



Minimizing the Effect of Sample Preparation on Measurement Uncertainty

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The measurement uncertainty of an analytical procedure depends upon many parameters, including analyte concentration, matrix properties, sample preparation technique, and measurement principle. Analysts can decrease the uncertainty that comes from sample handling by understanding the principles of uncertainty propagation. Guest author Veronika Meyer covers techniques to decrease uncertainty such as minimizing working steps; choosing dilution steps carefully; using identical instruments for samples and reference materials, parallel working principles, and internal standards and artificial matrices; and increasing the number of determinations.

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Sample Preparation Perspectives
Editor

An increasing number of analytical laboratory customers and government regulation bodies require that an analytical result be accompanied by its measurement uncertainty. The knowledge of uncertainty allows a proper evaluation of the result with regard to specifications, batch-to-batch variations, tolerances, and regulatory limits. Eurachem, a network of European organizations with the objective of establishing a system for the international traceability of chemical measurements and the promotion of good quality practices, and the Cooperative on International Traceability in Analytical Chemistry (CITAC), have published a detailed guide that explains how to determine the measurement uncertainty of chemical analyses (1). A downloadable version of this guide is available free of charge at <http://www.measurementuncertainty.org/mu/guide>.

At first sight, uncertainty might be the number obtained by repeating an analysis several times and calculating the standard deviation of the results. This approach could be correct, but it depends upon whether this number is based upon simple repeatability or reproducibility, which covers a much broader number of influencing parameters. In addition, any result can be prone to bias, but determining this contribution is difficult.

Analysts should study the aforementioned guide and learn its principles, which requires the identification of all uncertainty components that contribute to the combined standard uncertainty of the analytical procedure. Given that approximately two-thirds of a typical analytical chromatographic procedure is spent on sample preparation, it can contribute markedly to the overall uncertainty (2). In this "Sample Prep Perspectives" column, I will focus on parameters that contribute to measurement uncertainty that arises from the sample preparation component. I will discuss repeatabilities, although bias problems also can occur in the sample preparation step of the analysis.

Independent of the analytical procedure, the reproducibility of an analysis increases disproportionately with decreasing analyte concentration. Horwitz (3) demonstrated this relationship more than 20 years ago, and nobody could refute his famous empirical curve, which is reproduced in Figure 1. Users can get an estimate of measurement uncertainty from this curve alone. But the analytical procedure used undoubtedly influences the measurement uncertainty; for example, a titration can be performed with higher repeatability than a chromatographic analysis. Additional deviations from the Horwitz curve are caused by the matrix and the sample preparation technique itself. Taken together, the repeatability of an analytical result is a combination of all these parameters, and it is difficult to differentiate them. However, these difficulties should not be an excuse for sloppy sample preparation. All working steps in an analytical method, including the sampling step, should be optimized for cost and measurement uncertainty.

An Example of Measurement Uncertainty Caused by Sample Preparation

It is not uncommon that the uncertainty of a given analytical method is dominated by the repeatability of sample preparation. I chose a high performance liquid chromatography–mass spectrometry (HPLC–MS) procedure at random from the literature to describe the determination of olanzapine in serum (4). In this analysis, samples were mixed with an internal standard, cleaned up by solid-phase extraction (SPE) with recovery at 50 $\mu\text{g/L}$ = $87 \pm 4.0\%$ for three assays, and separated by reversed-phase HPLC on a C18 column. The calibration function was established with five data points and a correlation coefficient (r) of 0.9998. Determined by 10 injections from the same vial, the repeatability of the HPLC analysis was 1.2%. With these data it was possible to estimate the measurement uncertainty under repeatability conditions,

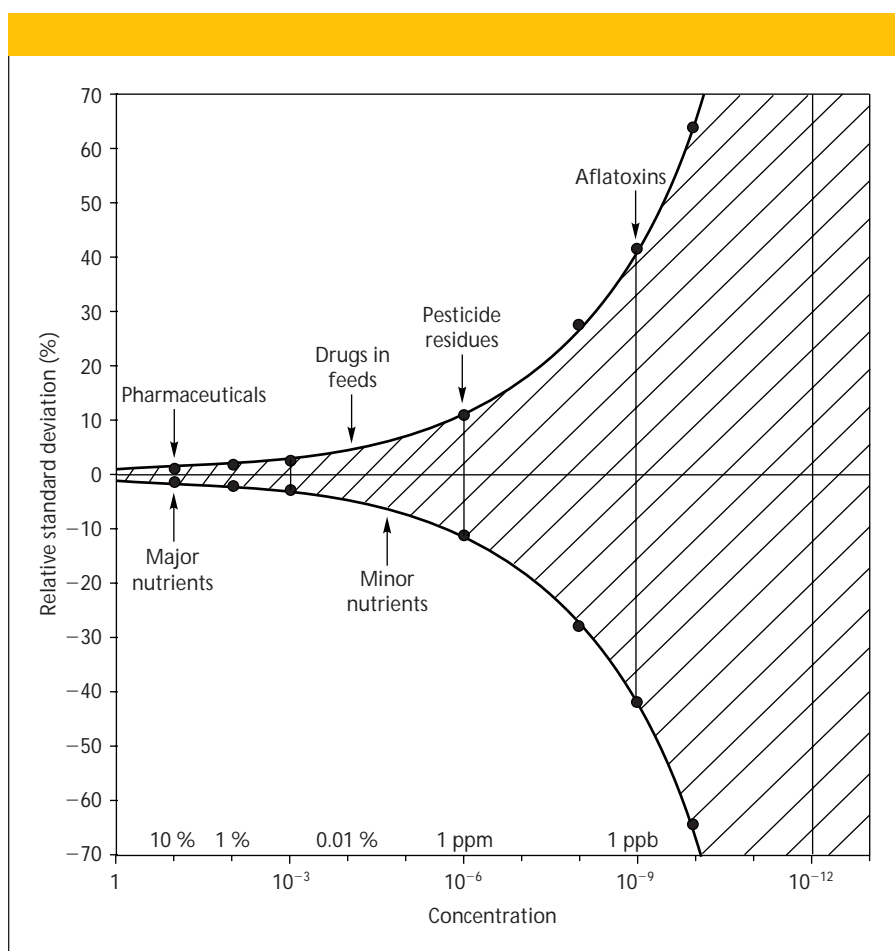


Figure 1: The Horwitz curve shows that the interlaboratory standard deviation of analytical results increases with decreasing analyte concentration. The curve was designed from the results of more than 50 interlaboratory studies. (Adapted from reference 3).

in accordance with the guide's principles (1), as 5.3%. In fact, the long-term variation of the method can be expected to be approximately 23% (expressed as a $\pm 2\sigma$ or 95% probability) because the dominating uncertainty of the extraction was calculated from only three determinations during validation. This small number of measure-

ments makes it necessary to expand the standard uncertainty with a coverage factor of 4.3; that is, the Student's t factor for two degrees of freedom (see Table I and Useful Hint 7 below).

In this example the main part of the total uncertainty — 75% — comes from the solid-phase extraction (SPE) step. The uncertainty of the analysis data calculated with the aid of the calibration function adds another 24%, and the remaining 1% comes from the uncertainties of the serum volume and the amount of added internal standard. These numbers are more or less typical for these types of analyses. (It is not the purpose of this column to show how this uncertainty calculation is performed, but readers can see reference 1 for more details.)

This result is rather disappointing because it could be difficult to improve the repeatability of SPE. Some of its uncertainty components are the type and batch of the SPE column, the preparation of this column, the washing and extraction steps, and the subsequent drying and redissolution of the eluate. However, it is possible to

Table I: Coverage factors for a limited number of measurements*

Number of Measurements	Coverage Factor (95%)
2	12.7
3	4.3
4	3.2
5	2.8
6	2.6
8	2.4
10	2.3
50	2.01
∞	1.96

* A standard deviation calculated from n determinations must be multiplied by $t(n)$ to expand it to a confidence limit of 95%.

quantify all these steps and to calculate their influence in the form of *sensitivity coefficients* (∂ repeatability/ ∂ influence); see the papers by Barwick and Ellison (5–7). Unfortunately, the necessary amount of work to quantify these uncertainty components is too involved for routine laboratory analyses, even when analysts use statistical experimental design.

So what everyday work procedures can be useful to decrease measurement uncertainty? Below, I will provide some hints that might be useful, although the effect of a particular contribution could be minimal with certain analytical procedures. It is important to understand the general thought process that might lead to an adequate working technique.

Seven Useful Hints

1 Use adequate working techniques: Several beneficial working techniques can be looked at in their inverse forms; that is, procedures that should be avoided.

For the transfer of volumes less than 200 μL , it can be better to use a syringe instead of a pipette. An experimental verification can provide the answer, so weigh the required volume of the solvent in use with an analytical balance and make at least 10 repetitions (20 experiments would be even more reliable).

Weighing operations might be more repeatable than volumetric ones for liquid samples, but they are more labor intensive. Analysts also must distinguish between volatile and nonvolatile analytes. If a dissolved analyte is nonvolatile, solvent evaporation is not a problem during many steps of sample preparation procedure; for example, after transfer to a volumetric flask that is not yet filled to the mark.

With samples that contain volatile analytes, the ratio of liquid to gas phase in a vial or a sampling bottle can be extremely important. Keep the gas volume small if an aliquot is taken from the liquid phase. Headspace sampling is a technique of its own, and analysts must know its pitfalls (8). Workers must consider the solvent's properties, including boiling point and viscosity.

Using instruments such as autosamplers and diluters or robots instead of handling samples manually can dramatically improve the standard deviation of volumetric operations.

Every additional step in sample handling is an additional source of uncertainty. Therefore, sample preparation protocols must involve as few steps as necessary. It is

unwise to blindly adopt a sample preparation protocol that was designed for another analytical problem without any thought or experimental verification. In trace analysis, it is even more important to avoid contamination from any source, but this concern depends upon the analyte and the specificity of the analysis.

2 Use large volumes: Small volumes are more difficult to handle and prone to loss of material, but they yield high analyte concentrations in dilution steps. On the other hand, large volumes allow more precise handling. Therefore, analysts should check carefully which procedures are adequate for a given problem. The uncertainty of a volumetric operation depends upon the calibration of the instrument, the repeatability that is attainable when using the instrument properly, and the temperature (9).

For example, compare the repeatabilities found with a piston-operated pipette when pipetting different volumes. A Transferpette (Brand, Wertheim, Germany) designed for variable volumes between 50 and 250 μL provided standard deviations of 7.8% for 12 repeated operations of 50 μL but only 2.3% for 250- μL operations. The test solvent was 2-ethyl-1-hexanol (isooctanol) with a boiling point of 182–185 $^{\circ}\text{C}$, which reduced the problem of evaporation. The pipetted amount of liquid was determined with an analytical balance. It is obvious that this type of pipette should not be used in its lower range for operations that are critical with regard to measurement uncertainty. Its use should be restricted to noncritical steps such as reagent addition.

3 Minimize the number of working steps: If a 1000-fold dilution is necessary, should it be performed in one step or in three 10-fold dilutions? The answer can be calculated easily in accordance to the approach presented in reference 9. Figure 2 illustrates the results.

The first case requires a 1-mL pipette and a 1000-mL measuring flask. The volume uncertainties of the pipette ($u[V_p]$) and the flask ($u[V_f]$) with water are 0.0033 and 0.39 mL, respectively. (I have assumed calibration uncertainties, which obey a triangular distribution, of 0.0029 mL and 0.163 mL, respectively; a temperature range of ± 3 $^{\circ}\text{C}$, which obeys a rectangular distribution; and repeatabilities of 0.0015 mL and 0.05 mL, respectively.) The relative uncertainty of the dilution is the com-

bination of the individual relative uncertainties according to the Gaussian law of error propagation, which applies also to the propagation of uncertainties:

$$\begin{aligned} \frac{u_c(\text{Dil})}{\text{Dil}} &= \sqrt{\left[\frac{u(V_p)}{V_p}\right]^2 + \left[\frac{u(V_f)}{V_f}\right]^2} \\ &= \sqrt{\left(\frac{0.0033}{1}\right)^2 + \left(\frac{0.39}{1000}\right)^2} \quad [1] \\ &= 3.3 \times 10^{-3} = 0.3\% \end{aligned}$$

$u_c(\text{Dil})$ is the absolute combined standard uncertainty of the dilution step, which usually is of no interest. Its division by the value of the dilution step (the dilution step Dil is only a written operation, so its numeric value needs not be known) directly gives the relative combined standard uncertainty and the multiplication with 100 yields its value in percentage.

The second case requires three 1-mL pipettes (also with uncertainties of 0.0033 mL) and three 10-mL volumetric flasks. The flasks have an uncertainty with water of 0.011 mL. (I have assumed a calibration uncertainty of 0.010 mL and repeatability of 0.002 mL.) These data combine to provide the following equation:

$$\begin{aligned} \frac{u_c(\text{Dil})}{\text{Dil}} &= \sqrt{3\left[\frac{u(V_p)}{V_p}\right]^2 + 3\left[\frac{u(V_f)}{V_f}\right]^2} \\ &= \sqrt{3\left(\frac{0.0033}{1}\right)^2 + 3\left(\frac{0.11}{10}\right)^2} \quad [2] \\ &= 6 \times 10^{-3} = 0.6\% \end{aligned}$$

The first approach, which performs the dilution in one step, yields a lower uncertainty, so it is preferable. Analysts must decide if it makes ecological and economical sense to prepare a large volume of solution when most of it is not used for the analysis.

4 Make sample and reference measurements in close time proximity and use the same instrument:

Most quantitative analytical instrumental techniques used today require the comparison of sample and reference signals. Chromatographic analyses are typical examples of this approach. (A reference is unnecessary in titrimetric and gravimetric analyses.) If two runs are performed using the same instrument and in close temporal vicinity, analysts can expect that all instrumental measurement parameters

will be identical. For example, the wavelength of the UV detector is identical because the lamp was not switched off between the two runs. In reality, it is unimportant if the detector is operating at the desired wavelength or has drifted slightly because both measurements are made under the same conditions. Similar considerations can be made with regard to the autosampler — its repeatability is important, but its accuracy is not — and even for pipettes. It is advantageous to use the same pipette to add an internal standard solution to the sample and the reference because its actual volume will not influence the result.

If the work is performed within a short time and if all solutions are at room temperature, then the temperature uncertainty is small, irrespective of the actual temperature, and even can be neglected for the calculation of volume uncertainties.

5 Use an internal standard: As soon as an identical amount of internal standard is added to both the sample and the reference, most subsequent operations become noncritical. This is especially true for all volumetric working steps. However, it is not true for an extraction if analysts have no proof that the analyte and the internal standard behave identically with regard to their recoveries. As a consequence, these operations do not appear in the measurement uncertainty calculation.

Further, workers do not need to know the true amount of internal standard. If it is added in dissolved form with a pipette, the same pipette is used for the sample and the reference, and all solutions have the same temperature, then the only parameter that the uncertainty calculation will exhibit is the repeatability of this pipette. Even when calculating an analytical result such as the analyte concentration or amount, workers do not need to know a true internal standard mass. The number might be wrong, but this error is compensated for because mass ratios are compared; therefore, neither the weighing operation of the internal standard nor its purity are considered for uncertainty calculation.

6 Prepare an artificial matrix or use a certified matrix reference material: If the sample and the reference are accompanied by the same mixture of matrix and other interfering compounds, the analytical result will be more reliable. The influence of these compounds upon sample preparation —

on pH values, extraction recoveries, and coprecipitation — will be similar in both cases. Additional, and probably unwanted, peaks also will appear in the reference chromatogram, thus forcing analysts to take care of optimum chromatographic resolution of the analyte peaks. If the resolution is still suboptimal, the group of peaks will look similar and errors caused by peak overlap will compensate for each other to some degree (10).

7 Perform multiple analyses: If a worker repeats an analysis and calculates the standard deviation of the mean, this number is not very reliable for a small database, and it covers only approximately 67% of the normal distribution curve. If a 95% confidence limit is required, the standard deviation needs to be expanded with the Student's *t*-factor, which is called coverage factor (*k*) in the context of measurement uncertainty (11–13). Table I shows the relevant data. If three determinations were performed dur-

ing validation, the standard deviation of the mean must be multiplied by a factor of 4.3; this factor is 2.8 for five determinations, and it still is 2.3 for 10 determinations. It is not forbidden to keep the number of analyses low; however, it is necessary to expand the uncertainty by the appropriate factor.

With regard to calibration functions, which are determined by linear regression from *n* data points, the data uncertainty calculated with this function decreases as *n* increases and more repeated analyses of the same sample (repeated injections from the same vial) are performed (14).

Conclusion

The hints provided in this “Sample Prep Perspectives” column are basic common sense. Most of the hints are not new, and they already should be considered standard procedures in well-organized, professional laboratories. It is a new challenge to put them in the context of measurement uncertainty where they get a rational base.

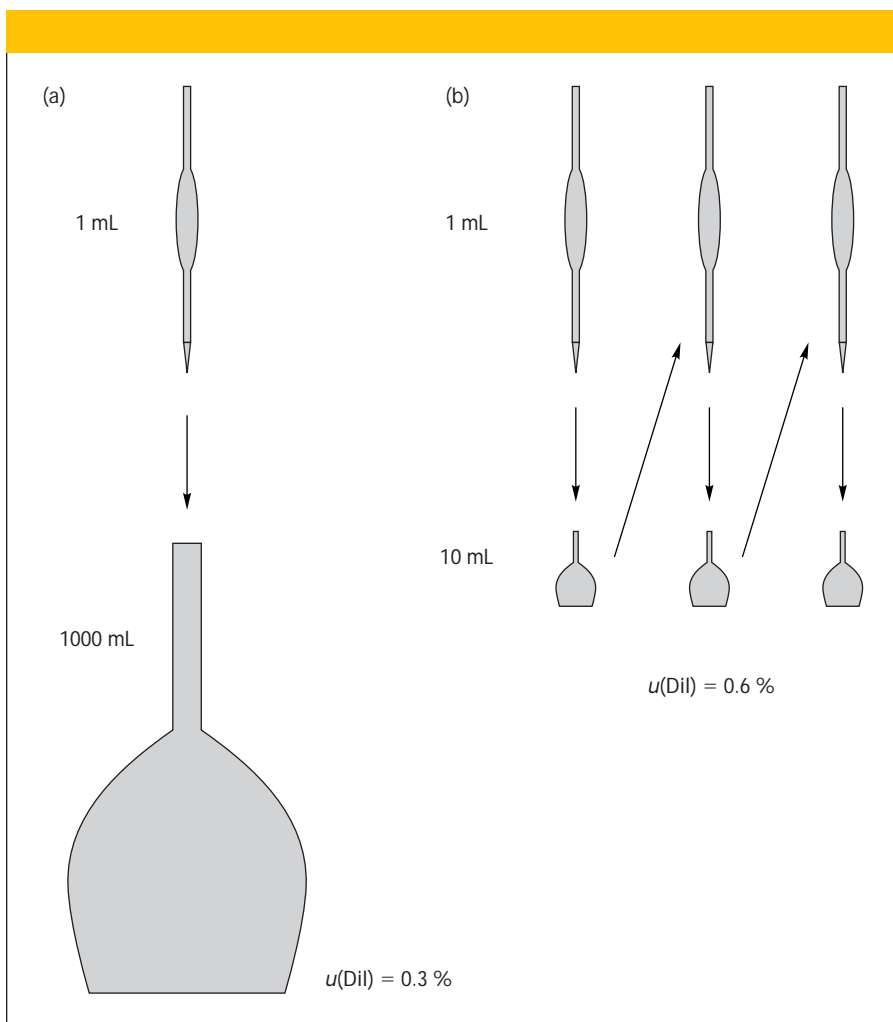


Figure 2: The combined standard uncertainty resulting from the dilution of 1 mL to 1000 mL with water in (a) one and (b) three steps.

The knowledge of measurement uncertainty is an added value to any analytical result.

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