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EVALUATION OF MEASUREMENT UNCERTAINTY
ANNEX 2.3

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Annex 2 to Guideline "Evaluation of Measurement Uncertainty"
PA/PH/OMCL (18) 145 (in its current version)

Estimation of measurement uncertainty using Top-down approach

Annex 2.3 Use of Certified Reference Materials for the estimation of MU

This annex provides an example of how to use the uncertainty of the certified reference material in top-down approach for estimation of measurment uncertainty of the testing result. According to Ph. Eur. 5.12, a Certified Reference Material (CRM) is a reference material characterised by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that states the value of the specified property, its associated uncertainty, and a statement of metrological traceability.

CRMs are available as pure substances, in solution in a pure solvent, or as substances in a matrix. They are typically used for qualification and calibration of equipment, validation and quality control of analytical procedures, in particular to evaluate the precision and bias (i.e. uncertainty) of a method.

The uncertainty of the CRM can be expressed as:

a) *Expanded uncertainty (in units of the test result)*

For example:
A buffer used for calibration of pH-meters has a certified value of 7.00 ± 0.02 pH units (the expanded uncertainty given in the certificate is U(pH) = 0.02, for a coverage factor k = 2).
The standard uncertainty, $u(pH)$, i.e. a standard deviation (in units of the test), can be calculated from the expanded uncertainty as:

$$u(pH) = \frac{U(pH)}{k} = \frac{0.02}{2} = 0.01 \text{ pH units}$$

The relative standard uncertainty is calculated as:

$$\frac{u(pH)}{pH\ (Certified\ value)} = \frac{0.01}{7.00} = 0.0014$$

(corresponding to 0.14%)

b) *Expanded uncertainty (as relative value in percent i.e. a relative standard deviation in % of the mean of measurements in cases where sources of variation have different units)*

For example:
1. The certificate of analysis of Rafoxanide Reference Material includes a declared content (C_{cert}) of 99.7% and the sentence: *"The estimated uncertainty of a single measurement of the assay can be expected to be 0.5% relative (level of confidence P= 95%, n=6), whereby the assay measurements are calculated by 100% minus found impurities".*
Since the certificate provides the number of determinations (n), the coverage factor (k = 2) can be replaced by the Student t-value considering the level of confidence and number of replicates given in the certificate. Therefore, the Student t-value is equal to 2.571 for P=95% and n = 6.
The value of 0.5% given in the certificate is the relative expanded uncertainty ($U(C_{cert}rel)$. The standard uncertainty $u(\mathrm{C_{cert}})$ can be calculated as follows:

a. The expanded uncertainty (units of the test) is calculated as:

$$U(\mathrm{C_{cert}}) = \frac{U(\mathrm{C_{cert}}rel)}{100} \cdot \mathrm{C_{cert}} = \frac{0.5}{100} \cdot 0.997 = 0.004985\%$$

b. The standard incertainty $u(\mathrm{C_{cert}})$ in units of the test can be calculated from the expanded uncertainty as:

$$u(\mathrm{C_{cert}}) = \frac{U(\mathrm{C_{cert}})}{t - value} = \frac{0.004985}{2.571} = 0.001939\%$$

2. Perchloric acid solution 0.1 mol/L in acetic acid is provided with a certificate which contains the following information: $\mathrm{C_{cert}}$ *= 0.1003 mol/L ± 0.2%, 95% level of confidence.*

Since the number of determinations (n) is not mentioned in the certificate, the expanded uncertainty is calculated using a coverage factor k = 2 for the given level of confidence, P = 95%.

The expanded uncertainty (units of the test, mol/L) is calculated as:

$$U(\mathrm{C_{cert}}) = \frac{U(\mathrm{C_{cert}}rel)}{100} \cdot \mathrm{C_{cert}} = \frac{0.2}{100} \cdot 0.1003 = 0.0002\ \mathrm{mol/L}$$

The standard uncertainty (for a coverage factor k = 2, P = 95%) is:

$$u(\mathrm{C_{cert}}) = \frac{U(\mathrm{C_{cert}})}{k} = \frac{0.0002}{2} = 0.0001\ \mathrm{mol/L}$$

The relative standard uncertainty is:

$$\frac{u(\mathrm{C_{cert}})}{\mathrm{C_{cert}}} = \frac{0.0001}{0.1003} = 0.001$$

(corresponding to 0.1%)

Example: Estimation of uncertainty of measurement of recovery experiments during method validation study for determination of carprofen

1. Description of the analytical procedure

The content of the Carprofen in tablets was determined using a previously validated in house HPLC method, by performing two determinations from a pool of 5 grounded tablets.

2. Estimation of measurment incertainty

2.1 Specification of measurand

The measurand is the content of Carprofen in tablets, expressed as mg/tablet.

2.2 Quantification of the uncertainty of measurement using data from Certified Reference Materials

The Carprofen reference material was used during the method validation to carry out recovery experiments. The following information is reported in the certificate of analysis:

Purity ($\mathrm{C_{cert}}$) is 99.7 %, U = ± 0.2 % (for k = 2).

The standard uncertainty (in units of the test) is calculated as:

$$u(\mathrm{C_{cert}}) = \frac{U(\mathrm{C_{cert}})}{k} = \frac{0.2}{2} = 0.1\%$$

The relative standard uncertainty is calculated as:

$$\frac{u(\mathrm{C_{cert}})}{\mathrm{C_{cert}}} = \frac{0.1}{99.7} = 0.001 \text{ (corresponding to 0.1\%)}$$

Uncertainty of recovery

Recovery experiments were performed by spiking samples of carprofen at concentration levels of 80, 100 and 120% of the nominal content (corresponding to 24.20, 30.25 and 36.30 mg/g of carprofen) by adding to a placebo appropriate volumes of a solution made using the Carprofen CRM.
The recovery (R) is calculated as a ratio of the obtained results (C_{obs}) and the nominal value (C_{nom}) and reported in Table 1.

Table 1. Results from recovery experiments

Concentration level (%)	C_{nom}	C_{obs}	Recovery R (%)	Number of det.	Mean of R (%)	S (%)
80	24.20	23.75	98.14	3	98.18	0.559
	24.20	23.63	97.64			
	24.20	23.90	98.76			
100	30.25	30.12	99.57	3	99.01	0.618
	30.25	29.98	99.11			
	30.25	29.75	98.35			
120	36.30	35.65	98.21	3	98.89	0.689
	36.30	35.89	98.87			
	36.30	36.15	99.59			
			Overall	9	98.69	0.624

The overall mean of the 9 recoveries ($\bar{R}$) is a weighted mean calculated as:

$$\overline{\mathrm{R}} = \frac{N_{80\%} \cdot \bar{R}_{80\%} + N_{100\%} \cdot \bar{R}_{100\%} + N_{120\%} \cdot \bar{R}_{120\%}}{N_{80\%} + N_{100\%} + N_{120\%}}$$

$$\overline{\mathrm{R}} = \frac{3 \cdot 98.18 + 3 \cdot 99.01 + 3 \cdot 98.89}{3 + 3 + 3} = 98.69\%$$

The overall standard deviation (S) is a weighted standard deviation calculated as:

$$S = \sqrt{\frac{(N_{80\%} - 1) \times S_{80\%}^2 + (N_{100\%} - 1) \times S_{100\%}^2 + (N_{120\%} - 1) \times S_{120\%}^2}{(N_{80\%} - 1) + (N_{100\%} - 1) + (N_{120\%} - 1)}}$$

$$S = \sqrt{\frac{(3-1) \times (0.559)^2 + (3-1) \times (0.618)^2 + (3-1) \times (0.689)^2}{(3-1) + (3-1) + (3-1)}} = 0.624\ \%$$

The corresponding relative standard deviation (RSD) is calculated as:

$$RSD = \frac{S}{\bar{R}} \cdot 100 = \frac{0.624}{98.69} \cdot 100 = 0.632\%$$

The above formulae calculate a mean recovery and mean standard deviation across the different spike levels. They can be used only if the mean recoveries, in one hand, and standard deviations, on the other hand, of the spike levels are close enough. Otherwise, the uncertainty should be specifically calculated at each spike level.

Relative standard uncertainty of the mean recovery

The standard uncertainty $u(\bar{R})$ of the mean recovery $\bar{R}$**,** is calculated as:

$$u(\bar{R}) = \frac{S}{\sqrt{n}} = \frac{0.624}{\sqrt{9}} = 0.208\%$$

The relative standard uncertainty of the mean recovery $\bar{R}$**,** is calculated as:

$$\frac{u(\bar{R})}{\bar{R}} = \frac{0.208}{98.69} = 0.00211 \text{ (corresponding to 0.211\%)}$$

The relative combined standard uncertainty of $\bar{R}$ is calculated by combining the relative standard uncertainty of $\bar{R}$ $(\frac{u(\bar{R})}{\bar{R}})$ and relative standard uncertainty of the CRM $(\frac{u(\mathrm{C_{cert}})}{\mathrm{C_{cert}}})$ given in the certificate:

$$\frac{u_c(\bar{R})}{\bar{R}} = \sqrt{(\frac{u(\bar{R})}{\bar{R}})^2 + (\frac{u(\mathrm{C_{cert}})}{\mathrm{C_{cert}}})^2}$$

$$\frac{u_c(\bar{R})}{\bar{R}} = \sqrt{(0.00211)^2 + (0.001)^2} = 0.00233 \text{ (corresponding to 0.233\%)}$$

The uncertainty related to the preparation of the spiking solutions has not been taken into consideration in this calculation since is mostly considered negligible. However, detailed information is provided in Annex 1 if this contribution is to be considered.

Significance of bias

The bias is the difference between the mean value obtained and the nominal value. It can be expressed as absolute or relative (%). It is important to evaluate if the estimated bias is statistically significant or not, in order to evaluate the need for a potential correction of the final result.

The significance of the bias is evaluated by comparison of a calculated Student's t-value (t_{calc}) to a critical Student's t-value. t_{calc} is calculated as:

$$t_{calc} = \frac{\bar{R} - E}{\sqrt{(u_c(\bar{R})^2 + u(\mathrm{C_{cert}})^2}}$$

When the recoveries are expressed in % of the expected concentration level, the expected value E = 1 (or 100%). In the example of carprofen CRM:

$$t_{calc} = \frac{0.9869-1}{\sqrt{0.00208^2+0.001^2}} = \text{ - } 5.69.$$

The sign of the calculated t-value informs about the bias, which is negative (i.e. the tablet content is underestimated on average).

The critical Student's t-value is equal to 2.45 considering the sum of degrees of freedom of each concentration level (6 in total, i.e. 2 degrees of freedom times 3 concentration levels) and a level of confidence P = 95%.

The bias is therefore statistically significant, as the calculated t-value, in absolute (5.69), is greater than the critical value (2.45).

Case 1: Significant bias with correction

If the bias is significant, the final assay result may be corrected by dividing the mean assay value by the mean recovery (in % divided by 100).
The assay result of Carprofen tablets is 48.60 mg/tablet (RSD = 0.98%), obtained as mean of 2 determinations from a pool of 5 grounded tablets. The corrected mean is calculated as:

$$Corrected\ mean\ value = \frac{48.60}{0.9869} = 49.25\ mg/tablet$$

The relative combined standard uncertainty of the corrected mean can be calculated by combining $\frac{u_c(\bar{R})}{\bar{R}}$ and RSD² divided by the number of assay determinations as given below:

$$\frac{u_c(Corrected\ mean)}{Corrected\ mean} = \sqrt{\frac{RSD^2}{n} + (\frac{u_c(\bar{R})}{\bar{R}})^2}$$

$$\frac{u_c(Corrected\ mean)}{Corrected\ mean} = \sqrt{\frac{0.0098^2}{2} + (0.00233)^2} = 0.00767$$

Alternatively, the relative combined standard uncertainty can be calculated using the variability from the spiking experiments (as given in paragraph 4) rather than the variability calculated during the assay.

$$\frac{u_c(Corrected\ mean)}{Corrected\ mean} = \sqrt{\frac{0.0632^2}{9} + (0.00233)^2} = 0.00314$$

The expanded uncertainty (coverage factor k = 2, 95 % level of confidence) is calculated as:

$$U = k \cdot \frac{u_c(Corrected\ mean)}{Corrected\ mean} \cdot Corrected\ mean$$

Considering the two assay determinations:

$$U = 2 \cdot 0.00767 \cdot 48.60 = 0.75\ mg/table$$

Considering the spiking experiments:

$$U = 2 \cdot 0.00314 \cdot 48.60 = 0.31\ mg/tablet$$

Case 2: Significant bias without correction

If the bias is significant but no correction of the assay is applied, the calculation of the relative combined standard uncertainty is as:

$$\frac{u_c(\,mean)}{mean} = \sqrt{\frac{RSD^2}{n} + (\frac{u_c(\bar{R})}{\bar{R}})^2 + \text{bias}^2}$$

n= number of assay determinations.

Considering the two assay determinations:

$$\frac{u_c(\,mean)}{mean} = \sqrt{\frac{0.0098^2}{2} + (0.00233)^2 + (1 - 0.9869)^2} = 0.0150$$

Considering the spiking experiments:

$$\frac{u_c(\,mean)}{mean} = \sqrt{\frac{0.0632^2}{9} + (0.00233)^2 + (1 - 0.9869)^2} = 0.0135$$

The expanded uncertainty (coverage factor k = 2, 95 % level of confidence) is calculated as:

$$U = k \cdot \frac{u_c(\,mean)}{mean} \cdot\ mean$$

Considering the two assay determinations:

$$U = 2 \cdot 0.0150 \cdot 48.60 = 1.46\ mg/tablet$$

Considering the spiking experiments:

$$U = 2 \cdot 0.0135 \cdot 48.60 = 1.31\ mg/tablet$$

Case 3: Non significant bias

Assuming that the spiking experiments led to a mean recovery of 99.95 % (n = 9) and a standard deviation of 0.253 %, the combined uncertainty of the mean recovery is calculated in two steps:
The standard uncertainty $u(\bar{R})$ of the mean recovery $\bar{R}$**,** is calculated as:

$$u(\bar{R}) = \frac{S}{\sqrt{n}} = \frac{0.253}{\sqrt{9}} = 0.0843\%$$

The relative standard uncertainty of the mean recovery $\bar{R}$**,** is calculated as:

$$\frac{u(\bar{R})}{\bar{R}} = \frac{0.0843}{99.95} = 0.000843$$

The relative combined standard uncertainty is obtained by:

$$\frac{u_c(\bar{R})}{\bar{R}} = \sqrt{(\frac{u(\bar{R})}{\bar{R}})^2 + (\frac{u(\mathrm{C_{cert}})}{\mathrm{C_{cert}}})^2}$$

$$\frac{u_c(\bar{R})}{\bar{R}} = \sqrt{(0.000843)^2 + (0.001)^2} = 0.00131$$

Then, the significance of the bias is evaluated by the mean of a Student's t-test:

$$t_{calc} = \frac{\bar{R}-E}{\sqrt{u_c(\bar{R})^2 + u(\mathrm{C_{cert}})^2}} = \frac{0.9995-1}{\sqrt{0.000843^2+0.001^2}} = -0.381$$

The sign of the calculated t-value informs about the bias, which is negative (i.e. the tablet content is underestimated on average) but found to be not significant. Indeed, the t-value, in absolute, is lower than the critical value, 2.45, calculated for 6 degrees of freedom (2 degrees of freedom times 3 concentration levels) and a level of confidence of P = 95%.

The correction is not needed but the uncertainty of the mean bias should be included in the combined uncertainty. Then the relative combined standard uncertainty of the reported mean value will be calculated as:

$$\frac{u_c(\,mean)}{mean} = \sqrt{\frac{RSD^2}{n} + (\frac{u_c(\bar{R})}{\bar{R}})^2}$$

Considering the two assay determinations:

$$\frac{u_c(\,mean)}{mean} = \sqrt{\frac{0.0098^2}{2} + (0.00131)^2} = 0.00705$$

Considering the spiking experiments:

$$\frac{u_c(\,mean)}{mean} = \sqrt{\frac{0.00632^2}{9} + (0.00131)^2} = 0.0025$$

The expanded uncertainty (coverage factor k = 2, 95 % level of confidence) is calculated as:

$$U = k \cdot \frac{u_c(\,mean)}{mean} \cdot\, mean$$

Considering the two assay determinations:

$$U = 2 \cdot 0.00705 \cdot 48.60 = 0.69\, mg/tablet$$

Considering the spiking experiments:

$$U = 2 \cdot 0.0025 \cdot 48.60 = 0.24\, mg/tablet$$

2.3 Reporting of results

Case 1: Significant bias with correction

(49.25 ± 0.75) mg/tablet considering n= 2 assay determination
(49.25 ± 0.31) mg/tablet considering n= 9 spiking experiments

Case 2: Significant bias without correction

(48.60 ± 1.46) mg/tablet considering n= 2 assay determination
(48.60 ± 1.31) mg/tablet considering n= 9 spiking experiments

Case 3: Non significant bias

(48.60 ± 0.69) mg/tablet considering n= 2 assay determination
(48.60 ± 0.24) mg/tablet considering n= 9 spiking experiments

Table 3. Summary of results for cases 1, 2 and 3

	U	Result to be reported
Case 1 – Significant bias with correction (assay)	0.75 mg/tablet	(49.25 ± 0.76) mg/tablet
Case 1 – Significant bias with correction (spiking experiments)	0.31 mg/tablet	(49.25 ± 0.31) mg/tablet
Case 2 – Significant bias without correction (assay)	1.46 mg/tablet	(48.60 ± 1.46) mg/tablet
Case 2 – Significant bias without correction (spiking experiments)	1.31 mg/tablet	(48.60 ± 1.31) mg/tablet
Case 3 – Insignificant bias (assay)	0.69 mg/tablet	(48.60 ± 0.69) mg/tablet
Case 3 - Insignificant bias (spiking experiments)	0.24 mg/tablet	(48.60 ± 0.24) mg/tablet

3. References

1. S L R Ellison and A Williams (Eds). Eurachem/CITAC guide: Quantifying Uncertainty in Analytical Measurement, Third edition, (2012) ISBN 978-0-948926-30-3. Available from www.eurachem.org.

Appendix 1

To follow the decision tree how to handle with the bias and $\frac{u(\bar{R})}{\bar{R}}$