Advanced HPLC: Simulator week 2

Prelab Assignment

Answer the following question without using the simulator. These questions are based on using liquid chromatography for the analytical separation of phenol, benzophenone, and *hexanophenone*.

1. a) Draw the chemical structures of three compounds. Upload or paste your drawing into your electronic lab notebook (ELN).

   b) List them in the order of least polar to most polar.

2. What order would you expect these compounds to elute on a C-18 column?

3. How would you expect the elution times for these molecules to change if the mobile phase was adjusted from 5% to 50% to 100% (%v/v) acetonitrile?

4. What would you expect to happen to the theoretical plate height (*HETP*) if:

   a. the flow rate was increased significantly (2.0 mL/min to 6.0 ml/min) ?

   b. the diameter of the stationary phase packing particles was doubled (3.0 μm to 6.0 μm) ?

   c. the length of the column was doubled (100 mm to 200 mm)

5. How would the look of the chromatogram be affected in each of above scenarios?

Lab exercise part 1: Retention order and theoretical plates[1]

Use the virtual HPLC (http://multidlc.org/hplcsim/hplcsim.html) to try all of the actions described in the pre-lab assignment questions 2 – 5, then answer the following questions in your ELN.

- Which of your six predictions in the prelab were correct (2, 3, 4a, 4b, 4c, 5)?

- Which of your pre-lab predictions were incorrect (2, 3, 4a, 4b, 4c, 5)?
  For these actions, explain why the action had the effect it did in the simulator.

Lab exercise part 2: Optimizing gradient separations

For this part, keep the three molecules from part 1, and add in five other molecules from last week (caffeine, N-benzylformamide, diethylformamide, methylparaben, and acetanilide) plus naphthalene and

[1] part 1 created by Professor Megan Nagel, Bethel University

"impurity E" for a total of 10 analytes to be separated. Remember that caffeine is a "custom compound" that must be added to the simulator using the following data:

Caffeine parameters	Mobile phase: Acetonitrile	Mobile phase: Methanol
ln k_w intercept	1.5608	2.0909
ln k_w slope	-0.0150	-0.0217
S intercept	-9.056	-6.2387
S slope	0.0699	0.0400

- Set the % acetonitrile in the mobile phase composition to the value that you optimized last week for getting the best baseline separation between the original seven peaks. (Note that adding in additional analytes does not change the separation of the original peaks, unless the added analytes overlap in the same region of the chromatogram.)
- Record your observations of the chromatogram. Are all 10 analytes eluting with baseline separation? If not, which peaks are overlapping? How long does the separation take? How many samples could you separate (in a 24-h day because of the autosampler) if each sample contained these analytes?
- Now switch to gradient elution. This allows you to change the mobile phase during the run in a smooth increase towards higher percentages of organic solvent. Have all group members manipulate the three adjustable parameters (starting %B, ending %B, and time to reach ending %B) on their own computers, trying to achieve *baseline resolution of all 10 peaks in the minimum amount of time*. Time is counted as the elution time of the last peak in the chromatogram or the time to reach ending %B, whichever is larger. (Tip: If a peak disappears from the chromatogram, check the retention time table to locate it.) You are playing in a 3-dimensional optimization space here, with a lot of room to explore. After 5 minutes, come together and see who has developed the fastest 10-peak baseline separation. Then have everyone start from there and optimize for 5 more minutes, and compare results again. Select the best set of gradient parameters that a member of your group was able to find – the one that achieves baseline separation of all peaks in the shortest possible time.
- Take another look at your group's best gradient separation that you've just selected. If the last peak elutes *before* your gradient elution reaches its final time, that difference is wasted gradient time. Try to eliminate this wasted gradient time (if you have it) to shorten your analysis time per sample even further. Keep in mind, though, that if you only change the time to reach ending %B, the slope of the gradient will increase, which may degrade the resolution of closely-spaced peaks. Instead, try to keep the slope (d%B / dt) constant as you shorten the gradient.
- Record these final, uber-optimized gradient parameters in your ELN, along with the retention times of the 10 analytes, and the identify of solvent B.
- Click the Export Chromatogram button in the simulator. This will download a file called "FullChromatogram.csv", with time in the first column and detector signal in the second. Change the name of this file to "FullChromatogramACN.csv" and save it to your Chem 220 folder.

- To calculate your **analysis time per sample**, you should take the larger of (retention time of the last peak) or (time to reach ending %B) and then add 10 minutes, since at the end of each gradient run, the pump must go back to the starting mixture and equilibrate the column for 10 minutes in preparation for the next run. How long is your analysis time per sample, including the column equilibration time? Calculate and record how many samples you could separate in a day using this gradient elution method.
- Switch the mobile phase solvent to methanol (this seems to work only in Chrome?), and record your answers to the following questions: What happened to the retention times in the table? Are the peaks in the same elution order as before? If not, which peaks have switched places? (2:50 pm)
- Adjust the starting and ending %B to optimize the gradient separation again, having all members of your group working simultaneously / independently to explore the 3-D parameter space, as before. Was your group able to create a separation that is better and faster than with acetonitrile?
- Record your optimized *methanol* gradient parameters and the retention times of the 10 analytes in your ELN. Export the optimized chromatogram generated using this gradient, change the name of the file to "FullChromatogramMeOH.csv" and save it to your Chem 220 folder.
- On a relative polarity scale (where alkanes = 0 and water = 1), methanol = 0.762 and acetonitrile = 0.460. Methanol is also a protic solvent (possessing H atoms capable of H-bonding), like water. In your ELN, explain how these facts are consistent with the solvent-swapping observations that you just wrote down, paying special attention to any peaks that swapped their elution order. You may need to look up the structure of these molecules to provide a good O-chem-level explanation of what is happening.
- If you had to analyze samples containing these 10 analytes in lab next week, which mobile phase system would you choose: acetonitrile / water gradient, methanol / water gradient, isocratic acetonitrile / water, or isocratic methanol / water? Justify your choice based on today's results.

Lab exercise part 3: Things you can't easily do on a non-virtual HPLC[2]

One of the advantages of a virtual HPLC is that you can vary parameters that normally aren't accessible, and column parameters like "void time" (t_m) and the number of theoretical plates (N) are calculated automatically by the software.

- In the simulator, set the mobile phase flow rate to 2.0 ml/min and the temperature to 25 ºC. Set the mobile phase composition to 50% methanol isocratic. Remove naphthalene from the compound list.
- Record the retention time for phenol under these conditions.
- Record the void time (under Column Properties).
- Write the equation for the retention factor, k, then calculate the retention factor for phenol, making sure that units cancel.
- Compare it to the one listed in the table under the chromatogram. Do they agree?

[2] Part 3 based on an exercise written by Prof. Mark Vitha, Drake University

Effect of C-term, and particle size on resolution

- Record your group's prediction for what would happen if you switched to a column with a higher *C*-term in the van Deemter equation.
- Now change the *C*-term from 0.05, to 0.25, to 1.0 and note the changes in the resolution. Record these observations, and also the plate height output (HETP under Chromatographic Properties) Do the changes in resolution agree with your prediction?
- Change the C-term back to 0.05. Note that on a non-virtual HPLC, you cannot enter in whatever van Deemter equation terms that you want. These are determined by the column dimensions, packing quality, and history of use. The default column packing particle size in the simulator is 3 μm. Determine by experimentation the column packing particle size (to 3 sig figs) that would result in the same plate height as setting the *C*-term to 0.25. Record this particle size.
- Let's say a separation requires a plate height of 5 x10^{-4} cm, and you are getting ready to purchase a column. What packing particle size should your column have to achieve this plate height?
- Let's also say that the separation requires 10,000 theoretical plates. What length of column should you order?
- Here is a list of Agilent "Zorbax" columns (taken from a British vendor's website). These columns are good for separating basic analytes at low and medium pH. If you needed this type of separation, a plate height of ~5 x10^{-4} cm, and ~10,000 theoretical plates, which column would you order?

Part Number	Short Description	Phase	Length	ID	Particle Size	Price
863608-901	Zorbax Bonus-RP 1 x 150mm 3.5um Column	Polar Embedded	150 mm	1 mm	3.5 um	£492.00
861608-901	Zorbax Bonus-RP 1 x 30mm 3.5um Column	Polar Embedded	30 mm	1 mm	3.5 um	£322.00
865608-901	Zorbax Bonus-RP 1 x 50mm 3.5um Column	Polar Embedded	50 mm	1 mm	3.5 um	£366.00
828768-901	Zorbax Bonus-RP 2.1 x 100mm 1.8um 600 Bar RRHT Column	Polar Embedded	100 mm	2.1 mm	1.8 um	£534.00
861768-901	Zorbax Bonus-RP 2.1 x 100mm 3.5um Column	Polar Embedded	100 mm	2.1 mm	3.5 um	£425.00
863700-901	Zorbax Bonus-RP 2.1 x 150mm 3.5um Column	Polar Embedded	150 mm	2.1 mm	3.5 um	£498.00
883725-901	Zorbax Bonus-RP 2.1 x 150mm 5um Column	Polar Embedded	150 mm	2.1 mm	5 um	£442.00
827768-901	Zorbax Bonus-RP 2.1 x 50mm 1.8um 600 Bar RRHT Column	Polar Embedded	50 mm	2.1 mm	1.8 um	£462.00
861700-901	Zorbax Bonus-RP 2.1 x 50mm 3.5um Column	Polar Embedded	50 mm	2.1 mm	3.5 um	£330.00

- How does the column packing particle size affect the price of the column? Why do you think this is the case?

Lab exercise part 4: Calculating resolution

- Upload the .csv file of your group's best gradient elution (either ACN or MeOH) into the Jupyter notebook for this experiment. Our Python coding goal today is to create peak-recognition algorithms that will locate and integrate all peaks, independently determine their retention times, and calculate their $W_{1/2}$ peak widths. We will then use this to determine the resolution between adjacent peaks, and the relative response of the virtual detector to the 10 analyte species. Our work to make our algorithms pick peaks will be made easier by the fact that the dataset created by the virtual HPLC instrument is noise-free!