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Making a Calibration Curve

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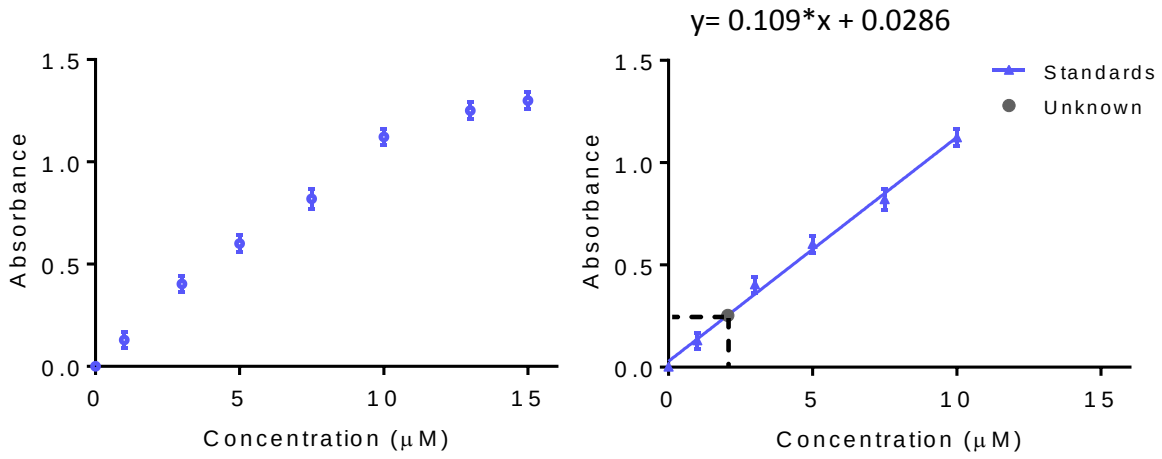

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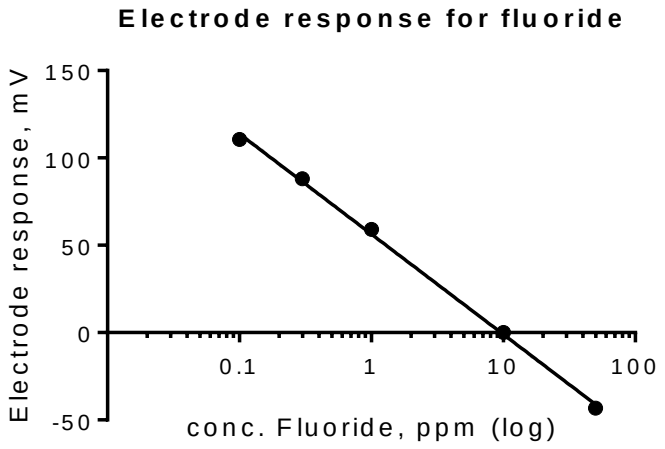

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Chemistry Science Education Title:

Making a Calibration Curve

Overview:

Calibration curves are used to understand the instrumental response to an analyte and predict the concentration in an unknown sample. Generally, a set of standard samples are made at various concentrations with a range than includes the unknown of interest and the instrumental response at each concentration is recorded. For more accuracy and to understand the error, the response at each concentration can be repeated so an error bar is obtained. The data are then fit with a function so that unknown concentrations can be predicted. Typically the response is linear, however, a curve can be made with other functions as long as the function is known. The calibration curve can be used to calculate the limit of detection and limit of quantitation.

When making solutions for a calibration curve, each solution can be made separately. However, that can take a lot of starting material and be time consuming. Another method for making many different concentrations of a solution is to use serial dilutions. With serial dilutions, ~~you start with~~ a concentrated sample ~~and then~~is diluted ~~it~~ down in a stepwise manner to make lower concentrations. The next sample is made from the previous dilution, and the dilution factor is often kept constant. The advantage is that ~~you~~ only ~~make~~ one initial solution is needed. The disadvantage is that any errors in solution making, pipetting, etc., get propagated as ~~you make~~ more solutions or made. Thus, ~~you must be very~~ care must be taken~~ful to make~~ when making the initial solution ~~correctly~~.

Principles:

Calibration curves can be used to predict the concentration of an unknown sample. To be completely accurate, the standard samples should be run in the same matrix as the unknown sample. A sample matrix is the components of the sample other than the analyte of interest, including the solvent and all salts, proteins, metal ions, ~~etc~~etc. that might be present in the sample. In practice, ~~this~~ running calibration samples in the same matrix as the unknown is sometimes difficult, as the unknown sample may be from a complex biological or environmental sample. Thus, many calibration curves are made in a sample matrix that closely approximates~~s~~ the real sample, such as artificial cerebral spinal fluid or artificial urine, but may not be exact. The range of concentrations of the calibration curve should bracket that in the expected unknown sample. Ideally you would run a few concentrations above and below the expected concentration sample.

Many calibration curves are linear and can be fit with the basic equation y=mx+b, where m is the slope and b is the y-intercept. However, not all curves are linear and sometimes to get a line, one or both set of axes will be on a logarithmic scale. Linear regression is typically

Commented [JJ1]: Discuss R squared, where it comes from and what it says about the curve.

Commented [JJ2]: Give a definition of this.

Commented [JJ3]: Are there any common ways to work around this issue?

performed using a computer program and the most common method is to use a least squares fitting. With a linear regression analysis, an R^2 value, called the coefficient of determination, is given. For a simple single regression, R^2 is the square of the correlation coefficient (r) and provides information about how far away the y values are from the predicted line. A perfect line would have an R^2 value of 1, and most R^2 values for calibration curves are over 0.95. When the calibration curve is linear, the slope is a measure of sensitivity: how much ~~of a~~the signal changes ~~in signal you get~~ for a change in concentration. A steeper line with a larger slope indicates a more sensitive measurement. A calibration curve can also help define the linear range, the range of concentrations that the instrument gives a linear response. Outside this range, the response may taper off due to instrumental considerations, and the equation from the calibration cannot be used. This is known as the limit of linearity.

Limit of detection is the lowest amount that can be statistically determined from the noise. Generally this is defined as a signal that is 3 times the noise. The limit of detection can be calculated from the slope of the calibration curve and is generally defined as LOD=3*S.D./m, where S.D. is the standard deviation of the noise. The noise is measured by taking the standard deviation of multiple measurements. Alternatively, in one trace, noise can be estimated as the standard deviation of the baseline. The limit of quantitation is the amount that can be differentiated between samples and is usually defined as 10 times the noise.

Procedure:

1. **Making the Sstandards: Sserial dDilutions.**

   1.1. Make a concentrated stock solution of the standard. Typically, the compound ~~will be~~is accurately weighed out and then ~~the~~ quantitatively transferred into a ~~solution made in a~~ volumetric flask. Add some solvent, mix so the sample dissolves, then ~~F~~fill to the line with the proper solvent.

   1.2. Perform serial dilutions. Take another volumetric flask and pipette the amount of standard needed for the dilution, then fill to the line with solvent and mix. A ten-fold dilution is typically made, so for a 10 mL volumetric flask, ~~you would~~ add 1 mL of the previous dilution.

   1.3. Continue as needed for more dilutions, pipetting from the previous solution to dilute it to make the next sample. For a good calibration curve, at least 5 concentrations are needed.

2. **Run the Ssamples for the Ccalibration Ccurve and the Uunknown.**

   2.1. Run the samples with the instrument to determine the instrumental response needed for the calibration curve.

Commented [JJ4]: How is this determined?

Commented [JJ5]: Can you mass this directly in the vol. flask to ensure all standard makes it into the solution? Also, include the step of mixing the solution before filling to the calibration mark.

Commented [JV6]: Not really. If you mass into a small volumetric flask (10-25 mL) and you get too much, you can't get the spatula back in to get any extra out. The top is too small. So this is not standard practice

Commented [JJ7]: 1) How many standards should be made for a proper calibration curve? 2) Are these ten-fold dilutions, or some other factor?

Commented [JJ8]: What instrument will you be using?

Commented [JV9]: Here, a UV-Vis spectrophotometer. I kept it general, because it could apply to anything. You can put the specific instrument I will be using in if you want.

2.2. Take the reading with the first sample. It is a good idea to run the samples in random order (*i.e.* not highest to lowest or lowest to highest) in case there are any systematic errors. In order to get an estimate of noise, repeat the reading on any given sample three to five times.

2.3. Run the additional standard samples, repeating the measurements for each sample to get an estimate of noise. Record the data ~~so you can~~to make a plot later.

2.4. Run the unknown sample(s). Use as similar conditions to running the standards as possible. Thus, the sample matrix or buffer should be the same, the pH should be the same, and the concentration should be in the range of the standards ~~you ran~~run.

3. Makinge the Ccalibration Ccurve.

3.1. Record the data in a spreadsheet and use a computer program to plot the data vs concentration~~points~~. If at least triplicate measurements were taken for each point, error bars can be plotted of the standard deviation of those measurements to estimate of the error of each point. For some curves, ~~you might need to~~the data might need to be plotted ~~the data~~ with an axis as a log to get a line. The equation that governs the calibration curve is generally known ahead of time, so a log plot is used when there is a log in the equation.

3.2. Examine ~~your~~ the calibration curve. Does it look linear? Does ~~the~~it have a portion that looks non-~~If the curve clearly has a portion that is not~~ linear (*i.e.* ~~you~~ reached the limit of the instrumental response)?~~, those points must be removed before performing a linear regression.~~ To check, fit all the data into a linear regression using the software. If the coefficient of determination (R2) is not high, remove some of the points at the beginning or ending of the curve that do not appear to fit the line and perform the linear regression again. It is not acceptable to remove points in the middle~~,~~ just because they have a large error bar. From this analysis, decide what portion of the curve is linear. ~~For some curves, you might need to plot the data with an axis as a log to get a line. If the curve clearly has a portion that is not linear (*i.e.* you reached the limit of the instrumental response), those points must be removed before performing a linear regression.~~

3.3. ~~Perform the linear regression using a computer~~the software. The output of the linear should be an equation of the format y=mx+b, where m is the slope and b is the y-intercept. The units for the slope are the y-axis unit/concentration, in this example (**Figure~~.~~ 1**) absorbance/μM. The units for the y-intercept are the y-axis units. A ~~correlation~~ coefficient of determination (R^2) ~~will be~~is obtained. The higher the R^2 the better the fit; a

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Commented [JJ10]: Provide a more step-by-step explanation for this.

Commented [JJ11]: Provide a more step-by-step explanation for this.

perfect fit gives an R^2 of 1. The program may also be able to give an estimate of the error on the slope and the intercept.

4. Results: Calibration Ceurve of Aabsorbance of Blue Dye #1.

4.1. The calibration curve for absorbance of blue dye #1 (at 631 nm) is shown below **(Figure 1).** The response is linear from 0 to 10 uM. Above that concentration the signal begins to level off because the response is out of the linear range of the UV-Vis spectrophotometer.

4.2. Calculate the LOD. From the slope of the calibration curve, the LOD is 3*s.d. (noise)/m. For this calibration curve, the noise was obtained by taking a standard deviation of repeated measurements and was 0.021. tThe LOD would be 3*0.021/.109=0.58 uM.

Commented [JJ12]: How and where was this calculated?

4.3. Calculate the LOQ. The LOQ is 10*s.d.(noise)/m. For this calibration curve, LOQ is 10*0.021/.109 =1.93 uM.

4.4. Calculate the concentration of the unknown. Use the line equation to calculate the concentration of the unknown sample. The calibration curve is only valid if the unknown falls into the linear range of the standard samples. If the readings are too high, dilution might be necessary. In this example, the unknown sports drink was diluted 1:1. The absorbance was 0.243 and this corresponded to a concentration of 2.02 uM. Thus the final concentration of blue dye #1 in the in the sports drink was 4.04 uM.

5. Applications:

Applications: Calibration curves are used in many fields of analytical chemistry, biochemistry, and pharmaceutical chemistry. It is common to use them with spectroscopy, chromatography, and electrochemistry measurements. A calibration curve can be used to understand the concentration of an environmental pollutant in a soil sample. It could be used determine the concentration of a neurotransmitter in a sample of brain fluid, vitamin in pharmaceutical samples, or caffeine in food. Thus, calibration curves are useful in environmental, biological, pharmaceutical, and food science applications. The most important part of making a calibration curve is to make accurate standard samples that are in a matrix that closely approximates ~~your~~ the sample mixture.

An example of an electrochemistry calibration curve is shown below **(Figure 2).** The data were collected with an ion-selective electrode for fluoride. Electrochemical data follow the Nernst equation $E=E^0 + 2.03*R*T/(nF) * \log C$. Thus, the concentration data (x-axis) must be plotted on a log scale to obtain a line. This calibration curve could be used to measure the concentration of fluoride in toothpaste or drinking water.

Legend:

Figure 1. Calibration curves for UV-Vis absorbance of blue dye. Left: The absorbance was measured of different concentrations of blue dye #1. The responses level off after 10 uM, when the absorbance is over 1. The error bars are from repeated measurements of the same sample and are standard deviations. Right The linear portion of the calibration curve is fit with a line, y=0.109*x + 0.0286. The unknown data is shown in black.

Figure 2. Calibration curve for an ion-selective electrode. The response of a fluoride selective electrode (in mV) to different concentrations of fluoride is plotted. The expected equation for the electrode response is y (in mV)=-59.2*log x+b at 25 °C. The actual equation is y=-57.4*log x +56.38. The R^2 value is 0.998.