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An Expression of Uncertainty in Calibration Using Stepwise or Separate Dilution of a Stock Solution

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The traditional method for linear calibration can estimate the confidence intervals of calibration lines from a set of experimental data for a single calibration line. However, the following situations, often encountered in laboratories, are out of reach of the method, since the concentrations of the standard solutions are not independent of each other: (A) a standard solution is diluted from a more concentrated one in a stepwise way (stepwise dilution); (B) every standard solution for a calibration experiment is prepared from a stock solution, but the stock solution is newly prepared for each calibration (separate dilution with the variable concentration of the stock solution). This paper puts forward a theory to calculate the confidence intervals of calibration lines in the above situations. Analyses made up of sample weighing, dilution, HPLC measurement and calibration with the linear least-squares fitting are taken as examples. The proposed theory is numerically compared to the traditional method.

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Introduction

Linear regression analysis is a procedure for assessing the mathematical relationship between one variable and another. The most common technique of the regression is the linear least-squares fitting which minimizes the residual (sum of squares about regression).[1] In analytical sciences, it is often used as a calibration line, $Y = aX + b$.

A series of experiments is carried out for the calibration line. If a number of calibration lines are drawn from a number of series under exactly fixed conditions, they more or less differ from each other due to the ubiquitous random error. The true calibration line would be obtained, only if the Y values are observed without error. The uncertainty of the calibration line is referred to as the confidence interval (CI).[1,2]

The observed calibration line can be an unbiased estimator and the CIs can be calculated correctly, if the following conditions are satisfied:[3]

1. The value of X is treated as if it is free of error.
2. The population of Y for an X value is independent of the population of Y for another X value.
3. There is a linear relationship between X and the mean of the population of Y.
4. The standard deviation (SD) of the population of Y is constant (called homoscedastic).

The violation of conditions 1 and 4 has been discussed so far.[2-11]

The error exists in every analytical step including preparation and measurement. However, the only means for observing the error is measurement and in theory, not only the error of instrumental analysis, but also the preparation error is assumed to be accumulated in the measurement, Y. This treatment is adopted by the common statistical approach as well as the present paper.

This paper focuses on the following examples violating condition 2 (Fig. 1):

A. The most concentrated calibration standard is made from the stock solution, and the less concentrated standard is diluted from the more concentrated one in a stepwise way (stepwise dilution).
B. Calibration standards are made separately from the stock solution (separate dilution), and the stock solution is prepared newly for each series of calibration experiments.

In stepwise dilution, the violation of condition 2 is obvious. The second highest concentration, X_2, of the standard depends strongly on the concentration, X_1, of the starting standard. Similarly, X_3 depends on X_2, *etc*. Therefore, the correlation appears between the populations of Y for any combination of X_1 – X_4.

In separate dilution, the actual concentration, X_0, of the stock solutions varies at random every time the stock solution is prepared for each series. If X_0 is higher than the expected value, the concentrations, X_1 – X_4, of the standard solutions are all higher except the preparation error. Therefore, X_1 – X_4 are dependent on each other within a calibration line and so are the populations of Y.

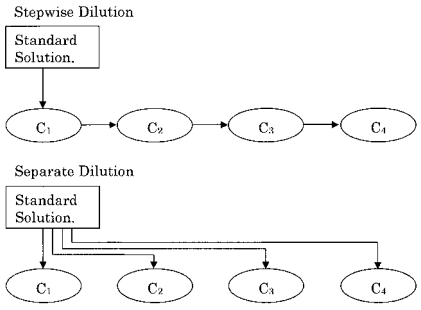

Fig. 1 Methods of dilution. The arrows denote the dilution from one to another. In the stepwise dilution, only the standard of X_1 is prepared directly from the stock solution. In separate dilution, every standard solution of concentrations X_1 – X_4 is prepared from the stock solution.

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Another interpretation of the separate dilution will be helpful for the understanding of this paper. In the traditional method, the 95% CIs (= $1.96\tilde{Y}$) are formulated as:

$$\bar{a}X + \bar{b} \pm 1.96\tilde{Y} \quad (1)$$

where $\tilde{Y}$ denotes the SD of all possible calibration lines at X and $\bar{a}X + \bar{b}$ is the true calibration line with mean slope, $\bar{a}$, and mean intercept, $\bar{b}$. The 95% CIs mean that 95% of all the possible calibration lines are included between the intervals, but the remaining 5% are not. The CIs theoretically cover all possibilities, although the experimental data of a single calibration line are the minimum requirements for the estimation of the CIs.[2]

If the stock solution is repeatedly used for the different series of experiments in the separate dilution, the data, Y, of a series scatter around the "true" calibration line and the population of Y can be completely related to all of the possibilities. That is, all of the influential errors occurring in the preparation and measurement of the standard solutions of X_1 – X_4 are reflected on the Y scattering. The complete relationship is essential, since the CIs are estimated from the single event (the observation of the single calibration line).

In the separate dilution with the variable X_0, the data of a series scatter around the "accidentally true" calibration line, but the scattering no longer represents all the possibilities. This is because the variation in X_0 is not reflected on the residual of the single calibration line. In this case, Eq. (1) is not applicable to example B (or example A) without a modification.

There have been published many studies on the confidence intervals of the linear calibration.[1-11] According to the authors' knowledge, however, this paper first proposes a theory to estimate the uncertainty of the total analysis with variable stock solutions and stepwise dilution.

Theory for $\tilde{Y}$

The SD, $\tilde{Y}$, is generally written as a function of concentration X:[4,5]

$$\tilde{Y}^2 = A_X^2(X - B_X)^2 + C_X^2 \quad (2)$$

In general, $\tilde{Y}$ is a U-shaped line.[1,2,4] The coefficients are:[4]

$$A_X = \tilde{a} \quad (3)$$

$$B_X = -\frac{\mathrm{Cov}(a,b)}{\tilde{a}^2} \quad (4)$$

$$C_X = \tilde{b}\left[1 - \frac{\mathrm{Cov}(a,b)^2}{\tilde{a}^2\tilde{b}^2}\right]^{1/2} \quad (5)$$

where $\tilde{a}$ is the SD of the slopes, a, and $\tilde{b}$ the SD of the intercepts, b, and Cov(a,b) the covariance of a and b. The variances and covariance, $\tilde{a}^2$, $\tilde{b}^2$ and Cov(a,b), for the unweighted linear least squares are listed in Table 1.[4]

If the concentration of the stock solutions varies, the "true" calibration line, $Y = \bar{a}X + \bar{b}$, for each stock solution is variable accordingly. Let ρ_S be the relative SD (RSD) of concentrations of the stock solutions. The SD of the "true" calibration lines on the Y-scale depends on concentration, X, as:

$$\rho_S(Y - \bar{b}) = \rho_S\bar{a}X \quad (6)$$

If the slope, $\bar{a}$, and calibration uncertainty, Y (= Eq. (2)), are probabilistically independent of each other, the calibration uncertainty in question can take the following form:

$$\tilde{Y}^2 = A_X^2(X - B_X)^2 + C_X^2 + (\rho_S\bar{a}X)^2 \quad (7)$$

where the additivity of variances, well-known concept of probability theory, is used. This equation concerns the separate dilution with the variable concentration, X_0, of the stock solution.

In the stepwise dilution, even if the concentration, X_0, of the stock solution is constant, it is impossible to use the traditional equation of calibration uncertainty (Eq. (2)) as mentioned above. If X_1 is accidentally higher than the desired one, the other concentrations, X_2 – X_4, will be higher with an appreciable probability. This situation resembles the separate dilution with the variable X_0. Therefore, the generalized interpretation of Eq. (7) can afford the estimation of the CIs in the stepwise dilution.

The concentration, X_1, of the starting solution affects the slope of the calibration lines even more strongly than the other concentrations, X_2 – X_4. Therefore, we can understand that ρ_S (Eq. (6)) denotes the variation in X_1 and the traditional part of Eq. (7) (= Eq. (2)) can be used as if X_1 is constant. The dependence of X_2 on X_1, X_3 on X_2, and X_4 on X_3, is neglected, since the most significant influence on the slope has already been contained in ρ_S. The scattering of the calibration data around the "true" calibration line is considered to be caused by the error from the instrumental analysis of the standard, X_1, and errors from the dilution and instrumental analyses of the standards, X_2 – X_4.

Table 1 Equations for linear least squares fitting with no weight

$$\tilde{a}^2 = \frac{1}{S_{xx}^2}\left\{\sum_{i=1}^{N}(X_i - \bar{X})^2\tilde{e}_i^2\right\} \quad (T1)$$

$$\tilde{b}^2 = \sum_{i=1}^{N}\left[\left\{\frac{1}{N} - \frac{\bar{X}}{S_{xx}}(X_i - \bar{X})\right\}^2\tilde{e}_i^2\right] \quad (T2)$$

$$\mathrm{Cov}(a,b) = \sum_{i=1}^{N}\left[\left\{\frac{(X_i - \bar{X})}{NS_{xx}} - \frac{\bar{X}}{S_{xx}^2}(X_i - \bar{X})^2\right\}\tilde{e}_i^2\right] \quad (T3)$$

$$\bar{X} = \frac{1}{N}\sum_{i=1}^{N}X_i \quad (T4)$$

$$S_{xx} = \sum_{i=1}^{N}(X_i - \bar{X})^2 \quad (T5)$$

$$a = \frac{\sum_{i=1}^{N}(X_i - \bar{X})Y_i}{S_{xx}} \quad (T6)$$

$$b = \left(\frac{1}{N}\sum_{i=1}^{N}Y_i\right) - a\bar{X} \quad (T7)$$

$\tilde{a}$ denotes the SD, $\{E[(a - E[a])^2]\}^{1/2}$, of the slopes, a; $\tilde{b}$ the SD, $\{E[(b - E[b])^2]\}^{1/2}$, of the intercepts, b; Cov(a,b) the covariance, $E[(a - E[a])(b - E[b])]$, of a and b; $\tilde{e}_i$ the measurement SD at concentration X_i; N the number of calibration standards (chromatograms).

Uncertainty for Preparation and Measurement

A total analysis made up of preparation, instrumental analysis and calibration is taken as an example. Here, the preparation comprises weighing and dilution. The high performance liquid chromatography (HPLC) is taken as an instrumental analysis.

This section introduces the methods for calculating the right side of Eq. (7). Noticing Eqs. (2) – (5) and T1 – T5 of Table 1, we can recognize that the independent variable of Y is the concentration, X:

$$\tilde{Y} = f(X,X_i,Y_i,\tilde{e}_i,\rho_S) \quad (8)$$

where Y_i denotes the instrumental response of the calibration standard of concentration, X_i, and $\tilde{e}_i$ is the SD of Y_i at concentration X_i.

The parameter, X_i, is known and Y_i is an observed value. The requirement for the estimation of Y is the mathematical description of $\tilde{e}_i$ and ρ_S. In HPLC analysis, $\tilde{e}_i$ can be written as the sum of squared SDs of responses which originate from the preparation, sample injection into the HPLC apparatus and baseline noise in the HPLC output:[12]

$$\tilde{e}_i^2 = (SD_{prep})^2 + (SD_{inj})^2 + (SD_{noise})^2 \quad (9)$$

The methods for calculating the right side as well as the uncertainty of the stock solution, ρ_S, are presented below.

Uncertainty of stock solution

The stock solution is assumed to be prepared by weighing an aliquot, m, of a powder sample and dissolving it in a volumetric flask of volume, V. The concentration, X_S, of the stock solution is:

$$X_S = \frac{m}{V} \quad (10)$$

Taking into account the propagation rule of indeterminate errors,[13,14] we can obtain the RSD, ρ_S, of concentration, X_S, of the stock solution:

$$\rho_S = r_m^2 + r_V^2 \quad (11)$$

where r_Z denotes the RSD of Z.

Uncertainty of dilution

It is assumed that an original solution of concentration, X_O, is diluted to the final solution of concentration, X_F. In the separate dilution, the original solution is pipetted in a volumetric flask. The final concentration, X_F, after the dilution takes the form:

$$X_F = \frac{V_1}{V_2} X_O \quad (12)$$

where V_1 denotes the volume of the pipette and V_2 the volume of the volumetric flask. The squared RSD, ρ_F^2, of X_F can be written as (see Appendix):

$$\rho_F^2 = \rho_O^2 + r_1^2 + r_2^2 \quad (13)$$

where ρ_O is the RSD of X_O and r_i is the RSD of V_i.

In the stepwise dilution, the pipetted original solution of volume V_1 is mixed with the pipetted solvent of volume V_2. The final concentration, X_F, takes the form:

$$X_F = \frac{V_1}{V_1 + V_2} X_O \quad (14)$$

The squared RSD, ρ_F^2, of X_F can be expressed as (see Appendix):

$$\rho_F^2 = \rho_O^2 + \left(\frac{V_2}{V_1 + V_2}\right)^2 (r_1^2 + r_2^2) \quad (15)$$

where r_2 denotes the RSD of the volumes of the pipetted solvent.

The difference of the dilution uncertainty (Eqs. (13) and (15)) comes from the correlation between the original and final volumes (the numerator and denominator of Eqs. (12) and (14)). There is no correlation in the pipette-flask dilution (V_1/V_2), but the pipette-pipette dilution has the positive correlation ($V_1/(V_1 + V_2.)$).

Table 2 Experimental plan of separate dilution

Sample to be weighed	20 mg			
Stock solution (100 mL)	X_0 = 200 (mg/L)			
Calibration standard solutions to be prepared from stock solution				
	X_1	X_2	X_3	X_4
Concentration/mg L^{-1}	40	20	10	4
Sample pipette/mL	20	10	5	2
Volumetric flask/mL	100	100	100	100
Peak area in HPLC	20000	10000	5000	2000

Uncertainty of measurement

Many spectrochemists have directed their efforts at a theory to predict the measurement uncertainty from the noise of analytical instruments.[12,15-20] A typical noise is known as 1/f noise, which is also called flicker noise or pink noise. This paper adopts the FUMI theory (function of mutual information)[12] which approximates the 1/f noise as the mixture of well-known random processes, white noise and Markov process. The purpose of the FUMI theory is to estimate the SD and RSD of measurements (peak area or height) from the noise and signal in the instrumental output.

Results

Tables 2 and 3 list the details of the experimental procedures for the separate dilution and stepwise dilution, respectively. It is assumed that the stock solution is newly prepared each time the calibration line is drawn. Table 4 lists the values of the operational uncertainty which mimic the realities of experiments.

Separate dilution

In this case, the variation in the slope of the true calibration line corresponds to the variation in the concentration of the stock solutions. A 200-mg/L stock solution is prepared by weighing a powder sample (20 mg) and dissolving it in a 100-mL volumetric flask as shown in Table 2. Noticing Eq. (11) and Table 4, we can write the concentration variation as RSD, ρ_S:

$$\rho_S^2 = \left(\frac{0.029}{20}\right)^2 + (0.00069)^2 = 0.0000026 \quad (16)$$

where the weighing SD is 0.029 mg and the RSD of the volumetric flask is 0.00069.

The calibration standard of the highest concentration X_1 (= 40 mg/L) is prepared with a 20-mL pipette and a 100-mL volumetric flask. The measurement uncertainty, $\tilde{e}_1$, of the standard of X_1 can be described under the assumption that the concentration of the stock solution is invariant (Eqs. (9) and (13) and Table 4):

$$\tilde{e}_1^2 = (200000)^2\left[\left(\frac{0.012}{020}\right)^2 + (0.00069)^2\right] + (200000 \times 0.003)^2 + (210)^2 = 219$$

where the first term in the right side denotes the dilution error (volume SD of the 20-mL pipette = 0.12 mL; RSD of the volumetric flask is 0.00069), the second the injection error (RSD = 0.003) and the third the error from the HPLC noise (SD_{noise} = 210; see Eq. (9)). The dimension of $\tilde{e}_i$ is the peak area and then the first and second terms are transformed from RSD to SD by multiplying the peak area (= 20000).

Table 3 Experimental plan of stepwise dilution

Sample to be weighed	10 mg			
Stock solution (100 mL)	X_0 = 100 (mg/L)			
Calibration standard solutions to be prepared from stock solution				
	X_1	X_2	X_3	X_4
Concentration/mg L^{-1}	50	25	12.5	6.25
Sample pipette/mL	1	1	1	1
Dilution pipette/mL	1	1	1	1
Peak area in HPLC	25000	12500	6250	3125

In the similar way, the uncertainty of the more diluted standards is written as:

$$\tilde{e}_2^2 = (10000)^2\left[\left(\frac{0.012}{10}\right)^2 + (0.00069)^2\right] + (10000 \times 0.003)^2 + (210)^2 = 213$$

$$\tilde{e}_3^2 = (5000)^2\left[\left(\frac{0.0058}{5}\right)^2 + (0.00069)^2\right] + (5000 \times 0.003)^2 + (210)^2 = 211$$

$$\tilde{e}_4^2 = (2000)^2\left[\left(\frac{0.0058}{020}\right)^2 + (0.00069)^2\right] + (2000 \times 0.003)^2 + (210)^2 = 210$$

where $\tilde{e}_2$ denotes the SD of area measurements for the 20-mg/L solution prepared with the 10-mL pipette and 100-mL volumetric flask, $\tilde{e}_3$ that of the 10-mg/L solution with the 5-mL pipette and 100-mL volumetric flask and $\tilde{e}_4$ that of the 4-mg/L solution with the 2-mL pipette and 100-mL volumetric flask (Tables 2 and 4). The values of SD_{noise} (= 210) in the above equations are equal, since the widths of the chromatographic peaks (= the number of noises) are constant.

The objective equation is Eq. (7) for which the above values of ρ_S and $\tilde{e}_i$ are substituted. The order of calculations is:

$$T4 \rightarrow T5 \rightarrow T1 \rightarrow T2 \rightarrow T3 \rightarrow 3 \rightarrow 4 \rightarrow 5 \rightarrow 2 \rightarrow T6 \rightarrow 7$$

where the equation numbers are indicated. The final equation (Eq. (7)) takes the form:

$$\tilde{Y}^2 = 7.9^2 \times (X - 18.1)^2 + 107^2 + (0.0016 \times 500 \times X)^2 \qquad (17)$$

where A_X = 7.9, B_X = 18.1, C_X = 107, ρ_S = 0.0016 and a = 500.

Stepwise dilution

In the example of stepwise dilution (Table 3), a stock solution of 100 mg/L is prepared by weighing 10-mg sample and dissolving it in a 100-mL volumetric flask. The RSD of concentration X_0 takes the form

$$\rho_S^2 = \left(\frac{0.029}{10}\right)^2 + (0.00069)^2 = 0.0000089$$

where the weighing SD is 0.029 mg and the RSD of the volumetric flask is 0.00069 (Table 4). As considered in the theoretical section, the uncertainty of the stock solution, ρ_S, involved in Eq. (7) corresponds to the RSD of starting concentration, X_1. Therefore, the uncertainty of the starting solution should be added to the above equation. The preparation process is made up of the pipetting (1 mL) of the stock solution and addition of the pipetted solvent (1 mL). Therefore, the RSD of X_1 (Eq. (6)) can be written as:

$$\rho_S^2 = \left(\frac{0.029}{10}\right)^2 + (0.00069)^2 + \left(\frac{1}{1+1}\right)^2\left[\left(\frac{0.0058}{1}\right)^2 + \left(\frac{0.0058}{1}\right)^2\right] = 0.000028 \qquad (18)$$

where the RSD for the pipette-pipette dilution (Eq. (15)) is used.

The piptte-based variation in X_1 is included in ρ_S, but for the sake of calculation, the error of the instrumental analysis of the solution, X_1, remains in the SD, $\tilde{e}_1$:

$$\tilde{e}_1^2 = (25000 \times 0.003)^2 + (210)^2 = 223$$

where the first term denotes the uncertainty from the sample injection and the second term the error from the instrumental noise.

The standard solution of the second highest concentration, X_2, is prepared directly from the solution of X_1 with 1-mL pipettes and the response SD, $\tilde{e}_2$, can be written as:

$$\tilde{e}_2^2 = (12500)^2 \times \left(\frac{1}{1+1}\right)^2\left[\left(\frac{0.0058}{1}\right)^2 + \left(\frac{0.0058}{1}\right)^2\right] + (12500 \times 0.003)^2 + (210)^2 = 219$$

where the first term denotes the uncertainty from the dilution, the second term that from the sample injection and the third term that from the baseline noise. For the first term, Eq. (15) is used in which ρ_O = 0 under the assumption that the actual variability in concentration X_1 is neglected in $\tilde{e}_1$, $\tilde{e}_2$, $\tilde{e}_3$, and $\tilde{e}_4$. That is, the traditional equation of CIs (Eq. (2)) can be used as if X_1 is constant.

The solution of X_3 is prepared from the solution of X_2 and the uncertainty of the solution of X_2 is accumulated in the uncertainty of the solution of X_3 as

$$\tilde{e}_3^2 = (6250)^2 \times 2 \times \left(\frac{1}{1+1}\right)^2\left[\left(\frac{0.0058}{1}\right)^2 + \left(\frac{0.0058}{1}\right)^2\right] + (6250 \times 0.003)^2 + (210)^2 = 214$$

For the first term of this equation, Eq. (15) is used. Here, ρ_O^2 and the second term of Eq. (15) are both

Table 4 Uncertainty for operations

	Interval	SD	RSD, %
Electronic balance	±0.05 mg	0.029 mg	
Volumetric flask (100 mL)	±0.12 mL	0.069 mL	0.069
Pipette (1, 2, 5 mL)	±0.01 mL	0.058 mL	
Pipette (10, 20 mL)	±0.02 mL	0.012 mL	
HPLC injection			0.3
HPLC peak area		210	

If the distribution of error is uniform, ranging from $-x$ to $+x$, its intervals, $\pm x$, divided by $\sqrt{3}$ correspond to the SD. Note that the SD of the uniform distribution from –1 to 1 (±1) is $1/\sqrt{3}$, since $\sqrt{\int_0^1\left(X - \frac{1}{2}\right)^2 dx} = \frac{1}{2\sqrt{3}}$.

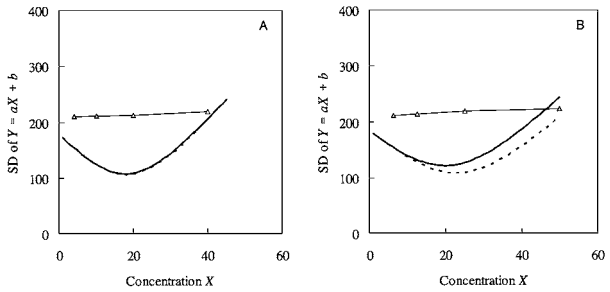

Fig. 2 SD, $\tilde{Y}$, of calibration lines for separate dilution (A) and stepwise dilution (B). ——, Eq. (7); ·····, Eq. (2); △, $\tilde{e}_i$. For actual calculation, Eqs. (17) and (19) (and also with Eq. (16) = Eq. (18) = 0) are used.

$$\left(\frac{1}{1+1}\right)^2\left[\left(\frac{0.0058}{1}\right)^2+\left(\frac{0.0058}{1}\right)^2\right].$$

For the solution of X_4, the accumulation of the uncertainty is enhanced furthermore in the stepwise way:

$$\tilde{e}_4^2 = (3125)^2 \times 3 \times \left(\frac{1}{1+1}\right)^2\left[\left(\frac{0.0058}{1}\right)^2+\left(\frac{0.0058}{1}\right)^2\right]+ (3125 \times 0.003)^2 + (210)^2 = 211$$

The final equation (Eq. (7)) can be obtained as:

$$\tilde{Y}^2 = 6.5^2 \times (X - 22.8)^2 + 108^2 + (0.0053 \times 500 \times X)^2 \qquad (19)$$

where A_X, B_X, C_X, ρ_S and a are calculated as mentioned in the previous example.

Discussion

Figure 2 shows the general property of the SD, $\tilde{Y}$, of calibration lines. The SD, $\tilde{Y}$, is large at the edges of the calibration line and takes the minimum at B_X (see Eq. (7)).

In Fig. 2A, the SD, $\tilde{Y}$, for the variable stock solutions (——) and that for the fixed stock solution (·····) overlap and we can see that the variation in the concentration of the stock solutions is not serious in the separate dilution. This is because the preparation error of the stock solution is so small that the resulting variation in the slope of the calibration lines is negligible. In fact, ρ_S = 0.16%, but another error in the experiments is even more crucial, *e.g.*, the RSD of 2-mL pipetting volume error is 0.29%; (see Table 4). The preciseness of the electric balance (RSD = 0.145%) and 100-mL volumetric flask (RSD = 0.069%) degrades the significance of the preparation error (see Eqs. (16) and (17)).

In the stepwise dilution, the difference between the traditional and new equations can be found (see Fig. 2B). The RSD of the stock solutions is 0.3% $(=\sqrt{(0.029/10)^2+(0.00069)^2})$ and RSD of the dilution to the starting solution is 0.41% $=\sqrt{(1/1+1)^2\,[(0.0058/1)^2+(0.0058/1)^2]}$. Consequently, ρ_S = 0.53% (Eqs. (18) and (19)), which means the uncertainty of the starting solution of concentration, X_1. The volume error of the small pipettes (1 mL) is usually large (= 0.58% here) and if a more precise method for dilution replaces it, the above effect on the calibration uncertainty, $\tilde{Y}$, can be reduced.

Figure 2 indicates that if the preparation error of the stock solution or starting solution of concentration, X_1, is unimportant, the traditional equation for the confidence intervals of calibration (Eq. (2)) is useful. However, if not, the strict equation (Eq. (7)) is necessary for the accurate estimation of the calibration uncertainty, $\tilde{Y}$, for the separate dilution with variable stock solutions and for the stepwise dilution.

In the well-known statistical approach, the SD, $\tilde{e}_1$, is calculated from the residual of the calibration line which is the least-squares fit to the calibration standard measurements.[1,2] On the other hand, our approach estimates the SD, $\tilde{e}_1$, from the causality of the uncertainty as mentioned above. A comparison of these approaches was made.[6]

In practice, the least-squares fitted calibration line is used in Eq. (1) instead of the true calibration line (in the above examples, Eqs. (17) and (19)). The interpretation of Eq. (1) is that the true calibration line exists inside the CIs with the specified probability. The different understanding of Eq. (1) is mentioned in the introductory section for convenience' sake.

Strictly, the independency assumption of $\bar{a}$ and Y (= Eq. (7)) is not correct. In realistic situations called heteroscedastic, the measurement SD, $\tilde{e}_i$, varies depending on the intensity of the measurement, Y_i. Therefore, the change in the slope, $\bar{a}$, affects $\tilde{e}_i$ and in turn, can exert an influence on Y. However, as long as the heteroscedasticity is not prominent like the system examined here (△ of Fig. 2), the uncertainty equation (Eq. (7)), though being approximate, will apply to a variety of situations in analytical chemistry.

Acknowledgements

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Appendix

Uncertainty of pipette-pipette dilution

The total differential of Eq. (14) is

$$dX_F = \frac{V_1}{V_1+V_2}dX_O + \frac{V_2}{(V_1+V_2)^2}X_O dV_1 - \frac{V_1}{(V_1+V_2)^2}X_O dV_2 \quad (A1)$$

Considering the independent randomness of differentials, dV_1 and dV_2, we can obtain

$$(dX_F)^2 = \frac{V_1^2}{(V_1+V_2)^2}(dX_O)^2 + \frac{V_2^2}{(V_1+V_2)^4}X_O^2(dV_1)^2 + \frac{V_1^2}{(V_1+V_2)^4}X_O^2(dV_2)^2 \quad (A2)$$

Dividing Eq. (A2) by Eq. (14), we can obtain Eq. (15) as:

$$\left(\frac{dX_F}{X_F}\right)^2 = \left(\frac{dX_O}{X_O}\right)^2 + \left(\frac{V_2}{V_1+V_2}\right)^2\left[\left(\frac{dV_1}{V_1}\right)^2+\left(\frac{dV_2}{V_2}\right)^2\right] \quad (A3)$$

where dZ denotes the SD of Z and dZ/Z the RSD of Z.

Uncertainty of pipette-flask dilution

The similar handling of Eq. (12) leads to Eq. (13):

$$\left(\frac{dX_F}{X_F}\right)^2 = \left(\frac{dX_O}{X_O}\right)^2 + \left(\frac{dV_1}{V_1}\right)^2 + \left(\frac{dV_2}{V_2}\right)^2 \quad (A4)$$

The uncertainty equations, (A3) and (A4), with constant X_O have

already been derived.[21]

References

1. N. Draper and H. Smith, "*Applied Regression Analysis*", 2nd ed., **1981**, John Wiley & Sons, Inc.
2. J. C. Miller and J. N. Miller, "*Statistics for Analytical Chemistry*", 2nd ed., **1988**, Ellis Horwood, West Sussex.
3. D. A. Schoeller, *Biomed. Mass Spectrom*., **1976**, *3*, 265.
4. Y. Hayashi, R. Matsuda, and R. B. Poe, *Analyst*, **1995**, *121*, 591.
5. L. A. Currie, *ACS Symp. Ser*., **1985**, *284*, 49.
6. R. Matsuda, Y. Hayashi, C. Yomota, Y. Tagashira, M. Katsumine, and K. Iwaki, *Analyst*, **2001**, *126*, 2061.
7. A. Hubaux and G. Vos, *Anal. Chem*., **1970**, *42*, 849.
8. J. F. K. Huber, J. A. R. J. Hulsman, and C. A. M. Meijers, *J. Chromatogr*., **1971**, *62*, 79.
9. D. A. Kurtz, *Anal. Chim. Acta*, **1983**, *150*, 105.
10. D. G. Mitchell, *ACS Symp. Ser*., **1985**, *284*, 115.
11. R. L. Watters, Jr., R. J. Carroll, and C. H. Spiegelman, *Anal. Chem*., **1987**, *59*, 1639.
12. Y. Hayashi and R. Matsuda, *Anal. Chem*., **1994**, *66*, 2874.
13. H. G. Hecht, "*Mathematics in Chemistry*", **1990**, Prentice Hall, New Jersey.
14. R. A. Day and A. L. Underwood, "*Quantitative Analysis*", 6th ed., **1991**, Pentice Hall, Engelwood Cliffs, New Jersey.
15. C. Th. J. Alkemade, W. Snelleman, G. D. Boutilier, B. D. Pollard, J. D. Winefordner, T. L. Chester, and N. Omenetto, *Spectrochim. Acta*, **1978**, *33B*, 383.
16. C. Th. J. Alkemade, W. Snelleman, G. D. Boutilier, and J. D. Winefordner, *Spectrochim. Acta*, **1980**, *35B*, 261.
17. P. W. J. M. Boumans, *Anal. Chem*., **1994**, *66*, 459A.
18. J. D. Ingle and Jr., S. R. Crouch, "*Spectrochemical Analysis*", **1988**, Prentice-Hall, Inc., New Jersey.
19. R. Matsuda, Y. Hayashi, S. Sasaki, K. Saito, K. Iwaki, H. Harakawa, M. Satoh, Y. Ishizuki, and T. Kato, *Anal. Chem*., **1998**, *70*, 319.
20. E. D. Prudnikov, J. W. Elgersma, and H. C. Smit, *J. Anal. At. Spectrom*., **1994**, *9*, 619.
21. Y. Haishima, C. Hasegawa, T. Yagami, O. Tuchiya, R. Matsuda, and Y. Hayashi, *J. Pharm. Biomed. Anal*., **2003**, *32*, 495.