Before it is possible to measure the concentration of an analyte in a solution using spectroscopy, a calibration curve is needed. The calibration curve acts to relate the measurement result (an absorbance value) to the quantity of interest (a concentration). According to the Beer-Lambert Law there is a linear relationship between concentration and absorbance. Note, however, that this relationship tends to deviate from linearity at higher absorbance values for most instruments due to stray light in the spectrometer or nonlinearity of the detector.

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Let’s say you have a lingering suspicion that your nephew poured a bottle of sports drink into your backyard swimming pool at yesterday’s gathering. Can you prove his guilt with spectroscopy? What is the lowest concentration of sports drink that could reliably be detected in a swimming pool? What degree of dilution can be measured? A hundredfold? A thousandfold? How far can we take this measurement?

In Part 1 of this two-part series, we looked at sources of noise that may arise from the spectrometer, as well as from the experimental method. In this installment, we will put that information to good use to measure low concentrations of sports drinks in water, developing a calibration curve and determining the limit of detection.

Keywords

- Spectrometer noise
- Limit of detection
- Beer-Lambert Law
- Optical density

Considerations

- Analyte concentration
- Calibration curve plotting
- System drift

Tech Note: Noise in Spectrometers, Part 2
Written by Dieter Bingemann, Ph.D.

In Part 1 of this two-part series, we looked at sources of noise that may arise from the spectrometer, as well as from the experimental method. In this installment, we will put that information to good use to measure low concentrations of sports drinks in water, developing a calibration curve and determining the limit of detection.

Calibration

Before it is possible to measure the concentration of an analyte in a solution using spectroscopy, a calibration curve is needed. The calibration curve acts to relate the measurement result (an absorbance value) to the quantity of interest (a concentration). According to the Beer-Lambert Law there is a linear relationship between concentration and absorbance. Note, however, that this relationship tends to deviate from linearity at higher absorbance values for most instruments due to stray light in the spectrometer or nonlinearity of the detector.

A calibration curve can be determined by measuring the absorbance of a series of solutions of known concentration (a set of “external stan-
The optical density is measured for a series of solutions over a concentration range spanning that of the expected unknown solutions. Ideally, ten or more standard solutions are measured to develop the standard curve, equally spaced in concentration over the range of interest. The absorbance value measured for each standard solution is then plotted as a function of the associated concentration, and a linear regression curve fitted through the points. The regression line constitutes the desired calibration curve.

Three measurements are needed to make each absorbance determination: the light intensity through the sample, the intensity through a reference, and the intensity without any light on the detector (often called the “dark”). To detect a low absorbance solution, as in the case of the sports drink spilled in the pool, we need to reduce the noise level and drift (in time) of these three signals (sample, reference, dark). This requires a spectrometer with a high signal-to-noise ratio (SNR), such as the thermo-electrically cooled CCD detector in the QE Pro spectrometer that we are using for the experiments in this application note. An STS spectrometer is another excellent candidate for absorbance measurements, featuring a CMOS detector with comparable SNR.

### Experiment

The first and simplest way to improve the signal-to-noise ratio in our low-absorbance measurement is to average over a significant number of spectra, keeping in mind that in order for this to be of benefit, we must also minimize system drift over the correspondingly longer measurement time. We can eliminate a significant contributor to system drift – light source drift – by using a dual-beam absorbance setup with one light source illuminating two solutions in two cuvette holders via a bifurcated fiber (one a reference and the other a sample). An inline shutter on each path is used to control which cuvette the light illuminates – the sample or the reference. A single spectrometer records the light intensity after the cuvettes by using a second bifurcated fiber to route light from each cuvette to the same spectrometer. This is made more efficient by rotating the two fibers at the common leg of the bifurcated cable to line up as best possible with the slit direction in the spectrometer. (Tip: To achieve best alignment of a bifurcated fiber to a spectrometer slit, attach each in turn to the same light source and rotate the fiber at the spectrometer junction until each leg delivers roughly the same amount of light to the spectrometer.)

This dual-beam setup (with alternate detection of the sample and reference cuvettes) makes it possible to correct for drift in the light source, as that drift is most likely to occur more slowly than the time scale needed to switch between the two light paths. Similarly, using a single spectrometer not only cuts cost, but also eliminates sources of slow drift in the spectrometer itself.

To collect data on the series of standard solutions, the three required signals (dark, reference, sample) are acquired in short sets (10 spectra each in this experiment) by opening and closing the inline shutters appropriately (both paths closed for “dark,” path A open for “sample,” and path B open for “reference”). The shutters may be controlled through software via the spectrometer’s GPIO port. A short C# program (using OmniDriver to interface with the spectrometer) was written to cycle through these measurements and average over multiple sets (100 sets of 10 spectra in this experiment, for a total of 1000 averages for each of the three signals).

Even though stray light in the spectrometer is more of an issue with high optical density measurements (in contrast to the low-absorbance measurements of this investigation), we limited the incoming lamp spectrum with an additional near-infrared blocking filter (KG-5) to just the visible region of interest (400-700 nm) to reduce the noise level in the signal. Finally, we eliminated ambient light by covering any open light path -- for example, in the shutters -- with a black cloth.

### Procedure

A dilution series of 10 samples was prepared, starting from a thousandfold diluted stock solution, with concentrations of $1 \times 10^{-4}$ to $10 \times 10^{-4}$ of the original sports drink concentration. A cuvette with the lowest concentration solution was inserted into one cuvette holder (light path “A”), and a cuvette with distilled water (reference) into the other cuvette holder (light
We started the measurement application developed to control the shutters to record 100 sets of 10 spectra each of “dark,” “A,” and “B” intensities. To account for any difference in the light paths A and B we exchanged the sample and reference cuvettes (now measuring sample in light path B and reference in light path A) and recorded another 100 sets of 10 spectra each of dark, A, and B.

When moving to the next concentration in the series we drained the sample cuvette and rinsed it once with the solution of the next higher concentration (known as an “analytical rinse”). This approach introduces a smaller error in the sample concentration than a rinse with distilled water. A separate “blank” measurement (with just water in the sample cuvette) should be added at the very beginning to account for any differences in the cuvettes themselves.

A total of six spectra were acquired for each concentration: three for the original placement of the cuvettes (dark, I_D, intensity in light path A, I_A, and intensity in light path B, I_B, with an unknown light intensity I_0), and three more for the swapped placement (dark, I_D*, intensity in light path A, I_A*, and intensity in light path B, I_B*, with an unknown light intensity I_0*).

As a first step in the analysis we subtracted the dark signal from all intensities, yielding the dark-corrected intensities, for example $I_{cA} = I_A - I_D$. Similarly we corrected the other three intensities $I_{cB}, I_{cA*},$ and $I_{cB*}$. Rather than the intensity itself, we are interested in the transmission (and the associated absorbance) of each solution, and eventually the change in absorbance in the sample due to the analyte (the sports drink). With some simple math one can show that the transmission of the sample relative to the transmission of the reference is related to the recorded (and corrected) intensities according to the following equation:

$$\frac{I_{cA}(S)}{I_{cB}(R)} \times \frac{I_{cB}^*(S)}{I_{cA}^*(R)} = T^2$$

With $I_{cA}(S)$ signifying the dark-corrected intensity (as defined above) measured in light path A with the sample cuvette (S) in the cuvette holder. Similarly, “R” stands for the reference cuvette and “T” is the desired transmission of the analyte.

This approach has a subtle advantage. For example, as we determine the square of the transmission out of two sets of measurements, we cut the error in transmission measurement by 50% compared to a procedure in which we leave the reference cuvette in place (in light path B) and cycle the sample cuvette against a second blank cuvette (in light path A).

This setup and measurement approach eliminates all drifts in the lamp, in imaging of the light path, and in the spectrometer that happen on a time scale slower than the acquisition of a single set of dark, A, and B intensities (the time scale being primarily defined by the time needed to open and close the shutter). Fluctuations on a time scale faster than such a set acquisition will be reduced through averaging over repeated sets.

Even though this setup accounts for many of the potential drifts and fluctuations in the measurement, a few errors cannot be corrected for, such as any random differences in the placement of the cuvette, or any bubbles, particles or dirt in the solutions or on the cuvettes.

Results

Figure 1 shows the recorded absorbance spectra for each calibration solution (with concentrations from 1 to $10\times10^{-4}$ of the original sports drink).

![Absorbance spectra](image)
We averaged the absorbance from 610 nm to 640 nm (corresponding to ~15 nm on either side of the absorbance maximum) for additional noise reduction, and plotted the average absorbance as a function of the concentration, thus yielding a calibration curve defined by the linear regression through the points (Figure 2).

### Limit of Detection

The regression procedure also yields the standard error of regression (the standard deviation of the measured points from the regression line), as an error estimate of the measurement setup in absorbance units. The limit of detection is defined as an absorbance signal that corresponds to three times this standard error above the signal extrapolated to a blank sample (zero concentration), signifying a signal level at which the chance of falsely claiming the presence of the analyte for a blank sample (a “false positive”) drops to 1%. If the observed absorbance falls below this signal level, it is below the limit of detection and we need to state that no drink could be detected in the sample at that dilution factor. If the actual concentration is right at the limit of detection, we therefore run a 50% risk of falsely claiming the absence of the drink in the sample (a so-called “false negative”).

Rather than specifying the absorbance level that represents the limit of detection, it is more useful to report the concentration corresponding to this absorbance. The identification of this limit of detection concentration is schematically shown in Figure 2: it is the concentration at which the calibration curve reaches the limit of detection absorbance (regression intercept plus three times the standard error).

### Conclusion

We find a detection limit of about $2 \times 10^{-4}$ of the original sports drink concentration for a dual-path measurement using a QE Pro spectrometer, corresponding to a dilution factor of roughly 5000-fold. Assuming your nephew poured one liter of your sports drink into the pool, you would still have a chance of detecting traces of the sports drink in a sample for a 5000 liter pool and prove him guilty. And at that point, provided your family has followed your explanation of the finer points of spectroscopic absorption measurements, they might just believe you!