

# 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 intro 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  $\mu\text{m}$  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  $\mu\text{m}$ , 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 ( $C_M$ ) 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 too 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:

$$H_{ex} = \frac{\pi r^2 \mu}{24 D_M}$$

$H_{ex}$  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 (cm<sup>2</sup>/in)

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<sub>2</sub> and N<sub>2</sub>

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 through 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  $\mu\text{m}$

Some new high-speed, high-performance

1 to 4.6 mm diameter

3-5  $\mu\text{m}$  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, 30-40 $\mu$ m 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 3-10 $\mu$ m 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  $\mu$ l 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 monochrometer  
 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 N<sub>2</sub> 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 matrix 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

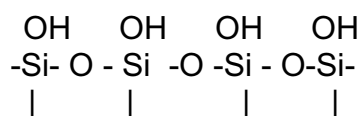
Solid support

silica or silica based

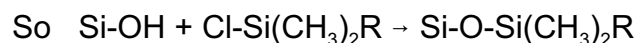
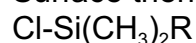
formed as mechanically sturdy particles uniform, porous

Diameter of 3,5 or 10 $\mu$ m

Surface fully hydrolyzed by heating in 0.1M HCl of a day or two



Surface then bonded to coating



Due to steric effect maximum density of coating is 4  $\mu\text{mol/m}^2$

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<sub>2</sub>H<sub>4</sub>CN (Least polar), C<sub>3</sub>H<sub>6</sub>OCH<sub>2</sub>CHOHCH<sub>2</sub>OH  
 C<sub>3</sub>H<sub>6</sub>NH<sub>2</sub> or C<sub>3</sub>H<sub>6</sub>N(CH<sub>3</sub>)<sub>2</sub> (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 fo 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

$$K_2'/k_1' = 5/64 = 10^{(x-8.7)/2}$$

$$.078 = 10^{(x-8.7)/2}$$

$$\text{Log}(.078) = (x-8.7)/2$$

$$-1.11 = (x-8.7)/2$$

$$-2.22 = (x-8.7)$$

$$-2.22 + 8.7 = x$$

$$X = 6.5$$

So what does our solvent composition have to be?

$$6.5 = X(5.1) + Y(10.2)$$

$$X + Y = 1$$

$$X = 1 - Y$$

$$6.5 = (1 - Y)5.1 + Y(10.2)$$

$$6.5 = 5.1 - 5.1Y + 10.2Y$$

$$6.5 - 5.1 = (10.2 - 5.1)Y$$

$$1.4 = 5.1Y$$

$$Y = 1.4 / 5.1$$

$$= 27.4, \text{ call it } 27\% \text{ water and } 73\% \text{ methanol}$$

Retention time is now  
 $K' = 5 = (X - .48) / .48$   
 $5(.48) = X - .48$   
 $2.4 = X - .48$   
 $2.4 + .48 = X$   
 $X = 2.88$   
 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 charge) 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  $\epsilon^0$  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 too weak

vary the ratio between the two  
 $\epsilon^\circ$  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  $\text{SO}_3\text{H}^+$

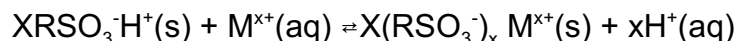
Weak acid  $\text{COO}^-\text{H}^+$

Anion exchangers

Tertiary amines  $-\text{N}(\text{CH}_3)_3^+\text{OH}^-$  (Strong base)

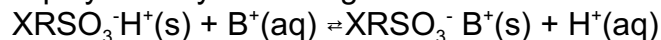
Primary amines  $-\text{NH}_3^+\text{OH}^-$  (weak base)

Use sulfonic acid (Strong cation exchanger) as example



Initially concentration of  $\text{M}^{x+}$  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  $\text{M}^{x+}$  is a monovalent base,  $\text{B}^+$  then we have



You can treat this like any old equilibrium

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

rearranging

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

We can assume that  $[\text{H}^+] > [\text{B}^+]$  and that there are lots more sites on the

resin for binding so that  $[XRSO_3^- H^+](s) > [XRSO_3^- B^+](s)$

This makes the right side of the equation a constant so

$$\frac{[XRSO_3^- B^+](s)}{[B^+]} = K'$$

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(large)

$Ba^{+2} > Pb^{+2} > Sr > Ca > Ni > Cd > Cu > Co > Zn > Mg > UO_2$  (All +2)

$Tl^+ > Ag^+ > Cs^+ > Rb^+ > K^+ > NH_4^+ > Na^+ > H^+ > Li^+$

Ks for anions on strong base exchangers

$SO_4^{2-} > C_2O_4^{2-} > I^- > NO_3^- > Br^- > Cl^- > CHO_2^- > CH_3COO^- > OH^- > F^-$

## 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, a 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 through a second column

This column designed to remove solvent ions

Example

For cation exchange, solvent is usually HCl (So lots of H<sup>+</sup>)

Suppressor column is an anion exchanger Resin+Cl<sup>-</sup>

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

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 from 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 5-10 $\mu$ m 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 2500 $\text{\AA}$

Surface treated with hydrophilic 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  $V_r$  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 if two molecules are with 10% of each

other's size

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