

Guided Inquiry for Chromatography and Internal Standard Methods

KEY! This is the KEY for both the course Chromatographic Parameters Guided Inquiry and the Internal Standard Lab Guided Inquiry.

Parameters Calculated from a Chromatogram and Separation Efficiency.

This exercise is aimed at getting you comfortable calculating parameters from a chromatogram which are used to measure the efficiency of the separation. Good efficiency allows for analytes in a mixture to be separated and easily quantitated. In a chromatographic method, a sample mixture is injected into the instrument's injector. The mobile phase will carry the mixture into the column which holds a stationary phase. The components partition themselves between the mobile phase and the stationary phase, and they are of opposite polarity. This partition is governed by the partition coefficient, K , and tells us the ratio of the concentration of the component in the stationary phase to that in the mobile phase (NOTE: K here is an equilibrium constant). As the mixture flows through the column, different components have different partition coefficients; if the method conditions are set properly, each component will exit to the detector by itself as a band of concentrations of the analyte from low-to-high-to-low. This results in a Gaussian shaped peak. We record the detector signal versus time, and this is termed a **chromatogram**. A liquid chromatogram showing the separation of reducing sugars in molasses will be used for this exercise. The chromatogram is on the last page. **The column used has a length, L , of 20.00 cm and the flow rate was 1.00 mL/min. You will need this information later!**

Please read in your text and the experimental handout to find out more about how chromatographic methods work. Videos describing our GC-14A gas chromatograph are posted on eLC.

1. A standard containing glucose analyzed under the same conditions as the unknown was performed. The lactose peak had its maximum intensity at 5.76 min in the chromatogram; this is called the *retention time*, t_r , for lactose. In Table 1, the peak ID column is empty. Using this retention time and the retention times for the peaks in the table, enter lactose in the correct row under the ID label.
2. In another experiment, lactose, fructose and glucose were analyzed giving retention time data of 3.65, 4.03 and 5.75 min (times don't necessarily correlate to compounds here). A third experiment had lactose and glucose giving retention time data of 5.75 min and 3.66 min. Now, identify all the peaks in the table and explain your reasoning.

In the first chromatogram, you know that the 5.75 min peak is lactose. In the third experiment you also know lactose was at 5.75 min. Thus, in the second and third experiment the 3.66 min peak was glucose. That means that 4.03 is fructose.
3. The linear velocity of the mobile phase, μ , in cm/s can be calculated by using the length of the column in centimeters and the time it takes an unretained peak to elute. The latter presents the time it takes the mobile phase to travel through the column.

$$\mu = \frac{L}{t_m}$$

Calculate the linear velocity in cm/sec using the data for the unretained peak in Table 1. (Show calculation here with units)

$$\mu = \frac{L}{t_m} = \frac{20.00 \text{ cm}}{0.47 \text{ min.}} \times \frac{1 \text{ min.}}{60 \text{ s}} = 0.71 \frac{\text{cm}}{\text{min}}$$

4. You can find the volume of mobile phase required to elute a compound by taking its retention time, t_r , and multiplying it by the flow rate. Find the volume of mobile phase required to elute lactose. Do your calculation below and show units:

$$1.00 \frac{\text{mL}}{\text{min}} \times 5.75 \text{ min} = 5.75 \text{ mL mobile phase}$$

5. The corrected retention time, t'_r , is calculated using the retention time of the peak, t_r , and the retention time of the unretained peak, t_m . This value tells you how long the analyte was actually interacting with the stationary phase in the column. Calculate the corrected retention time for the four sugar peaks using the equation, $t'_r = t_r - t_m$, and enter them in the Table 1. Show one example calculation here (with units).

$$\text{For glucose, } t'_r = t_r - t_m = 0.66 \text{ min.} - 0.47 \text{ min.} = 0.19 \text{ min.}$$

5. The retention factor (formally called the capacity factor), k , is calculated by taking the adjusted retention time, t'_r , and dividing by the retention time of the unretained peak, t_m . Calculate the retention factor for the four sugar peaks using the equation, $k = \frac{t_r - t_m}{t_m} = \frac{t'_r}{t_m}$, and enter them in the Table 1. Show one example calculation here (with units).

$$\text{For glucose } k = \frac{t_r - t_m}{t_m} = \frac{t'_r}{t_m} = \frac{0.19 \text{ min.}}{0.47 \text{ min.}} = 0.41$$

The retention factor can be looked at as the ratio of

- the time the analyte spends in the stationary phase to the time in the mobile phase.
- the moles of analyte in the stationary phase to moles in the mobile phase.
- the number of column volumes required to elute the analyte.

Is there a correlation between k and retention time? Yes, as t_r increases so does k

Which sugar spends the most time in the mobile phase? Glucose (small k) Stationary phase? Sucrose (large k)

Which sugar has more moles of analyte in the stationary phase? Sucrose (largest k)

Based on this, which sugar has the greatest partition coefficient between the mobile phase and stationary phase? In other words, which has the largest ratio of the concentrations in the stationary phase to the mobile stationary phase?

Sucrose because the partition coefficient is the ratio of the concentration in the stationary phase to that of the mobile phase. The retention factor, k , follows that trend!

6. The relative retention, α , is calculated by taking the ratio of the adjusted retention times for two adjacent peaks. The larger adjusted retention time goes in the numerator as this value is always greater than one. Calculate the retention factor for the pairs glucose/fructose, fructose/lactose, lactose/sucrose using the equation, $\alpha_{1,2} = \frac{t'_{r2}}{t'_{r1}}$, and enter them in the Table 1. Show one example calculation here (with units).

$$\text{For fructose/glucose: } \alpha_{1,2} = \frac{t'_{r2}}{t'_{r1}} = \frac{7.59 \text{ min.}}{6.78 \text{ min.}} = 1.12$$

If the relative retention is 1 for two peaks, what does that mean for their retention times?

The separation is useless because they come out at the same time (no separation!).

Visually examine the peak pairs and explain how relative retention changes with peak separation (retention times).

Peaks that are closer together have smaller relative retention values. So, we want larger relative retention for separation (but not too large because it may take too long to elute the analytes!).

Relative retention is a function of the mobile composition and stationary phase composition. Because it is the ratio of (1) the retention factors of the two peaks and (2) the partition coefficients for the two analytes we may also write:

$$\alpha = \frac{k_B}{k_A} = \frac{K_B}{K_A}$$

Thus, compound B will take longer to elute because it prefers the stationary phase over the mobile phase.

7. The resolution between two adjacent peaks, R_s , is a measure the number of “average” standard deviations by which the peaks are separated. The basic idea is to divide the separation of the two peaks by the average baseline width, w_{avg} . You can also use the peak width at half height, $w_{1/2}$. Quantitative analysis is best with baseline resolution which correlates to R values ≥ 1.5 .

$$R = \frac{[(t_r)_B - (t_r)_A]}{\frac{1}{2}(w_A + w_B)} = \frac{0.589[(t_r)_B - (t_r)_A]}{\frac{1}{2}(w_{A,1/2} + w_{B,1/2})}$$

Calculate the resolution for the pairs glucose/fructose, fructose/lactose, lactose/sucrose using the equation, and enter them in the Table 1. Show one example calculation here (with units).

We are using width at half height (pay attention to table column title!)

$$R = \frac{0.589[(t_r)_B - (t_r)_A]}{\frac{1}{2}(w_{A,1/2} + w_{B,1/2})} = \frac{0.589[7.59 - 6.78]}{\frac{1}{2}(0.16 + 0.21)} = 1.21$$

After **visually** examining the peak pairs, can you see who is resolved best? Explain.

Yes, the pair with the big valley between them will give the best R value.

Which pairs are baseline separated based on the value of R you calculated. How does that compare to your previous answer?

Only fructose/lactose has an R greater than 1.5.

What happens to the peak separation as R increases?

They get farther apart.

Is there a correlation between resolution and relative retention?

Yes! They parallel each other!

8. The number of theoretical plates, N, has its origins in fractional distillation. It is sometimes thought of as representing the number of times the analyte equilibrates between the stationary and mobile phase during the elution process (like in oil fractional distillation methods). N is measured by taking the square of the retention time (not corrected retention), t_r , and dividing it by the square of the baseline width. You can also use the half-width, but a constant is involved. N is related to resolution and is a measure of separation efficiency. Larger values of N are better for a given column length.

$$N = 16 \left(\frac{t_r}{w} \right)^2 = 5.55 \left(\frac{t_r}{w_{1/2}} \right)^2$$

Calculate the number of plates for each sugar peak, and enter them in the Table 1. Show one example calculation here (with units).

$$\text{For glucose: } N = 5.55 \left(\frac{t_r}{w_{1/2}} \right)^2 = 5.55 \left(\frac{3.66}{0.16} \right)^2 = 2904$$

Find the average number of plates for the separation by taking the average of all the plates for each sugar.

$$\text{Average} = 2042$$

Look at the N value for each peak, and then relate it to the resolution you calculated between peaks. Is there a relationship?

Yes, better N values relates to better efficiency of separation.

9. The height equivalent of a theoretical plate, H, is calculated from the length of the column in millimeters (usually) and the number of plates for the peak. More plates means a lower plate height and thus, better efficiency.

$$H = \frac{L}{N}$$

Calculate the number of H for each sugar peak, and enter them in the Table 1. Show one example calculation here (with units). Column length is given at the beginning of this inquiry. Show one example calculation here (with units).

$$H = \frac{L}{N} = \frac{200.0 \text{ mm}}{2904} = 0.0689 \text{ mm}$$

Look at the N value and relate it to the resolution you calculated between peaks.

Repeat Ignore

10. To find the optimum flow rate, the plate height is plotted versus linear velocity. This is done by taking several chromatograms at different mobile phase linear flow velocities. The minimum plate height in this curve indicates the best linear velocity and represents the best efficiency of separation. This is needed for proper quantitation.
11. An internal standard is a compound which is different from the analyte that is added to an unknown sample. The chemical properties of the internal standard are similar to those of the analyte. However, the signals for each will occur at different wavelengths (for optical methods) or at different times (for chromatographic methods). The signal from the analyte is radioed with signal from the internal standard to quantify the analyte. An example of an internal standard/analyte pair is acetaminophen and caffeine (HPLC) or lithium and sodium in optical emission methods.

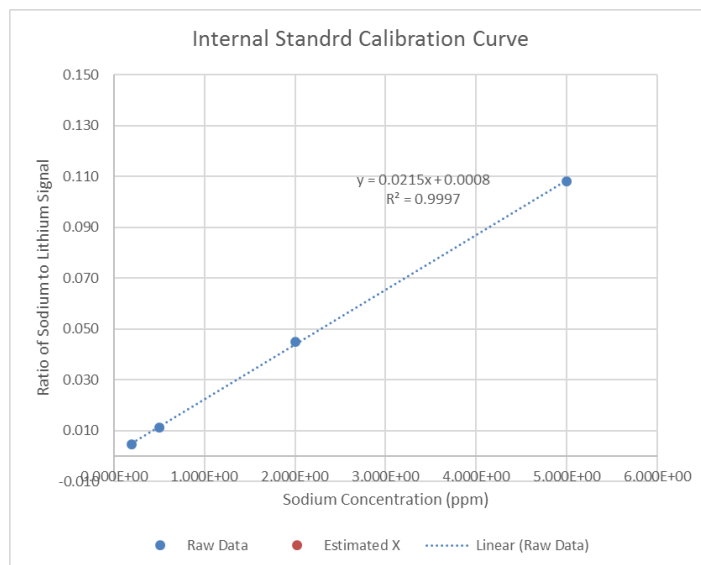
In the table we see data taken from the analysis of sodium using lithium as an internal standard.

Sodium Concentration, ppm	Sodium Emission Signal	Lithium Emission Signal	<i>Sodium Signal</i> <i>Lithium Signal</i>
0.20	0.22	48	0.00458
0.50	0.53	47	0.0112
2.00	2.30	51	0.0451
5.00	5.00	46	0.108

Calculate the ratio of the sodium signal to the lithium signal and place the values in the column. Show one example calculation here with units.

$$\text{For 0.20 ppm} = \frac{0.2}{48} = 0.00458$$

In EXCEL, plot this ratio versus the sodium concentration. Add a trend line and write down the equation for the best fit line and the R^2 value. *This is an internal standard plot.* Make sure it is labeled properly. Insert the plot here.



Other quantities from this regression are (you can easily calculate the others you need from your calculator or use the `linest()` function in EXCEL from Experiment #8)

$s_y = 0.00103$ (there was a typo here!)

$S_{xx} = 14.4675$

A measurement of an unknown gave a sodium signal of 0.88 and a lithium signal of 48, find the concentration and uncertainty of the unknown to the correct number of significant figures. The equation for the uncertainty is provided. Replications should be set to one (1).

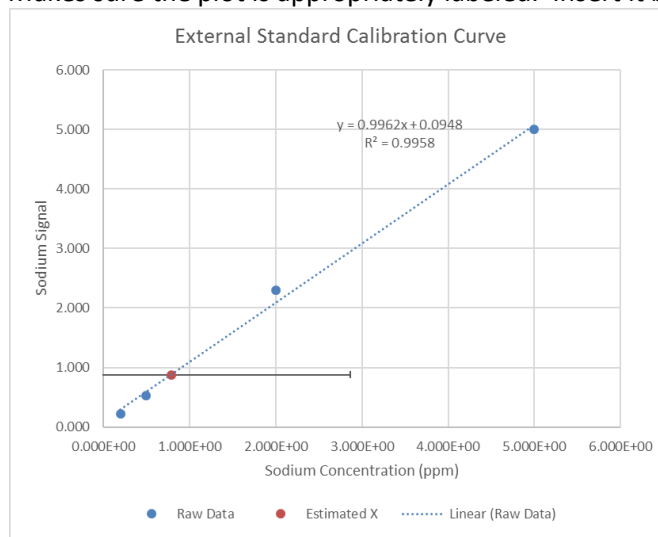
$$s_c = \frac{s_y}{|m|} \sqrt{\frac{1}{\text{Replications}} + \frac{1}{\text{Points}} + \frac{(\bar{y}_c - \bar{y})^2}{m^2 S_{xx}}}$$

I modified the Exp 8 guided inquiry worksheet to do this!

Use the ratio 0.99/49 for the unknown signal in the EXCEL file.

Concentration Mean $0.8_1 \pm 0.2_4$ from internal standard method

Repeat this experiment, but plot the sodium signal versus sodium concentration. Add a trend line to get the slope, intercept and R^2 . Makes sure the plot is appropriately labeled. Insert it below:



Other quantities from this regression line are (others you can calculate with your calculator)

$$s_y = 0.173879$$

$$S_{xx} = 14.4675$$

Using the same measurements for the unknown (sodium signal = 0.88; lithium signal = 48), find the concentration and uncertainty of the unknown to the correct number of significant figures about this regression. The equation for the uncertainty can be found above. Again, replications should be one.

Calculate the 95% confidence level the concentration determined from the internal standard plot and the external calibration plot.

Use 0.88 as the signal here.

Concentration Mean $0.8_1 \pm 0.2_4$ from Internal standard method (from previous)

Concentration Mean $0.7_8 \pm 0.8_6$ from External standard method

Compare and contrast the concentrations and confidence limits in the two cases. What are your conclusions as to which is superior?

We have a lower confidence limit and interval with the internal standard which is superior. Plus, the r^2 for the internal standard was much better than the external standard.

Let's say that the unknown you analyzed was made by adding 0.300 mL of toluene (internal standard), mixed with 15.00 mL of the original unknown and topped off with methanol in a 25 mL volumetric flask.

Using the concentration determined from the internal standard plot, use the dilution equation to find the concentration in the original 15.00 mL unknown (use dilution equation backwards). Show work and units here.

See below

To find the confidence interval for this new concentration we take the confidence interval for the unknown solution analyzed and find the percent relative error, Confidence Limit/Analyzed Unknown Concentration. We then multiply the new concentration by this percentage and that is the confidence interval for the original unknown. Show work and report the final unknown concentration and 95% confidence interval to the correct number of significant figures.

Using Internal standard result and $M_1V_1 = M_2V_2$ where $M_1 = 0.8_1$; $V_1 = 25$ mL ; $M_2 = ?$; and $V_2 = 15.00$ mL

We get $M_2 = 1.35$ ppm

Find relative error in M_1 : $0.2_4/0.8_1 = 0.296$

Error in original unknown = $0.296 * 1.35 = 0.3996$

Concentration in original unknown: $1.3 \pm 0.4_0$

These results are not good, but they should show that the internal standard methodology is superior to an external standard methodology when its hard to control all variables.

Peak ID	Retention Time, t_R (min)	Peak Half Width, $w_{1/2}$ (min)	Corrected Retention Time, t'_R (min)	Retention Factor, k (unitless)	Relative Retention, α (unitless)	Resolution, R (unitless)	Theoretical Plates, N	Height equivalent of a Theoretical Plate, H (mm)
Unretained	0.47 (t_m)	---	---	---	---	---	---	---
Glucose	3.66	0.16			---	---		
Fructose	4.04	0.21						
Lactose	5.75	0.32						
Sucrose	6.55	0.41						

Table 1:

