not overcome interferences caused by overlapping peaks from co-extracted compounds.

7.15 Standard addition is useful technique to use when a ‘blank’ tobacco matrix is not available or the type of tobacco being analysed is not known.

Use of internal standards

7.16 An internal standard is a chemical compound added to the sample test portion or sample extract in a known quantity at a specified stage of the analysis, in order to check the correct execution of (part of) the analytical method. The internal standard should be chemically stable and/or typically show the same behaviour as of the target analyte.

7.17 One internal standard may not be representative of all pesticides being monitored. It is advisable to use more than one internal standard in case the recovery or detection of the primary internal standard is comprised. When analysing a specific group of pesticides with similar properties an internal standard with similar behaviour can be chosen. However, an internal standard for every pesticide is too costly for multi-residue methods with partly over 100 pesticides.

7.18 It is recommended to use an isotopically labelled internal standard. An isotopically labelled internal standard is an internal standard with the same chemical structure and elemental composition as the target analyte, but one or more of the atoms of the molecule of the target analyte are substituted by isotopes. A prerequisite for the use of isotopically labelled internal standards is the use of mass spectrometry, which allows the simultaneous detection of the co-eluting non-labelled analytes and the corresponding isotopically labelled internal standards. Isotopically labelled internal standards can be used to accurately compensate for both analyte losses and volumetric variations during the procedure, as well as for matrix effects and response drift in the chromatography-detection system. Losses during extract storage will also be corrected for by the isotopically labelled internal standard. Use of isotopically labelled internal standards will not compensate for incomplete extraction of incurred residues.

7.19 Internal standards can be used to act as a quality control marker in order to monitor the whole sample preparation process. This is a simple and effective approach in determining whether samples were prepared correctly (i.e. no dilution steps were missed).

Multi-point calibration standards

7.20 When using multi-point calibration standards to generate a calibration curve:

- The curve should consist of between 3 and 6 standards of different concentration.
- The curve should not be forced through zero.
- Appropriate weighting should be applied to the curve.
- The difference between measured and theoretical concentration of each standard must be ≤ ± 20%.

Single-point calibration standards

7.21 Before a single-point calibration standard is used the detector linearity must be known. For quantitative work, the pesticides levels in the calibration must be near the level in the residue sample.

7.22 The use of a single-point calibration point is only justified if previous calibration curves using multi-point standards have demonstrated that the curve passes through zero.

Calibration of metabolites

7.23 Where a pesticide is determined as a degradation product/metabolite, the calibration standards should be prepared from a "pure" standard using the degradation product/metabolite, if available.

Chromatographic integration

7.24 Chromatograms must be examined by the analyst and baseline fitting checked and manually adjusted if necessary.

7.25 Peak height or area may be used, whichever yields the more accurate and repeatable results.

7.26 Calibration by mixed isomer standards may utilise summed peak areas or heights or measurement of a single component, whichever is the most accurate.

7.27 Modern instruments are capable of monitoring many pesticides concurrently. The data acquisition parameters of an instrument together with the number of pesticides monitored at any one time will dictate the number of the data points plotted across each chromatographic peak. On average, 12 - 15 data points are required to satisfactorily integrate a chromatographic peak.

8. METHOD VALIDATION

8.1 Method validation is the process of ensuring that an analytical method is accurate, reproducible, and robust within the specified analyte range for the intended application.

8.2 Within-laboratory method validation should be performed to provide evidence that a method is fit for the purpose for which it is to be used.

8.3 Methods that have been validated are generally more likely to provide reliable results than those that have not.

8.4 Method validation is a requirement of accreditation bodies, and must be supported and extended by performance verification (on-going analytical quality control).

8.5 All procedures that are undertaken in a method should be validated, if practicable.

8.6 The following parameters specify minimum validation requirements:

Table 2. Validation parameters and their definitions

Parameter	Definition
Scope	The number of different tobacco matrices to which the method can be successfully applied.
Accuracy	The closeness in agreement of the accepted true value or a reference value to the actual result obtained.
Precision	A measure of the ability of the method to generate reproducible results.
Linearity	A method's ability to obtain test results that are directly proportional to the sample concentration over a given range.
Limit of quantitation (LOQ)	The LOQ is defined as the lowest concentration at which an acceptable mean recovery (normally 70 - 120%) and acceptable relative standard deviation are obtained (normally ≤ 20%). It is also frequently defined as the concentration with a signal-to-noise ratio of 6 or 10 [Ref. 12.2].
Selectivity	Selectivity refers to the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour [Ref. 12.3].

8.7 Different accreditation bodies may demand different criteria for method validation but following these guidelines should place different laboratories on a similar accreditation status level.

8.8 For multi-residue methods, representative matrices may be used. Validation should be performed on at least 2 fortification levels (preferably at the LOQ and ten times that of the LOQ or at the GRL) using tobacco types that are routinely analysed.

8.9 The mean recovery rates should be within 70 and 120% with a relative standard deviation ≤ 20%.

8.10 Recoveries are generally fortified at the point of extraction. While they may not be reflective of the extractability of incurred field residues that may have penetrated the matrix or become bound to it during weathering they do indicate whether subsequent steps of the analytical method are operating with sufficient accuracy and precision.

8.11 Method validation should be performed using those tobacco types, which are analysed on a routine basis to gauge the extent of matrix effects occurring. For leaf tobacco this may typically include:

- Virginia flue cured
- Burley
- Oriental
- Dark tobacco (dark fire cured, dark air cured)

However, matrix effects may differ for the same tobacco type due to different crop years, different regions etc.

If finished tobacco products are also analysed typical types evaluated may include:

- Cigarette tobacco (American blend, dark blend, Virginia blend)
- Cigar tobacco
- Pipe tobacco
- Roll-your-own tobacco
- Smokeless tobacco
- Snuff

Accreditation

8.12 In addition to analytical method validation it is highly recommended that laboratory operations should meet the requirements of a recognised accreditation scheme, complying with ISO 17025. The quality requirements described in this document are intended as guidance for accreditation purposes.

Proficiency test

8.13 It is highly recommended that laboratories participate in proficiency tests. Not only is this a requirement for ISO 17025 accredited laboratories but also a valuable tool for laboratories to assess the accuracy and reliability of their methods over time.

9. CONFIRMATION OF RESULTS

9.1 Ideally there should be some overlap of residue detection between LC and GC methodologies. Analysing residues by different methods is a useful cross-check.

9.2 Negative results (residues below the reporting limit) can be considered confirmed if the recovery is acceptable.

9.3 Positive results (residues at or above the reporting limit) must be supported by concurrent calibration and recovery determinations.

9.4 Confirmation is not mandatory for all positive results and must be decided by the laboratory on a case-by-case basis. In general, confirmation should be performed in the following situations:

- Positive results for residues with no calibration or recovery performed with the batch of samples
- Positive results obtained using a non-selective method
- Unusual positive results or those suspected of exceeding the GRL

9.5 Detectors employed with GC or LC such as ECD, FPD, NPD, PDA and fluorescence offer only limited selectivity. Their use, even in combination with different selective columns, can only provide limited confirmatory evidence. These limitations may be acceptable for frequently found residues, especially if such results are also confirmed using a more specific technique. Such limitations in the degree of confirmation should be acknowledged when reporting the results.

9.6 The minimum acceptable retention time for the analyte under examination should be at least twice the retention time corresponding to the void volume of the column. The retention time of the analyte in the extract should correspond to that of the calibration standard with a tolerance of ± 0.1 min, for both GC and LC.

9.7 The term ‘confirmation by mass spectrometry’ normally refers to overwhelming evidence that a tobacco sample actually contains the residue, i.e. proof of identity. Confirmation of the quantity of residue present in a sample can only be achieved by analysis of a second test portion.

9.8 Using tandem mass spectrometric detection systems (e.g. LC-MS/MS) at least 2 specific mass transitions should be acquired.

9.9 The general minimum requirement for GC-MS is for data from two ions of *m/z* > 200; or three ions of *m/z* > 100, preferably including the molecular ion.

9.10 For full scan, MRM and SIM the relative intensities of the detected ions, expressed as a percentage of the most abundant ion or transition, should correspond to those of the calibration standard at comparable concentration and measured under the same conditions.

Table 3. Identification requirements for different MS techniques

MS detector / Characteristic		Acquisition	Requirements for identification	
Resolution	Typical system (example)		Minimum No. of ions	Other
Unit mass	Single MS quadrupole, ion trap, TOF	full scan, limited *m/z* range, SIM	3 ions	S/N ≥ 3 Analytes peaks from both product ions in the extracted ion chromatograms must fully overlap. Ion ratio from sample extracts should be within ± 30% (relative) of average of calibration standards from same sequence.
	MS/MS triple quadrupole, ion trap, Q-trap, Q-TOF	SRM, MRM, mass resolution for precursor-ion isolation equal or better than unit mass resolution	2 product ions	

Larger tolerances are more likely to lead to a higher percentage of false-positive results. Conversely, lower tolerances will lead to a higher number of false-negative results.

10. REPORTING OF DATA

Expression of results

10.1 Results should be expressed in mg/kg.

10.2 In instances where results have been corrected for a dry weight basis they should be expressed in mg/kg (dwb).

10.3 Results should not be adjusted for recovery.

10.4 The recovery data related to the reported samples should also be reported.

10.5 If results are adjusted for recovery, this must be stated. In this case they should be adjusted using the mean value from three recoveries performed in the same tobacco type and within the same analytical batch.

10.6 Residues below the reporting limit (RL) should be reported as < RL mg/kg.

10.7 The summing of results for independently determined but structurally related pesticides is to be discouraged. Summing independently determined pesticides results in less information and more uncertainty regarding the data. If the pesticide has its own chemical abstract number and is measured separately from other pesticides, it should be reported separately. Otherwise significant confusion results as to the true nature of the residue.

Note: Aldicarb and its metabolites are a good example. If aldicarb and its metabolites, the sulfoxide and sulfone, are reported as the sum, the resulting summed value now has less information. Was it all aldicarb? Was there any sulfoxide present? Since the toxicology of these compounds is different, such information may be important. In addition, by not summing analytes the confusing situation of trying to sum "less than" values is avoided.

Note: Some laboratories only determine the parent compound. In this case a summed value gives the false impression that all three analytes were measured. In another case the parent and sulfoxide are oxidised to the sulfone which is then determined and reported as parent. Here the determined value should have a separate title such as "Total Aldicarbs by Oxidation".

Rounding of data

10.8 It is essential to maintain uniformity in reporting results.

Table 4. Reporting results and significant figures

mg/kg	Significant Figures
≥ 0.01 - < 10	2
≥ 10.0 - < 100	3 *(or to a whole number)*
≥ 100	3

10.9 Percent recoveries should be rounded to two significant figures if less than 100 or to three significant figures if greater than 100.

10.10 Reporting limits < 10 mg/kg should be rounded to 1 significant figure and those ≥ 10 mg/kg should be rounded to 2 significant figures.

10.11 Additional significant figures may be recorded for the purpose of statistical analysis, or agreed with the customer.

11. MEASUREMENT UNCERTAINTY

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

Uncertainty can be determined from method validation (for example, based on 2 × SD of reproducibility at three levels of fortification), inter-laboratory studies (proficiency tests) and in-house quality control.

Typical uncertainty may be estimated using an ISO (JCGM 100:2008: Guide to the expression of uncertainty in measurement ISBN 92-67-10188-9) or Eurachem (EURACHEM/CITAC) Guide, Quantifying uncertainty in analytical measurement 3rd edition (2012) (https://eurachem.org/index.php/publications/guides/quam).

Uncertainty data relate primarily to the analyte and matrix used to generate the data and extrapolation should be used with caution.

Uncertainty tends to be greater at lower levels, it may therefore be necessary to generate data from a range of concentrations.

Proficiency test results provide an alternative to estimating method uncertainty as they offer important information about the contribution of inter laboratory bias to the MU of an individual laboratory.

Two approaches are explained in depth. In both examples, an expanded coverage factor of k = 2 is assumed to calculate the expanded MU represented by U' from the relative standard uncertainty u'.

1st Approach

Whenever a laboratory has participated in a number of Proficiency Tests (European Proficiency Tests [EUPTs] or other relevant PTs on pesticide residues) and achieved acceptable *z*-scores for all (or almost all) the pesticides present in the test material, this approach can be applied. In this approach, a default value of 50 % as expanded MU is applied. This default value is based on the mean relative standard deviations of results reported by the participating laboratories in a number of EUPTs for multi-residue methods on fruit and vegetables. This mean ranged around 25%, providing an expanded uncertainty of 50%.

$$U' = 2 \times 0.25 = 0.50 \qquad U' = 50\ \%$$

The first approach is to be adopted, providing that the MU of the laboratory is ≤ 50 % and in order to do this the 2nd approach can be undertaken.

2nd Approach

In this approach, the expanded MU is calculated using the within-laboratory reproducibility relative standard deviation combined with estimates of the method and the laboratory bias.

$$U' = \sqrt{u'(RSD_{wR})^2 + u'(bias)^2}$$

Where:

u'	is the combined standard uncertainty
$u'(RSD_{wR})$	is the within-laboratory reproducibility
u'(bias)	is the uncertainty component arising from method and laboratory bias, estimated from PT data

To calculate $u'(RSD_{wR})$ preferably long-term quality control recovery data should be used although recoveries coming from validation data can be included too.

12. REFFERENCES

12.1 Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed. European Commission, SANTE/11813/2017

12.2 T. Wenzl, J. Haedrich, A. Schaechtele, P. Robouch, J. Stroka; Guidance document on the estimation of LOD and LOQ for measurements in the field of contaminants in feed and food. European Commission, JRC Technical Report, 2016

12.3 J. Vessman, R. I. Stefan, J. F. van Staden, K. Danzer, W. Lindner, D. Thorburn Burns, A. Fajgelj, H. Müller; Selectivity in analytical chemistry (IUPAC Recommendations 2001). Pure and Applied Chemistry, Vol. 73, No. 8, pp. 1381-1386, 2001

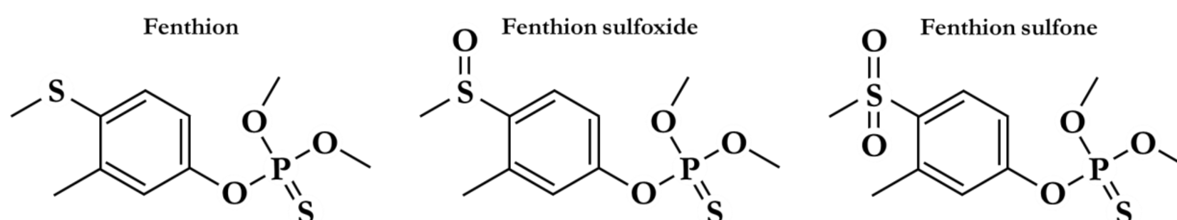
APPENDIX 1. CONVERSION FACTORS

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

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

Example 1

Residue definition: Sum of fenthion, fenthion sulfoxide and fenthion sulfone, expressed as fenthion



Example of calculating the conversion factor (Cf):

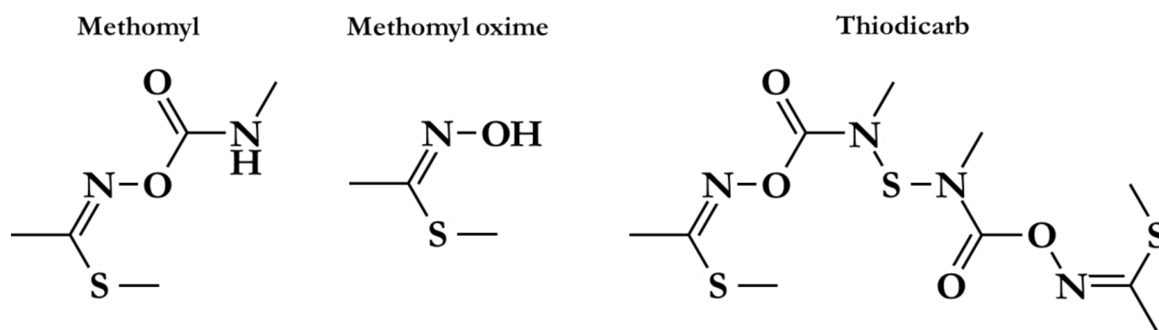
$Cf_{\text{Fenthion sulfoxide}} = Mw_{\text{Fenthion}} / Mw_{\text{Fenthion sulfoxide}} = 278.3/294.3 = 0.946$

Compound name	Molecular Weight (Mw)	Cf
Fenthion	278.3	1.00
Fenthion sulfoxide	294.3	0.946
Fenthion sulfone	310.3	0.897

$C_{\text{Fenthion (sum)}} = 1.00 \times C_{\text{Fenthion}} + 0.946 \times C_{\text{Fenthion sulfoxide}} + 0.897 \times C_{\text{Fenthion sulfone}}$

Example 2

Residue definition: Sum of methomyl, methomyl-oxime and thiodicarb expressed as methomyl



In this example, stoichiometry has to be applied to the calculation of the concentration, and the conversion factor of thiodicarb has to be multiplied by two.

Example of calculating the conversion factor (Cf):

$$Cf_{\text{Thiodicarb}} = 2 \times Mw_{\text{Methomyl}}/Mw_{\text{Thiodicarb}} = 2 \times 162.2/354.5 = 0.915$$

Compound name	Molecular Weight (Mw)	Cf
Methomyl	162.2	1.00
Methomyl oxime	105.2	1.54
Thiodicarb	354.5	0.915

$$C_{\text{Methomyl (sum)}} = 1.00 \times C_{\text{Methomyl}} + 1.54 \times C_{\text{Methomyl oxime}} + 0.915 \times C_{\text{Thiodicarb}}$$

Table 5. Conversion factors of agrochemicals listed in CORESTA Guide No. 1

Aldicarb (sum)	= Aldicarb + 0.922 × Aldicarb sulfoxide + 0.856 × Aldicarb sulfone
Benomyl + Carbendazim + Thiophanate-methyl (sum)	= 0.659 × Benomyl + Carbendazim + 0.558 × Thiophanate-methyl
Carbofuran (sum)	= Carbofuran + 0.933 × 3-Hydroxycarbofuran
Chlordane (sum)	= cis-Chlordane + trans-Chlordane
DDT (sum)	= o,p'-DDT + p,p'-DDT + 1.11 × o,p'-TDE (DDD) + 1.11 × p,p'-TDE (DDD) + 1.12 × o,p'-DDE + 1.12 × p,p'-DDE
Deltamethrin +Tralomethrin (sum)	= Deltamethrin + 0.760 × Tralomethrin
Demeton-S-methyl (sum)	= Demeton-S-methyl + 0.935 × Demeton-S-methyl sulfoxide + 0.878 × Demeton-S-methyl sulfone
Dichlorvos + Naled + Trichlorfon (sum)	= Dichlorvos + 0.580 × Naled + 0.859 × Trichlorfon
Dimethoate + Omethoate (sum)	= Dimethoate + 1.08 × Omethoate
Disulfoton (sum)	= Disulfoton + 0.945 × Disulfoton sulfoxide + 0.896 × Disulfoton sulfone
Endosulfan (sum)	= alpha-Endosulfan + beta-Endosulfan + 0.962 × Endosulfan-sulfate
Fenamiphos (sum)	= Fenamiphos + 0.949 × Fenamiphos sulfoxide + 0.905 × Fenamiphos sulfone
Fenthion (sum)	= Fenthion + 0.946 × Fenthion sulfoxide + 0.897 × Fenthion sulfone
HCH (alpha, beta, delta)	= alpha-HCH + beta-HCH + delta-HCH
Heptachlor (sum)	= Heptachlor + 0.959 × cis-Heptachlor epoxide + 0.959 × trans-Heptachlor epoxide
Iprodione (sum)	= Iprodione + Iprodione metabolite
Methiocarb (sum)	= Methiocarb + 0.934 × Methiocarb sulfoxide + 0.876 × Methiocarb sulfone
Methomyl + Methomyl oxime + Thiodicarb (sum)	= Methomyl + 1.54 × Methomyl oxime + 0.915 × Thiodicarb
Terbufos (sum)	= Terbufos + 0.947 × Terbufos sulfoxide + 0.899 × Terbufos sulfone

APPENDIX 2. GLOSSARY

Accuracy	Closeness of agreement between a test result and the true, or the accepted reference value.
Analyte	The chemical species of which the concentration is to be determined. For the purpose of this guideline: a pesticide residue or metabolite, breakdown product or derivative of a pesticide.
Batch (analytical batch)	For extraction, clean-up and similar processes, a batch is a series of samples dealt with by an analyst (or team of analysts) in parallel, usually in one day, and should incorporate at least one recovery determination.
Bias	The difference between the mean measured value and the true value, i.e. the total systematic error. *See accuracy.*
Blank (Matrix)	Tobacco matrix known not to contain detectable levels of the analyte(s) sought.
Blank (Reagent)	A complete analysis conducted using the solvents and reagents only, in the absence of any sample.
BUR	Burley *(tobacco type).*
Calibration	Determination of the relationship between the observed signal and known quantities of the analyte.
Calibration standard	A solution of analyte at known concentration (and ISTD if included). Typically several calibration standards at different concentrations are used to quantitate samples containing pesticides at unknown concentration. Standards may be matrix-matched.
CI	Chemical ionisation *(GC-MS ionisation technique).*
Collaborative	An analytical exercise where several laboratories analyse a sample for a specific analyte(s) within a given timeframe using a prescribed methodology. *See proficiency test.*
Confirmation	The process of generating sufficient evidence to ensure that a result for a specific sample is valid. It is impossible to confirm the complete absence of residues.
Contamination	Unintended introduction of the analyte into the sample, solutions or extract by any route and at any stage during sample analysis.
DAC	Dark air cured *(tobacco type).*
DAD	Diode array detector *(used with LC; also known as PDA).*
DFC	Dark fire cured *(tobacco type).*
DTCs	Dithiocarbamates *(class of fungicides analyse using a targeted residue method).*
dwb	dry weight basis. Indicates that tobacco weight has been corrected for moisture content.
ECD	Electron capture detector *(used with GC).*
EI	Electron ionisation *(GC-MS ionisation technique).*
ESI	Electrospray ionisation *(LC-MS ionisation technique).*
False negative	A result wrongly indicating that the analyte concentration does not exceed a specified value.
False positive	A result wrongly indicating that the analyte concentration exceeds a specified value.

FCV	Flue-cured Virginia *(tobacco type).*
FPD	Flame-photometric detector *(used with GC and may be specific to sulphur or phosphorus detection).*
GC	Gas chromatography.
GC-MS	Gas chromatography-mass spectrometry.
GRL	Guidance residue level. *Refer to CORESTA Guide no. 1 for more information.*
HPLC	High pressure liquid chromatography *(operates below 6000 psi). See also UHPLC.*
Interference	A positive or negative response produced by one or more compounds other than the analyte, contributing to the response measured for the analyte, or making integration of the analyte response less certain or accurate.
Internal standard (ISTD)	A chemical added, in known quantity, at a specified state in analysis to facilitate determination of the identity and/or quantity of the analyte. The ISTD should have similar physico-chemical characteristics to those of the analyte. Isotopically labelled analyse form ideal ISTDs, where available.
LC	Liquid chromatography. Instrumental technique that is also known as HPLC or UHPLC; supports the use of several different detector types.
LC-MS/MS	Liquid chromatography-tandem mass spectrometry.
LCL	Lowest calibrated level. The lowest level of analyte used successfully to quantitate a batch of samples.
LOD	Limit of detection. The minimum concentration of the analyte that can be detected with acceptable certainty, though not quantifiable with acceptable precision.
LOQ	Limit of quantitation / quantification. The minimum concentration of analyte that can be quantified with acceptable accuracy and precision. Variously defined but value must be greater than the LOD.
m/z	Mass-to-charge ratio.
Matrix effects	An influence of one or more undetected components from the sample on the measurement of the analyte concentration. Matrix effects can results in the analyte response being increased (enhancement) or lowered (suppression) compared with the analyte response in solvent.
Matrix-matched calibration	Calibration intended to compensate for matrix effects. A blank matrix should be prepared as for the analysis if samples. Matrix matched calibration may compensate for matrix effects but does not eliminate the underlying cause. Because the underlying cause remains, the intensity of effect may differ from one matrix or sample to another.
Method	A sequence of analytical procedures, from receipt of sample through to calculation of results.
Method development	The process of design and preliminary assessment of the characteristics of a method, including ruggedness.
Method validation	The process of characterising the performance to be expected of a method in terms of its scope, selectivity, accuracy, sensitivity, repeatability and reproducibility.
MH	Maleic hydrazide *(plant growth regulator analysed by targeted residue method)*
MRM (1)	Multi-residue method.

MRM (2)	Multiple reaction monitoring. An MS/MS term where several transitions are observed simultaneously.
MS/MS	Tandem mass spectrometry. An MS procedure in which a parent (or precursor) ion from the primary ionisation process, is isolated, fragmented, usually by collision, and the product ions separated. *See MRM, SRM and transition.*
MS^n	Ion trap mass spectrometry. Similar to tandem MS, however, procedure may be performed repetitively on a sequence of product ions, although this is not usually practical with low level residues.
NPD	Nitrogen phosphorus detector *(used with GC)*.
ORT	Oriental *(tobacco type)*.
PDA	Photo-diode array detector *(used with HPLC; also known as DAD)*.
Proficiency test	Analytical exercise where several laboratories analyse a sample for a given set of analytes within a prescribed time-frame using their own methods. *See Z-score and collaborative.*
'Pure' standard	A relatively pure sample of the solid or liquid analyte of known purity.
Recovery	The proportion of analyte remaining at the point of the final determination, following its addition (usually to a blank sample) immediately prior to extraction. Usually expressed as a percentage. Routine recovery refers to the determination(s) performed with the analysis of each batch of samples.
Reference material	Material characterised with respect to its notionally homogeneous content of analyte. Certified reference materials (CRMs) are normally characterised in a number of laboratories, for concentration and homogeneity of distribution of analyte.
Repeatability	The precision (standard deviation) of measurement of an analyte (usually obtained from recovery or analysis of reference materials), obtained using the same method on the same sample(s) in a single laboratory over a short period of time, during which differences in the materials and equipment used and/or the analysts involved will not occur.
Reporting limit	The lowest level at which at which a pesticide residue is reported as an absolute number. It is typically represented by the LOQ or LCL.
Reproducibility	The precision (standard deviation) of measurement of an analyte (usually by means of recovery or analysis of reference materials), obtained using the same method in a number of laboratories, by different analysts, or over a period in which differences in the material and equipment will occur. Internal reproducibility is that procedure in a single laboratory under these conditions.
RSD	Relative standard deviation.
Sample	A general term with many meaning but, for this guideline, refers to laboratory sample, test sample, test portion, or an aliquot of extract.
S/N	Signal-to-noise ratio *(often used in determining LOD and LOQ)*.
SD	Standard deviation.
SIM	Single ion monitoring.
SPE	Solid phase extraction.
Spike or spiking	Addition of analyte for the purpose of recovery determination or standard addition.
SRM	Single reaction monitoring. An MS/MS term were only one transition is recorded.

Stock standard	The most concentrated solution of the ‘pure standard material, from which aliquots are used to prepare working standards or calibration standards.
Targeted (or Single) residue method	An analytical method where all parameters are optimised for the extraction and analysis of the targeted analyte.
Transition	A precursor/product ion pair from the same analyte is known as a transition. *See MS/MS, MRM and SRM.*
UHPLC	Ultra-high pressure liquid chromatography *(typically operate in the range 10K - 15K psi). See also HPLC.*
Working standard	A general term used to describe dilutions prepared from the stock standard, which are used, for example, to prepare calibration standard or to spike recoveries.
Z-Score	A *z*-score is a statistical value obtained during a proficiency test. It is derived by comparing a participant’s result to an assigned value which is then standardised against a measure of acceptable analytical variation. Superficially, *z*-scores can be interpreted as: • ≤ 2 ‘satisfactory’ • ≤ 3 ‘questionable’ • > 3 ‘unsatisfactory’