Liquid Chromatography

First chromatographic method described (as a non-instrumental method).

Since samples don't need to be initially vaporized, potentially any compound can be assayed by this method.

Instrumental development lagged behind that of GC because of difficulties in creating a stable solvent flow.

Liquid Chromatography

There are several types of interaction that have been used to separate eluents.

Major Categories

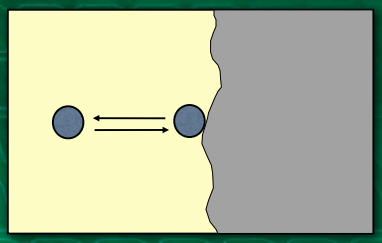
surface adsorption

solvent partitioning

ion exchange

relative solute size

Adsorption chromatography



The stationary phase is a solid. Separation is due to a series of adsorption/desorption steps.

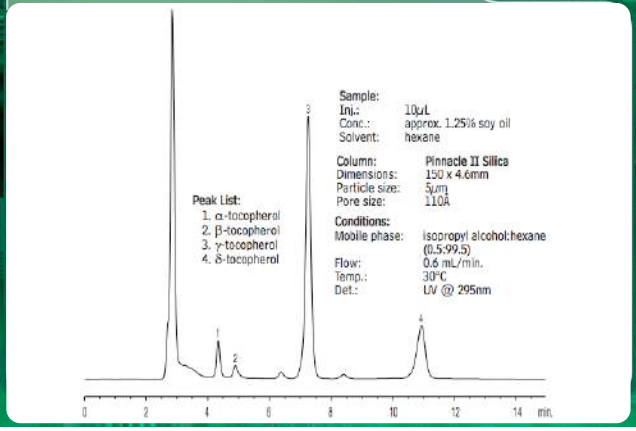
Adsorption chromatography

Silica and alumina are common stationary phases although there are a range of modified surfaces that have been evaluated.

Both solute and solvent are attracted to the polar sites on the stationary phase.

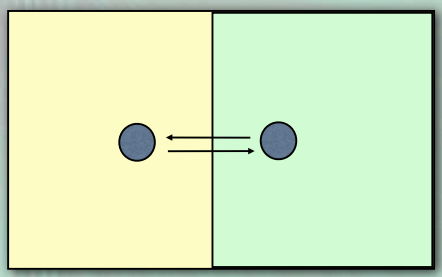
If solutes have differing degrees of attraction to the phase, a separation is possible.

Tocopherol in Soy Oil.



Partition chromatography

Separation is based on solute partitioning between two liquid phases. (relative solubility)



Partition chromatography

This approach comes closest to our countercurrent extraction model.

More highly retained species have a greater affinity (solubility) for the stationary phase - compared to the mobile phase (solvent)

Separation of solutes is based on differences in this relative solubility.

Partition chromatography

Two basic modes of operation

Normal phase.

- Polar stationary phase and non-polar solvent.

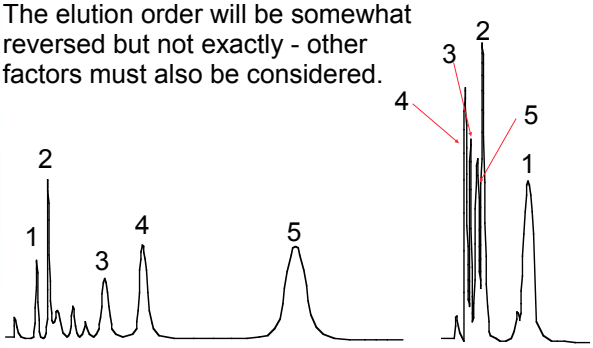
Reverse phase.

- Non-polar stationary phase and polar solvent.

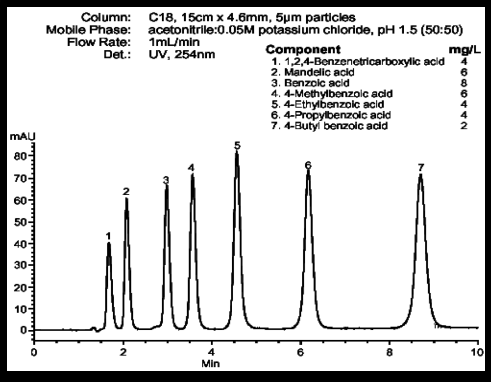
Normal phase was first described but reverse phase is now more common.

Partition chromatography

The elution order will be somewhat reversed but not exactly - other factors must also be considered.

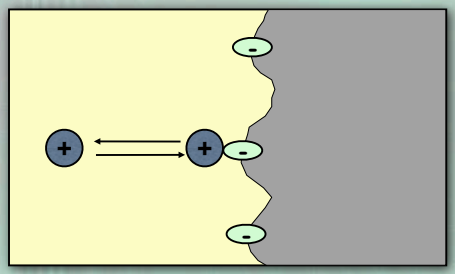


Partition chromatography



Ion exchange chromatography

The stationary phase has an ionically charged surface, opposite that of the eluents.



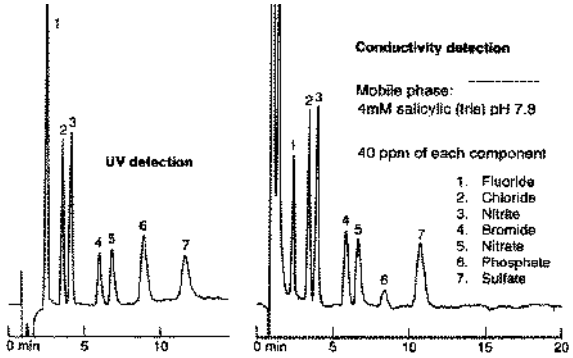
Ion exchange chromatography

For instrumental LC, weak exchange resins are typically used.

These are exchange groups bound to a support.

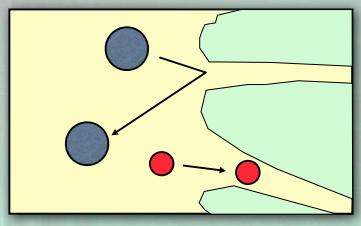
The traditional exchange resin beads would be crushed under normal HPLC conditions.

Ion exchange chromatography



Size exclusion chromatography

Separation is based on molecular size. Stationary phase is a material of controlled pore size. Also called gel permeation.



Size exclusion chromatography

Columns can be obtained that will separate specific size ranges.

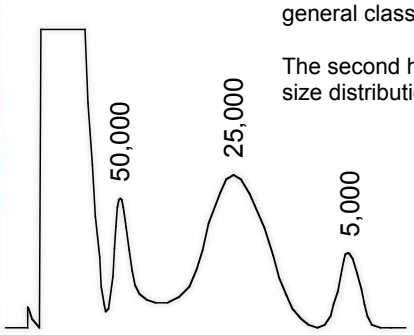
Larger species will elute first - they can't pass through as many pores so their path is shorter.

Useful for determining size and size range for polymers, proteins, ...

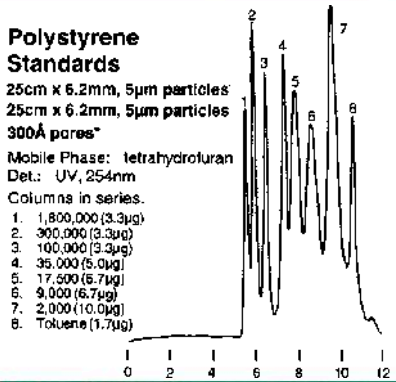
Size exclusion chromatography

This example shows three general classes of components.

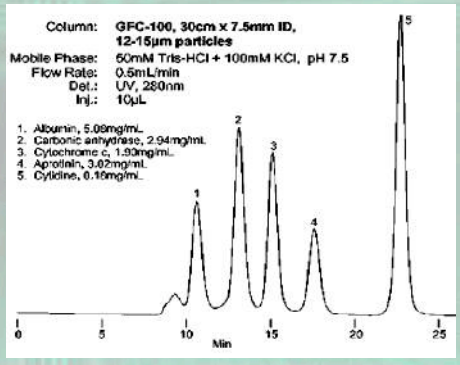
The second has a much larger size distribution.



Size exclusion chromatography

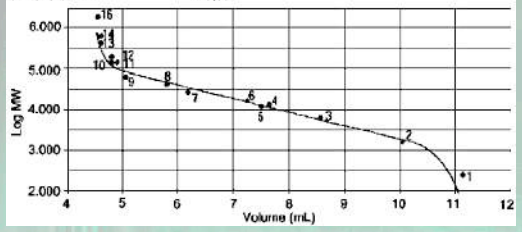


Size exclusion chromatography



Size exclusion chromatography

	Molecule	MW		Molecule	MW
1.	Cytidine	243	9.	Albumin	66,000
2.	Neurotensin	1673	10.	Alcohol dehydrogenase	150,000
3.	Aprotinin	6500	11.	β-Globulins	150,000
4.	Cytochrome c	12,400	12.	β-Amylase	200,000
5.	Ribonuclease A	13,690	13.	Apoferritin	443,000
6.	Myoglobin	16,900	14.	Thyroglobulin	669,000
7.	Carbonic anhydrase	29,000	16.	Blue dextran	2,000,000
8.	Ovalbumin	43,500			



LC solvents

We will limit our coverage to normal and reverse phase work.

Solvent selection is much more critical for these methods.

First step is to determine which mode to use.

Normal or Reverse phase?

Mode selection

In general

- If sample is water insoluble or non-polar - use normal phase
- If sample is water soluble or not soluble but polar - use reverse phase.
- It's not always this cut and dry but represents a good starting point.

Solvent selection

We seldom can find a 'pure' solvent do the job - typically use a blend of two or more.

Factors to consider

Solvent Strength - a measure of relative solvent polarity (ability to displace a solute). Scales based on silica or alumina.

Polarity Index - a related index used for reverse phase methods.

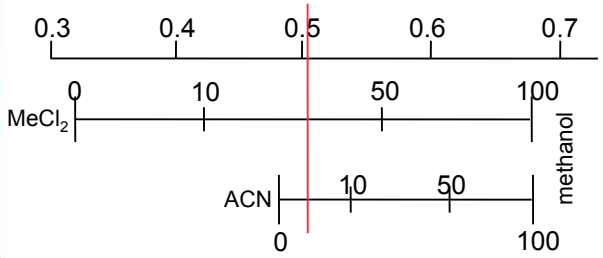
Solvent strength and polarity index

Solvent	ε°	P'	viscosity	RI	UV cutoff
n-pentane	0.00	~0.0	0.23	1.36	210
CCl_4	0.18	1.6	0.97	1.47	265
toluene	0.29	2.4	0.59	1.50	285
ethyl ether	0.38	2.8	0.32	1.35	220
THF	0.45	4.0		1.41	220
MEK	0.51	4.7		1.38	330
acetonitrile	0.65	5.8	0.37	1.34	210
methanol	0.95	5.1	0.60	1.33	210

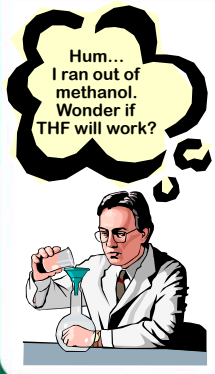
ε° is for alumina.

Mixing solvents

Optimum solvent strength or polarity can be obtained by mixing solvents.



Reverse phase solvent selection



- Snyder extended the method of solvent blending for reverse phase.
- He recognized that polarity is just one factor that you can play with.
- It relies on the use of 4 solvents in developing the optimum separation.

Reverse phase solvent selection

$$R_S = \frac{\sqrt{N}}{4}\,(\alpha - 1)\left(\frac{k'}{1+k'}\right)$$

selectivity term

polarity term

Polarity is just one factor. The other is solvent selectivity.

Reverse phase solvent selection

Polarity

Determines how long the eluents are retained - t_R

Selectivity

The relative retentions of the eluents.

Can affect the shape of the peaks.

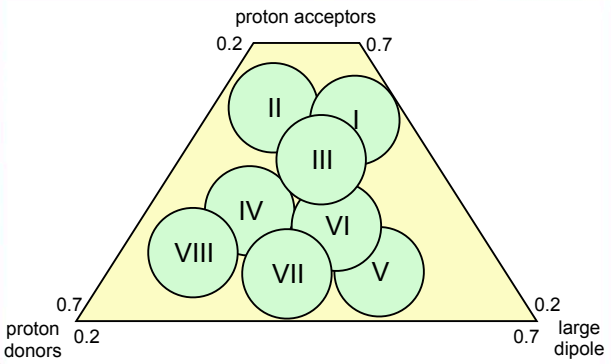
Not all eluents are affected the same.

Solvent classes

Snyder classed solvent using acid, base, dipole and chemical properties.

Class	Partial solvent list
I.	aliphatic ethers and alkyl amines
II.	aliphatic alcohols
III.	THF, pyridines, DMSO, amides
IV.	formamide, acetic acid, glycols
V.	$MeCl_2$, 1,2-DCE
VI.	alkyl halides, esters, ketones, nitriles
VII.	benzene and derivatives
VIII.	chloroform, m-cresol, water

Solvent classes



Solvent classes

Not all solvents are truly usable.

- Can't be mixed at any proportion
- May interact chemically
- UV absorption or viscosity is too high
- Toxic, too flammable
- High vapor pressure
- Too expensive

Common reverse phase solvents

methanol	- acid
acetonitrile	- base
tetrahydrofuran	- large dipole
water	- polarity adjustment

All are low viscosity
available in high purity
UV transparent
miscible in each other

The four solvent method

A simple series of steps can be followed to obtain the optimum reverse phase solvent blend.

1. Start with a single solvent and water. Adjust the % water from 0% on up until the best possible separation is obtained - optimum k' for peaks of interest.

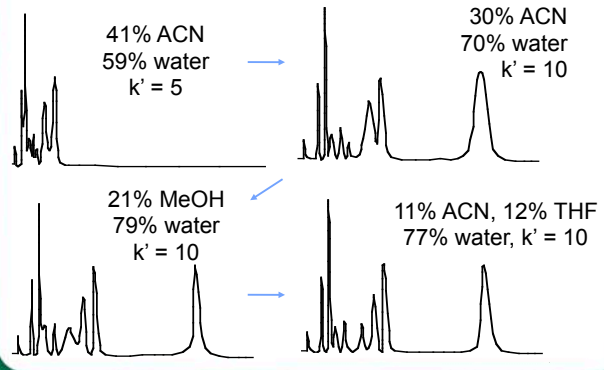
The four solvent method

2. Create blends using each of the other solvents and water that have the same solvent polarity.

3. Evaluate each solvent for improvements in peak shape or movement of selective peaks.

4. A mix of any of the blended solvents is then evaluated for optimum resolution.

The four solvent method



Gradient elution

Unlike GC, variations in temperature have minimal effect on an LC separation.

However, variations in solvent polarity can greatly affect retention.

This can be accomplished by altering the solvent mix during an analysis.

Gradient elution

Advantages of gradient elution

- total analysis time is reduced
- overall resolution of a mixture is improved
- better peak shapes are possible
- improved sensitivity

Gradients can result in baseline drift.

Gradient elution

Can be produced

Stepwise - change from one solvent to another during a run.

Continuous - ramped - comparable to a temperature program.

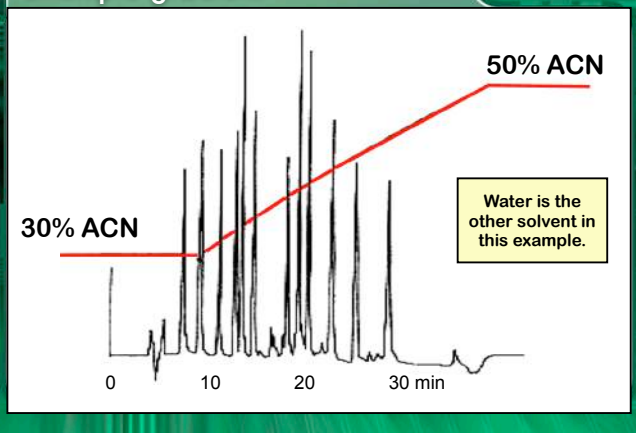
Most common type is 'mixed stream' - solvents are pumped together with turbulent mixing. Each solvent is controlled by a programmer and the total flow rate is held constant.

Gradient elution

Not all LC methods can make use of gradient elution

Ion exchange	- yes
liquid-liquid	- difficult
bonded phase	- yes
size exclusion	- no
adsorption	- yes

Example gradient



Gradient elution

Steps in developing a gradient

First - determine if a single solvent blend can be used - 4 solvent method.

If no single blend is suitable, then a gradient should be attempted.

The results from step one will help in determining the starting and final polarity.

Gradient elution

Starting solvent should have a polarity that adequately resolves the first few components.

Final polarity should adequately resolve the last few species in a timely manner.

Now you play connect the polarity - attempt various blending rates to separate the remaining components.