

Guided Inquiry for Chromatography: Parameters that determine Separation Efficiency

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 in Chapters 23 and 24 to find out more about how chromatographic methods work.

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.
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)

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:

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).

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).

The retention factor can be looked at as the ratio of

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

Is there a correlation between k and retention time?

Which sugar spends the most time in the mobile phase?

Stationary phase?

Which sugar has more moles of analyte in the stationary phase?

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?

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).

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

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

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).

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

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

What happens to the peak separation as R increases?

Is there a correlation between resolution and relative retention?

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).

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

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

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).

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

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.

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		0.16			---	---		
Fructose		0.21						
Lactose		0.32						
Sucrose		0.41						

Table 1:

