

Assessing Uncertainties Related to Linear Calibration Curves: A Case Study for Flame Atomic Absorption Spectrometry

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Abstract: Least square linear regression is widely used in analytical chemistry. In practice a linear relationship between substance content and measured value still has been assumed based on the correlation coefficient criterion, although not recommended. Textbooks provide the necessary formulas for the fitting process, based on the assumption that there is no error in the independent variable. In practice the ordinary least squares (OLS) textbook procedure is used even when the previously stated assumptions are not strictly fulfilled. In this paper, how to validate the calibration function is dealt with in detail using as an example based on measurements obtained for cadmium determination by flame atomic absorption spectrophotometry (FAAS). Assessing uncertainties related to linear calibration curves is also discussed. Considering uncertainties of weights and volumetric equipment and instrumental analytical signal it is observed that the most important factor that contributes to the final uncertainty is the uncertainty of the calibration function.

Keywords: uncertainty, calibration function, flame atomic absorption spectrophotometry

1. INTRODUCTION

Soil contamination by cadmium must not exceed a limit of $5 \mu\text{g g}^{-1}$. Thus, a measured value of $4 \mu\text{g g}^{-1}$ with an uncertainty of $1 \mu\text{g g}^{-1}$ can be considered as compliant with the requirements. That will not be the case if an uncertainty of $2 \mu\text{g g}^{-1}$ is associated with the same value.

Chemical analysis measurements provide a basis for important decisions concerning health, environmental protection, industrial processes, international trade, and commerce, among others. Therefore, chemical measurements must be *good* and have a *known quality* to be meaningful and to provide an adequate result for its intended purpose. Analysts could ask what “*good*” and “*of known quality*” means. This can be interpreted as a result of the “required accuracy.”

Accuracy of measurement means the closeness between the result of a measurand and its true value [1]. Because “accuracy” is a qualitative concept, one should not use it quantitatively. The results should instead be associated with their uncertainties. Uncertainties associated with analytical

measurements represent the doubt or level of reliability associated with the measurement.

Element determination by flame atomic absorption spectrophotometry (FAAS) is very used by analysts. One of the most widely applied statistical techniques is the fitting of a straight line to a set of (x,y) data. Most textbooks on statistical methods [2-4] provide the formula for this fitting process and many hand calculators provide rapid means to have these formulas solved. On the other hand, calibration uncertainties are recently focused due to the need to have analytical results associated with its uncertainties. This consideration can also be exploited for computation of the confidence interval for the prediction of a y -value at a given x -value. In order to calculate the uncertainties of a calibration function, one must go through the straight-line model validation.

Frequently analysts are concerned about improper uses of correlation coefficients [5]. They usually decide on linear adjust model considering the value obtained for the correlation coefficient.

Let us use (x_i, y_i) to denote the i th data pairs and suppose there are n pairs in total. The correlation coefficient, R , is defined as:

$$R = \frac{\sum(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum(x_i - \bar{x})^2 \sum(y_i - \bar{y})^2}}$$

where \bar{x} and \bar{y} are the averages of the x and y measurements and Σ denotes summation over all n observations.

When the points lie exactly on a straight line of positive slope $R = +1$; when the points lie exactly on a straight line of negative slope $R = -1$. Mathematically R lies between $+1$ and -1 . Maybe this fact has given rise to the idea that R being near ± 1 indicates a linear relationship between the x and y variables. However values of R which can be considered large can come from markedly non-linear relationships [5, 6]. Although it has been discussed by many authors, in practice analysts misunderstand this concept.

For analytical processes considering instrumental responses the calibration function is usually obtained by means of a calibration experiment; the observations usually represent the result of a physical measurement that must be converted into the analytical result [4]. The model equation

used is the straight line equation, $Y_i = \alpha + \beta X_i + \varepsilon_i$ (with $i = 1$ to N), where Y_i is the response variable, X_i the independent variable, α the intercept, β the slope and ε_i is the residual. The usual fitting procedure assumes that the x values have no error and the y values are subject to errors. In practice the ordinary least squares (OLS) textbook procedure is used even when the previously stated assumptions are not strictly fulfilled. If the x values are subject to errors, most of the users consider them as so small with respect to errors in y , that they are assumed as not significant [7].

Every calibration begins with the choice of a preliminary range which should contain the expected sample concentration as much as it is possible in the centre of the range. The measured values at the lower end of the range must be significantly different from the process blank. Since the imprecision of an analysis tends to increase with increasing substance content, the range must not be chosen too large. To ensure the applicability of the simple linear regression, the analytical precision over the entire range must be constant. This is known as the homoscedasticity assumption [7]. It can be understood that both the homogeneity of variances as the linearity of the calibration function should be tested and confirmed.

Fitting a calibration function by OLS requires several assumptions related to the residuals and to the model. The omission of the assumptions tests is an important source of errors in analytical chemistry.

This paper proposes to describe the various steps to demonstrate the validation of the linear regression model and a procedure for calculation of uncertainties components of an analytical result due to sample preparation (uncertainty of weights and volumetric equipment) and instrumental analytical signal (calibration uncertainty). A numerical example is carefully explained based on measurements obtained for cadmium determination by flame atomic absorption spectrophotometry (FAAS).

The calibration experiment

After establishing the preliminary range with the standard samples prepared so that their concentrations are distributed equidistantly as possible over the entire chosen range, the calibration function ($\hat{y}_i = a + bx_i$) is calculated from the measured values.

The regression parameters a and β are estimated by the least square estimators a and b considering the quantities that minimize the residual sum of squares,

$$\sum_{i=1}^n (y_i - \hat{y}_i)^2$$

where \hat{y}_i is the predicted dependent variable given by the estimated regression, x_i the known concentration, a the estimate of intercept

$$a = \bar{y} - b\bar{x}$$

and b is the estimate of slope (measure of sensitivity).

$$b = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sum (x_i - \bar{x})^2}$$

The measure of sensitivity results from the change in the measured value caused by a change in the

concentration values. If the calibration function for an analytical procedure is linear, the sensitivity is constant over the entire range and is equivalent to the regression coefficient b .

For each value x_i at which a y_i measured signal is available, the residual e_i is given as $e_i = y_i - \hat{y}_i$

The statistic R^2 is evaluated as the proportion of total variation about the mean of measurements explained by the regression [2,3].

Verification of Linearity

In order to perform the lack-of-fit test, ANOVA statistical test should be carried out. The total variability of the responses is decomposed into the sum of squares due to regression and the residual (about regression) sum of squares and the residual sum of squares is decomposed into lack-of-fit and pure error sums of square. The former is concerned to deviation from linearity and the latter from repeated points. Replications of each calibration point give information about the inherent variability of the response measurements (pure error). If the replicates are repetitions of the same reading or obtained by successive dilutions, the residual variance s_{res}^2 will tend to underestimate the variance σ^2 and the lack-of-fit test will tend to wrongly detect non-existence lack-of-fit. ANOVA table can be constructed from equations shown in Table 1.

Table 1 – ANOVA table for OLS

SOURCE	SUM OF SQUARE	d.f.	MEAN SQUARE
Total	SQT $\sum y_{ij}^2$	n	MQT=SQT / n
correction ("b")	FC $n \cdot y_{00}^2$	1	FC
Total corrected	SQC $\sum (y_{ij} - y_{00})^2$	$n - 1$	MQC=SQC / $(n - 1)$
Due to regression ("a")	SQR $\sum (y_i - y_{00})^2$	1	MQR=SQR
Residual	SQE $\sum (y_{ij} - y_i)^2$	$n - 2$	MQE=SQE / $(n - 2)$
Pure Error	SQE $\sum (y_{ij} - y_{i0})^2$	$n - n_i$	MQEP=SQEP / $(n - n_i)$
	P		
Lack-of-fit	SQL $\sum (y_i - y_{i0})^2$	$n_i - 2$	MQL=SQL / $(n_i - 2)$

d.f. = degrees of freedom; n = total number of i - calibration points; n_i = number of concentration levels; y_{ij} = measured signal; y_{00} = mean of the measured signals; y_i = predicted dependent variable; y_{i0} = mean of the replicates of i - concentration level; "i" index refers to x - independent variable; "j" refers to replicates in x - levels. First \sum is related from $i = 1$ to $i = n$. Second summation \sum in SQC, SQE e SQEP is from $i = 1$ to $j = n_i$

A significant MQR/MQE ratio confirms that there is regression. If the ratio MQL/MQEP is higher than the critical level, a the linear model appears to be inadequate. A non-significant lack-of-fit indicates that there appears to be no reason to doubt the adequacy of the model and both the pure error and lack-of-fit mean squares can be used as estimates of the variance σ^2 .

Test of Homogeneity of Variances

The described linear regression calculation requires each data point in the range has a constant (homogeneous) absolute variation. Inhomogeneity can lead to a higher imprecision and to a higher inaccuracy through possible change in the linear slope. In order to test the homogeneity of variances, replicates of n standard samples of each of the lowest and the highest concentrations of the preliminary range are analyzed separately. The means and the variances, for both set of data, are calculated. The variances of both

series of measurements are checked for homogeneity using the F-test. When the test statistic does not exceed the critical value, there is no reason to reject the null hypothesis and believe that there is not a significant difference between the variances. In the case of inhomogeneity of variances or non-linearity, the chosen range must be reduced so as to fulfill these conditions, or more complicated calibration methods must be chosen as the weighted regression equations or higher degree-regression functions [4,8].

EXPERIMENTAL

In the present study, FAAS was used for the cadmium detection and the uncertainty of the calibration function was assessed. Measurements were obtained by using a Perkin Elmer Flame Absorption Spectrometer, 5000 Model, with a cadmium lamp as the external source, at 247 nm wavelength and 0.7 slit width, and a deuterium lamp as the background corrector. All chemical reagents were analytical grade.

A solution of HNO₃ 0.1 M was prepared for the leaching step. The studied material was a sample of vermiculite containing cadmium as contaminant. The sample was dried at 60°C for two hours to remove water content. Adequate aliquots of a NIST certificated standard solution of 1.000 ±0.002 g.l⁻¹ of cadmium were diluted with deionised water to obtain five solutions (0.5, 0.75, 1.0, 1.5 and 2 mg.L⁻¹) for the calibration function. The cadmium responses were measured in acid solutions obtained from leaching 56.3 mg of the solid material with 15 ml 0.1 M HNO₃. After filtration of the leachate through a Whatman medium porosity filter paper, the filtrate was made up to 250 ml in a volumetric flask. Two ten-fold dilutions with deionised water were carried out to adjust cadmium concentration to the calibration curve working range. The analytical procedure is illustrated schematically in Figure 1.

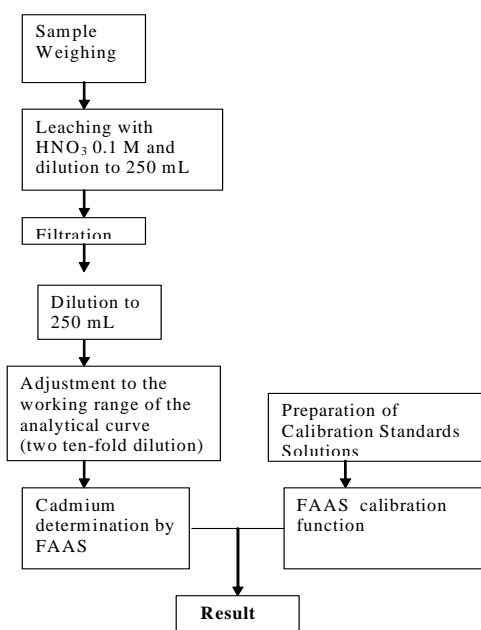


Figure 1 – The adopted analytical procedure

Uncertainty components (Figure 2) were quantified for each step of the analytical procedure as follows: weighing

operation, dilution effects, measuring cadmium by flame atomic absorption spectrometry using a linear calibration function, and calculation of the final result.

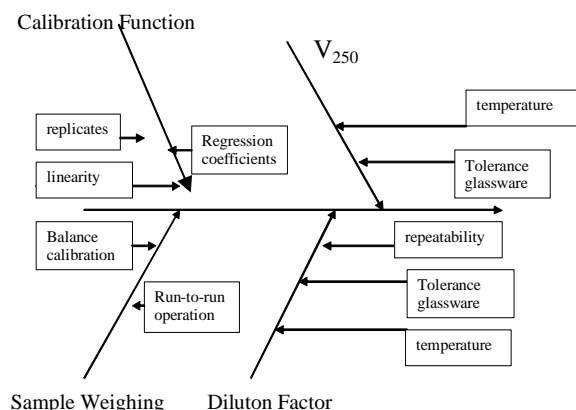


Figure 2 – Uncertainties Sources in Cadmium Determination

Investigation of the Contribution of Individual Steps

Step 1: Weighing

56.3 mg of the dried solid sample were weighted by the difference between container plus sample and container without sample. The uncertainty in the balance certificate was stated as ±0,1 mg at a 95% confidence level. A standard deviation of 0.0510 was calculated dividing 0.1 by 1.96. The run-to-run variability, ±0.1 mg, was estimated by means of a Shewhart control graph [9,10]. Combining these two components resulted in:

$$s(m_i) = \sqrt{2(0.0510)^2 + (0.09902)^2} = 0.1225$$

Step 2: Dilution

The uncertainty of the internal volume of the 250 ml volumetric flask was indicated by the manufacturer as ±0.15 ml [11,12]. Since this figure was not given with a confidence level and assuming a triangular distribution [13], the appropriate standard deviation was calculated as $0.15:6^{1/2} = 0.0612$ ml.

The effect due to temperature difference, from the moment of the flask calibration until the analysis time, was calculated as ±3 °C. Since the volume expansion coefficient of the liquid ($2.1 \times 10^{-4} \text{ °C}^{-1}$ at 20°C) was considerably greater than that of the flask ($10 \times 10^{-6} \text{ °C}^{-1}$ for borosilicate glass flasks), only the former was considered. So, the temperature effect for the dilution step resulted in $\pm 250 \times 3 \times 2.1 \times 10^{-4} = \pm 0.1575$ ml. The standard deviation was calculated as $0.1575:3^{1/2} = 0.09094$, assuming an approximated rectangular distribution [13].

Combining the two contributions to the uncertainty of the 250 ml volume (V_{250}) the result was:

$$s(V_{250}) = \sqrt{0.0612^2 + (0.09094)^2} = 0.1096$$

Two ten-fold dilutions were necessary to adjust the expected level of cadmium in the solution to the working

range of the analytical curve. Contributions due to repeatability and variation within specification limits were determined and combined for each type of glassware available (10 ml pipettes and 100 ml volumetric flasks). Table 2 summarizes the calculation of the uncertainties from repeatability run-to-run operation and arising from variation within specification limits and temperature difference.

Table 2 - Uncertainties due to run-to-run operations, manufacturer's specifications and temperature effect

VOLUMETRIC MATERIAL v.mL	s*	tolerance/3 ^{1/2} + Temperature effect	Combined standard deviation	Standard uncertainty (1s - mL)	Relative Standard Uncertainty (1s/V)
pipeta 10 mL	0,012	0,00894	(0,012 ² + 0,0894) ^{1/2}	0,0150	0,00150
balão 100 mL	0,010	0,0547	(0,010 ² + 0,0547) ^{1/2}	0,0556	0,000556
balão 250 mL	0,020	0,1096	(0,020 ² + 0,1096) ^{1/2}	0,1114	0,000446

* run-to-run operations estimates by Shewhart Control Charts

There was an uncertainty associated with the initial and final volumes taken, so the dilution factor uncertainty was associated with them. Dilution factors were calculated as:

$$\frac{s_{\text{factor } 10}}{\text{factor } 10} = \sqrt{\left(\frac{s_{V_{10}}}{10}\right)^2 + \left(\frac{s_{V_{100}}}{100}\right)^2}$$

$$\frac{s_{\text{factor } 10}}{10} = \sqrt{(0,00150)^2 + (0,000556)^2}$$

$$s_{\text{factor } 10} = 0.0160$$

where $s_{\text{factor } 10}$ = the standard deviation of the dilution factor.

Step 3: Measuring Cadmium by Flame Atomic Absorption Spectrometry Using a Linear Calibration Function

The calibration experiment was started with the choice of a preliminary linear working range from 0.5 to 2 mg.L⁻¹ cadmium solutions. Five analytical solutions, with concentrations of 0.5mg.L⁻¹, 0.75mg.L⁻¹, 1mg.L⁻¹, 1.5mg.L⁻¹, and 2mg.L⁻¹ were prepared from a 1.000 ± 0.002g.L⁻¹ cadmium solution. The analytical curve was prepared and measured four times in order to estimate day-to-day variation. Four replicates of each of the lowest and the highest concentration of the working range were submitted to a linear regression analysis to obtain the coefficients "a" and "b".

Homogeneity of variances and linearity were verified by a statistical significance test. Tables 3 and 4 summarize analysis of variance data and tests for linearity and regression efficiency [14]; R² = 0.9992 and R²_{max} = 0.9994 were large enough to demonstrate efficiency. Linear regression parameters were estimated for sensitivity, the slope of the calibration function (a = 0.2358), and for the ordinate intercept (b = 0.01419).

Confidence limits for the intercept were calculated as within -0.0094 to +0.0185. Zero is within these calculated limits so we assumed a regression line passing through the origin. The combination of the uncertainties of the measured

values and the uncertainties of the regression coefficients resulted in the uncertainty of an analytical result.

Table 3 - Analysis of variance parameters
S_b² = 1.91511 × 10⁻⁶; S_a² = 7.09549 × 10⁻⁷

Sources of Variation	Sum of Square (SQ)	Degrees of Freedom	Mean Square (MQ)	
<i>totals</i>	1,95053	20	0,0975265	<i>MQT</i>
<i>correction</i>	0,08139	1	0,0813960	<i>FC</i>
<i>corrected</i>	0,32260	19	0,01697937	<i>MQC</i>
<i>regression</i>	0,32235	1	0,32235276	<i>MQR</i>
<i>residual</i>	0,00025	18	1,4191E-05	<i>MQE</i>
<i>pure error</i>	0,00018	15	1,26333E-05	<i>MQEP</i>
<i>linearity</i>	0,00006	3	2,19792E-05	<i>MQL</i>

Table 4 - Results of linearity and regression efficiency tests

LINEARITY	Is "a" different from zero?
Fcalculated = MQL / MQEP = 0,1795	Fcalculated = MQR / MQE = 22 715
F _{3; 15; 0,025} = 4,15	F _{1; 18; 0,025} = 5,98
Fcalculated < F critical	Fcalculated >> F critical
Linearity is accepted	"a" ? zero
EFFICIENCY	MAXIMUM EFFICIENCY
R ² = SQR / SQC = 0,9992	R ² _{max} = (SQC - SQEP) / SQC = 0,9994
Efficiency is confirmed.	

Uncertainty due to variability in "y" was estimated by calculating [2,4,14]:

$$s_{y_i} = \sqrt{\frac{MQE}{r} + S_b^2 + S_a^2(x_i - x_m)^2}$$

where

r = number of sample replications,

S_b² = MQE / n (contribution due to "b"),

n = number of standard solutions (working range),

S_a² = MQE / S_{xx} (contribution due to "a"),

S_{xx} = Σ(x_i - x_m)²

x_m = Σx_i / n.

The diluted solution (one replication, r = 1) resulted in 0.273 units of absorbance. The following expression provided the amount of cadmium present in the diluted solution using the calibration function y = 0.2358x + 0.01419:

$$[\text{Cd}^{2+}] = \frac{\text{Absorbance} - b}{a} \pm \frac{tS_{y_i}}{a}$$

$$[\text{Cd}^{2+}] = \frac{0.273 - 0.01419}{0.2358} \pm \frac{2.101 \times 0,003861}{0.2358}$$

$$[\text{Cd}^{2+}] = (1.098 \pm 0.034) \text{ mg L}^{-1}$$

Due to the calibration function, $x_{\text{observed}} = 1.10 \text{ mg L}^{-1}$ is associated with the uncertainty of $\pm 0.03 \text{ mg L}^{-1}$ or 3%.

Step 4: Calculation of Final Result

The final result expressed as mg of cadmium per mg of solid sample was calculated as 27.4.

Uncertainty of the final result (27.4mg) was estimated by the combination of the components described in Table 5.

Table 5 - Intermediate values and uncertainties for cadmium determination

Sources of uncertainties	Value (v)	Standard Uncertainty (1s)	Relative standard uncertainty (1s/v)
x_{obs} (mg/L)	1,098	0,01689 *	0,01538
V_f (mL)	250	0,1096	0,0004384
Dilution factor	$2 \times 10^{**}$	0,02234 **	0,002234
Initial mass (mg)	56,3	0,1225	0,002176

* calculated using $Sy/a \text{ } Sx \text{ } obs = 0,003982 / 0,2358 = 0,01689$

** corresponds to 2 ten-folds dilutions; $s = \sqrt{2 \times (0,0158)^2} = 0,02234$

Accordingly new recommended nomenclature [15], total uncertainties as combined uncertainty, u_c , and expanded uncertainty, U , were calculated, as shown in the following equations:

$$u_c = \pm \sqrt{(0,01538)^2 + (0,0004384)^2 + (0,002234)^2 + (0,002176)^2} = \pm 0,01570$$

$$u_c = \pm 0,01570 \times 27,4$$

$$u_c = \pm 0,43$$

and

$$U = \pm 2 \times 0,43 = \pm 0,86$$

The final result and uncertainty was (27.4 \pm 0.9) grams cadmium per gram of solid sample or expressed as 27.4mg with associated uncertainty of 3%.

CONCLUSION

It can be observed that the uncertainty due to x_{observed} is much higher than the other figures. The measured value 1.10 mg L^{-1} is associated with an uncertainty of $\pm 0.03 \text{ mg L}^{-1}$, due to function calibration. This figure represents an uncertainty of $\pm 3\%$ ($0.034 : 1.098 \times 100 = 3\%$). The final result for the cadmium determination resulted in 27.4mg with an expanded uncertainty of ± 0.9 . In percentage, this represents 3% ($0.86 : 27.4 \times 100$) as the uncertainty due to the calibration procedure. Hence, the uncertainty estimate of the various steps of an analysis demonstrates that the calibration step might give an important contribution to the uncertainty of the final result. In the present case, it was the main factor. Such result claims analysts to a designed experiment planning for the calibration function, in order to obtain lower limits for uncertainty when linear least square

fit is considered. Frequently, analysts don't pay much attention to the analytical curve. They just prepare the analytical solutions, use the linear least square fit procedure and calculate the correlation coefficient R , assuming that if this figure is close to -1 and $+1$, the calibration was properly performed. In reality, the regression validity should be demonstrated. First the linear regression parameters are estimated for sensitivity by means of the slope of the calibration function and for the ordinate intercept. By means of ANOVA data F-tests for linearity and regression efficiency are carried out. In the present example, calculated values for R^2 and R^2_{max} demonstrate required efficiency.

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