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Basic principles of chromatographic separation techniques

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What is chromatography ?

Chromalography is a process which separates chemical species from one another.

The fundamental driving force of chromatography is the chemical equilibrium that results when a species distributes between two phases

Chromatography is similar to liquid-liquid extraction

In liquid-liquid extraction, solutes distribute themselves between two immiscible liquid phases until an equilibrium is established. Solutes will also distribute themselves between a liquid and a solid phase. Similarly, vapors establish equilibrium between gas and solid or between gas and liquid phases.

For any particular phase system, the equilibrium concentrations depend primarily on the chemical composition of the solute.

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In the chromalographic process species distribute between two immiscible phases.

- flowing
- stationary

The rate of migration of each species is determined by its distribution coefficient, K_d

Species which are distributed mainly into the **flowing phase** move **rapidly**.

Species which are distributed mainly in the **stationary phase** move **slowly.**

TLC – thin layer chromatography

- The stationary phase is supported on glass, plastic or alluminium layer
- The flowing phase climbs the layer by capillarity
- We need to use chemicals to reveal solute spot

LOW RESOLUTION TECNIQUE

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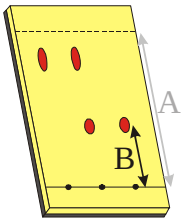

$$R_f = \frac{B}{A}$$

Using the same chromatographic condition

R_f is a characteristic of the solute

TLC

Analytical → Follow organic reaction
Follow Column Cromatography separation
First product analysis

Preparative → Isolate small amount of product.
Allow to purify solutes which are also very small R_f

All TLC tecniques need chemical detection

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Column Chromatography

Flowing phase

Stationary phase

R_f is replaced by Retention Volume:

V_r is the flowing phase volume required to eluite the solute

Column Chromatography

Advantages:

Better resolution than TLC
Higher loading than TLC
Detection can be done by TLC on a small amount of eluate

Good preparative chromatography tecnique

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Column Chromatography

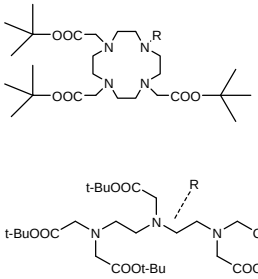
On the basis of nature of the stationary phase we have:

- Normal phase chromatography
- Reverse phase chromatography
- Ion exchange chromatography
- Size exclusion chromatography

Normal phase Column Chromatography

Common solvents

Hexane/Et_2O
Hexane/AcOEt
DCM/MeOH
…



DCM/MeOH/Water
DCM/MeOH/NH_3
DCM/MeOH/Water/NH_3
ACN/Water
….

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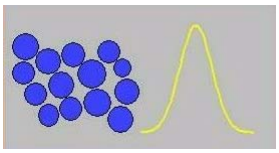
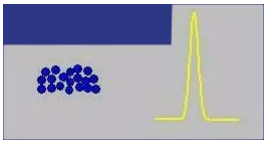
From CC (to flash chrom.) to HPLC



High performance refers to high speed, high resolution separations.
High performance is achieved by using very small diameter (< 20 μ) column packings.

The use of small diameter packings reduces band broadening and gives narrower peaks.



When small particle packings are used, **high pressures** are required to push the mobile phase (eluent) through the column. HPLC is sometimes called high pressure liquid chromatography.

Flash Chromatography

J. Org. Chem., Vol. 43, No. 14, 1978 **2923**

Rapid Chromatographic Technique for Preparative Separations with Moderate Resolution

W. Clark Still,* Michael Kahn, and Abhijit Mitra

Experimental Section

Chromatography columns and the flow controller valve were assembled as described in the text. The silica gel used was 40–63 μm (400–230 mesh) silica gel 60 (E. Merck No. 9385).[10] Solvents were distilled prior to use. Thin layer chromatograms (TLC) were run on glass supported silica gel 60 plates (0.25-mm layer, F-254) (E. Merck No. 5765).

Flash Chromatography. General Procedure. First a low viscosity solvent system (e.g., ethyl acetate/30–60 °C petroleum ether)[8]

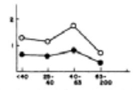


Figure 2. Silica gel particle size (μm).

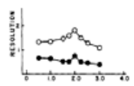


Figure 3. Eluant flow rate (in./min).

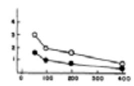


Figure 4. Sample size (mg).

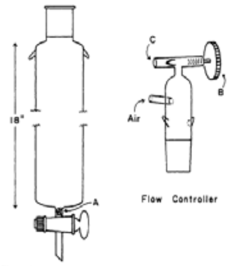


Figure 5.

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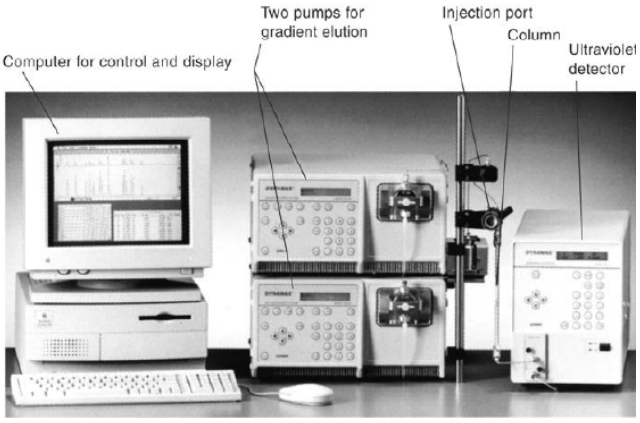
Automated Flash Chromatography

HPLC instrument



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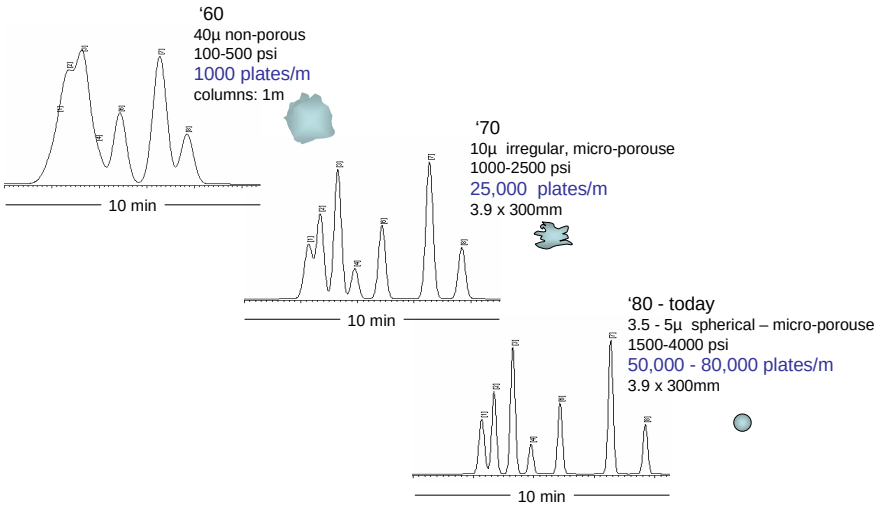

HPLC pump systems

Isocratic

Fixed composition of the flowing phase

Gradient (high or low pressure gradient)

Flowing phase composition changes during the separation

Better separation in less time and less solvent

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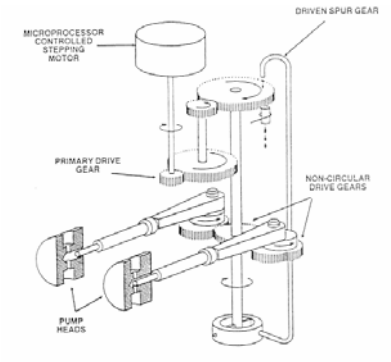

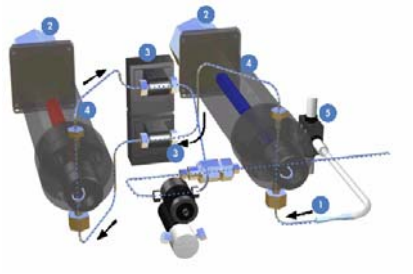

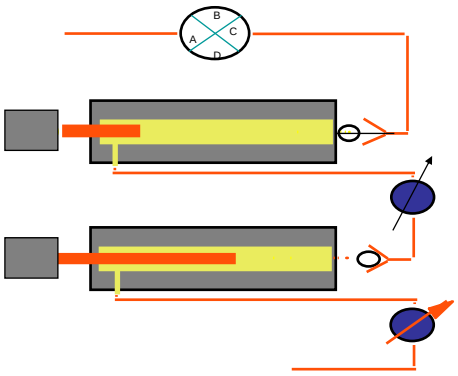

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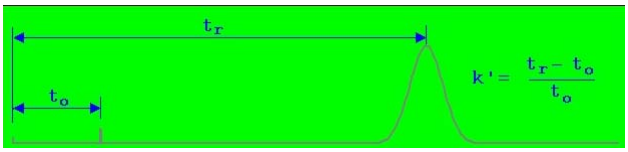
Retention time and capacity factor in HPLC



The retention time t_r is the time required to elute a peak

t_0 is the time required to elute an unretained species (K_d=0)

K_d is the distribution coefficient $K_d = \frac{[solute]_{mobile}}{[solute]_{stationary}}$

Retention is often expressed in terms of capacity factor k'

Retention time and retention volume in HPLC

Retention time is dependant on eluent flow rate.

Retention volume (V_r) is the volume of eluent passed through the column at the retention time

The retention volume of a unretained component is equal to V_m , the volume of mobile phase in the column

$V_r = t_r F$ and $V_m = t_0 F$

$$V_r = V_m + K_d V_s$$

F is flow rate
V_s is the stationary phase volume

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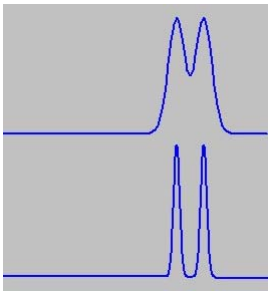
Column efficiency

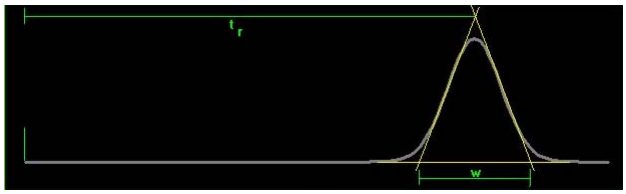
Column efficiency refers to peak width. An efficient column gives narrow peaks making it easier to separate sample components.

Efficiency is a function of:
- -column lenght
- -Particle size
- -Flow rate

Changing these parameters affects the pressure drop across the column. Column length and flow rate also affect the retention time



Column efficiency



Efficiency is measured in terms of the number of theoretical plates N

$$N = 16\left(\frac{t_r}{w}\right)^2$$

The height equivalent to a theoretical plate (HETP) is given by

$$HETP = \frac{L}{N}$$

L = column lenght

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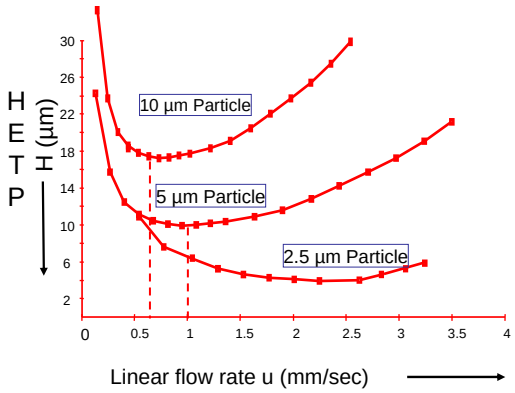
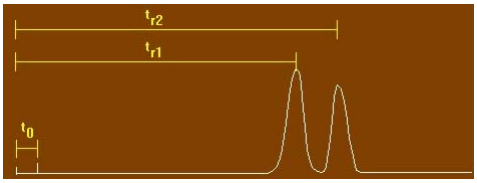
Van Deemter equation



Selectivity



The selectivity parameter α is a measure of peak spacing.

Selectivity is measured from retention:

$$\alpha = \frac{t_{r2} - t_0}{t_{r1} - t_0} = \frac{k'_2}{k'_1}$$

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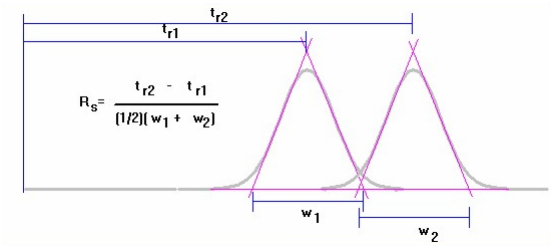
Resolution

The objective of chromatography is the separation of component mixtures.

Resolution is the term used to quantitatively describe how well the objective was met



Resolution

The objective of chromatography is the separation of component mixtures.

Resolution is the term used to quantitatively describe how well the objective was met

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

Selectivity Factor

Efficiency Factor

Retention Factor

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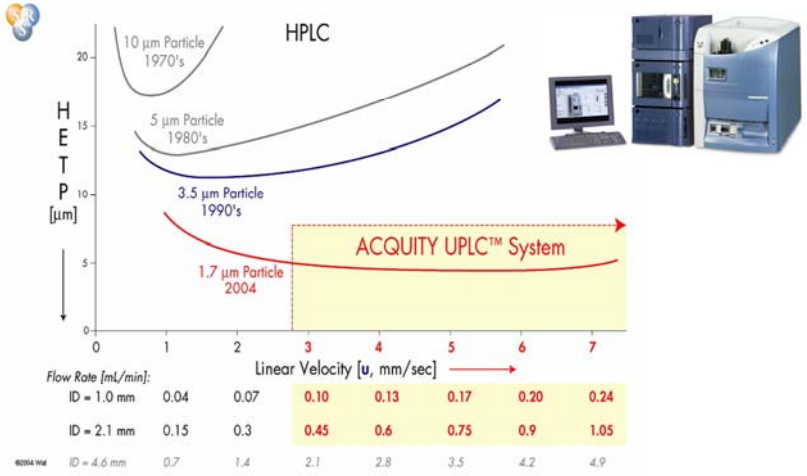
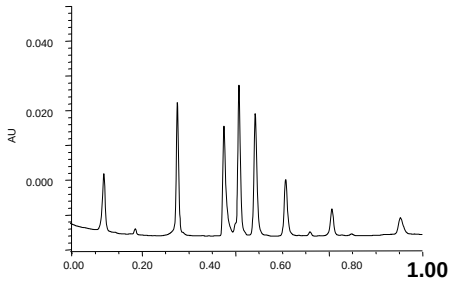
Ultra Performance Liquid Chromatography UPLC



Short time…. High resolution!!!



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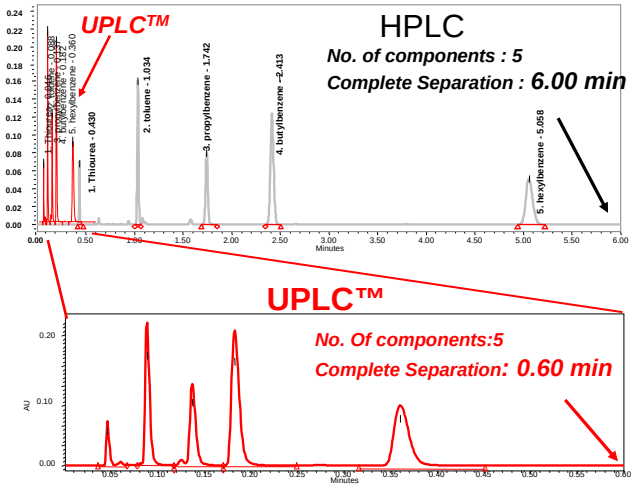
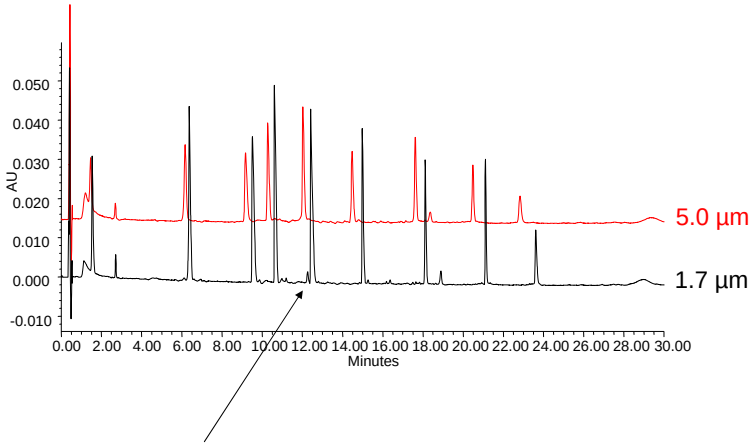


Same time, more information…



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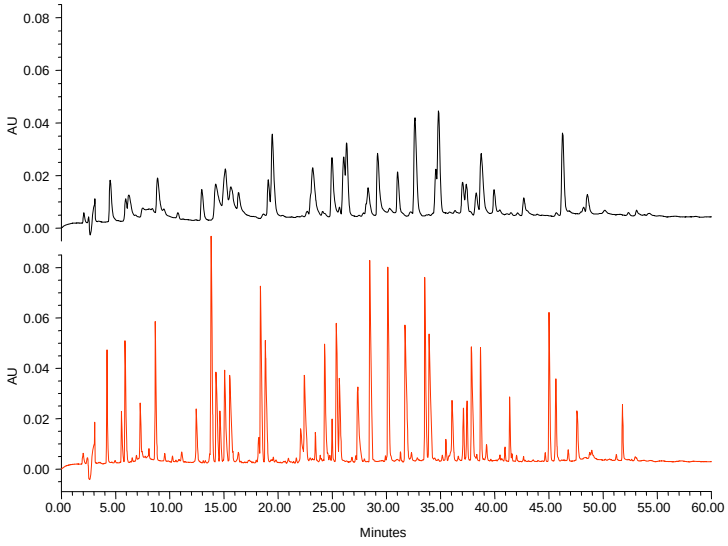
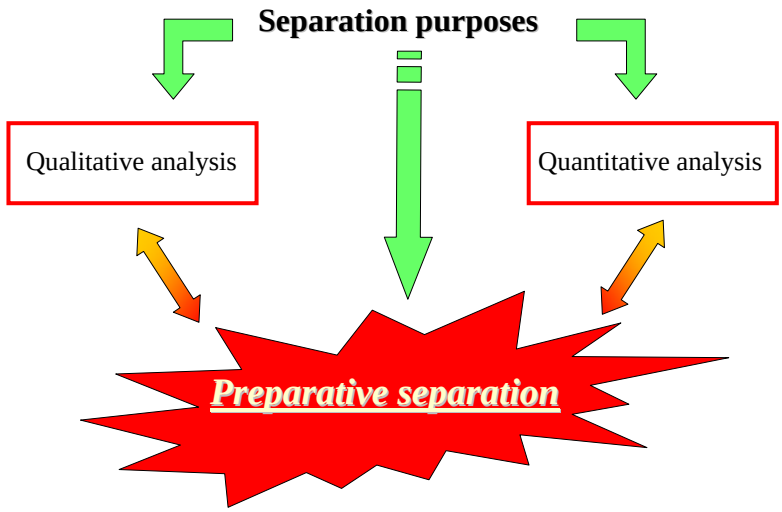


HPLC
4.8 µm
peaks = 70

UPLC™
1.7 µm
peaks = 168



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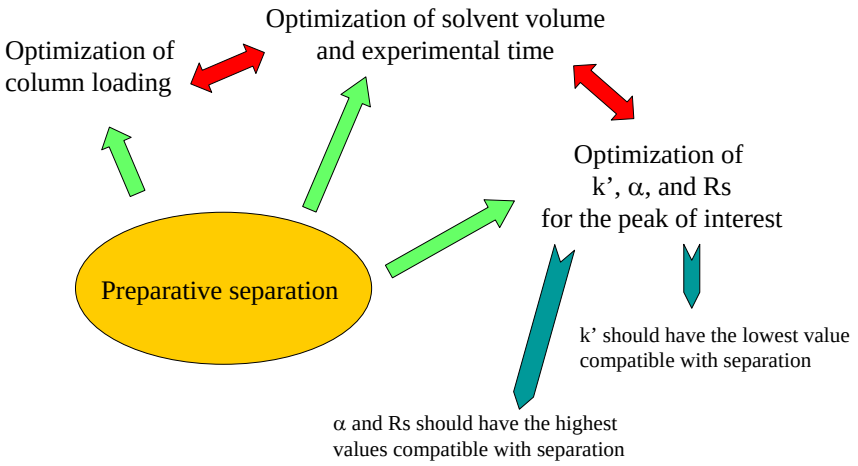
Qualitative analysis → Based on retention time

Relevant parameters: k'

Quantitative analysis → Based on evaluation of peak area

Relevant parameters: k', α, R_s



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Column and solvent selection

Normal phase chromatography

Normal phase refers to the use of polar column packings and low polarity eluents

Separation of low to moderate polarity compounds

Samples which have little solubility in aqueous eluents are candidates for normal phase chromatography. Normal phase is often successful at separating geometric and positional isomers.

Normal phase packings

Bare adsorbent: Silica gel and alumina

Bonded phases: silica or polymer supports onto which polar functional groups such as $-NH_2$ or -CN have been chemically bound.

Eluents:

Weak solvent: Hexane

Strong solvents: Methyl-tert-butyl ether
Methylene chloride
Acetonitrile
Methanol

Column and solvent selection

Reversed phase chromatography

Over 75% of all HPLC separations are carried out on reversed phase columns

The reversed phase is a good choice for mixtures with different numbers, types or locations of alkyl functional groups. It is also suitable for samples with different types of polar functional groups.

Reversed phase packings

Silica bonded C18 groups,
C8 groups,
Phenyl groups

Bare polystirenic resins

Eluents:

Weak solvent: Water

Strong solvents: Methanol
Acetonitrile
isopropanol
THF

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Column and solvent selection

Reversed phase chromatography

The reversed phase can even separate weak acids and bases, providing the pH of the eluent is buffered to keep them in their undissociated form.

Solvent additives

Weak	**Strong**
Water	CH_3CN
Water/TFA 0.1%	CH_3CN/TFA 0.1%
Water/C_3F_7COOH	CH_3CN/C_3F_7COOH
$AcONH_4$ 7 mM, pH= 7	CH_3CN
$AcONH_4$ 4 mM, pH= 4	CH_3CN
CF3COONH$_4$ 4mM pH=4	CH_3CN
NH_3 7 mM	CH_3CN

Not surprisingly, the reversed phase is the chromatographer's first choice when sample structure is unknown.

Reversed phase packings

Silica bonded C18 groups,
C8 groups,
Phenyl groups

Bare polystirenic resins

Eluents:

Weak solvent: Water

Strong solvents: Methanol
Acetonitrile
THF

Column and solvent selection

Ion Pair chromatography

Ion pair chromatography is a technique for the separation of ionizable organic compounds on reversed phase columns.

It is generally preferred over ion exchange because it offers higher efficiency and greater control over selectivity

Ion pair chromatography differs from reversed phase in that the eluent contains a hydrophobic counter ion called an *ion pairing agent.*

It is widely believed that ion pairing agents adsorb onto the stationary phase to form the equivalent of an ion exchange stationary phase.

Phase packings

Silica bonded C18 groups,
C8 groups,

Eluents:

Weak solvent: Water buffer
with ion pairing agents

Strong solvents: Methanol
Acetonitrile

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Column and solvent selection

Ion Pair chromatography

Ion pairing agents

Buffers are used to keep the sample compound ionized. Buffer concentrations of 0.02 - 0.20 M are typically used.

Buffer	pH range
Phosphate	1.1 – 3.1
Phospate	6.2 – 8.2
Acetate	3.8 – 5.8
Borate	8.2 – 10.2

Phase packings

Silica bonded C18 groups,
C8 groups,

Eluents:

Weak solvent: Water buffer
with ion pairing agents

Strong solvents: Methanol
Acetonitrile

Column and solvent selection

Ion Pair chromatography

Ion pairing agents

Ion pairing agents are added at concentration of 0.005 to 0.5 M.

For an anionic sample:
Tetrabutylammonium hydrogen sulfate
Tetrabutylammonium phosphate
Cetyltrimethylammounium bromide
Trioctyl amine

For a cationic sample:
Sodium octylsulfonate
Sodium dodecylsulfate

Phase packings

Silica bonded C18 groups,
C8 groups,

Eluents:

Weak solvent: Water buffer
with ion pairing agents

Strong solvents: Methanol
Acetonitrile

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Column and solvent selection

Ion exchange chromatography

The unique selectivity of ion exchange chromatography is most useful for the **separation of inorganic ions**. It is also useful for the separation of **proteins**, **peptides and amino acids**.

The retention in ion exchange is controlled by the **pH** and **ionic strength** of the eluent rather than its organic solvent content.

High capacity ion exchange packings are especially useful for preparative separations.

Ion exchange phase packings

Polymer bound: Ammonium salt
Sulfonate salt

Eluents:

Weak solvent: Water buffer low concentration

Strong solvent: Water buffer high concentration;
acid or basic solution

Column and solvent selection

Size exclusion chromatography

Size exclusion chromatography (SEC) is used for samples which contain high molecular weight compounds and for samples whose components are significantly different in molecular size.

SEC is sometimes used for purified metal complexes from salts

Size exclusion phase packings

Cross-linked sugar (sepharose, dextrane, …)

Eluents:

Water or buffer.

In preparative conditions water or volatile buffer are preferred

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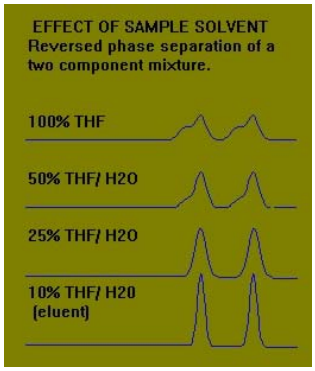
Sample preparation

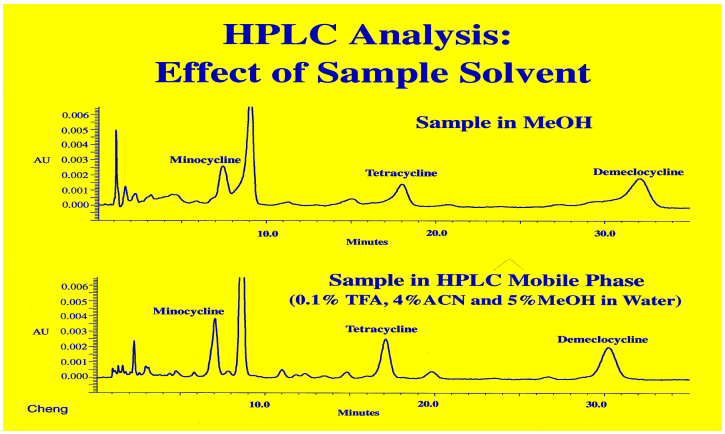
- The sample must first be dissolved or diluted in a suitable solvent.
- Concentrations up to about 5 mg/ml are typical for analytical separations.
- Preparative separations use higher concentrations.
- Sample solvents which produce a detector response should be avoided because they introduce large peaks which may interfere with the analysis.
- Sample solvents which are strong eluents should be avoided because they can cause band broadening or band splitting.
  In general, the eluent strength of the sample solvent should be no greater than that of the eluent.
  Thus the best sample solvent is often the eluent itself.



Sample solution must be filtered on a 0.45µm filter

Sample preparation

Peak Distortion
due to Solvent Choice



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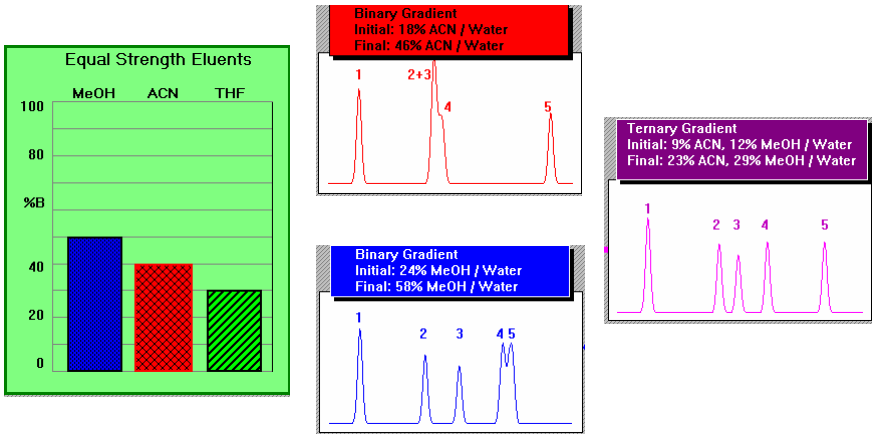
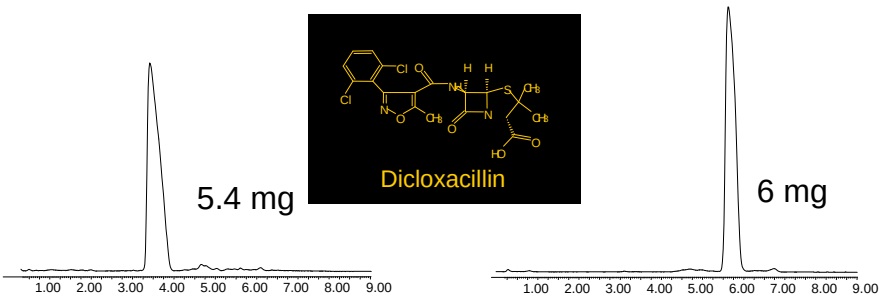
Something more about solvents



Acetonitrile, pH 2

Methanol, pH 2



Applying the same gradient with both organic solvents, sample elutes later with methanol than with acetonitrile.

Changing the organic solvent may improve peak shape due to the additional interaction with the sample.

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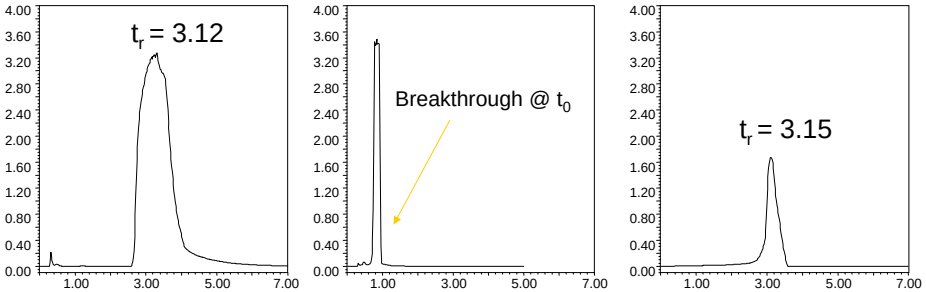
Use of Buffers

Sample: Propranolol

25 mM Buffer, pH 7

DI water, pH 7

DI water, pH 7



20% ACN
3 mg

20% ACN
3 mg

7% ACN
0.5 mg

Buffered mobile phases enhance retention and mass loading. **There is a high risk of breakthrough and retention loss leading to recovery problems when buffers are left out!**

UV Detector set-up

Diode array:

-multiple wavelength selection

-3D chromatogram can be acquired

Single wavelength UV detector:

-generally set on 190 – 220 nm

-if the compound of interest shows an absorption peak in a well defined spectrum region the detector can be set on this wavelength

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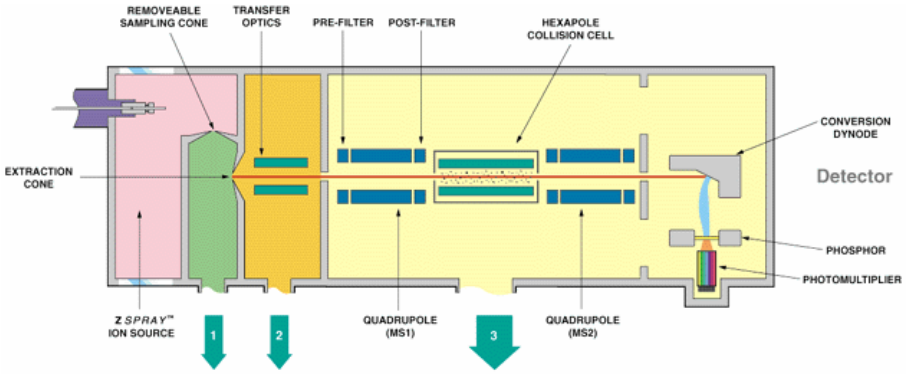
LC-MS

Instrument Schematic



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API Ionisation Techniques

- ESI and APcI differ in…
  - How ions are generated
    - ESI - solution phase ionization
    - APcI - gas phase ionization
  - Analyte compatibility
    - ESI - polar compounds and large biomolecules
    - APcI - less polar, smaller compounds (relative to those ionized by ESI) that have some volatility
  - Flow rate compatibility
    - ESI - 0.001 to 1 mL/min
    - APcI - 0.2 to 2 mL/min

ESI vs APcI

Technique	Flow Rate (ml/min)	MW Range	Species Produced
ESI	0.001 – 0.3	<200,000 Da	$(M+H)^+$ $(M-H)^-$ $(M+nH)^{n+}$
APcI	0.2 – 2.0	<1000 Da	$(M+H)^+$ $(M-H)^-$

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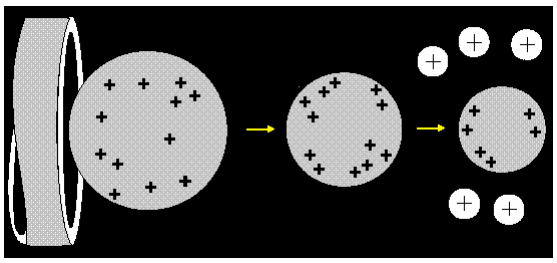
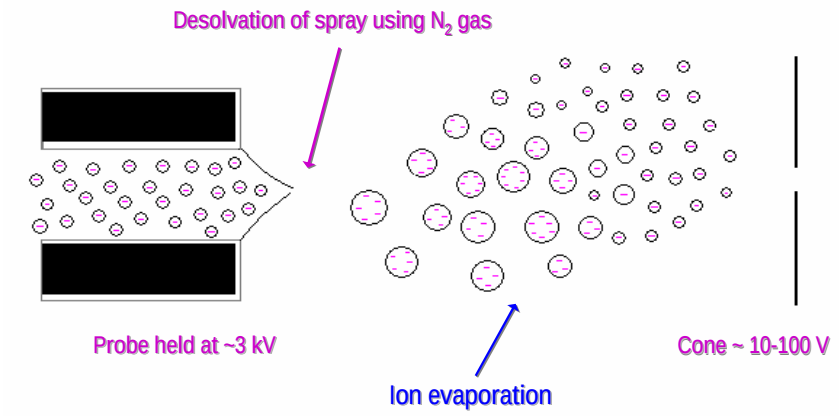
Electrospray Ionisation Theory



As droplets evaporate, the electric field increases and ions move towards the surface.

Electrospray Ionisation



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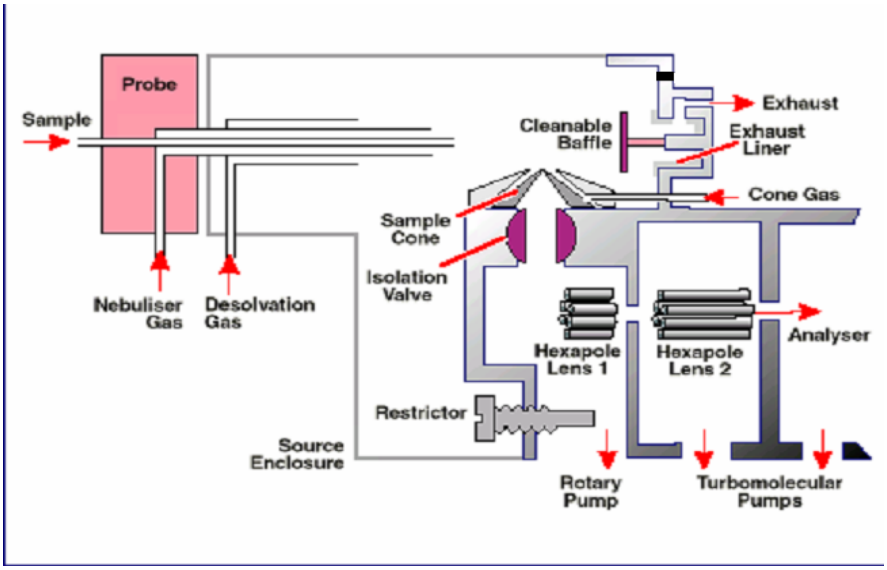

Recognising Multiply Charged Ions

- Mass spectrometers operate on the basis of mass-to-charge ratio (m/z). Mass assignments are normally made assuming a single charge per ion (i.e. m/z = m)

- Single charge — Mass = (M+H)
- Double charge — Mass = $^1/_2$ (M+2H)
- n charge — Mass = $^1/_n$ (M+nH)

- Isotopes of doubly charged ions are separated by 0.5 Da

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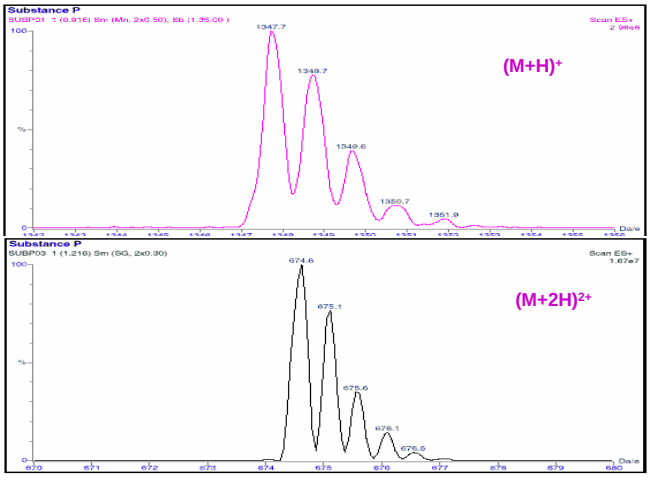
Multiply Charged Ions



Atmospheric Pressure Chemical Ionisation (APcI)

- Low molecular weight (<1000 Da)
- Singly charged species
- In-source fragmentation can occur, even at low cone voltages - caused by increased temperature
- Mobile phase can be non-polar (normal-phase chromatography)

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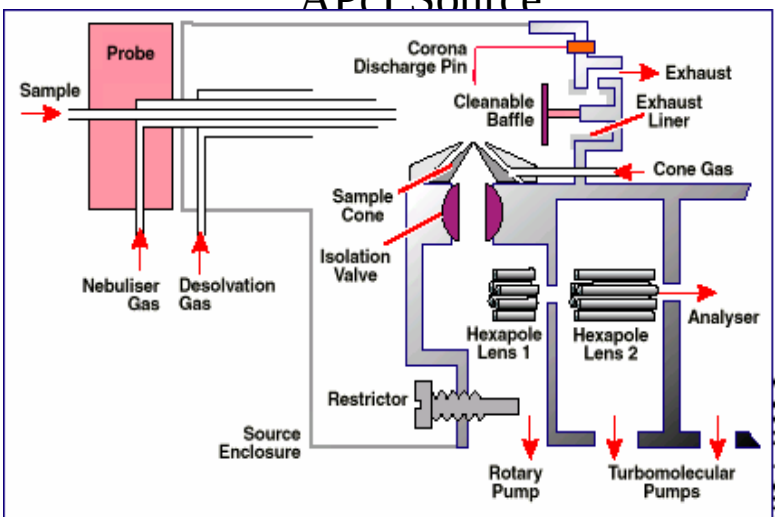
APcI Source



APcI Theory

- The probe is heated to aid desolvation and a gaseous vapour forms.
- Mobile phase vapour enters the source and solvent ions react with ions formed by the corona discharge pin to produce reactive reagent ions.
- Analyte molecules react with these reagent ions and usually gain or lose a hydrogen (protonation or deprotonation) for positive or negative ion mode.
- The ions then pass into the Z-Spray source and are analysed as in ESI mode.

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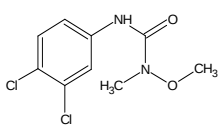
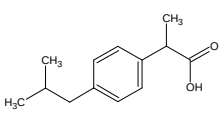
Positive or Negative?

Basic Compounds ($-NH_2$) $(M+H)^+$

Acidic Compounds ($-CO_2H$, $-OH$) $(M-H)^-$



Linuron +ve ion



Ibuprofen -ve ion

pH Considerations

Positive ion mode - Analysis of basic compounds
- Lower pH with an acid
  - e.g. Formic or acetic acid

Negative ion mode - Analysis of acidic compounds
- Raise pH with a base
  - e.g. Ammonium hydroxide/Ammonia soln.

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Commonly Used Solvents and Additives

Solvents
- Water
- Acetonitrile
- Methanol
- Isopropanol

Additives
- Acetic acid
- Formic acid
- Ammonium hydroxide
- Ammonium acetate*

* Salt concentrations should be kept to 10 mM or less.

Solvents and Additives to be Used with Discretion

- TFA - Used with Proteins and Peptides
  - Will suppress (to some extent) positive ion electrospray at levels > 0.1%.
  - Will greatly suppress negative ion electrospray.
- TEA
  - Readily ionized to give an intense $(M + H)^+$ ion at m/z 102.
  - Will suppress positive ion electrospray of less basic compounds. May enhance negative ion electrospray of less basic compounds.
- THF
  - 100% THF is highly flammable.
  - Should not be used with APCI if air is being used as the nebuliser gas.

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Unsuitable Solvents and Buffers

- Non-volatile salts (phosphate, borate, citrate, etc.)
- Surface active agents/detergents (suppress ionisation)
- Inorganic acids (sulphuric acid, phosphoric acid etc.)