Application Note 2a

ERM
European Reference Materials

Using Reference Materials for Calibration. Background

As the first in a series dedicated to calibration, this note explains principles for the use of reference materials in analytical calibration, that is, calibration for the purpose of determining the response behaviour of analytical instruments. Issues addressed include the estimation and use of calibration uncertainty, and requirements on reference materials for calibration. ERM® are perfectly suited for this purpose, because they fulfil the stated requirements.

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INTRODUCTION

One of the basic requirements of ISO/IEC 17025 is that all equipment having a significant effect on the accuracy or validity of measurement results provided by a laboratory shall be calibrated before being put into service. To this end, laboratories, especially accredited ones, must have an established calibration programme which ensures that measurements are traceable [1] to the International System of Units (SI) or to other agreed references.

The term "calibration" is currently defined in reference [1] as the

"operation that, under specified conditions, in a first step, establishes a relation between the **quantity values** with measurement uncertainties provided by **measurement standards** and corresponding **indications** with associated **measurement uncertainties** and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication".

For a chemist in an analytical laboratory this general definition means that calibration determines the relationship between measured instrumental response and amount of the substance to be determined (the analyte) using appropriate calibration materials, and also the calculation used to obtain the result from a new observation. Establishing this relationship is key to establishing traceability to SI units or other appropriate references.

ANALYTICAL CALIBRATION: FOCUS ON INSTRUMENTAL RESPONSE

Analytical instruments are typically calibrated using one or more calibration materials containing known amounts of the analyte. Most often these are synthetic materials such as calibration solutions, prepared from pure substances, but for special analytical techniques (e.g. direct analysis of solid samples by SS-AAS or XRF) matrix materials are used for calibration.

PRINCIPLES OF CALIBRATION: SINGLE-POINT CALIBRATION

1) Calibration

The basic principles of calibration can be illustrated by the simplest case: single-point calibration. This is used when the instrumental response (such as the peak area in a chromatogram) is, perhaps after baseline correction, strictly proportional to the amount of analyte. Then calibration may be carried out at a single level, by replicate analyses of a single calibration material. From the calibration data ($\bar{y}_{cal}$ is the mean value of response; x_{cal} is the reference value of analyte amount) the response factor is determined according to

$$F = \frac{\bar{y}_{cal}}{x_{cal}} \qquad (1)$$

The response factor is then used to convert instrumental response data y_{samp} obtained on other samples into analyte amounts x_{samp} according to

$$x_{samp} = \frac{y_{samp}}{F} \qquad (2)$$

Equations (1) and (2) illustrate the two steps in calibration; establishing a relationship between reference value x_{cal} and response y, and then using this to derive a calculation to predict values x_{samp} from new measured values y_{samp}.

2) Calibration uncertainty

Like any other measurement result the result of a calibration – whether calculated using a factor or a function summarising the calibration data – has an associated uncertainty. This uncertainty has to be included in the uncertainty budget of any result obtained using the calibrated equipment [2,3]. In the case of single-level calibration the uncertainties directly associated with calibration may be expressed as follows:

$$\frac{u_{cal}(x_{samp})}{x_{samp}} = \sqrt{\left(\frac{s(y_{samp})}{y_{samp}}\right)^2 + \left(\frac{s(\overline{y}_{cal})}{\overline{y}_{cal}}\right)^2 + \left(\frac{u(x_{cal})}{x_{cal}}\right)^2} \quad (3)$$

In this expression, all the terms are in the form of relative standard uncertainties. $u_{cal}(x_{samp})$ is the uncertainty in x_{samp} arising from calibration. The different terms on the right represent, respectively:

- the uncertainty arising from variation in the response obtained on a sample,
- the uncertainty arising from variation in the response from calibration material,
- the uncertainty associated with the reference value used in calibration.

All of these uncertainty contributions are combined as a root sum of squares.

Note: There are usually other sources of uncertainty in a complete measurement. These can often be combined with the calibration uncertainty by simple extension of equation (3). Details can be found in references [2] and [3].

The structure of the uncertainty budget described here can be used for other calibration designs, by combining the following contributions to the standard uncertainty of the result:

- the variability of measurements for the given sample, expressed as a standard deviation;
- the variability of measurements on calibration materials, expressed as a standard deviation;
- the standard uncertainty of the reference values attributed to the calibration materials.

The standard uncertainty reported to the customers additionally requires the analyst to consider the standard uncertainty associated with other effects not covered by the calibration.

MATRIX EFFECTS

The sample matrix can bias the analyte signal through spectral and non-spectral interference effects. Non-spectral interferences or matrix effects as they are often known, are characterized by changes in signal intensity that are matrix-induced and not related to spectral overlap. Matrix effects can lead to signal suppression, as well as signal enhancements. An important issue is that matrix effects often depend on the absolute matrix concentration not on the relative concentration of matrix to analyte. Matrix effects can be reduced by simply diluting the sample (if permitted by analyte concentration) or corrected for by certain calibration strategies such as internal standardisation, standard addition, matrix-matched calibration or isotope dilution mass spectrometry. More details on matrix effects and calibration strategies compensating for that can be found in the following literature [4,5].

REQUIREMENTS FOR CALIBRATION MATERIALS

Calibration materials have to be sufficiently homogeneous and stable so as to ensure that the assigned property values (including uncertainty) are valid for any portion of the calibration sample taken and utilised according to the supplier´s specification. In addition, technical requirements for calibration materials are case-dependent and cannot be summarised in a single application note. The only generic requirements that can be addressed here concern the information provided for a calibration material:

- For each reference value (analyte concentration) x_{ref} the standard uncertainty $u(x_{ref})$ has to be specified, either directly or by way of specifying an expanded uncertainty $U(x_{ref})$ with the associated coverage factor k. In addition a statement of traceability is required (Application Note 3 "Using Reference Materials to Establish Traceability").

- For matrix materials, the matrix needs to be specified in sufficient detail to enable comparison with sample matrices where analyte-matrix interferences may occur.

EXAMPLES

Examples are explained in Application Note 2b "Using Reference Materials for Calibration. Examples".

[1] International Vocabulary of Metrology — Basic and General Concepts and Associated Terms, 3rd edition (VIM 3) available from http://www.bipm.org or as ISO/IEC Guide 99-12:2007

[2] Hässelbarth W, Guide to the Evaluation of Measurement Uncertainty for Quantitative Test Results, Eurolab Technical Report No. 1/2006, www.eurolab.org

[3] Ellison SLR, Williams A, Roesslein M (Eds),: Quantifying Uncertainty in Analytical Measurement 2nd Ed. (2000). Eurachem/CITAC Guide, available at www.eurachem.org.

[4] Thompson M, Ellison SLR, Analytical Methods Committee Report: A review of interference effects and their correction in chemical analysis with special reference to uncertainty, Accred Qual Assur (2005) 10:82–97

[5] Vogl J, *Calibration strategies and quality assurance,* in Nelms S (ed.) "ICP Mass Spectrometry", Blackwell Publishing Ltd. (2005) 147-181

Application Note 2b

ERM
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Using Reference Materials for Calibration. Examples - Determination of Kavain by HPLC-UV

ERM®-AC020a, kavain, is a CRM with a certified purity of 99.8 ± 0.2 mass %. The intended use of this material is for the calibration of methods for the determination of kavain in herbal products, foodstuffs and other relevant matrices. This example shows how ERM®-AC020a can be used as a calibrant to determine the amount of kavain in a solution of unknown concentration, and estimates the uncertainty of the calibration.

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CALIBRATION DATA

Five calibration standards from a certified reference material (CRM) of well-defined purity and uncertainty (ERM®-AC020a kavain) plus a blank were prepared. Standards were prepared in 1 % formic acid in acetonitrile. Chrysin was used as internal standard (IS) with a fixed mass fraction for all calibration standards and samples. The calibration standards were approximately equally spaced across the intended calibration range.

The peak height ratios (compared to the chrysin internal standard) were plotted against the corresponding kavain mass fractions and the regression parameters for the calibration line were calculated. The regression line and the regression parameters can be obtained, when plotting the data in Microsoft Excel® and using the tool "regression".

Table 1:   Observed calibration data

Calibration standard	Kavain mass fraction in µg/g	Observed peak height relative to IS
	x_{cal}	y_{cal}
1	0.0000	0.00000
2	20.000	0.53576
3	40.000	1.06537
4	60.000	1.58447
5	80.000	2.11463
6	100.00	2.65250

With this plot (Fig. 1) the regression line can be obtained in the following form:
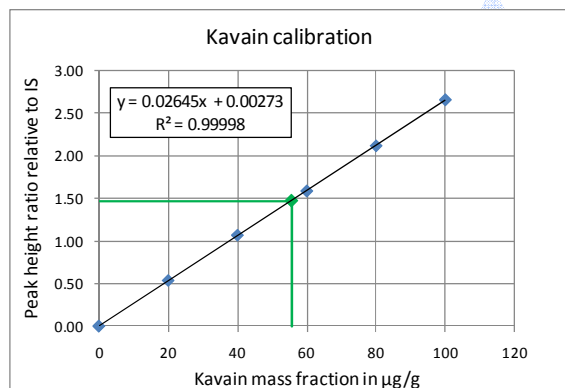
$$y = a \cdot x + b \tag{1}$$

Fig. 1: Plot of the calibration data (blue) together with the regression line (black) and the results obtained for the unknown sample (green)

For the kavain calibration the parameters a and b are as follows:

Slope a: 0.02645 g/µg
Intercept b: 0.00273

Inspection of the calibration line gave no reason to suspect non-linearity, and inspection of the residuals showed no obvious trend. The calibration achieved the required correlation coefficient of at least 0.999.

The prepared solution of an unknown sample was analysed and a peak height ratio of 1.47200 was obtained. Applying the regression equation from above and introducing the peak height ratio as $\bar{y}_{samp}$ = 1.47200 a kavain mass fraction of x_{samp} = 55.5490 µg/g can be calculated.

CALIBRATION UNCERTAINTY

To calculate the uncertainty contribution for the kavain mass fraction in the sample deriving from the calibration, we start with the following equation (equation 3 in Application Note 2a):

$$\frac{u_{cal}(x_{samp})}{x_{samp}} = \sqrt{\left(\frac{s(y_{samp})}{y_{samp}}\right)^2 + \left(\frac{s(\bar{y}_{cal})}{\bar{y}_{cal}}\right)^2 + \left(\frac{u(x_{cal})}{x_{cal}}\right)^2} \tag{2}$$

To estimate the uncertainty associated with random variation in the observations in the calibration and in the observation of the peak height for the unknown sample, the prediction interval $s_{x_{samp}}$ for predicted values of x is used.

This is calculated from the following equation:

$$s_{x_{samp}} = \frac{s(r)}{a} \sqrt{\frac{1}{N} + \frac{1}{n} + \frac{(\bar{y}_{samp} - \bar{y}_{cal})^2}{a^2 \sum_{i=1}^{n}(x_i - \bar{x}_{cal})^2}} \tag{3}$$

N is the number of observations used to obtain the value $\bar{y}_{samp}$

$\bar{y}_{samp}$ is the arithmetic mean of peak height ratios determined for the unknown sample

$\bar{y}_{cal}$ is the arithmetic mean of the observed peak height ratios y_{cal} of the calibration standards in Table 1

$\bar{x}_{cal}$ is the arithmetic mean of the kavain mass fractions in the calibration standards in Table 1

x_i is the kavain mass fraction in the calibration standard i

n is the number of (x, y) pairs used in the regression

a is the estimated slope of the regression line.

The terms $s(y_{samp})$ and $s(\bar{y}_{cal})$ can be identified in equation (3) and therefore equation (2) can be modified as follows:

$$\frac{u_{cal}(x_{samp})}{x_{samp}} = \sqrt{\left(\frac{s_{x_{samp}}}{x_{samp}}\right)^2 + \left(\frac{u(x_{cal})}{x_{cal}}\right)^2} \tag{4}$$

The residual standard deviation $s(r)$ in equation (3) is given by

$$s(r) = \sqrt{\sum_{i=1}^{n} r_i^2 / (n-2)} \tag{5}$$

where the residuals r_i are the differences between observed and predicted value for the peak height. The predicted values can be obtained by inserting the kavain mass fractions of the calibration standards x_{cal} (Table 1) in the equation (1) of the regression line and calculate y_{cal}. These predicted values y_{cal} and the corresponding residuals are given in the Table 2.

Table 2: Predicted values for the peak height ratios and resulting residuals

Observation	Predicted y_{cal}	Residuals r_i
1	0.00273	-0.00273
2	0.53182	0.00394
3	1.06091	0.00446
4	1.59000	-0.00553
5	2.11909	-0.00446
6	2.64819	0.00431

With the residuals r_i from the table above $s(r)$ can be calculated as follows:

$$s(r) = \sqrt{\frac{0.000112}{(6-2)}} = 0.00529$$

This result is being inserted together with the other required data in equation (3):

$$s_{x_{samp}} = \frac{0.00529}{0.02645} \sqrt{\frac{1}{1} + \frac{1}{6} + \frac{(1.47200 - 1.32546)^2}{0.02645^2 \times 7000}} = 0.21643 \, \mu g / g$$

This gives the uncertainty associated with variability in observations in the calibration and in the observation of the peak height for the unknown sample. Dividing by the calculated mass fraction in the sample we can express this as a relative standard uncertainty as follows:

(0.21643 µg/g) / (55.5490 µg/g) = 0.00390.

The term $u(x_{cal})$ has uncertainty contributions from the CRM used to prepare the calibration standards and the preparation of these standards; here only dilution occurs:

$$\frac{u(x_{cal})}{x_{cal}} = \sqrt{\left(\frac{u_{CRM}}{x_{CRM}}\right)^2 + \left(\frac{u(x_{dil})}{x_{dil}}\right)^2} \tag{6}$$

u_{CRM} is obtained from the calibration material certificate. The CRM has a certified value of 99.8 mass % kavain with a value for the standard uncertainty, u_{CRM}, of 0.075 mass % or expressed as a relative standard uncertainty (and an expanded uncertainty, U_{CRM}, of 0.2 mass %, calculated by $U_{CRM} = k \cdot u_{CRM}$ with a coverage factor $k = 2.45$):

(0.075 %) / (99.8 %) = 0.00075.

For the preparation of the calibration standards, we assume that the relative standard uncertainty associated with volumetric operations in preparing the calibration solutions is 0.001. Combining all the terms as relative standard uncertainties, gives the relative calibration uncertainty:

$$\frac{u_{cal}}{55.5490} = \sqrt{0.00390^2 + 0.00075^2 + 0.00100^2} = 0.00410$$

Converting this relative uncertainty to the units in which the result is expressed gives the calibration uncertainty u_{cal} as:

55.5490 µg/g x 0.00410 = 0.22775 µg/g

Uncertainties and uncertainty contributions are usually rounded to a maximum of two significant digits. Subsequently the quantity value, here the kavain mass fraction in the sample, is rounded so that the total number of digits agrees with the uncertainty.

This results in a kavain mass fraction x_{samp} with the uncertainty contribution for the calibration u_{cal}:

x_{samp} = 55.55 µg/g

u_{cal} = 0.23 µg/g

Finally, note that this estimates the uncertainty associated with calibration. It does not include the (usually much larger) uncertainties associated with extraction efficiency, test sample preparation, matrix effects, test material inhomogeneity or operator effects. However, it is useful in deciding whether the calibration procedure is suitable for its intended use.

NOTE

In addition to calibration using pure substances, it is also possible to use matrix calibrants. Often this is preferred for non-destructive testing, solid sampling techniques or when strong matrix effects occur. Examples are the quantification of toxic metals in plastic by XRF or the quantification of sulfur in fuel applying the combustion-UV-fluorescence technique.

Suitable CRMs for toxic metals in plastic are ERM®-EC680k and ERM®-EC681k; suitable CRM for sulfur in fuel are ERM®-EF211, ERM®-EF212a and ERM®-EF213.