Practice Problem Set 12
HPLC

28-1. List the types of substances to which each of the following chromatographic methods is most applicable:
(a) gas-liquid
(b) liquid - partition
(c) reversed-phase partition
(d) ion exchange liquid-liquid partition
(e) gel permeation
(f) gel filtration
(g) gas-solid
(h) liquid adsorption
(i) ion-pair

a. Volatile, thermally stable substances
b. Nonvolatile, thermally unstable compounds of low to moderate molecular weights
c. Nonvolatile, thermally unstable compounds of low to moderate molecular weights
d. Ionic species
e. High molecular weight non polar compounds
f. High molecular weight polar compounds
g. Low molecular weight gases
h. Low to moderate polarity solutes of low to moderate molecular weight
i. ionic species using RPLC stationary phases


Resolution in partition chromatography can be improved when:

a. Changing the pH of the mobile phase
b. Changing the percentage of organic modifier in the mobile phase
c. Changing the type of organic modifier in the mobile phase
d. Changing the temperature
e. Increasing column length

f. Adjusting factors that will minimize band broadening

28-3. Describe a way to manipulate the retention factor of a solute in partition chromatography.

The retention factor in partition chromatography can be manipulated by:

a. Changing the pH of the mobile phase

b. Changing the percentage of organic modifier in the mobile phase

c. Changing Temperature

d. Changing the thickness or chain length of stationary phase

e. Increasing column length

28-4. a. How can the selectivity factor be manipulated in (a) gas chromatography and (b) LC?

The selectivity factor in gas chromatography can be manipulated by changing the temperature.

b. The selectivity factor in liquid chromatography can be manipulated by:

1. Changing the nature of the stationary phase.

2. Changing the mobile phase composition

3. Changing the type of organic modifier in the mobile phase

4. Adjusting the temperature

28-5. In preparing a hexane-acetone gradient for an alumina HPLC column, is it desirable to increase or decrease the proportion of hexane as the column is eluted?

This is a liquid solid chromatography where the stationary phase is polar. For such a gradient, one should start with the less polar solvent and increase the percentage of the more polar solvent with time in order to force late eluting solutes (more strongly adsorbed) to leave the column.
28-6. Define
(a) isocratic elution.
(b) gradient elution.
(c) extra column band broadening
(d) reversed-phase packing.
(e) normal-phase packing.
(f) bulk property detector.
(g) solute property detector.

a. Isocratic elution is an elution process under constant mobile phase composition throughout the whole process

b. Gradient elution is a process through which the strength of the mobile phase is changed during the separation process

c. Extra-column band broadening occur due to following reasons:
1. Incompatible fittings
2. Tubing length and diameters
3. Detector cell volume
4. Sample volume

d. Reversed-phase packing material is the packing which has a non polar bonded stationary phase in the range from C_{1}-C_{18}

e. Normal phase packing material is the packing which has a polar group terminated stationary phase (like ~OH, NH_{2}, CN, etc)
f. A bulk property detector is a detector that responds to changes in a mobile phase property due to presence of solute (like refractive index)
g. A solute property detector is a detector that responds to a solute property (like absorption of UV-Vis radiation, fluorescence, etc)

28-7. What is meant by the linear-response range of a detector?

The linear response range of a detector is the range of analyte concentrations to which the calibration curve is linear

28-8. What is a guard column in partition chromatography?
A guard column is a short column of the same packing material as the analytical column where it is connected before the analytical column in order to protect it from any contamination from sample matrix. This will extend the lifetime of the analytical column

28-9. What are the properties of a desirable HPLC detector?

Properties of an ideal detector includes:

1. High sensitivity
2. Minimum drift
3. Wide dynamic range
4. Fast response time
5. Same response factor for all solutes
6. Good reliability (no fooling)
7. Responds to all solutes (universal)
8. Very small volume (2-10 µL)

28-12. Compare between the mobile phases used in GC and HPLC.

The mobile phase in gas chromatography should be of high viscosity to decrease longitudinal diffusion and should also be compatible with the detector. It serves to carry the solutes through the column. In liquid chromatography, the mobile phase composition must be optimized as solutes partition between mobile and stationary phases. Also changing the nature of the organic modifier in the mobile phase will change the selectivity factor. The viscosity of the mobile phase in LC is chosen as low as possible to increase diffusion coefficient of solute in the mobile phase and thus decrease $H_M$

28-13. In a normal phase partition column, a solute was found to have a retention time of 29.1 min, while an unretained solute had a retention time of 1.05 min when the mobile phase was 50% chloroform:50% hexane. Calculate $K'$ for the solute and find the mobile phase composition that will bring $K'$ to 10. ($P'_{\text{hexane}} = 0.1$, $P'_{\text{chloroform}} = 4.1$)

$k'_1 = (29.1 - 1.05)/1.05 = 26.7$
\[ P'_1 = 0.50 \times 4.1 + 0.50 \times 0.1 = 2.1 \]

\[ \log(K'_2/K'_1) = (P'_1 - P'_2)/2 \text{ (the equation is set up this way since we deal with NPC not RPLC)} \]

\[ \log(10/26.7) = (2.1 - P'_2)/2 \]

\[ P'_2 = 2.95 \]

\[ 2.95 = x \times 4.1 + (1-x) \times 0.1 \]

\[ x = 0.71 \]

\% Chloroform = 71\% and \% Hexane = 29\%

28-14. What can you do if K' was satisfactory but you still can not get acceptable resolution?

In case the retention factor required (10) was satisfactory, an attempt to exchange chloroform by another solvent will certainly change selectivity and may lead to better separation.

28-15. Predict the order of elution of the following compounds on a C\textsubscript{18} column using RPLC:

a. n-hexane, hexanol, and benzene
diethyl ether, nitrobutane, and ethyl acetate

In RPLC, solutes will be eluted in a reverse order to NPC where retention will increase with decreasing polarity. Therefore in terms of retention time from highest to lowest we have:

a. n-Hexane>benzene>hexanol

b. diethylether>ethylacetate>Nitrobutane

28-16. Estimate the distribution constant for compounds B and C in the figure below, if the retention volumes for compounds A and D were 5.1 and 14.2 mL.
For very large molecules, $K = 0$

$$K = \frac{(V_e - V_o)}{V_i}$$

$V_o = 5.1$

For very small molecules, $K = 1$, and:

$$V_i = 14.2 - 5.1 = 9.1$$

From the graph, $V_e (B) = 8.6$

$$K = \frac{(V_e - V_o)}{V_i}$$
\[ K = \frac{(8.6 - 5.1)}{9.1} = 0.38 \]

From the graph, \( V_e(C) = 11.7 \)

\[ K = \frac{(V_e - V_o)}{V_i} \]

\[ K = \frac{(11.7 - 5.1)}{9.1} = 0.73 \]

**Questions from the Internet**

**basic components for a HPLC system?**

A workable HPLC system must include a sampler, a pump, a column, a detector, and data processor (computer with software). An degasser and a column oven may also be used for better quality of analysis. Basically there are two types of designs. One is integrated design which build all the components into one box. The other type is modular design which allows swap and change of components after installation.

**2. There are so many brands of HPLC available in the market, how do I judge which one is the best for me?**

Although the specification and price are important, they should not be the only standard. For many chemists, the meaning of the specification may not be easy to understand. Sometimes the sales person may mislead you using their specification. Here are some other items you may consider: a) feedback from other users, b) hands-on experience or an instrument demo, c) support capability of the vendor, d) cost of maintenance, e) possibility of future upgrade.

**3. What are the important specifications for a sampler?**

Reproducibility, range of injection volume, linearity over the injection range, and carry over. A good linearity is very helpful when your sample concentration varies a lot or when you want to make a calibration curve using only one or two standard solutions. The carry over property is very critical in trace analysis. Sometimes a very complicated washing program has to be used before each sample run to avoid carry over problem.

**4. What are the difference between a high pressure gradient pump and a low pressure gradient pump?**

In a low pressure gradient pump, different mobile phase is mixed using a valve before entering the pump head. In a high pressure gradient pump, each different mobile phase is delivered by an individual pump head and then mixed at the pump outlet. The low-pressure gradient design is of lower cost as it only use one pump head. It can also use more types of mobile phase without significant increase of cost. The high-pressure gradient design provides a much faster gradient since solvent mixing point is much closer to the column head. This is measured using delay volume. The value can be 50-300 uL for high pressure gradient pump and can be 2 to 3 times larger for a low pressure gradient pump. A small
delay volume is important when the analysis time is short or the flow rate is low. If the delay volume is too large, it become impossible to obtain reproducible gradient run since the planned composition cannot reach the column head before a run is finished.

5. What are the important specifications for an isocratic pump?

Maximum working pressure, flow rate range, flow reproducibility and accuracy, pressure pulsation, pressure monitoring and over pressure shut-off function.

6. What are the important specifications for a gradient pump?

Maximum working pressure, flow rate range, flow reproducibility and accuracy, gradient reproducibility and accuracy, delay volume, pressure pulsation, pressure monitoring and over pressure shut-off function.

7. Why is an on-line degasser always recommended for a gradient pump?

Permanent gas has different solubility in different solvents. When different solvents are mixed on line, the dissolved gas can be released from solvent due to changed solubility. To verify this, you may add some water to methanol and observe the large amount of bubbles released from methanol. The bubble formed can cause higher baseline noise and shift of retention time. If you are doing an isocratic LC, a degasser is not so critical since most bubble has been removed when you filter the mobile phase under vacuum.

8. How many types of detectors are available for HPLC?

UV detector, fluorescence detector, electrochemical detector, conductivity detector, refractive index detector, evaporative light scattering detector, chiral detector, radioactive detector, mass spectrometry detector (MSD).

9. How to select a suitable detector according to my application?

If your compounds absorb UV light within the range above 200 nm, a UV type detector could be the best choice. It is the most robust and most widely used HPLC detector. Fluorescence detector can have 10 to 100 times better sensitivity for some compounds of large aromatic ring in the structure (such as poly aromatic hydrocarbons). Electrochemical detector is useful for compounds that can be easily electrochemically reduced or oxidized, such as phenols, aromatic amines, and some carbohydrates. It can produce better method sensitivity for such compounds than a UV detector due to better selectivity. However, as the electrodes have direct contact with column effluent and involve reaction with sample matrix, the response may not be as stable as a UV detector. Conductivity detector is mainly for inorganic ions in an ion exchange based liquid chromatographic system. Normally a suppressor is needed to remove background ions from mobile phase for a satisfactory detection sensitivity. Refractive index detector can detect any compounds but the sensitivity is around 100-1000 times lower than a UV detector. Another limit of this detector is that it cannot do gradient LC. This detector is mainly used for organic compounds that do not have reasonable UV absorbance, such as small organic acids, carbohydrates, and some polymers. Evaporative light scattering detector is of similar application range as refractive index detector and it can allow gradient HPLC. The disadvantage of this detector is consumption of large amount of nitrogen for evaporating the mobile phase. MSD is getting more and more popular although the price is 10-20 times higher than other
HPLC detectors. It can act as a general purpose detector using scan mode or as an highly selective detector using selective ion monitoring. The most important advantage of MSD is in identity confirmation.

10. How to choose the flow cell volume for a UV detector?

If the flow cell is too large, two adjacent peaks may get mixed in the cell. If it is too small, the noise may be higher due to less light reaching the photo diodes. The higher back pressure could also limit the flow rate range. The peak size may be used for the selection. A narrow peak need a smaller flow cell. The rule of thumb is the flow cell volume should not be more than 1/3 of the peak volume. For example, if the peak width is 0.1 min at 1 mL flow rate, the flow cell volume should not be more than 33 uL. If your flow rate is 0.01 mL/min, the flow cell volume should be around 0.3 uL.

11. How to choose an equivalent C18 column for my HPLC?

The situation in HPLC column is quite different from GC columns. C18 or ODS columns from different supplier can give very different elution profile. This is because the silica properties, the carbon coverage and the surface area varies considerably. If you want to be sure of the same elution pattern without changing the mobile phase, the column from the same manufacturer should be used. If you want to try a column of similar properties, you can look at columns that have similar surface area and carbon loading for the packing material.

12. How to choose a guard column?

Its volume should not be more than 10% of the analytical column. The packing should be similar to the one in the analytical column. If this is not available, you may use a guard column which has weaker retention to your analytes than the analytical column.

13. What are important specifications for a column?

Separation efficiency, inertness, durability, pH range, and batch to batch reproducibility.

14. How to choose a chiral column?

It is difficult to predict if a chiral column will give good separation to a pair of chiral isomers. So the best way is to search literature to see if it has been done before. Or you may send your sample to the column supplier for a sample testing.

15. Some columns are end capped. What is it good for?

When the silica is bonded with C18, or C8, there are still some active sites on the silica left over. An end capping using a more active short chain can make the column more inert to some basic compounds and make the column more resistant to basic dissolution to the silica material. However such capping is not stable at low pH (<3). So normally such column is not very suitable for mobile phase of low pH.

16. How to make a column last longer?
Filter your sample and mobile phase. Make sure the pH of the mobile phase is within the working range of the column. Flush the column with methanol or acetonitrile if it is not going to be used for sometime.

17. Why acetonitrile and methanol are most commonly used solvent for reverse phase HPLC?

These two solvents can mix well with water at any ratio and do not have significant UV absorption within the UV detection range. They are also easy to obtain.

18. What are the differences between acetonitrile and methanol as mobile phase?

Acetonitrile has lower UV absorbance at short UV region (200-210 nm) and is better choice if your UV detection is in this region (such as for some small organic acids). Acetonitrile also generate lower back pressure than methanol. This is good when you are trying to use higher flow rate or when your column is partially clogged. On the other hand, methanol is of lower cost. It is also more stable than acetonitrile. When acetonitrile is dry and is exposed to ambient light, some polymers may be produced which may block your HPLC valves or filters. It is a good idea to use amber bottles for acetonitrile.

19. What are the differences between reverse phase HPLC and normal phase HPLC?

In the reverse phase LC, the mobile phase is polar and the column packing is non-polar. So compounds of high polarity will elute faster than compounds of low polarity. In case of normal phase HPLC the order is opposite. The column packing is polar and the mobile phase is of low polarity. Compounds of low polarity will elute faster. Nowadays most people use reverse phase LC due to its better reproducibility and ease in solvent handling. Normal phase LC is easier to have retention time shift due to moisture build up in the silica packing. Normally a gradient elution cannot be used for normal phase LC. However normal phase LC do have its advantages. It is good for separation of isomers and compounds of very low polarity. Normally reverse phase LC is not good for such situations.

20. Which parameter can tells the instrument status well?

The pump pressure. It reflects the status of column and the system. A very low pressure (e.g. <10 bar) indicates leak in the system. A very high pressure tells some block in the column or tubing. If the pressure fluctuate widely (e.g. change from 10 bar to above 50 bar), the pump inlet may have been blocked, some bubble could be in the system, or the inlet valve is malfunctioning. It is a good habit to keep an eye on the pressure while the instrument is running.

Try working out these questions
1. Given the HPLC chromatogram above for a mixture of nucleosides, calculate the resolution between the Guanosine and Adenosine peaks using your best estimate of the required parameters from the chromatogram.

2. What is a theoretical plate? What are some factors that determine N?

3. What is gradient elution and why would you need to use it? Give two examples of types of gradients used in chromatography.

4. Given the HPLC chromatogram above for a mixture of barbiturates, calculate the number of theoretical plates based on the Amobarbital peak using your best estimate of the required parameters from the chromatogram.

5. Referring to the chromatogram at the top of the page, assuming that barbital is more polar than phenobarbital which is more polar than talbutal, etc, was this experiment run under normal or reverse phase conditions?

6. Short Answer:
   a. In liquid chromatography, the mobile / stationary phase is always liquid. (circle one).
   b. Which HETP is better, 0.5 mm or 1.0 mm?
   c. The main purpose of chromatography is _____________________________.
   d. The most common HPLC detector is: ___________________________

7. Give the best match for the terms in the first list with the characteristics in the second:

   Peak Identification
   1. Barbital (0.25 mg/ml)
   2. Phenobarbital (0.1 mg/ml)
   3. Talbutal (0.3 mg/ml)
   4. Amobarbital (0.25 mg/ml)
   5. Mephobarbital (0.1 mg/ml)
A. One common stationary phase is DEAE

B. Mobile phase is more polar than stationary phase

C. Water in sample may cause interference because of its attraction to the stationary phase.

D. Smallest solute particles elute slowly.

8. Give the best match for the terms in the first list with the characteristics in the second:

<table>
<thead>
<tr>
<th>Term</th>
<th>Characteristic</th>
</tr>
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<tbody>
<tr>
<td>normal phase chromatography</td>
<td>A. Detects the current change as the eluent passes through a flame.</td>
</tr>
<tr>
<td>ion-exchange chromatography</td>
<td>B. Detects the current change as the eluent passes through a stream of high energy electrons.</td>
</tr>
<tr>
<td>reverse phase chromatography</td>
<td>C. Detects the current change due to the difference in heat conductance of mobile phase and analyte.</td>
</tr>
<tr>
<td>size exclusion chromatography</td>
<td></td>
</tr>
</tbody>
</table>

All of the above are: ____________________________________________ .

9. Give definitions of and describe the differences between WCOT, SCOT, and PLOT columns.

10. Sketch a simple diagram of a GC. Identify all components and comment on how the temperature is adjusted for the three temperature zones.