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High Performance Liquid Chromatography Monitoring Reaction Kinetics

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Abstract

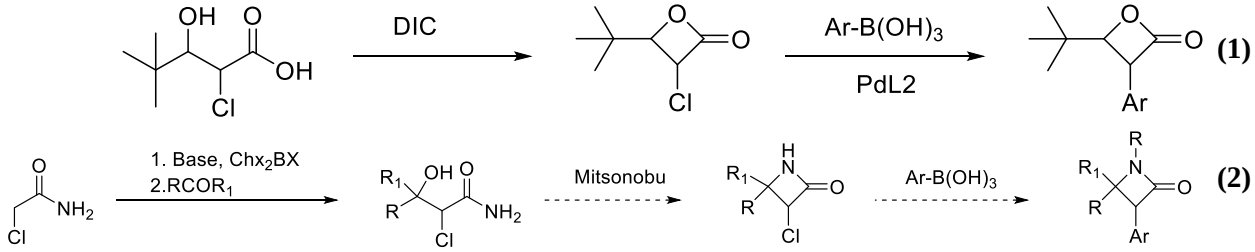
To develop a chromatographic method to characterize enantioselective kinetics, the reaction between aromatic aldehydes and ephedrine will be monitored with High Performance Liquid Chromatography (HPLC). First, develop kinetics analysis procedure with the HPLC, the decomposition of aspartame was observed with citrate and phosphate buffers. The procedure produced an activation energy in the presence of phosphate buffer of 71.9 kJ/mol with a -0. 126 % error. The aromatic aldehyde, benzaldehyde, and ephedrine were run through a stereospecific column to separate peaks with various mobile phases. A more nonpolar mobile phase is required for better peak and retention time separation.

Introduction

High performance liquid chromatography (HPLC) is a chromatographic method that was developed in the early 20th century as analytical tool for qualitive and quantitative characterization of mixtures. The BC Chemistry department recently received a Waters Alliance HPLC with a quaternary solvent pump and a dual wavelength UV detector. Standard operating procedures needed to be developed for this instrument before faculty and students could make use of it for their research agendas.

To develop operating procedures around quantitating kinetics, my project involved first determining the kinetics on the Water Alliance HPLC, from a previously written lab procedure (*1*). Once a general operating procedure is produced for the HPLC, the project will shift its focus to analyzing the enantioselective kinetics by working with aromatic aldehyde and ephedrine (*2*) (*3*).

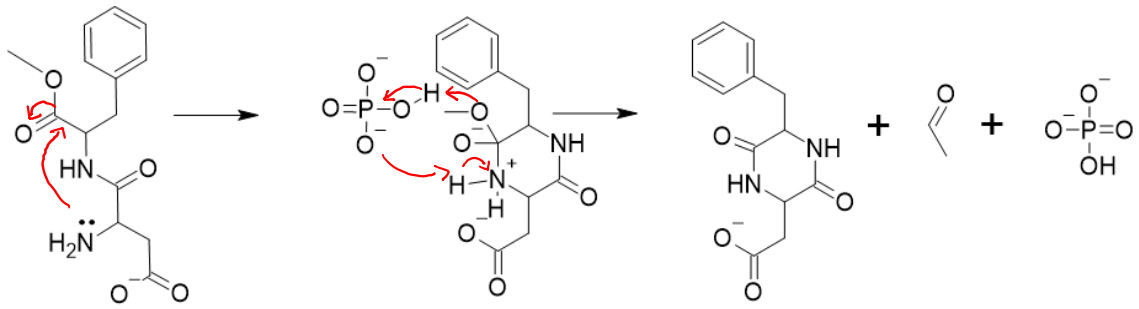
Ultimately, this project lays the groundwork for the development of a chromatographic method for characterizing the products of a boron-mediated lactonization **(1)** and lactamization **(2)** synthesis by Dr. Barnabas Otoo, who is trying to synthesize penicillin analogs as part of an NIH grant.



The products of Reactions **(1)** and **(2)** are chiral and the products are expected to be a mixture of enantiomers. Characterization of the enantiomer mixtures under various reaction conditions would help to guide Otoo toward his desired outcome. Chiral compounds present a particular challenge to chromatographic methods since enantiomers share the same physiochemical properties and would elute with the same retention time when using conventional stationary phases. Chromatographic separation of chiral compounds requires a chiral stationary phase, such as cyclodextrin, which provides enantioselective interactions with the analytes. The Chemistry Department has a Cyclobond HPLC column that has not been used by the current faulty, so a preliminary experiment to evaluate the enantioselectivity of the column is required before a method can be developed for the penicillin analogs described above. Therefore, this project represents a stepwise method development protocol for the ultimate goal of

synthetic penicillin analog analysis – first, to develop a chromatographic kinetics analysis, and second, to extend this method to an enantioselective kinetic analysis.

First, the HPLC was used to measure the rate for decomposition of aspartame. When aspartame decomposes at pH 7.0, roughly 50% of the chemical produces the product 3-carboxymethyl-6-benzyl-2,5-dioxopiperazine (DKP). Additional molecules such as citrate or phosphate in the buffer system help catalyze the reaction. The decomposition of aspartame in the presence of phosphate is shown below in reaction 3 (*1*).



(3)

Aspartame's decomposition is already known to be first order in the presence of citrate and phosphate (Equation 4) (*1*). Using the equation for first order reactions and plotting the natural log of concentration over time, allows for the rate constant, k_r, to be found as the opposite sign of the plot's slope (*4*).

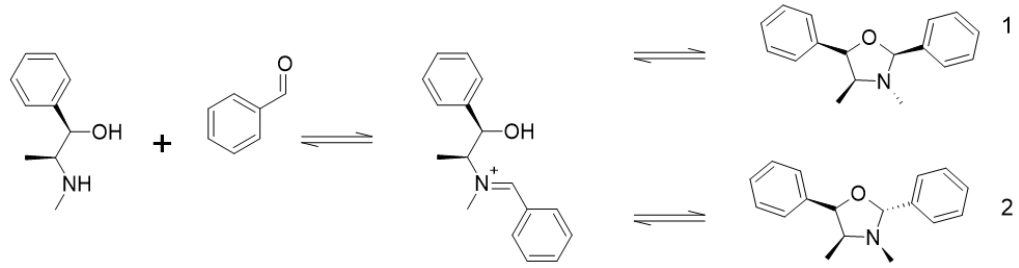
$$\ln[A] = \ln [A]_0 - k_r t \quad \textbf{(4)}$$

To find the activation energy, $E_{a,}$, of this reaction, the Arrhenius equation can be used (Equation 5) (*4*). It requires k_r values from reactions performed at two different temperatures.

$$ln\frac{k_r'}{k_r} = \frac{E_a}{R}(\frac{1}{T} - \frac{1}{T'}) \quad \textbf{(5)}$$

Once a method was developed on the HPLC to quantify the k_r and E_a for the decomposition of aspartame, a similar method would be applied to enantioselective kinetics for the reaction between the aromatic aldehyde and ephedrine.

The reaction between aromatic aldehydes and ephedrine was shown by Neelakantan to form oxazolidines (*3*). Neelakantan was able to show the product of the aromatic aldehyde with the ephedrine produced a diastereomer mixture (Reaction 6) (*3*).



(6)

Diastereomers form when a reaction is driven by both kinetic and thermodynamic controls. The thermodynamic control products are more stable as the reaction is overall slower and requires higher temperatures (*5*). The kinetic control products are less stable as they form from a faster reaction at lower temperatures (*5*). Reaction controls allows for the reaction mechanism to be determined and which

product is more abundant. To pin point which control a reaction undergoes, products are analyzed under specific the reaction conditions. Agami and Rizk used Neelakantan's work to determine the stability of the diastereomers formed. Using thermodynamic control conditions and ^{1}H-NMR, they showed that isomer 1 was the major product and therefore more stable (*2*). Even under these conditions, isomer 2 still is present; however, when the starting aromatic aldehyde was changed, the abundance of isomer 2 increased (*2*). Being able to understand how the conditions and mechanism lead to the formation of a stereospecific product allows for better control of the final product.

$$Rate = \frac{|\Delta J|}{\Delta t} \quad \textbf{(7)}$$

$$v = k_r[A]^x[B]^y \quad \textbf{(8)}$$

A way to moniter the reaction of the aromatic aldehyde with the ephedrine is by observing its reaction rate. The reaction rate measures the change in concentration of products or reactants over time in a fixed volume (Equation 7) (*4*). Rate can also be determined using the rate law, which describes relationship between concentrations of reactants to the power of their order and the rate constant, k_r (Equation 8) (*4*). The rate constant describes reaction speed and only depends on temperature (*4*). Often times the Eqn. 8 and the rate constant are used as Eqn. 4 cannot account for the formation and speed of intermediates formed. To use the Eqn. 8 experimentally, a pseudo rate law is utilized when there is more than one reactant, to find the reaction order. This involves one reactant being in excess while the other is changed to determine the order. Once one of the reactant's orders is determined the other can be experimental found. With this information and using the slope to represents the rate constant the rate of the reaction can be determined (*4*). Pairing this information with the HPLC kinetics analysis procedure will allow for the initial reactants' concentrations of an aromatic aldehyde, in this case benzaldehyde, and ephedrine to be monitored. A HPLC procedure will be created to separate the reactants which will then allow for a pseudo rate laws to determine reactions rate law and complete the kinetics analysis.

Experimental

Aspartame Decomposition:

The first part of the research was focused on quantitating the decomposition kinetics of aspartame at different temperatures and with different buffer conditions to determine its energy of activation. Using the "Decomposition of Aspartame" paper as a guide to preparing the reaction (*1*). The first phosphate buffer was prepared by mixing 660.3 mg $(NH_4)HPO_4$, 1.461 g NaCl, and about 10 mL of deionized water in a 50-mL flask. Drops of 1.0 M HCl were added till the pH was 6.98 and transferred to a 25.00 mL flask and filled with deionized water so the final concentrations were 0.20 M phosphate and 1.0 M NaCl. A second phosphate buffer was prepared by adding 1.6509 g $(NH_4)HPO_4$ and about 50 mL deionized water to a 100 mL beaker. Drops of 1.0 M HCl were added till the pH was 3.05 and transferred to a 500.00 mL flask and filled with deionized water so the final concentration was 0.025 M. This buffer was used as a mobile phase. The citrate buffer was prepared by mixing 1.4711 g $Na_3C_6H_5O_7 \bullet 2H_2O$ and about 15 mL deionized water in a 50 mL beaker. Drops of 1.0 M HCl were added till the pH was 7.07 and transferred to a 25.00 mL flask and filled with deionized water so the final concentration was 0.20 M citrate.

Before adding aspartame to the buffers to start the decomposition, the Waters Alliance 2695 HPLC parameters was prepared. The column used was an XBridge C-18 (15cm length, 4.6 diameter). The instrument method Aspartame40_mrs had the following parameters: an isocratic flow of 55/45 0.025M phosphate buffer pH 3.05/methanol, 1.00 min/mL flow rate, 2.00 min acceleration, sample temperature and column temperature of 40ºC ± 1ºC, and wavelength set at 200nm (*1*). This instrument method was uploaded to the method set, Aspartame40MS_mrs.

To start the aspartame decomposition in the presence of citrate, 10.00 mL of the 0.20 M citrate buffer and 5.3 mg of aspartame were mixed in a 50 mL beaker. After the solution was mixed for a minute, 1.000

mL of the solution was pipetted into a HPLC vial and placed into the sample chamber. The HPLC was previously equilibrated for 10.0 minutes with the Aspartame_40MS_mrs method set. Then the sample set, Asparame40_citrate_mrs was started. Injections of 10 µL were made every 15 minutes, starting at time zero and ending at time 225 minutes. The processing method Aspartame40PS_citrate_mrs was used to find the area under the peak for aspartame and 3-carboxymethyl-6-benzyl-2,5-dioxopiperazine (DKP). Using the injection time and retention time, the time of the reaction was paired to the concentration of aspartame in excel for the first order relationship to be plotted and analyzed with regression statistics. This process was repeated with the 0.20 M phosphate buffer pH 6.98 except only 4.9 mg of aspartame was added and the phosophate buffer was filtered for impurities before being mixed with the aspartame. The sample set phosphate_40 last injection was at time 120 minutes instead of 225 minutes and was processed with Aspartame40PS_phosphate2_mrs.

To measure the kinetics at a different temperature, the instrument method Aspartame35_mrs was created with the same parameter as Aspartame40_mrs except the sample and column temperature were set at 35ºC ± 1ºC, instead of 50ºC, as this HPLC max temperature is 40ºC. This instrument method was uploaded to the method set Aspartame35_mrs. Again 4.9 mg of aspartame was mixed with 10.00 mL of the filtered 0.20 M phosphate buffer pH 6.98 and added to the HPLC. The Asartame35_phosphate_mrs sample took injections every 15 minutes starting at time zero and ending at time 90 minutes. It was processed with Aspartame40PS_phosphate4_mrs and then analyzed.

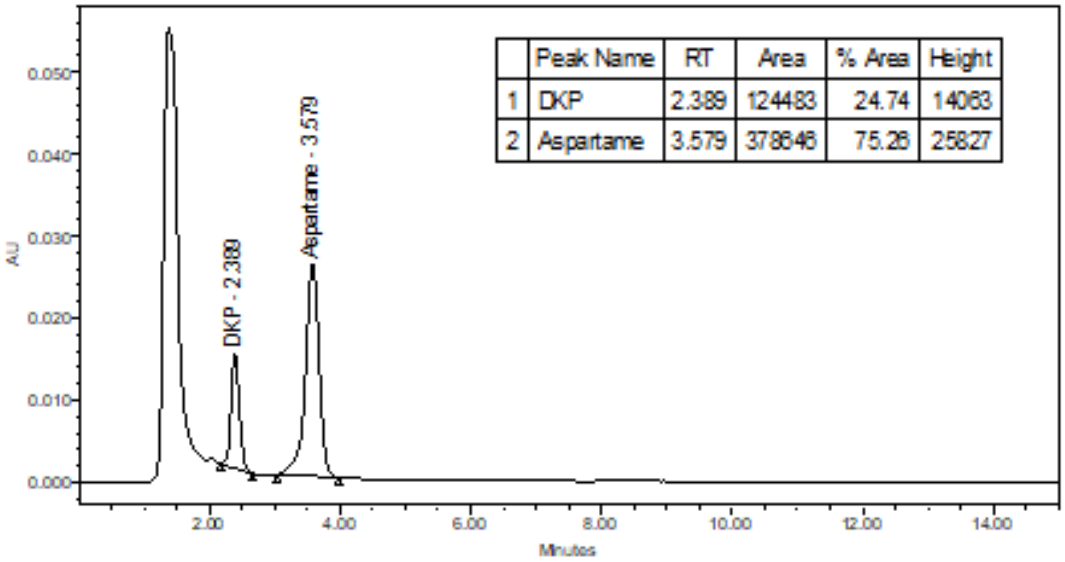
Seperation of Stereochemical Molecules:

The next part of the research was to apply the HPLC technique to the reaction between ephedrine and an aromatic aldehyde. The Waters Alliance 2695 HPLC was again used for this, but the 250 mm Cyclobond stereoselective column was used instead. Before the reaction could be observed the instrument parameters had to be experimentally determined. The wavelength for detection for all of the parameters was set at 258 nm. Benzaldehyde was chosen as the first aromatic aldehyde to observe. Originally, the benzaldehyde was not found, so almond extract was used a source of benzaldehyde. The first few instrument methods created were held under the pressure of 1000 psi with mobile phases of methanol, isopropanol, or a 50/50 methanol/isopropanol mix. The solution was too concentrated and the benzaldehyde solution was found. The benzaldehyde stock solution was prepared by adding 100 µL benzaldehyde to a 10.00 mL flask and filling the rest with methanol. A HPLC vial was prepare by adding 30 µL of the benzaldehyde stock solution to the vial with 1.000 mL of methanol. The ephedrine vial was prepared by adding 500 µL of the 1mg/1mL solution to an HPLC vial. R-(-)-carvone and S-(+)- carvone stocks solutions were prepared by adding 5 µL of each to a separate 10.00 mL flask and then filling with methanol. A R,S-carvone mixture was prepared by adding 5 µL or each to one 10.00 mL flask. Each stock solution had a 1.000 mL of the solution to 3 difference HPLC vials.

With the prepared HPLC vials, various instrument methods were prepared to see what parameters are needed to separate the stereoisomers. At first the parameter was set up with a pressure limit of 1000 psi. Most instrument methods AEX, used a mobile phase of 100% isopropanol or 50/50 methanol/isopropanol while the flow rate was adjusted between 0.100 mL/min to 0.200 mL/min. The pressure would not raise above 0 psi so the inline filter was removed and cleaned. A dry and wet purge were performed as well to remove bubbles in solvent line. The pressure limit was too low and it was raised to 2500 psi. Eight instrument methods were created with this pressure limit. Benz_IM1 was a pure methanol mobile phase with a 1.00 mL/min flow rate. Benz_IM2 was also a pure methanol mobile phase with a 1.20 mL/min flow rate. The next four had 1.00 mL/min flow rate and had 30/50 (benz_IM3), 50/50 (benz_IM4), 70/30 (benz_IM5), and 40/60 (benz_IM6) acetonitrile/methanol isocratic mobile phase. Benz_IM7 had a mobile phase of ethyl-acetate and a 0.500 mL/min flow rate. The last instrument method created was benz_IM8 with a 50/50 ethyl-acetate/methanol mobile phase with a 0.200 mL/min flow rate. A CYCLOBOND shutdown instrument method and method set were created as well, but never worked as it would cause the pressure to become greater than the limit and shut down the sample set.

Results

The data for the decomposition of aspartame involved generating a chromatogram for each buffer and temperature for every injection. A sample of chromatogram at time 6 for the decomposition in the citrate buffer is shown below in Figure 1. It contains the aspartame and DKP peaks.



	Peak Name	RT	Area	% Area	Height
1	DKP	2.389	124483	24.74	14063
2	Aspartame	3.579	378646	75.26	25827

Figure 1. Time 6 chromatograph of aspartame's decomposition in the presences of the 0.20 M citrate buffer pH 7.04 at 40ºC.

Using the area under the curve function of the Water's Alliance System, the area of each peak was determined for every injection. Since aspartame's decomposition is first order, the natural log of the area under the aspartame peak can be plotted against the time (Figure 2). The time was determined by adding the retention time of the aspartame peak to the injection time. While the injections were set up to run every 15 minutes there was a slight time buffer before the next injection started.

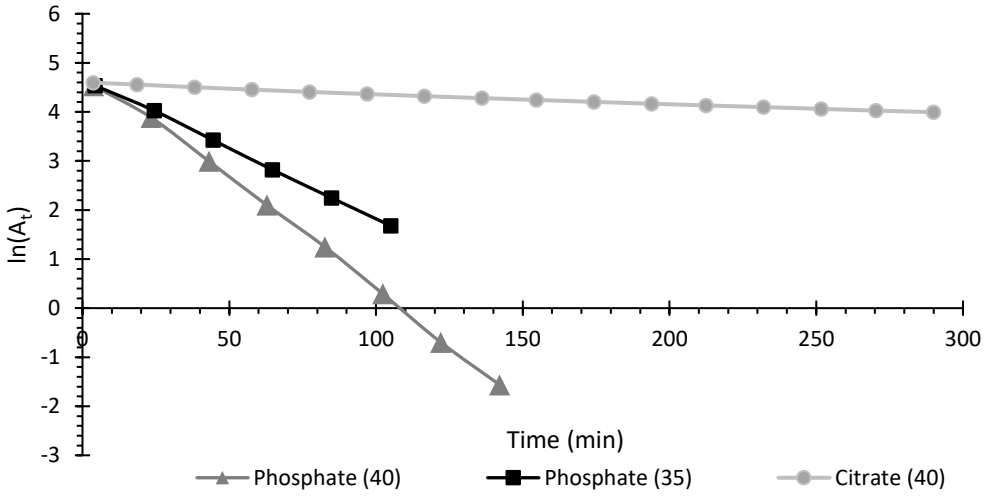


Figure 2. First order plot of $\ln(A_t)$ verse time, where A represents the peak area of aspartame under various temperature and buffer conditions. The slope of the plot's times negative 1, is equal to the k_r.

The slope of the natural log of aspartame's peak area verse time allowed for k_r to be determined as it equals the slope multiplied by negative in first order kinetics. To determine the standard deviation in k_r a regression analysis was used for each line. The k_r values for the decomposition in the citrate and phosphate buffer at 40ºC was compared to the average of the paper values. Using the deviating temperatures and k_r values for the decompositions with the phosphate buffer, the energy of activation was calculated (Table 1).

Table 1. Decomposition of Aspartame Kinetics. *(1)*

	k_r (1/min)			E_a (kJ/mol)
	0.20 M Citrate, pH 7.07, 40ºC	0.20 M Phosphate, pH 6.98, 40ºC	0.20 M Phosphate, pH 6.98, 35ºC	
Data	0.00208 ± 0.000040	0.04491 ± 0.00076	0.02868 ± 0.00030	71.9
Lit. Value (*1*)	???	???	???	???
Percent Error (%)	-27.0	142.7	N/A	-0.126

The next part of the project, using the Cyclobond column to separate stereoisomers, many chromatograms were produced. The chromatograms produced when the pressure limit was only 1000 psi all had very broad and tailing peaks. These peaks where also above 1.00 AU as the samples had not yet been diluted. For example, partially diluted benzaldehyde, R-(-)-carvone, and S-(+)-carvone all produced the wide peaks in Figure 3.

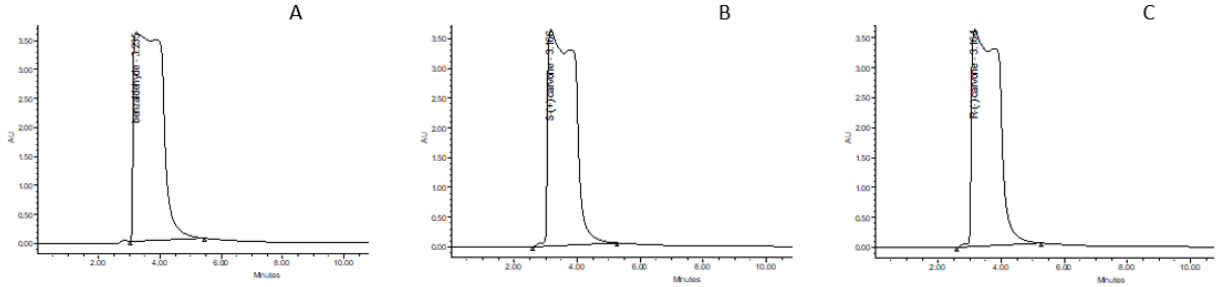


Figure 3. Chromatograms of benzaldehyde (A), S-(+)-carvone (B), and R-(-)-carvone (C) with the 1.00 mL/min flow rate of methanol. The peaks were very broad and the retention times for each chemical were similar as benzaldehyde's was 3.235 min, 3.266 min for S-(+)-carvone, and 3.164 min for R-(-)-carvone.

After dilution the challenge was determining the HPLC parameters needed to produce separate stereoisomer peaks and between benzaldehyde and ephedrine. The following figures show the chromatograms with the retention times. The first figure has a 1.20 mL/min flow rate and mobile phase that is only methanol (Figure 4). The flow rate was slowed to 1.00 mL/min and then isocratic flows of 40/60, 50/50, and 70/30 acetonitrile/methanol were used (Figure 5, 6, and 7).

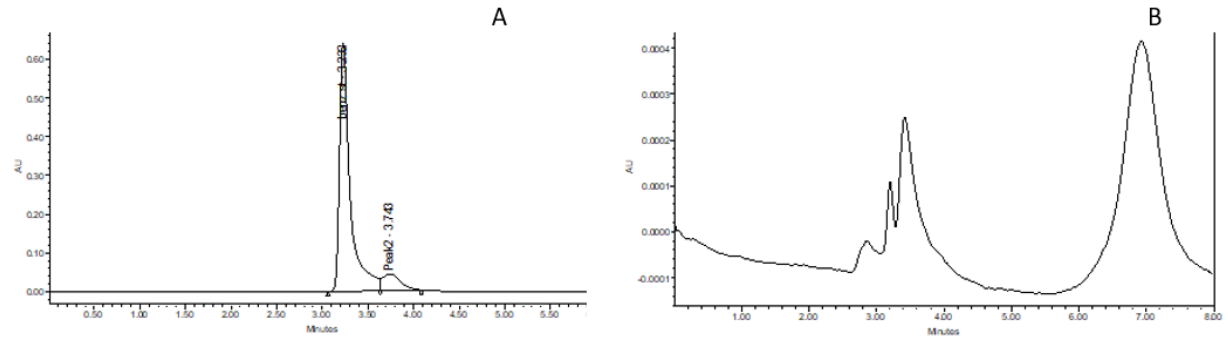


Figure 4. Chromatography of benzaldehyde (A) and ephedrine (B) with a flow rate of 1.20 mL/min of methanol. Benzaldehyde's retention time was 3.232 min. Ephedrine's retention time was not solved for.

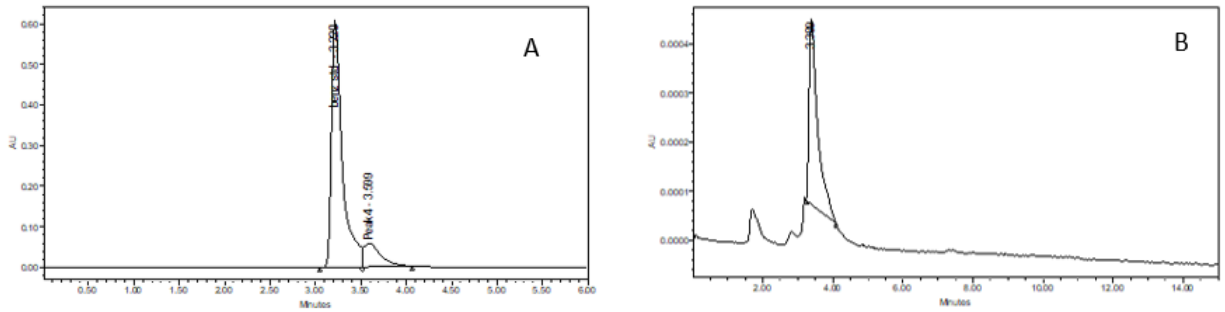


Figure 5. Chromatography of benzaldehyde (A) and ephedrine (B) with a flow rate of 1.00 mL/min and a ratio of 40/60 acetonitrile methanol. Benzaldehyde's retention time was 3.220 min. Ephedrine's retention time was 2.992 min.

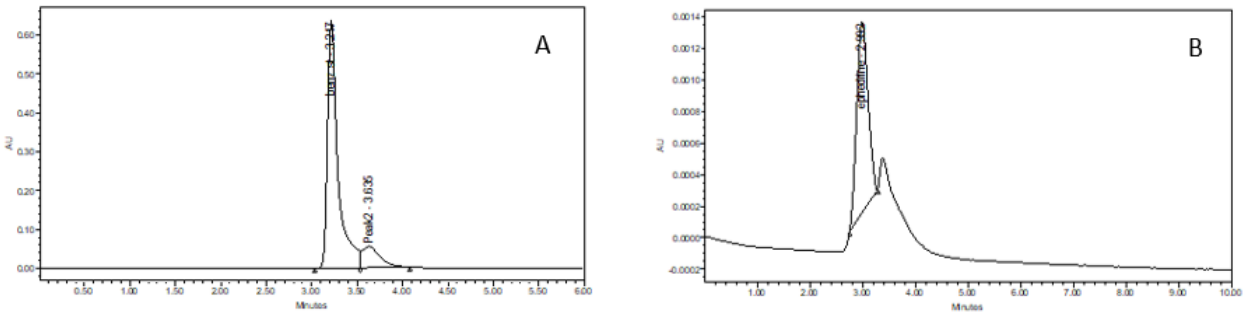


Figure 6. Chromatography of benzaldehyde (A) and ephedrine (B) with a flow rate of 1.00 mL/min and a ratio of 50/50 acetonitrile methanol. Benzaldehyde's retention time was 3.217 min. Ephedrine's retention time was 2.992 min.

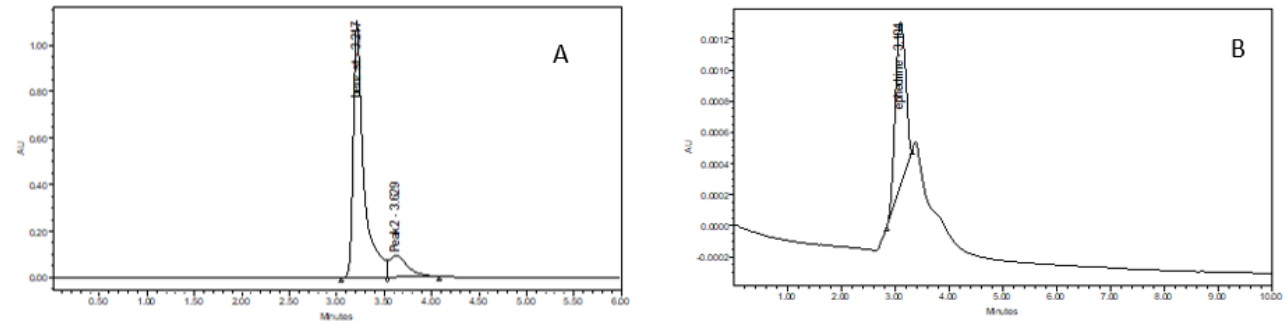


Figure 7. Chromatography of benzaldehyde (A) and ephedrine (B) with a flow rate of 1.00 mL/min and a ratio of 70/30 acetonitrile methanol. Benzaldehyde's retention time was 3.217 min. Ephedrine's retention time was 3.104 min.

Since the coating of the Cyclobond column is polar, switching to a more nonpolar mobile should cause a greater difference in retention times. A flow rate of 0.500 mL/min was used with an ethyl-acetate mobile phase (Figure 8).

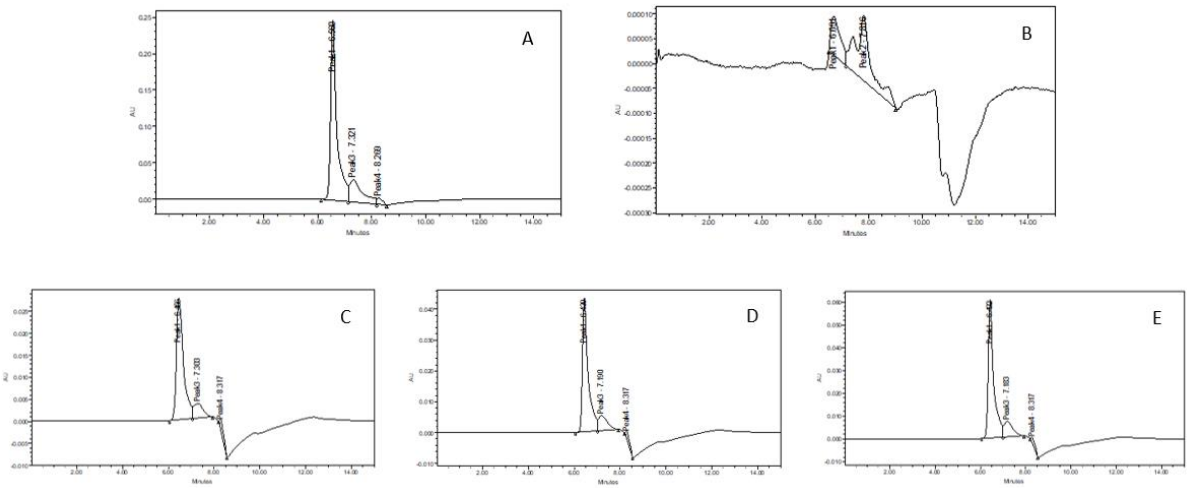


Figure 8. Chromatography of benzaldehyde (A), ephedrine (B), S-(+)-carvone (C), R-(-)-carvone (D), and R,S-carvone (E) with a flow rate of 0.500 mL/min and ethyl-acetate. Benzaldehyde's retention time was 6.560 min, ephedrine's was 6.691 min, S-(+)-carvone's was 6.456 min, R-(-)-carvone 6.439 min, and R,S-carvone 6.432 min.

Discussion

The results from the first part of the project, determining the kinetics of aspartame's decomposition provide a way to measure kinetics on the HPLC. The chromatography in Figure 1 shows how the peak area is determined for the retention time. Using the peak area for each retention and injection time allowed for the decomposition's first order kinetics to be observed (Figure 1). Plotting the relationship between time and $\ln(A_t)$ allow for the slope, and k_r to be found (Figure 2). The citrate buffer at 40ºC had a k_r of 0.00208 ± 0.000040 min^- with a -27.0 % error from the average of the 2 groups in the paper method (Table 1) (*1*). The phosphate buffer at 40ºC had a k_r of 0.04491 ± 0.00076, which was a 142.7 % error (Table 1) (*1*). The percent error with the citrate buffer was much lower than the error in k_r for the phosphate buffer. A possible explanation of citrate buffer increased accuracy compared to the phosphate buffer, is that citrate did not require filtration and it did not need salt to be added.

While both k_r values had a high percent error, the error for the activation energy was -0.126% for the activation energy value of 71.9 kJ/mol for the phosphate buffer (Table 1) (*1*). The low percent error in activation energy shows that the overall process of using the HPLC to calculate k_r at different temperatures is accurate. The inaccuracy in the k_r value is not as concerning considering the activation energy value. Possible reasons for the high percent error in the k_r is the difference in heating methods. The paper method used water baths to heat the sample. Once in the HPLC the sample was no longer heated (*1*). The method used for this project did not use a hot water bath and instead used the sample chamber and column oven to control the reaction heat. Inconsistencies between these two methods would alter the k_r value. Since this would be the main reason for the variation in the k_r value, and the energy of activation was accurate, this method of heating the sample chamber and column was adopted and used for the next part of the procedure.

Before the method of finding k_r and activation energy for the reaction between an aromatic aldehyde and ephedrine, the retention times of the reactants need to be separated with distinct retention times and peaks. After fixing the pressure to the correct max range, 2500 psi, of the 250 mm column the various mobile phases were experimented with. The methanol mobile phase and high concentrations R,S-carvone and benzaldehyde had peaks that were too wide (Figure 3). The benzaldehyde chromatography for the pure methanol mobile phase with a 1.20mL/min flow rate was resolved with a 3.232 min retention time (Figure 4). The ephedrine chromatogram for the same conditions was less resolute and appeared to have baseline error, so its peak retention times were not solved (Figure 4). The mixtures of acetonitrile and methanol tested had retention times that were too similar (Figure 5, 6, and 7). The mixed mobile phase with the largest difference in retention time was the 50/50 acetonitrile/methanol with a difference of 0.225 min (Figure 6). The reason for the overlap of retention times and peaks is due to the use of most polar mobile phase.

The difficulty using mostly polar mobile phases is that the column matrix is composed of β-cyclodextrin (Figure 9). The cyclodextrin is a polar, hydrophilic matrix. Having a polar mobile phase would affect the mass transport, or the equilibrium of analyte affinity for the mobile or stationary phase. If both the mobile and stationary phase are polar then the analytes like benzaldehyde and ephedrine would have the same affinity for both the mobile and stationary phase and elute around the same time. A more nonpolar mobile phase of ethyl-acetate was experimented with. Using a mobile phase of only ethyl-acetate, the retention times of benzaldehyde, ephedrine, S-(+)-carvone, R-(-)-carvone, and R,S-carvone were still all almost identical (Figure 8). These chromatograms displayed that an even more nonpolar mobile phase is required. Difficulties arose with staying under the pressure limit of 2500 psi when using ethyl-acetate. The flow rate had to be lowered to only 0.500 mL/min. Additionally, the designed CYCLOBOND shutdown method that would flush the column of ethyl-acetate and store the column in isopropanol, never ran to full completion as the pressure got too high. The column had to manually monitored to flush and store the column in isopropanol.

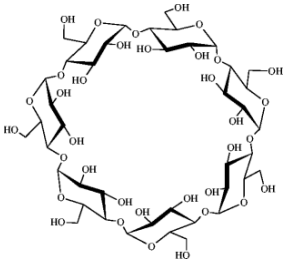


Figure 9. β-cyclodextrin. *(6)*

In the future, this research will need to move to a more nonpolar mobile phase, such as hexanes or even diethyl ether. This theoretically will provide a better difference for the analyte's affinity for the stationary and mobile phase to allow for difference in retention time and peaks. An alternative, or in case the non-polar solvent does not result in the desired peak resolution, a 7.5 cm C-18 column could be installed just before the Cyclobond column in order to provide peak resolution of the achiral compound. Once the peak separation between benzaldehyde and ephedrine are achieved with the column, the kinetics can finally be derived for the formation of the mostly thermodynamically controlled product, isomer 1 (Figure 1). With the kinetics of the benzaldehyde and ephedrine reaction, additional aromatic aldehydes and solvents can be used to find the kinetics that led to the formation of the kinetically controlled isomer 2 (Figure 1). With the kinetics for both products, the reaction between the aromatic aldehyde and ephedrine can be more accurately controlled to produce the more desired isomer in higher percent yield.

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