Chapter 28 High Performance Liquid Chromatography

Problems: 1, 2, 3, 4, 6, 7, 9, 12, 13, 15, 18

HPLC
Includes 4 basic types of chromatography with liquid mobile phase
- Partition
- Adsorption or Liquid-Solid
- Ion exchange
- Size exclusion or gel permeation

Will also have brief into to planar chromatography because good diagnostic tool

Earliest, like that done by Tswett
- Glass column 1-5 cm diameter 50-500 cm length
  - for good flow particles in 150-200 um diameter range
  - flow fraction of an ml/min
- if tried to speed up with vacuum or pressure plate height increases, and separation efficiency decreased

1960's figured out how to drop packing material diameters down to 3-10 um, then could increase flow rate with P and not lose efficiency. HPLC refers to these newer methods, to distinguish from older methods that are still used for preparative purposes.

28A Scope of HPLC
most widely used separation method
annual sales of HPLC equipment in the billion dollar range
- sensitivity
- easily adapted
- quantitative
- can be used for all sorts of nonvolatile substances

Figure 28-1
HPLC includes several different complementary techniques

28B Column efficiency
Will Go back to chapter 26 to review column efficiency.
Will also introduce some additional sources of band spreading that are unique to liquid chromatography

28B-1 Effects of Particle Size of packing
- mass transfer coefficient ($C_n$) related to square of particle size
- smaller the particle, the more efficient the column

see figure 28-2
Should see a minimum in flow rate, but usually this minimum flow rate is to low to be practical

28B-2 Extra-Column Band Broadening in LC
in LC significant band broadening can occur OUTSIDE the column occurs as solvent moves through tubes connecting things together difference in flow rate between materials in center and at walls of tubing

Didn’t see this in GC, because was masked by diffusion. In LC diffusion is 10x slower so can now see this problem

Equation:

\[ H_{ex} = \frac{\pi r^2 \mu t}{24 D_m} \]

\( H_{ex} \) is plate height due to external broadening
\( r \) is tubing diameter
\( \mu \) is flow rate
\( D_m \) is diffusion coefficient of solute in mobile phase (cm² /min)

Most serious in small bore columns
need to reduce radius of extra column tubing to < .1 inch

(Typical HPLC ID from .005" to .08")

28B-3 Effect of Sample Size on Column Efficiency

figure 28-3
sample size measured in ug sample/ g packing material
In general plate height increases with sample size
But note that reversed-phase bonded packing show little effect
(Poor figure doesn’t get that point across)

28C Instruments
for reasonable flow rate with 2-10um particles need pumps that operate to several thousand psi

This high pressure requirement makes HPLC equipment more expensive

typical system shown in figure 28-4

1 or more solvent reservoirs
Usually equipped with means for removing dissolved gases \( O_2 \) and \( N_2 \)
Interfere by sometimes forming bubbles
Bubbles can cause band spreading
Can interfere with detector
Several ways to remove
1. Apply vacuum
2. Sparge (bubble a low solubility gas through)
3. Pre filter though a millipore filter (temporary)
4. (Ours) run solvent through gortex tubing under vacuum as flows into HPLC

Separation can be
‘isocratic’ a single solvent composition
‘Gradient’ Solvent composition changes during run
Usually greatly enhances separation
Can be 2 or three solvents
Can be changes in steps or gradually
Utility illustrated in figure 28-5

28C-2 Pumping Systems
Should be:
need to generate up to 6000 psi
(That’s what mine do, but generally start blowing leaks about 4000)
Pulse free output
0.1 to 10 mls /min flow rate
need flow rate reproducibility to .5% error
must be corrosion resistant
While at high pressure, no explosion hazard
Will just get an annoying leak
Solvent may be flammable, however.
3 different pump designs

Reciprocating pumps Figure 28-6
Small chamber with a plunger that moves back and forth to pump solvent
Used in 90% of all HPLC systems
Used in my system
Need 2 ball and check valves to control flow into and out of plunger cylinder

Advantages
Small volume
High output pressure
Flow independent of pressure
Disadvantage
Get pulses of pressure or flow
Needs to be dampened or shows up in detector output
**Displacement Pump**
Essentially a large screw driven plunger
Advantage- no pulses in flow
Disadvantage - once empty (maybe 250 ml) nothing more to give

**Pneumatic Pumps**
Mobile phase in a container that is pressurized to
Advantages - cheap pulse free
Dis- limited capacity and pressure, flow rate depends on solvent viscosity

**Flow control and Programming systems**
Use computer to monitor pumps
Use computer to mix gradients
Use computer to keep P at set levels

**28C-3 Sample Injection Systems**
Often limiting factor in quantitative work is getting sample on column reproducibly.

Want limited volumes, 5ul to 2 ml

Most common is use of injection loop with syringe figure 28-7
can get good reproducibility (<1%) if careful

**28C-4 Liquid Chromatographic Columns**
usually steel walled tubes
filled with hundreds of different kinds of material
prices in 200-$500 range

**Analytical Columns**
10-30 cm long
If you want longer, hook them together
Inside diameter 4 to 10 mm
Particle size 5 or 10 μm

Some new high-speed, high-performance
1 to 4.6 mm diameter
3-5 μm packing
3-7.5 cm length
High speed, minimal volume
So don’t waste solvent
Can get separations in < 1 minute!
Guard Columns
Short column containing same material as analytical column
Placed before regular column
Acts as filter, and will bind anything that binds to column irreversibly
Cheaper to replace this than the entire column

Column Thermostats
For most applications column T is not critical and can be at ambient T
Sometimes more critical or just gives better reproducibility if control T so add a column thermostat/heater

28C-5 Types of Column Packings
two basic types
Pellicular
Spherical, nonporous, glass or polymer beads, 30-40μm diameter
Thin layer of silica, alumina or polystyrene resin or ion exchange resin deposited on surface
Sometimes an additional coating as well
Usually for guard columns not analytical columns
Porous
Porous microparticles ranging from 3-10μm diameter
Try to have as uniform size as possible
Silica, alumina or resin
Silica most common
  Made by agglomerating submicron particles together
  Sometimes coat outside with organic film

28C-6 Detectors
No universal detector
2 major types of detectors
Bulk property - respond to mobile-phase bulk properties like refractive index, dielectric constant, or density which change slightly in the presence of solute
Solute Property - responds to some property of solute itself (UV or vis, fluorescence etc.)

Table 28-1
1982 74% of detectors were UV absorbance 15% fluorescence

Absorbance detectors
Figure 28-9
Typical Z-shaped flow cell
Volume only about 1 to 10 μl so broadening minimized
Can get pathlength of 2-10 mm!
Can only take about 600psi
Many times set up as double beam so have 2 cells or cell and filter

Some detectors simple, use Hg lamp for a few lines
And filters to isolate lines
($3,000)
Other more complicated, actual lamp and monochromometer
Set to a precise wavelength
($5,000-8,000)
Best use photodiode array
So get complete UV spectrum for every second that HPLC runs

**IR detectors**

**Fluorescence Detectors** ($8,600)

**Refractive Index Detectors**

*Figure 28-11*
Refractive index? How much light is bent entering or leaving a medium

Good in that responds to nearly every solute so almost universal
Bad in that not very sensitive and very T sensitive

**Evaporative Light Scattering (ESLD)**
New, just hit market in last 3-5 years
Column effluent passed into a nebulizer,
Converted to a mist with N₂ gas
Solvent evaporates, leaving mist of solute
Solute mist passes through laser beam and amount light scattered from solute mist is measured

Good- responds nearly the same for all nonvolatile solutes
More sensitive than Refractive index
Detects down to 5 ng/25 µl
Cost $10,000-15,000

**Electrochemical -skip**

**Mass Spectrometric**
Just like GC/MS, but additional sample problem
1. Reduce volume - split column effluent - take only a fraction of column output
2. vaporize sample - various designs

**28D Partition Chromatography**
Partition - most widely used type
in past used for nonionic, polar compounds up to 3000 MW
new techniques extent to ionic compounds as well

2 subdivision
Liquid-liquid liquid stationary phase retained on solid matrix by adsorption
Bonded-phase liquid stationary phase chemically bonded to matric surface

Originally was all liquid-liquid. Now almost all Bonded phase, to that is all we will look at

**28D-1 Columns for Bonded-Phase Chromatography**
Solid support
silica or silica based
formed as mechanically sturdy particles uniform, porous
Diameter of 3.5 or 10μm
Surface fully hydrolyzed by heating in 0.1M HCl of a day or two

\[
\text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OH} \\
\text{-Si-} \quad \text{O-} \quad \text{Si-} \quad \text{-O-} \quad \text{-Si-} \\
\bigg| \quad \bigg| \quad \bigg| \quad \bigg| \quad \bigg|
\]

Surface then bonded to coating
Cl-Si(CH₃)₂R

So Si-OH + Cl-Si(CH₃)₂R → Si-O-Si(CH₃)₂R

Due to steric effect maximum density of coating in 4 μmol/m²
Unreacted OH groups make slightly polar and add unfavorable polar interaction that creates tailing artifacts
So add a second step to react with chlorotrimethyl silane to cap the free OH’s (This reagent physically smaller so can get in and react.)

**Reversed-Phase and Normal Phase Packings**
Early chromatography used polar stationary phase and nonpolar solvents. This was termed Normal phase

Later started using nonpolar stationary phase and polar solvent
This then called reverse phase
Normal phase (stationary is polar, mobile is nonpolar)
least polar elutes first
Increasing polarity of mobile decreases elution time

Reverse phase (Stationary nonpolar, mobile is polar)
Most polar elutes first
Increasing polarity of solvent increases elution time

Most columns are reverse phase (roughly 75%)
R usually
C8 or C18

Mobile phase a mixture of water and miscible, less polar solvents like acetonitrile, methanol, tetrahydrofuran

Don’t want pH > 7.5 because this can lead to hydrolysis of Bond between R and Si

Normal phase
R = C$_2$H$_4$CN (Least polar), C$_3$H$_6$OCH$_2$CHOHCH$_2$OH
C$_3$H$_5$NH$_2$ or C$_3$H$_6$N(CH$_3$)$_2$ (most Polar)
Solvent ethylether, chloroform, n-hexane

28D-2 Method Development
More complicated than GC
In GC carrier gas was largely irrelevant was inert
In HPLC solute interact with both solvent and stationary phase
Need proper balance between solute, solvent and stationary phase

Relative polarities
Least polar
hydrocarbons<ethers<esters<ketones<aldehydes<amides<alcohols<water
Try to match column polarity to polarity of solute, then use solvent of different polarity

No good overall theory, so have to use trial and error

Mobile-Phase Selection in Partition Chromatography
To improve resolution can change N, k’ and α
k’ strongly depends on solvent composition so can be easily changed experimentally
k’ best between 2 and 5, but still works if .5 to 20
can also change α with mobile phase composition or with changing column material
Effect of Solvent Strength on Retention Factors

Solvent that interact strongly with solutes
- Called ‘strong’ solvents
- Are polar

P’ polarity index developed by Snyder for ranking solvents (Table 28-2)

If you want a polarity in between use a mixture of solvent

\[ P'_{AB} = \phi_A P'_{A} + \phi_B P'_{B} \]

\( \phi \) is volume fraction

Change in p’ of 2 units is about equal to a 10-fold change in k’

\[ \text{Ie } K'_2/k'_1 = 10^{(P_{2}-P_{1})/2} \]

Practical example (Example 28-1 from text)

Using a solvent system of 30% methanol and water
- Unretained fraction comes out in .48 minute
- The fraction you want comes out in 31.3 minutes

What is k’

How should you change solvent to get k’=5

When will the solute elute in this system?

\[ K' = (31.3-.48)/.48 = 64 \]

P’ for current solvent is:

\[ .3(5.1)+.7(10.2) = 8.7 \]

Want new k’ to be 5 so

\[ K'_2/k'_1 = 5/64 = 10^{(x-8.7)/2} \]

\[ .078=10^{(x-8.7)/2} \]

\[ \log(.078)=(x-8.7)/2 \]

\[ -1.11 = (x-8.7)/2 \]

\[ -2.22 = (x-8.7) \]

\[ -2.22 + 8.7 = x \]

\[ X=6.5 \]

So what does our solvent composition have to be?

\[ 6.5= X(5.1) + Y(10.2) \]

\[ X+Y=1 \]

\[ X=1-Y \]

\[ 6.5=(1-Y)5.1 + Y(10.2) \]
\[
6.5 = 5.1 \cdot -5.1Y + 10.2Y \\
6.5 - 5.1 = (10.2 - 5.1)Y \\
1.4 = 5.1Y \\
Y = \frac{1.4}{5.1} \\
= 27.4, \text{ call it } 27\% \text{ water and } 73\% \text{ methanol}
\]

Retention time is now
\[
K' = 5 = (X - 0.48)/0.48 \\
5 \cdot 0.48 = X - 0.48 \\
2.4 = X - 0.48 \\
2.4 + 0.48 = X \\
X = 2.88
\]
So you have shortened your run from 31.3 minutes to 2.88 minutes!

**Graphic example figure 28-6a and b**

A \( K' = 5 \), 41\% acetonitrile-59\% water separation in about 2 min, poor resolution

Increase % water to 70, \( k' \) increases to about 10, time on column about doubled, and start to see better resolution, but still not the greatest

**Effect of Mobile phase Selectivities**

In above example need more work to get early band separated
Need to change \( \alpha \) (selectivity factor)
Try a different solvent system with \( k' \) about the same
4 compatible solvents are:
- Water, methanol, acetonitrile, and tetrahydrofuran (THF)
Essentially get \( k' \) where you want it they try the 2 other solvents to see if you can get better resolution of the close peaks

Can do same in Normal Phase chromatography
- Here the solvents to mix are: ethyl ether, methylene chloride, chloroform,
- and the solvent strength is adjusted with n-hexane

**28D-3 Applications of Partition Chromatography**

Reverse phase packings and highly polar solvents almost ideal, universal system wide range of applications Find all over the place

**Derivative Formation**

Sometimes make derivatives of components
- Reduce polarity so works better in adsorption chromatography
Increase detector response
(Make fluorescent)
To selectively enhance detector response for a component

**Ion Pair Chromatography**
Special reverse phase technique used for ionic species
Mobile phase - aqueous buffer with organic solvent
   - Like methanol or acetonitrile
   - And ionic compound containing a counter-ion (opposite change) to analyte
Counterion and solute form neutral ion pair that separates on reversephase column
Applications overlap ionexchange
Ion exchange usually better for small ions
   - But doesn’t do as well with large ions

**Chiral Stationary Phases**
Use chiral stationary phase to separate enantiomers
Works better in HPLC than in GC

**28E Adsorption Chromatography**
liquid-solid chromatography
the original chromatography by Tswett
has been adapted for HPLC
Silica or alumina solid phase, but alumina has higher capacity and wider range of useful forms
Generally silica and alumina are similar
Retention
   - olefin < aromatic < halides, sulfides < ethers < nitrocompounds < esters, aldehydes, ketones < alcohols, amines < sulfones < sulfoxides < amide < carboxylic acids

**28E-1 Solvents**
again wide variety of solvent and solvent mixtures to optimize separation

**Solvent Strength**
the polarity index \( P' \) used for reverse phase works fairly well here
but eluent strength \( e^o \) on same table is actually better
parameters in table for alumina
   - Multiply by .8 for silica

**Choice of solvent systems**
similar system to one used in partition systems
choose 2 compatible solvents one too strong, and one to weak
vary the ratio between the two
c" does not vary linearly with volumes, so can’t use the same math, have
to experiment a bit more
if you end up with overlapping peaks, change one strong solvent for
another but keep k’ for the mixture the same to see if you can get resolution

28E-2 Applications
most suited for nonpolar compounds with MW < about 5000
some overlap with partition, but largely complementary
some differentiation between isomers!

28F Ion-Exchange Chromatography (IC)
separates ions based on ion-exchange resin

28F-1 Ion Exchange Equilibria
Based on exchange of ions of same sign between soluble ions in solutions and
ions on the surface of an insoluble high MW solid

first produced in 1930's for water softening
Most common
cation exchangers
Strong acid SO₃⁻H⁺
Weak acid COO⁻H⁺
Anion exchangers
Tertiary amines -N(CH₃)₃⁺OH⁻ (Strong base)
Primary amines -NH₃⁺OH⁻ (weak base)

Use sulfonic acid (Strong cation exchanger) as example

\[ \text{XRSO}_3\text{H}^+(s) + M^{x+}(aq) = \text{XR}(\text{SO}_3)^{-} + M^{x+}(s) + x\text{H}^+(aq) \]

Initially concentration of M^{x+} is high, so equilib shifts left and cation binds
later as was material off column, concentration of M is low so equilib can
shift back and the cation can begin to move down the column

Let’s simplify a bit by assuming that M^{x+} is a monovalent base, B⁺ then we have

\[ \text{XRSO}_3\text{H}^+(s) + B^+(aq) = \text{XR}(\text{SO}_3)^{-} B^+(s) + \text{H}^+(aq) \]

You can treat this like any old equilibrum

\[ K = \frac{[\text{XR}(\text{SO}_3)^{-} B^+](s)[\text{H}^+]}{[\text{XRSO}_3\text{H}^+](s) + [B^+]} \]

rearranging
\[ [\text{XRSO}_3\text{B}^+](s) / [B^+] = K [\text{XRSO}_3\text{H}^+](s)/[\text{H}^+] \]

We can assume that [H^+]>[B^+] and that there are lots more sites on the
resin for binding so that \([\text{XRSO}_3\text{H}^+](s) > \text{[XRSO}_3\text{B}^-](s)\)

This makes the right side of the equation a constant so
\([\text{XRSO}_3\text{B}^-](s)/[\text{B}^-] = K'\)
So we are back to a straightforward binding constant like we had earlier
for partition chromatography, so all the math and parameters that were
based on distribution coefficients can be applied to ion exchange as well

Note that in this treatment the K for affinity represents the relative affinity
for B with respect to H^+

Thus we will work with relative affinities (much like we worked with relative
polarities)

In General polyvalent ions have a much higher affinity than monovalent
ions
If you are looking at ions of the same net charge then
Size of hydrated ion and other properties come into play

For Sulfonated cation exchangers
\[K(\text{large})\]
\[\text{Ba}^{2+} > \text{Pb}^{2+} > \text{Sr} > \text{Ca} > \text{Ni} > \text{Cd} > \text{Cu} > \text{Co} > \text{Zn} > \text{Mg} > \text{UO}_2 \text{ (All +2)}\]
\[\text{Tl}^+ > \text{Ag}^+ > \text{Cs}^+ > \text{Rb}^+ > \text{K}^+ > \text{NH}_4^+ > \text{Na}^+ > \text{H}^+ > \text{Li}^+\]

Ks for anions on strong base exchangers
\[\text{SO}_4^{2-} > \text{C}_2\text{O}_4^{2-} > \text{I}^- > \text{NO}_3^- > \text{Br}^- > \text{Cl}^- > \text{CHO}_2^- > \text{CH}_3\text{COO}^- > \text{OH}^- > \text{F}^-\]

**28F-2 Ion-Exchange Packings**
Originally ion exchange resins were small porous beads of styrene and
divinylbenzene with a typical structure shown in figure 28-21
Can see linear polymer and crosslinks and functional groups
Works great for gravity filtration, but not for HPLC
Slow diffusion of ions through spheres
Spheres were compressible

New generation
Pellicular beads - glass or polymer
30-40\(\mu\)m diameter
Ion exchange coated on the bead
Use silica microparticles
Coat with a thin film of exchanger

**28F-3 Inorganic applications**
mobile phase water or water mixed with miscible organic solvents
mobile phase also has ionic species as buffer and also to enhance separation

Since this chromatography deals with ions, an conductivity detector is close to ideal, since it would sense every ion coming off the column

One major drawback. Can you think of it?
Ions in solvent, usually much higher concentration!

Need a way to get rid of solvent ions

**Eluent Suppressor Columns**
1975
Pass though a second column
This column designed to remove solvent ions

Example
For cation exchange, solvent is usually HCl (So lots of H+ )

Suppressor column is an anion exchanger  Resin+Cl-

\[
H^+(aq) + Cl^-(aq) + \text{resin} \rightarrow \text{Resin} + \text{Cl}^- \quad (\text{Cl bound}) \quad H^+ + OH^- \rightarrow H_2O
\]

So get rid of both!
Works but inconvenient because have to replace frequently
Ways to get around this

**28F-4 Organic and Biochemical Applications of Ion exchange**
Lots

**28F-5 Ion-Exclusion** - skip

**28G Size Exclusion**
Gel permeation or gel filtration

Packing is 10µm silica beads
each bead is filled with network of uniformly sized pores
when molecule enters pore it is removed form external solvent flow
ability of solute to enter pore depends on relative size of solute and the pore
If solute > pore - can’t enter - come right through in void volume
If solute < pore it enters and spends increased time inside the pore comes out last
If solute intermediate, get fractionation according to size
No chemical or physical interactions, in fact try to avoid interactions

28G-1 Column Packings
Silica beads - stronger so can stand higher pressure and wider range of solvents
- but also can adsorb solutes and degrade molecules
Polymer beads

Generally 5-10μm diameter

Again the first beads were styrene-divinyl benzene, but this is hydrophobic so didn’t work with water!

Now hydrophilic gels
Sulfonated divinyl benzenes
Polyacrylamides
Carbohydrates (?) At least in non-HPLC

Porous glass and silica
Pores from 40 to 2500Å
Surface treated with hydrophillic substance to make inert

28G-2 Theory
\( V_{t} \) total volume packed into a column
\( V_{t} = V_{g} + V_{i} + V_{o} \)
- \( V_{g} \) vol of gel (solid matrix)
- \( V_{i} \) = volume of solvent inside the pores
- \( V_{o} \) = volume of solvent outside of pores

Solutes too large to enter will come off in \( V_{o} \)
Solutes small enough to enter will come off in \( V_{i} + V_{o} \)

Solutes of intermediate size come off at some elution volume that reflect amount of material that can actually enter the pore
\( V_{e} = V_{o} + KV_{i} \)

Equation actually applies to all solutes
But where too large \( K=0 \)
When too small \( K=1 \)

Can now make \( K \) into a distribution coefficient
\( K = (V_{e} - V_{o})/V_{i} = c_{S}/c_{M} \)

Again, once we have a \( K \), all the math learned in previous chapters
applies

**General use is shown in figure 28-27**
- When have intermediate size, get $V$, proportional to $\log MW$
- Run a few standards and get $MW$ as well as separation

**Exclusion limit** - size beyond which molecule doesn't penetrate
**Penetration limit** - size below which all molecules penetrate equally

**28G-3 Applications**
- Gel filtration - aqueous solvents, hydrophilic packings
- Gel permeation - nonpolar solvents- hydrophobic packings

Desalting columns - pore a sample through a short column, just long enough that all salts are retained but all large $MW$ species pass through

Separation of oligimers

Separation of proteins DNA, polysaccharides

**Overall Good**
- Short, well defined separation times
- Narrow bands so good sensitivity
- No sample loss

**Problems**
- Short elution time, so limited # of bands can be resolved
- Not useful for separation it two molecules are with 10% of each other's size

**28H TLC - Skip, but you might read for background**