

INSTRUMENT FACILITIES FOR CHEM401 L AT DUKE



Instrument Facilities for CHEM401L at Duke

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Licensing

A detailed breakdown of this resource's licensing can be found in [Back Matter/Detailed Licensing](#).

CHAPTER OVERVIEW

1: Analytical Equipment and Methods for Calibration

1.1: Analytical Lab Equipment

1.2: Preparing Solutions

1.3: Methods of Calibration

1.4: Uncertainty in values determined from a Calibration Curve

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1.1: Analytical Lab Equipment

There is a vast array of equipment available for making analytical measurements and working with analytical samples. The equipment can range from the simple and inexpensive, to the complex and expensive. This section will describe some of the equipment that you will use in lab during this module, or equipment that you should be knowledgeable about so that you can assess the error in your data.

Equipment for Measuring Mass

An object's mass is measured using a digital electronic analytical balance (Figure 1.1.1). An electromagnet levitates the sample pan above a permanent cylindrical magnet. When we place an object on the sample pan, it displaces the sample pan downward by a force equal to the product of the sample's mass and its acceleration due to gravity. The balance detects this downward movement and generates a counterbalancing force by increasing the current to the electromagnet. The current needed to return the balance to its original position is proportional to the object's mass. A typical electronic balance has a capacity of 100–200 g, and can measure mass to the nearest ± 0.01 mg to ± 1 mg.



Figure 1.1.1 : The photo shows a typical digital electronic balance capable of determining mass to the nearest ± 0.1 mg. The sticker inside the balance's wind shield is its annual calibration certification. For a review of other types of electronic balances, see Schoonover, R. M. *Anal. Chem.* **1982**, 54, 973A-980A.

Although we tend to use interchangeably, the terms “weight” and “mass,” there is an important distinction between them. Mass is the absolute amount of matter in an object, measured in grams. Weight, W , is a measure of the gravitational force, g , acting on that mass, m :

$$W = m \times g$$

An object has a fixed mass but its weight depends upon the acceleration due to gravity, which varies subtly from location-to-location.

A balance measures an object's weight, not its mass. Because weight and mass are proportional to each other, we can calibrate a balance using a standard weight whose mass is traceable to the standard prototype for the kilogram. A properly calibrated balance gives an accurate value for an object's mass; see [Appendix 9](#) for more details on calibrating a balance.

If the sample is not moisture sensitive, a clean and dry container is placed on the balance. The container's mass is called the **tare** and most balances allow you to set the container's tare to a mass of zero. The sample is transferred to the container, the new mass is measured and the sample's mass determined by subtracting the tare. A sample that absorbs moisture from the air is treated differently. The sample is placed in a covered weighing bottle and their combined mass is determined. A portion of the sample is removed and the weighing bottle and the remaining sample are reweighed. The difference between the two masses gives the sample's mass.

Several important precautions help to minimize errors when we determine an object's mass. To minimize the effect of vibrations, the balance is placed on a stable surface and in a level position. Because the sensitivity of an analytical balance is sufficient to measure the mass of a fingerprint, materials often are handled using tongs or laboratory tissues. Volatile liquid samples must be weighed in a covered container to avoid the loss of sample by evaporation. To minimize fluctuations in mass due to air currents, the balance pan often is housed within a wind shield, as seen in Figure 1.1.1 . A sample that is cooler or warmer than the surrounding air will create a convective air currents that affects the measurement of its mass. For this reason, bring your samples to room temperature before determining their mass. Finally, samples dried in an oven are stored in a desiccator to prevent them from reabsorbing moisture from the atmosphere.

Equipment for Measuring and Transferring Volume

Analytical chemists use a variety of glassware to measure volume, including graduated cylinders, volumetric pipets, and volumetric flasks. The choice of what type of glassware to use depends on how accurately and how precisely we need to know the sample's volume and whether we are interested in containing or delivering the sample.

Transfer Pipet

A **transfer pipet** is a device used to transfer small amounts of volume. These are also known as "eye droppers" and "Pasteur pipets", and they are usually made of either glass or plastic (Figure 1.1.2). Some transfer pipets have graduated markings to indicate the amount of volume being transferred. You should not use a transfer pipet to attempt to measure precise volumes because these devices are not accurate or precise. They are useful for transferring small volumes. For example, you might use a transfer pipet to move an approximate volume of liquid from a flask to a cuvette, or for adding solution drop wise to reach the calibration mark on a graduated cylinder or volumetric flask.



Figure 1.1.2 : The photo shows several plastic transfer pipets stored in a beaker. ([Pualuu](#), [Transfer pipette](#), [CC BY-SA 4.0](#))

Graduated cylinder

A **graduated cylinder** is the simplest device for delivering a known volume of a liquid reagent (Figure 1.1.3). The graduated scale allows you to deliver any volume up to the cylinder's maximum. Typical accuracy is $\pm 1\%$ of the maximum volume. A 100-mL graduated cylinder, for example, is accurate to ± 1 mL. In this course, you should not use graduated cylinders to measure volume; rather a volumetric flask or volumetric pipette is the preferred tool because they are more precise than a graduated cylinder.



Figure 1.1.3 : An example of a 250-mL graduated cylinder.

Volumetric Pipet

A **volumetric pipet** (with one "t") provides a more accurate method for delivering a known volume of solution. Several different styles of pipets are available, two of which are shown in Figure 1.1.4 . **Volumetric pipets provide the most accurate means for delivering a known volume of solution.** A transfer pipet delivering less than 100 mL generally is accurate to the hundredth of a mL. Larger transfer pipets are accurate to a tenth of a mL. For example, the 10-mL transfer pipet in Figure 1.1.3 will deliver 10.00 mL with an accuracy of ± 0.02 mL.



Figure 1.1.4 : Two examples of 10-mL volumetric pipets. The pipet on the top is a transfer pipet and the pipet on the bottom is a Mohr measuring pipet. The transfer pipet delivers a single volume of 10.00 mL when filled to its calibration mark. The Mohr pipet has a mark every 0.1 mL, allowing for the delivery of variable volumes. It also has additional graduations at 11 mL, 12 mL, and 12.5 mL.

Scientists at the Brookhaven National Laboratory used a germanium nanowire to make a pipet that delivers a 35 zeptoliter (10^{-21} L) drop of a liquid gold-germanium alloy. You can read about this work in the April 21, 2007 issue of *Science News*.

To fill a volumetric pipet, use a rubber suction bulb to pull the solution up past the calibration mark (*Never use your mouth to suck a solution into a pipet!*). After replacing the bulb with your finger, adjust the solution's level to the calibration mark and dry the outside of the pipet with a laboratory tissue. Allow the pipet's contents to drain into the receiving container with the pipet's tip touching the inner wall of the container. A small portion of the liquid remains in the pipet's tip and is not be blown out. With some measuring pipets any solution remaining in the tip must be blown out. There are some videos that describe the use of volumetric pipettes in a later section of this module.

How to use Volumetric Pipettes and pipette bulbs



Pipettes

Delivering microliter volumes of liquids accurately is not possible using the devices described above. Digital pipettes and micropipettes (spelled with two "t" and an "e"), which come in a variety of volume ranges, provide for the routine measurement of milliliter and microliter volumes (Figure 1.1.5).



Figure 1.1.5 : A set of two digital micropipettes. The pipet on the top delivers volumes between 0.5 mL and 10 μ L and the pipet on the bottom delivers volumes between 10 mL and 100 μ L.

How to use an automatic pipette (aka "pipetter" or "pipetteman")



Volumetric Flask

A **volumetric flask** is calibrated to contain (T.C.) or to deliver (T.D.) a specific volume of solution at room temperature (Figure 1.1.6). Most volumetric flasks have one calibration marking that, unless indicated otherwise, is a T.C. calibration. These flasks are used to create solutions with accurate and precise concentration. When filled to its calibration mark, a volumetric flask that contains less than 100 mL generally is accurate to the hundredth of a mL, whereas larger volumetric flasks are accurate to the tenth of a mL. For example, a 10-mL volumetric flask contains $10.00 \text{ mL} \pm 0.02 \text{ mL}$ and a 250-mL volumetric flask contains $250.0 \text{ mL} \pm 0.12 \text{ mL}$.



Figure 1.1.6 : A collection of volumetric flasks with volumes of 10 mL, 50 mL, 100 mL, 250 mL, and 500 mL.

To use a volumetric flask to create a solution, first transfer the reagent to the volumetric flask. Then add enough solvent to dissolve the reagent. Continue to add solvent in several portions, mixing thoroughly after each addition, and then adjust the volume to the flask's calibration mark using a dropper. Finally, complete the mixing process by inverting and shaking the flask at least 10 times (some recommend 20 times!).

How to use volumetric flasks



Correct Use of Volumetric (Class A) Glassware

Volumetric flasks and volumetric pipets fall into the category of "**Class A Glassware**". These are devices for creating solutions with highly accurate concentrations.

If you look closely at a volumetric pipet or a volumetric flask you will see markings similar to those shown in Figure 1.1.7 . The text of the markings in Figure 1.1.7 , reads:

10 mL T.D. at 20 °C $\pm$ 0.02 mL

This text indicates that the pipet is calibrated to deliver (T.D.) 10 mL of solution with an uncertainty of ± 0.02 mL at a temperature of 20 °C. The temperature is important because glass expands and contracts with changes in temperatures; thus, the pipet's accuracy is less than ± 0.02 mL at a higher or a lower temperature. For a more accurate result, you can calibrate your volumetric glassware at the temperature you are working by weighing the amount of water contained or delivered and calculating the volume using its temperature dependent density.



Figure 1.1.7 : Close-up of the 10-mL transfer pipet from Figure 1.1.3 .

Volumetric glassware uses the abbreviation T. C. for “to contain” and T. D. for "to deliver".

You should take three additional precautions when you work with pipets and volumetric flasks. First, the volume delivered by a pipet or contained by a volumetric flask assumes that the glassware is clean. Dirt and grease on the inner surface prevent liquids from draining evenly, leaving droplets of liquid on the container's walls. For a pipet this means the delivered volume is less than the calibrated volume, while drops of liquid above the calibration mark mean that a volumetric flask contains more than its calibrated volume. Commercially available cleaning solutions are available for cleaning pipets and volumetric flasks.

Second, when filling a pipet or volumetric flask the liquid's level must be set exactly at the calibration mark. The liquid's top surface is curved into a meniscus, the bottom of which should align with the glassware's calibration mark (Figure 1.1.8). When adjusting the meniscus, keep your eye in line with the calibration mark to avoid parallax errors. If your eye level is above the calibration mark you will overfill the pipet or the volumetric flask and you will underfill them if your eye level is below the calibration mark.

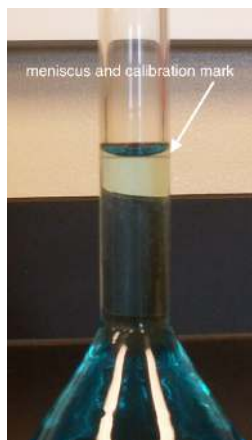


Figure 1.1.8 : Proper position of the solution's meniscus relative to the volumetric flask's calibration mark.

Finally, before using a pipet or volumetric flask rinse it with several small portions of the solution whose volume you are measuring. This ensures the removal of any residual liquid remaining in the pipet or volumetric flask.

Tolerance Limits Quick Reference

Tolerance limits on balances are assumed to be in the last digit of the digital reading. For other measuring devices, the tolerance limits are found below (and also in the [P-chem Lab manuals](#) (Characterizing Experimental Error Modules), the [Harvey Text](#), and elsewhere).

Tolerance Limits for Class A Volumetric Glassware

Table 3.2.1 : Measurement Errors for **Class A Volumetric Glassware**

Volumetric Pipets		Volumetric Flasks		Burets	
Capacity (mL)	Tolerance (mL)	Capacity (mL)	Tolerance (mL)	Capacity (mL)	Tolerance (mL)
1	± 0.006	5	± 0.02	10	± 0.02
2	± 0.006	10	± 0.02	25	± 0.03
5	± 0.01	25	± 0.03	50	± 0.05
10	± 0.02	50	± 0.05		
20	± 0.03	100	± 0.08		
25	± 0.03	250	± 0.12		
50	± 0.05	500	± 0.20		
100	± 0.08	1000	± 0.30		
		2000	± 0.50		

Tolerance limits for Digital Pipettes

Table 3.2.2 : Measurement Errors for Digital Pipets

Pipet Range	Volume (mL or μL)	Percent Measurement Error
10–100 μL	10	$\pm 3.0\%$
	50	$\pm 1.0\%$
	100	$\pm 0.8\%$
100–1000 μL	100	$\pm 3.0\%$
	500	$\pm 1.0\%$
	1000	$\pm 0.6\%$
1–10 mL	1	$\pm 3.0\%$
	5	$\pm 0.8\%$
	10	$\pm 0.6\%$

The tolerance values for the volumetric glassware in Table 3.2.1 are from the ASTM E288, E542, and E694 standards. The measurement errors for the digital pipets in Table 3.2.2 are from www.eppendorf.com.

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1.2: Preparing Solutions

Preparing a solution of known concentration is perhaps the most common activity in any analytical lab. The method for measuring out the solute and the solvent depend on the desired concentration and how exact the solution's concentration needs to be known. Pipets and volumetric flasks are used when we need to know a solution's exact concentration; graduated cylinders, beakers, and/or reagent bottles suffice only when *approximate* concentrations are OK. Two methods for preparing solutions are described in this section.

Preparing Stock Solutions

A **stock solution** is prepared by weighing out an appropriate portion of a pure solid or by measuring out an appropriate volume of a pure liquid, placing it in a suitable flask, and diluting to a known volume. Exactly how one measures the reagent depends on the desired concentration unit. For example, to prepare a solution with a known molarity (M), you weigh out an appropriate mass of the reagent, dissolve it in a portion of solvent, and bring it to the desired volume. To prepare a solution where the solute's concentration is a volume percent, you measure out an appropriate volume of solute and add sufficient solvent to obtain the desired total volume.

Estimating Uncertainty (Error) in a Stock Solution

Remember that chemistry lab course you took - CHEM301L or CHEM310L - where you spent two weeks learning to do error analysis, and then practiced using it on each experiment afterward? Time to dig up that knowledge again! The error associated with a stock solution is propagated from the error in the weighed mass of the solute and the error of the volume from the volumetric flask. The errors in mass (from an electronic balance) and volume measurements (from Class A Volumetric glassware or automatic pipettes) were discussed previously (In [CHEM301L](#) or [CHEM310L](#) - See Characterization of Experimental Error for the errors in measurements, and Propagation of Error for determining errors when measurements are used in a function).

? Exercise 1.2.1

Provide instructions for preparing 500 mL of 0.0125 M KBrO_3 and for calculating the error associated with the stock solution concentration. Assume you have a balance with $d=0.0005$ g and a 500 mL volumetric flask (Class A).

Answer

Preparing 500 mL of 0.0125 M KBrO_3 requires

$$0.5000 \text{ L} \times \frac{0.0125 \text{ mol KBrO}_3}{\text{L}} \times \frac{167.00 \text{ g KBrO}_3}{\text{mol KBrO}_3} = 1.04375 \approx 1.044 \text{ g KBrO}_3$$

Because the concentration has four significant figures, we must prepare the solution using volumetric glassware. The error associated with a Class A volumetric flask is ± 0.2 mL (see [Measurement Errors for Type A Volumetric Glassware](#) - these tables are also in the P-Chem Lab Manuals).

Instructions for preparation: Weigh a 1.044 g sample of KBrO_3 on an analytical balance. Record the actual mass weighed. Transfer the sample quantitatively to a clean 500-mL volumetric flask and fill part way with ultrapure water. Swirl to dissolve the KBrO_3 and then dilute with ultrapure water to the flask's calibration mark, and invert 20 times. Calculate the actual concentration based on the actual mass weighed.

Error: The error in mass is ± 0.0005 g and the error in volume is ± 0.2 mL or ± 0.0002 L. The function for calculating the concentration (C) from the mass and volume is a simple one:

$$C = \frac{\text{Mass (1.044 g)}}{\text{Volume (0.500 L)}}$$

The propagation of error can be approximated using a "shortcut": $\Delta(C) = C \times \sqrt{\frac{\Delta^2(\text{Volume})}{\text{Volume}^2} + \frac{\Delta^2(\text{Mass})}{\text{Mass}^2}}$

Let's assume the actual mass is 1.0440 g. Then, the calculated error is:

$$\Delta(C) = (0.0125\text{M}) \times \sqrt{\frac{(0.0002\text{L})^2}{(0.5000\text{L})^2} + \frac{(0.0005\text{g})^2}{(1.0440\text{g})^2}} = 0.00000779995 = 7.79995 \times 10^{-6} \text{ M}$$

The concentration would be reported as $0.012500 \pm 0.000008 \text{ M}$ or $12.500 \pm 0.008 \text{ mM}$

Preparing Solutions by Dilution

Solutions are often prepared by diluting a more concentrated stock solution. A known volume of the stock solution is transferred to a new container and brought to a new volume. Since the total amount of solute is the same before and after dilution, we know that

$$C_o \times V_o = C_d \times V_d \quad (1.2.1)$$

where C_o is the stock solution's concentration, V_o is the volume of stock solution being diluted, C_d is the dilute solution's concentration, and V_d is the volume of the dilute solution. Again, the type of glassware used to measure V_o and V_d depends on how precisely we need to know the solution's concentration.

Note that Equation 1.2.1 applies only to those concentration units that are expressed in terms of the solution's volume, including molarity, formality, normality, volume percent, and weight-to-volume percent. It also applies to weight percent, parts per million, and parts per billion if the solution's density is 1.00 g/mL. We cannot use Equation 1.2.1 if we express concentration in terms of molality as this is based on the mass of solvent, not the volume of solution. See Rodríguez-López, M.; Carrasquillo, A. *J. Chem. Educ.* **2005**, *82*, 1327-1328 for further discussion.

✓ Example 1.2.2

A laboratory procedure calls for 10 mL of a 0.0250 M solution of NH_3 . Provide instructions for how you would prepare this solution using a stock solution of concentrated NH_3 ($0.8002 \pm 0.0005 \text{ M}$). Report the error associated with the diluted concentration.

Answer

First of all - note that ammonia is a strong base; all SOP's should be followed carefully and PPE should be worn.

Substituting known volumes into Equation 1.2.1 gives the following:

$$0.8002 \text{ M} \times V_o = 0.0250 \text{ M} \times 10 \text{ mL} = 0.31242 \dots \text{mL}$$

To make this solution as precisely as possible, we could use a 10 mL volumetric flask and an automatic pipette. The errors in these analytical tools are $\pm 0.02 \text{ mL}$ for the flask and $\pm 1 \mu\text{L}$ for a 500 μL automatic pipette. (see [Measurement Errors for Type A Volumetric Glassware](#) - these tables are also in the P-Chem Lab Manuals).

Instructions for preparation: Put 312 μL of NH_3 into a 10.00 mL volumetric flask. Fill the flask part way with ultrapure water, and swirl to mix. Then dilute with ultrapure water to the flask's calibration mark, and invert 20 times.

Error: The error in the measured volumes are (± 0.02) mL for the flask and $\pm 1 \mu\text{L}$ for a 500 μL automatic pipette. The function for calculating C_d is:

$$C_d = \frac{C_o \times V_o}{V_d}$$

The propagation of error can be approximated using a "shortcut": $\Delta(C_d) = C_d \times \sqrt{\frac{\Delta^2(C_o)}{C_o^2} + \frac{\Delta^2(V_o)}{V_o^2} + \frac{\Delta^2(V_d)}{V_d^2}}$

$$\Delta(C_d) = 0.0250 \text{ M} \times \sqrt{\frac{(0.0005 \text{ M})^2}{(0.8002 \text{ M})^2} + \frac{(0.001 \text{ mL})^2}{(0.312 \text{ mL})^2} + \frac{(0.02 \text{ mL})^2}{(10.00 \text{ mL})^2}} = 0.000096 = 9.6 \times 10^{-5}$$

The concentration would be reported as $0.0250 \pm 0.0001 \text{ M}$ or $25.0 \pm 0.1 \text{ mM}$

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1.3: Methods of Calibration

Single Point Calibration vs Multiple-point Calibration

The simplest calibration is a single-point calibration using a standard. For example, using a standard solution of known concentration of component X , we could measure its signal (eg absorption), and then calculate the response (eg the ϵ_X at the wavelength of maximum absorbance).

Limitations to Single-point calibration

A single-point standardization is the least desirable approach for standardizing a method. There are two reasons for this. First, any error in our determination of the response, (eg ϵ_X), carries over into our calculation of concentration (C_X). Second, our experimental value for each response (eg ϵ) is based on a single concentration of analyte. To extend this value of ϵ to other concentrations of analyte requires that we assume a linear relationship between the signal and the analyte's concentration, **an assumption that often is not true** [Cardone, M. J.; Palmero, P. J.; Sybrandt, L. B. *Anal. Chem.* **1980**, 52, 1187–1191]. Figure 1.3.1 shows how assuming a constant value of response leads to a determinate error in concentration if the response becomes smaller at higher concentrations of analyte. Despite these limitations, single-point standardizations find routine use when the expected range for the analyte's concentrations is small.

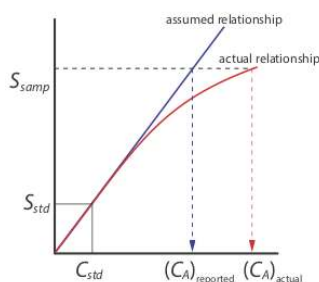


Figure 1.3.1 : Example showing how a single-point standardization leads to a determinate error in an analyte's reported concentration if we incorrectly assume that the response (slope) is constant. The **assumed** relationship between the sample signal (S_{samp}) and concentration of the analyte (C_A) is based on a single standard and is a straight-line; the **actual** relationship between S_{samp} and C_A becomes curved for larger concentrations of analyte.

Multi-point Calibration

The better way to standardize a method is to prepare a series of standards, each of which contains a different concentration of analyte. Standards are chosen such that they bracket the expected range for the analyte's concentration. A **multiple-point standardization** should include at least three standards, although more are preferable. A plot of the signal from the standards (S_{std} versus the concentrations of the standards (C_{std}) is called a **calibration curve**. The exact standardization, or calibration relationship, is determined by an appropriate curve-fitting algorithm.

There are two advantages to a multiple-point standardization. First, although a determinate error in one standard introduces a determinate error, its effect is minimized by the remaining standards. Second, because we measure the signal for several concentrations of analyte, we no longer must assume that the response (ϵ in this case) is independent of the analyte's concentration. Instead, we can construct a calibration curve similar to the “actual relationship” indicated in Figure 1.3.1.

External Calibration

The most common method of standardization uses one or more **external standards**, each of which contains a known concentration of analyte. We call these standards “external” because they are prepared and analyzed separate from the samples.

Figure 1.3.2 shows a typical **multiple-point external standardization**. The volumetric flask on the left contains a reagent blank and the remaining volumetric flasks contain increasing concentrations of Cu^{2+} . Shown below the volumetric flasks is the resulting calibration curve. Because this is the most common method of standardization, the resulting relationship is called a **normal calibration curve**.

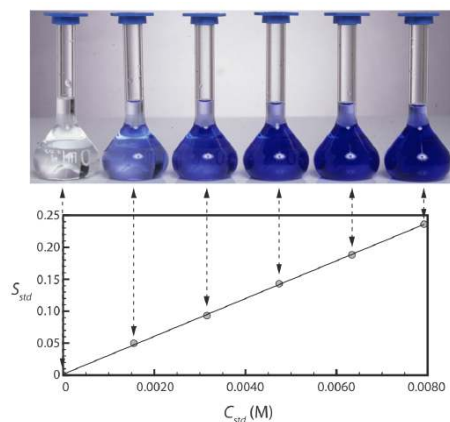


Figure 1.3.2 : The photo at the top of the figure shows a reagent blank (far left) and a set of five external standards for Cu^{2+} with concentrations that increase from left-to-right. Shown below the external standards is the resulting normal calibration curve. The absorbance signal of each standard, S_{std} is shown by the filled circles.

When a calibration curve is a straight-line, as it is in Figure 1.3.2 , the slope of the line gives the value of $b\epsilon$ (where b is path length) This is the most desirable situation because the method's sensitivity remains constant throughout the analyte's concentration range. When the calibration curve is not a straight-line, the method's sensitivity is a function of the analyte's concentration. In Figure 1.3.1 , for example, the value of $b\epsilon$ is greatest when the analyte's concentration is small and it decreases continuously for higher concentrations of analyte. The value of $b\epsilon$ at any point along the calibration curve in Figure 1.3.1 is the slope at that point. In either case, a calibration curve allows us to relate S_{samp} to the analyte's concentration.

Limitations to External Calibration

There is a serious limitation, however, to an external standardization. When we determine an analyte's response (ϵ) in this case) using external calibration, the analyte is present in the external standard's matrix; that is usually a much simpler matrix than that of "real" samples. When we use an external standardization we assume the matrix does not affect the value of the response (ϵ). **However, if the matrix does affect the response, we introduce a proportional determinate error into our analysis.** This is not the case in Figure 1.3.3 , for instance, where we show calibration curves for an analyte in the sample's matrix and in the standard's matrix. In this case, using the calibration curve for the external standards leads to a negative determinate error in analyte's reported concentration. If we expect that matrix effects are important, then we try to match the standard's matrix to that of the sample, a process known as **matrix matching**. If we are unsure of the sample's matrix, then we must show that matrix effects are negligible or use an alternative method of standardization. Both approaches are discussed in the following section.

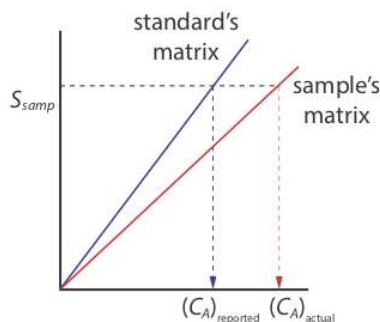


Figure 1.3.3 : Calibration curves for an analyte in the standard's matrix and in the sample's matrix. If the matrix affects the response (slope) as is the case here, then we introduce a proportional determinate error into our analysis if we use a normal calibration curve.

Validating an external calibration

Calibration by Standard Additions

We can avoid the complication of matching the matrix of the standards to the matrix of the sample if we carry out the standardization in the sample. This is known as the **method of standard additions**.

There are several methods of standard addition, and some are described in detail in [5.3: Determining the Sensitivity](#) by David Harvey. We will focus on standard addition by adding standard analyte solution directly to the sample, measuring the signal both before and after the spike (Figure 1.3.5). In this case the final volume after the standard addition is $V_o + V_{std}$ and the relationship between the absorbance and concentration of an analyte "X" becomes (the standard is a standard solution of the analyte X):

$$A_{sample} = \epsilon b C_X$$

$$A_{spiked} = \epsilon b \left(C_X \frac{V_o}{V_o + V_{std}} + C_{std} \frac{V_{std}}{V_o + V_{std}} \right) \quad (1.3.1)$$

$$\frac{A_{sample}}{C_X} = \frac{A_{spiked}}{C_X \frac{V_o}{V_o + V_{std}} + C_{std} \frac{V_{std}}{V_o + V_{std}}} \quad (1.3.2)$$

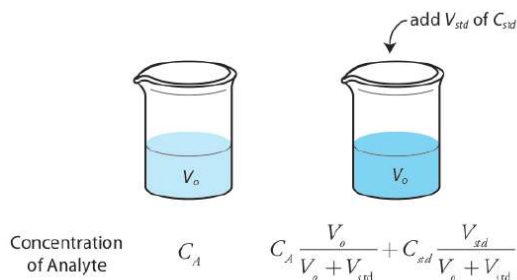


Figure 1.3.5 : Illustration showing an alternative form of the method of standard additions. In this case we add the spike of external standard directly to the sample without any further adjust in the volume.

Multiple Standard Additions

We can adapt a single-point standard addition into a multiple-point standard addition by preparing a series of samples that contain increasing amounts of the external standard. Figure 1.3.6 shows two ways to plot a standard addition calibration curve based on equation 1.3.1. In Figure 1.3.6, plot (a) we plot A_{spike} (or more generally S_{spike}) against the volume of the spikes, V_{std} . In plot (b) we plot $C_{std} \times \frac{V_{std}}{V_f}$ on the x axis instead (where $V_f = V_o + V_{std}$ is total volume after the spike). If k_A is constant, then the calibration curve is a straight line in both cases. It is easy to show that the slope is related to the response of the analyte in the given matrix (k_A and that x-intercept is related to the concentration of the analyte C_A ; the x-intercept is equivalent to $\frac{-C_A V_o}{C_{std}}$ in the case of plot (a) or $\frac{-C_A V_o}{V_f}$ in the case of plot (b) (see Figure 1.3.6).

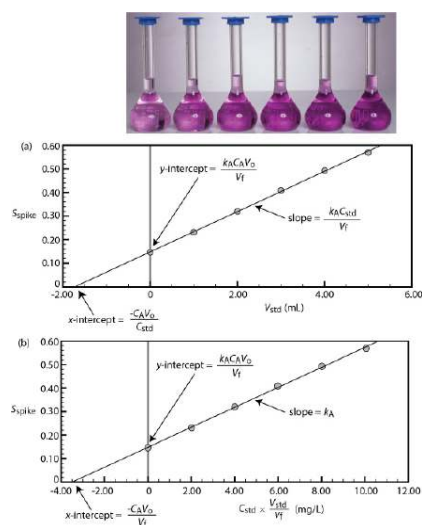


Figure 1.3.6 : Shown at the top of the figure is a set of six standard additions for the determination of Mn^{2+} . The flask on the left is a 25.00 mL sample diluted to 50.00 mL with water. The remaining flasks contain 25.00 mL of sample and, from left-to-right, 1.00, 2.00, 3.00, 4.00, and 5.00 mL spikes of an external standard that is 100.6 mg/L Mn^{2+} . Shown below are two ways to plot the standard additions calibration curve. The absorbance for each standard addition, S_{spike} , is shown by the filled circles.

For Mixtures with overlapping signals: The two-analyte Generalized Standard Addition Method (GSAM)

Suppose we need to determine the concentration of two analytes, X and Y , that are mixed in a sample. If each analyte has a wavelength where the other analyte does not absorb, then we can proceed using Beer's Law to determine the concentration. Unfortunately, UV/Vis absorption bands are so broad that it is sometimes impossible to find suitable wavelengths. Because Beer's law is additive the mixture's absorbance, A_{mix} , at a given wavelength will be a sum of the absorbance of each analyte:

$$(A_{\text{mix}})_{\lambda_1} = (\varepsilon_X)_{\lambda_1} b C_X + (\varepsilon_Y)_{\lambda_1} b C_Y \quad (1.3.3)$$

where λ_1 is the wavelength at which we measure the absorbance. Because Equation 1.3.3 includes terms for the concentration of both X and Y , the absorbance at one wavelength does not provide enough information to determine either C_X or C_Y . If we measure the absorbance at a second wavelength, λ_2

$$(A_{\text{mix}})_{\lambda_2} = (\varepsilon_X)_{\lambda_2} b C_X + (\varepsilon_Y)_{\lambda_2} b C_Y \quad (1.3.4)$$

then we can determine C_X and C_Y by solving simultaneously Equation 1.3.3 and Equation 1.3.4. Of course, we also must determine the value for ε_X and ε_Y at each wavelength. For a mixture of n components, we must measure the absorbance at n different wavelengths.

✓ Example 1.3.1

The concentrations of Fe^{3+} and Cu^{2+} in a mixture are determined following their reaction with hexacyanoruthenate (II), $\text{Ru}(\text{CN})_6^{4-}$, which forms a purple-blue complex with Fe^{3+} ($\lambda_{\text{max}} = 550 \text{ nm}$) and a pale-green complex with Cu^{2+} ($\lambda_{\text{max}} = 396 \text{ nm}$) [DiTusa, M. R.; Schlitt, A. A. *J. Chem. Educ.* **1985**, 62, 541–542]. The molar absorptivities ($\text{M}^{-1} \text{cm}^{-1}$) for the solutions of the individual metal complexes at the two wavelengths are summarized in the following table.

analyte	ε_{550}	ε_{396}
Fe^{3+} standard	9970	84
Cu^{2+} standard	34	856

When a sample that contains Fe^{3+} and Cu^{2+} is analyzed in a cell with a pathlength of 1.00 cm, the absorbance at 550 nm is 0.183 and the absorbance at 396 nm is 0.109. What are the molar concentrations of Fe^{3+} and Cu^{2+} in the sample?

Solution

Substituting known values into Equation 1.3.3 and Equation 1.3.4 gives

$$A_{550} = 0.183 = 9970C_{\text{Fe}} + 34C_{\text{Cu}}$$

$$A_{396} = 0.109 = 84C_{\text{Fe}} + 856C_{\text{Cu}}$$

To determine C_{Fe} and C_{Cu} we solve the first equation for C_{Cu}

$$C_{\text{Cu}} = \frac{0.183 - 9970C_{\text{Fe}}}{34}$$

and substitute the result into the second equation.

$$\begin{aligned} 0.109 &= 84C_{\text{Fe}} + 856 \times \frac{0.183 - 9970C_{\text{Fe}}}{34} \\ &= 4.607 - (2.51 \times 10^5) C_{\text{Fe}} \end{aligned}$$

Solving for C_{Fe} gives the concentration of Fe^{3+} as 1.8×10^{-5} M. Substituting this concentration back into the equation for the mixture's absorbance at 396 nm gives the concentration of Cu^{2+} as 1.3×10^{-4} M.

Let's consider the example of a mixed Co and Cr solution. To obtain results with good accuracy and precision **the two wavelengths should be selected so that $\epsilon_X > \epsilon_Y$ at one wavelength and $\epsilon_X < \epsilon_Y$ at the other wavelength**. It is easy to appreciate why this is true. Because the absorbance at each wavelength is dominated by one analyte, any uncertainty in the concentration of the other analyte has less of an impact. Figure 1.3.1 shows the spectra of a Cr standard and a Co standard, as well as a spectrum of the mixture. From the spectra of the standards, we can see that 400 nm is a reasonable choice for one of the wavelengths because it is a point of maximum absorption for Cr^{3+} , and $\epsilon_{\text{Cr}} > \epsilon_{\text{Co}}$. A reasonable choice for a second wavelength is 505 nm; it is a point of maximum absorbance for Co^{2+} where $\epsilon_{\text{Co}} > \epsilon_{\text{Cr}}$. These two wavelengths are used in Practice Exercise 1.3.1 (below). When the choice of wavelengths is not obvious, one method for locating the optimum wavelengths is to plot ϵ_X/ϵ_Y as function of wavelength, and determine the wavelengths where ϵ_X/ϵ_Y reaches maximum and minimum values [Mehra, M. C.; Rioux, J. J. *Chem. Educ.* **1982**, 59, 688–689].

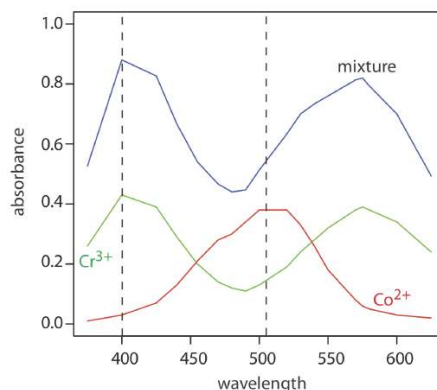


Figure 1.3.1 . Visible absorption spectra for 0.0250 M Cr^{3+} , 0.0750 M Co^{2+} , and for a mixture of Cr^{3+} and Co^{2+} . The two wavelengths used to analyze the mixture of Cr^{3+} and Co^{2+} are shown by the dashed lines. The data for the two standards are from Brewer, S. *Solving Problems in Analytical Chemistry*, John Wiley & Sons: New York, 1980.

? Exercise 1.3.1

The absorbance spectra for Cr^{3+} and Co^{2+} overlap significantly. To determine the concentration of these analytes in a mixture, its absorbance is measured at 400 nm and at 505 nm, yielding values of 0.336 and 0.187, respectively. The individual molar absorptivities ($\text{M}^{-1} \text{cm}^{-1}$) for Cr^{3+} are 15.2 at 400 nm and 0.533 at 505 nm; the values for Co^{2+} are 5.60 at 400 nm and 5.07 at 505 nm.

Answer

Substituting into Equation 1.3.3 and Equation 1.3.4 gives

$$A_{400} = 0.336 = 15.2C_{\text{Cr}} + 5.60C_{\text{Co}}$$

$$A_{400} = 0.187 = 0.533C_{Cr} + 5.07C_{Co}$$

To determine C_{Cr} and C_{Co} we solve the first equation for C_{Co}

$$C_{Co} = \frac{0.336 - 15.2C_{Cr}}{5.60}$$

and substitute the result into the second equation.

$$0.187 = 0.533C_{Cr} + 5.07 \times \frac{0.336 - 15.2C_{Cr}}{5.60}$$

$$0.187 = 0.3042 - 13.23C_{Cr}$$

Solving for C_{Cr} gives the concentration of Cr^{3+} as 8.86×10^{-3} M. Substituting this concentration back into the equation for the mixture's absorbance at 400 nm gives the concentration of Co^{2+} as 3.60×10^{-2} M.

We can extend the standard addition method to quantitate multiple components of a mixture using the **Generalized Standard Addition Method (GSAM)**. This is the preferred method of calibration when quantitation of multiple components are necessary and matrix effects are suspected. This is the method you will use to quantitate two components of a mixture (eg Co and Cr) that have overlapping spectral features (see Figure 3.4.1) and when matrix effects are suspected. For a sample with components X and Y, the initial absorbance of the sample at λ_1 is:

$$A_{\lambda_1}^0 = \varepsilon_{X_{\lambda_1}} b C_X^0 + \varepsilon_{Y_{\lambda_1}} b C_Y^0 \quad (1.3.5)$$

Where $\varepsilon_{X_{\lambda_1}}$ is the response factor (aka sensitivity, molar extinction coefficient, or the molar absorptivity) for component X at λ_1 , and $\varepsilon_{Y_{\lambda_1}}$ is the response factor for component Y at λ_1 .

In this method, the volume of total sample will change as standard solution of X or Y is added to the sample to create the standard addition calibration for X and Y. When volume changes, concentrations are not additive. But we can correct for this inconvenient truth by multiplying by total volume, V_t . When C is multiplied by V, the result is total moles (n) of each analyte; this value is additive. Multiplying by V gives the equation below.

$$V^0 A_{\lambda_1}^0 = \varepsilon_{X_{\lambda_1}} b n_X^0 + \varepsilon_{Y_{\lambda_1}} b n_Y^0 \quad (1.3.6)$$

As standards are added to the sample to create the calibration, the moles of analyte will change by Δn_x and Δn_y , and the new volume-corrected absorption for any point on the calibration would be:

$$V^i A_{\lambda_1}^i = \varepsilon_{X_{\lambda_1}} b (\Delta n_x + n_X^0) + \varepsilon_{Y_{\lambda_1}} b (\Delta n_Y + n_Y^0) \quad (1.3.7)$$

where Δn_X is the moles of X added and n_X^0 is the initial amount of analyte in the sample. The same follows for analyte Y.

The initial volume-corrected absorption, $V^0 A^0$ (Equation 1.3.6) can be factored from Equation 1.3.7 to give the change in absorbance upon standard addition:

$$\Delta(V A_{\lambda_1}) = V^i A_{\lambda_1}^i - V^0 A_{\lambda_1}^0 = \varepsilon_{X_{\lambda_1}} b (\Delta n_X) + \varepsilon_{Y_{\lambda_1}} b (\Delta n_Y) \quad (1.3.8)$$

For a mixture of two analytes, absorbance would be measured at two wavelengths as described above. The same model described here for λ_1 , also applies for λ_2 :

$$\Delta(V A_{\lambda_2}) = V^i A_{\lambda_2}^i - V^0 A_{\lambda_2}^0 = \varepsilon_{X_{\lambda_2}} b (\Delta n_X) + \varepsilon_{Y_{\lambda_2}} b (\Delta n_Y) \quad (1.3.9)$$

Using a known volume of the sample, we can add known amounts of one analyte to the solution to create a calibration line with at least 3 points. For example, suppose we have a sample containing two analytes, X, and Y in a complex matrix where matrix effects are unknown; and suppose we want to determine the concentrations of [X] and [Y] in the sample. This is a problem that can be solved by GSAM. First, we would obtain standard solutions of X and Y, collect their absorption spectra, and select appropriate wavelengths for analysis. Then, we would collect a spectrum of the analyte solution containing unknown amounts of X and Y.

To create a standard curve for X, we could add a standard solution of X into a known quantity of the sample while monitoring the solution mixture at the two wavelengths. Under these conditions, Δn_Y is zero, and so equation 1.3.8 is simplified to a

$\Delta(VA_{\lambda_1}) = \varepsilon_{X_{\lambda_1}} b(\Delta n_X)$; this is the form of a linear equation with the slope that is $\varepsilon_{X_{\lambda_1}} b$. By plotting $\Delta(VA_{\lambda_1})$ at one wavelength vs Δn_X we can determine the molar response factor of X at that wavelength (ε_{λ_1}). And, by plotting $\Delta(VA_{\lambda_2})$ at the second wavelength vs Δn_X we can determine the molar response factor of X at the second wavelength (ε_{λ_2}).

Then, using the same sample as above, we can add two aliquots of Y and follow a similar strategy plotting Δn_Y against $\Delta(VA_{\lambda_1})$ and $\Delta(VA_{\lambda_2})$ to find the response factors of Y at the two wavelengths.

Once the response factors of both analytes X and Y are determined at the two different wavelengths, the concentrations of each analyte in the original sample can be determined by simultaneously solving equation 1.3.5 and the analogous equation at λ_2 :

$$A_{\lambda_1}^0 = \varepsilon_{X_{\lambda_1}} bC_X^0 + \varepsilon_{Y_{\lambda_1}} bC_Y^0 \quad \text{and} \quad A_{\lambda_2}^0 = \varepsilon_{X_{\lambda_2}} bC_X^0 + \varepsilon_Y bC_{Y_{\lambda_2}}^0 \quad (1.3.10)$$

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1.4: Uncertainty in values determined from a Calibration Curve

You learned how to propagate error in the prerequisite laboratory courses, [CHEM301L](#) or [CHEM310L](#); thus, if you are here, we expect you know the sources of experimental error and how to propagate of errors in measurements (review as necessary). **However, when we determine an analyte concentration from a calibration curve (a linear regression), a simple propagation of error is not sufficient.** You can read more about this in [5.4: Linear Regression and Calibration Curves](#) by [David Harvey](#). A brief summary is below.

Obtaining the Analyte's Concentration From a Regression Equation and determining error

When a calibration curve is a straight-line, we represent it using the following mathematical equation

$$y = b + mx \quad (1.4.1)$$

where y is the analyte's signal, S_{std} , and x is the analyte's concentration, C_{std} . The constants b and m are, respectively, the calibration curve's expected y -intercept and its expected slope. An example of such a calibration curve is shown below in the figure.

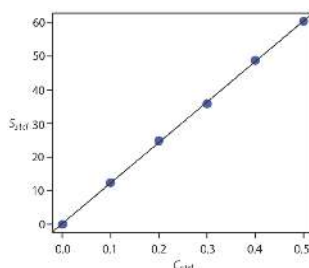


Figure 1.4.1 : Calibration curve for the data in [Table 1.4.1](#) and Example 1.4.1 .

Once we have our regression equation, it is easy to determine the concentration of analyte in a sample. When we use a normal calibration curve, for example, we measure the signal for our sample, S_{samp} , and calculate the analyte's concentration, C_A , using the regression equation.

$$C_A = \frac{S_{samp} - b}{m} \quad (1.4.2)$$

What is less obvious is how to report a confidence interval for C_A that expresses the uncertainty in our analysis. To calculate a confidence interval we need to know the **standard deviation in the analyte's concentration, s_{C_A} , which is given by the following equation**

$$s_{C_A} = \frac{s_r}{|m|} \sqrt{\frac{1}{k} + \frac{1}{n} + \frac{(S_{samp} - \bar{S}_{std})^2}{m^2 \sum_{i=1}^n (C_{std_i} - \bar{C}_{std})^2}} \quad (1.4.3)$$

where k is the number of replicate measurements we use to establish the sample's average signal, S_{samp} , n is the number of calibration standards (the number of points in the calibration curve), S_{std} is the average signal for the calibration standards, and C_{std_i} and $\bar{C}_{std}$ are the individual and the mean concentrations for the calibration standards. Knowing the value of s_{C_A} , the confidence interval for the analyte's concentration is

$$\mu_{C_A} = C_A \pm t s_{C_A}$$

where μ_{C_A} is the expected value of C_A in the absence of determinate errors, and with the value of t is based on the desired level of confidence and $n - 2$ degrees of freedom.

Equation 1.4.3 is written in terms of a calibration experiment. A more general form of the equation, written in terms of x and y , is given below.

$$s_x = \frac{s_y}{|m|} \sqrt{\frac{1}{k} + \frac{1}{n} + \frac{(y - \bar{y})^2}{m^2 \sum_{i=1}^n (x_i - \bar{x})^2}} \quad (1.4.4)$$

The same equation with all the variables labeled is shown in Figure 1.4.3.

A close examination of Equation 1.4.3 should convince you that the uncertainty in C_A is smallest when the sample's average signal, $\bar{S}_{samp}$, is equal to the average signal for the standards, $\bar{S}_{std}$. When practical, you should plan your calibration curve so that S_{samp} falls in the middle of the calibration curve. For more information about these regression equations see (a) Miller, J. N. *Analyst* **1991**, *116*, 3–14; (b) Sharaf, M. A.; Illman, D. L.; Kowalski, B. R. *Chemometrics*, Wiley-Interscience: New York, 1986, pp. 126–127; (c) Analytical Methods Committee “Uncertainties in concentrations estimated from calibration experiments,” *AMC Technical Brief*, March 2006.

✓ Example 1.4.3

Three replicate analyses for a sample that contains an unknown concentration of analyte, yield values for S_{samp} of 29.32, 29.16 and 29.51 (arbitrary units). Using the results from Example 1.4.1 and Example 1.4.2, determine the analyte's concentration, C_A , and its 95% confidence interval.

Solution

The average signal, $\bar{S}_{samp}$, is 29.33, which, using Equation 1.4.2 and the slope and the y-intercept from Example 1.4.1, gives the analyte's concentration as

$$C_A = \frac{\bar{S}_{samp} - b}{m} = \frac{29.33 - 0.209}{120.706} = 0.241$$

To calculate the standard deviation for the analyte's concentration we must determine the values for $\bar{S}_{std}$ and for $\sum_{i=1}^2 (C_{std_i} - \bar{C}_{std})^2$. The former is just the average signal for the calibration standards, which, using the data in Table 1.4.1, is 30.385. Calculating $\sum_{i=1}^2 (C_{std_i} - \bar{C}_{std})^2$ looks formidable, but we can simplify its calculation by recognizing that this sum-of-squares is the numerator in a standard deviation equation; thus,

$$\sum_{i=1}^n (C_{std_i} - \bar{C}_{std})^2 = (s_{C_{std}})^2 \times (n - 1)$$

where $s_{C_{std}}$ is the standard deviation for the concentration of analyte in the calibration standards. Using the data in Table 1.4.1 we find that $s_{C_{std}}$ is 0.1871 and

$$\sum_{i=1}^n (C_{std_i} - \bar{C}_{std})^2 = (0.1872)^2 \times (6 - 1) = 0.175$$

Substituting known values into Equation 1.4.3 gives

$$s_{C_A} = \frac{0.4035}{120.706} \sqrt{\frac{1}{3} + \frac{1}{6} + \frac{(29.33 - 30.385)^2}{(120.706)^2 \times 0.175}} = 0.0024$$

Finally, the 95% confidence interval for 4 degrees of freedom is

$$\mu_{C_A} = C_A \pm t s_{C_A} = 0.241 \pm (2.78 \times 0.0024) = 0.241 \pm 0.007$$

Figure 1.4.2 shows the calibration curve with curves showing the 95% confidence interval for C_A .

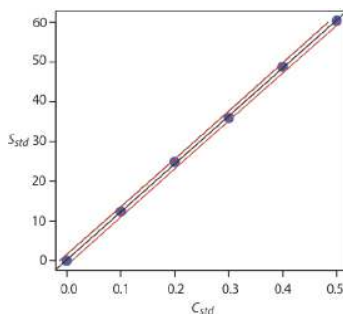


Figure 1.4.2 : Example of a normal calibration curve with a superimposed confidence interval for the analyte's concentration. The points in blue are the original data from Table 1.4.1 . The black line is the normal calibration curve as determined in Example 1.4.1 . The red lines show the 95% confidence interval for C_A assuming a single determination of S_{sample} .

In a standard addition we determine the analyte's concentration by extrapolating the calibration curve to the x -intercept. In this case the value of C_A is

$$C_A = x\text{-intercept} = \frac{-b}{m}$$

and the standard deviation in C_A is

$$s_{C_A} = \frac{s_r}{m} \sqrt{\frac{1}{n} + \frac{(\bar{S}_{std})^2}{(m)^2 \sum_{i=1}^n (C_{std_i} - \bar{C}_{std})^2}}$$

where n is the number of standard additions (including the sample with no added standard), and $\bar{S}_{std}$ is the average signal for the n standards. Because we determine the analyte's concentration by extrapolation, rather than by interpolation, s_{C_A} for the method of standard additions generally is larger than for a normal calibration curve.

? Exercise 1.4.1

Figure 1.4.2 shows a normal calibration curve for the quantitative analysis of Cu^{2+} . The data for the calibration curve are shown here.

$[\text{Cu}^{2+}] \text{ (M)}$	Absorbance
0	0
1.55×10^{-3}	0.050
3.16×10^{-3}	0.093
4.74×10^{-3}	0.143
6.34×10^{-3}	0.188
7.92×10^{-3}	0.236

Complete a linear regression analysis for this calibration data, reporting the calibration equation and the 95% confidence interval for the slope and the y -intercept. If three replicate samples give an S_{sample} of 0.114, what is the concentration of analyte in the sample and its 95% confidence interval?

Answer

We begin by setting up a table to help us organize the calculation

x_i	y_i	$x_i y_i$	x_i^2
0.000	0.000	0.000	0.000
1.55×10^{-3}	0.050	7.750×10^{-5}	2.403×10^{-6}

x_i	y_i	$x_i y_i$	x_i^2
3.16×10^{-3}	0.093	2.939×10^{-4}	9.986×10^{-6}
4.74×10^{-3}	0.143	6.778×10^{-4}	2.247×10^{-5}
6.34×10^{-3}	0.188	1.192×10^{-3}	4.020×10^{-5}
7.92×10^{-3}	0.236	1.869×10^{-3}	6.273×10^{-5}

Adding the values in each column gives

$$\sum_{i=1}^n x_i = 2.371 \times 10^{-2} \quad \sum_{i=1}^n y_i = 0.710 \quad \sum_{i=1}^n x_i y_i = 4.110 \times 10^{-3} \quad \sum_{i=1}^n x_i^2 = 1.378 \times 10^{-4}$$

When we substitute these values into Equation ??? and Equation ???, we find that the slope and the y-intercept are

$$m = \frac{6 \times (4.110 \times 10^{-3}) - (2.371 \times 10^{-2}) \times 0.710}{6 \times (1.378 \times 10^{-4}) - (2.371 \times 10^{-2})^2} = 29.57$$

$$b = \frac{0.710 - 29.57 \times (2.371 \times 10^{-2})}{6} = 0.0015$$

and that the regression equation is

$$S_{std} = 29.57 \times C_{std} + 0.0015$$

To calculate the 95% confidence intervals, we first need to determine the standard deviation about the regression. The following table helps us organize the calculation.

x_i	y_i	$\hat{y}_i$	$(y_i - \hat{y}_i)^2$
0.000	0.000	0.0015	2.250×10^{-6}
1.55×10^{-3}	0.050	0.0473	7.110×10^{-6}
3.16×10^{-3}	0.093	0.0949	3.768×10^{-6}
4.74×10^{-3}	0.143	0.1417	1.791×10^{-6}
6.34×10^{-3}	0.188	0.1890	9.483×10^{-6}
7.92×10^{-3}	0.236	0.2357	9.339×10^{-6}

Adding together the data in the last column gives the numerator of Equation ??? as 1.596×10^{-5} . The standard deviation about the regression, therefore, is

$$s_r = \sqrt{\frac{1.596 \times 10^{-5}}{6 - 2}} = 1.997 \times 10^{-3}$$

Next, we need to calculate the standard deviations for the slope and the y-intercept using Equation ??? and Equation ???.

$$s_m = \sqrt{\frac{6 \times (1.997 \times 10^{-3})^2}{6 \times (1.378 \times 10^{-4}) - (2.371 \times 10^{-2})^2}} = 0.3007$$

$$s_b = \sqrt{\frac{(1.997 \times 10^{-3})^2 \times (1.378 \times 10^{-4})}{6 \times (1.378 \times 10^{-4}) - (2.371 \times 10^{-2})^2}} = 1.441 \times 10^{-3}$$

and use them to calculate the 95% confidence intervals for the slope and the y-intercept

$$m = m \pm t s_m = 29.57 \pm (2.78 \times 0.3007) = 29.57 \text{ M}^{-1} \pm 0.84 \text{ M}^{-1}$$

$$b = b \pm ts_b = 0.0015 \pm (2.78 \times 1.441 \times 10^{-3}) = 0.0015 \pm 0.0040$$

With an average S_{samp} of 0.114, the concentration of analyte, C_A , is

$$C_A = \frac{S_{\text{samp}} - b}{m} = \frac{0.114 - 0.0015}{29.57 \text{ M}^{-1}} = 3.80 \times 10^{-3} \text{ M}$$

The standard deviation in C_A is

$$s_{C_A} = \frac{1.997 \times 10^{-3}}{29.57} \sqrt{\frac{1}{3} + \frac{1}{6} + \frac{(0.114 - 0.1183)^2}{(29.57)^2 \times (4.408 \times 10^{-5})}} = 4.778 \times 10^{-5}$$

and the 95% confidence interval is

$$\begin{aligned} \mu &= C_A \pm ts_{C_A} = 3.80 \times 10^{-3} \pm \{2.78 \times (4.778 \times 10^{-5})\} \\ \mu &= 3.80 \times 10^{-3} \text{ M} \pm 0.13 \times 10^{-3} \text{ M} \end{aligned}$$

Automate analysis using a calibration curve

You will create many calibration curves this semester, and you'll need to perform quality control checks to know whether your calibration curve is reasonable. The process of building a calibration curve, doing error analysis, and performing quality controls can become much more efficient when you build an automated tool to help you. In this example, we will use Excel. Below is an example of an excel spreadsheet template that you could build. Your TA's can help you through the creating of such a template. Alternatively, you could build your own script in MatLab or another data analysis software of your choice to perform the same functions. You can download an example to help get you started here: ([Link for Duke Students](#)).

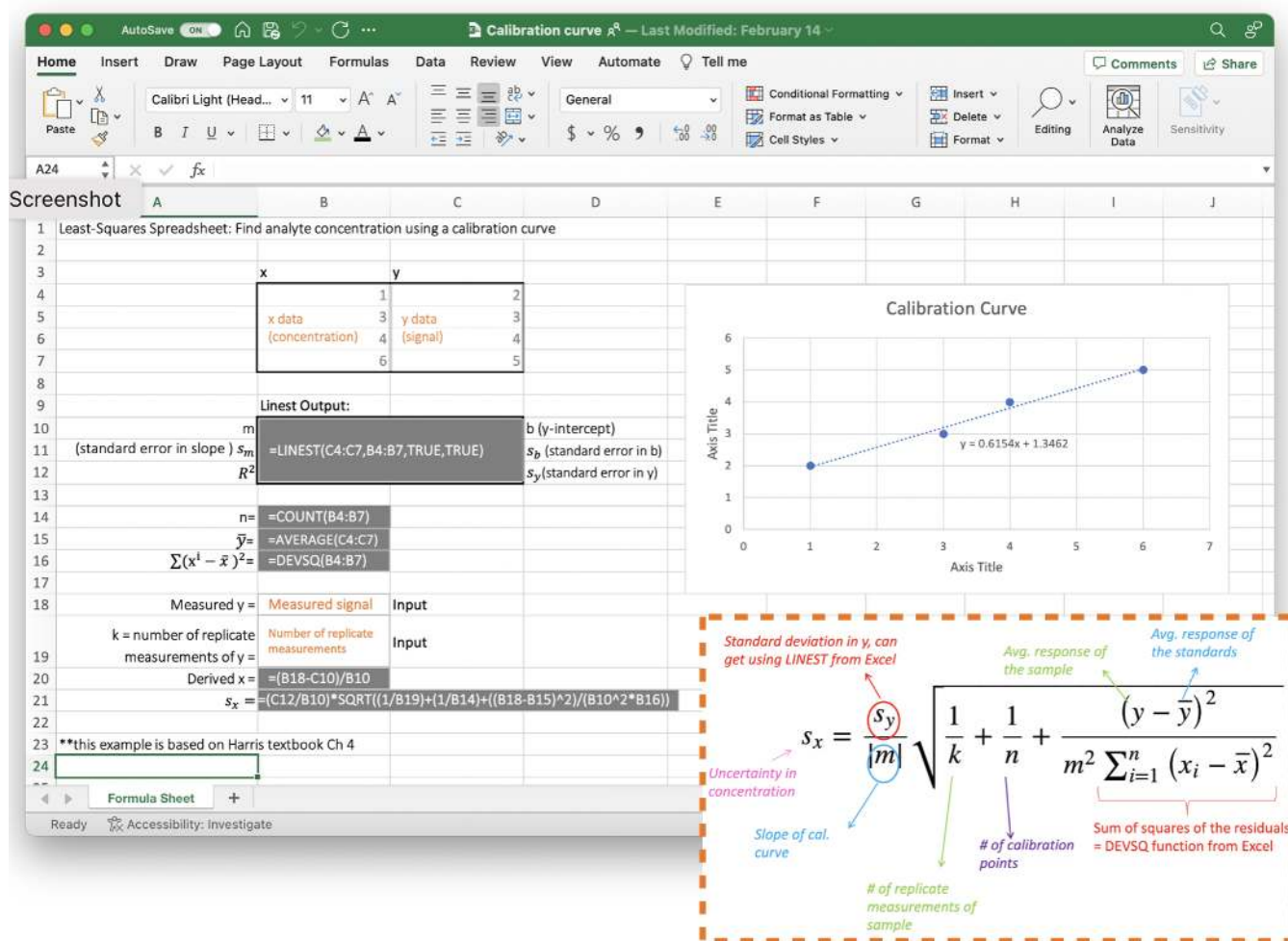


Figure 1.4.3: This example was inspired by Harris's Qualitative Chemical Analysis textbook, Figure 4-15. After entering the correct formulas, you should find that the Derived x = 2.2325 and the s_x is 0.3735. (Kathryn Haas; CC-BY-SA)

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CHAPTER OVERVIEW

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2.1: Absorbance and Beer's Law

2.2: UV/Vis Instrumentation

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2.1: Absorbance and Beer's Law

In absorption spectroscopy a beam of electromagnetic radiation passes through a sample. Much of the radiation passes through the sample without a loss in intensity. At some wavelengths, however, the radiation's intensity is attenuated. This process of attenuation is called absorption.

Absorption Spectra

There are two general requirements for an analyte's absorption of electromagnetic radiation. First, there must be a mechanism by which the radiation's electric field or magnetic field interacts with the analyte. For ultraviolet and visible radiation, absorption of a photon changes the energy of the analyte's valence electrons. A bond's vibrational energy is altered by the absorption of infrared radiation.

Figure 10.1.3 provides a list of the types of atomic and molecular transitions associated with different types of electromagnetic radiation.

The second requirement is that the photon's energy, $h\nu$, must exactly equal the difference in energy, ΔE , between two of the analyte's quantized energy states. Figure 10.1.4 shows a simplified view of a photon's absorption, which is useful because it emphasizes that the photon's energy must match the difference in energy between a lower-energy state and a higher-energy state. What is missing, however, is information about what types of energy states are involved, which transitions between energy states are likely to occur, and the appearance of the resulting spectrum.

We can use the energy level diagram in Figure 2.1.1 to explain an absorbance spectrum. The lines labeled E_0 and E_1 represent the analyte's ground (lowest) electronic state and its first electronic excited state. Superimposed on each electronic energy level is a series of lines representing vibrational energy levels.

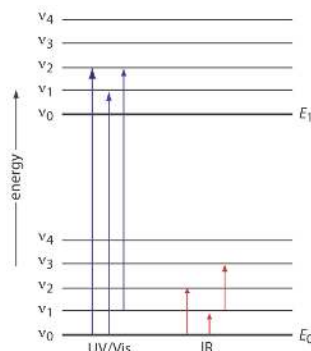


Figure 2.1.1 . Diagram showing two electronic energy levels (E_0 and E_1), each with five vibrational energy levels ν_0 – ν_4 . Absorption of ultraviolet and visible radiation (shown by the blue arrows) leads to a change in the analyte's electronic energy levels and, possibly, a change in vibrational energy as well. A change in vibrational energy without a change in electronic energy levels occurs with the absorption of infrared radiation (shown by the red arrows).

Infrared Spectra for Molecules and Polyatomic Ions

The energy of infrared radiation produces a change in a molecule's or a polyatomic ion's vibrational energy, but is not sufficient to effect a change in its electronic energy. As shown in Figure 2.1.1 , vibrational energy levels are quantized; that is, a molecule or polyatomic ion has only certain, discrete vibrational energies. The energy for an allowed vibrational mode, E_ν , is

$$E_\nu = \left(\nu + \frac{1}{2}\right)h\nu_0$$

where ν is the vibrational quantum number, which has values of 0, 1, 2, ..., and ν_0 is the bond's fundamental vibrational frequency. The value of ν_0 , which is determined by the bond's strength and by the mass at each end of the bond, is a characteristic property of a bond. For example, a carbon-carbon single bond (C–C) absorbs infrared radiation at a lower energy than a carbon-carbon double bond (C=C) because a single bond is weaker than a double bond.

At room temperature most molecules are in their ground vibrational state ($\nu = 0$). A transition from the ground vibrational state to the first vibrational excited state ($\nu = 1$) requires absorption of a photon with an energy of $h\nu_0$. Transitions in which $\Delta\nu = \pm 1$ give rise to the fundamental absorption lines. Weaker absorption lines, called overtones, result from transitions in which $\Delta\nu$ is ± 2 or ± 3 . The number of possible normal vibrational modes for a linear molecule is $3N - 5$, and for a non-linear molecule is $3N - 6$, where N is the number of atoms in the molecule. Not surprisingly, infrared spectra often show a considerable number of absorption bands. Even a relatively simple molecule, such as ethanol ($\text{C}_2\text{H}_6\text{O}$), for example, has $3 \times 9 - 6$, or 21 possible normal modes of vibration, although not all of these vibrational modes give rise to an absorption. The IR spectrum for ethanol is shown in Figure 2.1.2.

Why does a non-linear molecule have $3N - 6$ vibrational modes? Consider a molecule of methane, CH_4 . Each of methane's five atoms can move in one of three directions (x , y , and z) for a total of $5 \times 3 = 15$ different ways in which the molecule's atoms can move. A molecule can move in three ways: it can move from one place to another, which we call translational motion; it can rotate around an axis, which we call rotational motion; and its bonds can stretch and bend, which we call vibrational motion. Because the entire molecule can move in the x , y , and z directions, three of methane's 15 different motions are translational. In addition, the molecule can rotate about its x , y , and z axes, accounting for three additional forms of motion. This leaves $15 - 3 - 3 = 9$ vibrational modes. A linear molecule, such as CO_2 , has $3N - 5$ vibrational modes because it can rotate around only two axes.

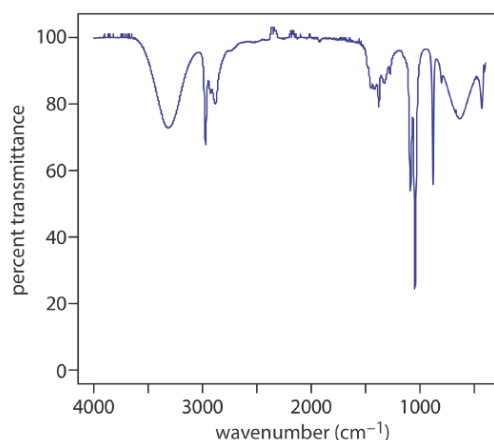


Figure 2.1.2. Infrared spectrum of ethanol.

UV/Vis Spectra for Molecules and Ions

The valence electrons in organic molecules and polyatomic ions, such as CO_3^{2-} , occupy quantized sigma bonding (σ), pi bonding (π), and non-bonding (n) molecular orbitals (MOs). Unoccupied sigma antibonding (σ^*) and pi antibonding (π^*) molecular orbitals are slightly higher in energy. Because the difference in energy between the highest-energy occupied MOs and the lowest-energy unoccupied MOs corresponds to ultraviolet and visible radiation, absorption of a photon is possible.

Four types of transitions between quantized energy levels account for most molecular UV/Vis spectra. Table 2.1.1 lists the approximate wavelength ranges for these transitions, as well as a partial list of bonds, functional groups, or molecules responsible for these transitions. Of these transitions, the most important are $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ because they involve important functional groups that are characteristic of many analytes and because the wavelengths are easily accessible. The bonds and functional groups that give rise to the absorption of ultraviolet and visible radiation are called **chromophores**.

Table 2.1.1. Electronic Transitions Involving n , σ , and π Molecular Orbitals

transition	wavelength range	examples
$\sigma \rightarrow \sigma^*$	<200 nm	C—C, C—H
$n \rightarrow \sigma^*$	160–260 nm	H_2O , CH_3OH , CH_3Cl
$\pi \rightarrow \pi^*$	200–500 nm	C=C, C=O, C=N, C≡C

transition	wavelength range	examples
$n \rightarrow \pi^*$	250–600 nm	C=O, C=N, N=N, N=O

Many transition metal ions, such as Cu^{2+} and Co^{2+} , form colorful solutions because the metal ion absorbs visible light. The transitions that give rise to this absorption are valence electrons in the metal ion's d -orbitals. For a free metal ion, the five d -orbitals are of equal energy. In the presence of a complexing ligand or solvent molecule, however, the d -orbitals split into two or more groups that differ in energy. For example, in an octahedral complex of $\text{Cu}(\text{H}_2\text{O})_6^{2+}$ the six water molecules perturb the d -orbitals into the two groups shown in Figure 2.1.3. The resulting $d \rightarrow d$ transitions for transition metal ions are relatively weak.

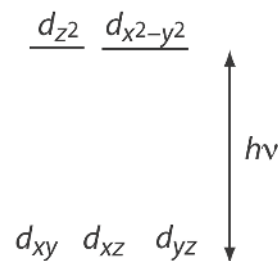
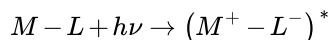


Figure 2.1.3. Splitting of the d -orbitals in an octahedral field.

A more important source of UV/Vis absorption for inorganic metal–ligand complexes is charge transfer, in which absorption of a photon produces an excited state in which there is transfer of an electron from the metal, M , to the ligand, L .



Charge-transfer absorption is important because it produces very large absorbances. One important example of a charge-transfer complex is that of o -phenanthroline with Fe^{2+} , the UV/Vis spectrum for which is shown in Figure 2.1.4. Charge-transfer absorption in which an electron moves from the ligand to the metal also is possible.

Why is a larger absorbance desirable? An analytical method is more sensitive if a smaller concentration of analyte gives a larger signal.

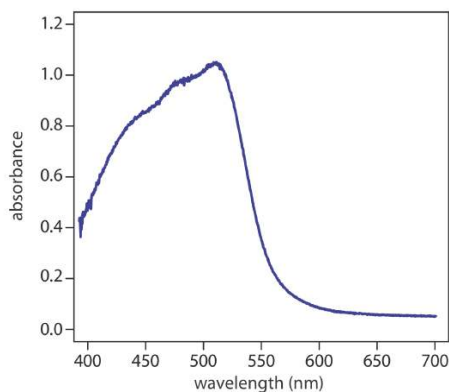


Figure 2.1.4. UV/Vis spectrum for the metal–ligand complex $\text{Fe}(\text{phen})_3^{2+}$, where phen is the ligand o -phenanthroline.

Comparing the IR spectrum in Figure 2.1.2 to the UV/Vis spectrum in Figure 2.1.4 shows us that UV/Vis absorption bands are often significantly broader than those for IR absorption. We can use Figure 2.1.1 to explain why this is true. When a species absorbs UV/Vis radiation, the transition between electronic energy levels may also include a transition between vibrational energy levels. The result is a number of closely spaced absorption bands that merge together to form a single broad absorption band.

UV/Vis Spectra for Atoms

The energy of ultraviolet and visible electromagnetic radiation is sufficient to cause a change in an atom's valence electron configuration. Sodium, for example, has a single valence electron in its $3s$ atomic orbital. As shown in Figure 2.1.5, unoccupied,

higher energy atomic orbitals also exist.

The valence shell energy level diagram in Figure 2.1.5 might strike you as odd because it shows that the 3p orbitals are split into two groups of slightly different energy. The reasons for this splitting are unimportant in the context of our treatment of atomic absorption. For further information about the reasons for this splitting, consult the chapter's additional resources.

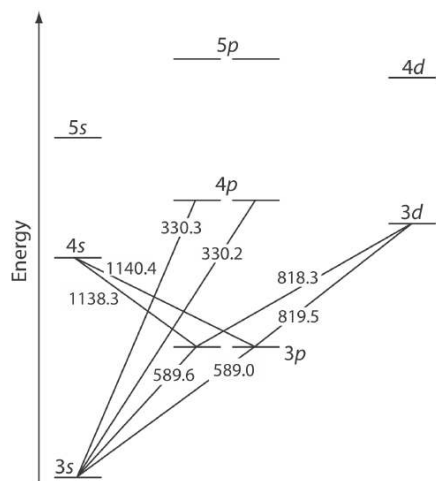


Figure 2.1.5 . Valence shell energy level diagram for sodium. The wavelengths (in wavenumbers) corresponding to several transitions are shown.

Absorption of a photon is accompanied by the excitation of an electron from a lower-energy atomic orbital to an atomic orbital of higher energy. Not all possible transitions between atomic orbitals are allowed. For sodium the only allowed transitions are those in which there is a change of ± 1 in the orbital quantum number (l); thus transitions from $s \rightarrow p$ orbitals are allowed, but transitions from $s \rightarrow s$ and from $s \rightarrow d$ orbitals are forbidden.

The atomic absorption spectrum for Na is shown in Figure 2.1.6 , and is typical of that found for most atoms. The most obvious feature of this spectrum is that it consists of a small number of discrete absorption lines that correspond to transitions between the ground state (the 3s atomic orbital) and the 3p and the 4p atomic orbitals. Absorption from excited states, such as the $3p \rightarrow 4s$ and the $3p \rightarrow 3d$ transitions included in Figure 2.1.5 , are too weak to detect. Because an excited state's lifetime is short—an excited state atom typically returns to a lower energy state in 10^{-7} to 10^{-8} seconds—an atom in the excited state is likely to return to the ground state before it has an opportunity to absorb a photon.

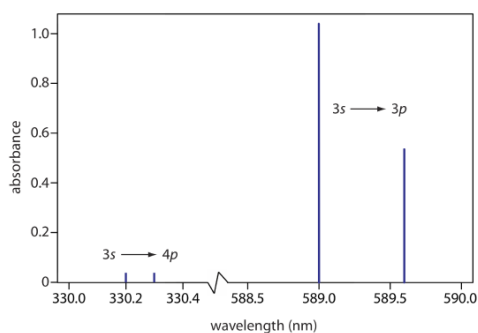


Figure 2.1.6 . Atomic absorption spectrum for sodium. Note that the scale on the x-axis includes a break.

Another feature of the atomic absorption spectrum in Figure 2.1.6 is the narrow width of the absorption lines, which is a consequence of the fixed difference in energy between the ground state and the excited state, and the lack of vibrational and rotational energy levels. Natural line widths for atomic absorption, which are governed by the uncertainty principle, are approximately 10^{-5} nm. Other contributions to broadening increase this line width to approximately 10^{-3} nm.

Transmittance and Absorbance

As light passes through a sample, its power decreases as some of it is absorbed. This attenuation of radiation is described quantitatively by two separate, but related terms: transmittance and absorbance. As shown in Figure 2.1.7 a, transmittance is the ratio of the source radiation's power as it exits the sample, P_T , to that incident on the sample, P_0 .

$$T = \frac{P_T}{P_0} \quad (2.1.1)$$

Multiplying the transmittance by 100 gives the percent transmittance, % T , which varies between 100% (no absorption) and 0% (complete absorption). All methods of detecting photons—including the human eye and modern photoelectric transducers—measure the transmittance of electromagnetic radiation.

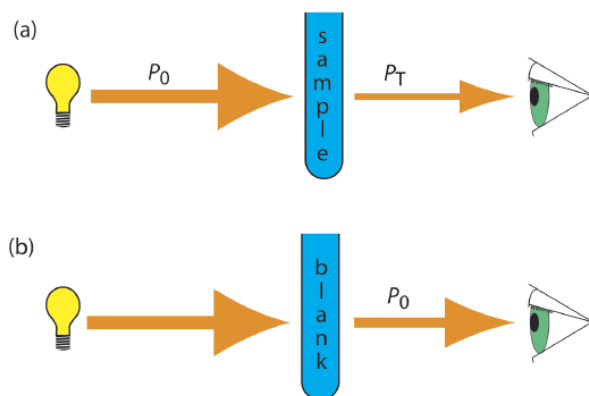


Figure 2.1.7 . (a) Schematic diagram showing the attenuation of radiation passing through a sample; P_0 is the source's radiant power and P_T is the radiant power transmitted by the sample. (b) Schematic diagram showing how we redefine P_0 as the radiant power transmitted by the blank. Redefining P_0 in this way corrects the transmittance in (a) for the loss of radiation due to scattering, reflection, absorption by the sample's container, and absorption by the sample's matrix.

Equation 2.1.1 does not distinguish between different mechanisms that prevent a photon emitted by the source from reaching the detector. In addition to absorption by the analyte, several additional phenomena contribute to the attenuation of radiation, including reflection and absorption by the sample's container, absorption by other components in the sample's matrix, and the scattering of radiation. To compensate for this loss of the radiation's power, we use a method blank. As shown in Figure 2.1.7 b, we redefine P_0 as the power exiting the method blank.

An alternative method for expressing the attenuation of electromagnetic radiation is absorbance, A , which we define as

$$A = -\log T = -\log \frac{P_T}{P_0} \quad (2.1.2)$$

Absorbance is the more common unit for expressing the attenuation of radiation because it is a linear function of the analyte's concentration.

We will show that this is true in the next section when we introduce Beer's law.

✓ Example 2.1.1

A sample has a percent transmittance of 50%. What is its absorbance?

Solution

A percent transmittance of 50.0% is the same as a transmittance of 0.500. Substituting into Equation 2.1.2 gives

$$A = -\log T = -\log(0.500) = 0.301$$

? Exercise 2.1.1

What is the % T for a sample if its absorbance is 1.27?

Answer

To find the transmittance, T , we begin by noting that

$$A = 1.27 = -\log T$$

Solving for T

$$\begin{aligned} -1.27 &= \log T \\ 10^{-1.27} &= T \end{aligned}$$

gives a transmittance of 0.054, or a %T of 5.4%.

Equation 2.1.1 has an important consequence for atomic absorption. As we learned from Figure 2.1.6, atomic absorption lines are very narrow. Even with a high quality monochromator, the effective bandwidth for a continuum source is $100 - 1000\times$ greater than the width of an atomic absorption line. As a result, little radiation from a continuum source is absorbed when it passes through a sample of atoms; because $P_0 \approx P_T$ the measured absorbance effectively is zero. For this reason, atomic absorption requires that we use a line source instead of a continuum source.

Absorbance and Concentration: Beer's Law

When monochromatic electromagnetic radiation passes through an infinitesimally thin layer of sample of thickness dx , it experiences a decrease in its power of dP (Figure 2.1.8).

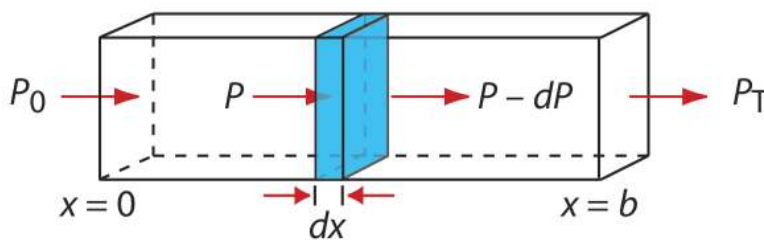


Figure 2.1.8. Factors used to derive the Beer's law.

This fractional decrease in power is proportional to the sample's thickness and to the analyte's concentration, C ; thus

$$-\frac{dP}{P} = \alpha C dx \quad (2.1.3)$$

where P is the power incident on the thin layer of sample and α is a proportionality constant. Integrating the left side of Equation 2.1.3 over the sample's full thickness

$$\begin{aligned} -\int_{P=P_0}^{P=P_T} \frac{dP}{P} &= \alpha C \int_{x=0}^{x=b} dx \\ \ln \frac{P_0}{P_T} &= \alpha b C \end{aligned}$$

converting from $\ln$ to $\log$, and substituting into Equation 2.1.2, gives

$$A = abC \quad (2.1.4)$$

where a is the analyte's **absorptivity** with units of $\text{cm}^{-1} \text{conc}^{-1}$. If we express the concentration using molarity, then we replace a with the **molar absorptivity**, ϵ , which has units of $\text{cm}^{-1} \text{M}^{-1}$.

$$A = \epsilon b C \quad (2.1.5)$$

The absorptivity and the molar absorptivity are proportional to the probability that the analyte absorbs a photon of a given energy. As a result, values for both a and ϵ depend on the wavelength of the absorbed photon.

✓ Example 2.1.2

A 5.00×10^{-4} M solution of analyte is placed in a sample cell that has a pathlength of 1.00 cm. At a wavelength of 490 nm, the solution's absorbance is 0.338. What is the analyte's molar absorptivity at this wavelength?

Solution

Solving Equation 2.1.5 for ϵ and making appropriate substitutions gives

$$\epsilon = \frac{A}{bC} = \frac{0.338}{(1.00 \text{ cm})(5.00 \times 10^{-4} \text{ M})} = 676 \text{ cm}^{-1} \text{ M}^{-1}$$

? Exercise 2.1.2

A solution of the analyte from Example 2.1.2 has an absorbance of 0.228 in a 1.00-cm sample cell. What is the analyte's concentration?

Answer

Making appropriate substitutions into Beer's law

$$A = 0.228 = \epsilon bC = (676 \text{ M}^{-1} \text{ cm}^{-1})(1 \text{ cm})C$$

and solving for C gives a concentration of 3.37×10^{-4} M.

Equation 2.1.4 and Equation 2.1.5, which establish the linear relationship between absorbance and concentration, are known as Beer's law. Calibration curves based on Beer's law are common in quantitative analyses.

As is often the case, the formulation of a law is more complicated than its name suggests. This is the case, for example, with Beer's law, which also is known as the Beer-Lambert law or the Beer-Lambert-Bouguer law. Pierre Bouguer, in 1729, and Johann Lambert, in 1760, noted that the transmittance of light decreases exponentially with an increase in the sample's thickness.

$$T \propto e^{-b}$$

Later, in 1852, August Beer noted that the transmittance of light decreases exponentially as the concentration of the absorbing species increases.

$$T \propto e^{-C}$$

Together, and when written in terms of absorbance instead of transmittance, these two relationships make up what we know as Beer's law.

Beer's Law and Multicomponent Samples

We can extend Beer's law to a sample that contains several absorbing components. If there are no interactions between the components, then the individual absorbances, A_i , are additive. For a two-component mixture of analyte's X and Y , the total absorbance, A_{tot} , is

$$A_{tot} = A_X + A_Y = \epsilon_X bC_X + \epsilon_Y bC_Y$$

Generalizing, the absorbance for a mixture of n components, A_{mix} , is

$$A_{mix} = \sum_{i=1}^n A_i = \sum_{i=1}^n \epsilon_i bC_i \quad (2.1.6)$$

Limitations to Beer's Law

Beer's law suggests that a plot of absorbance vs. concentration—we will call this a Beer's law plot—is a straight line with a y-intercept of zero and a slope of ab or ϵb . In some cases a Beer's law plot deviates from this ideal behavior (see Figure 2.1.9), and such deviations from linearity are divided into three categories: fundamental, chemical, and instrumental.

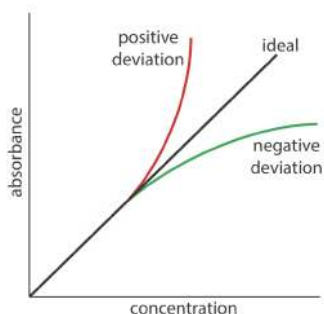


Figure 2.1.9 . Plots of absorbance vs. concentration showing **positive** and **negative** deviations from the ideal Beer's law relationship, which is a straight line.

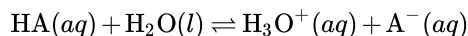
Fundamental Limitations to Beer's Law

Beer's law is a limiting law that is valid only for low concentrations of analyte. There are two contributions to this fundamental limitation to Beer's law. At higher concentrations the individual particles of analyte no longer are independent of each other. The resulting interaction between particles of analyte may change the analyte's absorptivity. A second contribution is that an analyte's absorptivity depends on the solution's refractive index. Because a solution's refractive index varies with the analyte's concentration, values of a and ϵ may change. For sufficiently low concentrations of analyte, the refractive index essentially is constant and a Beer's law plot is linear.

Chemical Limitations to Beer's Law

A chemical deviation from Beer's law may occur if the analyte is involved in an equilibrium reaction. Consider, for example, the weak acid, HA. To construct a Beer's law plot we prepare a series of standard solutions—each of which contains a known total concentration of HA—and then measure each solution's absorbance at the same wavelength. Because HA is a weak acid, it is in equilibrium with its conjugate weak base, A^- .

In the equations that follow, the conjugate weak base A^- is written as A as it is easy to mistake the symbol for anionic charge as a minus sign.



If both HA and A^- absorb at the selected wavelength, then Beer's law is

$$A = \epsilon_{HA} b C_{HA} + \epsilon_A b C_A \quad (2.1.7)$$

Because the weak acid's total concentration, C_{total} , is

$$C_{total} = C_{HA} + C_A$$

we can write the concentrations of HA and A^- as

$$C_{HA} = \alpha_{HA} C_{total} \quad (2.1.8)$$

$$C_A = (1 - \alpha_{HA}) C_{total} \quad (2.1.9)$$

where α_{HA} is the fraction of weak acid present as HA. Substituting Equation 2.1.8 and Equation 2.1.9 into Equation 2.1.7 and rearranging, gives

$$A = (\epsilon_{HA} \alpha_{HA} + \epsilon_A - \epsilon_A \alpha_{HA}) b C_{total} \quad (2.1.10)$$

To obtain a linear Beer's law plot, we must satisfy one of two conditions. If ϵ_{HA} and ϵ_A have the same value at the selected wavelength, then Equation 2.1.10 simplifies to

$$A = \epsilon_A b C_{total} = \epsilon_{HA} b C_{total}$$

Alternatively, if α_{HA} has the same value for all standard solutions, then each term within the parentheses of Equation 2.1.10 is constant—which we replace with k —and a linear calibration curve is obtained at any wavelength.

$$A = kbC_{\text{total}}$$

Because HA is a weak acid, the value of α_{HA} varies with pH. To hold α_{HA} constant we buffer each standard solution to the same pH. Depending on the relative values of α_{HA} and α_{A} , the calibration curve has a positive or a negative deviation from Beer's law if we do not buffer the standards to the same pH.

Instrumental Limitations to Beer's Law

There are two principal instrumental limitations to Beer's law. The first limitation is that Beer's law assumes that radiation reaching the sample is of a single wavelength—that is, it assumes a purely monochromatic source of radiation. As shown in Figure 10.1.10, even the best wavelength selector passes radiation with a small, but finite effective bandwidth. Polychromatic radiation always gives a negative deviation from Beer's law, but the effect is smaller if the value of ϵ essentially is constant over the wavelength range passed by the wavelength selector. For this reason, as shown in Figure 2.1.10, it is better to make absorbance measurements at the top of a broad absorption peak. In addition, the deviation from Beer's law is less serious if the source's effective bandwidth is less than one-tenth of the absorbing species' natural bandwidth [(a) Strong, F. C., III *Anal. Chem.* **1984**, 56, 16A–34A; Gilbert, D. D. *J. Chem. Educ.* **1991**, 68, A278–A281]. When measurements must be made on a slope, linearity is improved by using a narrower effective bandwidth.

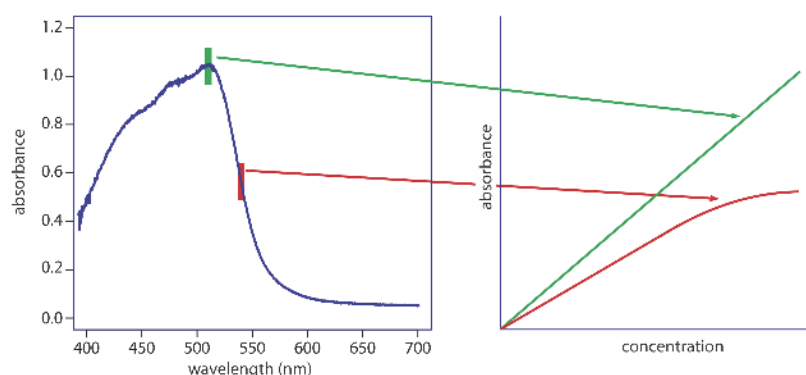


Figure 2.1.10. Effect of wavelength selection on the linearity of a Beer's law plot. Another reason for measuring absorbance at the top of an absorbance peak is that it provides for a more sensitive analysis. Note that the green Beer's law plot has a steeper slope—and, therefore, a greater sensitivity—than the red Beer's law plot. A Beer's law plot, of course, is equivalent to a calibration curve.

Stray radiation is the second contribution to instrumental deviations from Beer's law. Stray radiation arises from imperfections in the wavelength selector that allow light to enter the instrument and to reach the detector without passing through the sample. Stray radiation adds an additional contribution, P_{stray} , to the radiant power that reaches the detector; thus

$$A = -\log \frac{P_{\text{T}} + P_{\text{stray}}}{P_0 + P_{\text{stray}}}$$

For a small concentration of analyte, P_{stray} is significantly smaller than P_0 and P_{T} , and the absorbance is unaffected by the stray radiation. For higher concentrations of analyte, less light passes through the sample and P_{T} and P_{stray} become similar in magnitude. This results in an absorbance that is smaller than expected, and a negative deviation from Beer's law.

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2.2: UV/Vis Instrumentation

This section describes several different instruments for U-vis absorption spectroscopy, with an emphasis on the specific instruments used in our teaching labs at Duke, and their advantages and limitations. Methods of sample introduction are also covered in this section for more information about absorption spectroscopy, or for a description of the clinical, environmental, and other applications of absorption spectroscopy, see [10.3: UV/Vis and IR Spectroscopies](#) by David Harvey.

Instrument Designs for Molecular UV/Vis Absorption

Single-Beam Spectrophotometer.

An instrument that uses a monochromator for wavelength selection is called a **spectrophotometer**. The simplest spectrophotometer is a single-beam instrument equipped with a fixed-wavelength monochromator (Figure 2.2.2). Single-beam spectrophotometers are calibrated and used in the same manner as a photometer. One example of a single-beam spectrophotometer is Thermo Scientific's Spectronic 20D+, which is shown in the photographic insert to Figure 2.2.2 . *This is similar to the Genesys Spec 20 that you used in the previous module.*

The Spectronic 20D+ has a wavelength range of 340–625 nm (950 nm when using a red-sensitive detector), and a fixed effective bandwidth of 20 nm. Battery-operated, hand-held single-beam spectrophotometers are available, which are easy to transport into the field. Other single-beam spectrophotometers also are available with effective bandwidths of 2–8 nm. Fixed wavelength single-beam spectrophotometers are not practical for recording spectra because manually adjusting the wavelength and recalibrating the spectrophotometer is awkward and time-consuming. The accuracy of a single-beam spectrophotometer is limited by the stability of its source and detector over time.

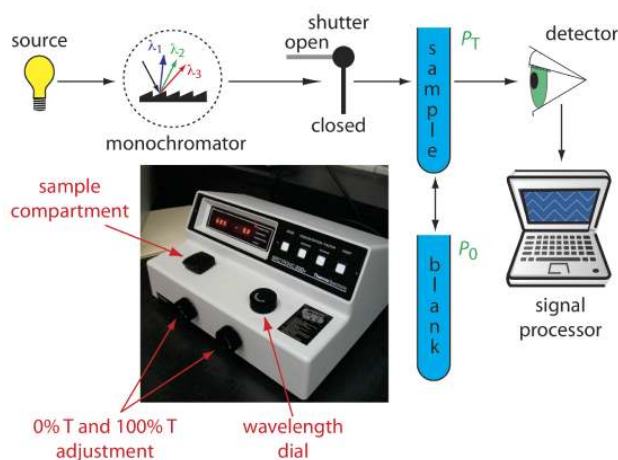


Figure 2.2.2 . Schematic diagram of a fixed-wavelength, single-beam spectrophotometer. The photographic inset shows a typical instrument. The shutter remains closed until the sample or blank is placed in the sample compartment. The analyst manually selects the wavelength by adjusting the wavelength dial. Inset photo modified from: Adi (www.commonswiki.org).

Double-Beam Spectrophotometer.

The limitations of a fixed-wavelength, single-beam spectrophotometer is minimized by using a **double-beam** spectrophotometer (Figure 2.2.3). A chopper controls the radiation's path, alternating it between the sample, the blank, and a shutter. The signal processor uses the chopper's speed of rotation to resolve the signal that reaches the detector into the transmission of the blank, P_0 , and the sample, P_T . By including an opaque surface as a shutter, it also is possible to continuously adjust 0%T. The effective bandwidth of a double-beam spectrophotometer is controlled by adjusting the monochromator's entrance and exit slits. Effective bandwidths of 0.2–3.0 nm are common. A scanning monochromator allows for the automated recording of spectra. Double-beam instruments are more versatile than single-beam instruments, being useful for both quantitative and qualitative analyses, but also are more expensive and not particularly portable. **There is one double-beam spectrometer in our instrument facility at Duke, and the instrument you will use is the same as that pictured in Figure 2.2.3 ; it is an Agilent Cary UV-vis double beam spectrometer.**

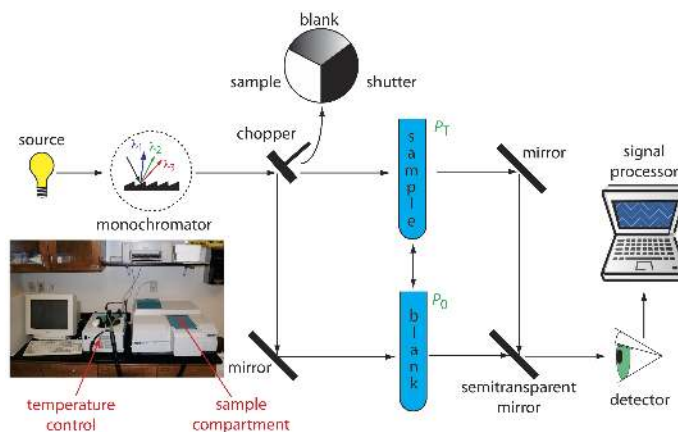


Figure 2.2.3 . Schematic diagram of a scanning, double-beam spectrophotometer. A chopper directs the source's radiation, using a transparent window to pass radiation to the sample and a mirror to reflect radiation to the blank. The chopper's opaque surface serves as a shutter, which allows for a constant adjustment of the spectrophotometer's 0%T. The photographic insert shows a typical instrument. The module in the middle of the photo is a temperature control unit that makes it possible to heat or cool the sample to a constant temperature.

Diode Array Spectrometer

An instrument with a single detector can monitor only one wavelength at a time. If we replace a single photomultiplier with an array of photodiodes, we can use the resulting detector to record a full spectrum in as little as 0.1 s. In a diode array spectrometer the source radiation passes through the sample and is dispersed by a grating (Figure 2.2.4). The photodiode array detector is situated at the grating's focal plane, with each diode recording the radiant power over a narrow range of wavelengths. Because we replace a full monochromator with just a grating, a diode array spectrometer is small and compact. **This is the kind of spectrometer that is integrated into the Duke teach labs' HPLC system as an in-line detector. It is also the type of detector that is used in our Agilent Cary 60 spectrometer.**

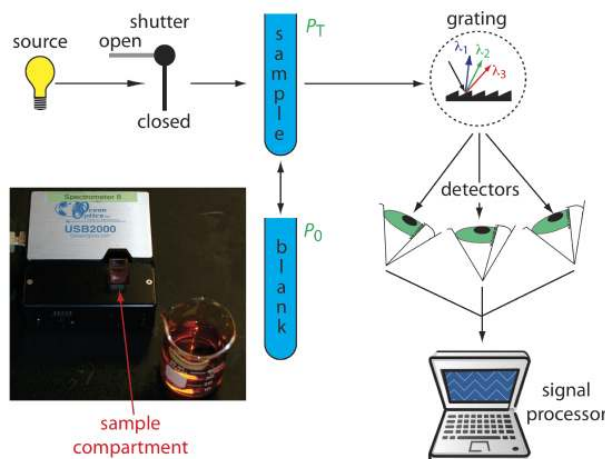


Figure 2.2.4 . Schematic diagram of a diode array spectrophotometer. The photographic insert shows a typical instrument. Note that the 50-mL beaker provides a sense of scale. Because the spectrometer is small and compact, it is easy to transport into the field.

One advantage of a diode array spectrometer is the speed of data acquisition, which allows us to collect multiple spectra for a single sample. Individual spectra are added and averaged to obtain the final spectrum. This **signal averaging** improves a spectrum's signal-to-noise ratio. If we add together n spectra, the sum of the signal at any point, x , increases as nS_x , where S_x is the signal. The noise at any point, N_x , is a random event, which increases as $\sqrt{n}N_x$ when we add together n spectra. The **signal-to-noise ratio** after n scans, $(S/N)_n$ is

$$\left(\frac{S}{N}\right)_n = \frac{nS_x}{\sqrt{n}N_x} = \sqrt{n} \frac{S_x}{N_x}$$

where S_x/N_x is the signal-to-noise ratio for a single scan. The impact of signal averaging is shown in Figure 2.2.5. The first spectrum shows the signal after one scan, which consists of a single, noisy peak. Signal averaging using 4 scans and 16 scans decreases the noise and improves the signal-to-noise ratio. One disadvantage of a photodiode array is that the effective bandwidth per diode is roughly an order of magnitude larger than that for a high quality monochromator.

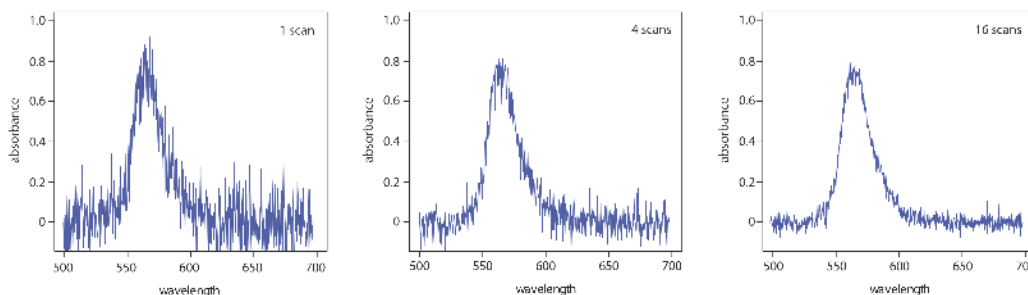


Figure 2.2.5. Effect of signal averaging on a spectrum's signal-to-noise ratio. From top to bottom: spectrum for a single scan; average spectrum after four scans; and average spectrum after adding 16 scans.

For more details on signals and noise, see Introduction to Signals and Noise by Steven Petrovic, an on-line resource that is part of the Analytical Sciences Digital Library.

Sample Cells.

Samples that are in liquid or solution state are placed in cells (called **cuvettes**) constructed with materials that are transparent to UV and/or Visible light. Common cuvette materials are quartz (fused silica), glass, and plastic (Figure 2.2.6). Glass and plastic are inexpensive, and they can be useful for analysis of samples that require the cell to be transparent only to visible light. However, glass is not transparent to light below about 380 nm. Some plastics are also not transparent to UV light. On the other hand, Quartz (or fused silica) is more expensive, but it is transparent to both UV and visible light. A quartz or fused-silica cell is required when working at a wavelength <300 nm where other materials show a significant absorption.

By far, the most common pathlength is 1 cm (10 mm), as is the case for the "standard" size cuvette pictured at the far left of Figure 2.2.6. Cells with shorter (as little as 0.1 cm) and longer pathlengths (up to 10 cm) are also available. Longer pathlength cells are useful when analyzing a very dilute solution or for gas samples. The highest quality cells allow the radiation to strike a flat surface at a 90° angle, minimizing the loss of radiation to reflection. A test tube often is used as a sample cell with simple, single-beam instruments, although differences in the cell's pathlength and optical properties add an additional source of error to the analysis.



Figure 2.2.6. Examples of sample cells for UV/Vis spectroscopy. From left to right (with path lengths in parentheses): rectangular plastic cuvette (10.0 mm), rectangular quartz cuvette (5.000 mm), rectangular quartz cuvette (1.000 mm), cylindrical quartz cuvette (10.00 mm), cylindrical glass cuvette with quartz windows (100.0 mm). Cells often are available as a matched pair, which is important when using a double-beam instrument.

If we need to monitor an analyte's concentration over time, it may not be possible to remove samples for analysis. This often is the case, for example, when monitoring an industrial production line or waste line, when monitoring a patient's blood, or when monitoring an environmental system, such as stream. With a **fiber-optic probe** we can analyze samples *in situ*. An example of a remote sensing fiber-optic probe is shown in Figure 2.2.7. The probe consists of two bundles of fiber-optic cable. One bundle transmits radiation from the source to the probe's tip, which is designed to allow the sample to flow through the sample cell.

Radiation from the source passes through the solution and is reflected back by a mirror. The second bundle of fiber-optic cable transmits the nonabsorbed radiation to the wavelength selector. Another design replaces the flow cell shown in Figure 2.2.7 with a membrane that contains a reagent that reacts with the analyte. When the analyte diffuses into the membrane it reacts with the reagent, producing a product that absorbs UV or visible radiation. The nonabsorbed radiation from the source is reflected or scattered back to the detector. Fiber optic probes that show chemical selectivity are called optrodes [(a) Seitz, W. R. *Anal. Chem.* **1984**, 56, 16A–34A; (b) Angel, S. M. *Spectroscopy* **1987**, 2(2), 38–48].

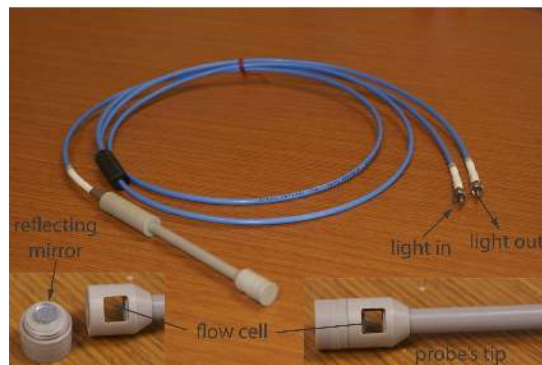


Figure 2.2.7 . Example of a fiber-optic probe. The inset photographs at the bottom of the figure provide close-up views of the probe's flow cell and the reflecting mirror.

Evaluation of UV/Vis and IR Spectroscopy

Accuracy

Under normal conditions a relative error of 1–5% is easy to obtained with UV/Vis absorption. Accuracy usually is limited by the quality of the blank. Examples of the type of problems that are encountered include the presence of particulates in the sample that scatter radiation, and the presence of interferents that react with analytical reagents. In the latter case the interferent may react to form an absorbing species, which leads to a positive determinate error. Interferents also may prevent the analyte from reacting, which leads to a negative determinate error. With care, it is possible to improve the accuracy of an analysis by as much as an order of magnitude.

Precision

In absorption spectroscopy, precision is limited by indeterminate errors—primarily instrumental noise—which are introduced when we measure absorbance. Precision generally is worse for low absorbances where $P_0 \approx P_T$, and for high absorbances where P_T approaches 0. We might expect, therefore, that precision will vary with transmittance.

We can derive an expression between precision and transmittance by applying the propagation of uncertainty as described in Chapter 4. To do so we rewrite Beer's law as

$$C = -\frac{1}{\epsilon b} \log T \quad (2.2.1)$$

Table 4.3.1 in Chapter 4 helps us complete the propagation of uncertainty for Equation 2.2.1; thus, the absolute uncertainty in the concentration, s_C , is

$$s_C = -\frac{0.4343}{\epsilon b} \times \frac{s_T}{T} \quad (2.2.2)$$

where s_T is the absolute uncertainty in the transmittance. Dividing Equation 2.2.2 by Equation 2.2.1 gives the relative uncertainty in concentration, s_C/C , as

$$\frac{s_C}{C} = \frac{0.4343 s_T}{T \log T}$$

If we know the transmittance's absolute uncertainty, then we can determine the relative uncertainty in concentration for any measured transmittance.

Determining the relative uncertainty in concentration is complicated because s_T is a function of the transmittance. As shown in Table 2.2.3 , three categories of indeterminate instrumental error are observed [Rothman, L. D.; Crouch, S. R.; Ingle, J. D. Jr. *Anal.*

Chem. **1975**, *47*, 1226–1233]. A constant s_T is observed for the uncertainty associated with reading %T on a meter's analog or digital scale. Typical values are ± 0.2 – 0.3% (a k_1 of ± 0.002 – 0.003) for an analog scale and $\pm 0.001\%$ (a k_1 of ± 0.00001) for a digital scale.

Table 2.2.3 . Effect of Indeterminate Errors on Relative Uncertainty in Concentration

category	sources of indeterminate error	relative uncertainty in concentration
$s_T = k_1$	%T readout resolution noise in thermal detectors	$\frac{s_C}{C} = \frac{0.4343k_1}{T \log T}$
$s_T = k_2 \sqrt{T^2 + T}$	noise in photon detectors	$\frac{s_C}{C} = \frac{0.4343k_2}{\log T} \sqrt{1 + \frac{1}{T}}$
$s_T = k_3 T$	positioning of sample cell fluctuations in source intensity	$\frac{s_C}{C} = \frac{0.4343k_3}{\log T}$

A constant s_T also is observed for the thermal transducers used in infrared spectrophotometers. The effect of a constant s_T on the relative uncertainty in concentration is shown by curve A in Figure 2.2.16 . Note that the relative uncertainty is very large for both high absorbances and low absorbances, reaching a minimum when the absorbance is 0.4343. This source of indeterminate error is important for infrared spectrophotometers and for inexpensive UV/Vis spectrophotometers. To obtain a relative uncertainty in concentration of ± 1 – 2% , the absorbance is kept within the range 0.1–1.

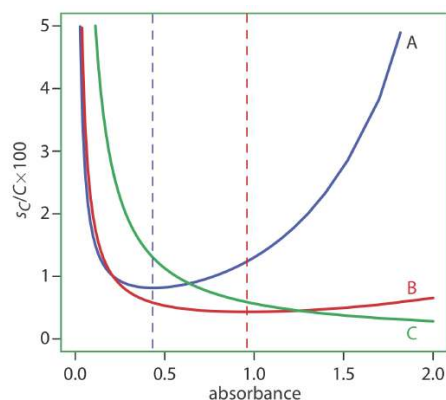


Figure 2.2.16 . Percent relative uncertainty in concentration as a function of absorbance for the categories of indeterminate errors in Table 2.2.10.3. A: $k_1 = \pm 0.0030$; B: $k_2 = \pm 0.0030$; and C: $k_3 = \pm 0.0130$. The dashed lines correspond to the minimum uncertainty for curve A (absorbance of 0.4343) and for curve B (absorbance of 0.963).

Values of s_T are a complex function of transmittance when indeterminate errors are dominated by the noise associated with photon detectors. Curve B in Figure 2.2.16 shows that the relative uncertainty in concentration is very large for low absorbances, but is smaller at higher absorbances. Although the relative uncertainty reaches a minimum when the absorbance is 0.963, there is little change in the relative uncertainty for absorbances between 0.5 and 2. This source of indeterminate error generally limits the precision of high quality UV/Vis spectrophotometers for mid-to-high absorbances.

Finally, the value of s_T is directly proportional to transmittance for indeterminate errors that result from fluctuations in the source's intensity and from uncertainty in positioning the sample within the spectrometer. The latter is particularly important because the optical properties of a sample cell are not uniform. As a result, repositioning the sample cell may lead to a change in the intensity of transmitted radiation. As shown by curve C in Figure 2.2.16 , the effect is important only at low absorbances. This source of indeterminate errors usually is the limiting factor for high quality UV/Vis spectrophotometers when the absorbance is relatively small.

When the relative uncertainty in concentration is limited by the %T readout resolution, it is possible to improve the precision of the analysis by redefining 100% T and 0% T. Normally 100% T is established using a blank and 0% T is established while preventing the source's radiation from reaching the detector. If the absorbance is too high, precision is improved by resetting 100% T using a standard solution of analyte whose concentration is less than that of the sample (Figure 2.2.17 a). For a sample whose absorbance is too low, precision is improved by redefining 0% T using a standard solution of the analyte whose concentration is greater than that of the analyte (Figure 2.2.17 b). In this case a calibration curve is required because a linear relationship between absorbance and

concentration no longer exists. Precision is further increased by combining these two methods (Figure 2.2.17 c). Again, a calibration curve is necessary since the relationship between absorbance and concentration is no longer linear.

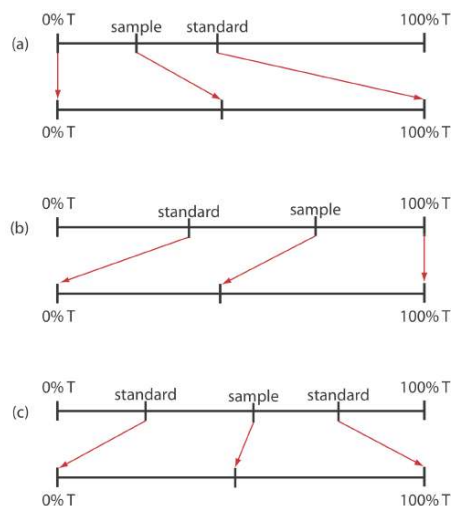


Figure 2.2.17 . Methods for improving the precision of absorption methods: (a) high-absorbance method; (b) low-absorbance method; (c) maximum precision method.

Sensitivity

The sensitivity of a molecular absorption method, which is the slope of a Beer's law calibration curve, is the product of the analyte's absorptivity and the pathlength of the sample cell (ϵb). You can improve a method's sensitivity by selecting a wavelength where absorbance is at a maximum or by increasing pathlength.

See Figure 10.2.10 for an example of how the choice of wavelength affects a calibration curve's sensitivity.

Selectivity

Selectivity rarely is a problem in molecular absorption spectrophotometry. In many cases it is possible to find a wavelength where only the analyte absorbs. When two or more species do contribute to the measured absorbance, a multicomponent analysis is still possible, as shown in Example 2.2.2 and Example 2.2.3 .

Time, Cost, and Equipment

The analysis of a sample by molecular absorption spectroscopy is relatively rapid, although additional time is required if we need to convert a nonabsorbing analyte into an absorbing form. The cost of UV/Vis instrumentation ranges from several hundred dollars for a simple filter photometer, to more than \$50,000 for a computer-controlled, high-resolution double-beam instrument equipped with variable slit widths, and operating over an extended range of wavelengths. Fourier transform infrared spectrometers can be obtained for as little as \$15,000–\$20,000, although more expensive models are available.

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- 10.3: UV/Vis and IR Spectroscopy by David Harvey is licensed CC BY-NC-SA 4.0.

2.3: Single Wavelength Spectrometer (Genesy 20)

GENESYS 20 spectrophotometer

The Genesys 20 spectrophotometers are able to read single wavelength absorption of visible light. The instruments may look like the model shown in Figure 2.3.1 or may be grey in color.

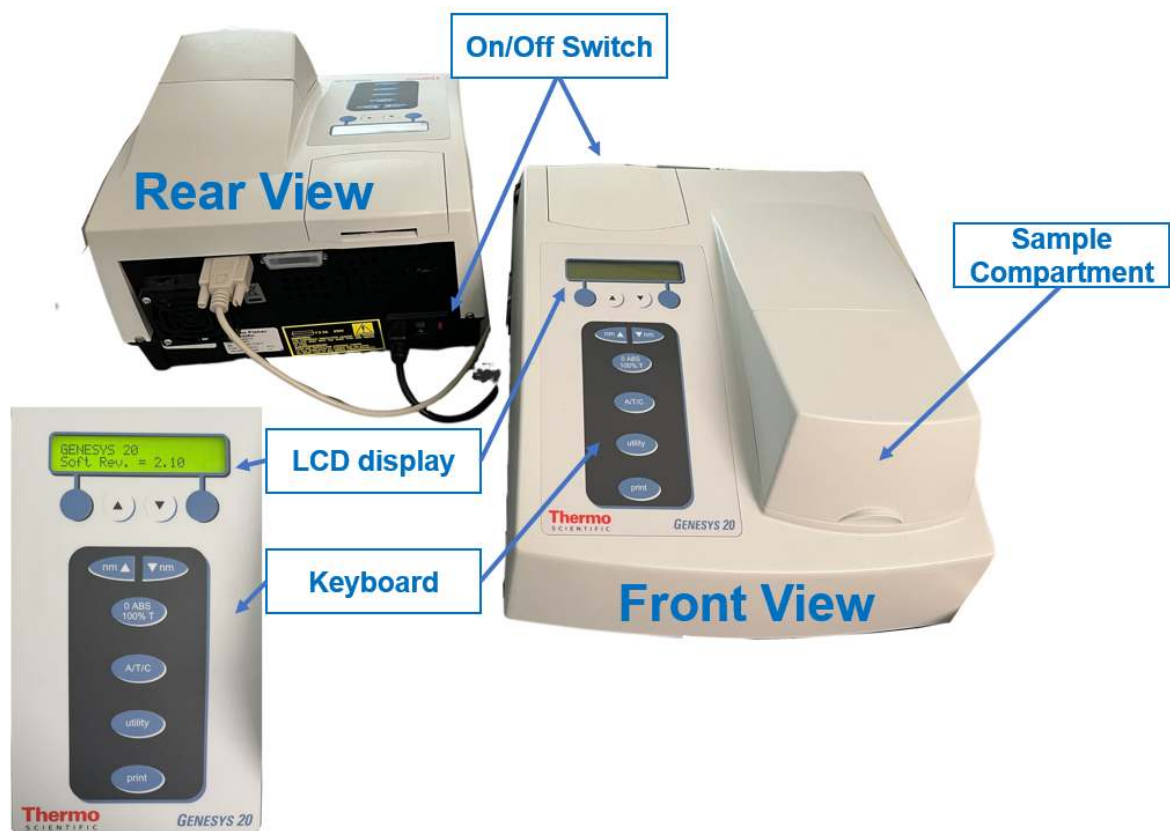


Figure 2.3.1: Copy and Paste Caption here. (CC-BY-SA; Kathryn Haas)

Basic Operating Procedure

1. Turn on the instrument with the ON-OFF power switch, located at the back of the instrument (see Figure 2.3.1). **Warm up the instrument for at least 20 minutes.**
2. Set the wavelength using the "nm arrow" buttons on the main keyboard (see Figure 2.3.1). Do not use the arrows that are just below the LCD display; those are to toggle the LCD screen display.
3. Set the instrument to read absorbance (A) using the A/T/C button on the keyboard. T is for transmittance.
4. To "zero" the instrument on a blank sample, take a clean cuvette and fill the cuvette 3/4 with blank solution. Wipe the outside of the cuvette with a Kimwipe®. Place the cuvette in the cuvette holder inside the sample compartment so that the light path (front to back) is passing through the sample. Close the sample compartment cover. Zero the instrument with the "0 ABS, 100% T" button.
5. The LCD screen should display the correct wavelength and 0.000 A. If it is not, check with your TA.
6. Using the instrument is as simple as placing the sample to be measured into the cuvette holder, closing the sample compartment cover, waiting about 10 seconds for the reading to stabilize and recording the absorbance off the display.
7. It is a good idea to check, from time to time, to make sure the instrument has not drifted. Do this by checking that a "blank" cuvette (one containing only blank solution) still has an absorbance of 0.000. If not, then the absorbance of samples cannot be trusted. If a blank does not read 0.000 then re-zero the instrument and repeat the previous samples. If the absorbance readings are drifting more than the expected uncertainty of the instrument, notify your TA.
8. When you are finished using the instrument, remove the final sample from the sample compartment and turn off the instrument

Video Instructions for operating a Genesys 20 Spec.

The video below is an excellent demonstration for how to use the Genesee 20 Spectrophotometer.



Specifications

The manufacturer states that the uncertainty in the measured absorbance is ± 0.003 absorbance units for absorbance readings from 0.001 to 0.300, and $\pm 1\%$ for absorbance reading from 0.301 to 2.50. More [detail from the manufacturer is below](#).

Product Line	GENESYS 20
Depth	13 in. 33 cm
Width (Metric)	12 in 30 cm
Height (English)	7 in. 19 cm
Weight (English)	10 lb. 4.5 kg
Drift	0.003 A/hr.
Hertz	50/60 Hz
Interface	RS-232-C (serial)
Noise	$\leq 0.001A$ at 0A $\leq 0.004A$ at 2A, 500 nm
Photometric Accuracy	$\pm 0.003 A$ from 0.0 to 0.3A 1.0% from 0.301 A to 2.5 A, NIST 930D filters
Detector Type	Silicon Photodiode
Light Source	Tungsten Halogen
Spectral Bandwidth	≤ 8 nm
Wavelength Range	325 to 1100 nm
Electrical Requirements	Selected automatically, 120 V
Lamp Life	1000 hrs.
Optical Design	Single Beam
Photometric Range	-0.1-2.5A; 0-125%T, 0-1999C
Stray Light	$< 0.1\%T$ at 340 nm and 400 nm

Wavelength Accuracy	± 2.0 nm

<https://www.fishersci.com/shop/product/ter-2/14385445>, accessed April 2, 2025

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SECTION OVERVIEW

2.4: Multi-wavelength Spectrometers (Agilent Cary Models)

There are two types of multi wavelength spectrometers in our instrument facility — both are *Agilent Cary* models, and both use the same software.

- The **Agilent Cary 100** instruments have tungsten and deuterium lamps that must be warmed up for approximately 1 hour prior to use.
- The **Agilent Cary 60** instrument has a xenon flash lamp, which does not require warm up.

Be sure you discuss which instrument you will use with your TA, and if you are using a **Cary 100**, be sure it is on for 1 hour prior to use.

2.4.1: Validation of Cary UV-Vis

2.4.2: Scan Method Set Up

2.4: Multi-wavelength Spectrometers (Agilent Cary Models) is shared under a [not declared](#) license and was authored, remixed, and/or curated by LibreTexts.

2.4.1: Validation of Cary UV-Vis

Prior to using any instrument, you should run checks to ensure the instrument is functioning properly. While the Cary UV-vis instruments run initialization procedures upon startup, there is a "Validate" program that will validate the instrument's performance. You should run a wavelength validation after the instrument has warmed up (if applicable). For Cary 100 instruments, use the deuterium (D2) lamp to validate wavelength accuracy. For the Cary 60, use the xenon (Xe) lamp to calibrate wavelength accuracy. The figure below gives a summary of the steps involved in validating the instrument.

1. Find the "Cary WinUV" shortcut on the computer desktop and open it. The linked folder contains all the programs that operate the Cary spectrophotometer. **Open the "Validate" Program.**
 - The Validate window should open. At the top left corner of the window, it should say "Validate-Online". If it says otherwise (ie "Validate-Offline, as pictured below) then it is not connected to the instrument. Wait a few moments to see if it connects. In the meantime, make sure the instrument is powered on. If it does not connect, try pressing "connect" (its where the start button is), and if that fails, get help.
2. On the left hand side of the Validate Window, choose "**Tests**".
 - The Tests window will open. Choose "Instrument Performance Tests".
 - There should be a list of several available tests in the "Test Order" box. If there is not, double check that the instrument is "online". Then, press the "Reset all limits to Default button" - this usually regenerated the list of available tests.
3. It is sufficient to run one wavelength validation test. In the "Test Order" box, choose the **wavelength accuracy test** that uses the instrument's own lamp (either D2 or Xe). To do this, you should remove all other tests by selecting each and pressing the "remove test" button that is just to the right of the box (it looks like an exclamation point with a red "X". Do this until the test you want is the only one remaining.
4. **Press "OK"** after you have specified the validation test to be run.
 - The USP Tests box will close.
5. Check to make sure there are no samples in the light path. Close the sample compartment. Then **Press "Start"**.
 - This should initiate data collection. It will take a few minutes, and during this time you should be able to see the collected spectrum recorded on the screen. If not, get help.

Figure [Math Processing Error]: A visual summary of steps involved in validating the Agilent Cary UV-vis spectrophotometers. (CC-BY-NC-SA; Kathryn Haas)

After the validation, copy and paste the relevant data and images from the report into your notebook.

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2.4.2: Scan Method Set Up

The figure below gives a visual summary of the steps to set up a method to collect a scan. After all settings have been chosen, press "OK". The instrument is ready for you to use at this point.

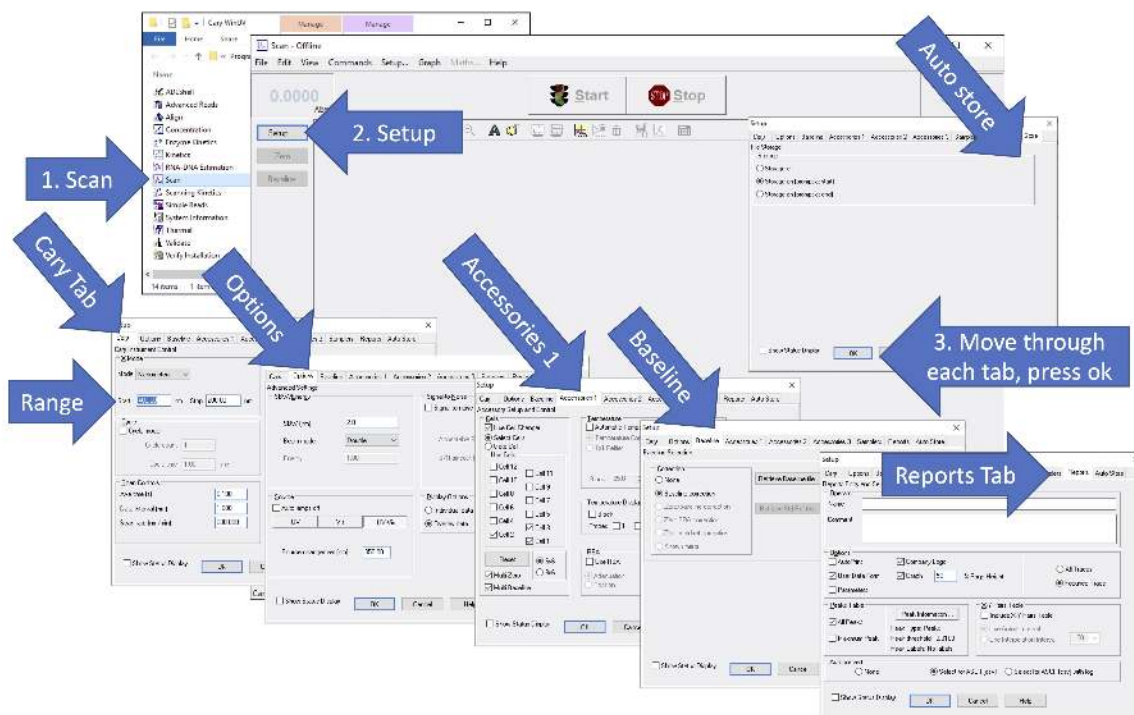


Figure 2.4.2.2: A visual summary of steps involved in setting up the Scan application for the Agilent Cary UV-vis spectrophotometers. (CC-BY-NC-SA; Kathryn Haas)

Steps:

1. Open the "Scan" program from the Cary WinUV folder.
2. Then go to "Setup". Go to each tab and modify the method settings in the following order.
 - o Cary Tab:
 - Set the scan range to include wavelengths of 400-200 nm (or a wider range if you prefer).
 - Set the data interval to 1 nm and the average time to 0.1 s.
 - o Options:
 - SBW/Energy: Use double beam mode
 - Source: Choose either UV or UV-vis
 - Display Options: Choose "Overlay Data"
 - o Accessories 1: This is the multi-cell, temperature-controlled sample holder. If this accessory is attached, follow the instructions below. Otherwise, uncheck the "Use cell changer" option.
 - If the multi-cell changer is installed, select "Use cell changer". Note that the cell changer must be installed correctly and aligned to operate correctly.
 - Check only the cell positions that you will use during the experiment.
 - Select multi-zero
 - Select multi-baseline
 - o Baseline:
 - Select Baseline correction
 - o Reports:
 - Select Report format
 - Select Report options

- Select for ASCII (csv). This option saves the data as a comma delineated format that can be opened in excel or and plotted using your own computer.
 - Auto Store:
 - "Storage on (prompt at start)" should be selected
3. After all settings have been chosen, press "OK". The instrument is ready for you to use at this point.

2.4.2: Scan Method Set Up is shared under a [not declared](#) license and was authored, remixed, and/or curated by LibreTexts.

CHAPTER OVERVIEW

3: Inductively Coupled Plasma Optical Absorption Spectrometer (ICP-OES)

[3.1: Introduction to ICP-OES](#)

[3.2: ICP-OES Instrument at Duke](#)

[3.3: Writing a method for the Perkin Elmer Avio 220 Max](#)

[3.4: ICP-OES Operation Instructions](#)

[3.5: Instructions for Viewing and Exporting Data using Syngistics Software](#)

3: Inductively Coupled Plasma Optical Absorption Spectrometer (ICP-OES) is shared under a [not declared](#) license and was authored, remixed, and/or curated by LibreTexts.

3.1: Introduction to ICP-OES

ICP-OES (Inductively Coupled Plasma–Optical Emission Spectroscopy) is a powerful tool for detecting trace metals in water, food, soil, and biological samples. By the end of this module, you should be able to:

- Explain the principle of atomic emission.
- Identify major components of an ICP-OES instrument.
- Recognize common matrix effects and strategies to mitigate them.
- Justify the choice of calibration strategy for a given sample.

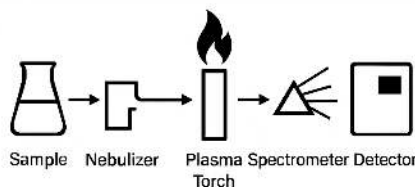
For an on-line introduction to much of the material in this section, see [Atomic Emission Spectroscopy \(AES\)](#) by Tomas Spudich and Alexander Scheeline, a resource that is part of the Analytical Sciences Digital Library.

Principles of Atomic Emission

When atoms are excited (by heat or electrical energy), electrons jump to higher energy levels. As they relax back down, they emit photons at element-specific wavelengths.

- Each element has a unique “fingerprint” of emission lines.
- The intensity of the emission is proportional to the number of atoms in the excited state (and thus the concentration in the sample).
- ICP-OES detects these emissions across many wavelengths simultaneously, enabling multielement analysis.

ICP-OES Instrument Overview



Nebulizer and Spray Chamber

- Converts liquid samples into an aerosol (fine mist).
- Carries aerosol droplets into the plasma.
- Transport efficiency (how much sample makes it into the plasma) depends on solution properties like viscosity and dissolved solids.

Plasma Torch

- Argon gas is ionized inside a quartz torch by a radio-frequency (RF) coil, creating a plasma at ~6000–10,000 K.
- The plasma serves as both atomizer (breaking samples into free atoms) and excitation source (promoting electrons to higher energy states).

Optical System

- A monochromator or polychromator separates emitted light by wavelength.
- Detectors measure intensity at selected wavelengths corresponding to target analytes.

Sample Introduction & Matrix Effects

Understanding and correcting for Sample Introduction and Matrix Effects is critical for collecting reliable data from ICP-OES.

- **Sample Introduction Effects:** The delivery of sample to the plasma at a constant rate and the maintenance of a constant plasma temperature are critical to getting reliable results in ICP-OES.
- **Matrix Effects:** In real samples, the matrix (everything in the sample besides the analyte of interest) can alter signal intensity in a number of ways. These are called matrix effects.

Effect (category)	Mechanism (what's happening)	Typical example	Primary fixes
Physical (transport/nebulization)	Viscosity/density/surface tension differences alter aerosol delivery; high TDS or organics load the plasma	Hard water ($\text{Ca}^{2+}/\text{Mg}^{2+}$); organics from filters	Dilute; matrix/acid match; optimize sample-intro hardware/flows; internal standard for physical drift only.
Chemical (plasma / ionization / loading)	Concomitant elements change excitation/ionization equilibria, or matrix consumes energy	High Na^+/K^+ suppressing analyte; high Fe loading	Dilute; matrix match; ionization buffer (e.g., Cs); standard additions when matching is impractical. (<i>Internal standard is generally not reliable here unless it behaves identically to the analyte.</i>)
Spectral overlap / background	Another line or structured background overlaps analyte wavelength; background slope/curvature	Fe line near analyte line; carbon-rich backgrounds	Choose alternate wavelength; background correction; inter-element correction (IEC) if unavoidable; verify with spike recovery .
Acid strength mismatch (a special physical case)	Different $\%\text{HNO}_3$ changes viscosity/transport vs. standards	Standards at 2% HNO_3 ; samples at 0.2%	Acid match standards and samples; then confirm with spike recovery .
Other sources of information: Thermo Fisher Scientific , Inorganic Ventures , Agilent			

Key takeaway: Calibration only works if the standards and samples behave the same in the instrument. Sample Introduction problems and Matrix effects disrupt this assumption.

ICP-OES Instruments: More Details

Plasma Source

A **plasma** is a hot, partially ionized gas that contains an abundant concentration of cations and electrons. The plasma used in atomic emission is formed by ionizing a flowing stream of argon gas, producing argon ions and electrons. A plasma's high temperature results from resistive heating as the electrons and argon ions move through the gas. Because a plasma operates at a much higher temperature than a flame, it provides for a better atomization efficiency and a higher population of excited states.

A schematic diagram of the inductively coupled plasma source (ICP) is shown in Figure 3.1.2 . The ICP torch consists of three concentric quartz tubes, surrounded at the top by a radio-frequency induction coil. The sample is mixed with a stream of Ar using a nebulizer, and is carried to the plasma through the torch's central capillary tube. Plasma formation is initiated by a spark from a Tesla coil. An alternating radio-frequency current in the induction coil creates a fluctuating magnetic field that induces the argon ions and the electrons to move in a circular path. The resulting collisions with the abundant unionized gas give rise to resistive heating, providing temperatures as high as 10000 K at the base of the plasma, and between 6000 and 8000 K at a height of 15–20 mm above the coil, where emission usually is measured. At these high temperatures the outer quartz tube must be thermally isolated from the plasma. This is accomplished by the tangential flow of argon shown in the schematic diagram.

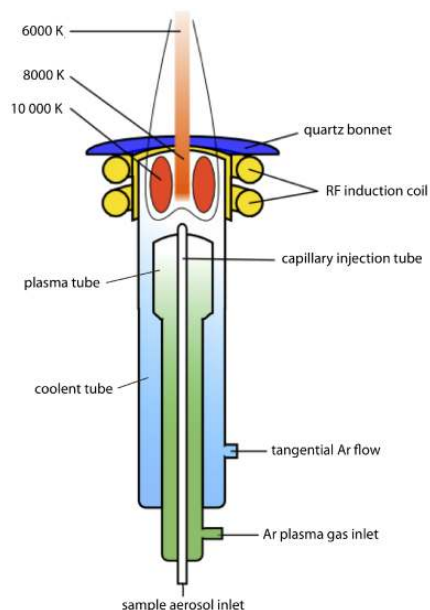


Figure 3.1.2 . Schematic diagram of an inductively coupled plasma torch.

Types of Multielemental Analysis

Atomic emission spectroscopy is ideally suited for a multielemental analysis because all analytes in a sample are excited simultaneously. If the instrument includes a scanning monochromator, we can program it to move rapidly to an analyte's desired wavelength, pause to record its emission intensity, and then move to the next analyte's wavelength. This **sequential analysis** allows for a sampling rate of 3–4 analytes per minute.

Another approach to a multielemental analysis is to use a multichannel instrument that allows us to monitor simultaneously many analytes (**simultaneous analysis**). A simple design for a multichannel spectrometer, shown in Figure 3.1.3 , couples a monochromator with multiple detectors that are positioned in a semicircular array around the monochromator at positions that correspond to the wavelengths for the analytes.

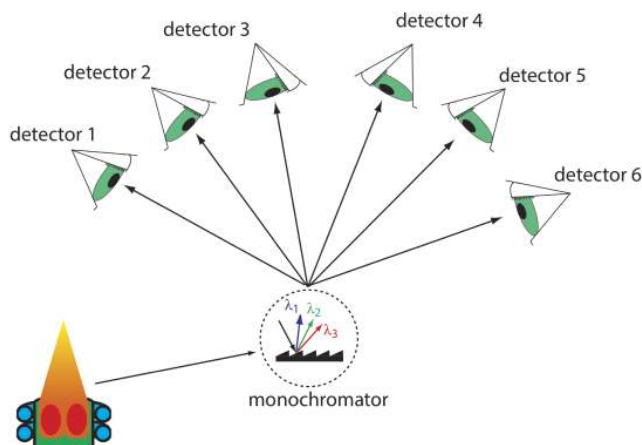


Figure 3.1.3 . Schematic diagram of a multichannel atomic emission spectrometer for the simultaneous analysis of several elements. Instruments may contain as many as 48–60 detectors.

Detection Limits for ICP-OES

ICP-OES can be used to detect elements across the periodic table; however, it is most sensitive to elements with low ionization energies such as metallic elements and heavier elements. The figure below shows the published detection limits for ICP-OES under ideal conditions using the most sensitive instruments available.

Optimization and Analysis of ICP-OES Data

Atomic emission is used widely for the analysis of trace metals in a variety of sample matrices. The development of a quantitative atomic emission method requires several considerations, including choosing a source for atomization and excitation, selecting a wavelength and slit width, preparing the sample for analysis, minimizing spectral and chemical interferences, and selecting a method of standardization.

Settings and Method Choices

- **Choice of Atomization and Excitation Source (Plasma vs Flame):** Except for the alkali metals, detection limits when using an ICP are significantly better than those obtained with flame. Plasmas also are subject to fewer spectral and chemical interferences than flame. For these reasons a plasma emission source is usually the better choice.
- **Selecting the Wavelength and Slit Width:** The choice of detection wavelength is dictated by the need for sensitivity and the need to avoid interferences from the emission lines of other constituents in the sample. Because an analyte's atomic emission spectrum has an abundance of emission lines—particularly when using a high temperature plasma source—it is inevitable that there will be some overlap between emission lines. For example, an analysis for Ni using the atomic emission line at 349.30 nm is complicated by the atomic emission line for Fe at 349.06 nm. A narrower slit width provides better resolution, but at the cost of less radiation reaching the detector. The easiest approach to selecting a wavelength is to record the sample's emission spectrum and look for an emission line that provides an intense signal and is resolved from other emission lines.
- **Sample Preparation:** Flame and plasma sources are best suited for samples in solution and in liquid form. Although a solid sample can be analyzed by directly inserting it into the flame or plasma, they usually are first brought into solution by digestion or extraction.

Data Analysis Choices

- **Background Correct to Minimize Spectral Interferences:** The most important spectral interference is broad, background emission from the flame or plasma and emission bands from molecular species. This background emission is particularly severe for flames because the temperature is insufficient to break down refractory compounds, such as oxides and hydroxides. Background corrections for flame emission are made by scanning over the emission line and drawing a baseline (Figure 3.1.4). Because a plasma's temperature is much higher, a background interference due to molecular emission is less of a problem.

Although emission from the plasma's core is strong, it is insignificant at a height of 10–30 mm above the core where measurements normally are made.

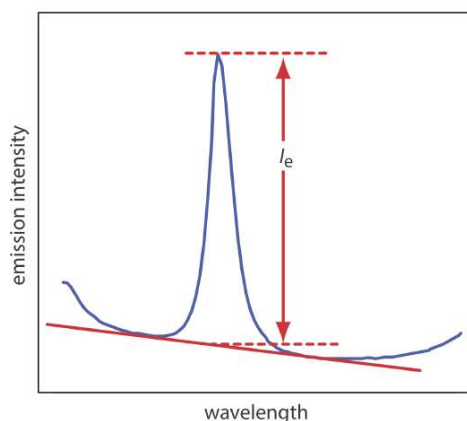


Figure 3.1.4 . Method for correcting an analyte's emission for the flame's background emission.

Minimizing Chemical Interferences

Flame emission is subject to the same types of chemical interferences as atomic absorption; they are minimized using the same methods: by adjusting the flame's composition and by adding protecting agents, releasing agents, or ionization suppressors. An additional chemical interference results from **self-absorption**. Because the flame's temperature is greatest at its center, the concentration of analyte atoms in an excited state is greater at the flame's center than at its outer edges. If an excited state atom in the flame's center emits a photon, then a ground state atom in the cooler, outer regions of the flame may absorb the photon, which decreases the emission intensity. For higher concentrations of analyte self-absorption may invert the center of the emission band (Figure 3.1.5).

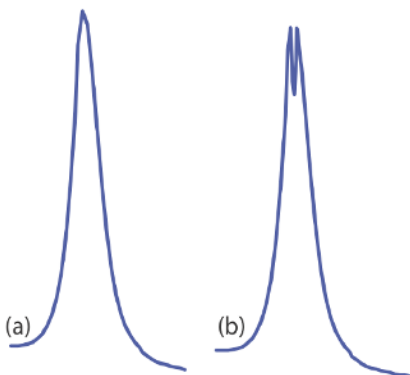


Figure 3.1.5 . Atomic emission lines for (a) a low concentration of analyte, and (b) a high concentration of analyte showing the effect of self-absorption.

Chemical interferences when using a plasma source generally are not significant because the plasma's higher temperature limits the formation of nonvolatile species. For example, **[Math Processing Error]** is a significant interferent when analyzing samples for Ca^{2+} by flame emission, but has a negligible effect when using a plasma source. In addition, the high concentration of electrons from the ionization of argon minimizes ionization interferences.

Standardizing the Method

From Equation **[Math Processing Error]** we know that emission intensity is proportional to the population of the analyte's excited state, **[Math Processing Error]**. If the flame or plasma is in thermal equilibrium, then the excited state population is proportional to the analyte's total population, N , through the Boltzmann distribution (Equation **[Math Processing Error]**).

A calibration curve for flame emission usually is linear over two to three orders of magnitude, with ionization limiting linearity when the analyte's concentrations is small and self-absorption limiting linearity at higher concentrations of analyte. When using a plasma, which suffers from fewer chemical interferences, the calibration curve often is linear over four to five orders of magnitude and is not affected significantly by changes in the matrix of the standards.

Emission intensity is affected significantly by many parameters, including the temperature of the excitation source and the efficiency of atomization. An increase in temperature of 10 K, for example, produces a 4% increase in the fraction of Na atoms in the 3p excited state, an uncertainty in the signal that may limit the use of external standards. The method of internal standards is used when the variations in source parameters are difficult to control. To compensate for changes in the temperature of the excitation source, the internal standard is selected so that its emission line is close to the analyte's emission line. In addition, the internal standard should be subject to the same chemical interferences to compensate for changes in atomization efficiency. To accurately correct for these errors the analyte and internal standard emission lines are monitored simultaneously.

Representative Method: Determination of Sodium in a Salt Substitute

The best way to appreciate the theoretical and the practical details discussed in this section is to carefully examine a typical analytical method. Although each method is unique, the following description of the determination of sodium in salt substitutes provides an instructive example of a typical procedure. The description here is based on Goodney, D. E. *J. Chem. Educ.* **1982**, 59, 875–876.

Description of Method

Salt substitutes, which are used in place of table salt for individuals on low-sodium diets, replaces NaCl with KCl. Depending on the brand, fumaric acid, calcium hydrogen phosphate, or potassium tartrate also are present. Although intended to be sodium-free, salt substitutes contain small amounts of NaCl as an impurity. Typically, the concentration of sodium in a salt substitute is about 100 µg/g. The exact concentration of sodium is determined by flame atomic emission. Because it is difficult to match the matrix of the standards to that of the sample, the analysis is accomplished by the method of standard additions.

Procedure

A sample is prepared by placing an approximately 10-g portion of the salt substitute in 10 mL of 3 M HCl and 100 mL of distilled water. After the sample has dissolved, it is transferred to a 250-mL volumetric flask and diluted to volume with distilled water. A series of standard additions is prepared by placing 25-mL portions of the diluted sample into separate 50-mL volumetric flasks, spiking each with a known amount of an approximately 10 mg/L standard solution of Na⁺, and diluting to volume. After zeroing the instrument with an appropriate blank, the instrument is optimized at a wavelength of 589.0 nm while aspirating a standard solution of Na⁺. The emission intensity is measured for each of the standard addition samples and the concentration of sodium in the salt substitute is reported in µg/g.

Questions

1. Potassium ionizes more easily than sodium. What problem might this present if you use external standards prepared from a stock solution of 10 mg Na/L instead of using a set of standard additions?

Because potassium is present at a much higher concentration than is sodium, its ionization suppresses the ionization of sodium. Normally suppressing ionization is a good thing because it increases emission intensity. In this case, however, the difference between the standard's matrix and the sample's matrix means that the sodium in a standard experiences more ionization than an equivalent amount of sodium in a sample. The result is a determinate error.

2. One way to avoid a determinate error when using external standards is to match the matrix of the standards to that of the sample. We could, for example, prepare external standards using reagent grade KCl to match the matrix to that of the sample. Why is this not a good idea for this analysis?

Sodium is a common contaminant in many chemicals. Reagent grade KCl, for example, may contain 40–50 µg Na/g. This is a significant source of sodium, given that the salt substitute contains approximately 100 µg Na/g.

3. Suppose you decide to use an external standardization. Given the previous questions, is the result of your analysis likely to underestimate or to overestimate the amount of sodium in the salt substitute?

The solid black line in Figure 3.1.6 shows the ideal calibration curve, assuming we match the standard's matrix to the sample's matrix, and that we do so without adding any additional sodium. If we prepare the external standards without adding KCl, the emission for each standard decreases due to increased ionization. This is shown by the lower of the two dashed red lines. Preparing the standards by adding reagent grade KCl increases the concentration of sodium due to its contamination. Because we underestimate the actual concentration of sodium in the standards, the resulting calibration curve is shown by the other dashed red line. In both cases, the sample's emission results in our overestimating the concentration of sodium in the sample.

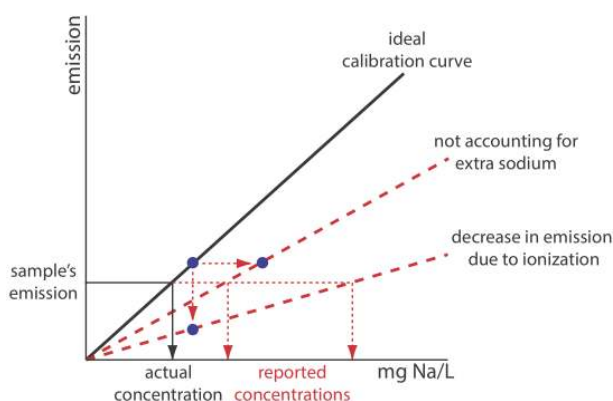


Figure 3.1.6 . External standards calibration curves for the flame atomic emission analysis of Na in a salt substitute. The solid black line shows the ideal calibration curve assuming we match the matrix of the samples and the standards using pure KCl. The lower of the two dashed red lines shows the effect of failing to add KCl to the external standards, which decreases emission. The other dashed red line shows the effect of using KCl that is contaminated with NaCl, which causes us to underestimate the concentration of Na in the standards. In both cases, the result is a positive determinate error in the analysis of samples.

4. One problem with analyzing salt samples is their tendency to clog the aspirator and burner assembly. What effect does this have on the analysis?

Clogging the aspirator and burner assembly decreases the rate of aspiration, which decreases the analyte's concentration in the flame. The result is a decrease in the emission intensity and a negative determinate error.

✓ Example 3.1.1

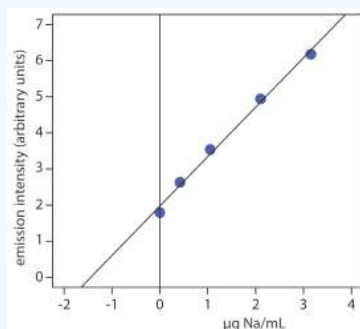
To evaluate the method described in Representative Method 10.7.1, a series of standard additions is prepared using a 10.0077-g sample of a salt substitute. The results of a flame atomic emission analysis of the standards is shown here [Goodney, D. E. *J. Chem. Educ.* **1982**, 59, 875–876].

added Na ($\mu\text{g/mL}$)	I_e (arb. units)
0.000	1.79
0.420	2.63
1.051	3.54
2.102	4.94
3.153	6.18

What is the concentration of sodium, in $\mu\text{g/g}$, in the salt substitute.

Solution

Linear regression of emission intensity versus the concentration of added Na gives the standard additions calibration curve shown below, which has the following calibration equation.



[Math Processing Error]

The concentration of sodium in the sample is the absolute value of the calibration curve's x -intercept. Substituting zero for the emission intensity and solving for sodium's concentration gives a result of $1.44 \mu\text{gNa/mL}$. The concentration of sodium in the salt substitute is

[Math Processing Error]

References & Resources

- Harvey, D. Analytical Chemistry 2.1. LibreTexts. Chapter 10: Spectroscopic Methods.
- Atomic Emission Spectroscopy (AES) module by Tomas Spudich and Alexander Scheeline (Analytical Sciences Digital Library).
- CHEM401L Module 5: Tainted Tap! What's in Your Water? (course manual).

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- [10.7: Atomic Emission Spectroscopy](#) by [David Harvey](#) is licensed [CC BY-NC-SA 4.0](#).

3.2: ICP-OES Instrument at Duke

The ICP-OES instrument in the Duke Teaching Labs is a Pekin Elmer Avio 220 Max ICP-OES. The instrument performs sequential multi-element analysis using either radial or axial monitoring.

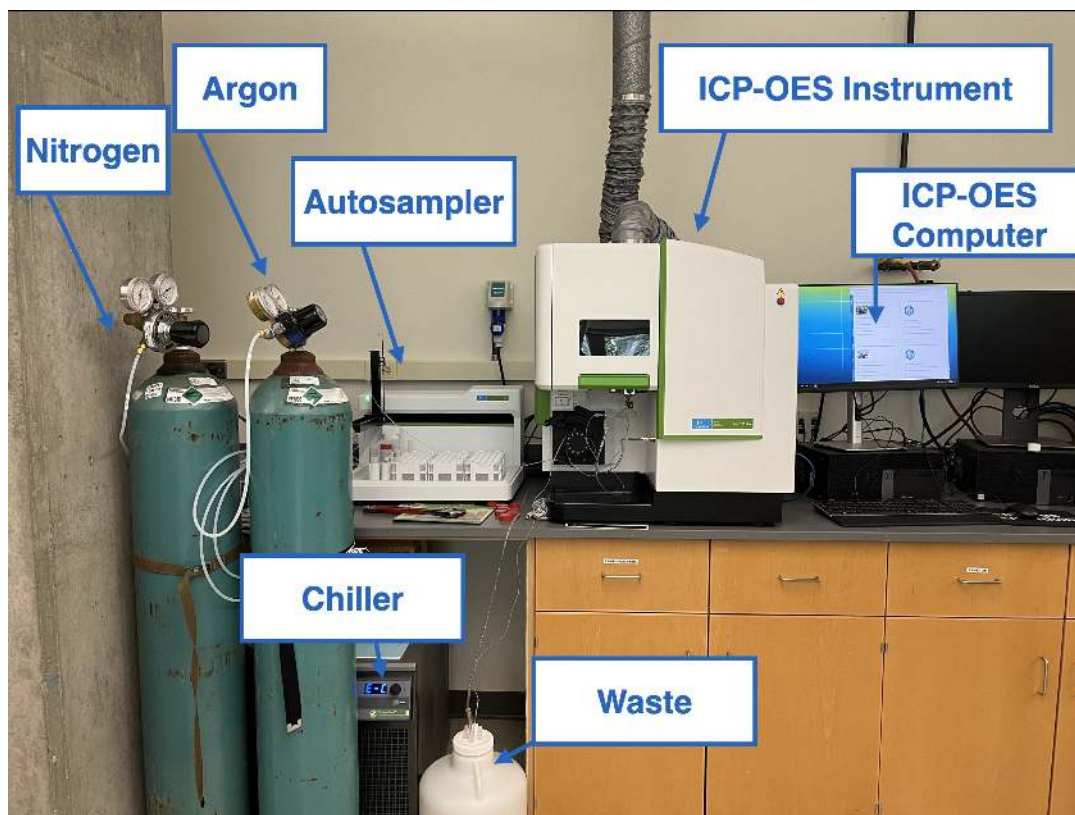


Figure [Math Processing Error]: Avio 220 Max ICP-OES Instrument at Duke Undergraduate Learning Labs

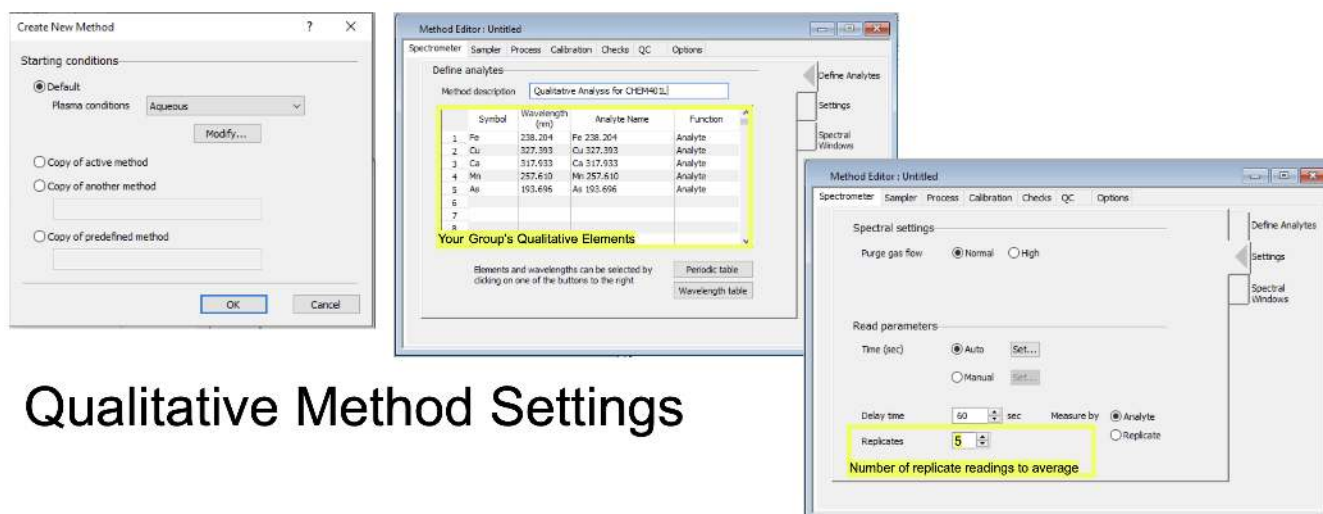
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3.3: Writing a method for the Perkin Elmer Avio 220 Max

I. Create a method for qualitative analysis

Follow the instructions below to set up a method for qualitative analysis. This method will not measure the concentration of any analyte. If you wish to quantitate an analyte, use the instructions for one of the quantitative methods.

- In the **Method tool box menu**, choose **New** to create a new method.
 - Accept the default starting conditions for Aqueous plasma conditions. (press OK)
 - In the Method Editor window, in the **Spectrometer tab**, give your new method a description (eg "Qualitative Analysis") and choose the elements for analysis. Use the default wavelengths for each element (note, all students will use the same method, add all elements for the group)
 - Choose the **Settings** tab at the right side of the Spectrometer tab. In making measurements for atomic emission, it is common to average the signal over a specified time interval (integration) and to report measurements that are actually averages of a collection of individual integrations (replicates). Flame atomic spectroscopy, as well as plasma and furnace techniques, have inherently noisy components. The flame is not perfectly stable, nor is the nebulizer completely consistent over a short time frame. This noise can be very effectively diminished by the averaging several replicates. The default integration time is 0.5-2 minutes for each element; and that will suffice. **Set the number of Replicates to 5.**
 - On the Calibration tab, you can choose to enter a calibration blank or not. If you do, you must specify the location of the blank in the autosampler.
 - Save the method and give it a name in the format "YYMMDD_QUAL_GROUPNAME"



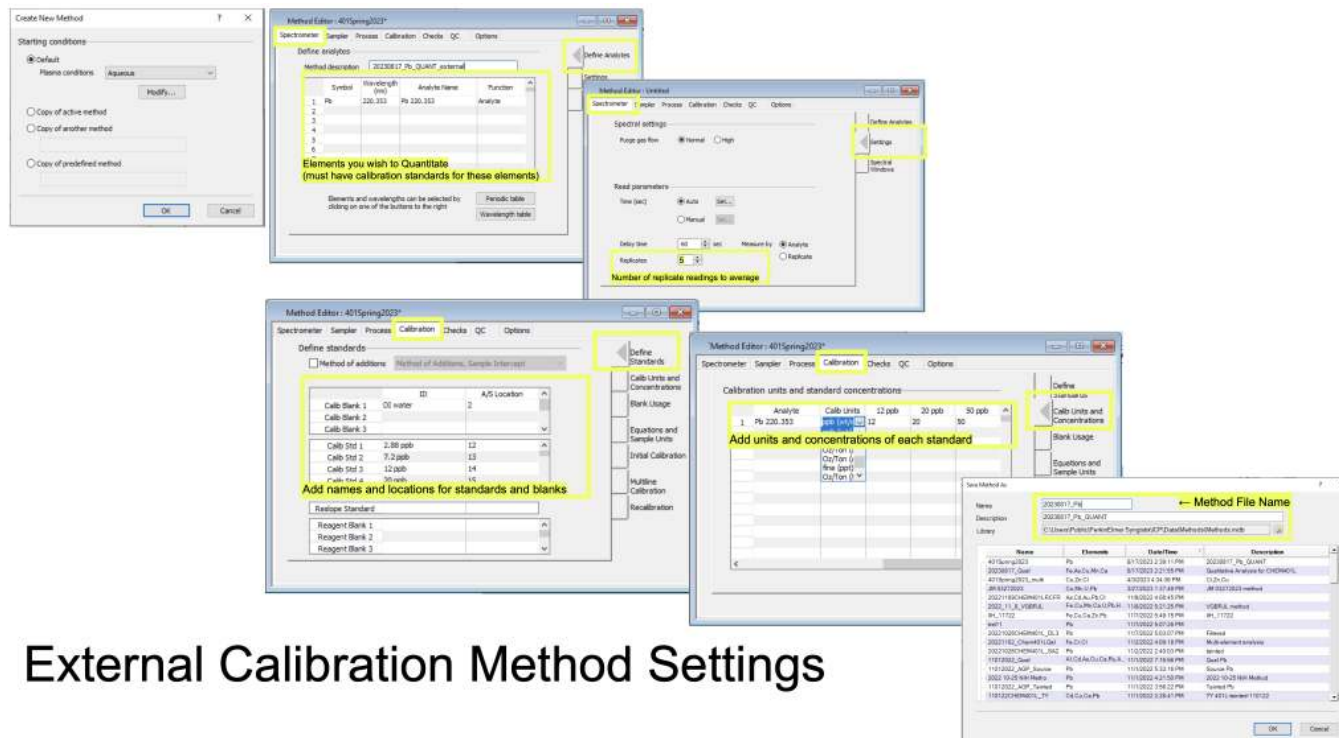
Qualitative Method Settings

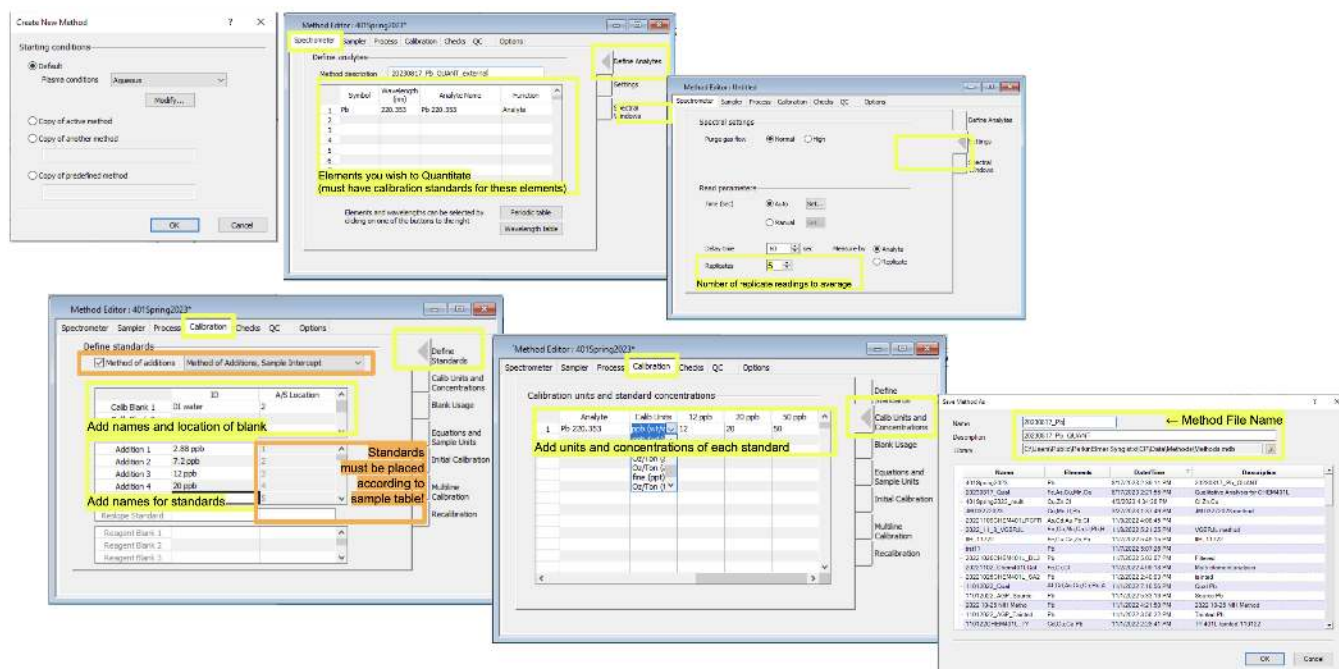
Figure 3.3.1: Qualitative method settings. (Kathryn Haas; CC-NC-BY-SA)

II. Create a method for quantitative analysis using external calibration

- Follow the instructions above, except for the following.
 - In the Method Editor window, in the Spectrometer tab, choose only the element(s) for quantitative analysis (only Pb). Use the default wavelengths for each element (note, all students will use the same method).
- Go to the **Calibration Tab (top)**.
 - In **Define Standards Tab (right)**, enter your standard solutions names and their locations in the auto sampler. Be sure to enter the correct rack positions for the calibration blank and the standard solutions in the A/s Location (Autosampler location).
 - In the **Calib Units and Concentrations** tab at the left, choose the units and enter the concentrations of each element in each standard solution.
 - Specify Blank Usage in the **Blank Usage** tab at the left. If you choose a specific blank, the software will subtract the blank's signal from the signal of all calibration standards and analyte solutions.

- d. If you would like to repeat calibration after some sample analysis, or if you prefer calibration to be done after all unknowns are analyzed, you can indicate that in the **Recalibration** tab at the left. This is necessary if there are many samples to run.
3. Go to the **Method tool box menu**, at the top of the main window and choose **Check Method**. Edit the method as necessary according to the check method instructions.
4. When your method is complete and has passed checks, go to the **Method tool box menu** and Save your method. Give your method a name in the format "**YYYYMMDD_Pb_GROUPNAME**".





Standard Additions Calibration Method Settings

Figure 3.3.3: Standard addition quantitative method settings. (Kathryn Haas; CC-NC-BY-SA)

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3.4: ICP-OES Operation Instructions

This page contains operating instructions for the **Perkin Elmer Avio 220 Max** at Duke Teaching Labs

I. Prepare the instrument

- **Check the rinse solution** on the auto sampler. The two 2L carboys should be full of rinse solution (5% nitric acid) to ensure efficient rinsing. If the rinse containers are not filled, add dilute nitric acid solution to the 2L mark on each container.
- **Check the waste container.** If the container is fuller than the shoulders of the bottle, it should be emptied. Consult your instructor if the waste is full.
- **Adjust the peristaltic pump tubing** so that the pump moves liquid from the auto sampler into the nebulizer, and from the nebulizer drain into the waste. Ask your TA for help with this step.
- **Open the argon gas tank to 90 psi.** Be sure there is sufficient pressure to operate the instrument. The ICP-OES instrument consumes argon at a rate of about 20 L/minute; one full compressed argon cylinder will provide about 6-8 hours of running time.
- If you plan to measure absorbance below 190 nm, the optics should be purged with nitrogen gas and the compressed nitrogen tank should be opened.

ALERT

It is **CRITICAL** that the samples and calibration standards are placed in the autosampler tray correctly, and that their position matches the position specified in the method and/or sample table.

- Inspect the sample position number on Figure 3.4.1 carefully and note that the numbers run consecutively from the back to the front of each row.
- Pick up one of the sample racks and note that there are feet on the bottom of each that must fit into place on the tray - always double check that the sample rack is seated correctly into the tray.

NEVER put a *closed* tube on the tray - it will destroy the sampling probe if the probe runs into a cap or encounters parafilm (parafilm clogs the probe). All sealed tubes should be stored in a separate rack under the bench.

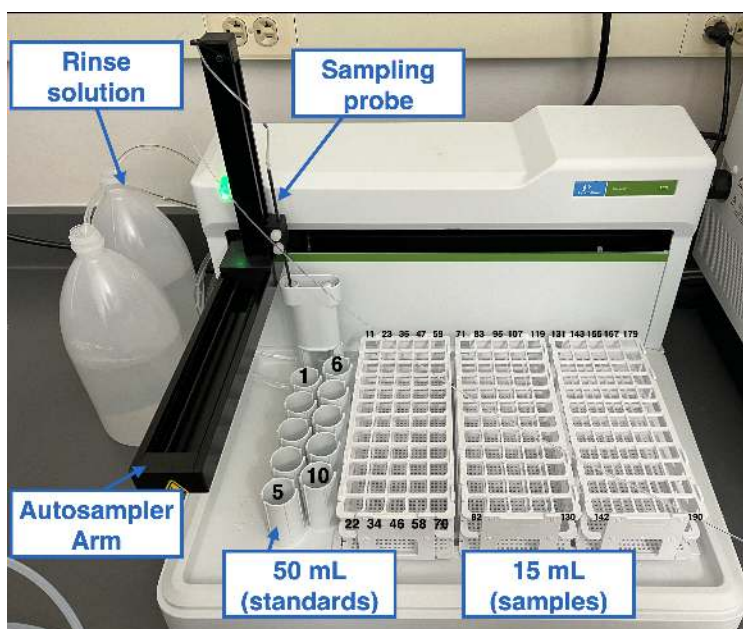


Figure 3.4.1: The autosampler with rack positions labeled.

- **Placing tubes in the racks** **Read the Alert box and the Figure.**
 - The auto sampler tray has a built in rack for ten 50 mL tubes (Positions 1-10) that are meant for standard solutions. Find this rack in Figure 3.4.1).

- The auto sampler tray also has three positions for removable sample racks that each accommodate 60 smaller, 15 mL tubes. Find the three removable racks in Figure 3.4.1)
 - Position 11 is in the back left corner of rack position R1, and the numbering of each row starts at the back of the autosampler rack (Figure 3.4.1).
- The way you must place tubes depends on what you are doing!
 - **Qualitative analysis:** Place the samples wherever is convenient and note the sample location in your notebook. When you create your method, you must enter the correct position in the method settings.
 - **External calibration:** Place the calibration solutions and the sample solutions in the auto sampler however is convenient. Record the position of each sample in your notebook so that you can enter them into your method settings. When you create your method, you must enter the correct position in the method settings. (we recommend putting calibration standards into the rack in organized fashion and in order of increasing concentration.

II. Open the software

1. Start the **Synergistix for ICP Online** instrument software by double clicking on the pink and purple "S" icon located on the desktop:



2. At the top of the window, choose the **Analysis tab**. At the top of the Analysis tab menu, there are several tool boxes.

Use "Online" Software for Operation

There are two copies of the **Synergistix for ICP** instrument software on the computer. The "online" version directly controls the instrument and data collection. Methods and data cannot be manipulated in the "online" software while the instrument is actively collecting data or conducting performance checks. The "offline" version is meant for editing methods and performing data analysis without interfering with the "online" instrument operation.

III. Enter Sample Information

1. Go to the **Sample Information tool box menu** at the top of the main **Analysis** tab and choose New to create a new sample list.
2. Enter a file description. Then enter the auto sampler location (A/S Location) and sample information in the table. (enter only the *samples*, not the blanks or calibration standards).
 - a. As you are entering the samples in this table, be sure to enter only the sample positions, and be sure to number them correctly.
 - b. If you are calibrating using the method of standard addition, please note that in the Autosample locations, each sample must be followed by its calibration standards, in consecutive order and increasing concentration of analyte. (see table with examples below)
 - c. Put all samples for your group in the sample tab see (below).

Example Sample locations for **External Calibration** (*samples can be in consecutive order*).

Example Sample locations for **Standard Additions with five calibration solutions** (*samples cannot be in consecutive order*).

d.

Example Sample locations for **External Calibration** (samples *can* be in consecutive order).

Sample No	A/S Location	Sample ID	Initial Sample Wt.	Sample Prep. Vol.	Aliquot Volume	Diluted To Vol.
1	11	water from FFSC				
2	12	spiked water				
3	13	filtered water				
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						
22						
23						

The image above is for an external calibration where the samples are in position 11,12,and 13. The locations of external calibration standards are set in the method editor.

...the method shown to the right would be appropriate for external calibration by simply uncheck the "Method of additions box" and leaving the A/S positions as they are.

Example Sample locations for **Standard Additions with five calibration solutions** (samples *cannot* be in consecutive order).

Sample No	A/S Location	Sample ID	Initial Sample Wt.	Sample Prep. Vol.	Aliquot Volume	Diluted To Vol.
1	11	Source				
2	17	Tainted				
3	23	Filtered				
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						
22						

The image above is for a 5 point calibration where the samples are in position 11,17,and 23. All intervening positions should contain the standard addition solutions for calibration.

...for a method of additions that looks like this (with 5 calibration solutions for each sample)

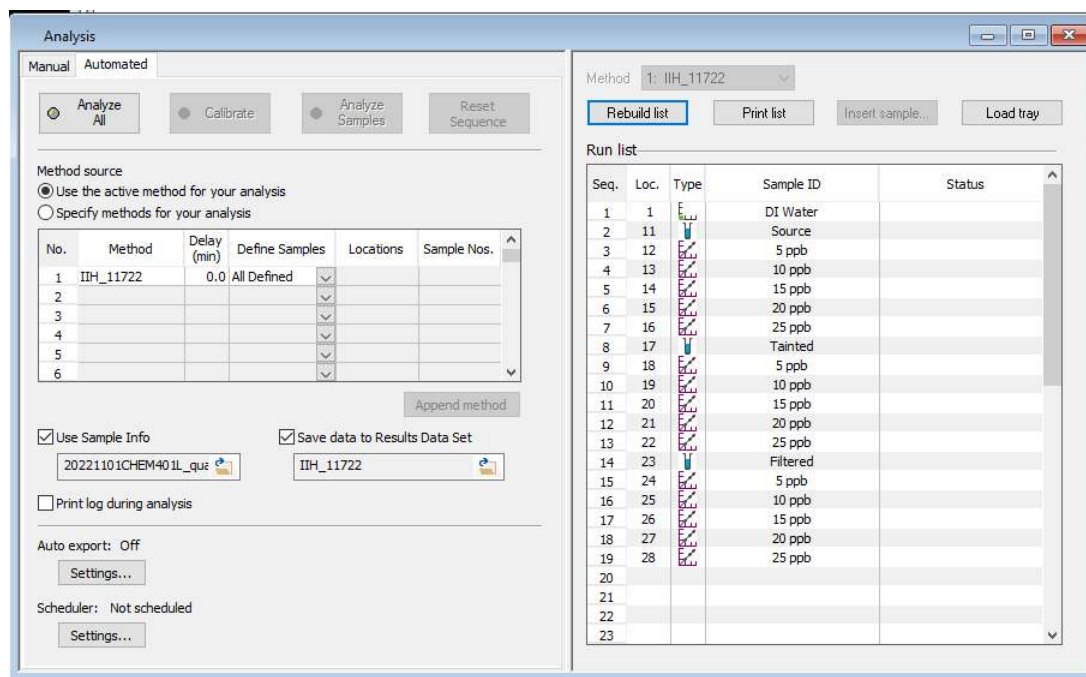
ID	A/S Location
Calib Blank 1	DI Water
Calib Blank 2	
Calib Blank 3	
Addition 1	5 ppb
Addition 2	10 ppb
Addition 3	15 ppb
Addition 4	20 ppb
Addition 5	25 ppb
Reslope Standard	
Reagent Blank 1	
Reagent Blank 2	
Reagent Blank 3	

- Save your sample list using the convention "YYYYMMDD_Qual_YOURINITIALS" for qualitative analysis or "YYYYMMDD_Pb_YOURINITIALS" for quantitative analysis.

IV. Run the Calibration and Samples

Before proceeding, be sure your method and sample list are listed in the "Status" window on the right of the software window.

1. Go to the Analysis tab in the top menu and choose **Analysis** from the Analysis box. The Analysis window will open and it will use the current method and sample list to populate a "Run list". Check the list and be sure that the positions for each solution are correct.
2. Set a new file name under which to store your data under "Save data to Results Data Set". Use the same convention used for the method and sample list.

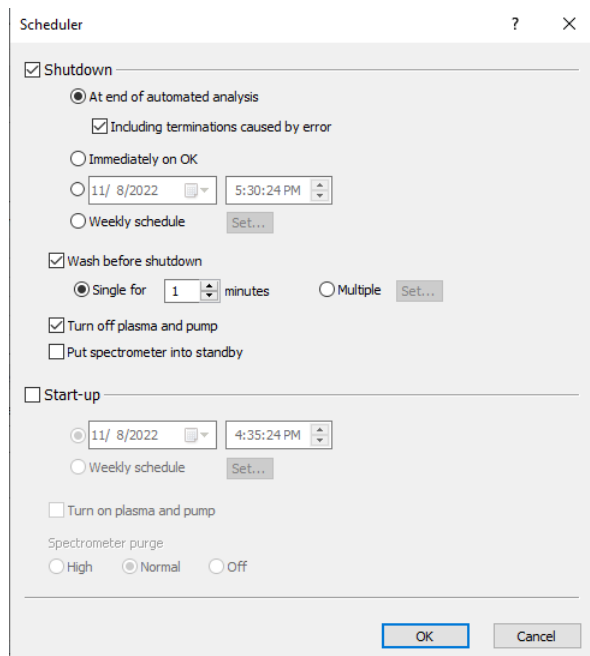


Using the scheduler to automatically shut down after an analysis.

To use the instrument overnight, make sure it is set up to turn itself off.

Go to **Scheduler settings** in the Analysis window or Scheduler under the Instrument tab. The following options will cause the instrument to completely shut down after the next analysis.

- a. select shutdown
- b. select at the end of automated analysis,
- c. select including terminations caused by errors
- d. select wash before shutdown for 1 minute
- e. select turn off plasma and pump



Turn on the Plasma

The plasma should be turned on after you have set up everything else. This is to conserve argon gas, which is quite expensive and consumed quickly by the plasma.

1. Go to the **Instrument Tab** and select **Plasma Control**
2. In the **Instrument Tab**, use the menu at the top of the screen to do the following:
 - a. Move the auto sampler to the wash location (if it is not already there).
 - b. Turn the peristolic pump on. (Visually inspect to ensure the tubing is installed correctly and that it is pumping smoothly in the correct direction.)
 - c. allow the wash solution to flow for at least 30 seconds.
 - d. Move the sampling probe to a container with deionized water and flow for 30 seconds.
3. Open the **Plasma Control** from the Instrument tab, then press the plasma on/off button to turn the plasma on. If the instrument has not been used recently, the plasma may be unstable due to air being in the lines. If the plasma goes out soon after the first attempt at lighting, re-start the plasma after a few moments.
4. After a stable plasma is attained, check the nebulizer for proper operation. The easiest way to do this is to shine a bright light on the nebulizer and observe the mist that is created when the nebulizer is functioning properly.
5. choose the measure workspace ("ball menu" and **select workspace--> measure**) go to the Analysis tab to initiate analysis.

Run the list

After the plasma is on and stable, you have confirmed the instrument is performing as expected, and the run list has been generated, do the following.

1. Check again that the run list matches the locations of all solutions in the autosampler.
2. Check that the scheduler is set if you plan to run overnight.
3. Press "Analyze all" to initiate analysis of the solutions in the sample run list.

V. Manual operation

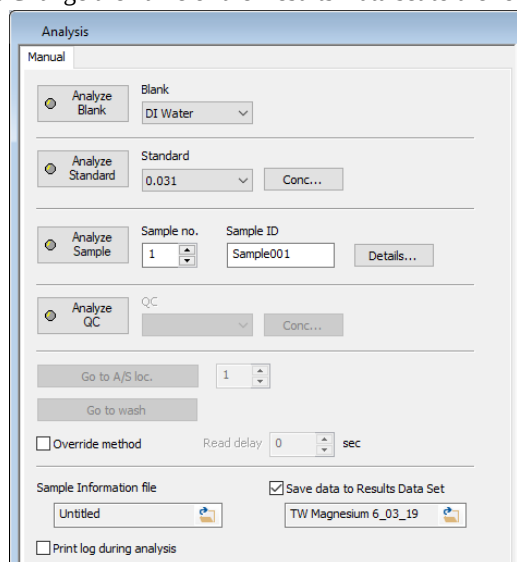
Manual operation is not typically used in the context of this course because it is more convenient to use the autosampler.

Manual Calibration the instrument

1. Go to the **Analyze tool box menu** at the top of the main **Analysis** tab and choose **Analysis**.



- In the Analysis window, choose the **Manual** tab to open the Manual Analysis Control.
(Or consult with your TA and decide whether if Automatic Analysis is a better option).
- Change the name of the Results Data set to the format YYYYMMDDCHEM401L_YOURINITIALS.



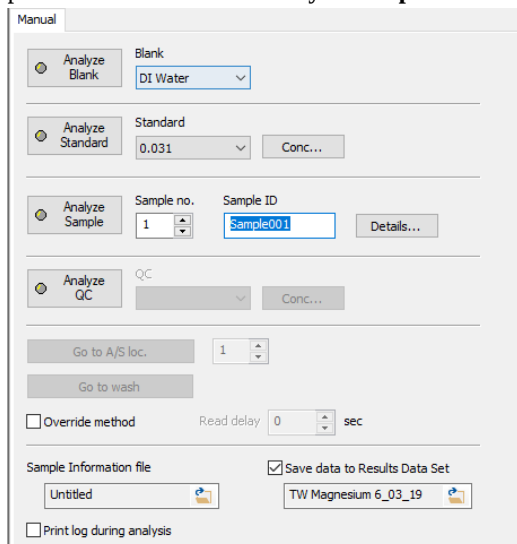
- Start the calibration with DI water.; this is the blank. Go to the A/S location containing DI water using the "Go to A/S location" option in the Analysis window. Click on **Analyze Blank** in the **Manual Analysis Control** window to make the measurement. The display in the **Results** window will show each of the three replicates and then report a mean, ABS(MEAN), standard deviation, SD, and relative standard deviation, RSD. The instrument can auto zero the detector based on this blank if it is specified in your method. You can watch the readings in real-time in the Results Tab, Spectra window.
- Next, move the sample probe to the location of the least concentrated standard. Choose the correct standard in the dropdown menu and then click the **Analyze Standard** button in the **Manual Analysis Control** window to run the analysis of the standard solution. Analyze the remaining standard solution from least-to-most concentrated in the same manner. (Give each group member a chance to operate the instrument.)
- At the end of running your standards you have a graph of the absorbance vs concentration on the screen. You have the regression statistics for the graph either at the bottom of the graph and/or in the **Results** window. Export a pdf and an excel file of these results.

Manually Measure Samples

(If applicable)

Use the **Analyze Sample** button of the **Manual Analysis Control** window. The ID for each sample was already specified in the Sample Information table (instructions above). Choose the sample for analysis and move the sample probe to the correct

position. Then click the **Analyze Sample** button. Repeat for each sample. Aspirate deionized water when finished.



The screenshot shows a software interface with a 'Manual' tab. It contains several sections for setting up an analysis:

- Analyze Blank:** A dropdown menu set to 'Blank' and a text field containing 'DI Water'.
- Analyze Standard:** A dropdown menu set to 'Standard', a text field with '0.031', and a 'Conc...' button.
- Analyze Sample:** A dropdown menu set to 'Sample no.', a text field with '1', a 'Sample ID' field with 'Sample001', and a 'Details...' button.
- Analyze QC:** A dropdown menu set to 'QC', a text field with '0.031', and a 'Conc...' button.
- Go to A/S loc.:** A button and a text field with '1'.
- Go to wash:** A button.
- Override method:** A checkbox and a 'Read delay' field set to '0' with a 'sec' unit.
- Sample Information file:** Two text fields, one with 'Untitled' and another with 'TW Magnesium 6_03_19'.
- Save data to Results Data Set:** A checked checkbox.
- Print log during analysis:** An unchecked checkbox.

The results of the sample runs will automatically be saved in your data file. **You should print a pdf copy of the Results Window at the end of the run.** To "print" a pdf, click on the Results window to select it. Next, click on the  button at the top left of the main software screen and select **Print Active Window**. Select **CutePDF Writer** from the list of available printers. Save the pdf file and note the location where it is saved. Feel free to create your own folder on the desktop where you can save all of your results over the two-week experiment.

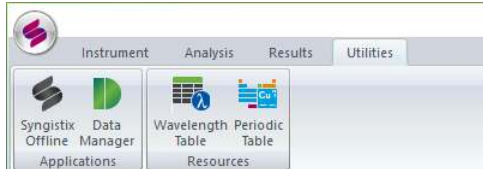
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3.5: Instructions for Viewing and Exporting Data using Syngistics Software

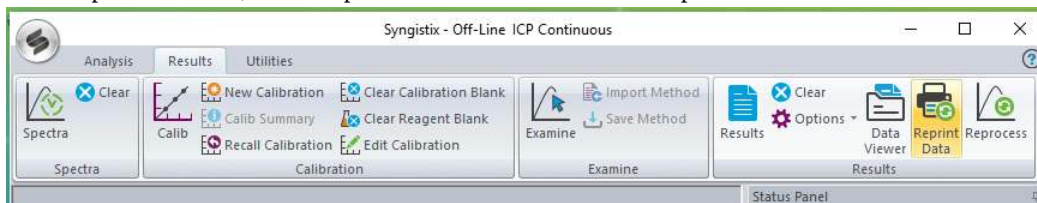
It is a good habit to use the "offline" version of the software to view and export data that has already been collected. If you use the "online" version (the version used to control the instrument) you could be "locked out" of some features or you can accidentally interfere with someone else's data collection. Follow the steps below to view, capture, and export data collected using the Avio 220 max.

Open Results in the Offline Syngistics

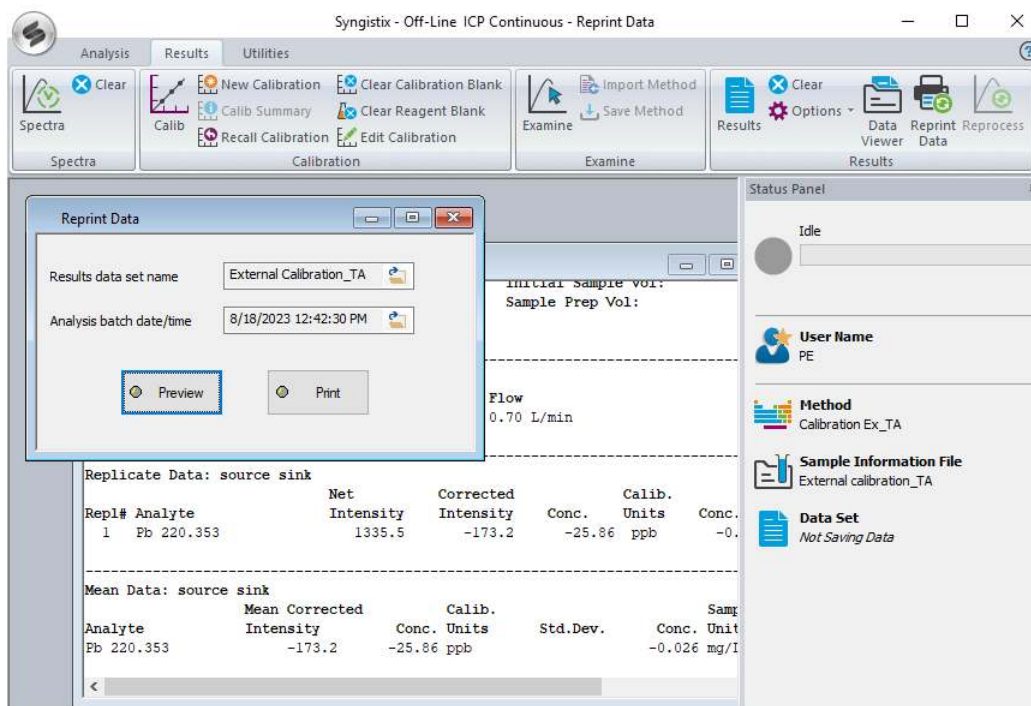
1. Open the Offline version of the Syngistics software. You can do this by selecting the grey Syngistics icon  from the desktop or by selecting it from the Utilities tab in the online software.



2. In the top ribbon menu, choose open the Results tab and select Reprint Data.

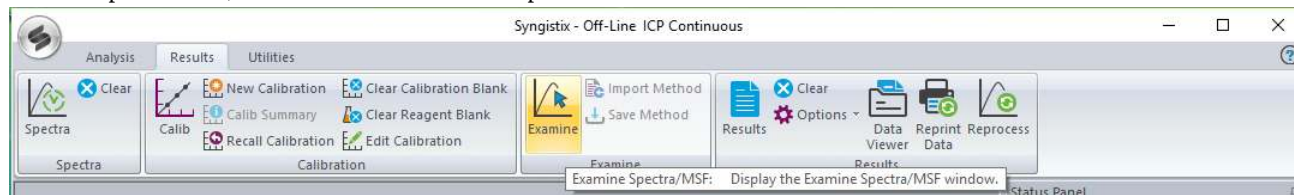


3. Choose the Data Set and the batch date/time that you wish to view by clicking on the folder icon  in the Reprint Data window. Then press **Preview** and wait until the data has been compiled. The Results window will open with a typed list of each standard and sample result.

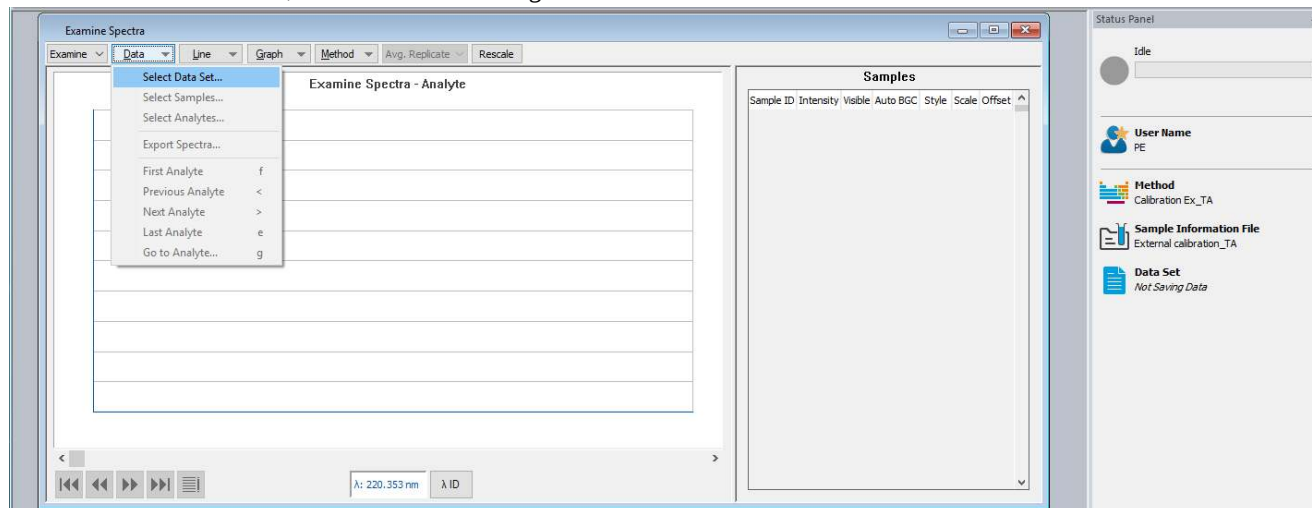


View, Capture, and Export Spectra and Qualitative Data

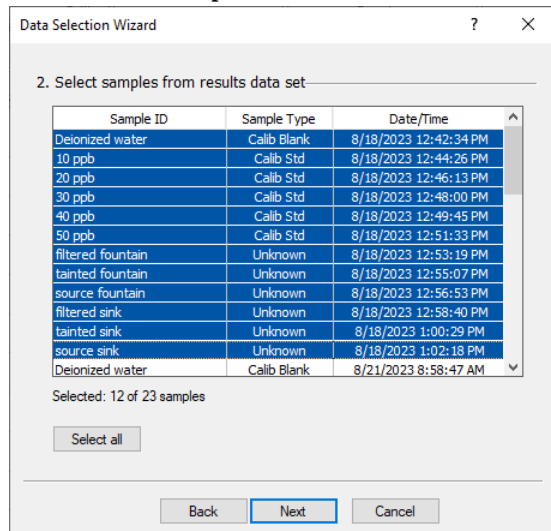
1. To view spectral data, use the Examine window. Open the Examine window from the Results tab on the ribbon toolbar.



2. From the Examine window, select the data set using **Data --> Select Data Set**.

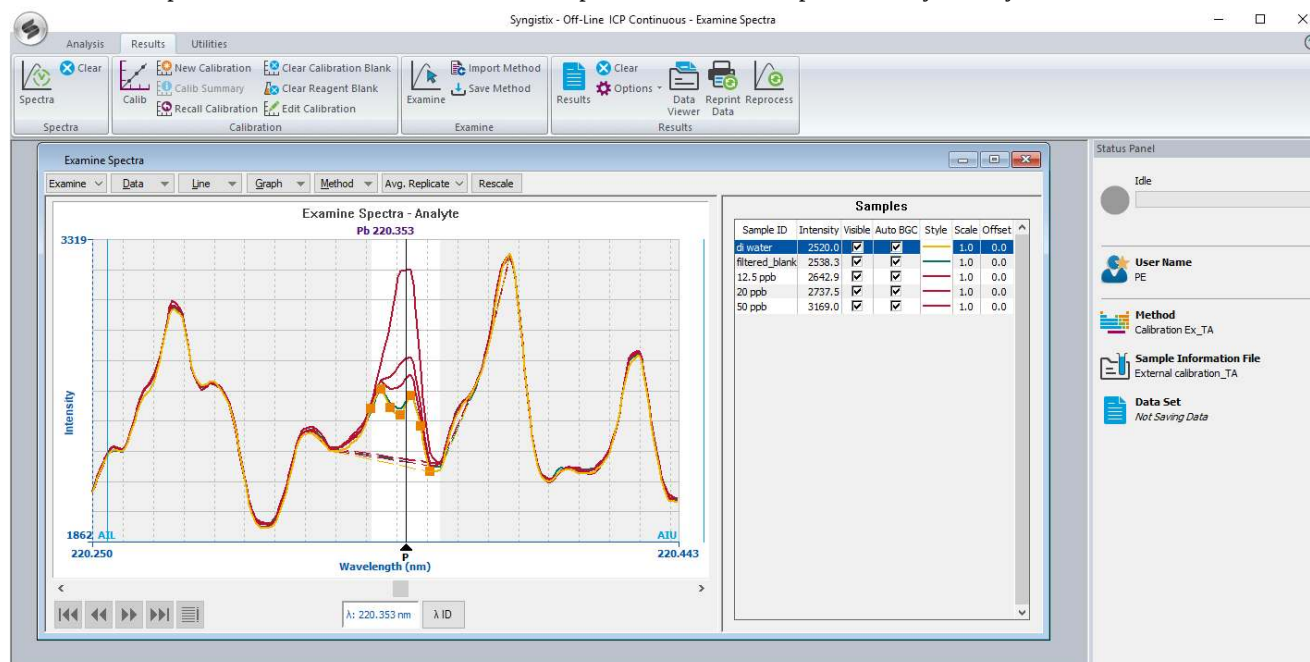


3. Choose which samples from the data set to view. Then press **Next**.



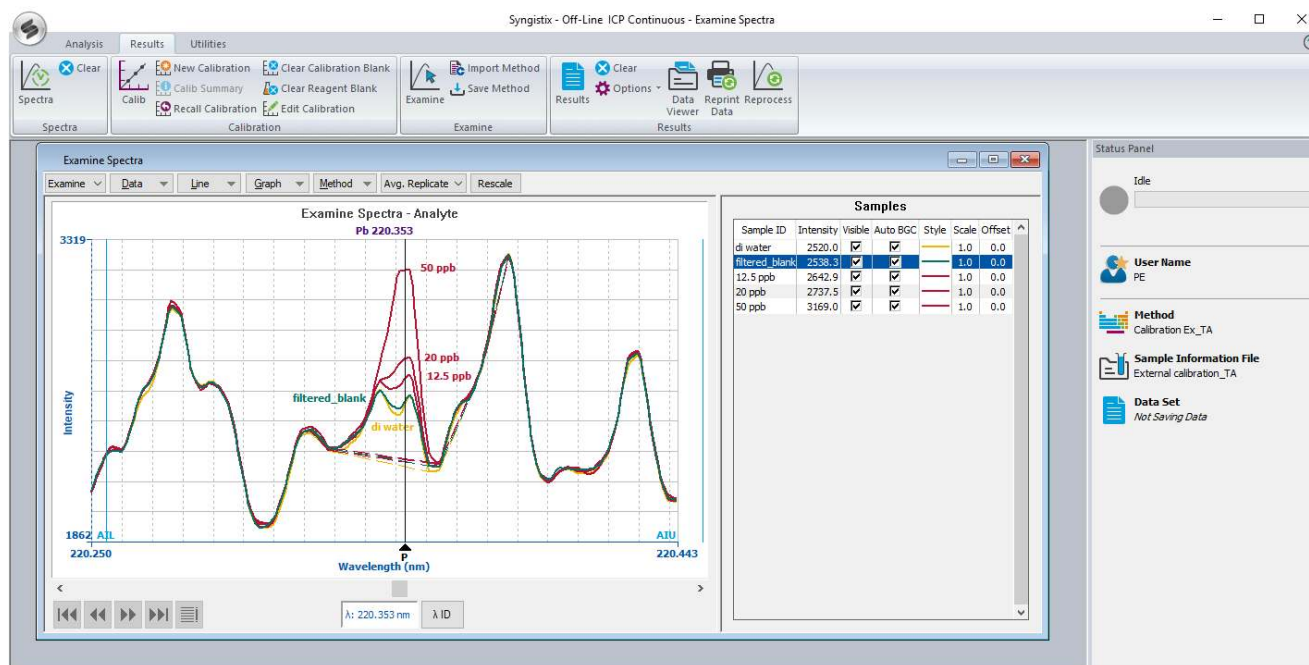
4. Select the analytes from each sample that you want to view. Then select **Finish**.

5. The Examine Spectra window should now contain the spectra from each sample and analyte that you choose.



6. Adjust view options in Examine Spectra window:

- The spectrum of each sample is shown in the window. Each spectrum also has a dashed line that is the "automatic background correction" - this shows how each peak is integrated.
- There is a list of samples at the right side of the window, and you can click on each sample to make it the "active" sample.
- The highlighted trace should become active in the main spectral window when you click on the sample name.
- The analyte is listed at the top of the spectrum (eg Pb measured at 220.353 nm is shown in the example above).
- You can change the analyte shown using the arrow buttons at the bottom left of the window, or by using the "Data" drop-down menu at the top of the "Examine Spectra" window.
- If the options are chosen to show the "Analytical Points", small orange-yellow boxes called "analytical points" will appear on the active spectrum and the window will be "greyed out" on either side of the analyte peak. To change this, right click anywhere on the list of samples and deselect the "Show Analytical Points" option.
- You can annotate the data with the names of each sample using the Graph options: Graph --> Add Label. To move a label after it is added, highlight the sample to which the label is attached and press SHIFT + CTRL simultaneously, then click on the new location where you would like the label to appear.



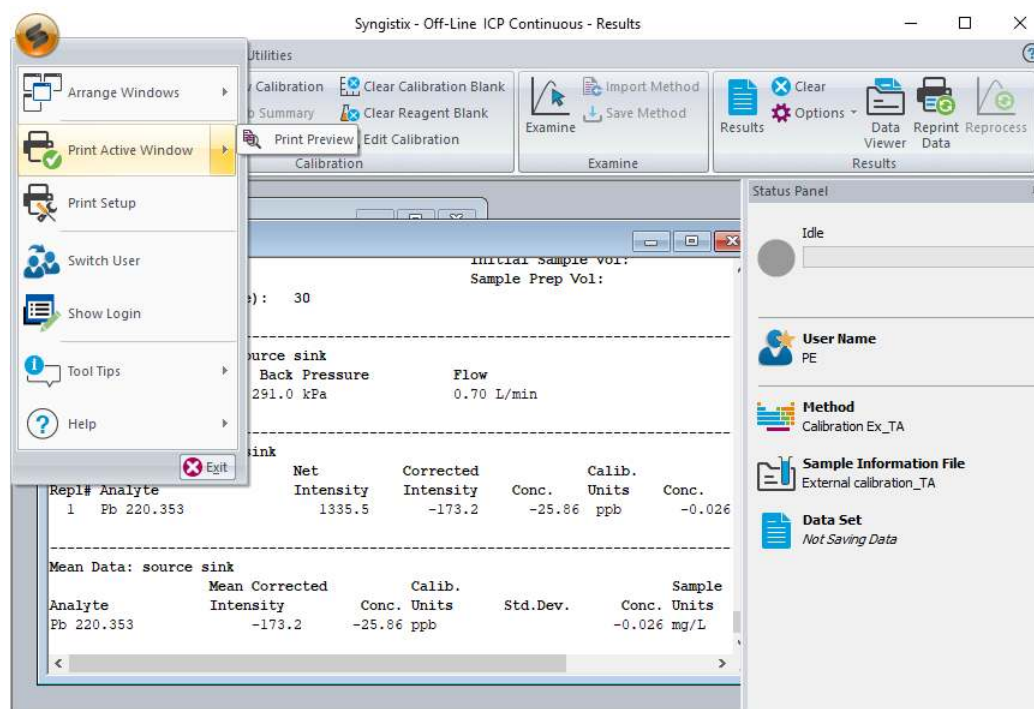
7. When you have a view that you think is appropriate for capturing your spectra, you can capture the view using the Snipping Tool .

8. You can export spectra into a comma delineated or text file using **Data--> Export Spectra** option. If you export as a comma delineated file, then you can import the file into excel or another similar program to create customized plots of the data.

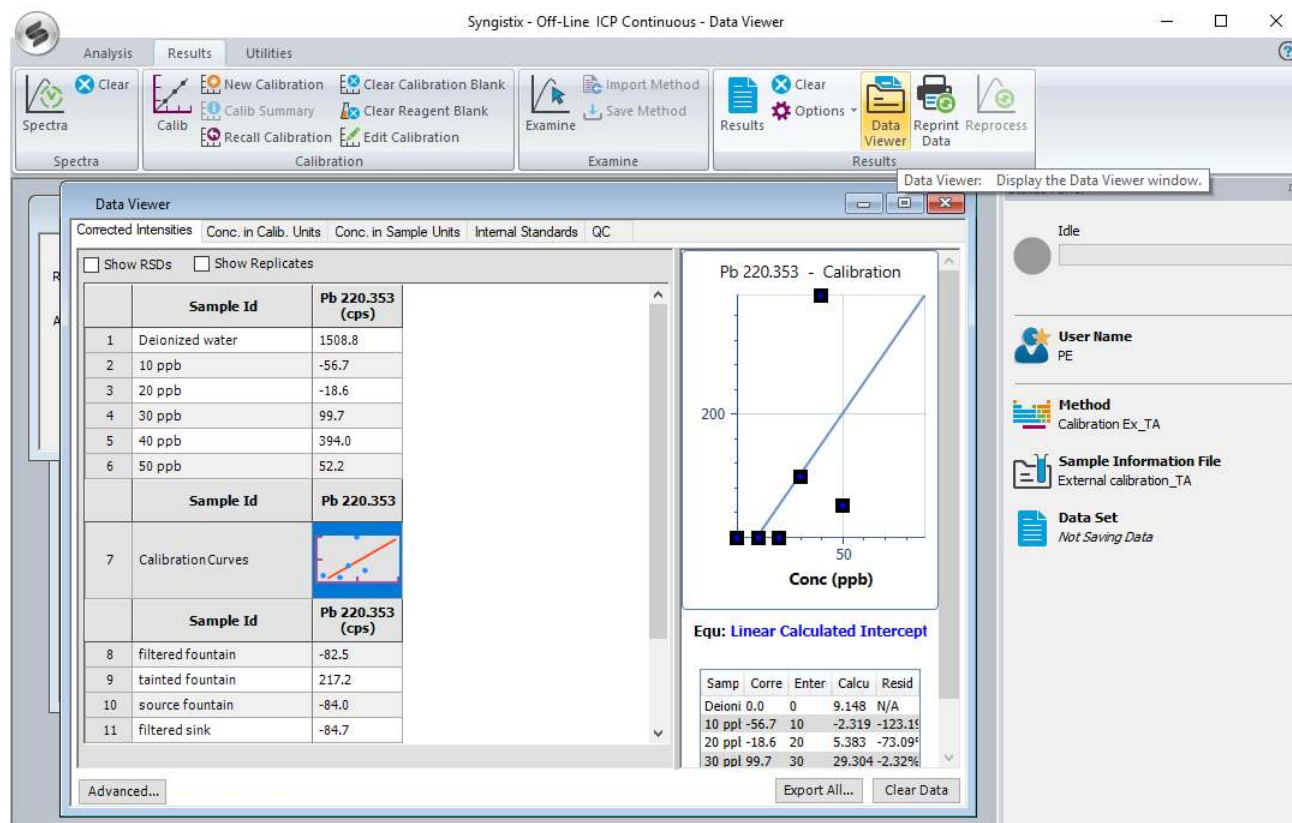
View and Export Quantitative Data

1. To export the Results window, click on it to make it active. Then go to the Ball icon at the top left-hand corner of the Syngistix window, and select Print Active Window. Then choose the printer as "Microsoft Print to PDF". Give the report a name beginning with "YYYYMMDD..." and a specifier that you recognize. Please specify the directory folder as **C:\Users\Public\PerkinElmer Syngistix\ICP\Data\Reports\YYYY_reportsCHEM401L** (YYYY is the current year). This will produce a pdf containing the

calculated results.



- After the results have been reprinted, you can also open them in the Data Viewer. Choose the **Data Viewer** from the Results tab. Notice that there are different views of the data available using the tabs at the top of this window. The data in each tab depends on how you set up your method. For a quantitative method, you can view calibration curves in some of the tabs.



- Export the data from the Data Viewer window into an .xlsx (Microsoft Excel) file by clicking the "Export All..." button at the bottom right of the Data Viewer window. Please choose the same directory specified above and name your .xlsx file using the

same root name as the pdf.

Creating figures for your Report

The simplest way to show the emission spectra is to use the Synergistics software, then capturing the figure as it appears in the software. To date this is the only way we have figured out to show spectra. On the other hand, calibration data can be exported and plotted in any software you prefer.

To create **overlaid emission spectra from multiple samples**, you can use the **Examine** feature and then use a screen capture or printing the active screen (ball menu, see Figure 3.5.1).

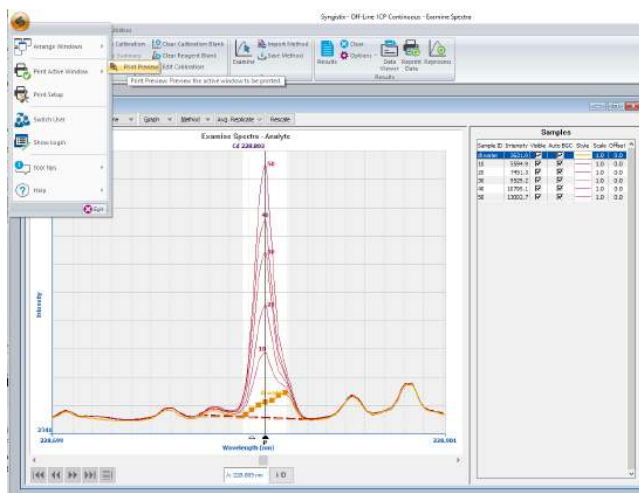


Figure 3.5.1: Spectra can be viewed using the Examine button in the Results Tab, and then printed to a pdf using the ball menu (top right) and then choose Print Active Window. Alternatively a screen shot can be taken using Shift + Windows + s. (CC-BY-SA; Kathryn Haas)

If you'd like to create multi-panel figures, or do more customized labeling of emission spectra, one way is to copy and past each figure into a powerpoint slide and arrange the plots, add annotations. An example spectral figure is given below in Figure 3.5.2. This was created by putting two screen captures in the same powerpoint slide, annotating the panels, and taking a screen shot.

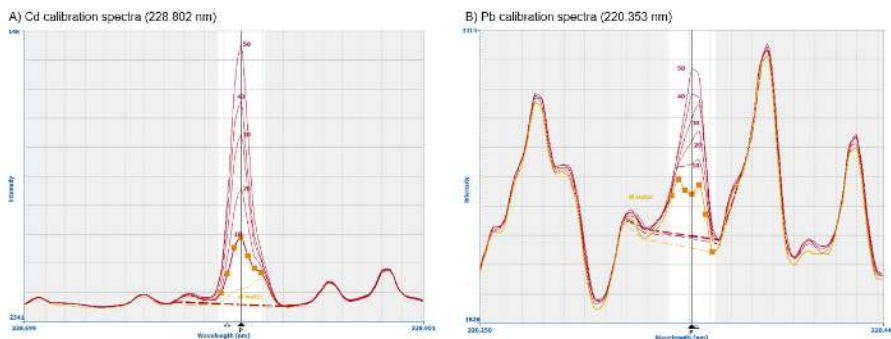


Figure 3.5.2: Emission spectra from Cd and Pb in calibration standards detected by ICP-OES, monitored at 228.802 and 220.353 nm, respectively. Spectra from deionized water (di water) are shown in yellow. Standards that contain 10, 20, 30, 40, and 50 ppb of Pb and Cd are shown in red and indicated using red labels (units in ppb = $\mu\text{g/L}$).

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CHAPTER OVERVIEW

4: Gas Chromatogram, Mass Spectrometer (GCMS)

- [4.1: Introduction to Gas Chromatography Instrumentation](#)
- [4.2: Quantitative and Qualitative GC and GC-MS](#)
- [4.3: Mass Spectrometers](#)
- [4.4: Interpreting Electron Ionization Mass Spectra](#)
- [4.5: GC-MS Instruments at Duke](#)
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4.1: Introduction to Gas Chromatography Instrumentation

GC Instrumentation

In **gas chromatography** (GC) we inject the sample, which may be a gas or a liquid, into a gaseous mobile phase (often called the carrier gas). The mobile phase carries the sample through a stationary phase (the column) that separates the sample's components based on their ability to partition between the mobile phase and the stationary phase. This is similar to liquid chromatography (see the [HPLC module](#)), except that the mobile phase is gas in this case.

The instrument is called a gas **chromatogram**. Figure 4.1.1 shows an example of a typical gas chromatograph, which consists of several key components:

- the mobile phase; a supply of compressed gas
- a heated injector, which rapidly volatilizes the components in a liquid sample
- an oven whose temperature we can control during the separation
- a column, which is placed within the oven
- a detector to monitor the eluent as it comes off the column.

Each component will be described in detail below.



Figure 4.1.1 . Example of a typical gas chromatograph with insets showing the heated injection ports—note the symbol indicating that it is hot—and the oven that houses the column. This particular instrument is equipped with an autosampler for injecting samples, a capillary column, and a mass spectrometer (MS) as the detector. Note that the carrier gas is supplied by a tank of compressed gas.

The Mobile Phase (Carrier Gas)

The mobile phase is an inert gas that does not react with the sample or the stationary phase; the most common mobile phases for gas chromatography are He, Ar, and N₂. The choice of carrier gas often is determined by the needs of instrument's detector. The flow rates depend on the type of column; it is usually 25–150 mL/min for a packed column and 1–25 mL/min for a capillary column.

The Stationary Phase (Column)

There are two broad classes of chromatographic columns: packed columns and capillary columns. In general, a packed column can handle larger samples and a capillary column can separate more complex mixtures. Only the capillary column is described here.

A capillary, or **open tubular column** is constructed from fused silica and is coated with a protective polymer coating. Columns range from 15–100 m in length with an internal diameter of approximately 150–300 μm . Figure 4.1.3 shows an example of a typical capillary column.



Figure 4.1.3 . Typical example of a capillary column for gas chromatography. This column is 30 m long with an internal diameter of 247 μm . The interior surface of the capillary has a 0.25 μm coating of the liquid phase.

Capillary columns are of three principal types. In a **wall-coated open tubular column** (WCOT) a thin layer of stationary phase, typically 0.25 nm thick, is coated on the capillary's inner wall. In a **porous-layer open tubular column** (PLOT), a porous solid support—alumina, silica gel, and molecular sieves are typical examples—is attached to the capillary's inner wall. A **support-coated open tubular column** (SCOT) is a PLOT column that includes a liquid stationary phase. Figure 4.1.4 shows the differences between these types of capillary columns.

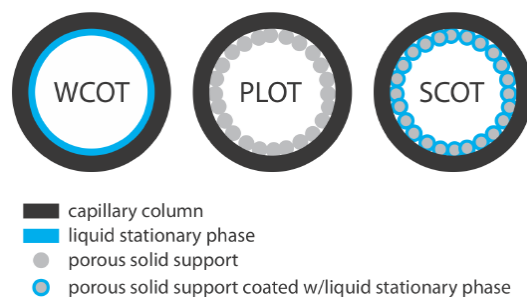


Figure 4.1.4 . Cross sections through the three types of capillary columns.

A capillary column provides a significant improvement in separation efficiency because it has more theoretical plates per meter and is longer than a packed column. For example, the capillary column in Figure 4.1.3 has almost 4300 plates/m, or a total of 129 000 theoretical plates. If we assume a $V_{\max}/V_{\min} \approx 50$, then it has a peak capacity of approximately 350. On the other hand, a packed column can handle a larger sample. Because of its smaller diameter, a capillary column requires a smaller sample, typically less than 10^{-2} μL .

Choice of stationary phase

Elution order in gas–liquid chromatography depends on two factors: the boiling point of the solutes, and the interaction between the solutes and the stationary phase. If a mixture's components have significantly different boiling points, then the choice of stationary phase is less critical. If two solutes have similar boiling points, then a separation is possible only if the stationary phase selectively interacts with one of the solutes. As a general rule, nonpolar solutes are separated more easily when using a nonpolar stationary phase, and polar solutes are easier to separate when using a polar stationary phase.

There are several important criteria for choosing a stationary phase: it must not react with the solutes, it must be thermally stable, it must have a low volatility, and it must have a polarity that is appropriate for the sample's components.

Many stationary phases have the general structure shown in Figure 4.1.5 a. A stationary phase of polydimethyl siloxane, in which all the $-\text{R}$ groups are methyl groups, $-\text{CH}_3$, is nonpolar and often makes a good first choice for a new separation. The order of elution when using polydimethyl siloxane usually follows the boiling points of the solutes, with lower boiling solutes eluting first. Replacing some of the methyl groups with other substituents increases the stationary phase's polarity and provides greater selectivity. For example, replacing 50% of the $-\text{CH}_3$ groups with phenyl groups, $-\text{C}_6\text{H}_5$, produces a slightly polar stationary phase. Increasing polarity is provided by substituting trifluoropropyl, $-\text{C}_3\text{H}_6\text{CF}$, and cyanopropyl, $-\text{C}_3\text{H}_6\text{CN}$, functional groups, or by using a stationary phase of polyethylene glycol (Figure 4.1.5 b).

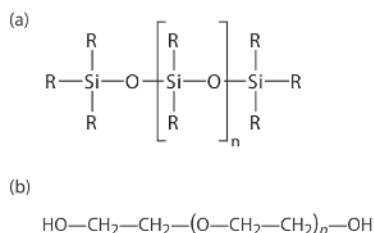


Figure 4.1.5 . General structures of common stationary phases: (a) substituted polysiloxane; (b) polyethylene glycol.

An important problem with all liquid stationary phases is their tendency to elute, or **bleed** from the column when it is heated. The temperature limits in Table 4.1.1 minimize this loss of stationary phase. Capillary columns with bonded or cross-linked stationary phases provide superior stability. A bonded stationary phase is attached chemically to the capillary's silica surface. Cross-linking, which is done after the stationary phase is in the capillary column, links together separate polymer chains to provide greater stability.

Another important consideration is the thickness of the stationary phase. From equation 12.3.7 we know that separation efficiency improves with thinner film of stationary phase. The most common thickness is 0.25 μm , although a thicker films is useful for highly volatile solutes, such as gases, because it has a greater capacity for retaining such solutes. Thinner films are used when separating low volatility solutes, such as steroids.

A few stationary phases take advantage of chemical selectivity. The most notable are stationary phases that contain chiral functional groups, which are used to separate enantiomers [Hinshaw, J. V. *LC .GC* **1993**, *11*, 644–648].

Sample Introduction

Three factors determine how we introduce a sample to the gas chromatograph. First, all of the sample's constituents must be volatile. Second, the analytes must be present at an appropriate concentration. Finally, the physical process of injecting the sample must not degrade the separation. Each of these needs is considered in this section.

Preparing a Volatile Sample

Not every sample can be injected directly into a gas chromatograph. To move through the column, the sample's constituents must be sufficiently volatile. A solute of low volatility, for example, may be retained by the column and continue to elute during the analysis of subsequent samples. A nonvolatile solute will condense at the top of the column, degrading the column's performance.

We can separate a sample's volatile analytes from its nonvolatile components using any of the extraction techniques described in Chapter 7. A liquid–liquid extraction of analytes from an aqueous matrix into methylene chloride or another organic solvent is a common choice. Solid-phase extractions also are used to remove a sample's nonvolatile components.

An attractive approach to isolating analytes is a **solid-phase microextraction** (SPME). In one approach, which is illustrated in Figure 4.1.6 , a fused-silica fiber is placed inside a syringe needle. The fiber, which is coated with a thin film of an adsorbent material, such as polydimethyl siloxane, is lowered into the sample by depressing a plunger and is exposed to the sample for a predetermined time. After withdrawing the fiber into the needle, it is transferred to the gas chromatograph for analysis.

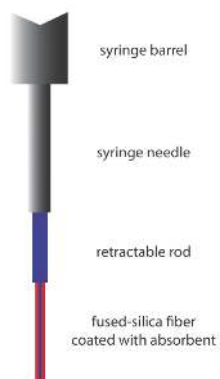


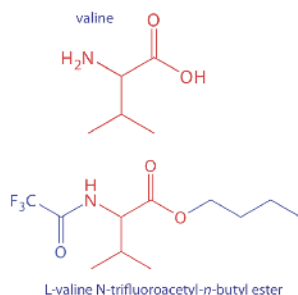
Figure 4.1.6 . Schematic diagram of a solid-phase microextraction device. The adsorbent is shown in red.

Two additional methods for isolating volatile analytes are a purge-and-trap and headspace sampling. In a **purge-and-trap**, we bubble an inert gas, such as He or N₂, through the sample, releasing—or purging—the volatile compounds. These compounds are carried by the purge gas through a trap that contains an adsorbent material, such as Tenax, where they are retained. Heating the trap and back-flushing with carrier gas transfers the volatile compounds to the gas chromatograph. In **headspace sampling** we place the sample in a closed vial with an overlying air space. After allowing time for the volatile analytes to equilibrate between the sample and the overlying air, we use a syringe to extract a portion of the vapor phase and inject it into the gas chromatograph. Alternatively, we can sample the headspace with an SPME.

Thermal desorption is a useful method for releasing volatile analytes from solids. We place a portion of the solid in a glass-lined, stainless steel tube. After purging with carrier gas to remove any O₂ that might be present, we heat the sample. Volatile analytes are swept from the tube by an inert gas and carried to the GC. Because volatilization is not a rapid process, the volatile analytes often are concentrated at the top of the column by cooling the column inlet below room temperature, a process known as **cryogenic focusing**. Once volatilization is complete, the column inlet is heated rapidly, releasing the analytes to travel through the column.

The reason for removing O₂ is to prevent the sample from undergoing an oxidation reaction when it is heated.

To analyze a nonvolatile analyte we must convert it to a volatile form. For example, amino acids are not sufficiently volatile to analyze directly by gas chromatography. Reacting an amino acid, such as valine, with 1-butanol and acetyl chloride produces an esterified amino acid. Subsequent treatment with trifluoroacetic acid gives the amino acid's volatile N-trifluoroacetyl-*n*-butyl ester derivative.



Adjusting the Analyte's Concentration

If an analyte's concentration is too small to give an adequate signal, then we must concentrate the analyte before we inject the sample into the gas chromatograph. A side benefit of many extraction methods is that they often concentrate the analytes. Volatile organic materials isolated from an aqueous sample by a purge-and-trap, for example, are concentrated by as much as 1000×

If an analyte is too concentrated, it is easy to overload the column, resulting in peak fronting (see Figure 12.2.7) and a poor separation. In addition, the analyte's concentration may exceed the detector's linear response. Injecting less sample or diluting the sample with a volatile solvent, such as methylene chloride, are two possible solutions to this problem.

Injecting the Sample

In Chapter 12.3 of Harvey's Analytical Text, he describes several explanations for why a solute's band increases in width as it passes through the column, a process we called band broadening. We also introduce an additional source of band broadening if we fail to inject the sample into the minimum possible volume of mobile phase. There are two principal sources of this precolumn band broadening: injecting the sample into a moving stream of mobile phase and injecting a liquid sample instead of a gaseous sample. The design of a gas chromatograph's injector helps minimize these problems.

An example of a simple injection port for a packed column is shown in Figure 4.1.7. The top of the column fits within a heated injector block, with carrier gas entering from the bottom. The sample is injected through a rubber septum using a microliter syringe, such as the one shown in in Figure 4.1.8. Injecting the sample directly into the column minimizes band broadening because it mixes the sample with the smallest possible amount of carrier gas. The injector block is heated to a temperature at least 50°C above the boiling point of the least volatile solute, which ensures a rapid vaporization of the sample's components.

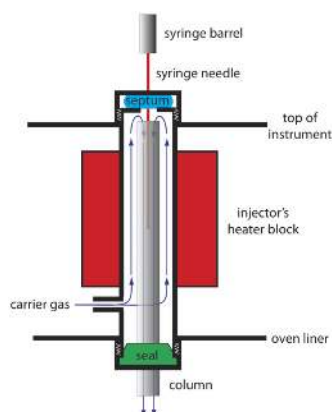


Figure 4.1.7. Schematic diagram of a heated GC injector port for use with packed columns. The needle pierces a rubber septum and enters into the top of the column, which is located within a heater block.



Figure 4.1.8 . Example of a syringe for injecting samples into a gas chromatograph. This syringe has a maximum capacity of 10 μL with graduations every 0.1 μL .

Because a capillary column's volume is significantly smaller than that for a packed column, it requires a different style of injector to avoid overloading the column with sample. Figure 4.1.9 shows a schematic diagram of a typical split/splitless injector for use with a capillary column.

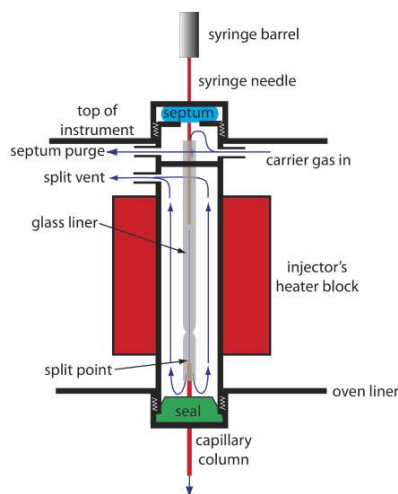


Figure 4.1.9 . Schematic diagram of a split/splitless injection port for use with capillary columns. The needle pierces a rubber septum and enters into a glass liner, which is located within a heater block. In a split injection the split vent is open; the split vent is closed for a splitless injection.

In a **split injection** we inject the sample through a rubber septum using a microliter syringe. Instead of injecting the sample directly into the column, it is injected into a glass liner where it mixes with the carrier gas. At the split point, a small fraction of the carrier gas and sample enters the capillary column with the remainder exiting through the split vent. By controlling the flow rate of the carrier gas as it enters the injector, and its flow rate through the septum purge and the split vent, we can control the fraction of sample that enters the capillary column, typically 0.1–10%.

For example, if the carrier gas flow rate is 50 mL/min, and the flow rates for the septum purge and the split vent are 2 mL/min and 47 mL/min, respectively, then the flow rate through the column is 1 mL/min ($= 50 - 2 - 47$). The ratio of sample entering the column is 1/50, or 2%.

In a **splitless injection**, which is useful for trace analysis, we close the split vent and allow all the carrier gas that passes through the glass liner to enter the column—this allows virtually all the sample to enter the column. Because the flow rate through the injector is low, significant precolumn band broadening is a problem. Holding the column's temperature approximately 20–25°C below the solvent's boiling point allows the solvent to condense at the entry to the capillary column, forming a barrier that traps the solutes. After allowing the solutes to concentrate, the column's temperature is increased and the separation begins.

For samples that decompose easily, an **on-column injection** may be necessary. In this method the sample is injected directly into the column without heating. The column temperature is then increased, volatilizing the sample with as low a temperature as is practical.

Oven with Temperature Control

In gas chromatography a sample is injected into a column containing a stationary phase composed either of a packed, liquid-coated solid support material (a "packed" column) or a high molecular weight, thermally stable polymer film coating the column inner wall (a capillary column). The sample is carried through the column by an inert "carrier gas" (the mobile phase). Because of their

different affinities for the stationary phase the individual components of the sample mixture pass through the column at different rates, eluting separately from the column at characteristic retention times. In a typical GC experiment the column temperature and carrier gas flow rate both affect the quality of the separation. With regard to the column temperature, two types of GC experiments should be considered, isothermal (constant temperature) and temperature programmed.

Isothermal Gas Chromatography

In an **isothermal** separation we maintain the column at a constant temperature. To increase the interaction between the solutes and the stationary phase, the temperature usually is set slightly below that of the lowest-boiling solute.

Consider, for example, the separation of members of a homologous series such as the n- alkanes. As shown in Figure 4.1.9, Panel A, in an isothermal experiment one expects the retention times of the solutes to vary as a function of boiling point. Indeed, the boiling points, and similarly, the heats of vaporization, ΔH_v , have been shown to increase linearly with increasing carbon number, which is related to molar volume. It has also been shown that the retention volume, V_r , is related to $-\Delta H_v$ by the equation below where R is the gas constant and T is the column temperature used in the chromatographic experiment. Based on the above equation it is evident that a linear relationship also exists between $\ln V_r$ and $1/T$, and that at constant temperature a linear relationship should exist between V_r and carbon number in the homologous series.

$$\ln V_r = (-\Delta H_v/RT) + \text{constant} \quad (4.1.1)$$

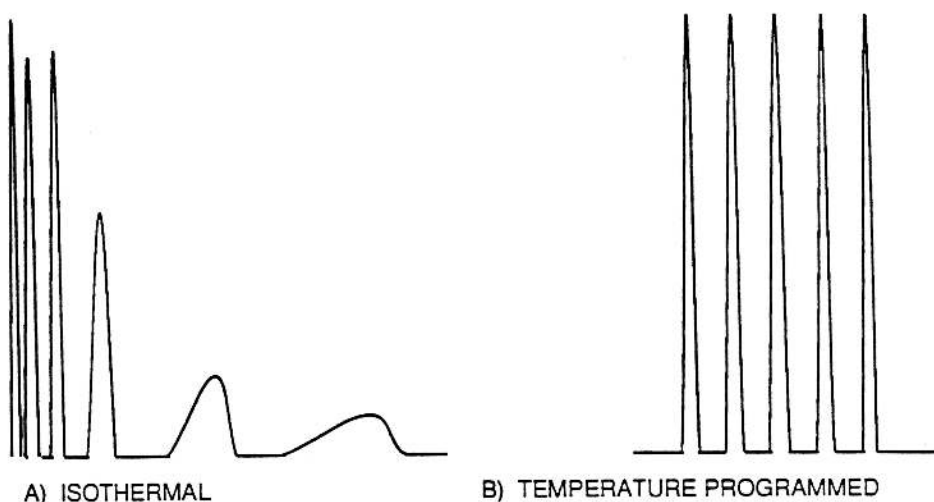


Figure 4.1.9: Example of an Isothermal Gas Chromatogram. (Copyright; author via source)

From the known relationship $V_r = V_o + KV_s$ and $k' = KV_s/V_o$ where V_o is the column dead volume, K is the partition coefficient for a particular volatile component, V_s is the volume of the stationary phase, and k' is the capacity factor, a net retention parameter, it can be shown that

$$k' = (V_r - V_o) / V_o \quad (4.1.2)$$

For a constant flow rate, F , $V_r = trF$ (tr = total retention time), and $V_o = toF$ (to = retention time of an un-retained species). Equation 4.1.2 can be rewritten as:

$$k' = (t_r - t_o) / t_o \quad (4.1.3)$$

Since k' is proportional to V_r , Equation 4.1.1 can then be written in the following form:

$$\ln[(t_r - t_o) / t_o] = (-\Delta H_v/RT) + \text{constant} \quad (4.1.4)$$

Thus, a linear relationship should result from plotting the measured retention times for a homologous series versus carbon number.

The advantage of an isothermal experiment is that the theory suggests that this linear relationship will hold for the separation of a homologous series of compounds. The disadvantage is that, in practice, when a large number of samples with a wide range in physical- chemical properties are separated, peaks for compounds of low boiling points tend to be crowded together at the beginning of the chromatogram while the slow moving, high boiling components tend to appear as severely broadened peaks with

impractically long retention times (Figure 4.1.9). The most common way to solve this problem is to use temperature programmed gas chromatography (TPGC).

Temperature Programmed Gas Chromatography (TPGC)

One difficulty with an isothermal separation is that a temperature that favors the separation of a low-boiling solute may lead to an unacceptably long retention time for a higher-boiling solute. **Temperature programming** provides a solution to this problem. At the beginning of the analysis we set the column's initial temperature below that for the lowest-boiling solute. As the separation progresses, we slowly increase the temperature at either a uniform rate or in a series of steps.

When a sample is injected into a gas chromatograph, individual components of the sample mixture pass through the column at different rates, eluting separately from the column at characteristic retention times depending on their affinity for the stationary phase, the temperature of the column, and the rate of flow of carrier gas. An optimized separation involves the correct balancing of these conditions. By slowly raising the temperature of the column during the separation process, each component may be eluted at its optimum temperature for the flow rate chosen. In general the minimum and maximum temperatures should bracket the boiling points of the most and least volatile components of the sample. The rate at which heating is to take place is dependent on the number and the retention spacing of the components to be separated. Figure 4.1.9 shows how typical isothermal chromatogram (Figure 4.1.9A) is optimized by TPGC (Figure 4.1.9B). It is found experimentally that when a constant heating rate is used the mathematical formulas described above in Equations 4.1.1 - 4.1.2 tend to hold and thus we tend to see the expected linear relationships between members of a homologous series of compounds.

Detectors for Gas Chromatography

The final part of a gas chromatograph is the detector. The ideal detector has several desirable features: a low detection limit, a linear response over a wide range of solute concentrations (which makes quantitative work easier), sensitivity for all solutes or selectivity for a specific class of solutes, and an insensitivity to a change in flow rate or temperature. This section will describe the two types of detectors that are available in the undergraduate teaching labs at Duke. If you are interested in learning what other types of detectors can be used with GC, see [12.4: Gas Chromatography](#) by David Harvey.

Flame Ionization Detector (FID)

The combustion of an organic compound in an H_2 /air flame results in a flame that contains electrons and organic cations, presumably CHO^+ . Applying a potential of approximately 300 volts across the flame creates a small current of roughly 10^{-9} to 10^{-12} amps. When amplified, this current provides a useful analytical signal. This is the basis of the popular **flame ionization detector**, a schematic diagram of which is shown in Figure 4.1.10 .

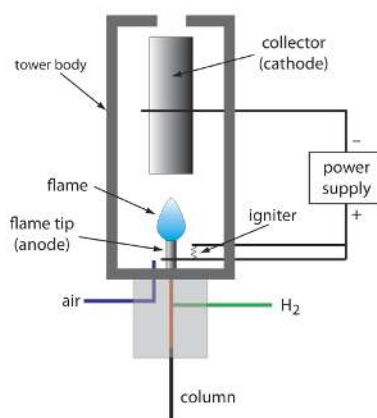


Figure 4.1.10 . Schematic diagram of a flame ionization detector. The eluent from the column mixes with H_2 and is burned in the presence of excess air. Combustion produces a flame that contains electrons and the cation CHO^+ . Applying a potential between the flame's tip and the collector gives a current that is proportional to the concentration of cations in the flame.

Most carbon atoms—except those in carbonyl and carboxylic groups—generate a signal, which makes the FID an almost universal detector for organic compounds. Most inorganic compounds and many gases, such as H_2O and CO_2 , are not detected, which makes the FID detector a useful detector for the analysis of organic analytes in atmospheric and aqueous environmental samples. Advantages of the FID include a detection limit that is approximately two to three orders of magnitude smaller than that for a

thermal conductivity detector, and a linear response over 10^6 – 10^7 orders of magnitude in the amount of analyte injected. The sample, of course, is destroyed when using a flame ionization detector.

Mass Spectrometer (MS)

A **mass spectrometer** is an instrument that ionizes a gaseous molecule using sufficient energy that the resulting ion breaks apart into smaller ions. Because these ions have different mass-to-charge ratios, it is possible to separate them using a magnetic field or an electrical field. The resulting mass spectrum contains both quantitative and qualitative information about the analyte. Figure 4.1.11 shows a mass spectrum for toluene.

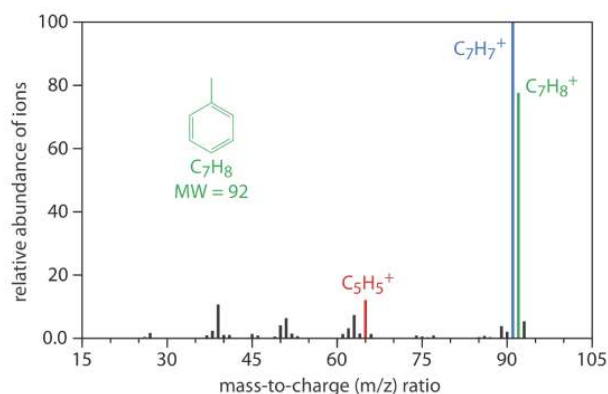


Figure 4.1.11 . Mass spectrum for toluene highlighting the molecular ion in green ($m/z=92$), and two fragment ions in blue ($m/z=91$) and in red ($m/z=65$). A mass spectrum provides both quantitative and qualitative information: the height of any peak is proportional to the amount of toluene in the mass spectrometer and the fragmentation pattern is unique to toluene.

Figure 4.1.14 shows a block diagram of a typical gas chromatography-mass spectrometer (GC–MS) instrument. The effluent from the column enters the mass spectrometer’s ion source in a manner that eliminates the majority of the carrier gas. In the ionization chamber the remaining molecules—a mixture of carrier gas, solvent, and solutes—undergo ionization and fragmentation. The mass spectrometer’s mass analyzer separates the ions by their mass-to-charge ratio and a detector counts the ions and displays the mass spectrum.

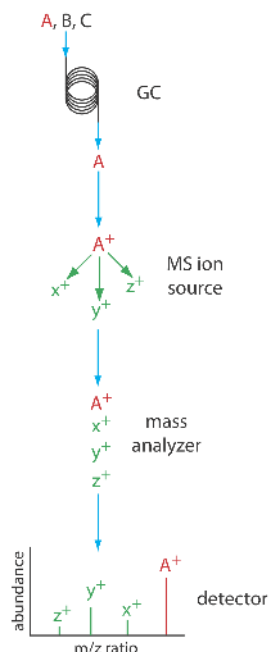


Figure 4.1.14 . Block diagram of GC–MS. A three component mixture enters the GC. When component A elutes from the column, it enters the MS ion source and ionizes to form the parent ion and several fragment ions. The ions enter the mass analyzer, which separates them by their mass-to-charge ratio, providing the mass spectrum shown at the detector.

There are several options for monitoring a chromatogram when using a mass spectrometer as the detector. The most common method is to continuously scan the entire mass spectrum and report the total signal for all ions that reach the detector during each

scan. This total ion scan provides universal detection for all analytes. We can achieve some degree of selectivity by monitoring one or more specific mass-to-charge ratios, a process called selective-ion monitoring. A mass spectrometer provides excellent detection limits, typically 25 fg to 100 pg, with a linear range of 10^5 orders of magnitude. Because we continuously record the mass spectrum of the column's eluent, we can go back and examine the mass spectrum for any time increment. This is a distinct advantage for GC-MS because we can use the mass spectrum to help identify a mixture's components.

For more details on mass spectrometry see [Introduction to Mass Spectrometry](#) by Michael Samide and Olujide Akinbo, a resource that is part of the Analytical Sciences Digital Library.

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4.2: Quantitative and Qualitative GC and GC-MS

QUALitative Analysis

Qualitative analysis in GC and GC-MS is the identification of analytes in a mixture. Qualitative analysis answers the question, "What is in this mixture?"

Using GC-MS:

Qualitative analysis of compounds is simple using a mass detector (MS), since the mass ion detected represents the molecular weight of the analyte. In addition, the fragmentation pattern observed in the mass spectrum can indicate the structure of the molecule.

Using GC:

If you do not have an MS detector (we do!), the retention time of a standard can be compared to that of an unknown to make a qualitative assignment. A more rigorous method of qualitative identification of a compound as follows; a member of a homologous series can be determined by comparing the capacity factor (k') of a series of standards and the k' value of the unknown. When more than one homologous series is being considered, a more positive identification can be made by measuring k' on two separate columns with different retention properties. For example, a non-polar column such as poly-silicone (column A) and a polar column such as diethylene-glycol succinate (column B) can be used. A linear plot of k' for the polar column versus k' for the non-polar column can be obtained for each homologous series. For example, an unknown could be identified as a member of a homologous series by its position on one of the lines of the A versus B plot shown below (Figure 4.2.1).

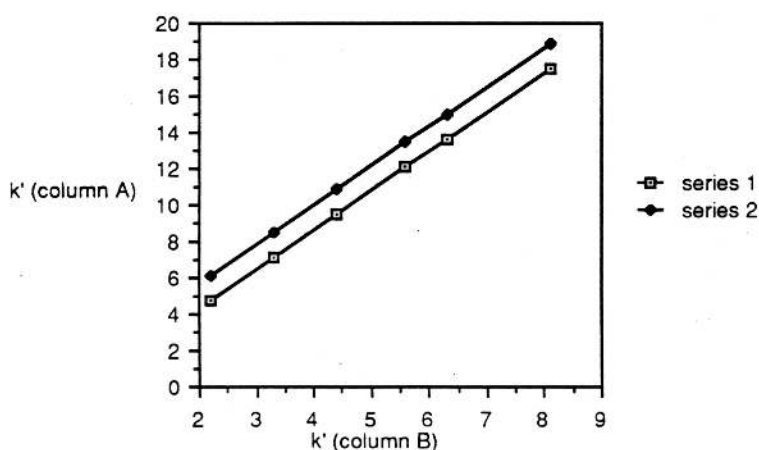


Figure 4.2.1: Copy and Paste Caption here. (Copyright; author via source)

QUANTitative Analysis

Quantitative analysis tells us how much of an analyte is in a mixture.

In an ideal GC or GC-MS analysis, the peak height and area under the peak are proportional to the amount of analyte injected onto the column. A peak's area is determined by integration, which usually is calculated by the instrument's computer. If two peak are resolved fully, the determination of their respective areas is straightforward.

Overlapping peaks, however, require a choice between one of several options for dividing up the area shared by the two peaks (Figure 4.2.15). Which method we use depends on the relative size of the two peaks and their resolution. In some cases, the use of peak heights provides more accurate results [(a) Bicking, M. K. L. Chromatography Online, April 2006; (b) Bicking, M. K. L. Chromatography Online, June 2006].

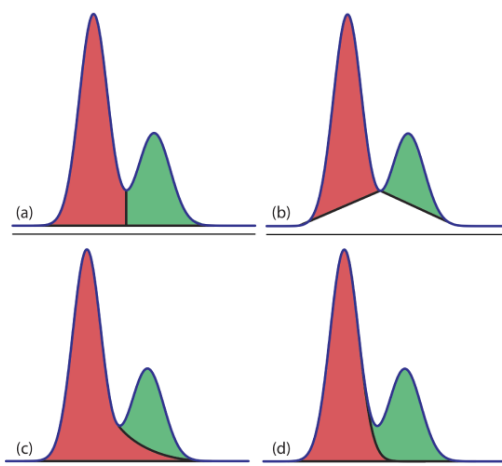


Figure 4.2.15 . Four methods for determining the areas under two overlapping chromatographic peaks: (a) the drop method; (b) the valley method; (c) the exponential skim method; and (d) the Gaussian skim method. Other methods for determining areas also are available.

For quantitative work we need to establish a calibration curve that relates the detector's response to the analyte's concentration. If the injection volume is identical for every standard and sample, then an external standardization provides both accurate and precise results. Unfortunately, even under the best conditions the relative precision for replicate injections may differ by 5%; often it is substantially worse. For quantitative work that requires high accuracy and precision, the use of internal standards is recommended (see below).

Another important consideration is the variation in detector sensitivity for different substances that may lead to inaccurate quantitative results. To overcome this problem, a response factor should be determined for the substance of interest relative to the internal standard. The molar response factor (r) for a component C_n is defined as:

$$r_{C_n} = \frac{\text{peak area}}{\text{moles of } C_n \text{ injected}} \quad (4.2.1)$$

Molar response factors must be determined from a calibration curve. Using standard solutions, known amounts of the analyte(s) are injected into the instrument, and the peak height and area are determined. By adding a known quantity of internal standard to every standard and to the sample, injecting it into the chromatogram, measuring peak areas, and incorporating response factors, the quantity of analyte may be determined.

Internal Standards

If injection volumes vary or if the concentration may change easily because the solvent is volatile, we can still create a calibration using an internal standard as a reference. The internal standard is a compound added to every solution that is used as a reference to compare to the analyte's signal. The species, which we call an **internal standard**, must be different than the analyte.

Because the analyte and the internal standard receive the same treatment, the ratio of their signals is unaffected by any lack of reproducibility in the injection procedure or changes in concentration due to evaporation. If a solution contains an analyte of concentration C_A and an internal standard of concentration C_{IS} , then the signals due to the analyte, S_A , and the internal standard, S_{IS} , are

$$S_A = k_A C_A$$

$$S_{IS} = k_{IS} C_{IS}$$

where k_A and k_{IS} are the sensitivities (aka response factors) for the analyte and the internal standard, respectively. Taking the ratio of the two signals gives the fundamental equation for an internal standardization.

$$\frac{S_A}{S_{IS}} = \frac{k_A C_A}{k_{IS} C_{IS}} = K \times \frac{C_A}{C_{IS}} \quad (4.2.2)$$

K is the relative sensitivity (relative response factor) for the analyte compared to the internal standard. Because K is a ratio of the analyte's sensitivity and the internal standard's sensitivity, it is not necessary to determine independently values for either k_A or k_{IS} .

Single Internal Standard

In a single-point internal standardization, we prepare a single standard that contains the analyte and the internal standard, and use it to determine the value of K in Equation 4.2.2.

$$K = \left(\frac{C_{IS}}{C_A} \right)_{std} \times \left(\frac{S_A}{S_{IS}} \right)_{std} \quad (4.2.3)$$

Having standardized the method, the analyte's concentration is given by

$$C_A = \frac{C_{IS}}{K} \times \left(\frac{S_A}{S_{IS}} \right)_{samp}$$

✓ Example 4.2.7

A sixth spectrophotometric method for the quantitative analysis of Pb^{2+} in blood uses Cu^{2+} as an internal standard. A standard that is 1.75 ppb Pb^{2+} and 2.25 ppb Cu^{2+} yields a ratio of $(S_A/S_{IS})_{std}$ of 2.37. A sample of blood spiked with the same concentration of Cu^{2+} gives a signal ratio, $(S_A/S_{IS})_{samp}$, of 1.80. What is the concentration of Pb^{2+} in the sample of blood?

Solution

Equation 4.2.3 allows us to calculate the value of K using the data for the standard

$$K = \left(\frac{C_{IS}}{C_A} \right)_{std} \times \left(\frac{S_A}{S_{IS}} \right)_{std} = \frac{2.25 \text{ ppb } Cu^{2+}}{1.75 \text{ ppb } Pb^{2+}} \times 2.37 = 3.05 \frac{\text{ppb } Cu^{2+}}{\text{ppb } Pb^{2+}}$$

The concentration of Pb^{2+} , therefore, is

$$C_A = \frac{C_{IS}}{K} \times \left(\frac{S_A}{S_{IS}} \right)_{samp} = \frac{2.25 \text{ ppb } Cu^{2+}}{3.05 \frac{\text{ppb } Cu^{2+}}{\text{ppb } Pb^{2+}}} \times 1.80 = 1.33 \text{ ppb } Pb^{2+}$$

Multiple Internal Standards

A single-point internal standardization has the same limitations as a single-point normal calibration. To construct an internal standard calibration curve we prepare a series of standards, each of which contains the same concentration of internal standard and a different concentrations of analyte. Under these conditions a calibration curve of $(S_A/S_{IS})_{std}$ versus C_A is linear with a slope of K/C_{IS} .

Although the usual practice is to prepare the standards so that each contains an identical amount of the internal standard, this is not a requirement.

✓ Example 4.2.8

A seventh spectrophotometric method for the quantitative analysis of Pb^{2+} in blood gives a linear internal standards calibration curve for which

$$\left(\frac{S_A}{S_{IS}} \right)_{std} = (2.11 \text{ ppb}^{-1} \times C_A) - 0.006$$

What is the ppb Pb^{2+} in a sample of blood if $(S_A/S_{IS})_{samp}$ is 2.80?

Solution

To determine the concentration of Pb^{2+} in the sample of blood we replace $(S_A/S_{IS})_{std}$ in the calibration equation with $(S_A/S_{IS})_{samp}$ and solve for C_A .

$$C_A = \frac{\left(\frac{S_A}{S_{IS}} \right)_{samp} + 0.006}{2.11 \text{ ppb}^{-1}} = \frac{2.80 + 0.006}{2.11 \text{ ppb}^{-1}} = 1.33 \text{ ppb } Pb^{2+}$$

The concentration of Pb^{2+} in the sample of blood is 1.33 ppb.

In some circumstances it is not possible to prepare the standards so that each contains the same concentration of internal standard. This is the case, for example, when we prepare samples by mass instead of volume. We can still prepare a calibration curve, however, by plotting $(S_A/S_{IS})_{std}$ versus C_A/C_{IS} , giving a linear calibration curve with a slope of K .

You might wonder if it is possible to include an internal standard in the method of standard additions to correct for both matrix effects and uncontrolled variations between samples; well, the answer is yes as described in the paper “Standard Dilution Analysis,” the full reference for which is Jones, W. B.; Donati, G. L.; Calloway, C. P.; Jones, B. T. *Anal. Chem.* **2015**, *87*, 2321-2327.

✓ Example 4.2.1

Marriott and Carpenter report the following data for five replicate injections of a mixture that contains 1% v/v methyl isobutyl ketone and 1% v/v *p*-xylene in dichloromethane [Marriott, P. J.; Carpenter, P. D. *J. Chem. Educ.* **1996**, *73*, 96–99].

injection	peak	peak area (arb. units)
I	1	48075
	2	78112
II	1	85829
	2	135404
III	1	84136
	2	132332
IV	1	71681
	2	112889
V	1	58054
	2	91287

Assume that *p*-xylene (peak 2) is the analyte, and that methyl isobutyl ketone (peak 1) is the internal standard. Determine the 95% confidence interval for a single-point standardization with and without using the internal standard.

Solution

For a single-point external standardization we ignore the internal standard and determine the relationship between the peak area for *p*-xylene, A_2 , and the concentration, C_2 , of *p*-xylene.

$$A_2 = kC_2$$

Substituting the known concentration for *p*-xylene (1% v/v) and the appropriate peak areas, gives the following values for the constant k .

$$78112 \quad 135404 \quad 132332 \quad 112889 \quad 91287$$

The average value for k is 110 000 with a standard deviation of 25 100 (a relative standard deviation of 22.8%). The 95% confidence interval is

$$\mu = \bar{X} \pm \frac{ts}{\sqrt{n}} = 111000 \pm \frac{(2.78)(25100)}{\sqrt{5}} = 111000 \pm 31200$$

For an internal standardization, the relationship between the analyte's peak area, A_2 , the internal standard's peak area, A_1 , and their respective concentrations, C_2 and C_1 , is

$$\frac{A_2}{A_1} = k \frac{C_2}{C_1}$$

Substituting in the known concentrations and the appropriate peak areas gives the following values for the constant k .

$$1.5917 \quad 1.5776 \quad 1.5728 \quad 1.5749 \quad 1.5724$$

The average value for k is 1.5779 with a standard deviation of 0.0080 (a relative standard deviation of 0.507%). The 95% confidence interval is

$$\mu = \bar{X} \pm \frac{ts}{\sqrt{n}} = 1.5779 \pm \frac{(2.78)(0.0080)}{\sqrt{5}} = 1.5779 \pm 0.0099$$

Although there is a substantial variation in the individual peak areas for this set of replicate injections, the internal standard compensates for these variations, providing a more accurate and precise calibration.

? Exercise 4.2.1

Figure 4.2.16 shows chromatograms for five standards and for one sample. Each standard and sample contains the same concentration of an internal standard, which is 2.50 mg/mL. For the five standards, the concentrations of analyte are 0.20 mg/mL, 0.40 mg/mL, 0.60 mg/mL, 0.80 mg/mL, and 1.00 mg/mL, respectively. Determine the concentration of analyte in the sample by (a) ignoring the internal standards and creating an external standards calibration curve, and by (b) creating an internal standard calibration curve. For each approach, report the analyte's concentration and the 95% confidence interval. Use peak heights instead of peak areas.

Answer

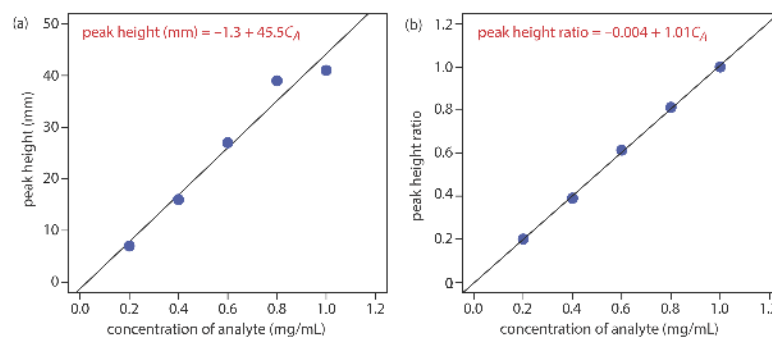
The following table summarizes my measurements of the peak heights for each standard and the sample, and their ratio (although your absolute values for peak heights will differ from mine, depending on the size of your monitor or printout, your relative peak height ratios should be similar to mine).

[standard] (mg/mL)	peak height of standard (mm)	peak height of analyte (mm)	peak height ratio
0.20	35	7	0.20
0.40	41	16	0.39
0.60	44	27	0.61
0.80	48	39	0.81
1.00	41	41	1.00
sample	39	21	0.54

Figure (a) shows the calibration curve and the calibration equation when we ignore the internal standard. Substituting the sample's peak height into the calibration equation gives the analyte's concentration in the sample as 0.49 mg/mL. The 95% confidence interval is ± 0.24 mg/mL. The calibration curve shows quite a bit of scatter in the data because of uncertainty in the injection volumes.

Figure (b) shows the calibration curve and the calibration equation when we include the internal standard. Substituting the sample's peak height ratio into the calibration equation gives the analyte's concentration in the sample as 0.54 mg/mL. The 95% confidence interval is ± 0.04 mg/mL.

To review the use of Excel or R for regression calculations and confidence intervals, see Chapter 5.5.



The data for this exercise were created so that the analyte's actual concentration is 0.55 mg/mL. Given the resolution of my ruler's scale, my answer is pretty reasonable. Your measurements may be slightly different, but your answers should be close to the actual values.

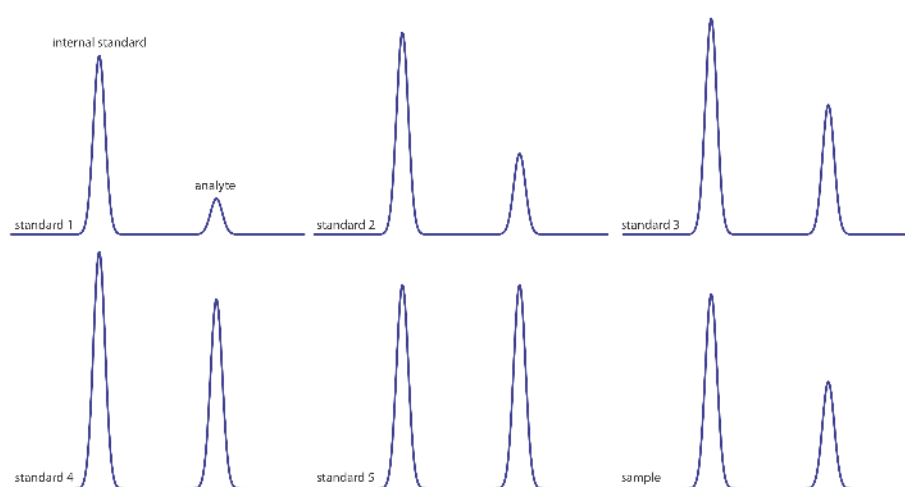


Figure 4.2.16 . Chromatograms for Practice Exercise 12.5.

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4.3: Mass Spectrometers

Mass Spectrometry is an analytical technique that can reveal specific, characteristic, structurally related information about a compound. Sometimes the questions that attract attention to mass spectrometry are qualitative in nature. For example, "Have I successfully synthesized a C19 sterol," or "What is the structure of the by-product from my synthesis reaction," or "Is the compound that I isolated from plasma really norepinephrine". Mass spectrometry can usually answer these questions. Other times a quantitative analysis is sought and then the ideas of molar response values, peak areas and the use of internal standards, mentioned above, are included in the determination.

A mass spectrometer has three essential needs: a means for producing ions, in this case (mostly) singly charged atoms; a means for separating these ions in space or in time by their mass-to-charge ratios; and a means for counting the number of ions for each mass-to-charge ratio. Figure 4.3.1 provides a general view of a mass spectrometer, and the requirements are described briefly below. Note that the mass spectrometer is held under vacuum as this allows the ions to travel great distances without undergoing collisions that might alter their charge or energy.

- **Sample Introduction:** The compound for mass spectroscopic analysis is injected into a high vacuum where the molecules can move freely in the evacuated space. In the case of GCMS, the GC column is attached directly to the MS.
- **Ion source:** The most common means for generating ions are plasmas of various sorts, lasers, electrical sparks, and other ions. In GC-MS, positive ions are commonly produced by electron ionization (EI), electron impact on the gaseous molecules of the sample.
- **Separating ions:** The most common way of separating ions is a quadrupole magnet (described in more detail below).
- **Counting ions:** The transducer for mass spectrometry must be able to report the number of ions that emerge from the mass analyzer.

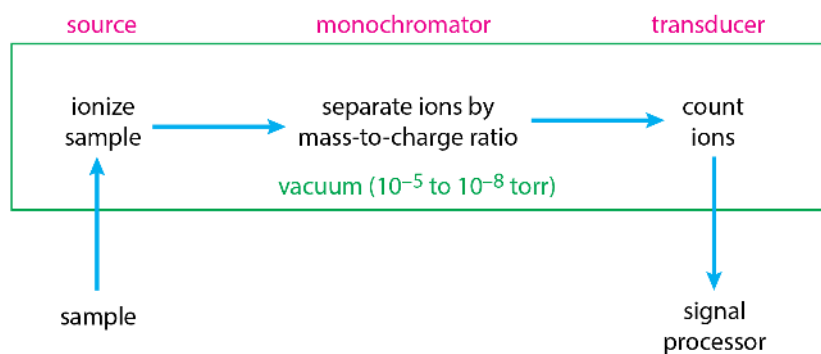


Figure 4.3.1: The basic components of a mass spectrometer. The blue arrows show the direction of sample-to-signal in which the sample is ionized, the ions separated by their mass-to-charge ratio, and ions with the same mass-to-charge ratio counted. The text in red at the top of the figure relates these components to their equivalent in optical spectroscopy.

In the Duke teaching labs, our mass spectrometers are quadrupole devices. They employ a combination of dc and radio frequency (rf) potentials as a mass "filter". The quadrupole typically consists of 4 cylindrical, parallel rods (10-25 cm in length) situated symmetrically in a square arrangement. Rods diagonally opposite each other are connected, in pairs, to dc and rf generators. Positive ions extracted from the ion source are accelerated into the quadrupole along the longitudinal axis of the four rods. The ions are influenced by the combined dc and rf fields. In order for an ion to reach the detector it must traverse the quadrupole without colliding with any one of the metal rods. For any level of rf/dc voltage only ions of a specific m/e avoid collision and reach the detector. The entire mass spectrum is obtained as voltages are swept from a pre-established minimum to a maximum.

Quadrupole Mass Analyzers

The quadrupole mass analyzer is probably the most important type of mass-selective devices; it is compact in size, low in cost, easy to use, and easy to maintain. As shown in Figure 4.3.3, a quadrupole mass analyzer consists of four cylindrical rods, two of which are connected to the positive terminal of a variable direct current (dc) power supply and two of which are connected to the power supply's negative terminal; the two positive rods are positioned opposite of each other and the two negative rods are positioned opposite of each other. Each pair of rods is also connected to a variable alternating current (ac) source operated such that the alternating currents are 180° out-of-phase with each other. An ion beam from the source is drawn into the channel between the quadrupoles and, depending on the applied dc and ac voltages, ions with only one mass-to-charge ratio successfully travel the length of the mass analyzer and reach the transducer; all other ions collide with one of the four rods and are destroyed.

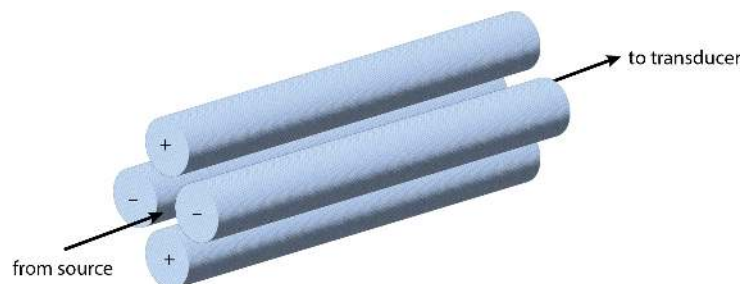


Figure 4.3.3: Basic arrangement of the quadrupole mass analyzer. The plus and the minus signs on each rod indicate which are connected to the positive terminal of the dc power supply. Not shown here is the circuitry for applying the alternating current. The ion beam enters the channel between the quadrupoles. Depending on the applied dc and ac voltages, some of the ions emerge from this channel and reach the transducer.

To understand how a quadrupole mass analyzer achieves this separation of ions, it helps to consider the movement of an ion relative to just two of the rods, as shown in Figure 4.3.4 for the poles that carry a positive dc voltage. When the ion beam enters the channel between the rods, the ac voltage causes the ion to begin to oscillate. If, as in the top diagram, the ion is able to maintain a stable oscillation, it will pass through the mass analyzer and reach the transducer. If, as in the middle diagram, the ion is unable to maintain a stable oscillation, then the ion eventually collides with one of the rods and is destroyed. When the rods have a positive dc voltage, as they do here, ions with larger mass-to-charge ratios will be slow to respond to the alternating ac voltage and will pass through the transducer. The result is shown in the figure at the bottom (and repeated in Figure 4.3.5a) where we see that ions with a sufficiently large mass-to-charge ratios successfully pass through the transducer; ions with smaller mass-to-charge ratios do not. In this case, the quadrupole mass analyzer acts as a high-pass filter.

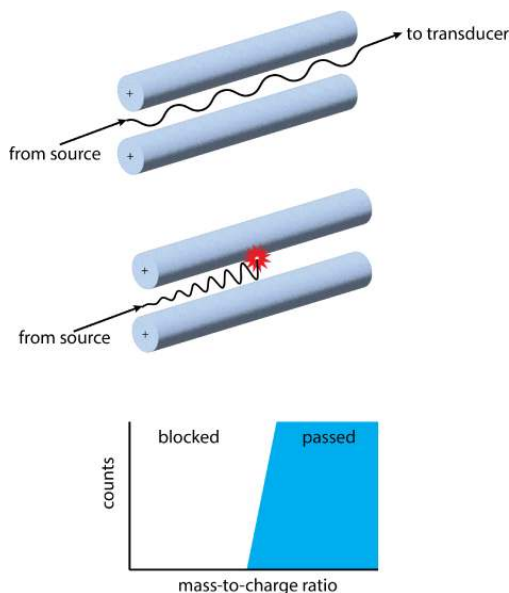


Figure 4.3.4: Illustration of how a quadrupole mass analyzer achieves separation of ions by their mass-to-charge ratio. See the text for additional details.

We can extend this to the behavior of the ions when they interact with rods that carry a negative dc voltage. In this case, the ions are attracted to the rods, but those ions that have a sufficiently small mass-to-charge ratio are able to respond to the alternating current's voltage and remain in the channel between the rods. The ions with larger mass-to-charge ratios move more sluggishly and eventually collide with one of the rods. As shown in Figure 4.3.5b in this case, the quadrupole mass analyzer acts as a low-pass filter. Together, as we see in Figure 4.3.5c, a quadrupole mass analyzer operates as both a high-pass and a low-pass filter, allowing a narrow band of mass-to-charge ratios to pass through the transducer. By varying the applied dc voltage and the applied ac voltage, we can obtain a full mass spectrum.

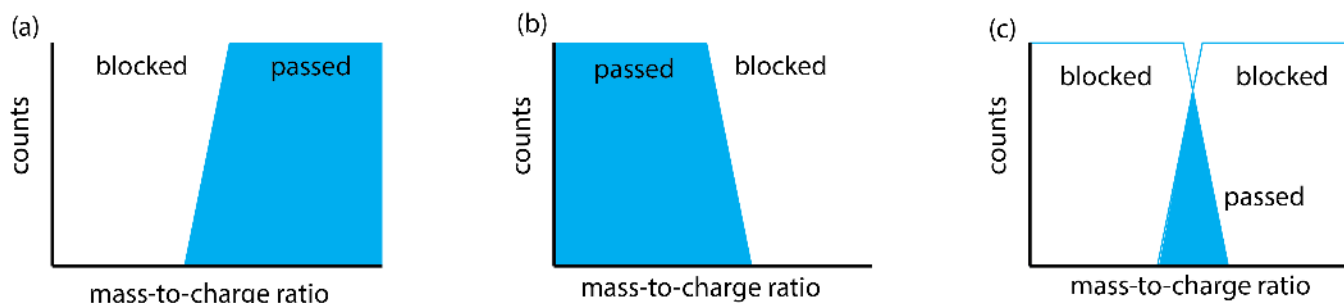


Figure 4.3.5: Illustration that shows how a quadrupole mass analyzer acts as both (a) a high-pass filter and (b) a low pass filter, with the result that it passes only ions with a narrow range of mass-to-charge ratios.

Quadrupole mass analyzers provide a modest mass-to-charge resolution of about 1 amu and extend to m/z ratios of approximately 2000. Quadrupole mass analyzers are particularly useful for sources based on plasmas.

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4.4: Interpreting Electron Ionization Mass Spectra

Different ionization methods will produce different types of mass spectra. This page describes the interpretation of the mass spectra derived from electron ionization mass spectrometry.

In order for mass spectrometry to be helpful the ions that are studied must originate from a pure source of the sample of interest, for to consider ions that are unrelated to the compound could be worse than having no mass spectral data at all. It is essential to ensure that a sample contains no material that will produce unexpected ions that could interfere with interpretation. Thus, the combination of gas chromatography and mass spectrometry (GCMS) is often the method of choice. The gas chromatograph can separate the compound of interest from other potentially interfering compounds and present it to the mass spectrometer in purified form over a time interval sufficient to obtain the mass spectrum.

Per unit quantity of sample, mass spectrometry perhaps delivers more information concerning a molecule than does any other spectroscopic technique. Although mass spectrometry rarely solves a problem "single-handedly", it often provides the critical complimentary data that finally "crack" an identification problem. For a complete identification of a sample compound 0.1 to 1 micrograms of the sample may be required. If all that is needed is detection and verification of the presence of an already-identified compound the sample requirements are less severe (sometimes requiring only femtomole quantities).

Although mass spectrometry has been used successfully in the determination of many compounds (usually having a molecular weight of less than 1,000), one consistent obstacle is whether a compound of interest is amenable to vapor phase analysis. However, techniques such as field desorption (where a sample molecule is desorbed from the surface of a probe or electrode as an ion when it enters a high electric field), and chemical derivatization to increase thermal stability and volatility, extend the applicability of mass spectrometry to increasing numbers and types of compounds.

The formation of Molecular Ions (M^+)

When the vaporized organic sample passes into the ionization chamber of a mass spectrometer, it is bombarded by a stream of electrons. These electrons have a high enough energy to knock an electron off an organic molecule to form a positive ion. This ion is called the **molecular ion - or sometimes the parent ion**. The molecular ion is often given the symbol M^+ or $M^{\cdot+}$ - the dot in this second version represents the fact that somewhere in the ion there will be a single unpaired electron. That's one half of what was originally a pair of electrons - the other half is the electron which was removed in the ionization process.

The most important ion sought in the mass spectrum is the **molecular ion (the ionized, intact molecule) because it is a direct indicator of the molecular weight of the compound**. While the molecular weight is usually sufficient to confirm the presence of a given compound, electron ionization mass spectrometry can give even more information from the fragmentation of ions. Since some compounds can have molecular weights that are nearly identical, it is much better to compare the major peaks in the experimental spectrum to those in a reference spectrum of the authentic compound.

Fragmentation

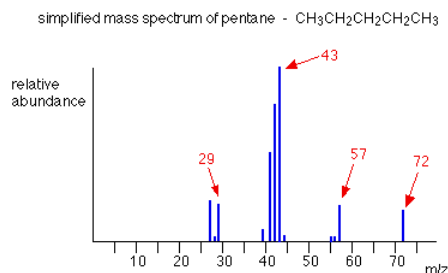
During the process of ionization considerable excess energy may be transferred to the molecular ion, which depending on its stability (related to its structure) may decompose into various fragment ions. The resulting fragment ions define subsets of atoms that may be related to functional groups or structural components of the original molecule. The array of fragment ions represented by peaks in a mass spectrum is often called a fragmentation pattern. Careful study of the fragmentation pattern is especially important when the mass spectrum is to be used to elucidate molecular structure or to distinguish between two or more structural isomers.

While some fragmentation of the sample molecules is necessary for structure evaluation, many compounds are so effectively fragmented by EI that no appreciable population of molecular ions survives to give an indication of the molecular weight in the mass spectrum. Chemical ionization (CI), involving ionization of the sample molecule by collisions with ions of a reagent gas (for example, the ions produced by electron ionization of methane), is a more "gentle" ionization technique that often produces abundant protonated molecular ions. The resultant spectrum provides a good indication of molecule weight but may have insufficient fragmentation for functional group and structural evaluation.

The simplest case is that a molecular ion breaks into two parts - one of which is another positive ion, and the other is an uncharged free radical.



The uncharged free radical won't produce a line on the mass spectrum. Only charged particles will be accelerated, deflected and detected by the mass spectrometer. These uncharged particles will simply get lost in the machine - eventually, they get removed by the vacuum pump. The ion, X^+ , will travel through the mass spectrometer just like any other positive ion - and will produce a line on the stick diagram. All sorts of fragmentations of the original molecular ion are possible - and that means that you will get a whole host of lines in the mass spectrum. For example, the mass spectrum of pentane looks like this:



Each line represents a different fragment produced when the molecular ion breaks up.

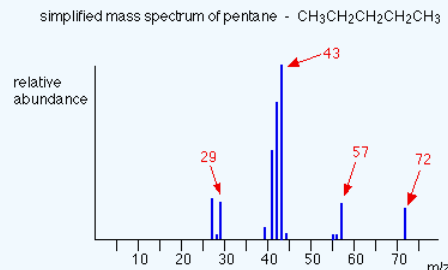
In the stick diagram showing the mass spectrum of pentane, the line produced by the heaviest ion passing through the machine (at $m/z = 72$) is due to the molecular ion. The tallest line in the stick diagram (in this case at $m/z = 43$) is called the **base peak**. This is usually given an arbitrary height of 100, and the height of everything else is measured relative to this. The base peak is the tallest peak because it represents the commonest fragment ion to be formed - either because there are several ways in which it could be produced during fragmentation of the parent ion, or because it is a particularly stable ion.

Interpreting fragmentation patterns

The following will demonstrate the interpretation of fragmentation patterns using examples of simple organic molecules.

Example 4.4.1: Mass Spectrum of Pentane

Let's have another look at the mass spectrum for pentane:

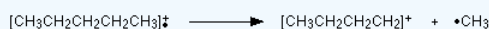


What causes the line at $m/z = 57$?

Solution

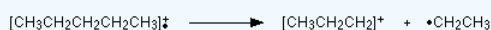
How many carbon atoms are there in this ion? There can't be 5 because $5 \times 12 = 60$. What about 4? $4 \times 12 = 48$. That leaves 9 to make up a total of 57. How about C_4H_9^+ then?

C_4H_9^+ would be $[\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2]^+$, and this would be produced by the following fragmentation:

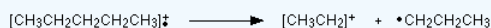


The methyl radical produced will simply get lost in the machine.

The line at $m/z = 43$ can be worked out similarly. If you play around with the numbers, you will find that this corresponds to a break producing a 3-carbon ion:



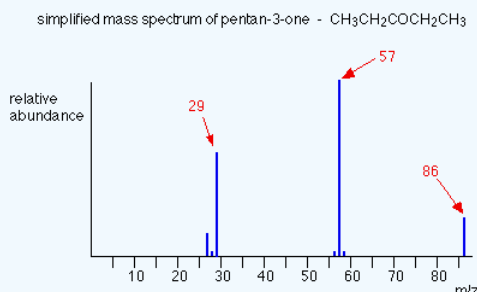
The line at $m/z = 29$ is typical of an ethyl ion, $[\text{CH}_3\text{CH}_2]^+$:



The other lines in the mass spectrum are more difficult to explain. For example, lines with m/z values 1 or 2 less than one of the easy lines are often due to loss of one or more hydrogen atoms during the fragmentation process.

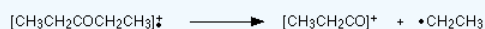
Example 4.4.2: Pentan-3-one

This time the base peak (the tallest peak - and so the commonest fragment ion) is at $m/z = 57$. But this isn't produced by the same ion as the same m/z value peak in pentane.

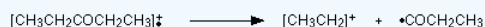


If you remember, the $m/z = 57$ peak in pentane was produced by $[\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2]^+$. If you look at the structure of pentan-3-one, it's impossible to get that particular fragment from it.

Work along the molecule mentally chopping bits off until you come up with something that adds up to 57. With a small amount of patience, you'll eventually find $[\text{CH}_3\text{CH}_2\text{CO}]^+$ - which is produced by this fragmentation:



You would get exactly the same products whichever side of the CO group you split the molecular ion. The $m/z = 29$ peak is produced by the ethyl ion - which once again could be formed by splitting the molecular ion either side of the CO group.



Peak heights and the stability of ions

The more stable an ion is, the more likely it is to form. The more of a particular sort of ion that's formed, the higher its peak height will be. We'll look at two common examples of this.

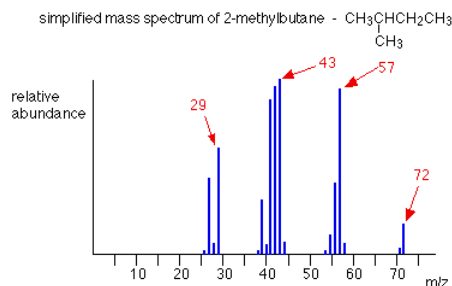
Examples involving carbocations (carbonium ions)

Summarizing the most important conclusion from the page on carbocations:

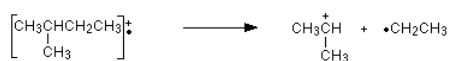
Order of stability of carbocations

primary < secondary < tertiary

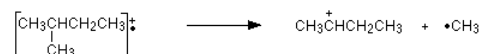
Applying the logic of this to fragmentation patterns, it means that a split which produces a secondary carbocation is going to be more successful than one producing a primary one. A split producing a tertiary carbocation will be more successful still. Let's look at the mass spectrum of 2-methylbutane. 2-methylbutane is an isomer of pentane - isomers are molecules with the same molecular formula, but a different spatial arrangement of the atoms.



Look first at the very strong peak at $m/z = 43$. This is caused by a different ion than the corresponding peak in the pentane mass spectrum. This peak in 2-methylbutane is caused by:



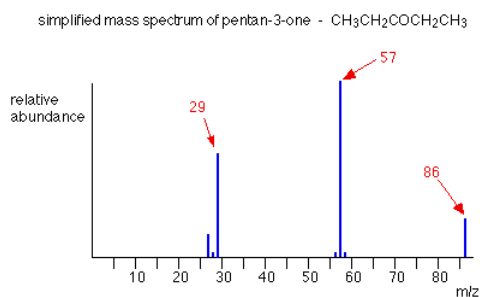
The ion formed is a secondary carbocation - it has two alkyl groups attached to the carbon with the positive charge. As such, it is relatively stable. The peak at $m/z = 57$ is much taller than the corresponding line in pentane. Again a secondary carbocation is formed - this time, by:



You would get the same ion, of course, if the left-hand CH_3 group broke off instead of the bottom one as we've drawn it. In these two spectra, this is probably the most dramatic example of the extra stability of a secondary carbocation.

Examples involving acylium ions, $[\text{RCO}]^+$

Ions with the positive charge on the carbon of a carbonyl group, $\text{C}=\text{O}$, are also relatively stable. This is fairly clearly seen in the mass spectra of ketones like pentan-3-one.



The base peak, at $m/z=57$, is due to the $[\text{CH}_3\text{CH}_2\text{CO}]^+$ ion. We've already discussed the fragmentation that produces this.

Using mass spectra to distinguish between compounds

Suppose you had to suggest a way of distinguishing between pentan-2-one and pentan-3-one using their mass spectra.

pentan-2-one	$\text{CH}_3\text{COCH}_2\text{CH}_2\text{CH}_3$
pentan-3-one	$\text{CH}_3\text{CH}_2\text{COCH}_2\text{CH}_3$

Each of these is likely to split to produce ions with a positive charge on the CO group. In the pentan-2-one case, there are two different ions like this:

- $[\text{CH}_3\text{CO}]^+$
- $[\text{COCH}_2\text{CH}_2\text{CH}_3]^+$

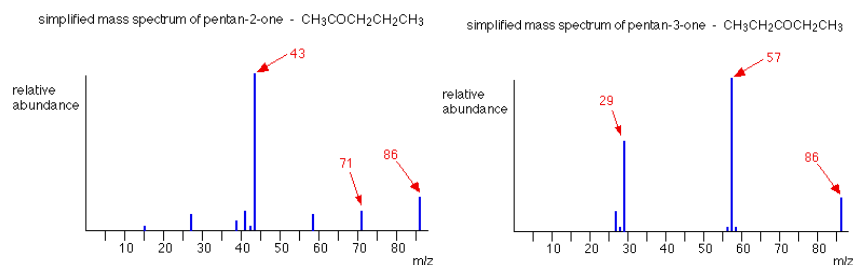
That would give you strong lines at $m/z = 43$ and 71 .

With pentan-3-one, you would only get one ion of this kind:

- $[\text{CH}_3\text{CH}_2\text{CO}]^+$

In that case, you would get a strong line at 57 . You don't need to worry about the other lines in the spectra - the 43 , 57 and 71 lines give you plenty of difference between the two. The 43 and 71 lines are missing from the pentan-3-one spectrum, and the 57 line is missing from the pentan-2-one one.

The two mass spectra look like this:



Computer matching of mass spectra

As you've seen, the mass spectrum of even very similar organic compounds will be quite different because of the different fragmentations that can occur. Provided you have a computer data base of mass spectra, any unknown spectrum can be computer analysed and simply matched against the data base.

Sources and Contributions

- Jim Clark (Chemguide.co.uk)
- Duke Chemistry

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4.5: GC-MS Instruments at Duke

The two GC-MS instruments in the teaching labs at Duke are Agilent 7820A and 5975. You will use the front column (DB5.625, non-polar) and the MS detector in this module.

- Each instrument is equip with two detectors.
 - The GC has a built in FID detector. Hydrogen and Air cylinders support the FID (H₂; 28 psi; air 58-60 psi)
 - A tandem quadrupole mass spectrometer (MS) sits to the left of the GC component.
- Each instrument's oven is equip with two column positions (front and back).
 - The front column is connected to the MS and is a DB5.625 (non-polar) column.
 - The back column (if installed) is connected to the FID and is a DBwax (polar) column
- The Carrier Gas is helium (He) from a compressed cylinder; feed pressure is 40-50 psi.

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4.5.1: Operating the Agilent GCMS

These are instructions for operating the Agilent GCMS equip with an automatic liquid sampler (ALS). Instructions for manual injections are not included here. *Please note that the Mass Hunter GCMS software is used only for data acquisition. Data analysis is done using different software packages (see next section).*

Prepare the GCMS for Operation

Check instrument consumables prior to use:

- Check to ensure that there is sufficient pressure of He mobile phase in the carrier gas tank.
- Check that the rinse solvent reservoirs in the ALS are full of ms-grade methanol.

1. Locate the Mass Hunter **GCMS software icon** located in the upper left corner of the computer desktop (Figure 4.5.1.1). Double click to open.
2. Allow a few minutes for the software to initialize. The green bars at the top left of the MassHunter window indicate when the instrument is ready (Figure 4.5.1.1).
3. Load the desired method using the **Method** tab (Figure 4.5.1.1). If the desired method is not listed in the quick access menu, you can find all methods in D:\MassHunter\GCMS1\methods.
 - Choose your method and load — loading takes a few moments.

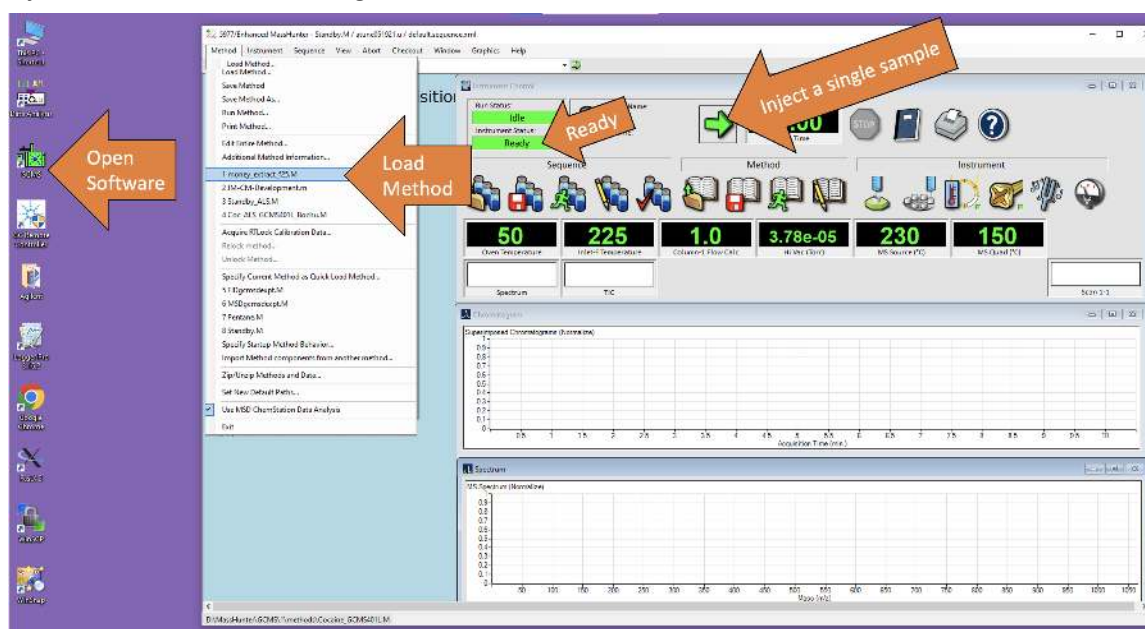


Figure 4.5.1.1: Infographic for how to open the GCMS MassHunter software, run a method, and prepare the instrument to inject a sample. (CC-BY-SA; Kathryn Haas for Duke Chem.)

- The green action bar will confirm when the method loading is complete (Figure 4.5.1.1).
 - The loaded method is displayed in small font across the top of the MassHunter window. For example, the method "Standby.M" is loaded as seen in Figure 4.5.1.1
4. When the method has been loaded and the instrument is ready, prepare to inject a sample;
 - Load a blank into vial position 1.
 - Load samples or calibration standards into other vial positions.

Inject a single sample

1. Press the green arrow button to "Run" a method on a single injection.

- After you press the "Run Method" icon (the green arrow), the Start Run window will open. You should choose the directory where you will save and retrieve your data files, enter the a file name, and sample information (Figure 4.5.1.2).
 - The data folder for your course is in the D: Drive, specifically D:\MassHunter\GCMS\1\data\CHEM401L....
 - Create a new folder inside the folder for your course if one does not exist. A good format for the file name is "D:\MassHunter\GCMS\1\data\CHEM401L-Fall2025"
 - Name your file making sure to retain the ".D" suffix. The recommended file naming format is using the date YYYYMMDD and underscores such as "20250902_01.D". Add the other information: user, sample name, info.
 - Specify the correct vial position in the ALS.
 - Record the directory, file name, and the identity of the sample in your notebook so that when you need to access the data later, you know where to find it and which file holds the data from which sample.

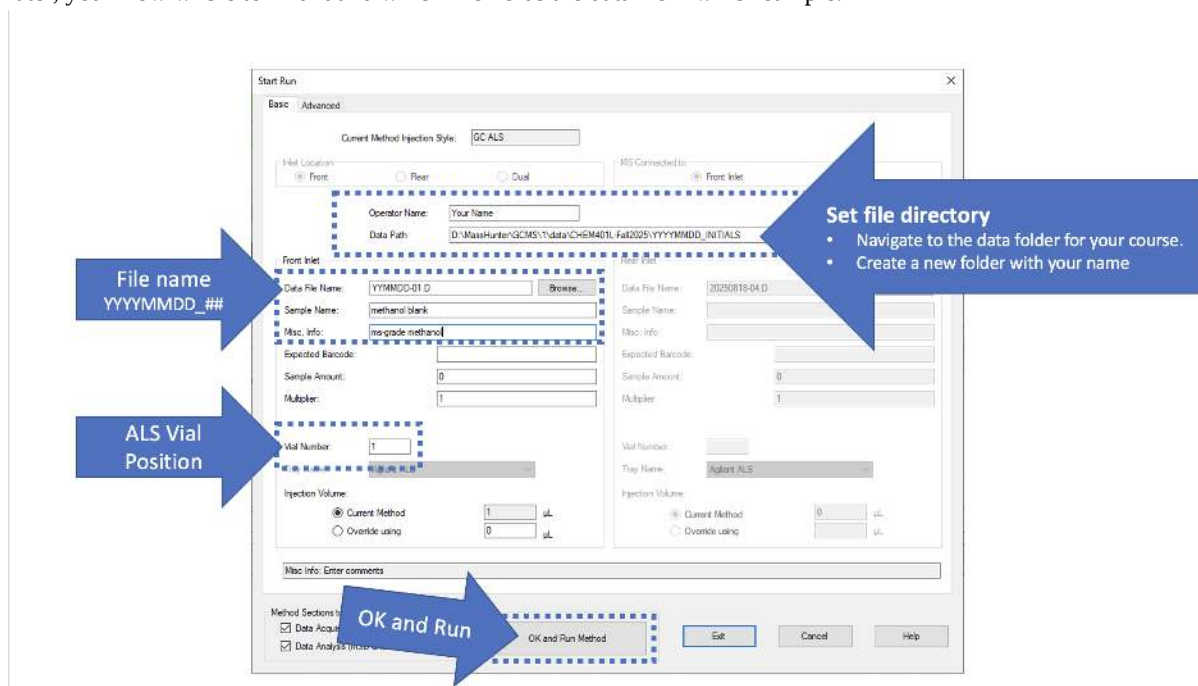


Figure 4.5.1.2: Infographic to show how to create a new folder to store your GCMS data. (CC-BY-SA; Kathryn Haas for Duke Chem.)

- When your file directory and file name are entered correctly, press the "OK and RUN METHOD" button. The GC/MS will prepare for your sample injection, and then the ALS will automatically inject your sample based on the settings in your method.



Warning

DO NOT override anything! You will get a message asking if you want to override the solvent delay. Keep your hands off the keyboard and mouse! **NEVER OVERRIDE THE SOLVENT DELAY**, doing so will drastically shorten the lifetime of the filaments inside the Mass Spec, potentially render the instrument inoperable, and likely result in increased chemistry laboratory fees.

- "Override Solvent Delay". **Do nothing**, or **Press NO!**

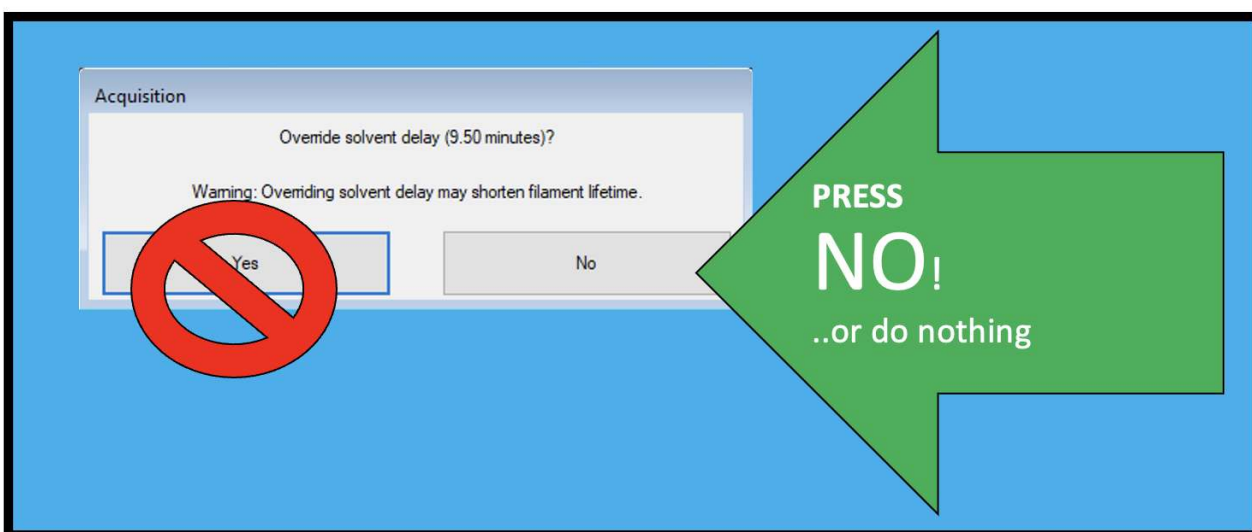


Figure 4.5.1.3: How to initiate a run after injection of sample in the inlet. Use the button at the front of the instrument to Start the run. Do NOT override the solvent delay. (CC-BY-SA; Kathryn Haas for Duke Chem.)

Create a Sequence for Multiple Injections

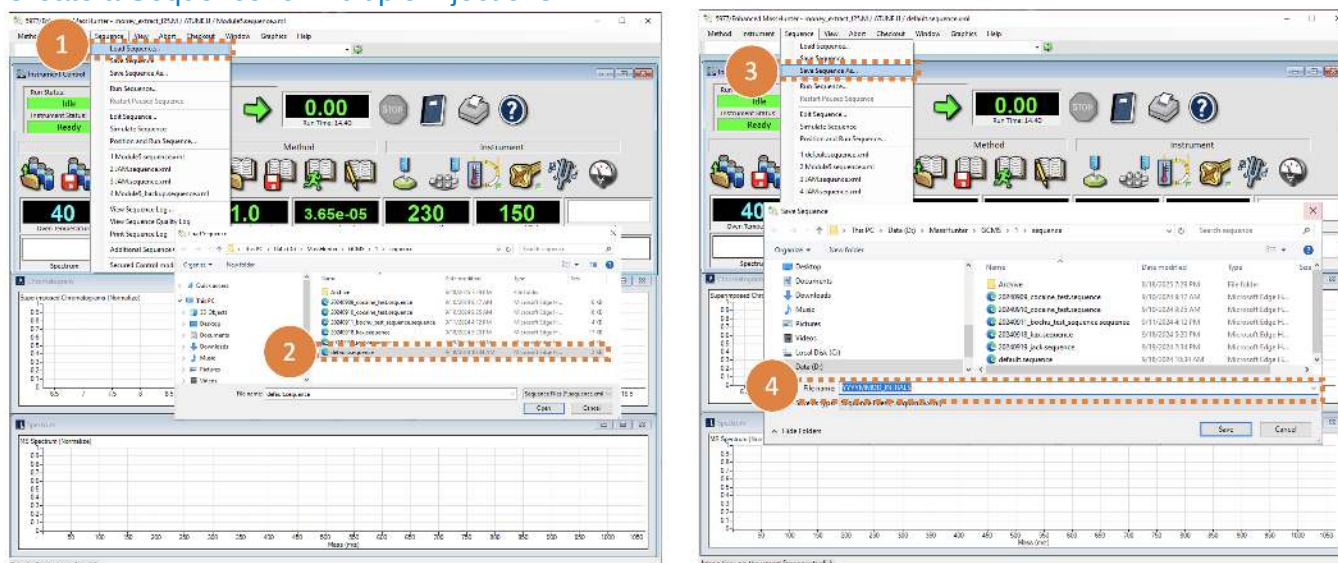


Figure 4.5.1.1: Copy and Paste Caption here. (Copyright; author via source)

1. At the top bar menu, go to Sequence and choose Load Sequence.
2. Choose the default sequence (default.sequence)
3. Go back to the top bar, go to Sequence and choose **Save Sequence As..**
4. **DO NOT OVERWRITE THE DEFAULT SEQUENCE.** Give the sequence a new name and save it in the sequence folder— this sequence may be used by your entire team (eg YYYYMMDD_TeamName).
 - o Record the location and name of the sequence file.
5. Go to the top bar, go to Sequence and choose **Edit Sequence.**

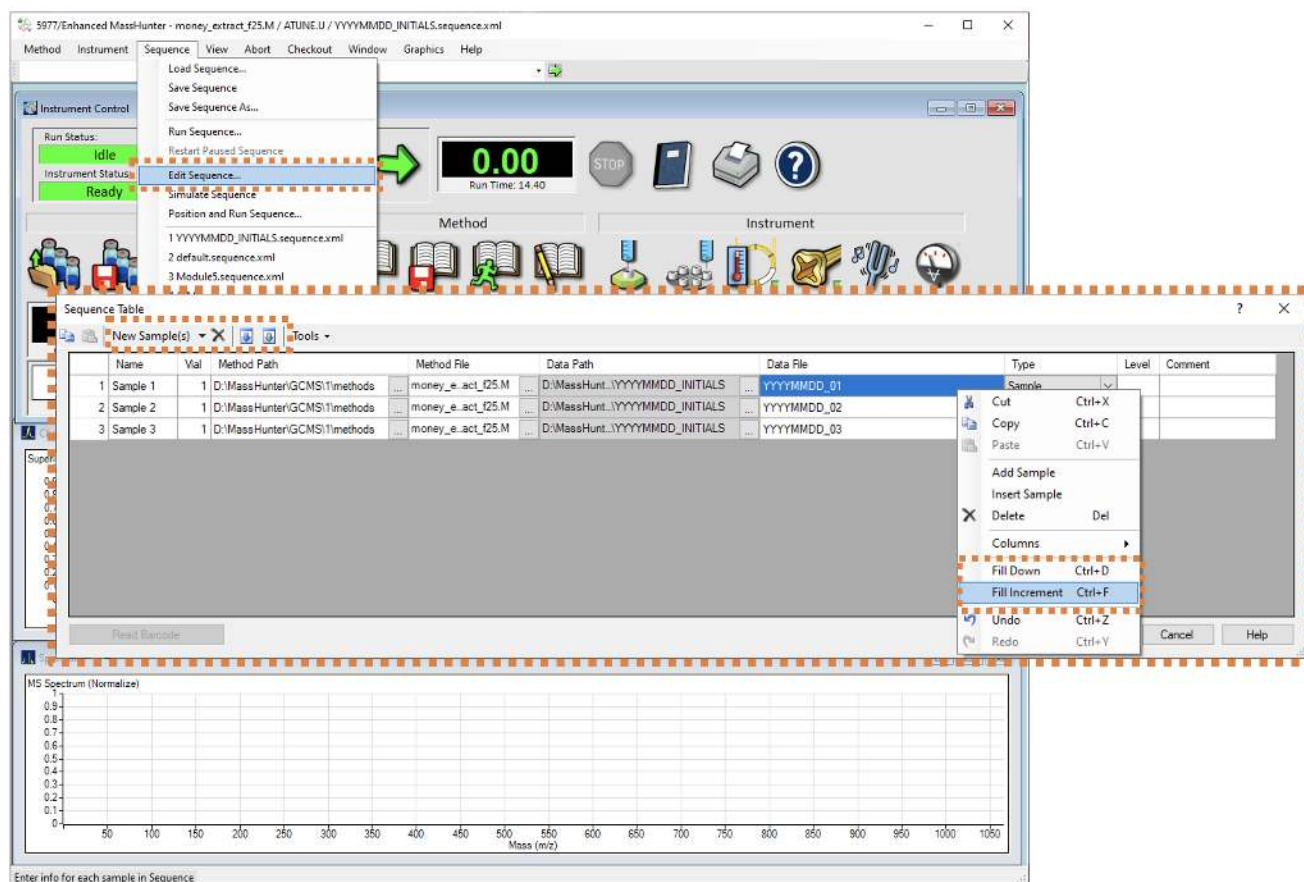


Figure 4.5.1.4: Edit Sequence and Sequence Table Window. (CC-BY-SA; Kathryn Haas for Duke Chem.)

- A Sequence Table window will appear. Each line corresponds to one injection in the sequence.
 - Add new lines using the **New Sample(s)** drop down at the top of the window.
 - Name the samples according to what they are (eg, Methanol blank | 0.1 ppm standard | \$1 extract YYYY)
 - Enter the correct ALS vial position for each sample.
 - Enter the path where the method is located
 - Enter the method file for each injection
 - Enter the path where data should be saved (your file folder within the course folder).
 - Enter a name for the first data file using the format YYYYMMDD_##. Then, right click in that cell and choose "Fill Increment" to automatically add unique names for each file.
 - Specify the Type (Cal, Sample, or Blank)
 - For each Calibration Standard, enter the Level (1 is the lowest concentration)
 - Enter comments if you wish (sample components)
- An example sequence is shown below:

Sequence Table

Name	Val	Method Path	Method File	Data Path	Data File	Type	Level	Comment
1 methanol blank	1	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_01	Blank	1	methanol ms-grade
2 0.1 ppm	2	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_02	Cal	1	cal meth. coc. dox is
3 0.2 ppm	3	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_03	Cal	2	cal meth. coc. dox is
4 0.3 ppm	4	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_04	Cal	3	cal meth. coc. dox is
5 0.4 ppm	5	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_05	Cal	4	cal meth. coc. dox is
6 0.5 ppm	6	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_06	Cal	5	cal meth. coc. dox is
7 1.0 ppm	7	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_07	Cal	6	cal meth. coc. dox is
8 2.0 ppm	8	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_08	Cal	7	cal meth. coc. dox is
9 4.0 ppm	9	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_09	Cal	8	cal meth. coc. dox is
10 6.0 ppm	10	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_10	Cal	9	cal meth. coc. dox is
11 10.0 ppm	11	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_11	Cal	10	cal meth. coc. dox is
12 15.0 ppm	12	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_12	Cal	11	cal meth. coc. dox is
13 20.0 ppm	13	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_13	Cal	12	cal meth. coc. dox is
14 1-20114h	16	D:\MassHunter\GCMS1\methods...	money_extract_Q25.M	D:\MassHunter\GCMS1\data\CHEM..YYYYMMDD_INITIALS...	YYYYMMDD_14	Sample		methanol extract from 1 us bil

OK Cancel Help

Figure 4.5.1.5: An example sequence. (CC-BY-SA; Kathryn Haas for Duke Chem.)

6. When your sequence is complete (or at least the first few lines are complete), ask someone else to double check your work.
 - Check that each column and each line is correct.
 - Check that each data file has a unique number
 - Check that the paths and method file is correct
 - Check that the samples are in the positions indicated in the sequence.
 - When you are sure that the sequence is set up correctly, press **OK**.
7. To run the sequence, go to the Sequence menu in the top bar and select "Run Sequence". Or use the hot button on the Instrument Control window.

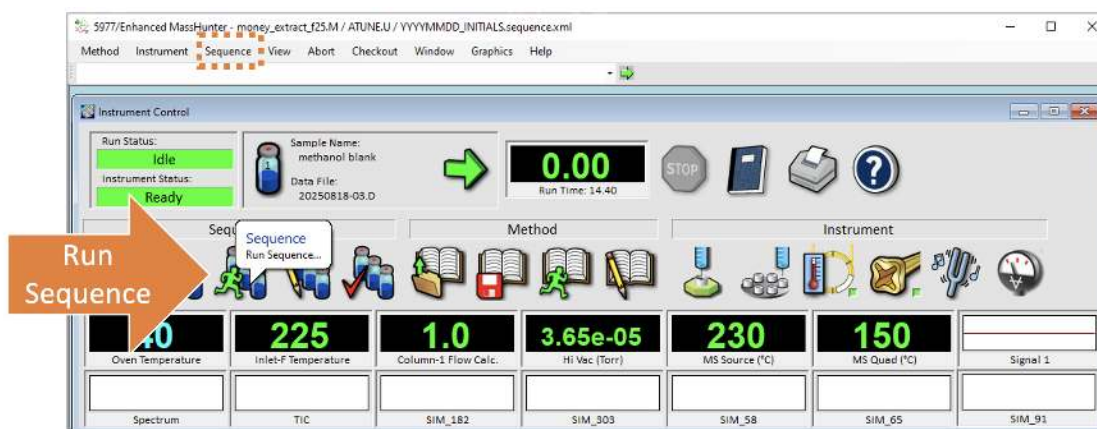


Figure 4.5.1.6: Running a sequence. (CC-BY-SA; Kathryn Haas for Duke Chem.)

8. A Yellow bar will appear at the top of the screen when the sequence is active. Wait while the sequence initializes and the first sample is injected.
9. To continue editing the sequence as the first samples are running, click Edit in the yellow bar and carefully make changes to your sequence. You can only add or edit lines that have not already been run, and that are at least one sample away from the active injection.

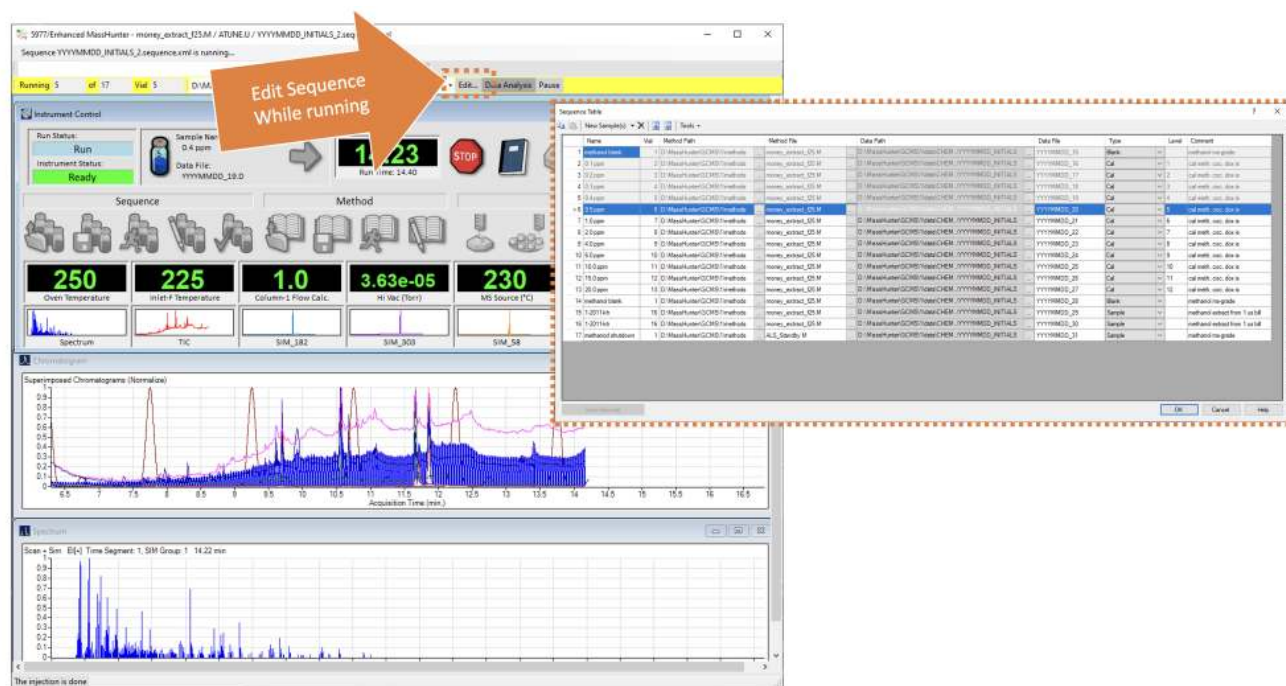


Figure 4.5.1.7: Editing an active sequence. (CC-BY-SA; Kathryn Haas for Duke Chem.)

- Be sure that you have assigned unique numbers to each new data file!
- Press OK when you are finished editing.
- Save your sequence after making edits.

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- **Lab 5: Gas Chromatography/Mass Spectrometry (GC/MS)** by W. R. Fawcett, John Berg, P. B. Kelley, Carlito B. Lebrilla, Gang-yu Liu, Delmar Larsen, Paul Hrvatin, David Goodin, and Brooke McMahon is licensed [CC BY-NC-SA 4.0](#).

4.5.3: Data Analysis using Agilent Mass Hunter Quantitative Analysis

Prerequisite step: Prior to data analysis, collect all of the data files in one directory on the computer's D: drive. If you followed instructions for data collection, they are already in a folder with the path D:\MassHunter\GCMS\1\data\CHEM401L-FallYYYY\YYYYMMDD_YourInitials. Know where your data directory is located on the computer hard drive!



To begin, open Agilent Mass Hunter Quantitative Analysis Program

Create a New Batch

A "Batch" is a data processing file that will eventually contain your samples, the method of analysis, and the results.

1. Create a new batch



- Click on **New Batch** () in the **Home Tab**.
- Select the directory containing your data files.
- Name the batch using the format **YYYYMMDD_CocaineQuantBatch_INITIALS**
- Click **Create** to create your new batch file within the same directory as your data files.
- Select the files that you would like to process in the batch. This should include all blanks, calibration standards, and samples.
- Select one calibration standard (10 or 15ppm is best) and one blank, then click "ok"

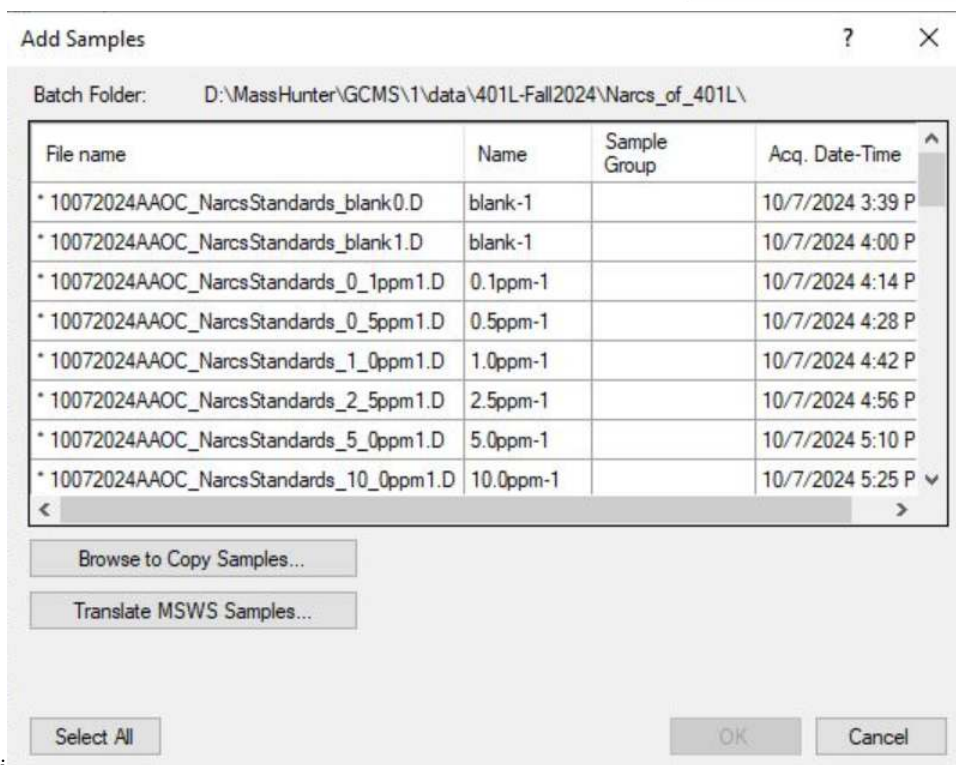
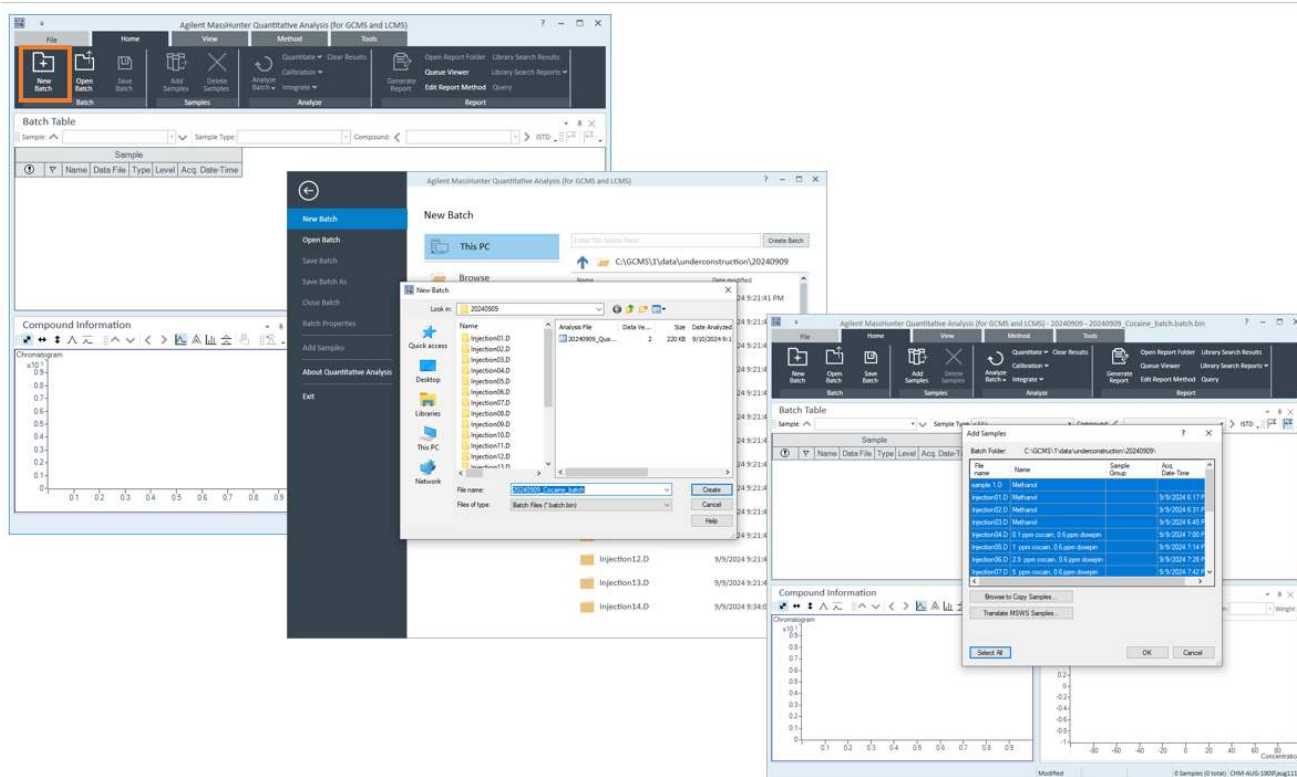


Figure 4.5.3.1: Copy and Paste Caption here. (CC-NC-BY-SA; Kathryn Haas, 2024)

2. In the Batch Table, change the "Type" to the appropriate option. If you already selected the appropriate type in the sequence for data collection, then the appropriate types may already be selected. Be sure they are correct for each line. The most common type selections are described below:

- *Blank*: select this type for your blank injection (MS-grade methanol)
- *Cal*: select this type for each of the calibration standards.

- **Sample:** select this type for each of the samples (eg. the extracts from currency).

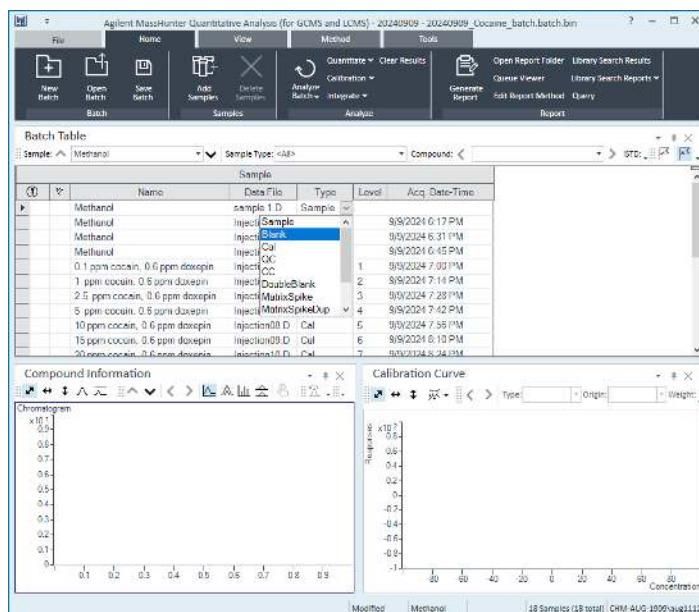


Figure 4.5.3.2: Copy and Paste Caption here. (CC-NC-BY-SA; Kathryn Haas, 2024)

3. In the Batch Table, change the "Level" to the appropriate value. The "level" refers to the calibration standards only. For the least concentrated standard, use level 1, and increase the level incrementally in integers for each additional calibration point. Replicate calibration points should have the same level assignment. (see Figure 4.5.3.2above.)

Create a Data Analysis Method

You should create a new method to analyze your data. You will use the NIST library to search for compounds.

4. In the **Method** Tab, expand the **New** menu and choose **New Method with Acquired Scan Data with Library Search**.

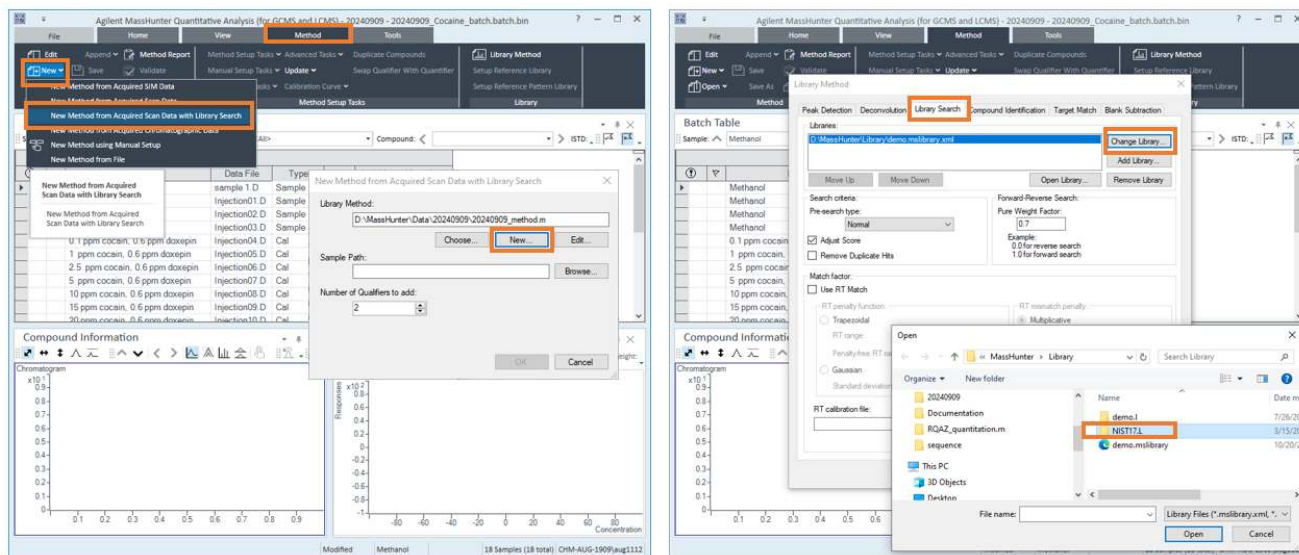


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

- In the new window that appears, under **Library Method**, choose **New...**
- In the new Library Method Window that appears, go to the **Library Search** Tab. Select **Change Library..**
- Select the **NIST17.L** library in the *MassHunter/Library* Directory. Then click **Open**.
- Peruse the other data analysis options in the other method tabs. For our purposes, the default options are usually sufficient.
- Click **Save & Exit**.

- You will be prompted to choose a new name for this new analysis method. Please save it within the same directory as your data and name it using the format YYYYMMDD_Cocaine_Quant_method_INITIALS

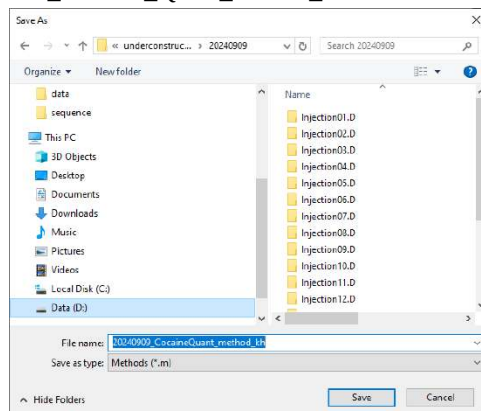


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

- The software will return you to the New Method Window, which now has the correct path to your new method in the Library Method dialogue box.
- Select "New" and "New Method from Acquired Scan Data with Library Search".
- In the New Method window, under Sample Path, choose Browse. Then navigate to your data directory and **choose a data folder containing medium to high levels of analyte for quantitation**. Typically, this would be a calibration standard with high concentration of analyte. Click **Open**.

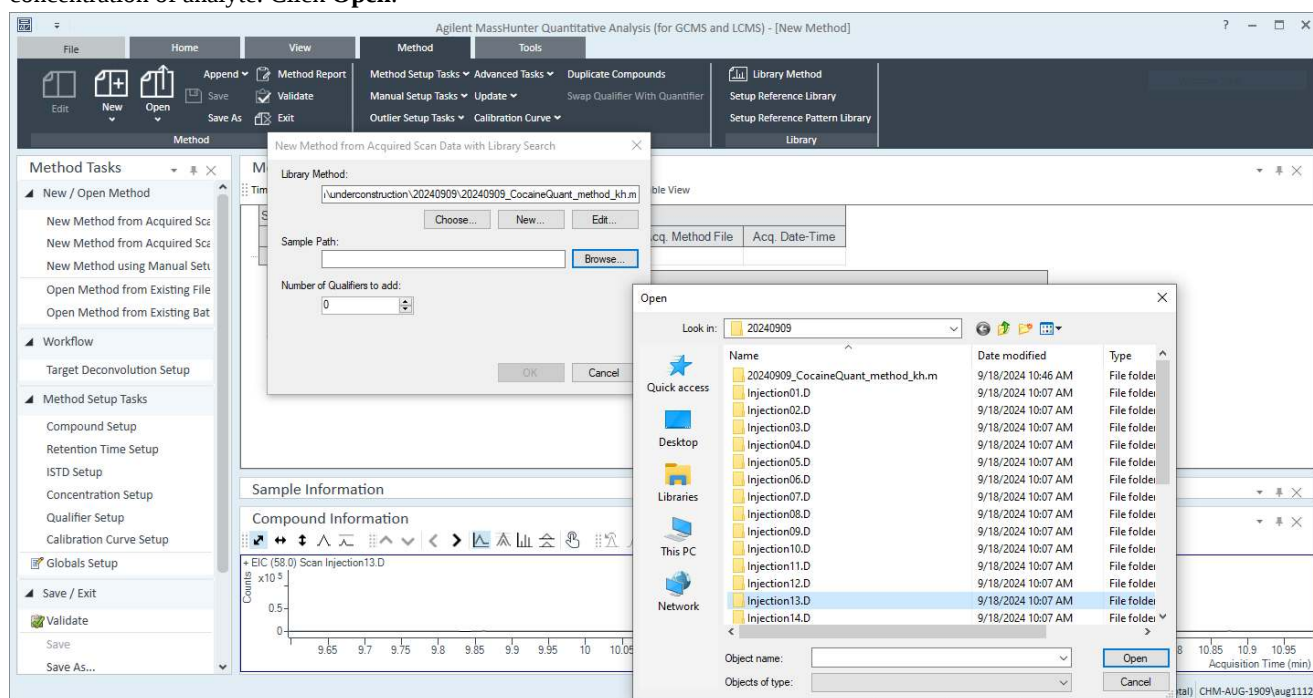


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

- Now your New Method is ready for creation. Click **OK** in the *New Method* window.

Set up Method

- In the *Method Table*, the sample that you selected in the previous set-up step should appear in the Sample Table. Underneath the Sample Table, there is a list of Quantifier compounds that will be used in quantitation.

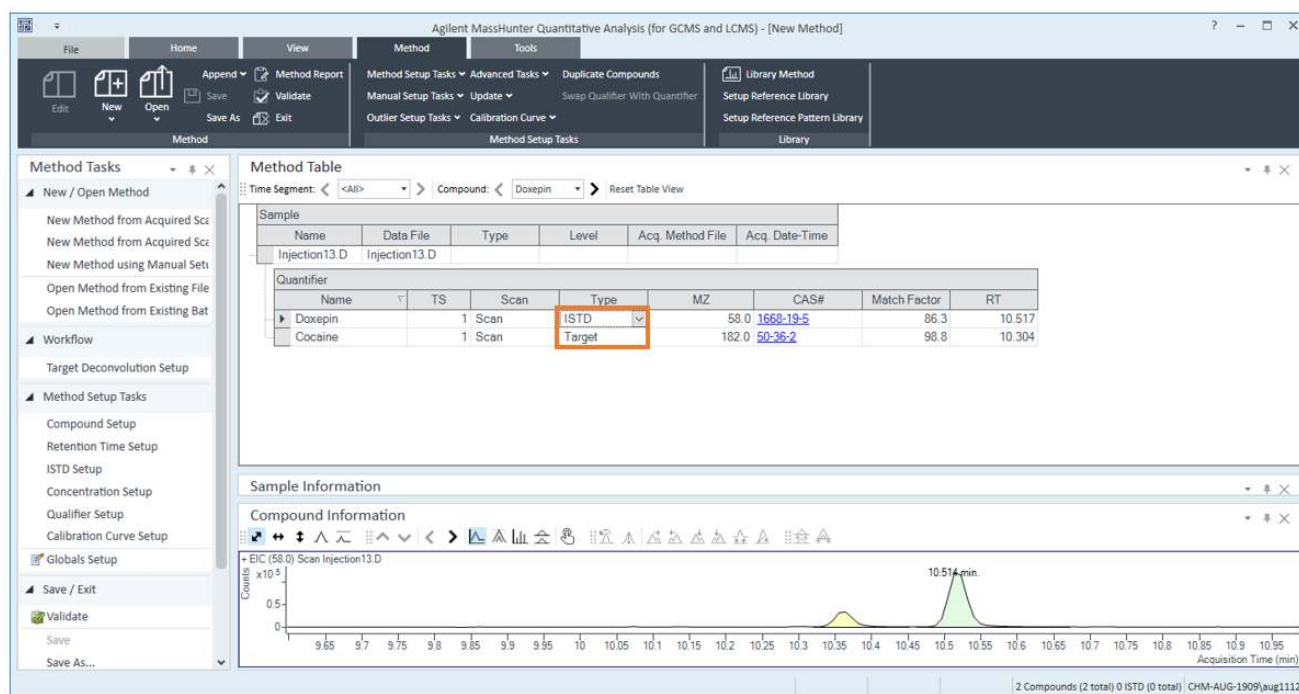


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

- If there are compounds in this list that are not of interest for quantitation, delete them by clicking on the line containing the compound information and press the delete key.
- Click on the name of compounds of interest to see their chromatogram in the Compound Information window. Check to be sure the data look reasonable and that the appropriate retention time is listed in the Quantifier table.
- Confirm that the MZ (mass/charge), CAS# are correct for any compounds of interest in the Quantifier table.
- Specify the Type in the Quantifier Table for each compound of interest.
 - Target: compounds for quantification and for which a calibration standards are in the batch.
 - ISTD: Compounds used as Internal Standard.

10. If you are not using an internal standard, skip to next step.

In the Method Setup Tasks (which can be accessed at the left menu or the top menu), go to ISTD setup to set up internal standard calibration.

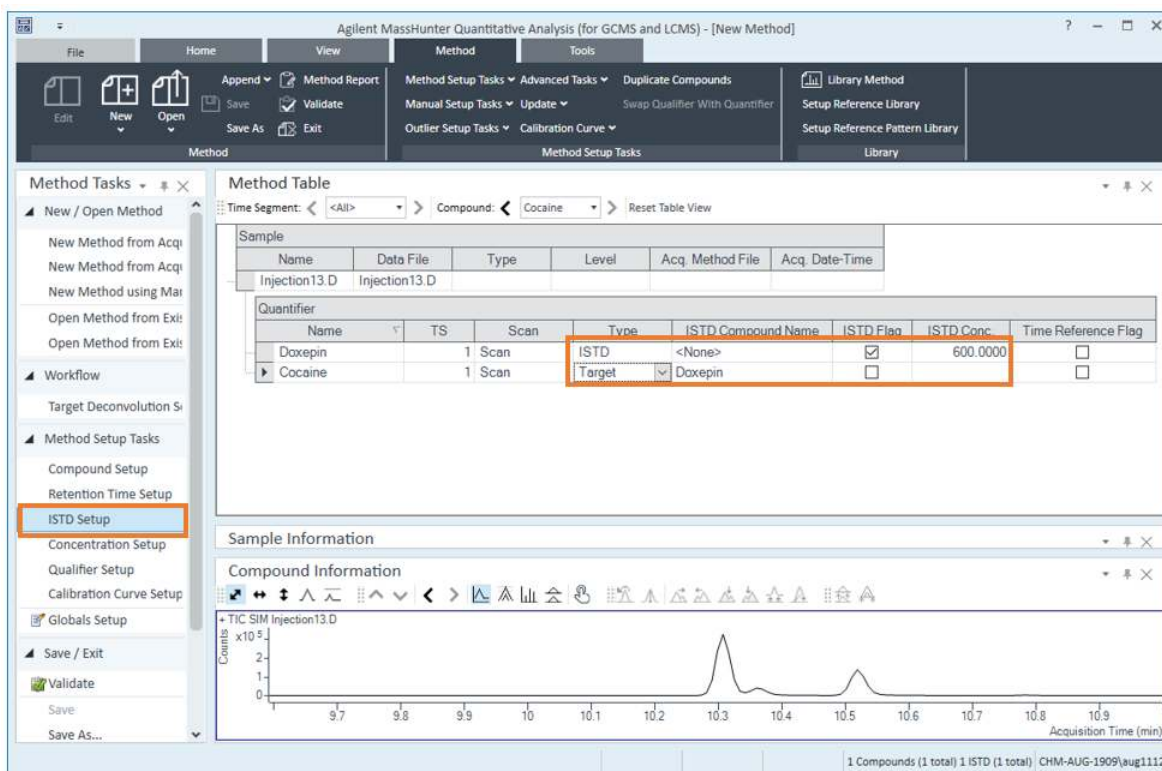


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

- Confirm the Type is correct for each compound.
 - In the line for the internal standard compound:
 - Check the box under the ISTD Flag column.
 - Enter the concentration of the internal standard in the ISTD Conc. column.
 - In the line for the target compound(s), choose the name of the internal standard in the ISTD Compound Name box.
 - In the line containing the compound information for the internal standard, enter the concentration of the Internal standard.
11. In the Method Setup Tasks, go to Concentration Setup and choose the most appropriate concentration units. The unit ng/mL is the same as ppb.

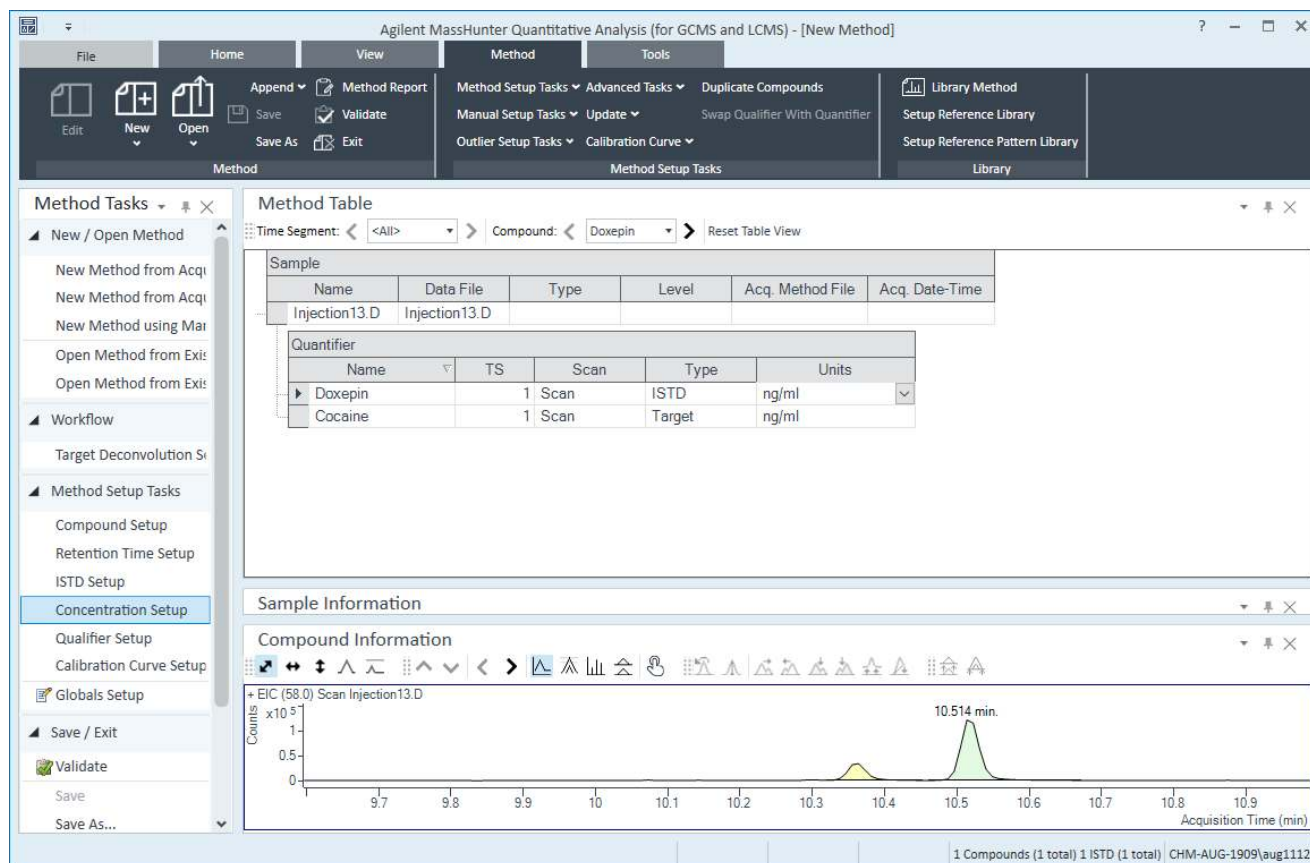


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

- Go to the top menu, in the *Method* tab. Expand the **Calibration Curve** menu and select **Create Levels from Calibration Samples**. This will create a *Calibration* table for each compound in the *Method Table* Window. Scroll down to see the calibration table for each compound in the Quantifier table.

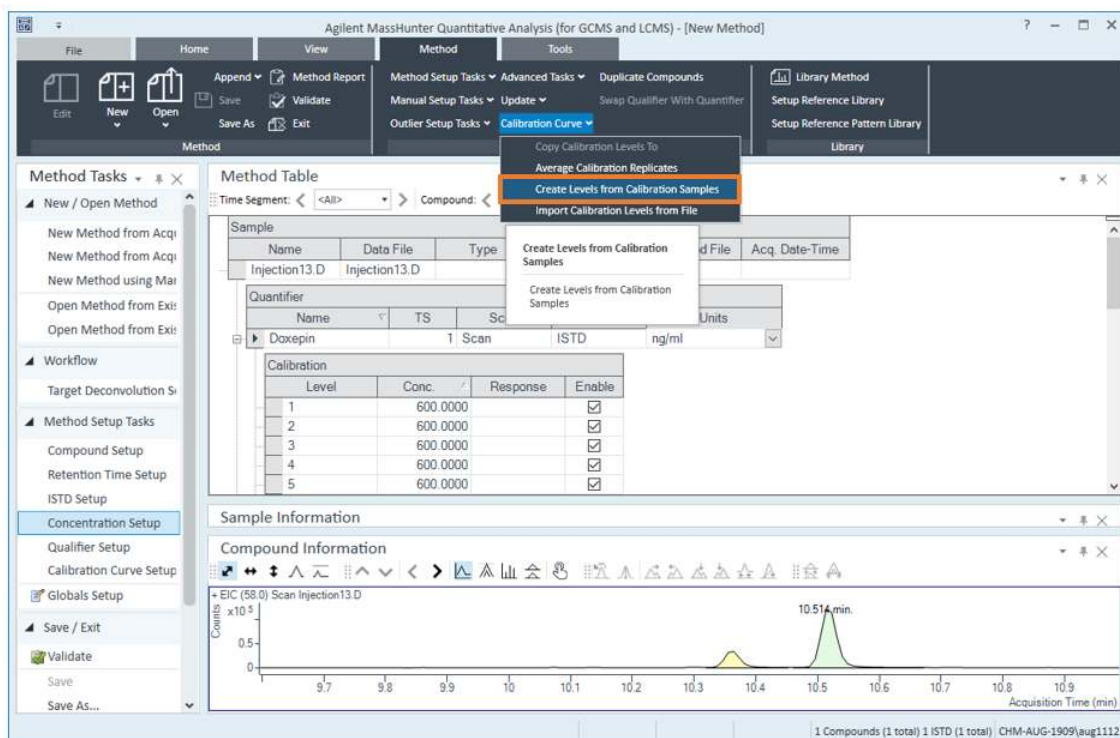
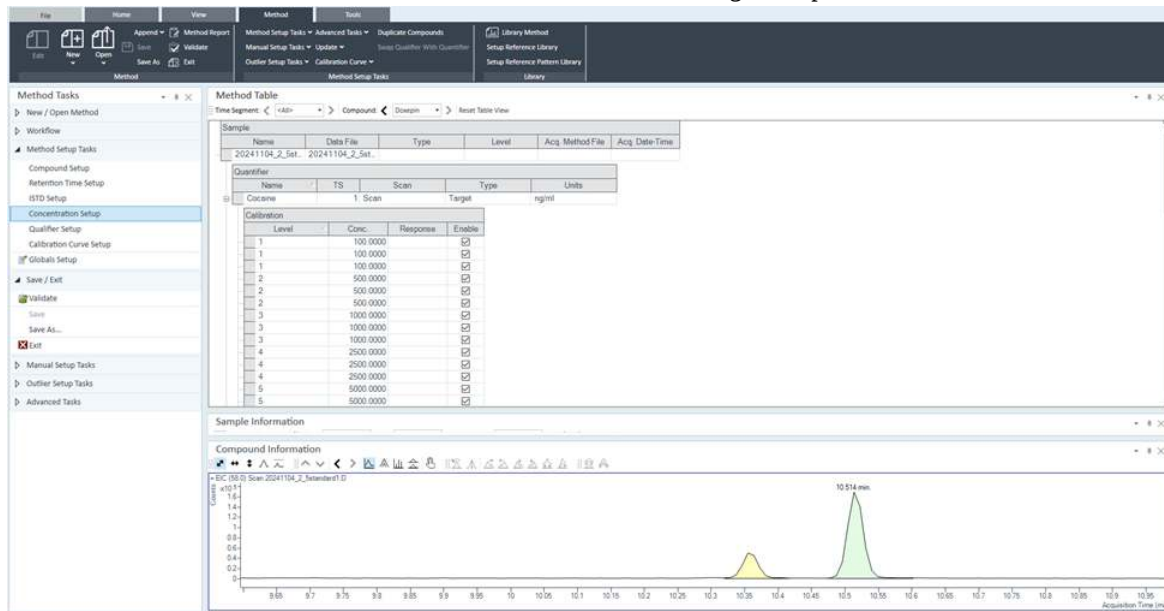


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

13. Enter *concentration of the target* compounds and *levels* for each calibration standard. You may have to type in the values for each sample manually. If you specified levels in your data collection sequence, then the Level assignments may already be done for you.

- Check to ensure the level assignments are correct for all data files in each Calibration table (scroll down to see all compounds and calibration tables).
- Check that the internal standard concentration is correct for each data file.
- Check that the calibration concentrations are correct for each of the target compounds in each of the calibration standards.



14. Click **Validate** method to check the method for errors.

- Correct any errors that are indicated.

15. Click **Exit**. Then select **Analyze** and **Yes**.

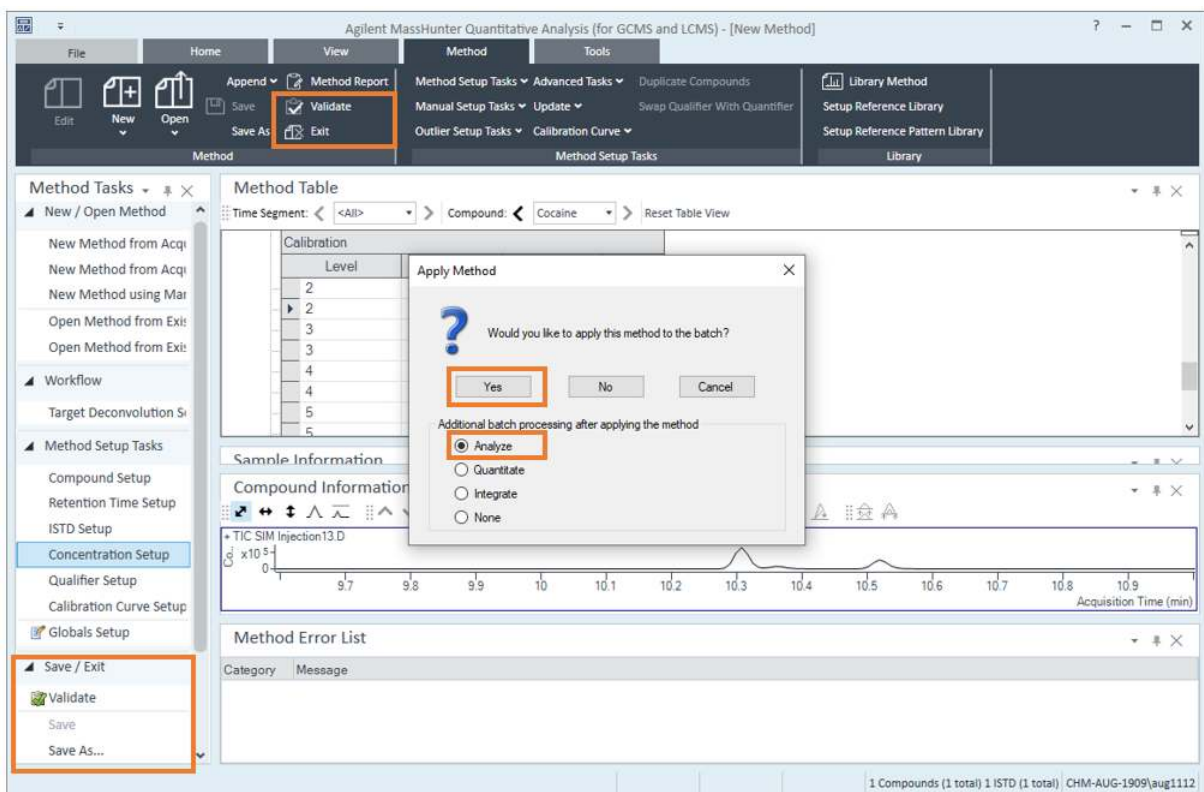


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

16. The software will then apply the data analysis method to all the samples in the batch. The result should look something like shown below. You can select individual data files in the Batch Table, and the Compound Chromatogram will be shown in the Compound Information window. You can screenshot this chromatogram for your report.

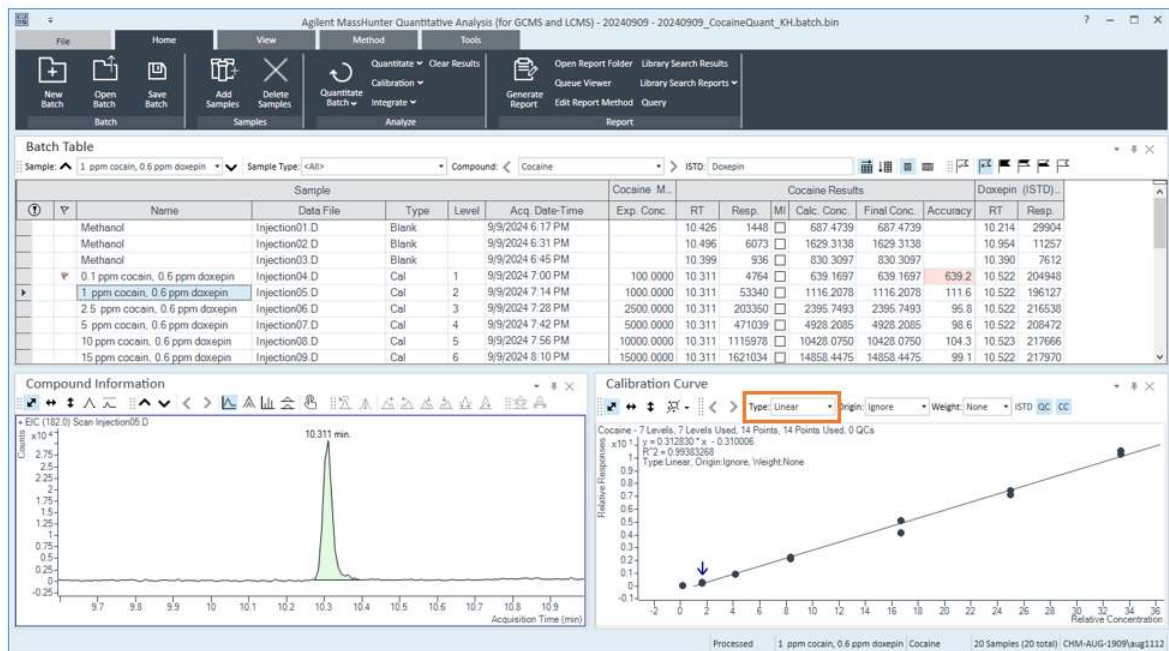
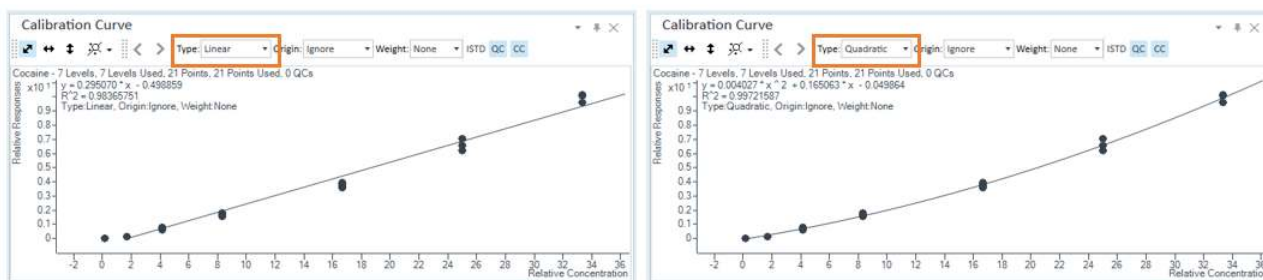


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

17. Note that you can change the Type of calibration in the *Home Tab* Calibration Curve window (see Figure above). It is possible that the best model for the data is not a straight line. To determine which fit is best for your data, a residuals analysis is necessary. However, this software does not perform such advanced tasks. To perform residuals analysis, use your training from

your prerequisite P-chem lab course and/or the corequisite Analytical Chemistry lecture course.



18. Press the Quantitate Batch button after any changes to the calibration type (see below).

Quantitate Samples using Quantitative Method

If you have already added your samples to your batch, select Quantitate Batch from the Home Tab. If you need to add samples to your batch, this is also a menu option under the Home tab.

After performing quantitation, the calculated sample concentrations will appear in the "Cocaine Results" column for each sample row.

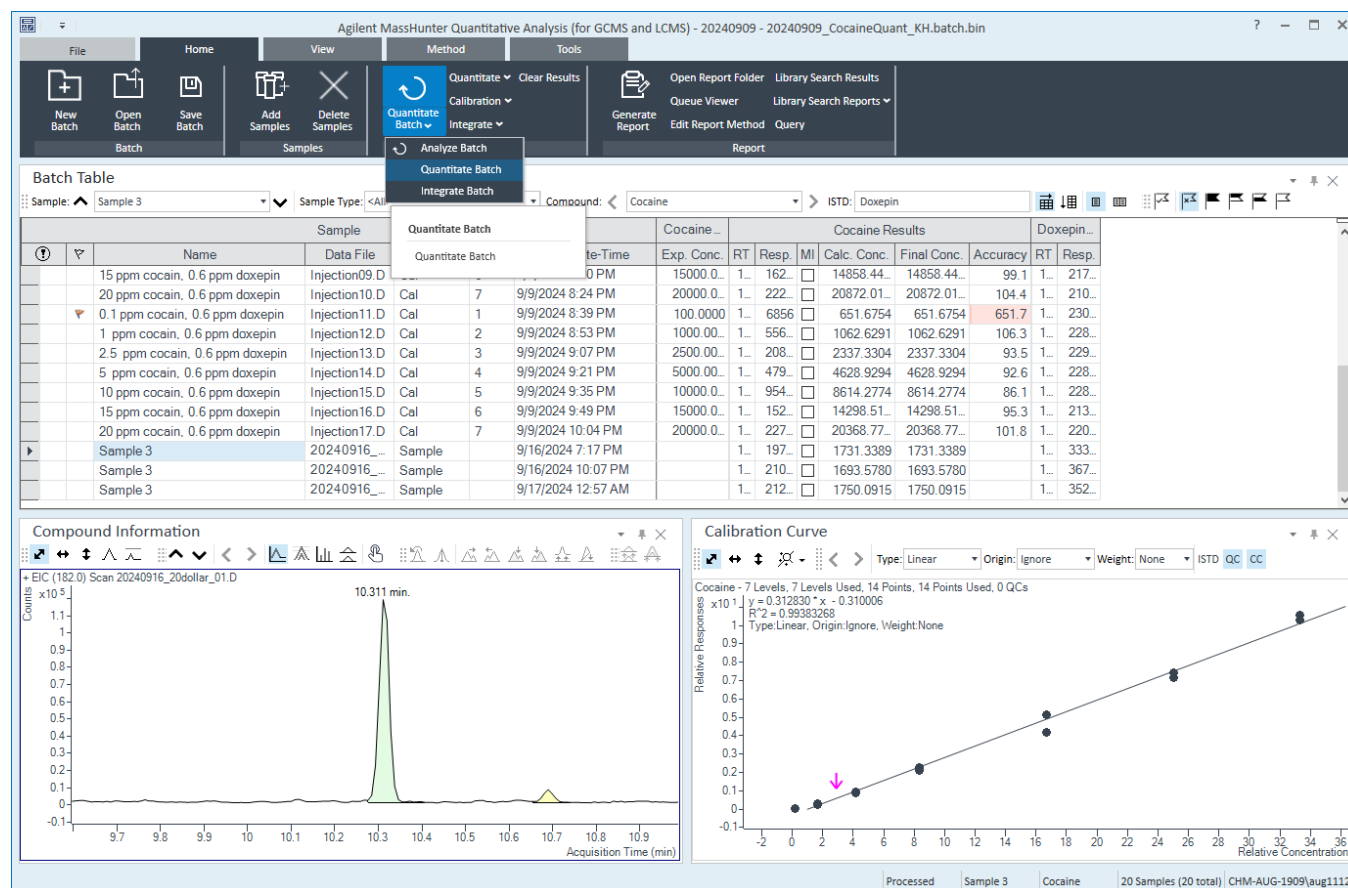


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

Save and Export your data

In the *Home Tab* toolbar, press the **Save Batch** button to save your work. You can recall the data processing at any time by opting this same batch file again at a later time.

Export the Batch Table

Save the results that are listed in the Batch table in a csv file that can be read by Excel or Matlab.

- **Right click** in the header of any column of the **Batch Table** (in the *Home Tab*, **Batch Table** window).
- Chose **Export Table...**
- Navigate to the directory where you would like to save this (your data directory) and specify a filename (eg YYYYMMDD_BatchTable.csv)
- Press Save
- Navigate to the same folder using a file explorer and ensure the file has been created, and that you can open the file using Excel.

Generate Report

Generate a pdf copy of your report using the following steps.

- In the Home Tab, select Generate Report.
- In the Generate Report window that appreas, select the folder where your would llike your report to be stored. Please select the same file directory in which your other files are stored. (For example, D:\MassHunter\GCMS\1\data\CHEM401L-FallYYYY\YYYYMMDD_YOURINITIALS)
- Choose the report Method. (in the data folder for your class, use the file 2024_Cocaine_Report_Template)
- Press OK.

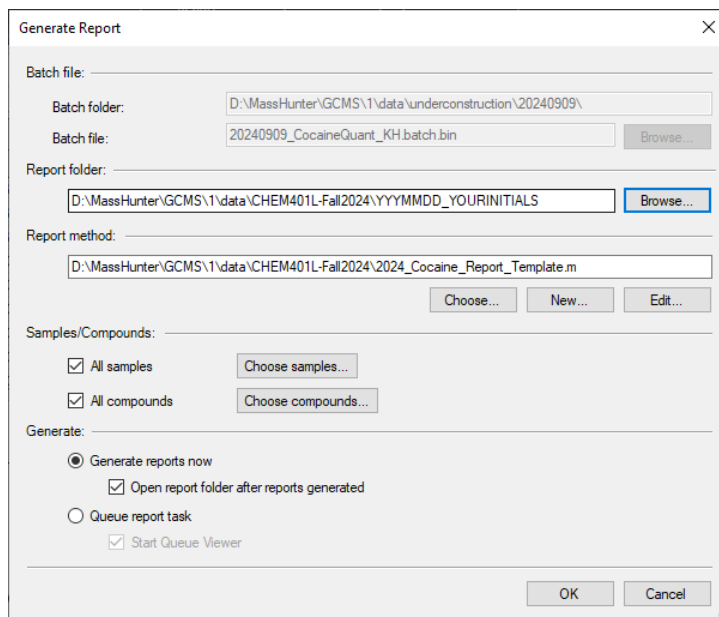


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

- Go to the file directory where your data is stored (or where you specified the Report Folder in the steps above) and find the report. It has the name "Cocaine_Report"

Note that this data analysis does not estimate error in the derived concentrations, therefore, you will need to plot the data from the report using excel, Matlab, or another program. Then perform linear least squares analysis and derive the concentration of your samples with error using the same methods you learned in the quant module (and also 401 lecture and in P-Chem's error analysis training).

Generate overlaid chromatograms

1. In the *View Tab*, Select **Chromatogram Information**.
2. In the Chromatogram Information window, put a check next to the appropriate data files. For example, if you would like to see the chromatogram of each calibration solution, check one file for each calibrant. When you select each file, it should appear in in the chromatogram panel. Make sure you select either all TIC scan files or all SIM files
3. In the *Chromatogram Information* window's Home tab, click the **Overlay** button. You may need to click this button more than once to rescale the chromatograms. Note that if properly overlaid on the same y-scale, the doxepin peak should look approximately the same in each sample, while the cocaine peak should show increasing response with increasing concentration.

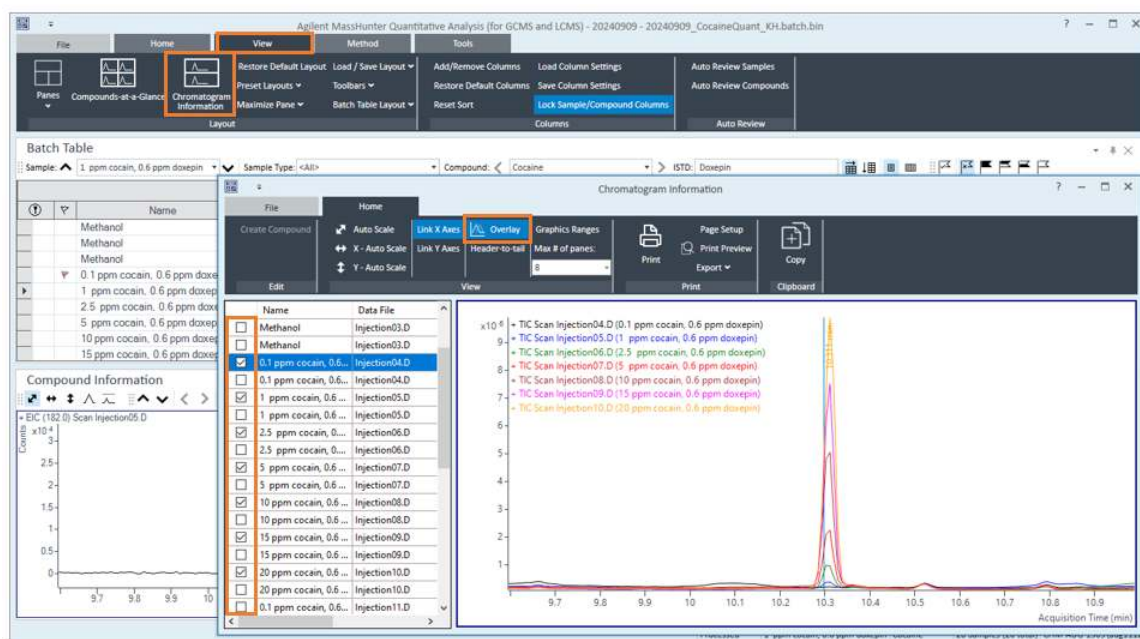


Figure 4.5.3.1: Copy and Paste Caption here. (Copyright; author via source)

4. When your chromatogram is ready, you can use the Export or Print buttons to save a file for use in your report. You can also right click on the chromatogram an copy/paste it into a document.

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CHAPTER OVERVIEW

5: High Performace Liquid Chromatography (HPLC)

- 5.1: High performance liquid chromatography
- 5.2: Chromatography simulation
- 5.3: HPLC Instruments at Duke
- 5.4: Ready the HPLC for Operation
- 5.5: Writing a Method for the Agilent 1100/1200 HPLC
- 5.6: Set up a sequence for the Agilent 1100/1200 HPLC
- 5.7: Running a Sequence
- 5.8: Data Analysis

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5.1: High performance liquid chromatography

Introduction

High-performance liquid chromatography (HPLC) is one of the most widely used techniques for separating, identifying, and quantifying compounds in a mixture. Chemists rely on HPLC in many areas, including pharmaceutical testing, food and beverage analysis, environmental monitoring, and forensic science. Because it is so versatile and precise, HPLC has become an essential tool for both research and industry.

At its core, HPLC works on a simple idea: different molecules spend different amounts of time moving with a **liquid (the mobile phase)** versus sticking to a **solid material (the stationary phase)**. The stationary phase is packed inside a narrow column, and a pump pushes the liquid mobile phase through the column at high pressure. When a mixture of compounds (a sample) is injected into the flowing mobile phase, its components interact differently with the stationary phase. Molecules that interact weakly move quickly and leave the column sooner, while molecules that interact strongly move slowly and leave later. This difference in travel time allows the mixture to be separated.

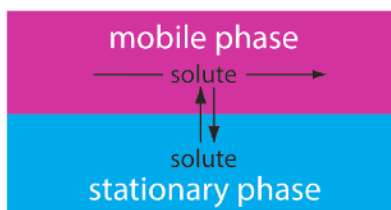


Figure 5.1.1. In chromatography we pass a mobile phase over a stationary phase. When we inject a sample into the mobile phase, the sample's components both move with the mobile phase and partition into the stationary phase. The solute that spends the most time in the stationary phase takes the longest time to move through the system, and vice versa. (From Harvey Ch 12.1)

The time it takes for each compound to travel through the column and reach the detector is called its **retention time**. On a chromatogram (the graph produced by the instrument), each compound appears as a peak. The position of the peak tells you when the compound eluted from the column, and the area under the peak is related to how much of the compound is present. By comparing retention times and peak areas to standards, chemists can identify and quantify the components of a sample.

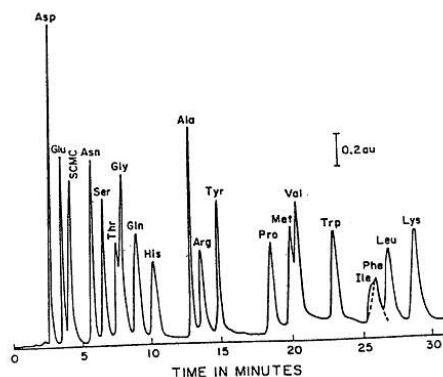


Figure 5.1.2: Chromatogram of Separation of Amino Acids by Reverse-Phase Chromatography.

HPLC Instrumentation

HPLC systems have several key parts:

- **Pump** – pushes the mobile phase through the column at high pressure.
- **Injector** – introduces the sample into the flowing mobile phase.
- **Column** – a tube packed with stationary phase particles where separation occurs.
- **Detector** – measures compounds as they leave the column and produces the chromatogram.

There are different ways to run HPLC. In **isocratic elution**, the mobile phase composition stays the same throughout the run. In **gradient elution**, the mobile phase changes over time (for example, starting with mostly water and gradually increasing the proportion of organic solvent). Gradient methods help separate mixtures that contain compounds with a wide range of polarities.

You may notice that HPLC is conceptually similar to other techniques you have already seen, such as thin-layer chromatography (TLC) or gas chromatography (GC). In all of these methods, compounds are separated based on how they partition between a stationary phase and a mobile phase. What makes HPLC unique is that it uses liquid as the mobile phase, solid particles packed in a column as the stationary phase, and high pressure to achieve fast, high-resolution separations.

In this course, you will learn how to operate an HPLC instrument, collect chromatograms, and interpret data to answer scientific questions. By the end of the module, you should be able to explain how HPLC works, run a basic separation, and use calibration methods to quantify analytes in real samples.

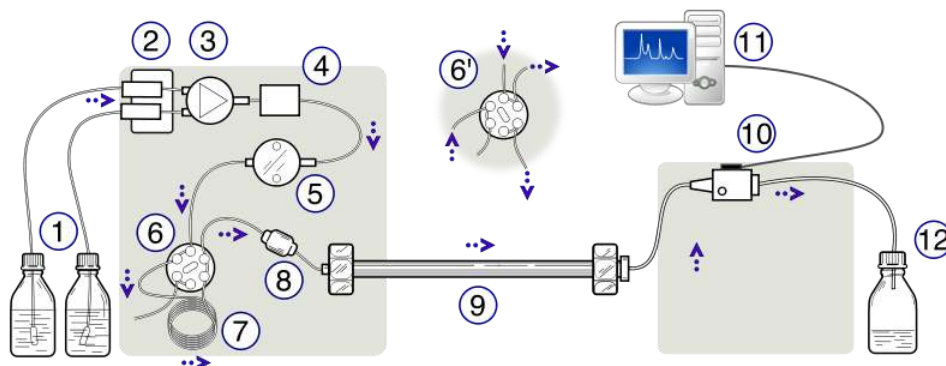


Figure 5.1.3: Schematic Diagram of a High-Performance Liquid Chromatograph. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column or guard column, (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector. Created by Yassne Mrabet.

UV-visible absorbance is the most commonly used mode of detection, and our instruments at Duke include a photodiode array (PDA) detector that monitors absorption of UV-vis light. Such detectors enable the eluent (or effluent) from the column to flow through an 8 to 10 μL spectrophotometric cell for detection of compounds at a particular wavelength (often in the ultraviolet, $< 400\text{nm}$, where many organic molecules absorb). Electrochemical and fluorescence detectors often are used to achieve lower detection limits. The other commonly used detector is based on a measurement of the differential refractive index.

Details about HPLC Components

The following is a list of common HPLC components. Details about the components you will use in this lab are found in the section on the [Agilent HPLC Components at Duke \(click here\)](#).

- The **mobile phase**, or solvent, is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample. It is critical that the mobile phase is kept free of particulate matter or dissolved gas. As the solvent is passed through a very narrow bore column, any particles could at worst plug the column, or at the very least add variability to the retention times during repeated different trials. Therefore HPLC solvent and sample should be filtered through sub-micron filters to remove solid particles. The solvent is degassed to keep it free of dissolved gases, which could bubble out of solution mid-separation under changing pressures. Bubbles in the column causes band spreading. In addition, both bubbles and dust interfere with detector performance.
- The **HPLC column**, separates components of the sample based on their differing interactions with the column packing (stationary phase). If a species interacts more strongly with the stationary phase in the column, it will spend more time adsorbed to the column's adsorbent and will therefore have a greater retention time. Columns can be packed with solids such as silica or alumina; these columns are called **homogeneous columns**. If stationary phase in the column is a liquid, the column is deemed a **bonded column**. Bonded columns contain a liquid stationary phase bonded to a solid support, which is again usually silica or alumina. The value of the constant C described in the van Deemter equation is proportional, in HPLC, to the diameter of the particles that constitute the column's packing material.
- The **HPLC pump** drives the solvent and sample through the column. The requirements for the pumps used in liquid chromatography are severe and include (1) the generation of pressures up to 6000 psi, (2) pulse-free output, (3) flow rates ranging from 0.1 to 10 mL/min, (4) flow reproducibility of 0.2% or better, and (5) resistance to corrosion by a variety of solvents. To reduce variation in the elution, the pump must maintain a constant, pulse free, flow rate; this is achieved with **multi-piston pumps**. The presence of two pistons allows the flow rate to be controlled by one piston as the other recharges.

- The **HPLC detector**, located at the end of the column, must register the presence of various components of the sample, but must not detect the solvent. For that reason there is no universal detector that works for all separations. A common HPLC detector is a **UV absorption detector**, as most medium to large molecules absorb UV radiation. Detectors that measure fluorescence and refractive index are also used for special applications. It is also common for an instrument to include many different kinds of detectors. For example, it is common for HPLC with a UV-vis detector to be coupled with a mass spectrometer so that analytes can be identified based on mass. A relatively new development is the combination of an HPLC separation with an **NMR detector**. This allows the pure components of the sample to be identified and quantified by nuclear magnetic resonance after having been separated by HPLC, in one integrated process.
- **Sample injection**: The ideal sample injection system places the sample at the top of the column in a sharp well-defined plug of minimum thickness. Injection can be done manually or automatically using an automated sample injection system (autosampler).

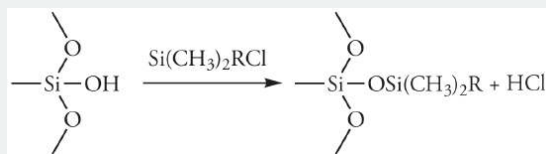
Classes of Liquid Chromatography

The conventional classification of separation modes in LC is that of liquid-solid, liquid-liquid, ion-exchange, and size-exclusion chromatography. Four general classes of LC can be distinguished from this perspective (listed below); these classes differ in the type of stationary phase and mobile phase used. **Reverse phase will be used in the in-lab HPLC module of this course.**

1. **Normal-phase chromatography** calls for the use of a polar stationary phase in conjunction with a non-polar (dispersive) mobile phase.
2. **Reverse-phase chromatography**, the opposite possibility, calls for the use of a non-polar stationary phase and a polar mobile phase (composed of one or more of the solvents water, methanol, acetonitrile, and tetrahydrofuran). The majority of chromatographic analyses fall into one of these two classes.
3. **Ion-exchange chromatography** involves ionic interactions. In this case the mobile phase must support ionization to ensure solubility of ionic solutes. The stationary phase must also be partially ionic to promote some retention. Consequently, the interactions with the stationary phase are strong, and this is usually reflected in longer analysis times and broad peaks.
4. **Size-Exclusion chromatography** involves separations based on molecular size alone and ideally requires that there be no energetic interaction of the solutes with the stationary phase.

Reverse Phase Chromatography

The most common class of chromatography used in HPLC is **reverse-phase** using C18-modified silica particles packed tightly into a metal cylinder. The alkyl group is bound covalently to the silica particles; **bonded stationary phases** are created by reacting the silica particles with an organochlorosilane of the general form $\text{Si}(\text{CH}_3)_2\text{RCl}$, where R is an alkyl or substituted alkyl group.



The properties of a stationary phase depend on the organosilane's alkyl group. If R is a polar functional group, then the stationary phase is polar. Examples of polar stationary phases include those where R contains a cyano ($-\text{C}_2\text{H}_4\text{CN}$), a diol ($-\text{C}_3\text{H}_6\text{OCH}_2\text{CHOHCH}_2\text{OH}$), or an amino ($-\text{C}_3\text{H}_6\text{NH}_2$) functional group. Because the stationary phase is polar, the mobile phase is a nonpolar or a moderately polar solvent. The combination of a polar stationary phase and a nonpolar mobile phase is called **normal-phase chromatography**.

In **reversed-phase chromatography**, which is the more common form of HPLC, the stationary phase is nonpolar and the mobile phase is polar. The most common nonpolar stationary phases use an organochlorosilane where the R group is an *n*-octyl (C_8) or *n*-octyldecyl (C_{18}) hydrocarbon chain. In this course, you will be using the most common type; a C_{18} -derivatized silica stationary phase (aka "C18")

The interactions of solutes with an alkyl-bonded phase, such as the C18 bonded-phase silica, are dispersive, and a polar mobile phase is conventionally used in conjunction with these stationary phases. The non-polar stationary phase makes these systems very useful for separating organic compounds with slight differences in the backbones or side-chains.

Separation Efficiency Depends on Affinities for Mobile and Stationary Phases

All chromatographic separations, including HPLC operate under the same basic principle; every compound interacts with other chemical species in a characteristic manner. Chromatography separates a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

Distribution Constant (K_c)

All chemical reactions have a characteristic equilibrium constant. For the reaction



There is a chemical equilibrium constant K_{eq} that dictates what percentage of compound **A** will be in solution and what percentage will be bound to the stationary compound **B**. During a chromatographic separation, there is similar relationship between compound **A** and the solvent, or **mobile phase, C**. This will yield an overall equilibrium equation which dictates the quantity of **A** that will be associated with the stationary phase and the quantity of **A** that will be associated with the mobile phase.

$$A_{mobile} \rightleftharpoons A_{stationary} \quad (5.1.2)$$

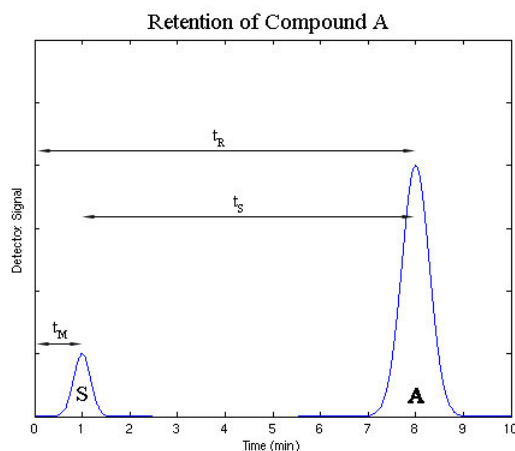
The equilibrium between the mobile phase and stationary phase is given by the constant **distribution constant, K_c** .

$$K_c = \frac{(a_A)_S}{(a_A)_M} \quad (5.1.3)$$

Where $(a_A)_S$ is the **activity** of compound **A** in the stationary phase, and $(a_A)_M$ is the activity of compound **A** in the mobile phase. In most separations, which contain low concentrations of the species to be separated, the activity of **A** in each is approximately equal to the concentration of **A** in that state ($(a_A) \approx [A]$); thus equation 5.1.3 can be approximated in terms of concentration:

$$K_c \approx \frac{[A]_S}{[A]_M} \quad (5.1.4)$$

The distribution constant indicates the amount of time that compound **A** spends adsorbed to the stationary phase as the opposed to the amount of time **A** spends solvated by the mobile phase. This relationship determines the amount of time it will take for compound **A** to travel the length of the column. The more time **A** spends adsorbed to the stationary phase, the more time compound **A** will take to travel the length of the column. The amount of time between the injection of a sample and its elution from the column is known as the **retention time**; it is given the symbol t_R .



The amount of time required for a sample that does not interact with the stationary phase, or has a K_c equal to zero, to travel the length of the column is known as the **void time, t_M** . No compound can be eluted in less than the void time.

Retention Factor

Since K_C is a factor that is wholly dependent on a particular column and solvent flow rate, a quantitative measure of the affinity of a compound for a particular set of mobile and stationary phases that does not depend on the column geometry is useful. The **retention factor**, k , can be derived from K_C and is independent of the column size and the solvent flow rate.

$$k = \frac{K_C V_S}{V_M} \quad (5.1.5)$$

The retention factor is calculated by multiplying the distribution constant by the volume of stationary phase in the column and dividing by the volume of mobile phase in the column.

Selectivity

In order to separate two compounds, their respective retention factors must be different, otherwise both compounds would be eluted simultaneously; the **selectivity** factor is the ratio of the retention factors.

$$\alpha = \frac{k_B}{k_A} \quad (5.1.6)$$

Where **B** is the compound that is retained more strongly by the column and **A** is the compound with the faster elution time.

Band Broadening

As a compound passes through the column it slowly diffuses away from the initial injection band, which is the area of greatest concentration. The initial, narrow, band that contained all of the sample becomes broader the longer the analyte remains in the column. This **band broadening** increases the time required for complete elution of a particular compound and is generally undesirable. It must be minimized so that overly broad elution bands do not overlap with one another. We will see how this is measured quantitatively when we discuss **peak resolution** momentarily.

Plate Height is a Measure of Separation Efficiency

In analytical chemistry, a quantitative metric of separation efficiency is *theoretical plates*. The concept of *plates* as a separation metric arose from the original method of **fractional distillation**, where compounds were separated based on their volatilities through many simultaneous **simple distillations**, each simple distillation occurred on one of many distillation plates. In chromatography, there are no actual plates, and you should think of the theoretical plates purely as a measure of separation efficiency. We say that a column is more efficient at a separation if it has a smaller theoretical plate height (H) or a higher number (N) of theoretical plates. And, for any given column, the H and N depend on an individual solute's diffusion coefficient.

Since there is no "real" height, nor are their "real" plates, we refer to the plates as **theoretical plates**, and their height is the **height equivalent of the theoretical plate (HETP or H)**. When a solute travels through a column, its peak width depends on its diffusion and the length or time that it has traveled on the column. Assuming that the peak is Gaussian in shape, the width of an elution peak at its base is approximately four standard deviations ($w = 4\sigma$). The theoretical plate height is given by the peak's **variance** (variance = standard deviation squared... σ^2) divided by the distance it has traveled (the length of the column, L).

$$H = \frac{\sigma^2}{L} \quad (5.1.7)$$

For a column of length L , the number of theoretical plates is the length divided by the plate height ($N = \frac{L}{H}$). Using the relationship between plate height and number of plates, and the relationship between peak width and standard deviation, the number of plates can also be found in terms of retention time and peak width (Equation 5.1.8). N is a dimensionless number, and in equation 5.1.8 both L and w are in units of length.

$$N = \frac{L}{H} = \frac{L^2}{\sigma^2} = \frac{16L^2}{w^2} \quad (5.1.8)$$

A more useful form of equation 5.1.8 is in terms of time; L can be expressed as retention time t_R and w is the width of the base of the elution peak in units of time.

$$N = \frac{16t_R^2}{w^2} = \frac{t_R^2}{\sigma^2} \quad (5.1.9)$$

In order to optimize separation efficiency, it is necessary to maximize the number of theoretical plates, which requires reducing the plate height. The plate height is related to the **flow rate** of the mobile phase, so for a fixed set of mobile phase, stationary phase, and analytes; separation efficiency can be maximized by optimizing flow rate as dictated by the **van Deemter equation**.

$$H = A + \frac{B}{v} + Cv \quad (5.1.10)$$

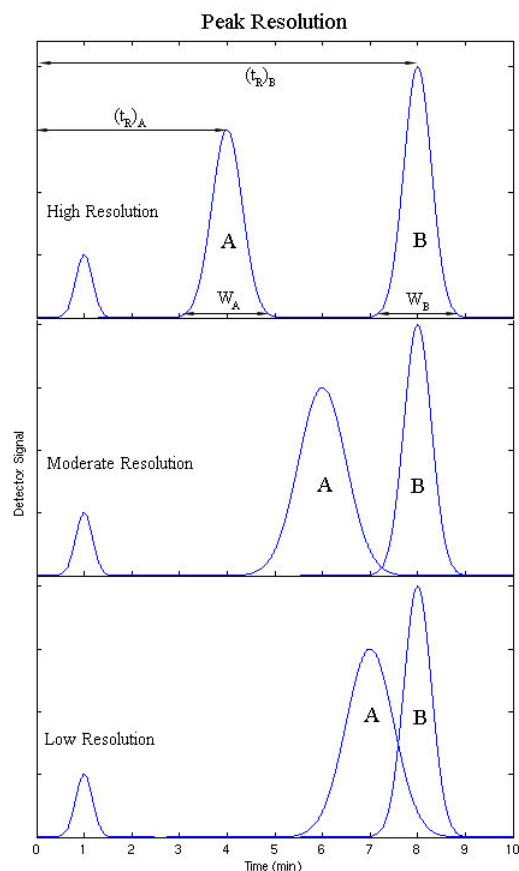
The three constants in the van Deemter equation are factors that describe possible causes of band broadening in a particular separation. A is a constant which represents the different possible paths that can be taken by the analyte through the stationary phase, it decreases if the packing of the column is kept as small as possible. B is a constant that describes the longitudinal diffusion that occurs in the system. C is a constant that describes the rate of adsorption and desorption of the analyte to the stationary phase. A , B and C are constant for any given system (with constant analyte, stationary phase, and mobile phase), so flow rate must be optimized accordingly. If the flow rate is too low, the **longitudinal diffusion factor** ($\frac{B}{v}$) will increase significantly, which will increase plate height. At low flow rates, the analyte spends more time at rest in the column and therefore longitudinal diffusion is a more significant problem. If the flow rate is too high, the **mass transfer term** (Cv) will increase and reduce column efficiency. At high flow rates the adsorption of the analyte to the stationary phase results in some of the sample lagging behind, which also leads to band broadening.

Resolution

The **resolution** of an elution is a quantitative measure of how well two elution peaks can be differentiated in a chromatographic separation. It is defined as the difference in retention times between the two peaks, divided by the combined widths of the elution peaks.

$$R_S = \frac{2 [(t_R)_B - (t_R)_A]}{W_B + W_A} \quad (5.1.11)$$

Where **B** is the species with the longer retention time, and **t_R** and **W** are the retention time and elution peak width respectively. If the resolution is greater than one, the peaks can usually be differentiated successfully.



Optimizing Separation using Reverse-Phase Chromatography

The objective of optimization in chromatography depends on the goals of a given project. For an analytic chemist, optimization usually means that the resolution is maximized while the time and solvent use is minimized. This means that individual peaks elute separately (with no overlapping peaks), the peak shapes are consistent and sharp across the entire chromatogram, and that this is accomplished in the shortest amount of time possible. By and large, and certainly in this experiment, optimization of the mobile phase composition is the primary objective. Your goal is to select the mobile phase composition and elution timetable that will achieve good resolution and peak shape within a reasonable analysis time.

Selection of mobile phase

Selecting the mobile phase (or solvent) is one of the most important steps when performing HPLC and is selected based on polarity. Solvent polarity relates to the ability of the components to partition into that phase. The polarity scale for different solvents can be found in Table 5.1.1. These solvents can be used exclusively or mixed to achieve the desired polarity.

Table 5.1.1: Relative polarities of common mobile phase solvent.

Solvent	Relative Polarity
Fluoroalkanes	<-2>
Cyclohexane	.04
1-Chlorobutane	1.0
Carbon tetrachloride	1.6
Toluene	2.4
Tetrahydrofuran (THF)	4.0

Ethanol	4.3
Methanol	5.1
Acetonitrile	5.8
Ethylene glycol	6.9
Water	10.2

When considering which factors affect the separation in a reverse phase HPLC experiment we should think not only about polarity, but also about hydrogen bonding ability (of both solvents and species being separated). The following data offer information on the dielectric constants (ϵ)¹ of the solvents we could use in this experiment; and, the hydrogen bonding donor (α , also known as hydrogen bonding acidity) and hydrogen bonding acceptor (β , also known as hydrogen bonding basicity)² values.

Solvent	ϵ	α	β
Water	78.5	1.17	0.47
Methanol	32.6	0.98	0.66
Acetonitrile	37.5	0.19	0.40

1. Common Organic Solvents: Table of Properties, http://www2.onu.edu/~b-myers/organic_solvents.html, accessed December 7, 2012.

2. Tables for Chemistry: Kamlet-Taft Solvent Parameters, <http://www.stenutz.eu/chem/solv26.php>, accessed December 7, 2012.

What we see from these numbers is that all three solvents are polar. While water has by far the highest dielectric constant, methanol and acetonitrile have high values (for comparison: hexane, toluene and methylene chloride have ϵ values of 1.89, 2.38 and 9.08 respectively). All three also have similar hydrogen bonding acceptor values, not surprising since all three have atoms with one or more lone pair of electrons that could interact with an electron deficient hydrogen from a solvent or a solute molecule. Notice that compared to water and methanol, acetonitrile has a very low hydrogen bonding donor number. This too is not surprising since acetonitrile is not considered a protic solvent. It has no hydrogen bound to an electronegative atom, that would participate in hydrogen bonding.

When considering how a molecule will behave in an HPLC experiment we want to consider not only solute-solvent interactions (i.e., is the sample strongly associated with the solvent or the column), but also solvent-solvent interactions (how will the sample associate with, or disrupt, the structure of the solvent)

Gradient Elution vs. Isocratic Elution

If the composition of the mobile phase remains constant throughout the HPLC separation, the separation is deemed an **isocratic elution**. Often the only way to elute all of the compounds in the sample in a reasonable amount of time, while still maintaining peak resolution, is to change the ratio of polar to non-polar compounds in the mobile phase during the sample run. Known as **gradient chromatography**, this is the technique of choice when a sample contains components of a wide range of polarities. For a **reverse phase gradient**, the solvent starts out relatively polar and slowly becomes more non-polar. The gradient elution offers the most complete separation of the peaks, without taking an inordinate amount of time. A sample containing compounds of a wide range of polarities can be separated by a gradient elution in a shorter time period without a loss of resolution in the earlier peaks or excessive broadening of later peaks. However, gradient elution requires more complex and expensive equipment and it is more difficult to maintain a constant flow rate while there are constant changes in mobile phase composition. Gradient elution tests the limitations of lower-quality experimental apparatus, making the results obtained less reproducible in equipment already prone to variation. If the flow rate or mobile phase composition fluctuates, the results will not be reproducible.

Applications

HPLC can be used in both qualitative and quantitative applications, that is for both compound identification and quantification. Normal phase HPLC is only rarely used now, almost all HPLC separation can be performed in reverse phase. Reverse phase HPLC is broadly effective, with the exception of only a few separation types; it cannot separate inorganic ions (they can be separated by ion exchange chromatography). It cannot separate polysaccharides (they are too hydrophilic for any solid phase adsorption to

occur), nor polynucleotides (they adsorb irreversibly to the reverse phase packing). Lastly, incredibly hydrophobic compounds cannot be separated effectively (there is little selectivity). Aside from these few exceptions, Reverse phase HPLC is used for the separation of almost all other compound varieties. It can be used to effectively separate similar simple and aromatic hydrocarbons, even those that differ only by a single methylene group. It also effectively separates simple amines, sugars, lipids, and even pharmaceutically active compounds. Reverse phase HPLC is also used in the separation of amino acids, peptides, and proteins. Finally reverse phase HPLC is used to separate molecules of biological origin.

The determination of caffeine content in coffee products is routinely done by reverse phase HPLC in commercial applications in order to guarantee purity and quality of ground coffee. HPLC is a useful addition to an analytical arsenal, especially for the separation of a sample before further analysis.

References

1. Skoog, D.; Holler, F.; Crouch, S. *Principles of Instrumental Analysis* **2007**
2. Swadesh, J.K. *HPLC: Practical and Industrial Applications* **2001**
3. Waters Corporation: History of Chromatography (accessed March 3, 2008)
4. Harvey, [Analytical Chemistry 2.1](#), Chapter 12: Chromatographic and Eletrophoretic Methods.
5. [High Performance Liquid Chromatography](#) (Supplemental Modules, Analytical Chemistry, Libretexts)

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5.2: Chromatography simulation

Get familiar with the simulator

1. Open the [Multi-Dimensional Separations Website](https://www.multisim.org/login) (do not use Safari)

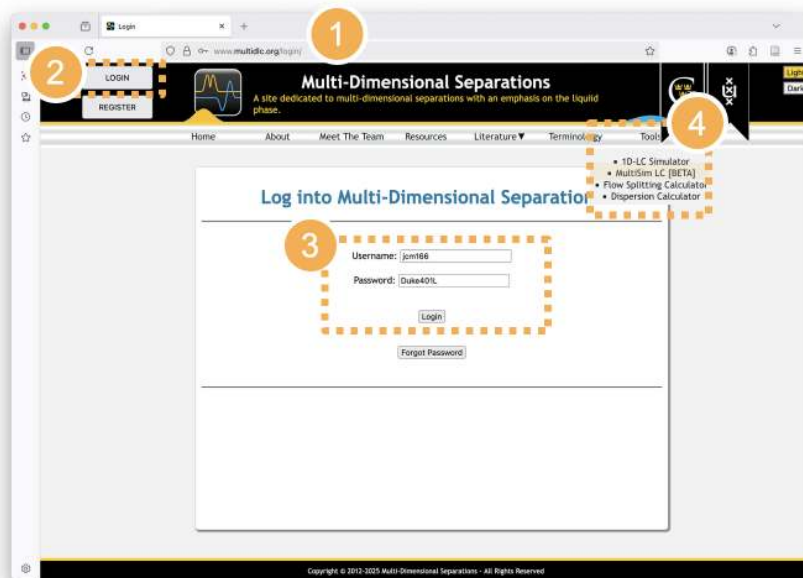


Figure 5.2.1: Access the Multi-Dimensional Separations Simulator. (CC-NC-BY-SA; Jack Malek 2025)

2. Click “login” on the top left (red arrow) .
 - Username: **jcm166**
 - password: **Duke401L**
3. Select tools, then **MultiSim LC [BETA]**
4. Click on Compounds to View options. Click “**add compound.**” In the Select Compounds popup window, deselect the default compounds and select **caffeine**, **acetophenone**, **phenol**, and **pyridine**

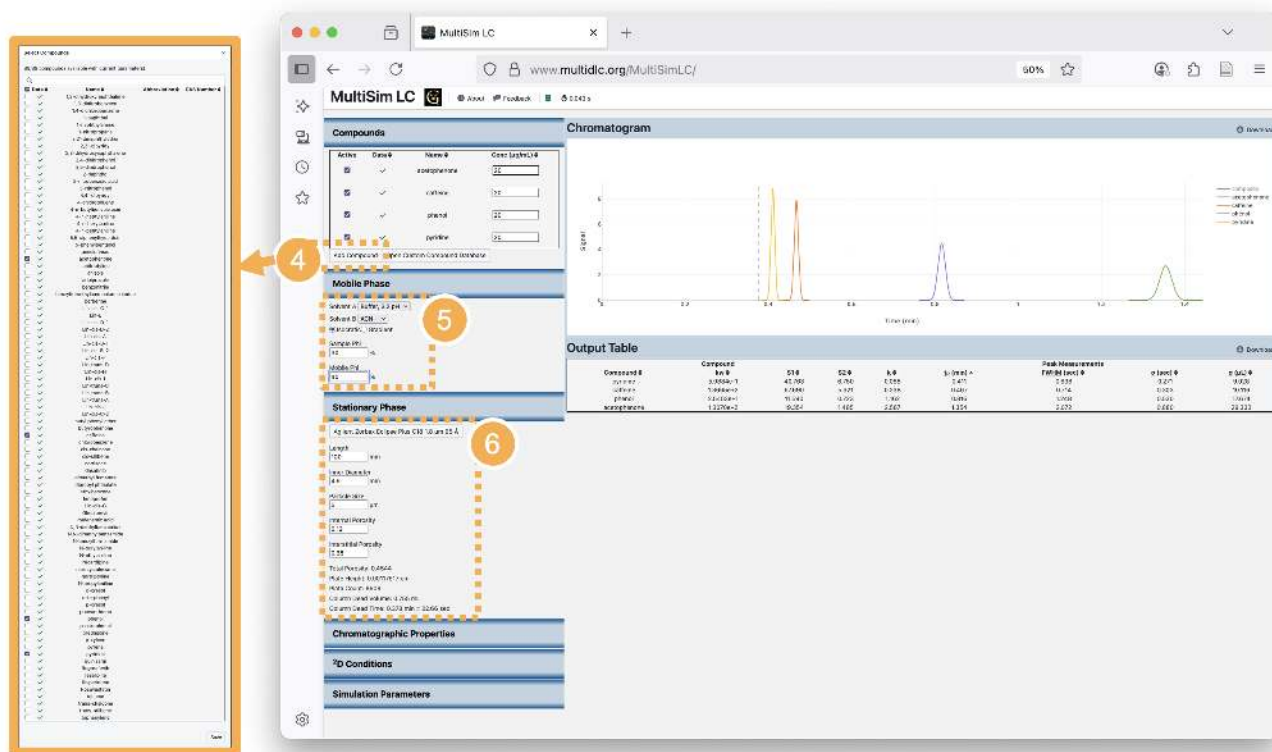


Figure 5.2.1: Copy and Paste Caption here. (Copyright; author via source)

- In the Mobile Phase options, you can adjust the type of method (isocratic and gradient); and you can adjust the mobile phase conditions.
 - Select ACN as Solvent B.
 - "Mobile Phi" is the percent of Solvent B in the mobile phase mixture.
- In the stationary phase tab, you can change the column parameters. For our applications, we will be using the Agilent Zorbax SB C18 column, with 100mm x 4.6mm dimensions with 5µm particle size and 0.12 internal porosity. The other parameters can be ignored for now.
- In the Chromatographic Properties tab, set the flow rate to 1mL/min and keep the rest of the parameters as they are.

Guidance for Exploring the HPLC Simulator

- Start with a Simple **Isocratic** method: Understand %B and Retention
 - Begin with an isocratic method in the Mobile Phase tab (constant %B).
 - Set %B to a low value (e.g., 10%) and observe: Are the peaks well separated? What happens to retention time as %B decreases?
 - Now increase %B (e.g., 50%, then 80%). How do the retention times change? Do peaks spread apart or crowd together?
- Introduce Gradients
 - Try a gradient that increases %B slowly over time (e.g., 10% → 40% over 5 min). How do retention times shift and how to peak shapes change compared to isocratic runs? Does it change whether peaks are co-eluting?
 - Compare a steep gradient (e.g., 10% → 90% over 5 min) with a shallow gradient (slow increase). Which gives better separation? Which is faster overall?

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5.3: HPLC Instruments at Duke

Duke Chem undergraduate teaching labs use Agilent 1000 series high performance liquid chromatographic systems. Important details of the components you will use are listed below in order of their position on the instrument (Figure 5.3.1) from top to bottom as you view the instrument.

- **MOBILE PHASE RESERVOIRS:** There are four 1-liter gas-tight glass solvent reservoir bottles labeled A1, A2, B1, and B2. Reservoir A1 contains deionized water. Reservoir B2 contains water with 10% acetonitrile. Reservoir B1 contains methanol, and reservoir A2 contains acetonitrile. All solvents have been filtered through 0.45 μ filters prior to introduction to the reservoirs. The solvents are degassed by a vacuum degasser (Agilent 1379B) that sits directly under the solvent reservoirs.
- **PUMP (AGILENT G1312A):** Our instrument uses a binary pump system. The Binary pump comprises two identical pumps integrated into one housing. Solvents from bottles A1 and A2 are assigned to one pump, while those from B1 and B2 are assigned to the other pump. Binary solvent mixtures are accomplished by proportional passage of solvent from each pump. The mixed solvent, or mobile phase, is then pumped through the column. Mobile phase changes can be accomplished easily and quickly, which is especially useful for re-equilibration following a gradient run.
- **AUTOMATED SAMPLE INJECTION SYSTEM (AGILENT G1367A):** This instrument uses a programmable and automated sample injection system. Samples for analysis are placed in 2 mL glass vials and sealed with a cap. The vials are placed at fixed locations in a 100-vial tray. A syringe system is used to withdraw a controlled volume of sample from the vial chosen for analysis. This sample is transferred to the sample loop of the injection valve. Rotation of the injection valve results in the sample loop being inserted in the flow path of the mobile phase just before it enters the head of the column. In this instrument, the sample volume is determined by the volume withdrawn by the needle up to a maximum of 100 μ L.
- **COLUMN:** The 10 cm x 4.6 mm i.d. stainless steel chromatographic column is packed with a homogeneous bed of uniform spherical silica gel particles which are 5 microns in diameter with a pore size of 120 Å. Some of the silanol (SiOH) groups on the surface of each particle have been derivatized with an eighteen-carbon alkane (C₁₈H₃₇) chain that gives the surface a certain degree of hydrophobic character. Octadecyl modified silica is one of the most common stationary phases used in reverse-phase LC. Chemical modification of the surface is preferred to coating by adsorption, because the latter surface may be removed by dissolution in the mobile phase. Separation in LC is achieved by means of differences in the interactions of the analytes with both the mobile and stationary phases. In this experiment, you will perform *reverse-phase LC* using a nonpolar stationary phase (C₁₈) and a polar mobile phase.
- **DIODE ARRAY DETECTOR (AGILENT G1315B):** Methods of detection used for HPLC include UV absorption, IR absorption, fluorescence, refractive index, electrical conductivity, mass spectrometry, electrochemical and radiochemical. Many modern LC instruments use diode array UV detectors. As well as the traditional two-dimensional presentation of detector signal against time, it is possible to display the UV spectra of the components giving rise to different chromatographic peaks. Differences in the spectrum between the front and tailing edges of a peak can be used to detect unresolved impurities. Such spectra can be captured in 'real time' without the need to interrupt the eluent flow. It is possible to construct pseudo-three-dimensional, or isometric plots, of absorbance, wavelength and time. (*Note:* This type of plot can be generated using the computer simulation used in your pre-lab assignment. You are encouraged to investigate this.) The Agilent instrument uses a diode array UV detector with a deuterium lamp source. All wavelengths in the range 190-950 nm are monitored simultaneously during elution. The detector can monitor an entire wavelength range or up to five discrete pre-selected wavelengths. To minimize the size of data files, complete spectrum scans are only saved when needed; typically monitoring at one or more select wavelengths is sufficient for most common analytical work. By careful selection of the wavelength(s) used for detection, the response of a particular analyte can be enhanced at the expense of possible interferences.
- **COMPUTER:** A desktop computer is interfaced to each LC module through an Ethernet interface. Instrument control, data acquisition and storage, and chromatographic peak integration are all initiated from the computer.

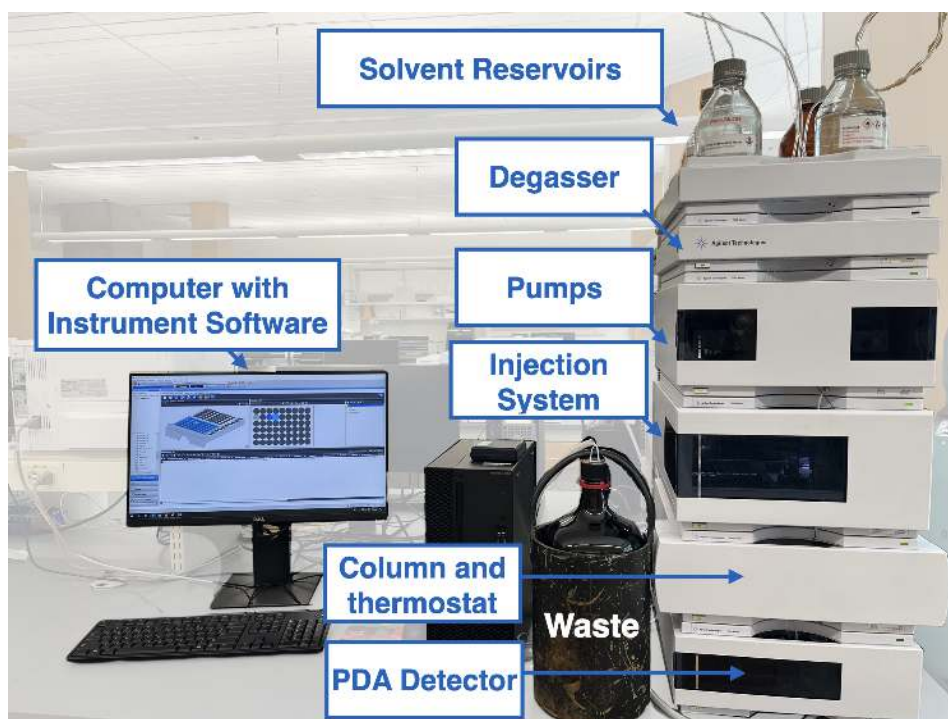


Figure 5.3.1: The Agilent HPLC instruments at Duke. Details of each component that you will use are listed in the text above.
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5.4: Ready the HPLC for Operation

1. Making sure the solvent reservoirs are full and the waste bottles have at least 1 Liter of volume available.
2. Make sure there is room in the waste container to accommodate about 2 L of waste solvent.
3. Launch Open Lab by clicking **HPLC (online)** (¶ [Figure 5.4.1](#)).



Figure 5.4.1: HPLC (online) desktop icon.

4. Use the Instrument Control Tab. Confirm that all components are green and indicate “ready.” (¶ [Figure 5.4.2](#))

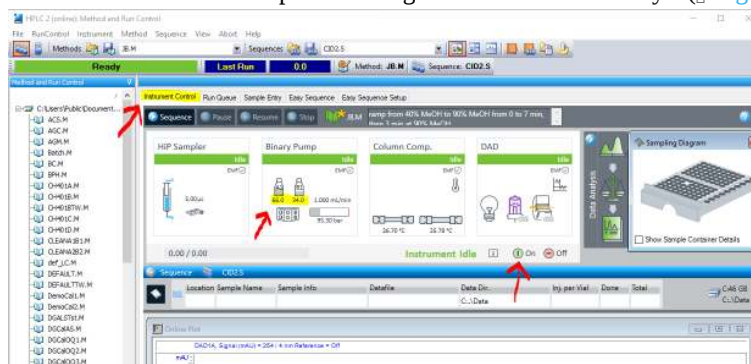


Figure 5.4.2: "Instrument Control" tab, "on" button, and solvent levels in the Binary Pump icon indicated by red arrows and yellow highlighting. The Ready Bar at the top left is green in this figure.

- a. If this is not the case, press the “On” button.
 - b. The DAD will take a few minutes to warm up; if there is a lightning bolt through the purple lamp, wait until it has gone away, and the ready bar has turned green.
 - c. Make sure that the solvent amounts listed under the Binary Pump module are accurate. Adjust them as necessary.
5. **Prime the pumps** to remove air bubbles from the lines (get your TA to help you with this).
 6. **Equilibrate the column for your first injection:** Begin pumping solvent through the column so that it is equilibrated prior to your first injection. If your first injection will use 100% methanol, pump 100% methanol through the column at a flow rate of 1 mL/min until the system is equilibrated.
Get your TA to help with this or follow these instructions:
 - a. Choose the **DEFAULT** method. You can do this by double clicking on **DEFAULT.M** in the **Method and Run Control Panel** (Left of instrument control screen) or by selecting **Method** from the top level menu, then **Load Method**, and selecting **DEFAULT.M**.
 - b. After the method is loaded, go to **Method --> Edit Entire Method**.
 - c. A box will appear in which you can select parts of the method to edit. Check only the Instrument/Acquisition option, and press OK. Then press OK in the next box also.
 - d. The Setup Method control box will appear. In Binary Pump Tab, you can choose the solvents and flow rate. So, if you wish to flow 100% methanol, you would choose solvent B1, and type 100 into the percentage of B1. The flow rate should be 1.00 mL/min.
 - e. After your solvent and flow rate are chosen, press apply, and the instrument should beginning to pump the solvent and flow rate you chose.

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5.5: Writing a Method for the Agilent 1100/1200 HPLC

A method is a list of steps that control the components of the instrument and create the chromatographic conditions required to perform an analysis. The instructions that follow are designed to set up a method for [Part 1 of the HPLC experiment](#).

Writing an isocratic method

1. Select **Load Method** from the Method menu, and then choose *default.M* from the list of methods.
2. Be sure to NOT to overwrite the default method. Immediately select **Save Method AS** from the **Method** menu. When the **Save Method As** box appears, type a name for the method file (eg *yourinitials_ic_40A60B.m*) in the text box. The name should indicate the ratios of A and B for the method you are about to create. Click on **OK** to save the method. There is no need to put anything in the **Comment for Method History** box.
3. You will now edit the default method. Select **Edit Entire Method** from the **Method** menu.
 - In the **Edit Method** box that appears (Figure 5.5.1), select all available options and then click on **OK** to continue. (Note: If you wish to discontinue editing at any time, select **CANCEL**.)

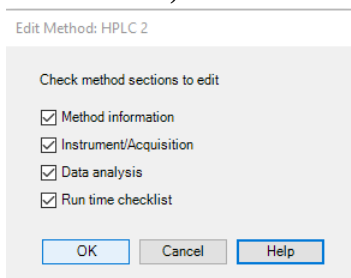


Figure 5.5.1: The Edit Method dialog box. Select all options and press OK.

2. The **Method Information** dialog box will appear. Move the cursor to the Method Comments text area and click on the left mouse button. Enter information about the method, for example: **60% METHANOL/40% WATER AT 1.000 ml/min**. Select **OK** to continue.
3. In the Injection Source/Location, accept the default, HipAls, which is the automatic sample injector. Press **OK**.
4. In the Setup Method Box, go to the **Binary Pump** tab (Figure 5.5.2). Check that the **Flow** is set to **1.000 ml/min**. This flow rate will be used during all subsequent experiments, unless specific contrary instructions are given.

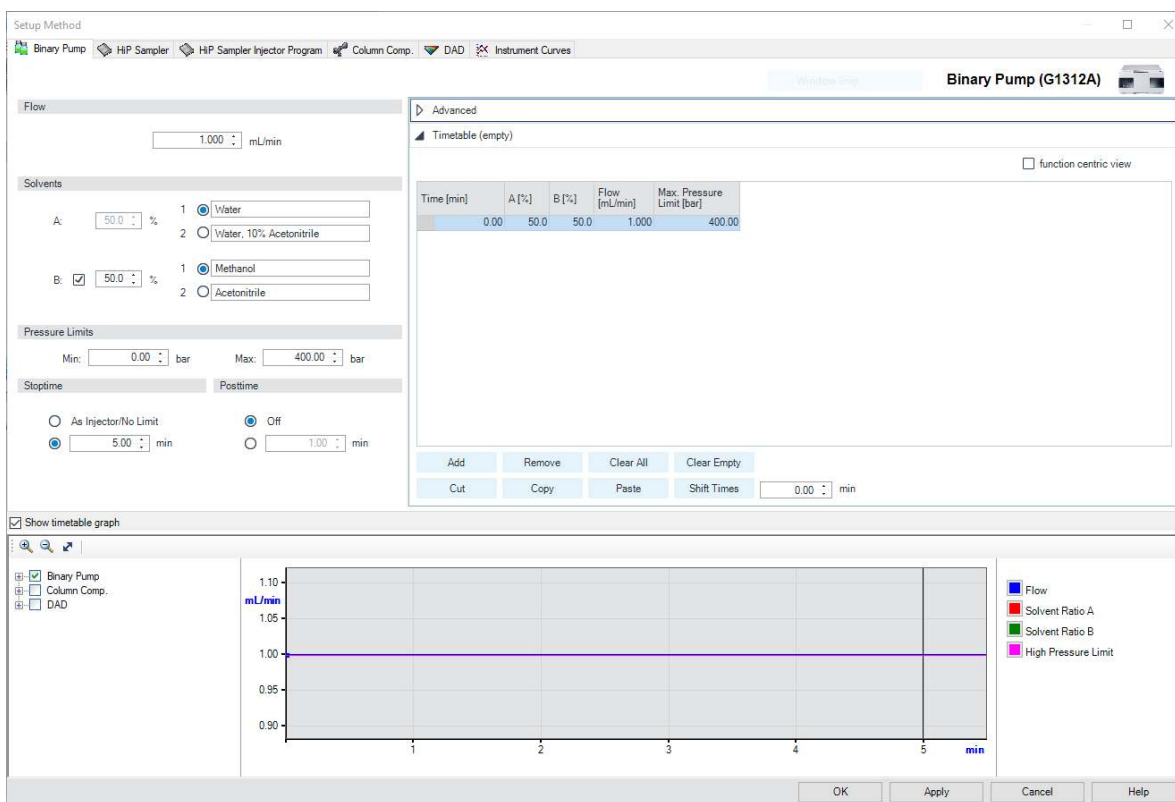


Figure 5.5.2: The Setup Method dialog box, on the Binary Pump Tab.

5. Also in the the **Binary Pump** tab check the following settings:

- **StopTime** (length of the analysis) should be **5 minutes**. The PostTime need not be used. (Figure 5.5.2)

- **Solvents** (mobile phase starting conditions): In this experiment, you will use solvents A1 (water) and B1 (methanol). Enter the appropriate solvent composition for your method, for example 50:50 A1:B1. (Figure 5.5.2)
 - **Pressure** limits: This setting protects the column and instrument from excessive pressure. The settings of **Min 0** and **Max 400** bar are appropriate. (Figure 5.5.2)
 - This will be an *isocratic* (constant mobile phase composition) analysis, and you do not need to modify the **Timetable** editor; the timetable is customized only for *gradient* runs. Delete any text that appears in the Timetable box other than the first line that sets initial conditions at time zero. (Figure 5.5.1).
6. Go to the **HiP Sampler** Tab (Figure 5.5.3). Check that the injection volume is **1.00 µl**. The other parameters should be left unchanged at the default values.

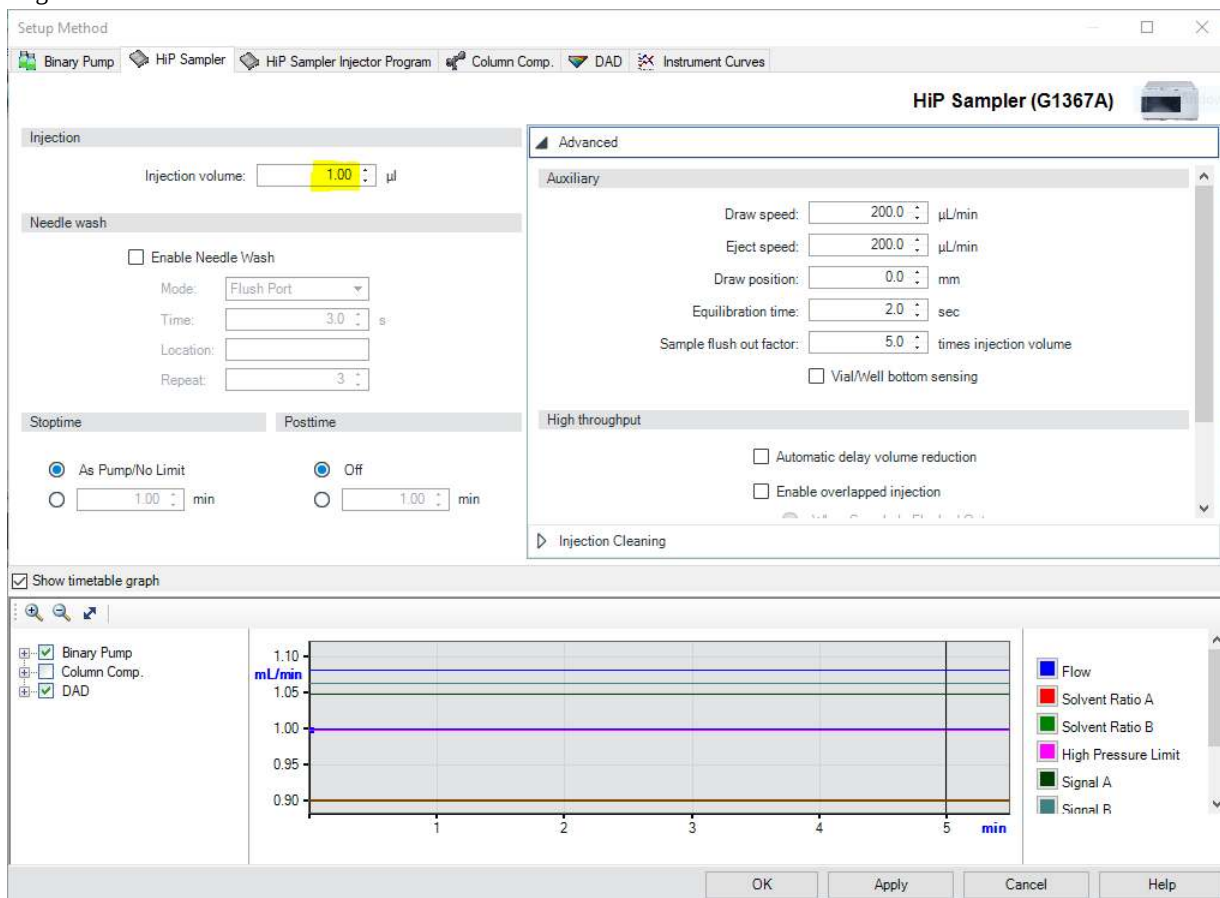


Figure 5.5.3: The Setup Method dialog box, on the HiP Sampler Tab.

7. Go to the **Column Components (Column Comp.)** tab (Figure 5.5.4). Select **Not Controlled** for both Left and Right temperature controls.

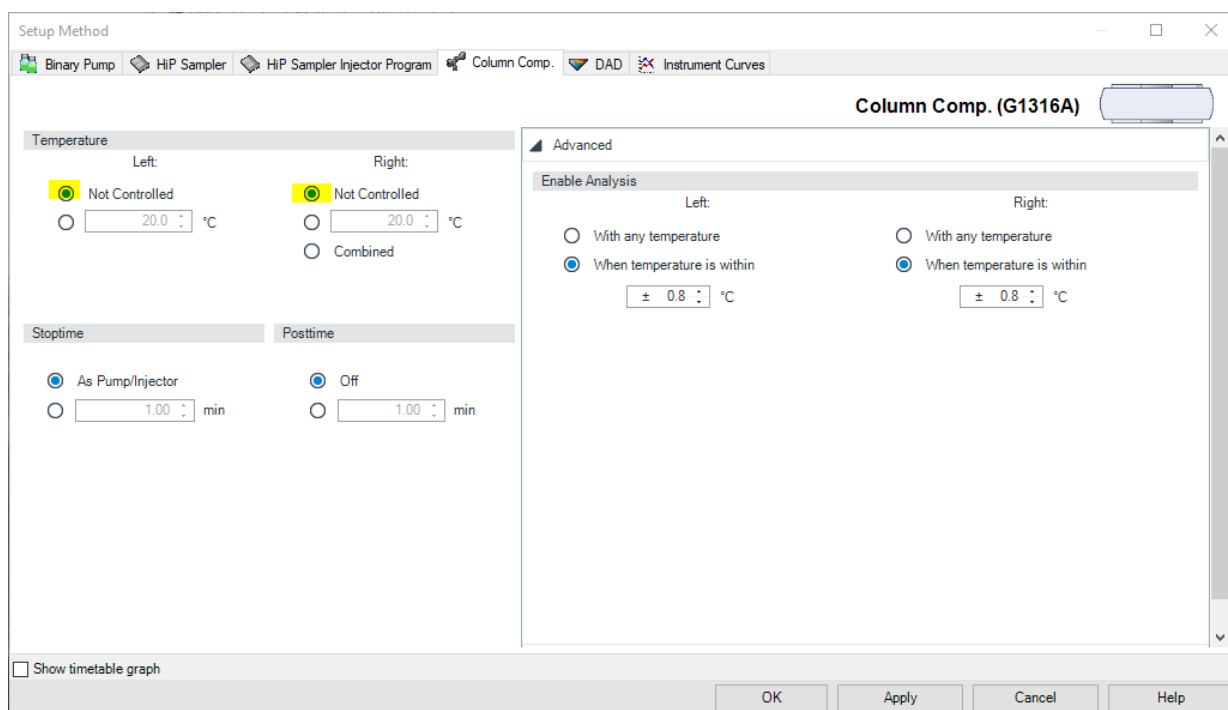


Figure 5.5.4: The Setup Method dialog box, on the Column Comp. Tab.

8. Go to the **Diode Array Detector (DAD)** tab (Figure 5.5.5). The DAD detector is used to monitor absorption of UV-vis light as analytes elute from the column.

- Set Signal A, B, and C to 254, 280 and 310 nm respectively.
- Check the box under "Acquire" to activate each signal, and enter the appropriate wavelength in the box.
- The default bandwidths of 4 nm need not be changed.
- All reference wavelengths should be set to **OFF** by leaving the Reference Wavelength box unchecked.
- All other parameters may be left at the default values.

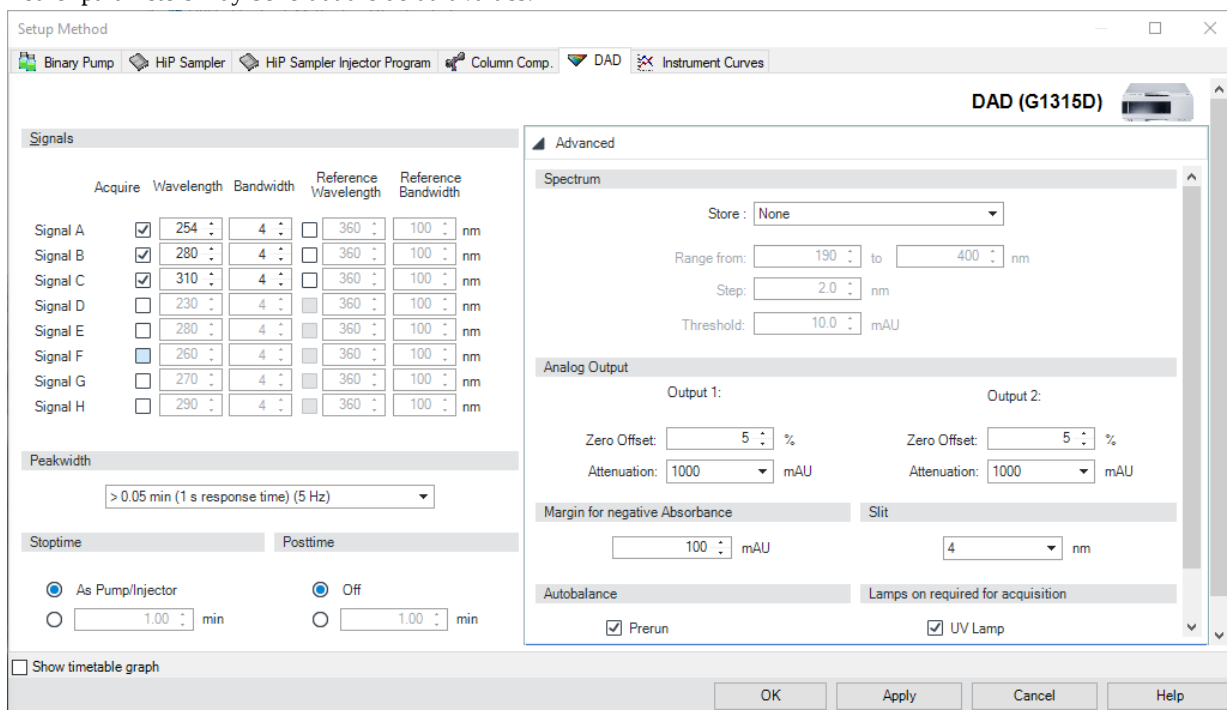


Figure Figure 5.5.5:
The Setup Method dialog box, on the DAD Tab.

9. Click **OK** at the bottom of the **Setup Method** box.
10. The Signal Details dialog box will appear. Delete any unnecessary rows. Select the signals you plan to use for qualitative analysis from the Available Signals and press the **Add to Method** button to add each of the available signals to the method. Then Press **OK**. (Figure 5.5.6)

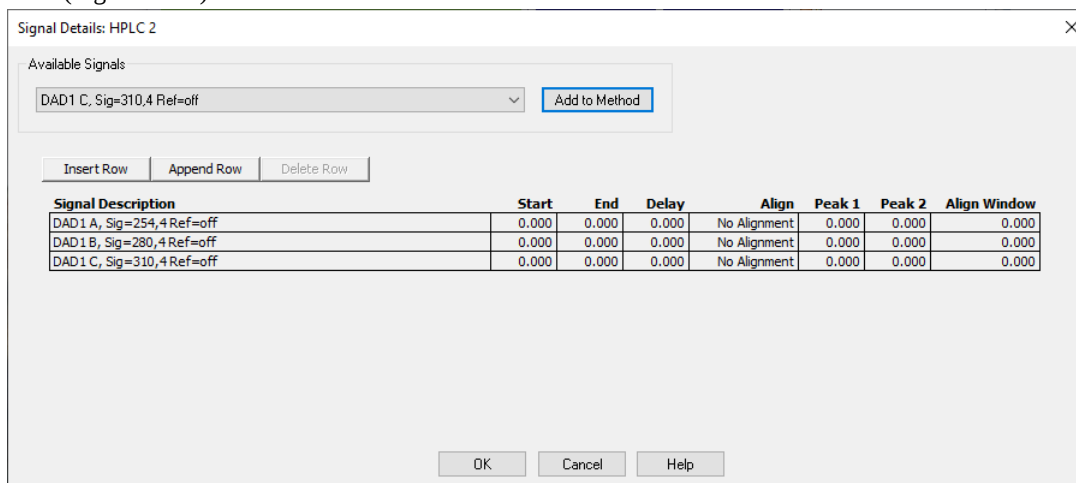


Figure 5.5.6: Signal Details box.

11. The **Integration Events** dialog box allows you to control how a chromatogram is integrated. Click on the icon with a green check mark. Then, make sure the values match those shown here. Figure 5.5.7

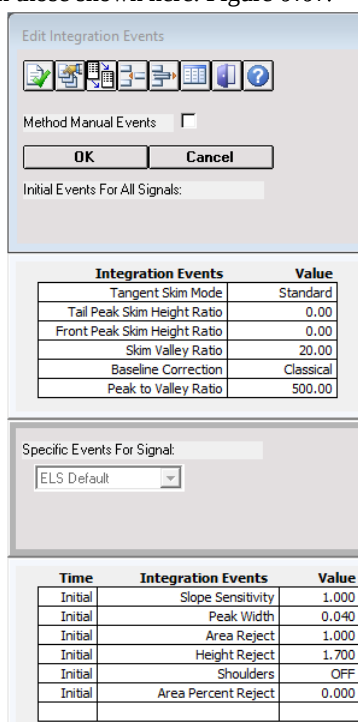
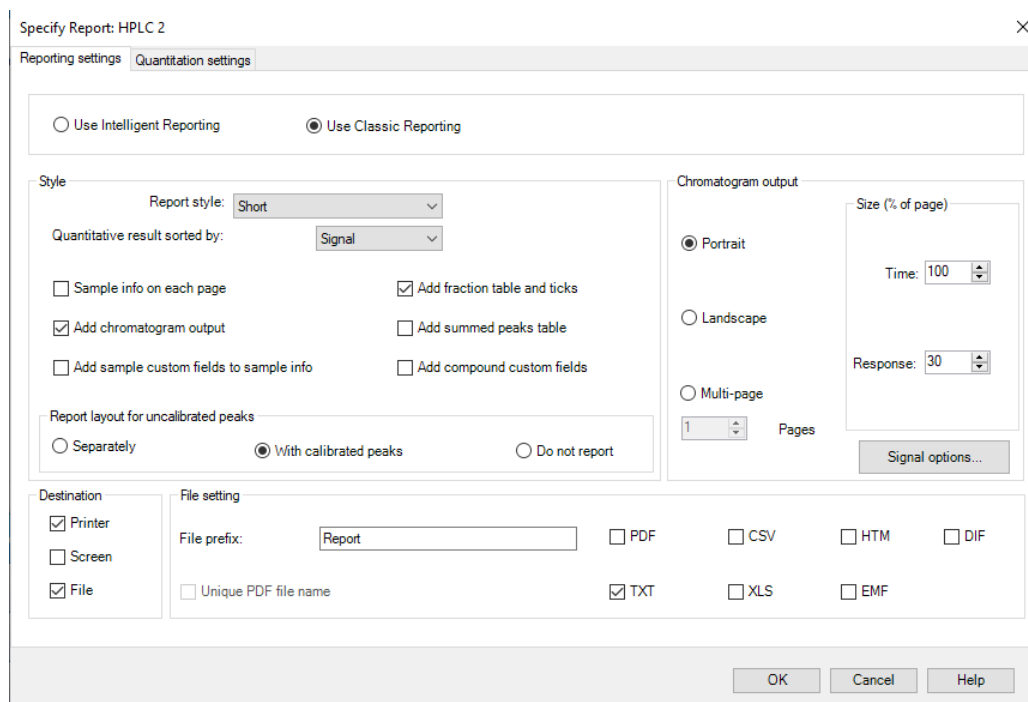


Figure 5.5.7: Integration Events box.

12. The Specify Report dialog box allows you to specify how the results should be reported. You will generate, and print, a chromatogram and an Area Percent report with calculations based on area and sorted by retention time. The raw data will be stored in a file and sent to the printer. Click **OK** to continue. Figure 5.5.8



Specify Report: HPLC 2

Reporting settings Quantitation settings

☐ Use Intelligent Reporting ☒ Use Classic Reporting

Style
Report style: Short
Quantitative result sorted by: Signal

☐ Sample info on each page ☒ Add fraction table and ticks
☒ Add chromatogram output ☐ Add summed peaks table
☐ Add sample custom fields to sample info ☐ Add compound custom fields

Report layout for uncalibrated peaks
☐ Separately ☒ With calibrated peaks ☐ Do not report

Chromatogram output
☒ Portrait ☐ Landscape ☐ Multi-page
 Size (% of page)
 Time: 100
 Response: 30
 Pages: 1
 Signal options...

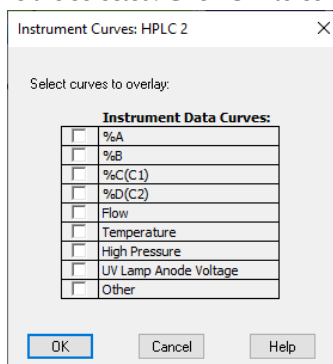
Destination
☒ Printer ☐ Screen ☒ File

File setting
 File prefix: Report
☐ Unique PDF file name
☐ PDF ☐ CSV ☐ HTM ☐ DIF
☒ TXT ☐ XLS ☐ EMF

OK Cancel Help

Figure 5.5.8: Specify Report box.

13. In the **Instrument Curves** dialog box, no options are selected. Click **OK** to continue.



Instrument Curves: HPLC 2

Select curves to overlay:

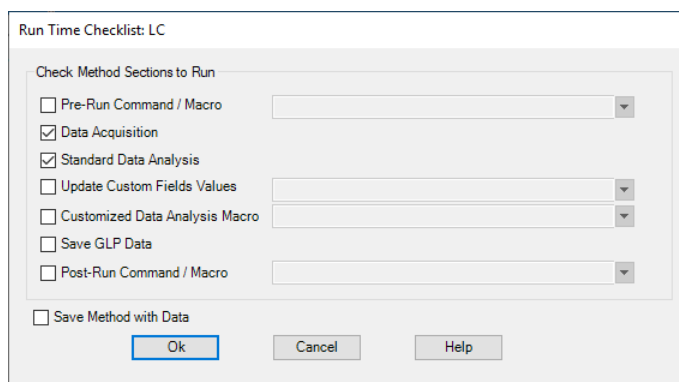
Instrument Data Curves:

☐	%A
☐	%B
☐	%C(C1)
☐	%D(C2)
☐	Flow
☐	Temperature
☐	High Pressure
☐	UV Lamp Anode Voltage
☐	Other

OK Cancel Help

Figure 5.5.9: Instrument Curves box.

14. Select only the **Data Acquisition** and **Standard Data Analysis** method sections in the **Run Time Checklist** dialog box and select **OK** to continue.



Run Time Checklist: LC

Check Method Sections to Run

☐	Pre-Run Command / Macro	
☒	Data Acquisition	
☒	Standard Data Analysis	
☐	Update Custom Fields Values	
☐	Customized Data Analysis Macro	
☐	Save GLP Data	
☐	Post-Run Command / Macro	

☐ Save Method with Data

Ok Cancel Help

Figure 5.5.10: Run Time Checklist box

15. Select **Save Method** from the **Method** menu. There is no need to put anything in the **Comment for Method History** box.

***TIP*:** When writing multiple methods with similar settings, you can save time by re-using a method you've already built instead of from the default.m method. (eg Go back to step 2). First, be absolutely sure the first method has all the correct settings before saving as a new method: Double check your work, then ask your TA to check it before proceeding.

Writing a gradient method

To write a gradient method, you can follow all the same steps as described above for an isocratic method; however, you would adjust the **Timetable** options in the **Binary Pump** tab of the *Edit Method* dialog box. When writing a gradient method, these are the recommended steps for the timetable:

1. Start conditions at a high fraction of the weak solvent (A, water). 97% is a good place to start - you will need to adjust this in the solvents options at the right of the **Binary Pump** tab, and you should see it reflected in the Timetable at the left.
2. Below the Timetable, press Add to add a line. The second line in the timetable should be a *pre-gradient elution step* - flow one column volume (void volume) of solvent at the starting condition through the column. This ensures that the sample is loaded onto the column under the starting conditions. The void volume of the column depends on the column diameter, length, and particle size.
3. Add a third line. The third line should result in a *Solvent Gradient* - Enter the fraction of B that you want to achieve at the end of the gradient and the time the gradient should take. A typical gradient is to start at 97%A/3%B and end at 3%A/97%B. The time of the gradient depends on the size of the column and the flow rate: we typically use a 4-minute gradient (so this should last until 5 min of the timetable).
4. Add another line for a post-gradient elution - flow one column volume of the strong solvent condition through the column.
5. Add another two lines for a post-gradient equilibration - this step sets you up for the next run. It is optional to build it into your method, and it only makes sense if the next injection will occur under the same starting conditions as this run. Return the condition to the starting solvent composition (weak solvent) and run for at least 5, and ideally 10 column volumes.
6. Make sure to edit the **stop time** under the Binary Pump tab in Setup Method window to match the duration of the run. (i.e. 11 min in Figure 5.5.11).

An example of a gradient table is shown below in Figure 5.5.11, where the option to Shot timetable graph is also selected.

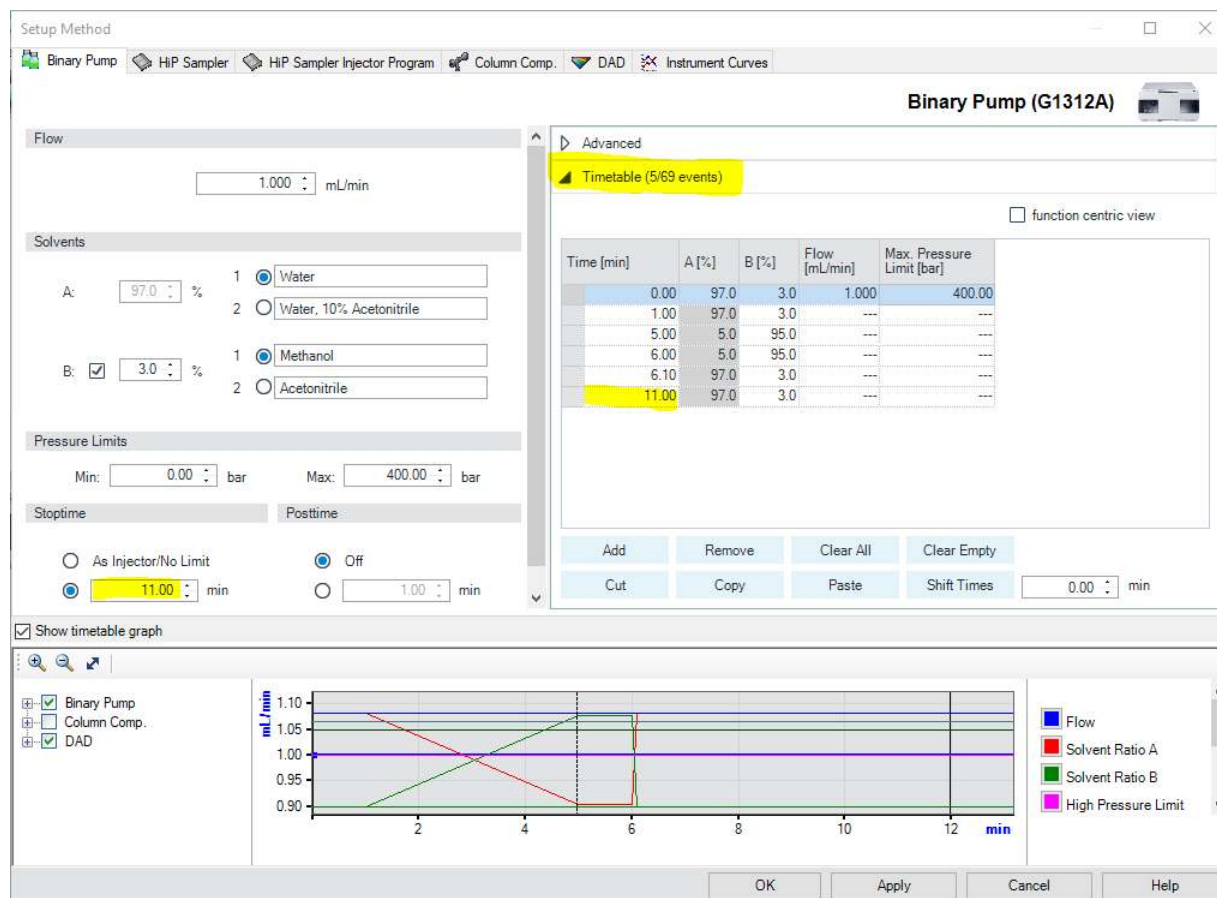


Figure 5.5.11: Setup Method box with an example of a gradient timetable.

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5.6: Set up a sequence for the Agilent 1100/1200 HPLC

A sequence tells the instrument which samples to analyze using which methods. Once the sequence is programmed and initiated, the user need not be present to monitor the analysis. If desired, the sequence can include steps for cleaning the system and shutting it down.

Before you begin, you must place the sample vials in the autosampler tray in the appropriate locations. The most critical part of this step is that you know the position of each sample, and that you enter the correct location in the sequence table. A map of the sample trays is below. There are two trays in the instrument. The front tray is P1 (tray position 1), while the back tray is P2 (tray position 2). Each tray has nine columns, labeled 1-9, and six rows, labeled A-F. You must know the position of your sample(s) so that you can correctly direct the injector robot to the correct sample location. For example, the position of the red checkered space in Figure 5.6.11 is P1-B2.

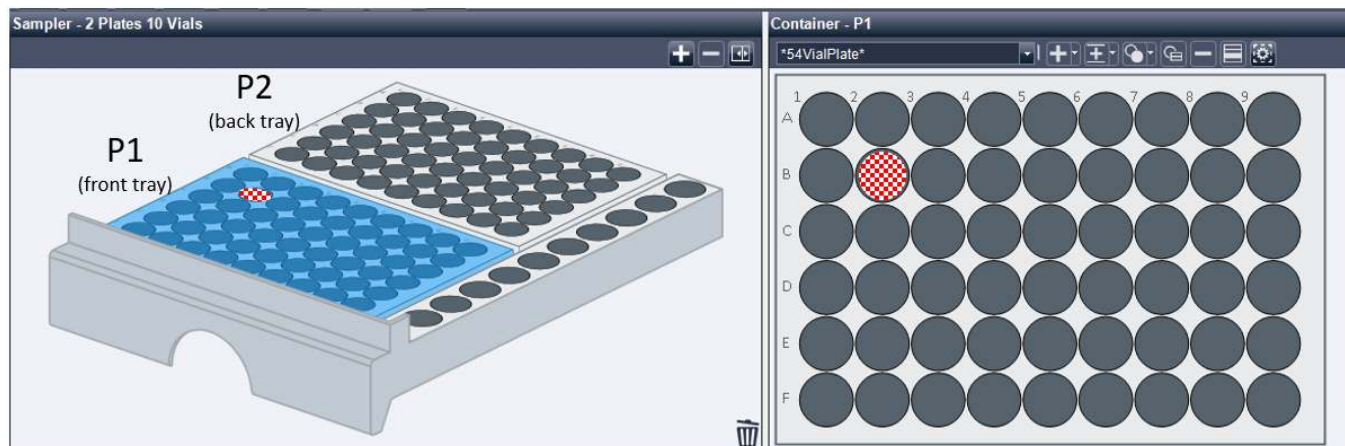


Figure 5.6.11: Map of the two sample trays. Left: The front is tray position P1, while the back is tray position P2. Right: Each tray holds 54 samples; rows are labeled A-F and columns 1-9. On the left, the front rack is highlighted in blue, and a map of its sample positions is shown on the right. The position of the red checkered circle shown in both the left and right diagrams is P1-B2. (Duke Chem)

Quick Start:

1. Go to the **Sequence** menu in the top tool bar and select **Load Sequence Template...**

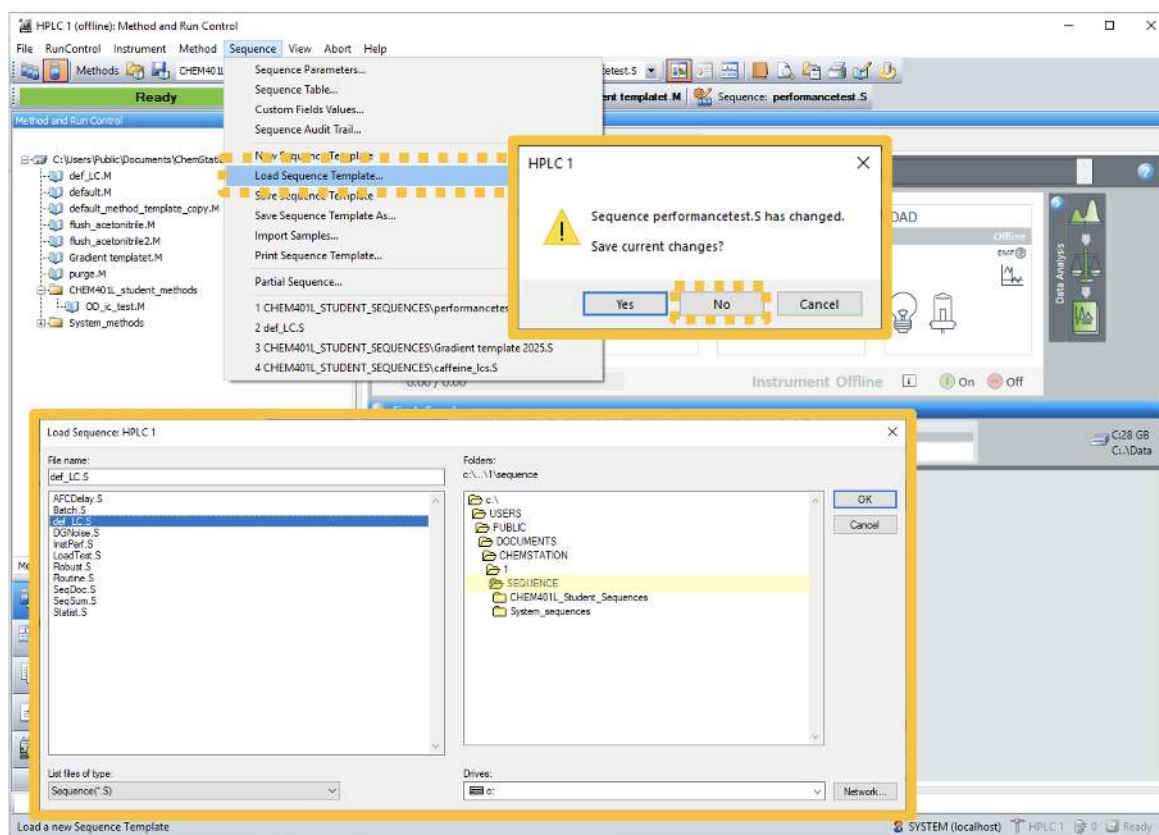


Figure 5.6.2: Copy and Paste Caption here. (Copyright; author via source)

- The software will prompt you to save changes to the current sequence file - select **NO** so that you do not save over another person's work.
- The Load Sequence window will appear
- Navigate to the main sequence folder

- Select the default sequence template called "def_LC.S"
2. Do not overwrite the default sequence! Go to **Sequence** in the top tool bar and select **Save Sequence Template As...**

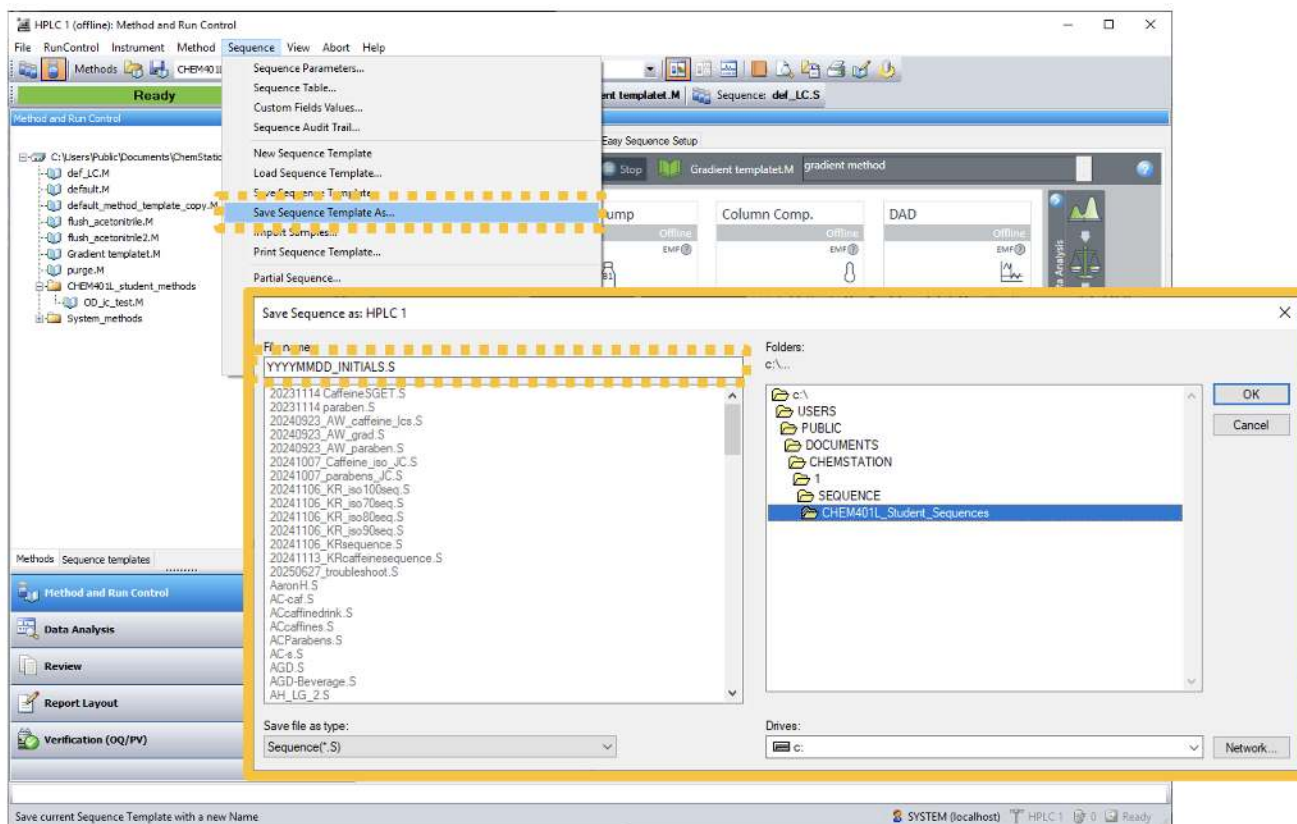


Figure 5.6.3: Copy and Paste Caption here. (Copyright; author via source)

- Use the convention YYYYMMDD_INITIALS to name your sequence; if this is a group sequence use the group name.
3. View and edit the sequence file by going to the Sequence menu at the top toolbar and selecting **Sequence Table...**

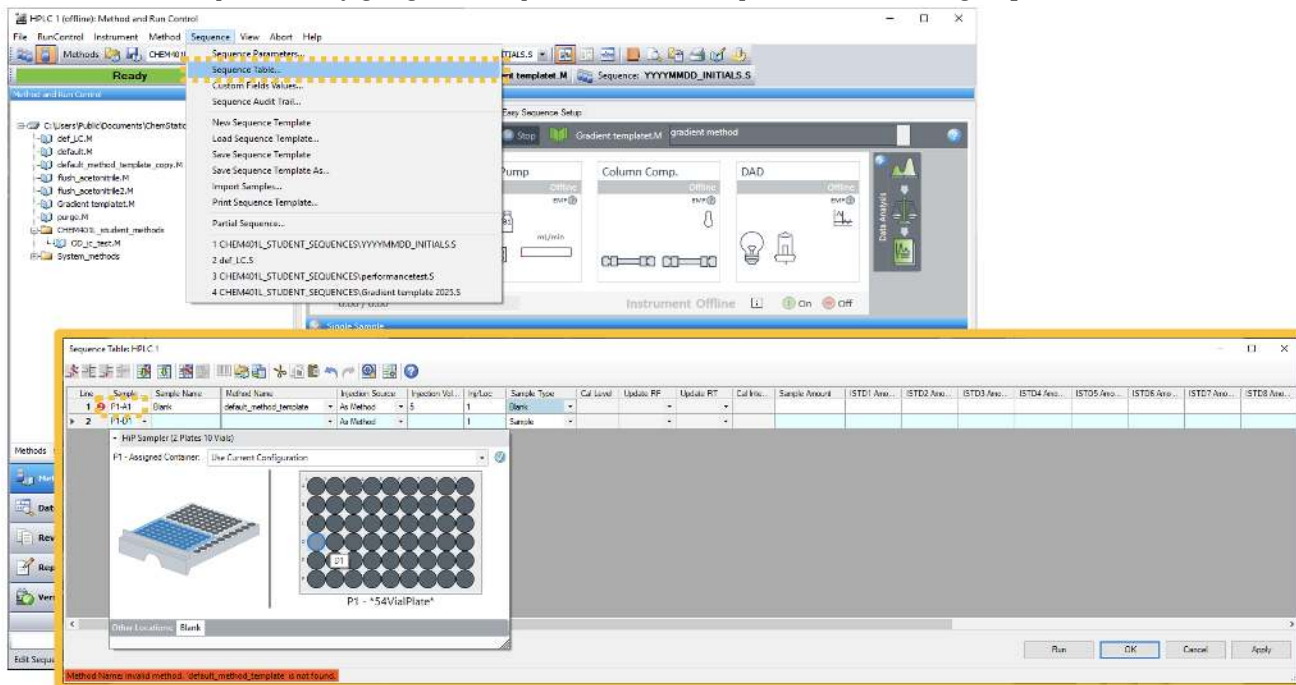


Figure 5.6.1: Copy and Paste Caption here. (Copyright; author via source)

- The sample tray diagram will open when you click the drop down arrow in the Sample Position cell.

Programming the first injection (or a single injection)

The following text describes how to create a sequence of methods for a single injection or the first injection in a series.

Note

- Make sure that the vials containing the samples to be analyzed are placed in the rack, and that their locations are known.
- Make sure the column is equilibrated before you run your sample! Consider running an equilibration method with a blank injection to accomplish this if you haven't equilibrated manually.

1. Select **Load Sequence Template** from the Sequence menu, and then choose a **default** template the list of sequences. Click **OK**.
2. Select **Save Sequence As** from the **Sequence** menu. When the **Save Sequence As** box appears, type **YYYYMMDD_ourinitials** in the text box. Click on **OK** to save the sequence.
3. You will now edit the sequence. Select **Sequence Table** from the Sequence menu. Select (highlight) the first line in the sequence table.
4. Enter the following information: **NOTE:** If there are any extra rows of information, delete them by clicking on the row # (in gray), which will highlight the row, and then right-clicking and choose *cut*.
 - **Location:** The tray location (eg. P1-B3) of the vial to be analyzed.
 - **Sample Name:** An area to enter comments (such as the composition of the mixture to be analyzed) that will appear on the report as Sample Comments.
 - **Method Name:** Enter the method you set up previously to run the sample use (*yourinitials*).
 - **Injection Vol.:** This is the volume of sample to be injected. If you do not enter a value, it will resort to the value programed in the method. Typically 1 uL is sufficient for the paraben mix. For caffeine, we typically use 5 uL injections.
*Please note that it is most convenient to use the same injection volume for calibration and sample injections. if you use different injection volumes for calibration and for sample injections, you will need to account for this in data analysis! In either case, take note of your injection volumes!
 - **Inj/Location:** Enter 1. This entry indicates how many injections (runs) should be made from the vial.
 - **Sample Type:** Choose the type of sample (optional)
5. When the table is complete, click **OK** to exit from the Sequence Table and return to the Top Level Menu. As long as you have already Saved using a new sequence name (step 2 above), select **Save Sequence** from the **Sequence** menu.
6. To run this single method sequence, proceed to the Run Analysis Section.

Programming additional injections

The following text describes how to create a sequence of methods for multiple injections.

1. Follow the first five steps given above as you would for a single injection. It is good practice to always begin a series of injections by running at least one "blank" injection to start with.
2. To run a second sample, using the same or a different method, click on the **Append Line** box at the bottom of the screen (there are also shortcut buttons at the top of the screen). This will insert a line after the first line and before the last line marker.
 - If you plan to add several sample injections using the same method, highlight the last line number and use the **Insert/Fill Down** button. Select **Increment** in the **Locations** box. Add **From Location** (eg P1-A3) and **To Location** (eg P1-B2) values. Add a **Method Name** (e.g. *default.M*); **Inj/Location** value (eg 1); and, **Sample Type** (eg Paraben Mixture). You will have to go back and enter the names of the samples in each line after the lines have been created.
3. After entering the sample names in the newly-created sequence lines you can select that range and use Copy and Paste to create new lines for each method. Remember to change the Method Name for each new method.

Important Tip

If you change the mobile phase composition between two injections, the column must be equilibrated with the new solvent conditions prior to the second injection. This can be accomplished by running one or two "blank" injections with the new method or with a custom "equilibration" method.

4. Clean and Flush the column at the end of each sequence:
 - After your samples have created a list of runs, add a final "blank" injection to be sure the column is clean.
 - Finally, the last line of your sequence table should flush the aqueous solvent out of the column and leave it in a high percentage of strong solvent. Use the **flush.M method** and a blank injection. This method flushes the column with 100% acetonitrile at a low flow rate of 0.10 ml/min for 1 hour.
5. When the entire table is complete, select **OK** to exit from the Sample Log Table and return to the Top Level menu. Select **Save Sequence As** from the **Sequence** menu. When the **Save Sequence As** box appears, type *yourinitials_yymmdd_#* in the text box. Click on **OK** to save the sequence.

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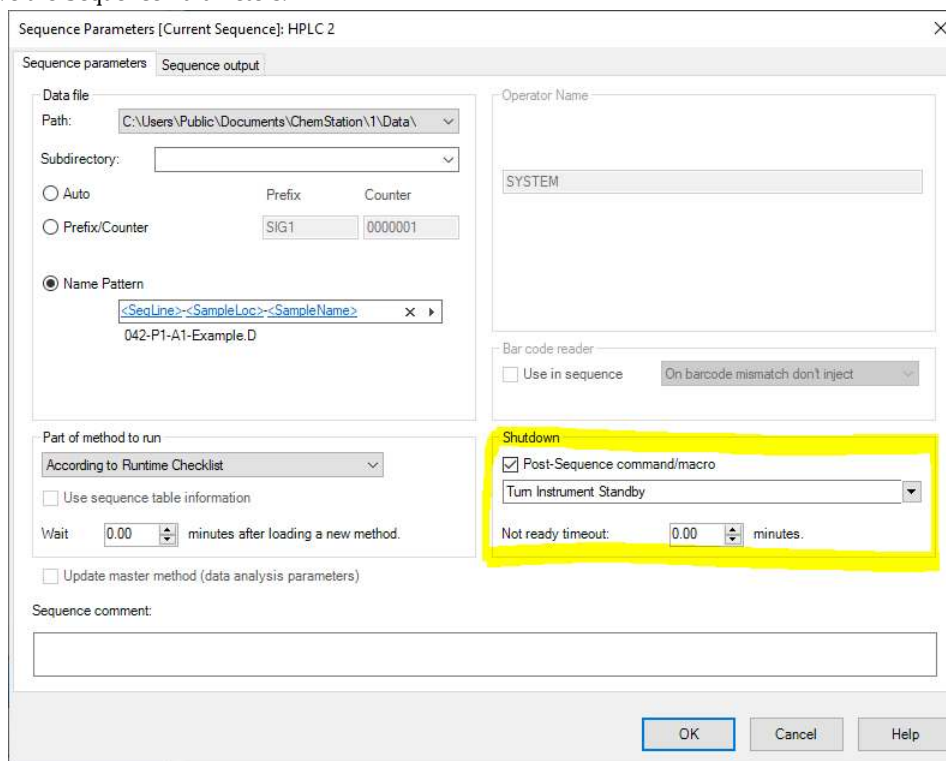
5.7: Running a Sequence

1. In the event that the pump and detector are off, select **System On** from the **Instrument** menu. The pump will begin to operate and the detector lamp will light.

Caution

- Make sure that all solvent reservoirs (top of instrument) are at least half full. If not, ask your instructors to show you how to refill the bottles.
- Make sure the waste bottle has space for at least 1 L of waste. If not, ask your instructors to remove the excess waste.
- Make sure that the waste solvent lines from the instrument are inserted all the way to the bottom of the 4L brown waste bottle on the floor.

2. Allow the column to equilibrate to the first mobile phase (eg 60/40 methanol/water) before beginning the analysis. When a method is first loaded, the method parameters are immediately sent to the instrument. You can consider the system equilibrated when the column pressure stabilizes. Consult your TA.
3. Within several minutes, a flat baseline should be achieved in the online plot window. Even using the most viscous mobile phase (50/50 acetonitrile/water), the operating pressure (displayed on the pump module and in the pump window on the screen) should not exceed 150 bar. **If you have any difficulties achieving a flat baseline or, if the pressure exceeds 150 bar - consult your TA immediately.** If flow is erratic or air bubbles are visible in the Teflon lines, your TA will assist you in re-purging the system.
4. Set the post-run sequence command: Go to **Sequence --> Sequence Parameters**. Find the **Shutdown** options and choose the most appropriate option for post-run macros.
 - If you are submitting just a few injections during the lab period, and you will be present at the end of the sequence, deselect (uncheck) the box for *Post-sequence command/macro*.
 - If you are running a longer sequence to run (eg overnight), please add a checkmark to *Post-sequence command/macro* and select *Turn Instrument Standby in the drop down menu* (See Figure 5.7.1).
 - Press OK to save the Sequence Parameters.



Sequence Parameters [Current Sequence]: HPLC 2

Sequence parameters | Sequence output

Data file
Path: C:\Users\Public\Documents\ChemStation\1\Data\
Subdirectory:
☐ Auto Prefix Counter
☐ Prefix/Counter SIG1 0000001
☒ Name Pattern
 <SeqLine><SampleLoc><SampleName>
 042-P1-A1-Example.D

Part of method to run
According to Runtime Checklist
☐ Use sequence table information
 Wait 0.00 minutes after loading a new method.
☐ Update master method (data analysis parameters)

Sequence comment:

Operator Name
SYSTEM

Barcode reader
☐ Use in sequence On barcode mismatch don't inject

Shutdown
☒ Post-Sequence command/macro
 Turn Instrument Standby
 Not ready timeout: 0.00 minutes

OK Cancel Help

Figure 5.7.1: Copy and Paste Caption here. (Copyright; author via source)

5. Go to Sequence --> Save Sequence Template As and give your sequence template a file name.
6. To begin the sequence, go to the Instrument Control Tab and press the button with the "play" icon (triangle inside a blue circle).
See Figure 5.7.2. Your sequence will begin - watch the sample injection system to ensure the first sample is injected.

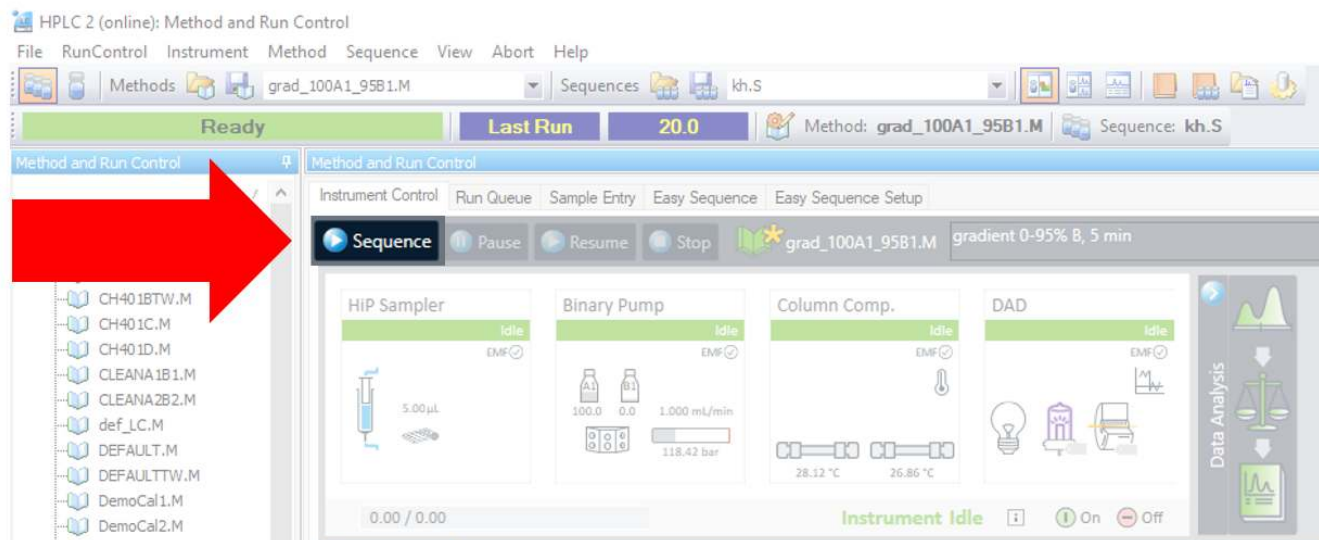


Figure 5.7.2: Copy and Paste Caption here. (Copyright; author via source)

An alternative option for starting a sequence is to use the **Run Control** menu from the top level toolbar, and then Select **Run Sequence**.

5. The chromatogram in progress will be displayed in the upper left box on the screen. You may change the attenuation of the signal and the time scale by clicking on the up and down arrows on the left side of the signal window. Remember this is done only for your viewing convenience. The final chromatographic output will be automatically scaled.
6. When each analysis is complete, the output will be sent to the screen, a file, and/or the printer as specified in your method. The text report of peak retention times and integrated areas will also be output as specified in the method.

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5.8: Data Analysis

Open the **offline** version of the ChemStation OpenLabs software to perform data analysis. Then follow the instructions below.

Open your data

1. In the Bottom Left-Hand side of the software window, go to the **Data Analysis Tab** (Figure 5.8.1).
2. In the Data Analysis window (Top Left), find and **select your data file** (Figure 5.8.1).

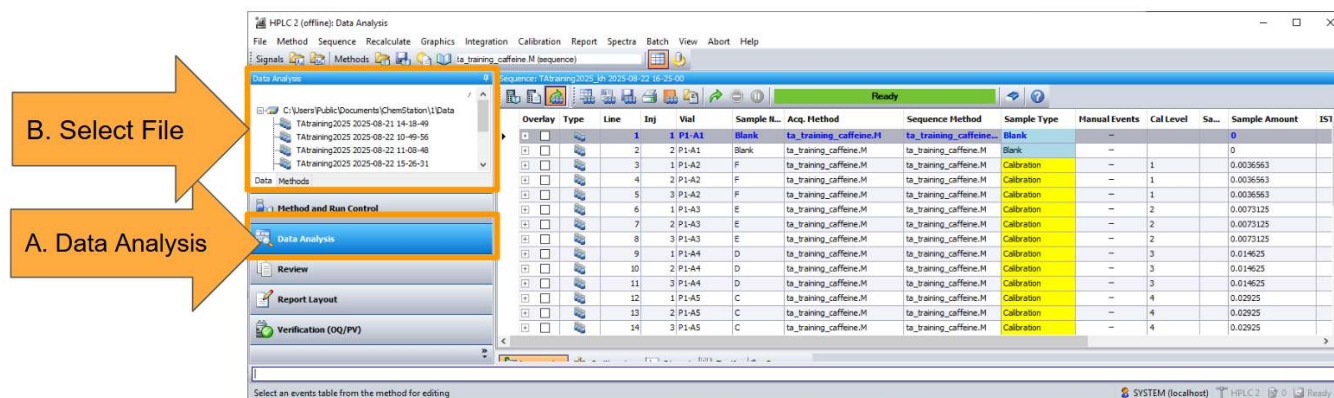


Figure 5.8.1: Copy and Paste Caption here. (Copyright; author via source)

Capture and Export Chromatograms

Capture a single chromatogram

- A. Go to the sequence table and select a sample for which you would like to show its chromatogram. Double click on the line to select it, then its chromatogram should appear in the window below the sample table (Figure 5.8.2).
- B. Show the Graphics Toolbar (Graph toolbar) by clicking the little button at the top of the chromatogram that looks like a paint palette with a "T" on it (🎨). This should open the graphics toolbar on the right side of the chromatogram window (Figure 5.8.2).
- C. To capture the chromatogram, click the "Copy Window to Clipboard" button (📄), (Figure 5.8.2). Then paste the captured image into a document (go to the CHEM401L Data Drive).

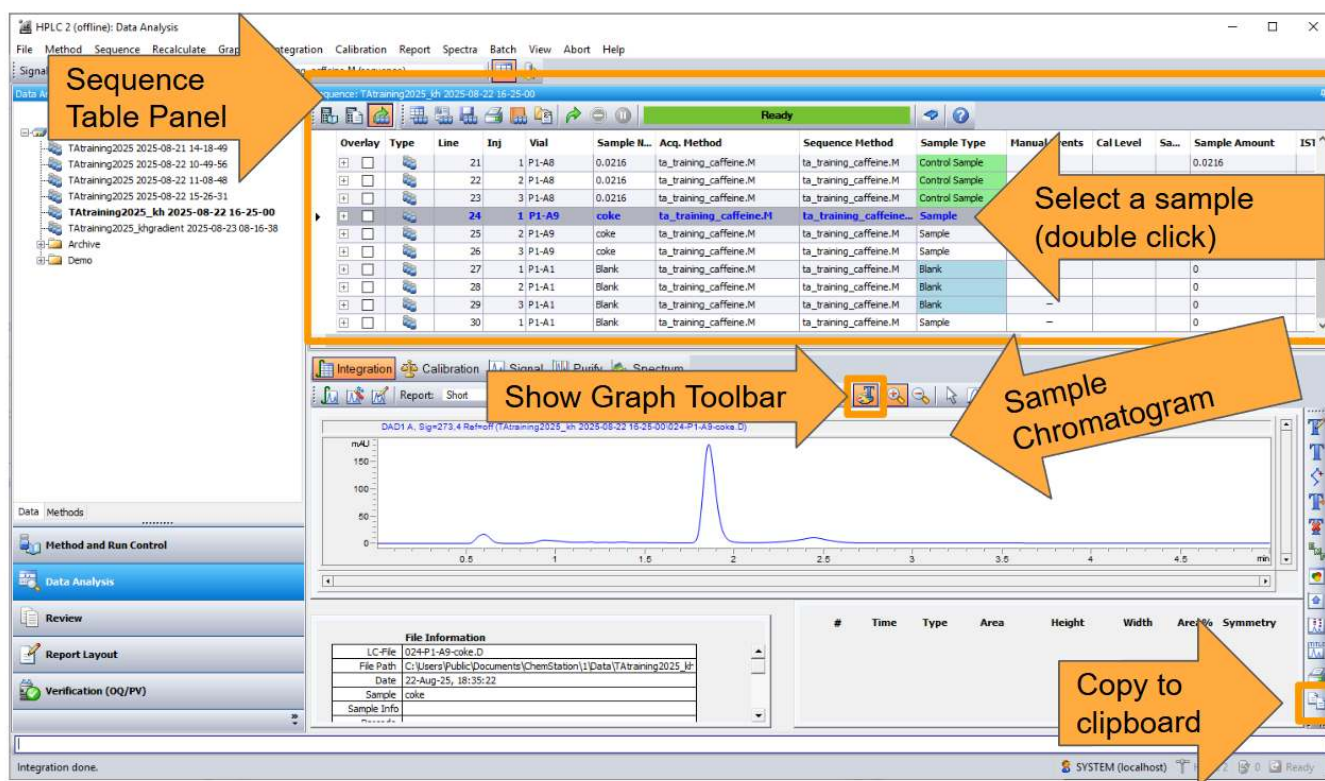


Figure 5.8.2: Copy and Paste Caption here. (Copyright; author via source)

Capture overlaid or stacked chromatograms

The instructions below show how to show multiple chromatograms stacked or overlaid:

- In the sequence table, check the "Overlay" box for each sample that you would like to overlay.
- Then, double click on the line for one of the selected injections. The chromatograms of all selected injections should appear stacked in the chromatogram window.

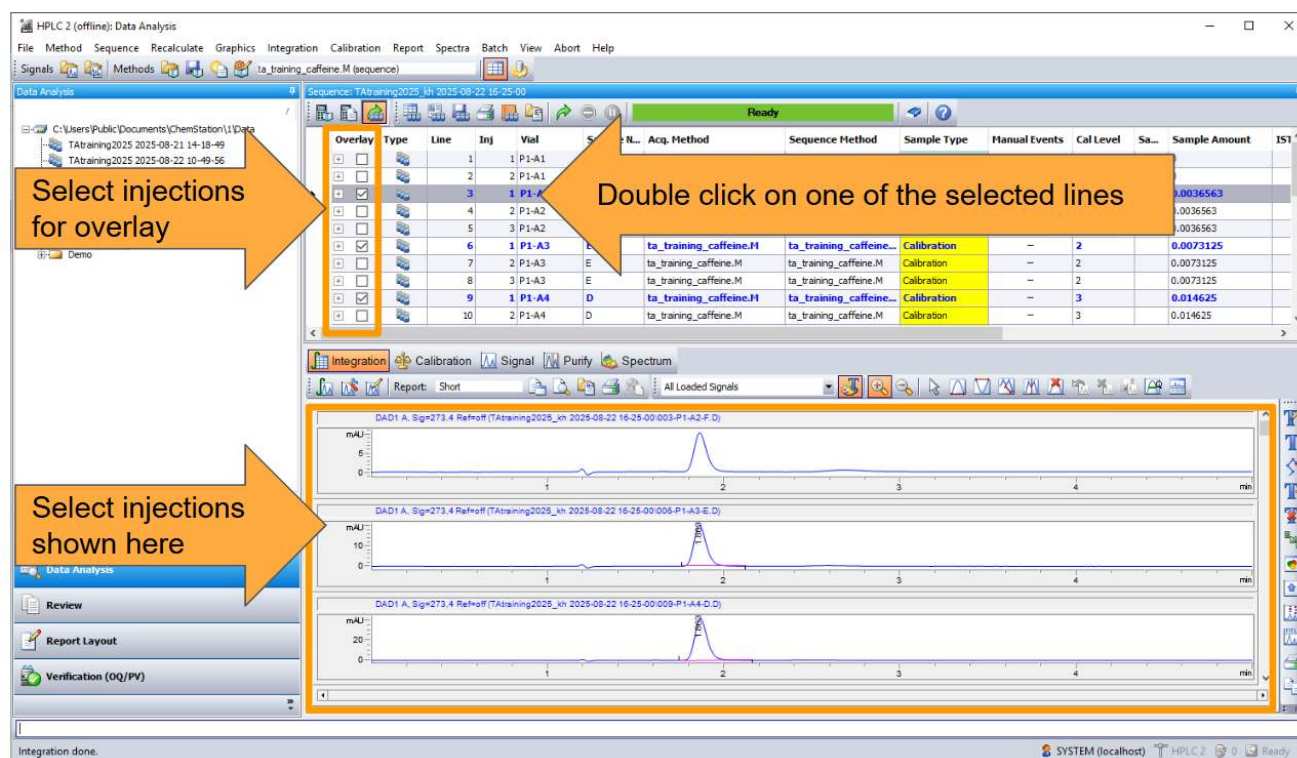


Figure 5.8.3: Copy and Paste Caption here. (Copyright; author via source)

C. To overlay them on the same set of axes:

- Show the Graphics Toolbar by clicking the little button at the top of the chromatogram that looks like a paint palette with a "T" on it (). This should open the graphics toolbar on the right side of the chromatogram window.
- To overlay the stacked chromatograms, click the button that looks like three colored circles ().
- To put the chromatograms on the same scale, click the button that looks like an up arrow ().

D. To capture the chromatogram view, click the "Copy Window to Clipboard" button (). Then paste the captured image into a document (go to the CHEM401L Data Drive).

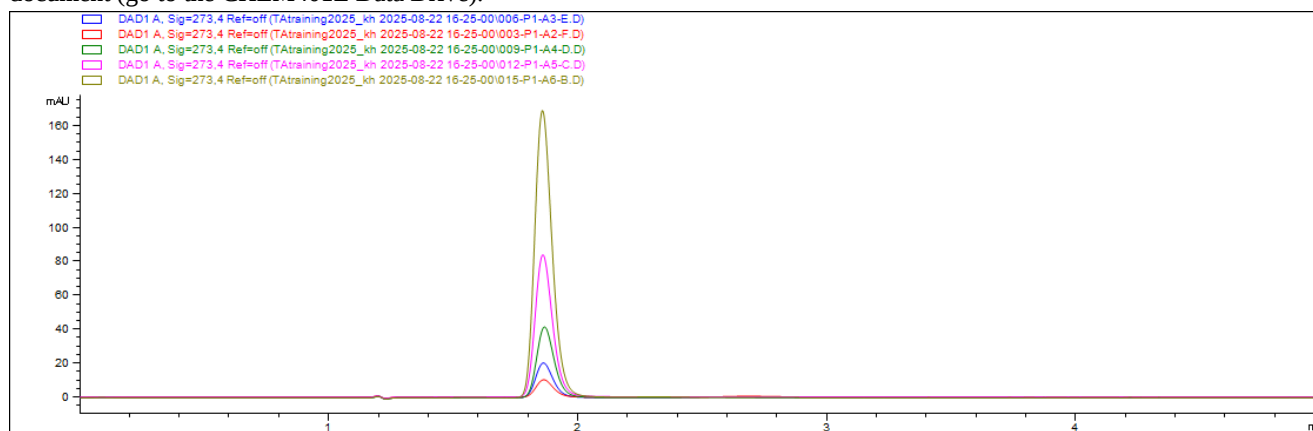


Figure 5.8.4: Copy and Paste Caption here. (Copyright; author via source)

Check Integration Parameters

- There is a list of sample injections in the Sequence Panel (top right, see Figure 5.8.2). Select the most dilute (least concentrated) standard and ensure that a peak is visible (See example in Figure 5.8.5).
- If there is no integration present (Compare Figure 5.8.5 to 5.8.6), or the integration is not correct, select **Integration** under the sequence table (). Then select the Edit Integration button ().

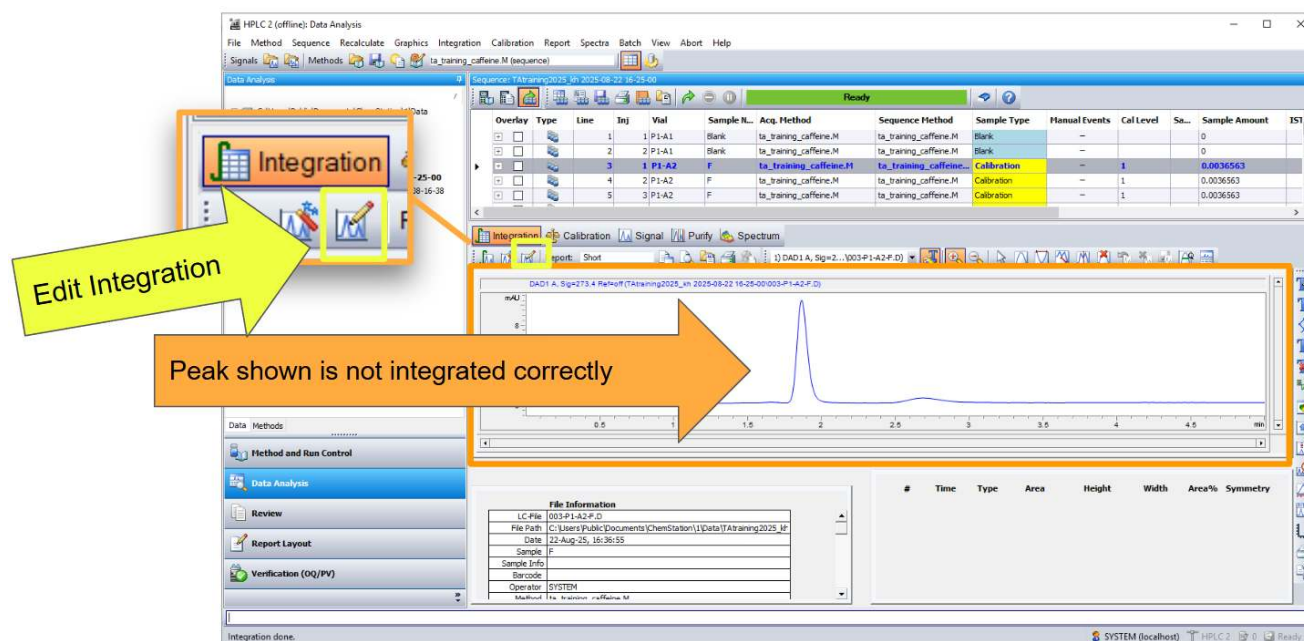


Figure 5.8.5: Copy and Paste Caption here. (Copyright; author via source)

C. In the integration panel, at the bottom, change the Integration Values as follows (see Figure 5.8.6) :

- Slope Sensitivity: 1
- Peak Width: 0.050
- Area Reject: 10
- Height Reject: 1
- Area Percent Reject: 0

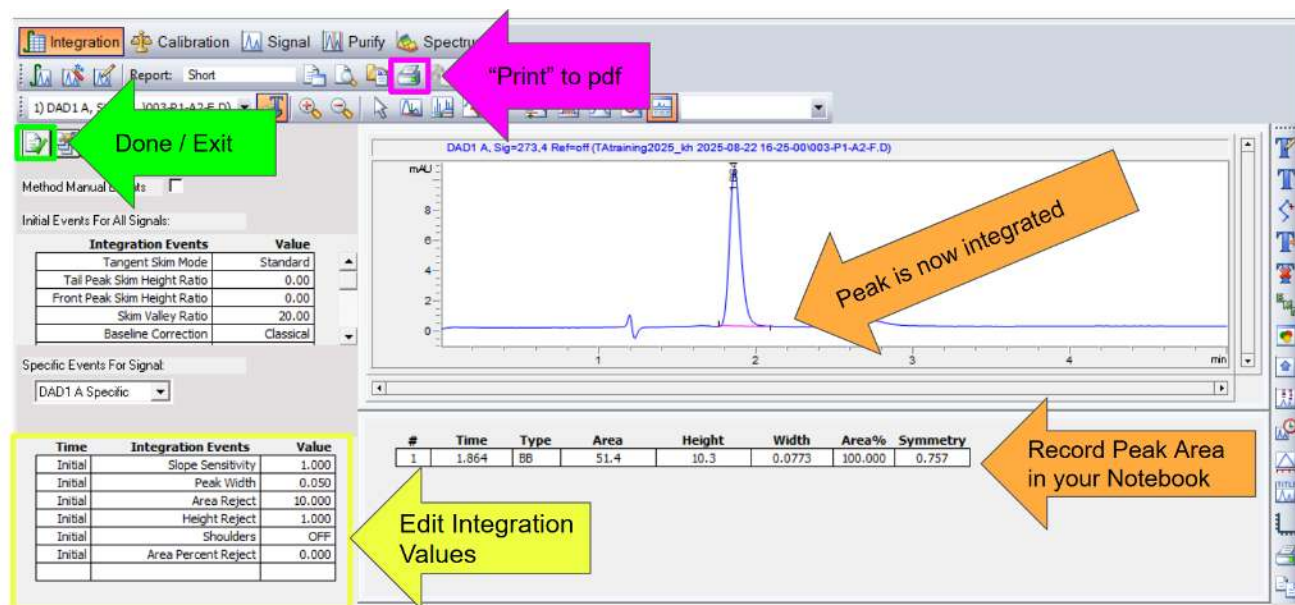


Figure 5.8.6: Copy and Paste Caption here. (Copyright; author via source)

D. After adjusting the integration values, the peak should be integrated. On the chromatogram, if you see a pink line under the peak that matches the expected baseline of the chromatogram, it is an appropriate integration.

- If there are no issues, Record the Peak Area in your notebook.
- You could also "print" the report for this injection. If you do this, please use the format YYYYMMDD_YourInitials. Note that you can append each additional sample report to this pdf if you are careful to check the correct settings in the pop up

box.

- E. Repeat these steps for each injection of calibration standards, controls, and samples. Record the areas of the caffeine peak in your notebook or in the shared data tables in each case.
 - F. When you are finished, you can exit the Edit Integration window with the "done" button (see Figure 5.8.6)
-

Optional Quantitative Analysis using the Open Labs Software

We recommend you walk away from the computer with the peak areas in your notebook and the chromatograms as instructed above.... and not bother to use this buggy, tedious software to conduct analysis. But, here are the instructions if you choose to use them.

- A. You can print the reports after each run or after all runs are complete. You can find the data reports by clicking the "data analysis" tab in the bottom left toolbar of the instrument control software window.
 - B. Before completing the lab, confirm that you have peaks for each of your runs. If you notice any issues with your data, talk with your TA.
 - C. Create a calibration curve using the caffeine standard peak height or area versus the concentration. This can be done using the Chemstation Sftware or using the calibration template that you created earlier this semester.
 - a. To create a calibration curve in *Chemstation*, start by clicking the "data analysis" tab in the bottom left corner of the window. This will open the HPLC (offline) window.
 - b. Find your data in the file navigator on the left panel, and highlight it.
 - c. Double click the first standard run in the sequence window.
 - d. Find the "Calibration" tab in the menu bar and select "New Calibration Table."
 - e. A new window "Calibrate: HPLC1" will appear and select "Automatic setup."
 - f. Set the "level" to 1 and put the concentration of the first run in the "Default Amount." Press "OK."
 - g. Double click the second run and go to the "Calibration" menu bar and click "Add Level."
 - h. Set the "Level" to 2 and enter the second run's concentration in the "Default Amount." Press "OK."
 - i. Repeat steps g and h for each of the remaining standard solutions.
 - j. The Calibration Table and the Calibration Curve can be viewed in the bottom of the window. Confirm that all the points are there.
 - D. Validate your calibration using a control sample provided by your instructors.
 - E. Calculate the concentrations of caffeine in each beverage sample. *Note that each beverage was diluted differently.
-

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