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Estimation of uncertainty of analytical results based on multiple peaks☆

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Abstract

The sources of the uncertainty of GC and HPLC analysis of pesticide residues, which consist of several components, and three methods applicable for the estimation of the uncertainty of the results are described. Different scenarios for estimating the uncertainty of measurements of multi-component residues are identified.
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1. Introduction

The residue definition of some 20% of pesticides elaborated within the Codex Committee on Pesticide Residues (CCPR) includes two to six components. These are either metabolites or isomers of the technical active ingredient. The maximum legally acceptable residue concentration (MRL, mg/kg) is expressed either as the sum of individually measured compounds or the residue components converted into a single substance, which is measured.

The sources of random and systematic errors and their effect on the overall uncertainty of the results were discussed previously [1]. The assessment of the random and systematic errors of the results based on the measurements of multiple peaks requires some special considerations. This paper discusses the problems related to the instrumental analytical component of the uncertainty of the results obtained with the GC and HPLC analysis of several compounds. The contribution to the combined uncertainty of the procedures performed before the instrumental analysis is not discussed here in detail. However, the methods described can be applied for the evaluation of the uncertainty of the whole procedure.

The uncertainty of the results (S_R) of residue analysis can be calculated as:

$$S_R = \sqrt{S_S^2 + S_L^2} \quad (1)$$

where S_S is the uncertainty of sampling and S_L is the uncertainty of laboratory phase of the determination of pesticide residues.

$$S_L = \sqrt{S_{Sp}^2 + S_A^2} \quad (2)$$

The analytical phase may include for instance the extraction, cleanup, evaporation, derivatisation, and instrumental determination. Its uncertainty, S_A, can be conveniently determined with recovery studies from the point of spiking the analytical portion. The combined uncertainty of sample processing and analysis, S_L, can be determined from the results of repeated analyses of analytical portions of samples containing detectable residues. Uncertainty of sample processing, S_{Sp}, can then be calculated.

The uncertainty of chromatographic analysis, S_{Ch}, is a component of the uncertainty of analytical phase. Experience shows [2] that it can be one of the major contributing factors to S_A. Therefore, it is worthwhile to estimate and regularly check the uncertainty of the chromatographic determination.

The uncertainty of the predicted analyte concentration (S_{Ch}) is calculated as [3]:

$$S_{Ch} = \sqrt{S_{x0}^2 + S_{As}^2} \quad (3)$$

where S_{x0} is the standard deviation of the analyte concentration calculated from the calibration data (see Eqs. (4a)

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and (4b)), and S_{AS} is the combined uncertainty of the analytical standard solutions.

The standard deviation of X_0 can be calculated [4] either from ordinary linear regression (OLR):

$$S_{s0} = \frac{S_{y/x}}{b}\left\{\frac{1}{m} + \frac{1}{nk} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}\right\}^{1/2} \quad (4a)$$

or for from weighted regression (WLR):

$$S_{x0w} = \frac{S_{(y/x)w}}{b}\left\{\frac{1}{mw_0} + \frac{1}{nk} + \frac{(y_0 - \bar{y}_w)^2}{b^2\left(\sum_i w_i x_i^2 - n\bar{x}_w^2\right)}\right\}^{1/2} \quad (4b)$$

The estimated S_{x0} has $nk-2$ degrees of freedom. The relative uncertainty of the predicted concentration is:

$$CV_{x0} = \frac{S_{x0}}{X_0} \quad (5)$$

The uncertainty of GC and HPLC measurements of residues can arise form different sources such as:

1. Re-isomerization, decomposition, transformation of the target analytes.
2. It may occur before and during sample preparation, processing, extraction, cleanup, and derivatisation as well as after injection (in the injector port or in the column). The extent of decomposition or transformation of compounds depends on the prevailing conditions and it is difficult to predict. However, certain precautions can be taken to reduce the alteration of the analyte(s) and the consequent variability of the results, for example, by careful selection of the sample processing procedure, use of deactivated liner in GC, etc.
3. Bad separation or non-selective detection of the target analytes (from each other or from the matrix).
4. Varying matrix blank response, and matrix effect.
5. Low or largely differing detection sensitivity for some of the analytes.
6. Integration error.
7. Varying conversion rate of derivatisation.
8. Uncertainty of standard preparation, injection, and calibration.

The uncertainty sources 1–6 may vary from day to day, and can be only partially assessed during method validation. They should be monitored with the regular performance verification performed during the routine use of the procedure.

The uncertainty of the analytical standard can be estimated taking into account the uncertainty of weighing the analytical standards (S_{wht}, $CV_{wht} = S_{wht}/\text{wht}$), and volumetric measurements CV_V, (pipetting, filling in the volumetric flask, temperature variation, and tolerance of volumetric glassware), and purity of analytical standards [3].

For preparing a working solution in three steps the analyte concentration, C_{AS}, is calculated as:

$$C_{AS} = \frac{wPV_{p1}V_{p2}}{V_{f1}V_{f2}V_{f3}} \quad (6)$$

where w is the mass of analytical standard; P its purity; V_{f1}, V_{f2}, and V_{f3} the volumes of the volumetric flasks; V_{p1}, V_{p2} the volumes of pipetted amounts. The combined uncertainty is calculated from the relative uncertainties (e.g. purity of analytical standard: CV_p) of the steps involved:

$$CV_{AS} = \sqrt{CV_{wht}^2 + CV_p^2 + CV_{V1}^2 + CV_{V2}^2 + CV_{V3}^2 + CV_{p1}^2 + CV_{p2}^2} \quad (7)$$

Under normal laboratory practice S_{AS} is usually much smaller than the S_{x0} and do not contribute to the uncertainty of instrumental analysis significantly. Nevertheless, the uncertainty of the preparation of the standard solutions should be estimated and the above assumption of $S_{x0} \gg S_{AS}$ (in practice $S_{AS} \leq 0.25 S_{x0}$) verified [5], as it is also the basic condition for the application of the linear regression.

2. Major scenarios of detecting multiple peaks

2.1. Scenario 1: "DDT"

The residue components are separated and individually detected, applying authentic analytical standards, within one chromatographic run. Their concentrations are calculated on the basis of the individual calibration plots. The residue, mostly expressed as the parent compound, is calculated as the sum of the detected amounts, taking into account the molecular weights, where necessary. Some examples are as follows.

2.1.1. Scenario 1 A: sum of specified compounds

- DDT (sum of *p,p′*-DDT, *o,p′*-DDT and metabolites *p,p′*-DDE and *p,p′*-TDE);
- endosulfan (sum of α, β and sulphate);
- chlorfenvinphos (sum of *cis* and *trans* isomers).

2.1.2. Scenario 1 B: sum of specified compounds expressed as a single compound

- phorate, (sum of phorate its oxygen analogue and their sulphoxides and sulphones expressed as phorate);
- disulfoton (sum of disulfoton, demeton-*S*, and their sulphoxides and sulphones expressed as disulfoton);
- aldicarb (sum of aldicarb, its sulphoxide, and sulphone expressed as aldicarb).

2.2. Scenario 2: "isomers"

The residue defined as the parent compound including its isomers (e.g. pyrethins, cypermethrin, permethrin, dicofol). In many cases the individual standards are not available. The peaks are integrated separately and the responses are summed. The total residue is calculated on the basis of summed peaks. This approach assumes that all components included in the residue definition have the same or

very similar response factors within the calibrated concentration range of the detection system. Under this condition the re-isomerization, which can often occur during the analysis, and/or different isomer ratios in the analytical standard and in the incurred residue would not cause an error during quantification.

2.3. Scenario 3: "single component"

The residue components are converted to a single component, for example by means of oxidation or hydrolysis, which is measured and then its amount calculated back to the parent compound. This is a special case of scenario 1 B, where the specified residue components included in the residue definition are measured as a single analyte.

Multiple peaks, especially those belonging to Scenario 2, may be quantified with the group integration function of the data processing softwares, provided that the ratio of response factors of the compounds included in the group remains the same at different concentration levels and it must be checked before quantitative determination. It is important to consider how different softwares treat the group of peaks.

For example, with Varian "Star" Chromatography Workstation for 3800 GC software it is possible to group the peaks if they are eluting one after another and exclude the irrelevant peak(s) from the group. However, as in the case of fenpropathrin, the group of peaks cannot be created if the peaks are separated far from each other and several other pesticide residues, which have to be considered, are eluting between them. In this case the peaks have to be separately integrated, their summed responses can then be used for preparing the calibration plot and performing the quantification.

The HP ChemStation Revision A.04.02 software allows to create a group of peaks even if they are far from each other and the group can include as many peaks as necessary. The software considers each peak share and quantifies the sample accordingly.

3. Experimental

The similarity of the ratio of response factors of different components at different concentration levels was checked with the mixtures containing chlorfenvinphos, propiconazole, etaconazol, cyfluthrin, and fenvalerate with approximate concentrations of 500, 800, and 1000 ng/ml. The high concentrations of the compounds were used purposely so that all isomers can be integrated and quantified at each concentration level. These compounds were selected in order to demonstrate different scenarios of multi-component substances. They cover the retention time range typical for the pesticide residues analyzed in the laboratory and contain different number of components and different ratio of peak sizes.

The experiment was performed with Varian GC with NPD detector, applying CPSIL 8CB 25 m × 0.32 mm × 0.25 μm column, 4 ml/min helium carrier gas, and temperature program 70°(1)–20 °C/min–160 °C(0)–4 °C/min–270 °C(3). The 1 μl of sample was injected on column. Injector port temperature was programmed as follows: 70 °C(0.1)–600 °C/min–270 °C(2)–200 °C/min–70 °C. Each mixture was injected three times.

Table 1
Concentration (ng/ml) of the compounds A, B, and C in the samples

Component	Sample		
	1	2	3
A	50	50	101
B	101	50	201
C	395	486	304
Total	547	588	609

Table 2
Concentration (ng/ml) of the compounds A, B, and C in the calibration solutions

Component	Calibration level			
	1	2	3	4
A	34	101	224	
B	30	101	302	
C	30	91	304	608

To illustrate the various options for estimating the uncertainty of chromatographic determinations of multiple peaks three samples were prepared. Each sample contained the same three stable and non-volatile components in different proportions to simulate different cases occurring in practice. The concentrations of the components A, B, and C in the samples are given in Table 1.

Analytical standard mixtures were prepared individually for each concentration of the three or four-levels calibration (Table 2), and injected three times.

Measurements were performed with GC coupled with quadrupole mass-spectrometric detector (G 1800A HP-MSD chromatograph electron ionization detector). Column HP-5 25 m × 0.25 mm × 0.25 μm was used with 1 ml/min helium carrier gas flow, and temperature program 70°(0)–60 °C/min–270 °C(10). The 1 μl of sample was injected in splitless mode. Injector port temperature was 270 °C. Selective ion monitoring program was used with three specific ions selected for each compound. One ion was used for quantification. Each sample was injected five times. The concentration of the components A, B, and C were determined with weighted linear regression.

4. Results and discussion

4.1. Repeatability of GC analysis

The relative uncertainty of the and GC/MSD measurements were tested with replicate injections. The relative un-

Table 3
Relative uncertainty (CV (%)) of measurements of isomer mixtures

Compound	Concentration level		
	1	2	3
Chlorfenvinfos 1	2.5	0.3	2.0
Chlorfenvinfos 2	3.1	0.4	1.2
Chlorfenvinfos total	3.0	0.3	1.3
Etaconazole 1	3.5	1.6	2.7
Etaconazole 2	5.7	3.0	2.5
Etaconazole total	5.0	2.5	2.5
Propiconazole 1	4.8	2.6	2.2
Propiconazole 2	4.7	1.3	1.2
Propiconazole total	4.8	1.7	1.6
Cyfluthrin 1	11.6	1.6	6.8
Cyfluthrin 2	3.1	1.9	8.5
Cyfluthrin 3	14.1	9.9	13.2
Cyfluthrin 4	14.4	5.2	15.1
Cyfluthrin total	8.0	0.9	9.3
Fenvalerate 1	5.0	5.8	11.2
Fenvalerate 2	4.9	0.3	18.0
Fenvalerate total	3.8	4.5	12.6

certainty (CV (%)) calculated from three replicate GC/NPD measurements of isomer mixtures was calculated on the basis of areas of single peaks and the sum of the areas of the isomers, which are marked in bold in Table 3.

The GC/MSD measurements of "samples" were performed in five replicates and the components quantified based on three or four-level calibrations injecting each standard solution three times. The calculated CVs are given in Table 4.

The calculated CVs vary substantially for both a given compound and concentration level, and among various concentration levels. This is the consequence of the fact that the estimation of the standard deviation based on a few measurements is not precise, and its confidence interval is very wide.

The relative uncertainty of the three replicate injections calculated using the summed areas was often smaller than uncertainty (CVs) calculated for some of the individual isomers. This finding is in accord with the theoretically expected value. For instance, the average area and corresponding CVs of the two etaconazole peaks were 5935 and 10833, and 2.7 and 2.45%, respectively. The standard deviations (S.D.) of three replicate injections of etaconazole peaks were 160.0 and 265.7. The calculated combined uncertainty of the sum of the measurements is 1.85%, while the measured value was 2.54% (Table 3). Similar tendency was observed when the uncertainty of the results was calculated for the sum of residues measured independently (Table 6).

Table 4
CV (%) calculated for replicate injections of calibration solutions and mixtures

Injected substance	Component		
	A	B	C
Calibration level 1	3	3	3
Calibration level 2	2	2	1
Calibration level 3	7	8	9
Calibration level 4			3
Sample 1	5	5	6
Sample 2	8	8	9
Sample 3	3	3	2

Table 5
Response ratios of the highest and smallest peaks at total concentration levels of 500, 800, and 1000 ng/ml

Compound	Concentration (ng/ml)	Replicates			Average
		1	2	3	
Chlorfenvinfos	954	8.89	8.94	9.08	8.97
	763	9.03	9.07	8.96	9.02
	477	9.11	8.98	9.05	9.05
Etaconazole[a]	891	**1.83**	**1.83**	**1.82**	***1.83***
	712	**1.94**	**1.88**	**1.87**	***1.90***
	445	**2.05**	**1.95**	**1.98**	***1.99***
Propiconazole[a]	845	**1.43**	**1.46**	**1.44**	***1.44***
	676	**1.44**	**1.50**	**1.47**	***1.47***
	422	**1.52**	**1.53**	**1.52**	***1.52***
Cyfluthrin	915	1.34	1.33	1.26	1.31
	732	1.23	1.24	1.23	1.23
	457	1.38	1.23	1.45	1.36
Cyfluthrin 1	915	2.25	2.10	2.53	2.29
	732	2.50	2.34	2.00	2.28
	457	1.83	1.72	2.14	1.90
Cyfluthrin 2	915	1.59	1.53	1.86	1.66
	732	1.62	1.63	1.72	1.66
	457	1.14	1.41	1.47	1.34
Fenvalerate	961	3.46	3.50	3.97	3.64
	768	3.50	3.24	3.64	3.46
	480	3.78	3.87	3.33	3.66

[a] ANOVA test demonstrated significant differences of mean response ratios.

4.2. *Testing the similarity of the response factors of multiple peaks along the concentration range*

As it was mentioned above, the calibration and quantification based on the summed responses of the analytes (Scenario 2) can only be applied when response factors of the multiple peaks are the same.

The response factors depend on the chemical structure of the components and mode of detection. The ratio of the isomers on each concentration level was calculated by dividing the highest peak by the smallest. (Table 5). The visual observation of the chromatograms or comparison of response ratios may not reveal the differences (Fig. 1). Therefore, one-way ANOVA test was applied to the calculated ratios in order to decide if the peak ratios were significantly different from each other at three concentrations levels. It is worth to note that the response ratios can be tested even if the authentic standards are not available for each of the components.

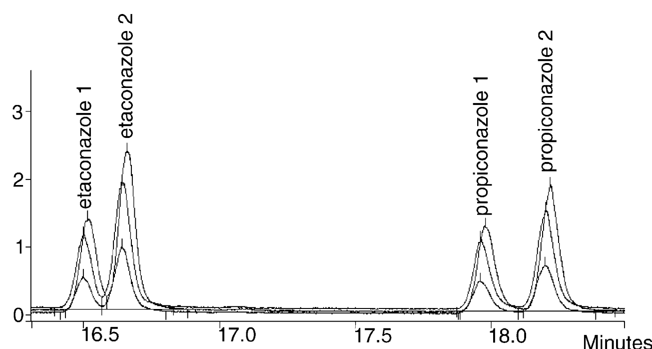

Fig. 1. Chromatographic peaks of etaconazole and propiconazole isomers at three concentration levels.

Of the compounds tested, significant difference was observed in the case of etaconazole and propiconazole. In such cases, authentic standards of the isomers should be used for quantitative determination as far as possible.

4.3. Calculation of uncertainty based on independently measured peaks (residue components) (Method 1)

Where the uncertainties of the chromatographic measurements of individual components (Scenario 1) are S_{Ch1}, S_{Ch2}, ..., S_{Chi}, then the combined uncertainty of the calculated result S_{Ch} is:

$$S_{Ch} = \sqrt{S_{Ch1}^2 + S_{Ch2}^2 + \cdots + S_{Chi}^2} \quad (8)$$

The average concentrations (C_{av}) and their standard deviations based on five replicate measurements were calculated for each component of the samples. The uncertainty (S_{Ch}) of the total residues was calculated according to Eq. (8).

Note: To keep the examples simple, it was assumed that the total residue is calculated as the sum of residue components without correction for molecular weight. Where correction for molecular weight shall be done and/or S_{AS} cannot be ignored, then the calculations should be carried out applying Eq. (3) in combination with Eq. (8), and/or with the corrected residue values, respectively.

Table 7
Calculation of the uncertainty of total residues based on the sum of predicted concentrations of the components (Method 2)

Replicate	Measured total residue in replicate samples (ng/ml)					C_{av} (ng/ml)	S.D.	CV (%)
	1	2	3	4	5			
Sample 1	523	547	567	588	595	564	29.47	5.22
Sample 2	575	686	712	677	676	665	52.47	7.89
Sample 3	671	699	702	689	681	688	12.94	1.88

The relative uncertainty (CV (%)) of the result was calculated by dividing the corresponding S_{Ch} with the sum of the average concentrations of the components $\left(\sum C_i\right)$. The results for samples 1–3 are presented in the Table 6.

4.4. Calculation of uncertainty from the sum of the residues (Method 2)

The concentrations of the three components were summed for each replicate sample. Average value, S.D., and CV of five "total residue" concentrations were calculated. The results for samples 1–3 are presented in the Table 7.

4.5. Calculation of the uncertainty of the results based on the uncertainties of predicted concentrations (Method 3)

Standard deviation of predicted concentration (S_{x0}) was calculated with Eq. (4b) from the calibration curve for each component of replicate samples. Each injection gave an estimate for the uncertainty of predicted concentration. Therefore, the typical uncertainty is calculated by pooling the S_{x0} values obtained from the five replicate measurements.

The uncertainty (S.D.) of total residue in the samples (sum of the concentrations of three components) is calculated with Eq. (8).

The results for mixtures 1–3 are presented in the Table 8.

Table 6
Calculation of the relative uncertainty of the total residue (CV (%)) in samples based on individually measured concentrations (Method 1)

Sample	Component	Concentration in replicate samples (ng/ml)					$C_{i,av}$ (ng/ml)	S.D.	S.D. of result	CV (%)
		1	2	3	4	5				
1	A	52	54	55	58	54	55	2.11	24.06	4.27
	B	100	103	107	108	112	106	4.55		
	C	371	389	406	422	429	403	23.53		
2	A	50	54	60	52	52	54	3.72	46.84	7.04
	B	52	56	62	59	59	58	3.80		
	C	473	576	590	566	565	554	46.53		
3	A	111	116	114	118	119	116	3.18	10.33	1.50
	B	226	230	238	230	224	230	5.54		
	C	333	353	349	341	337	343	8.12		

Table 8
Standard deviation of the predicted concentrations (S_{x0}) and the combined absolute and relative uncertainty (S.D., CV (%)) of the total residue in samples 1–3 (Method 3)

Sample	Component	Average Concentration (ng/ml)	S_{x0} values calculated for replicate injections					$S_{x0,av}$	S.D.	CV (%)
			1	2	3	4	5			
1	A	55	2.3	2.4	2.5	2.6	2.4	2.45	19.92	3.53
	B	106	3.1	3.3	3.4	3.4	3.6	3.37		
	C	403	17.8	18.7	19.6	20.4	20.8	19.48		
2	A	54	2.2	2.4	2.7	2.3	2.3	2.4	27.61	4.15
	B	58	1.4	1.6	1.8	1.7	1.7	1.62		
	C	554	23.1	28.5	29.3	28.0	28.0	27.46		
3	A	116	5.6	5.9	5.8	6.1	6.1	5.91	19.12	2.78
	B	230	8.0	8.1	8.4	8.1	7.9	8.10		
	C	343	15.8	16.8	16.6	16.2	16.0	16.27		

Table 9
Relative uncertainties of the total residues calculated with different methods

Sample	Component	Residue (ng/ml)	Relative uncertainty (%) estimated with Method		
			1	2	3
1	A	50	3.86		4.47
	B	101	4.29		3.18
	C	395	5.84		4.83
	A + B + C	547	4.27	5.22	3.53
2	A	50	6.95		4.48
	B	50	6.61		2.82
	C	486	8.40		4.95
	A + B + C	588	7.04	7.89	4.15
3	A	101	2.75		5.11
	B	201	2.41		3.53
	C	304	2.37		4.75
	A + B + C	609	1.50	1.88	2.78

4.6. Comparison of the results obtained with the three methods of estimating uncertainty

The relative uncertainties of the total residues and individual residue components are summarized in Table 9.

The Cochran test performed with the standard deviations of the sum of the results indicated that they are not significantly different. The differences between the results can be attributed to the different methods of estimation and to the uncertainty of the estimation of standard deviation and mean value based on five replicate measurements. Therefore, when sufficient number of replicates is done during the analysis each of the three described methods can be used for Scenario 1. For Scenarios 2 and 3, only Methods 2 and 3 are applicable since one measured or combined value is used for each compound for quantification of the uncertainty.

Where the response factors of the components are the same or similar along the working concentration range, the sum of the responses can be used to quantify mixtures of isomers, and there is no need to have authentic analytical standard for each component. Under these conditions the re-isomerization during the analysis does not cause any error.

As the residual standard deviation of the chromatographic peaks are usually proportional to the analyte concentration, the standard deviation of the larger peaks are numerically larger and contribute more to the uncertainty of the total residue concentration (Tables 6 and 8).

The uncertainty of results based on multiple peaks cannot be better than the uncertainty of any of the components. The relative uncertainty of the total residue can, however, be smaller than the relative uncertainty of its components (Tables 3 and 9).

During routine daily operation it is not possible to estimate the uncertainty of the measurement based on sufficient number of replicate analysis. However, applying for instance an Excel template, the standard deviation (S_{x0}) of the predicted concentration can be conveniently calculated and used, as an internal quality control check, to verify the performance of the chromatographic system, and to compare it to the uncertainty of the measurement obtained from replicate analysis of samples at the time of method validation.

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