



GC Connections

Chromatographers can reduce some side effects of nonlinear system performance by using chromatography data systems and their functions for quantitative and qualitative results calibration. This month, John Hinshaw focuses on using multilevel calibration for quantitative results computations.

Nonlinear Calibration

In a recent “GC Connections” column I discussed the sources of and remedies for nonlinear quantitative behavior in a gas chromatography (GC) system (1). In an ideal situation, a sample's composition would be unchanged as it passed through a chromatograph. In reality, all active system components to which a sample is exposed — inlets, columns, and detectors — can distort the apparent sample composition by adding or subtracting sample components, either wholly or partially. A linear relationship exists in theory between the injected amounts and the observed area counts recorded by a data system. Any residual systematic nonlinearity will distort this relationship, usually at the low and high ends of the chromatograph's dynamic range. Chromatographers often can eliminate most of these effects by observing common-sense maintenance and setup procedures and by choosing inlets, columns, and detectors that are suitable for their sample composition and solvent.

When no further improvements are attainable, chromatographers can use data-handling software to compensate for some of the remaining chromatographic system nonlinearity. Most data-handling systems include multilevel, nonlinear quantitation calibration routines that describe the relationships between measured area counts and apparent solute amounts throughout three or more levels of sample standards. These functions approximate the inverse of nonlinear chromatographic processes to linearize a system's overall response to changing solute amounts. Such mathematical legerdemain is appropriate only when chromatographers can do nothing else to improve a chromatograph's actual linearity. By misapplying multilevel nonlinear calibration to compensate for gross systematic errors, chemists can mask serious analytical malfunctions and risk compromising their results.

Data System Calibration

All chromatography data systems must be calibrated to deliver any meaningful results. Although operators might be unaware,

their chromatography data systems include default calibrations for solute quantitation and identification — even if they do nothing to generate calibration information. Chromatography data systems offer chromatographers several choices for quantitative and qualitative results calibration. This month's “GC Connections” focuses on quantitative computations; a future installment will address various peak-identification methods.

At the simplest level, a chromatography data system reports a table of detected peak retention times and raw (that is, uncorrected) area counts. At the next level, an operator can program a chromatography data system to report the area percentage of each peak in relationship to the sum of all the peaks' areas — the so-called *area-percent report* — or to list area percentages from the sum of the areas of selected peaks only — the so-called *normalized area-percent report*. I was surprised to find that, upon asking a group of 30 or so chromatographers attending a two-day course, roughly one-half of them used one of these reporting methods without a clear understanding that these uncalibrated area reports are almost meaningless.

The problems with calculations that use only uncalibrated areas stem from two effects. First is the implicit assumption that the chromatography system, including the detector, responds equally to the mass of each component in a chromatogram. This assumption is never exactly true, although it could be approximately correct within an acceptable error level for some nonpolar compounds, such as normal hydrocarbons larger than pentane, with a flame ionization detector. Because of injector adsorption and discrimination effects, however, this relationship quickly breaks down at trace levels or if a sample spans a wide range of molecular weights. A flame ionization detector responds somewhat differently to equal masses of dissimilar compounds such as normal, branched, unsaturated, or heterogenic hydrocarbons. Thermal conductivity detectors, electron-capture detectors, and other GC detectors exhibit widely varying

responses to different compounds. In addition, compounds can be adsorbed or otherwise lost to varying degrees as they pass through a chromatographic system. Even if solute losses in the system are minimized, chromatographers have no guarantees that equal injected amounts will yield equal area counts.

Second is the assumption that the chromatograph responds linearly to changing amounts of any individual compound. Ideally, measured area counts would double as the injected solute amounts double. In reality, and especially at the lower and upper ends of the system's response range, increas-

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ing or decreasing compound levels produces little or no corresponding response change. Adsorption at lower solute levels, inlet nonlinearity discrimination, and detector response changes give rise to a sometimes-complex relationship between injected amounts and recorded area counts. These effects occur in most real-world GC analyses. Even a simple present or not-present test that uses set area thresholds as the trigger for a hit requires knowledge of the area levels that correspond to the threshold amounts for each target compound. If an operator does nothing about it, a chromatography data system simply will assume that all compounds produce equal responses across all concentration ranges. A good chromatography data system will prompt users to choose a calibration method and then lead them through the process, thereby forcing them to calibrate their system in one way or another.

Chromatographers can minimize quantitative inaccuracies that stem from these two effects by carefully characterizing their system's responses to each target analyte across

the amounts that span the desired method dynamic ranges and minimum levels. The calibration process essentially develops a mathematical description of the measured relationships between peak responses and the corresponding injected solute amounts. The chromatography data system fits a curve to the measured solute amount and area count data points for a series of known standard samples. For each unknown sample analysis, it backcalculates the apparent solute amounts from their measured area counts.

This method of curve fitting and backcalculation is not foolproof. In fact, it requires a good deal of attention to obtain the best results. In particular, chromatographers should pay attention to the closeness of the calibration curve to the actual calibration data points and to the scatter — the standard deviations or variances — of the calibration data points throughout the entire method dynamic range, especially at the high and low ends of the range. Poor-quality calibration data will yield poor-quality results. Although many chromatography data system operators might fail to appreciate it, a little knowledge of the mathematics behind the calibration and calculations will go a long way toward a better understanding of the process and better quality results.

In addition to the fundamental calibration calculations discussed in this column, chromatography data systems must accommodate internal, external, and standard addition computations and unit conversions as part of a data-handling method. Interested readers can refer to a chromatography data system operator's manual or help files for information about these subjects.

Math Test

The mathematical functions that describe the relationships between amounts and areas can take several forms, which range from a simple linear relationship — the usual default setting — to more-complex nonlinear relationships.

Straight-line fits: A straight-line fit to the calibration data is the easiest relationship to understand. The chromatography data system calibration routine calculates the best slope and intercept values from the known amount and area count data using a simple formula that can be found in any high-school algebra text. The results are pairs of slope and intercept coefficients and correlation coefficients for each component under consideration. The chromatography data system then applies the following for-

mula to compute the amount of an unknown from its measured area counts.

$$\text{Amount} = x_1A + x_0 \quad [1]$$

In this equation, x_1 is the slope coefficient, x_0 is the amount axis (y axis) intercept, and A is the measured area count value for an unknown.

Figure 1 shows a plot of the measured calibration data points for the hypothetical solute response from the previous "GC Connections" installment on this topic overlaid with the best-fit line for that data (1). For the purposes of illustration, I restricted the range of calibration amounts to four orders of magnitude — from 10 pg to 100 ng — to eliminate the effects of system saturation at levels greater than 200 ng and to better illustrate the effects of calibration at the lower end of the range; few real-world analyses must cover more than three orders of magnitude.

The plotted points in Figure 1 show the measured area counts at each level of injected calibration amount for the hypothetical solute in question. At the low end of the plot, the area counts are less than what would be predicted by a simple constant uncalibrated response of 1×10^{-3} area count per nanogram, which is represented by the dashed line. For instance, the 10-pg calibration injection gave an area count of only 3, whereas an area count of 10 would be expected if the system were responding linearly to changing solute amounts at this low level. To see this result, find the 10-pg (0.01-ng) location on the vertical amount axis (y axis), follow it horizontally to the corresponding data point, and trace downward from that point to the horizontal area axis (x axis), as shown by the solid brown lines.

The solid blue line in Figure 1 shows the best-fit linear regression to the calibration data. The apparent curvature is caused by the logarithmic nature of the plot, which accentuates small errors exponentially as the levels go down. This effect, although present to the same degree, would not be apparent on a linear plot because the small errors would be plotted at the same scaling factor throughout the entire range and thus would be no more visible at high or low levels.

The correlation, or regression, coefficient (r) provides some information about how well the computed line fits the calibration data points. An r value of 1.0 is a perfect fit, to within the precision level of the calculation. In statistical analysis, regression values of less than 0.95 are considered too

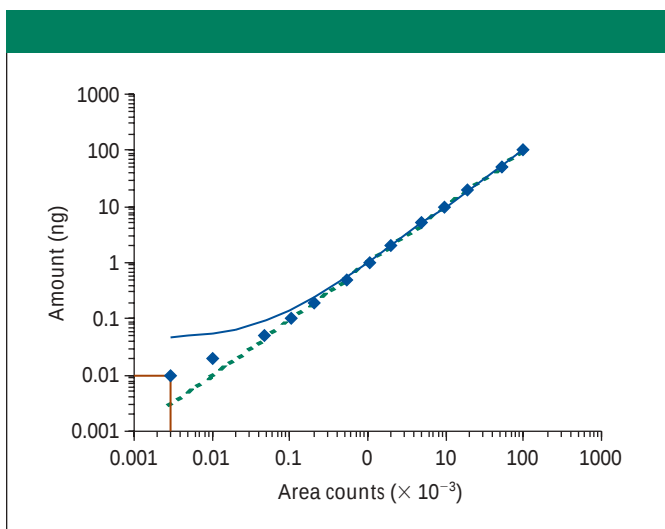


Figure 1: Plot of sample amounts versus area counts for linear calibration. The points represent measured pairs of amounts and areas from the calibration data. The dashed line represents an uncalibrated constant response factor of 1×10^{-3} area counts per nanogram. The solid line represents the best-fit linear regression to the calibration data, with $x_1 = 0.99112$, $x_0 = 0.04447$, and $r = 0.9994$.

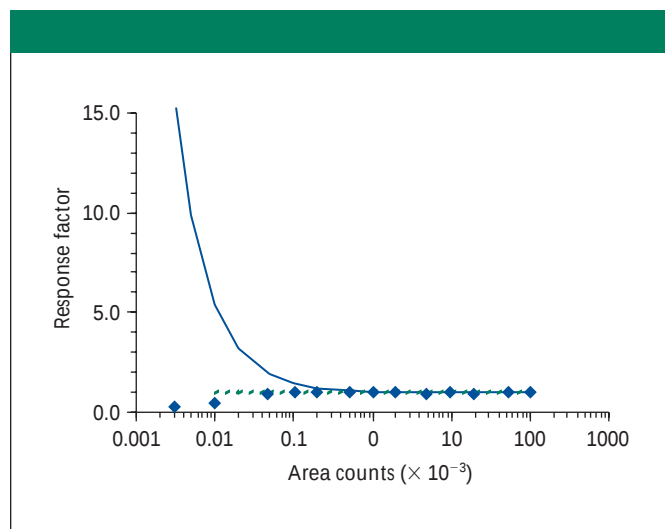


Figure 2: Plot of response factors versus area counts for linear calibration. The points represent measured pairs of amounts and apparent response levels for the area calibration data. The dashed line represents an uncalibrated constant response factor of 1×10^{-3} area counts per nanogram. The solid line represents the response factors calculated as a function of area counts from the best-fit linear regression.

low for good quality results. For chromatography data system calibration, however, it is important to examine plots similar to those shown in this column to assess the effects of small errors at low solute levels: the r value presents only an aggregate measure of the fit throughout the entire range. The best-fit linear regression has an r value of 0.9994, and yet the significant errors at low solute amounts are seen easily.

Figure 2 illustrates the response factors computed by the best-fit linear regression as shown by the solid line, which compensates for the apparent losses in calibration

response at low levels as shown by the plotted points. In this case, however, the calculated response factors overcompensated at the low end and increased the apparent solute amounts that would be reported by the chromatography data system to well beyond their actual values. Thus, the simple linear calibration seems to yield acceptable results at the higher levels, but it falls apart at lower levels.

Polynomial fits: Clearly, the simple linear regression fit leaves much to be desired at the low end of the scale. Most chromatography data systems can apply several

common alternative functions to calibration data, including logarithmic, exponential, and polynomial functions. The last function type works well for the hypothetical data in this column.

A polynomial function comprises the sum of two or more power functions of the independent variable and a zero-order coefficient. For a chromatography data system, they can be written in the form:

$$\text{Amount} = x_n A^n + x_{n-1} A^{n-1} + \dots + x_1 A + x_0 \quad [2]$$

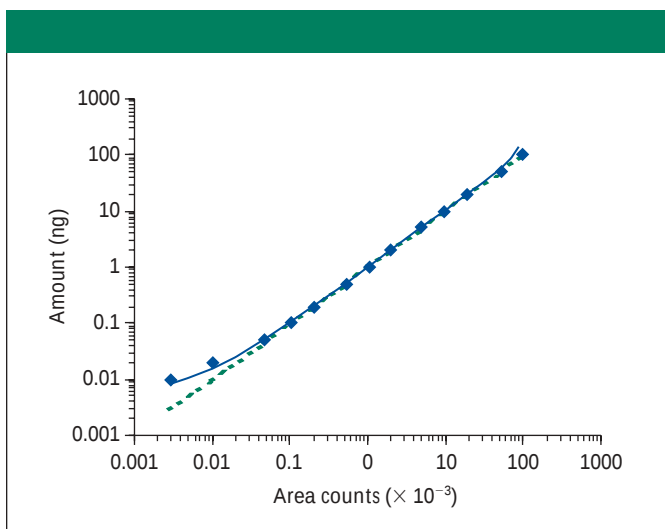


Figure 3: Same as Figure 1, except the solid line represents the best-fit fourth-order polynomial regression to the calibration data, with $x_4 = 2.0 \times 10^{-6}$, $x_3 = 0.0002$, $x_2 = 0.0077$, $x_1 = 0.9815$, $x_0 = 0.0056$, and $r > 0.99999$.

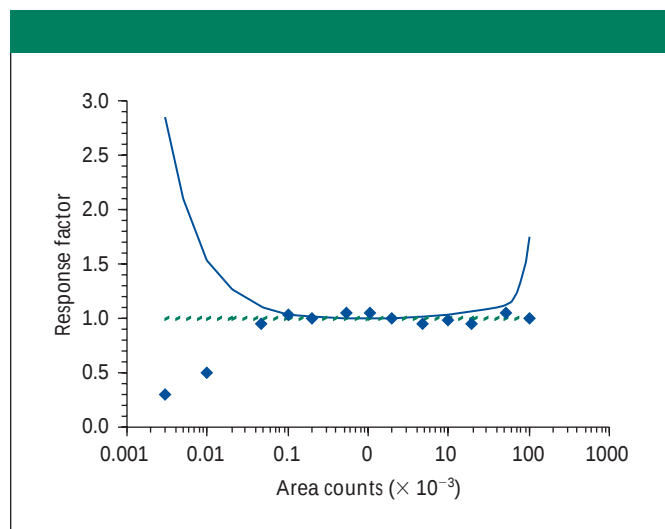


Figure 4: Same as Figure 2, except the solid line represents the response factors calculated as a function of area counts from the best-fit fourth-order polynomial regression.

A polynomial collapses to the same linear function as written in equation 1 when the value of n is 1. When n is 2, a polynomial is a quadratic equation. For calibration, most data systems include polynomial functions to the fourth order, where n is 4. Each of these polynomial orders can be fitted to experimental data with available formulas, although these formulas could be more difficult to locate than the simple linear case of the previous section.

Figures 3 and 4 illustrate the appearance of a fourth-order polynomial fit to the calibration data. Clearly, the accuracy at the low end is improved greatly, and the calibration curve now overlays the data almost exactly. At the high end, however, the polynomial introduces a new error and tends to overestimate calculated amounts relative to the calibration. This effect is seen most clearly in Figure 4 — the polynomial curve sweeps up too far at solute levels greater than 50 ng. However, it is a simple matter to calibrate the system to a higher level than the desired operating range and avoid this kind of problem by staying well within the observed range of accurate calibration.

This example is not meant to imply that a fourth-order polynomial function always

is the best one. Each situation is different, and each instrument system can deliver differing responses that vary from day to day. The best procedure is to calibrate often, examine each calibration curve for each component carefully, and ensure that the analyte amounts remain well within the most accurate calibrated ranges. In no case should chromatographers attempt to extrapolate calibration data beyond the measured minimum and maximum levels, because multilevel calibrations work only inside of their respective limits.

Conclusion

Nonlinear responses with changing solute amounts can be intrinsic to a chromatographic system because of detector responses or other effects. Despite their best efforts, chromatographers might be unable to eliminate nonlinear behavior entirely. In these cases, chromatography data systems offer a number of mathematical solutions to the problem of nonlinearity. Before relying on math functions to straighten out these data, however, examine the effects of such compensations and ensure that the result with nonlinear calibration is better than the result without.

It's never a good idea to assume that a chromatograph delivers equal responses to all components or at all levels. Always calibrate and check the system response throughout a range that encompasses the minimum and maximum levels for the analysis.

Reference

- (1) J.V. Hinshaw, *LCGC* **20**(2), 120–126 (2002).

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For an ongoing discussion of GC issues with John Hinshaw and other chromatographers, visit the Chromatography Forum discussion group at <http://www.chromforum.com>.

