I. Overview
As you know from your GC lab., chromatographic methods are used to separate the components of mixtures prior to detection, and can be used for both quantitative and qualitative analysis. Many of you used High Performance Liquid Chromatography (HPLC) to determine the caffeine content in a variety of beverages (your favorite Chemistry 365 lab!). You may recall that while GC is used for the separation of (primarily) volatile organic compounds, HPLC is used for the separation and detection of non-volatile organic and inorganic solutes. The technique is particularly applicable to biological, pharmaceutical, food, environmental, and industrial analyses.

All chromatographic separations utilize the same basic approach. First, the sample mixture is introduced into a flowing stream called the mobile phase. The mobile phase carries the sample through a column that contains a stationary phase. Next, the analytes partition between the mobile and stationary phases. Analytes with stronger attraction to the stationary phase take longer to travel through the column. Finally, a detector that responds to the analytes (placed at the downstream end of the column) monitors the concentration of the analytes as they elute from the column.

Different forms of liquid chromatography are classified by the nature of the stationary phase. Reversed-phase chromatography, the most popular mode (and the mode that will be employed in this exercise) utilizes a non-polar stationary phase. Other modes of liquid chromatography include normal phase (polar stationary phase), ion exchange, and size exclusion chromatography.

In reversed-phase HPLC, the stationary phase is nonpolar and the mobile phase is a mixed aqueous/organic solvent. The less polar (or more hydrophobic) an analyte is, the stronger its interaction with the stationary phase. Thus, solutes generally elute from a reverse-phase column with the most polar first and the most hydrophobic last. Unlike GC, the affinity of solutes for the stationary phase is strongly affected by the composition of the mobile phase. As the fraction of organic solvent (e.g. acetonitrile or methanol) is increased the affinity of all compounds for the reverse-phase stationary support will be reduced and retention times will decrease. A gradient of solvent composition with increasing fraction of organic solvent can be used to elute strongly retained solutes and overcome the general elution problem.

In this exercise, the separation of three parabens and a phthalate will be optimized. Parabens are common preservatives used in cosmetics, and phthalates are common plasticizers used to give
polymeric materials greater flexibility. Phthalates are partly responsible for the so-called “new car smell.” Your goal is to determine the best set of conditions (gradient) for the baseline resolution of your multi-component mixture in the shortest time period possible.

II. Procedure
A. Using the SII 440 or the Agilent 8453, obtain UV spectra of ethyl paraben and dipropyl phthalate in 40:60 acetonitrile:water. Be sure to use 40:60 acetonitrile:water for the blank. Note differences in the spectra, and record the best wavelengths for the analysis of parabens (they all have the same spectrum) and phthalates.

B. Starting the HPLC
1. From the pump control window, set the flow to 0.1 mL/min and wait about 30 minutes for the lamp to warm up. When the lamp is warmed up, the baseline on the plot should stop drifting and the back pressure should be constant.

2. Place ~ 5 x 10^-5 M mixed standard solution in a sample vial and cap it.

3. You will be adjusting parameters including the percent acetonitrile (gradient?), wavelength(s) of detection, and stop time. The one constant will be the injection volume (20 µL).

C. Develop and optimize a method
1. Inject the mixed standard using 80% acetonitrile as the mobile phase. Since there are four compounds, the optimized separation should have 4 peaks in addition to peak from the solvent front (i.e. t_m). If the initial separation does not have four analyte peaks, it is most likely because the concentration of acetonitrile is too high.

2. Change the method to use 70% acetonitrile. Repeat the injection and note any differences in the chromatograms. Repeat this process with lower and lower fractions of acetonitrile until you achieve baseline separation of the 4 components. Keep in mind that a 10% change in the acetonitrile concentration will have a large effect on t_r. You may use any fraction of acetonitrile (between 30 and 80%). After changing conditions, always confirm that the system has equilibrated.

3. Most likely there will be a long period between the first three peaks and the last peak. This classic situation is called the general elution problem and requires that a gradient elution
method be employed. Make some educated guesses about what the initial and final percentages of acetonitrile should be. Keep in mind that the rate of programming should not exceed 15%/min. Be sure to program a return to the initial conditions and a “post time” for the column to re-equilibrate before the next injection.

D. Remember to turn off the pump and the lamp.

III. Suggestions for Results and Discussion
A. Report the optimum separation conditions using both isocratic and gradient elution.

B. Using reversed phase retention theory and the spectral information for the parabens and phthalates, identify each of the four peaks in the mixed standard.

C. Calculate and report, using replicate results from the optimized isocratic method, the retention factors for methyl, ethyl and propyl parabens, and calculate the selectivity between each pair. Discuss the similarity or difference in the selectivity values. Also calculate and report the retention factors and selectivity for ethyl and propyl parabens using the chromatogram obtained using a higher percentage of acetonitrile, and discuss the results relative to those at the optimum conditions.

D. Calculate and report the resolution between methyl and ethyl paraben under optimum conditions and with the higher fraction of acetonitrile.

E. Calculate and report the average efficiency (N) for each peak for the optimized separation. Discuss any significant differences.

F. Comment on the gradient separation. Discuss the advantages and disadvantages of the approach, keeping such factors as sensitivity, baseline stability, and total analysis time in mind. Also comment on the width of the last peak using the isocratic and gradient methods.