Introduction to High Performance Liquid Chromatography

Klaus Unger, 2007

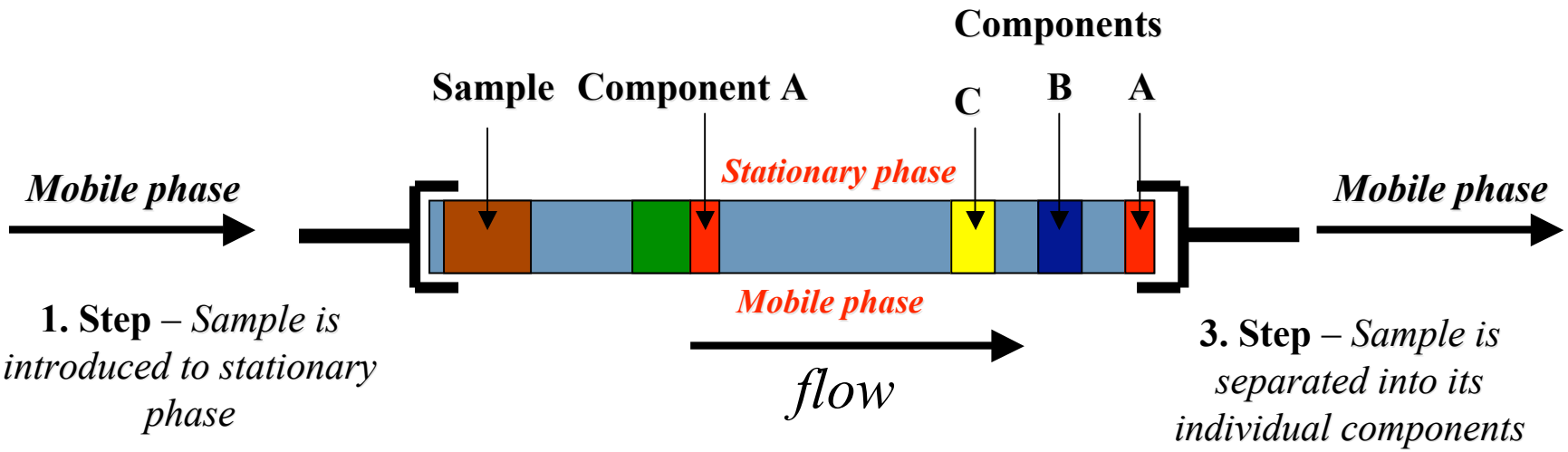
xxCourse Overview

- Introduction
- Historical perspective
- The HPLC system
- Separation criteria in HPLC
- Separation principles in HPLC
- Transport of solutes through the column
- Separation principles: selective dilution
- Selective retention
- Chromatographic parameters for characterizing separations
- Elution modes in HPLC
- Methodology and instrumentation
- Novel developments and approaches

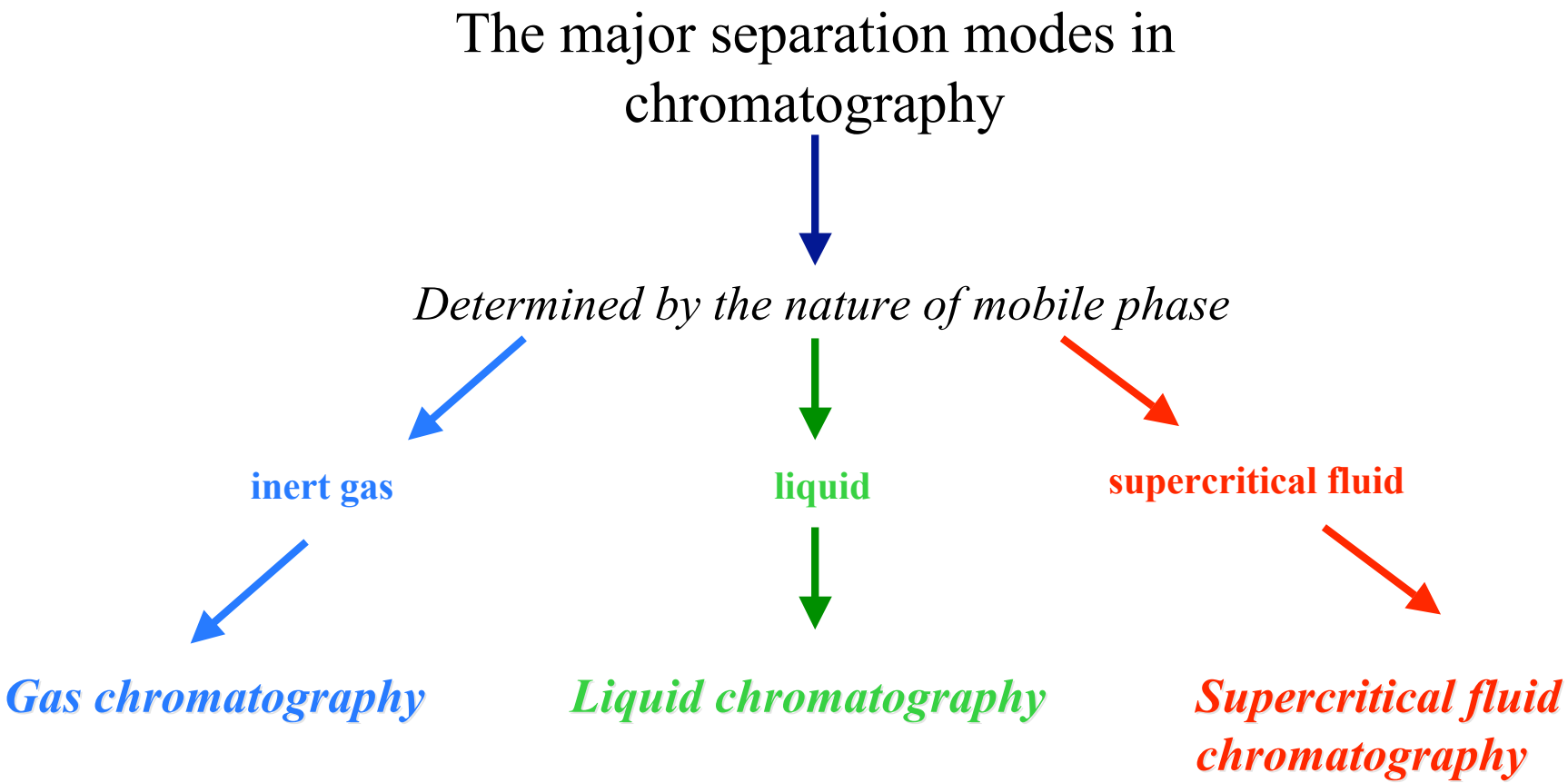
Introduction

Chromatography – *is an analytical technique whereby a sample is separated into its individual components.*

During separation the sample components are distributed between a stationary phase and a mobile phase. After separation the components can be quantified and even identified.



Introduction



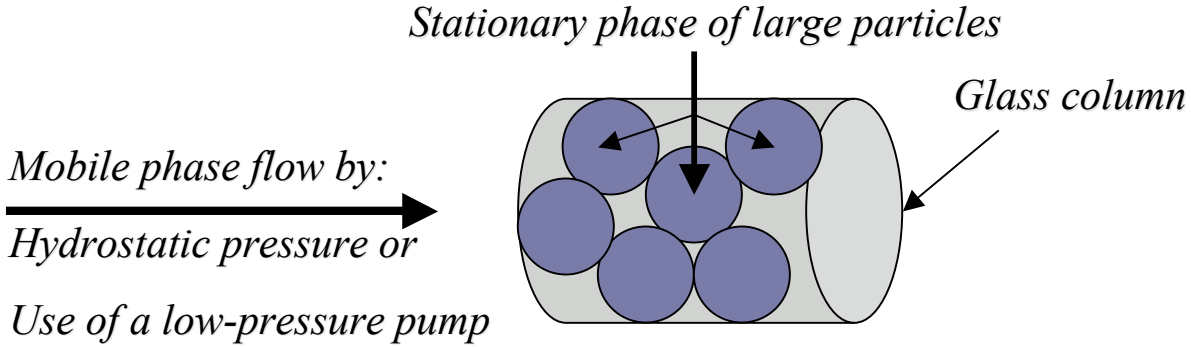
Historical perspective

- 1903 Tswett - plant pigments separated on chalk columns
- 1931 Lederer & Kuhn - LC of carotenoids
- 1938 TLC and ion exchange
- 1950 reverse phase LC
- 1954 Martin & Synge (Nobel Prize)
- 1959 Gel permeation
- 1965 instrumental LC (Waters)

Historical perspective

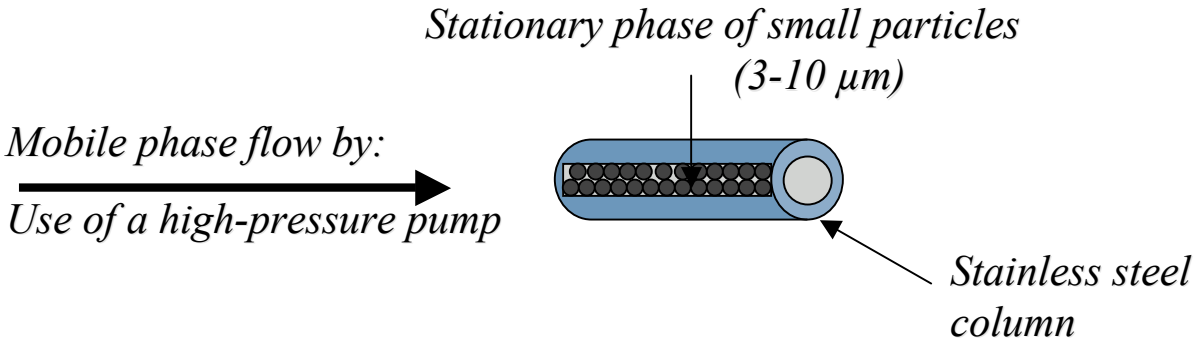
The term High Performance Liquid Chromatography (HPLC) was introduced in the 1970's to distinguish the modern high performance technique from classical low-pressure column chromatography, developed in the 1930's.

Classical column chromatography



- *poor separation*
- *long separation times*

High performance liquid chromatography



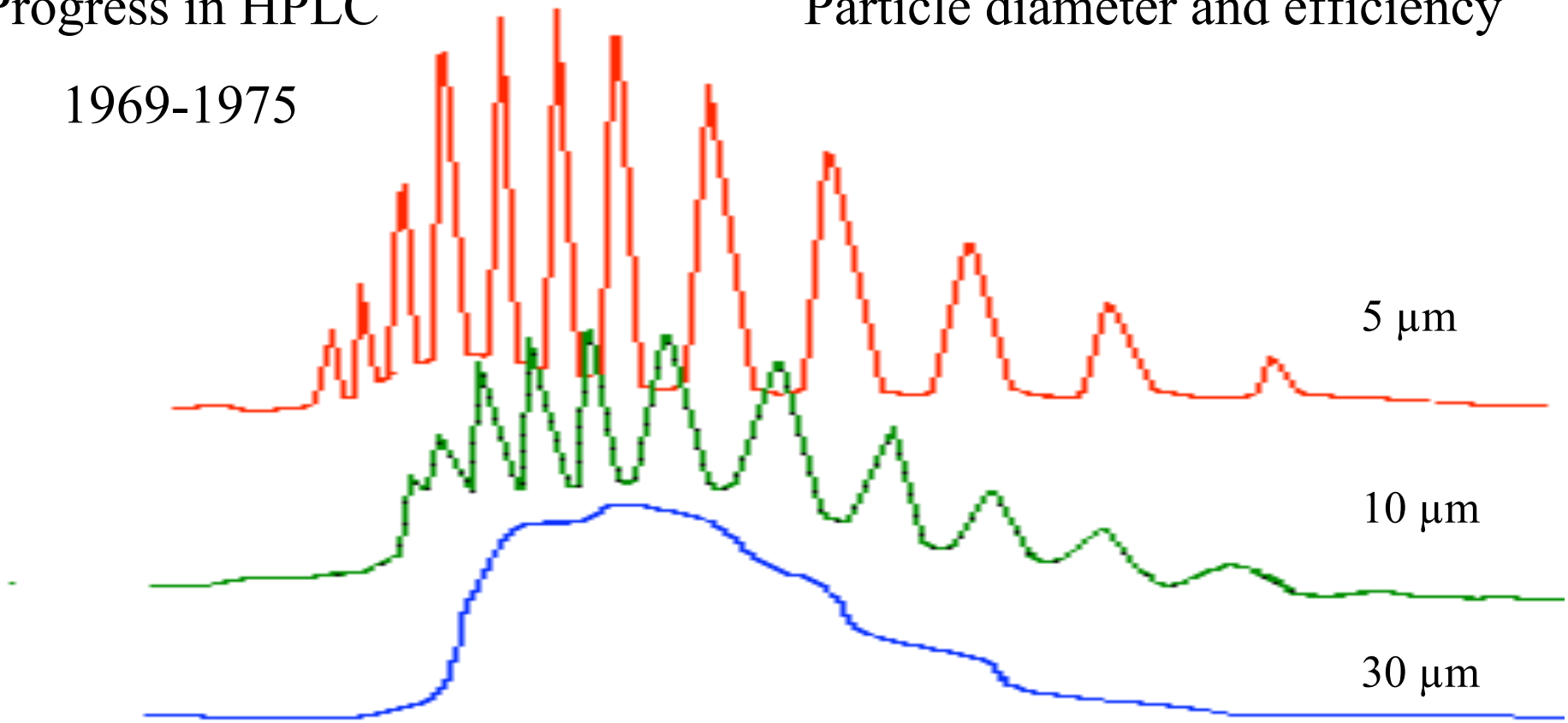
- *good separation*
- *short separation times*

Historical perspective

Progress in HPLC

1969-1975

Particle diameter and efficiency



Stat. Phase – Si 60

Sample – Polystyrene MW 600

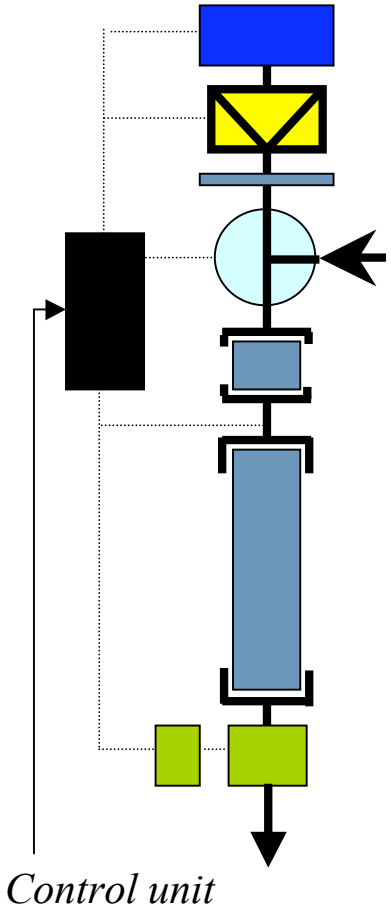
Eluent – n-Heptane-THF 92:8

Historical perspective

Improvement in separation efficiency

Year	Particle diameter	Separation power N/m
Classical LC	100 μm	100
1965	30 μm	10,000
1971	10 μm	30,000
1975	5 μm	60,000
1978	3 μm	100,000
1990	1.5 μm	360,000

The HPLC system

Scheme	Component	Function/feature
	***Mobile phase reservoir***	To ensure an adjustable, constant and reproducible flow of approx. 1.0 ml/min for analytical columns
	Pump →	
	Filter	To introduce a variable and reproducible sample volume (for instance 5-500 µl) as a narrow injection band or point injection onto the top of analytical column
	Injection valve →	
	Guard column	
	Analytical column	To reach optimum analyte separation per unit of time at an acceptable pressure drop and at acceptable column life time
	(thermostatted) →	
	Detector →	High sensitivity, low detection limit, high detector linearity and long term stability
Control unit	***Data acquisition/ evaluation*** →	To require and store data and perform calculations, to control and adjust operational and control system parameters and separation conditions

Separation criteria in HPLC

HPLC is a method of choise for the separation and analysis of:

nonvolatile compounds

strongly polar and ionic compounds, such as oligomers and polymers

high molecular mass compounds, such as oligomers and polymers

thermolabile and decomposable compounds

Separation criteria in HPLC

- Very applicable to separating substances
  - *Amino acids*
  - *Proteins*
  - *Nucleic acids*
  - *Carbohydrates*
  - *Terpenoids*
  - *Antibiotics*
  - *Steroids*
  - *Inorganic salts*

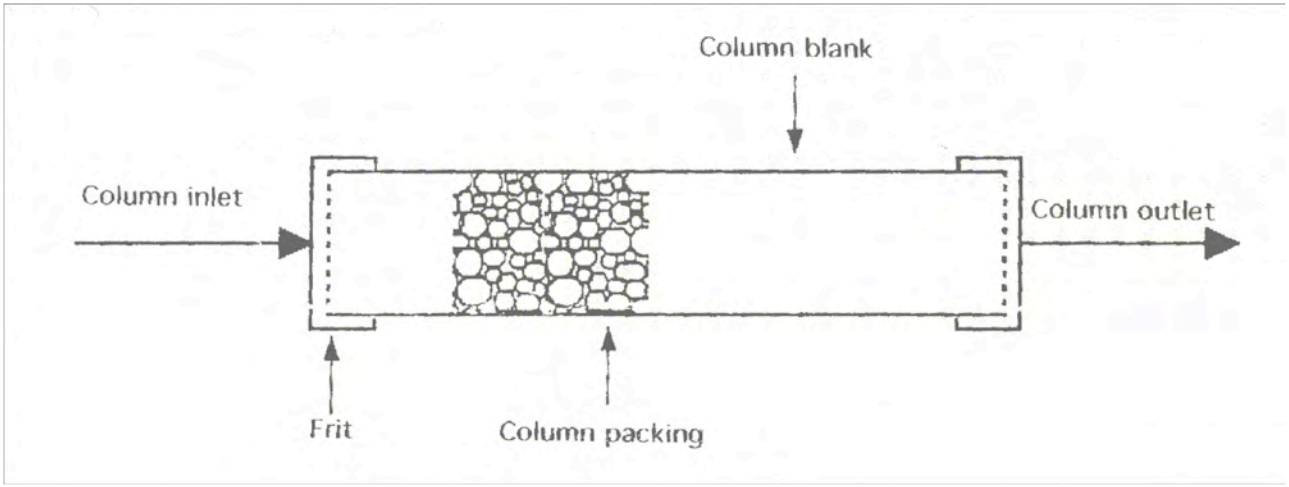
Separation criteria in HPLC

Major HPLC application areas are:

- Quality control in industry
- Determination of active compounds in drug research and the pharmaceutical industry
- Polymer analysis
- Determination of toxic compounds in environmental analysis
- Separation and isolation of biopolymers such as enzymes and nucleic acids
- Quality control in the food and cosmetics industry

Separation principles in HPLC

Separation of a mixture of compounds into its individual components takes place in the analytical column



Characteristic parameters for an analytical column are:

Column length L,

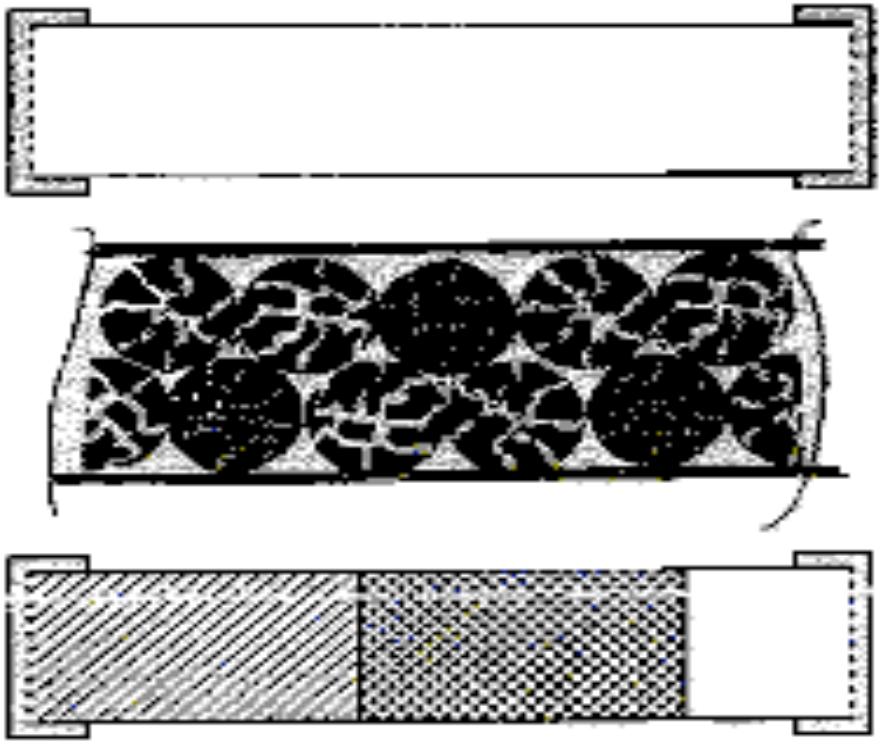
Column diameter d_c,

Average particle diameter d_p,

Pressure drop Δp

xxSeparation principles in HPLC

Volume ratio's in an analytical column



Void volume

$$V_{void} = \pi (d_c/2)^2 L$$

$V_0/V_{void} \sim 0{,}4$ $\quad V_0 = 40\%$

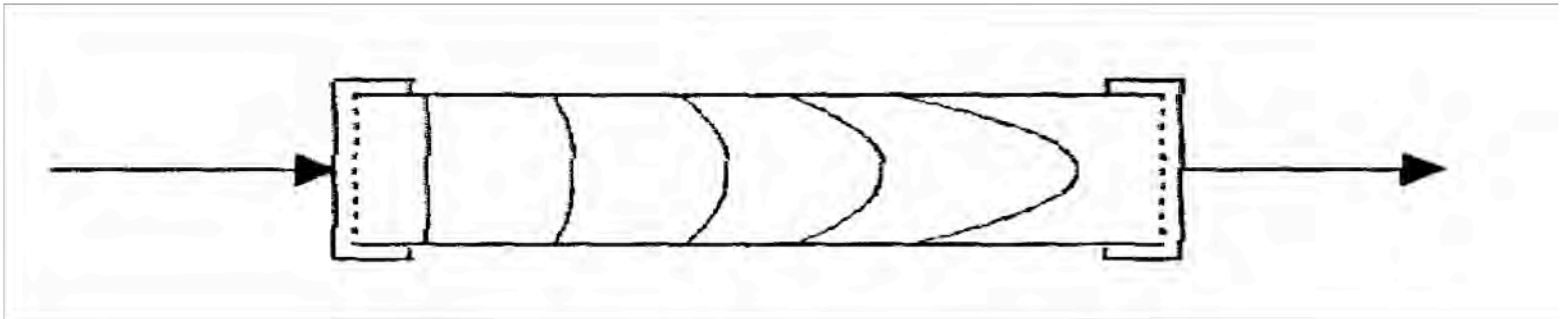
$V_p/V_{void} \sim 0{,}4$ $\quad V_p = 40\%$

$V_s/V_{void} \sim 0{,}2$ $\quad V_s = 20\%$

V_0 is the xxxx volume, V_{void} is the xxx volume and V_p vis the pore volume

Transport of solutes through the column

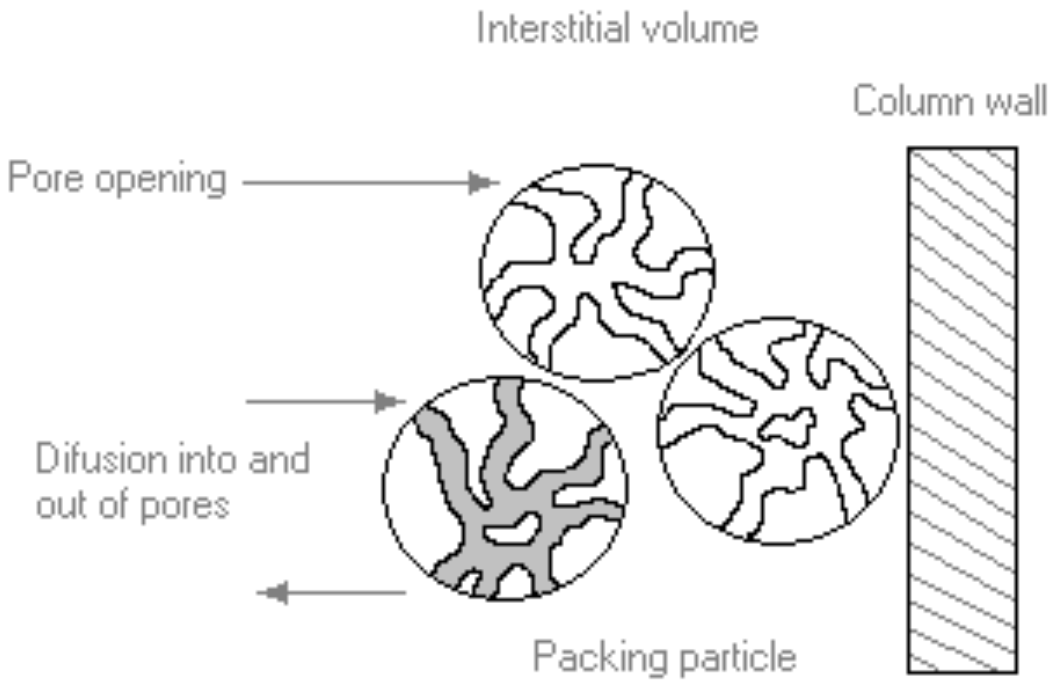
Flow profile of a solute, as it is transported through the column



The sample is introduced onto a column as a sharp rectangular profile. As the sample plug travels through the column, the initial rectangular flow profile becomes parabolic due to the irregular and random flow paths in the interstitial voids between the particles.

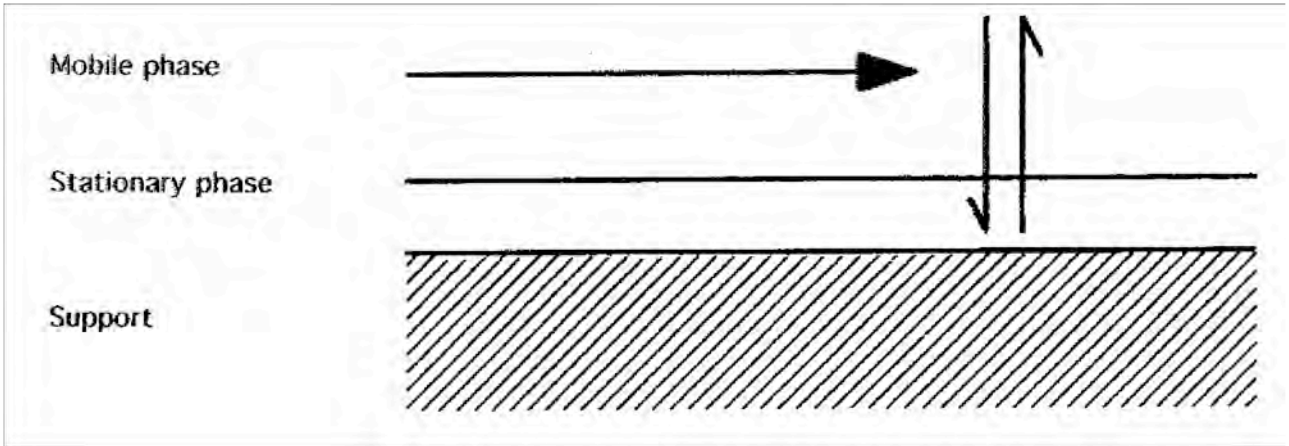
Transport of solutes through the column

Processes leading to broadening of the injection profile of a solute during transport through the column



Separation principles: selective dilution

Schematic drawing of the partitioning of a solute between stationary phase and mobile phase during chromatographic separation



The distribution coefficient K for a particular solute is defined as:

$$K = c_s / c_m$$

where c_s is the concentration of the solute in the stationary phase

c_m is the concentration of the solute in the mobile phase at equilibrium

Selective retention

All solute molecules are transported through the column at the same average mobile phase flow rate and spend the same amount of time in the mobile phase. This is defined as the column dead time t_m.

$$V_m = t_m f_v$$

V_m – dead volume

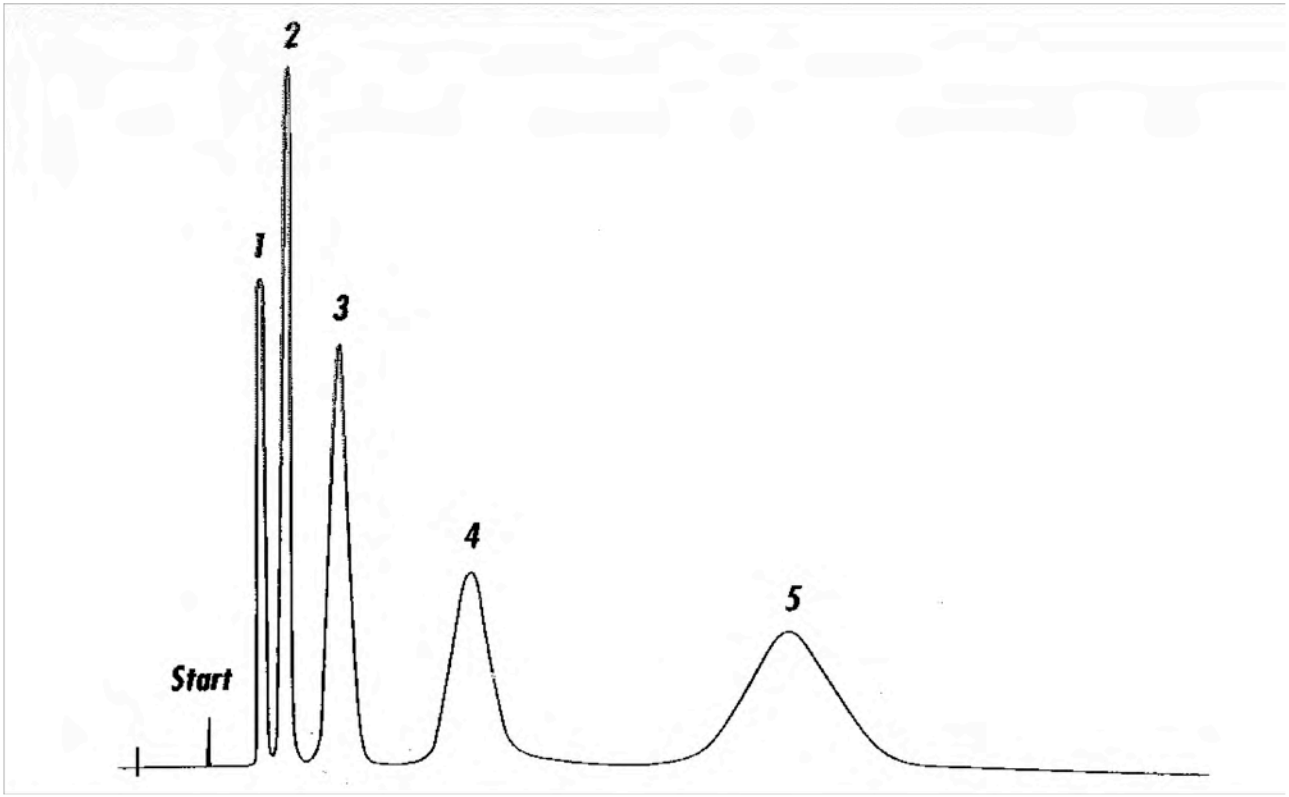
f_m – flow rate (ml/min)

The total time which a solute spends in a column is equal to the sum of the time spent in the mobile phase t_m, and the time spent in the stationary phase t_s. This is defined as the retention time t_r.

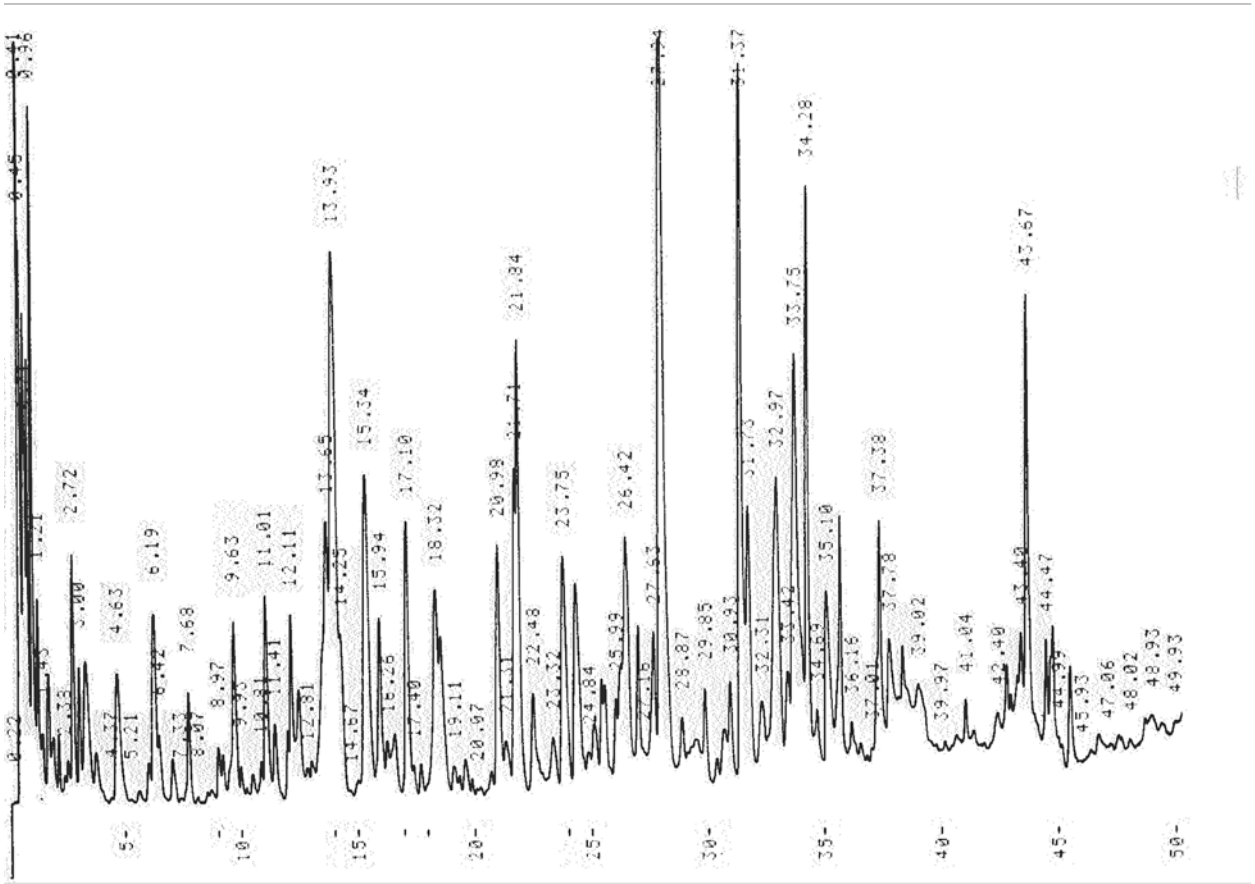
$$t_r = t_m + t_s$$

Selective retention

Chromatogram of a sample consisting of 5 compounds showing a complete separation



Selective retention



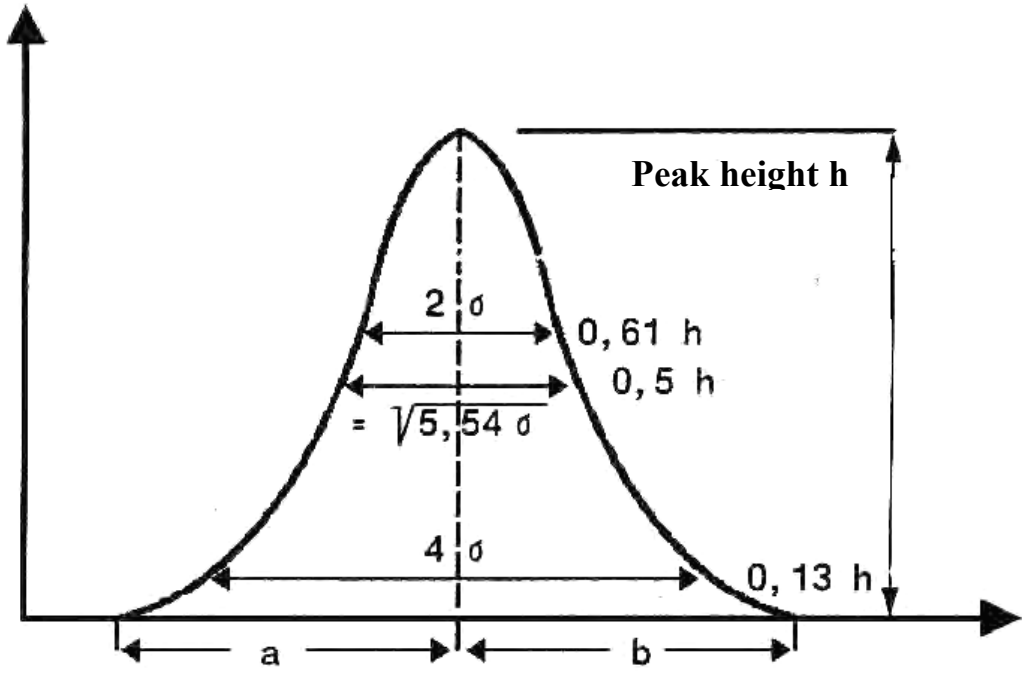
Chromatographic parameters

- The retention time is the time measured from the point of injection to the maximum point of the retained peak
- The dead time is the time the mobile phase front or an unretained peak travels through the column, measured from the point of injection until the time the peak maximum appears in the chromatogram
- The peak height is the distance measured from the base of the peak to the peak maximum
- The area under an analyte peak is proportional to the concentration of that analyte
- The peak width is usually measured at half height of the peak
- The peak symmetry factor is calculated by dividing the distance left (a) and right (b) of the vertical through the peak maximum (b/a)

Chromatographic parameters

Chromatographic peaks should possess Gaussian profiles in the optimal case and are characterised by:

- retention time t_R
- peak height h
- peak symmetry b/a
- standard deviation σ
at 61 % of peak height
- theoretical plate height H
(Peak width)



Chromatographic parameters

The theoretical plate height is a function of the peak width and the column length:

$$H = \sigma^2/L \approx 2\text{-}3\ d_p$$

With

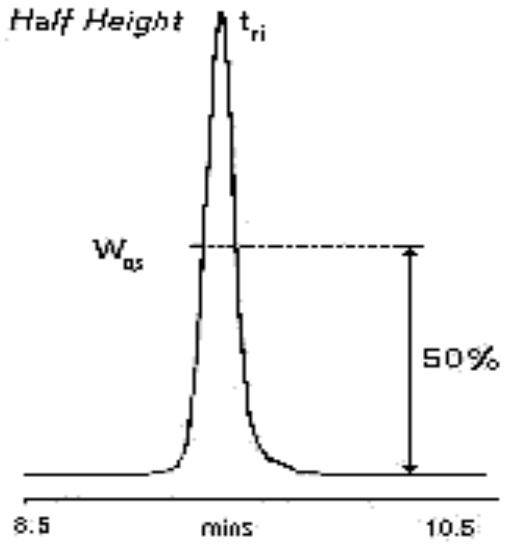
$$N = L/H$$

the theoretical plate height is converted into the dimensionless parameter N (plate number).

$$N = 5.54\ (t_{ri}/w_{t,0.5})^2$$
$$N = 5.54\ (V_{Ri}/w_{t,0.5})^2$$

Chromatographic parameters

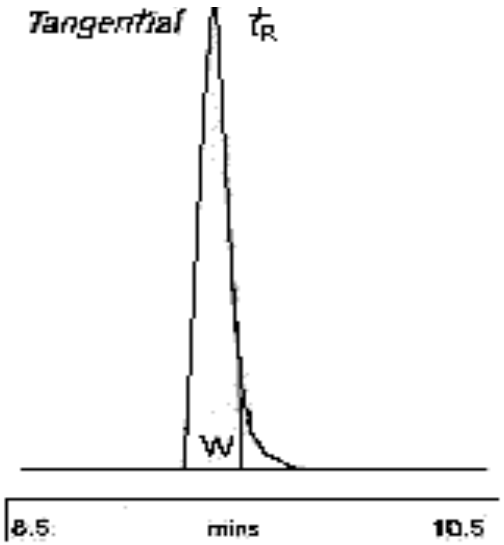
Methods for Plate number determination



$$N = 5.54 \left(\frac{t_{ri}}{W_{0.5}} \right)^2 \Bigg/ L$$

N = 82300 plates/m

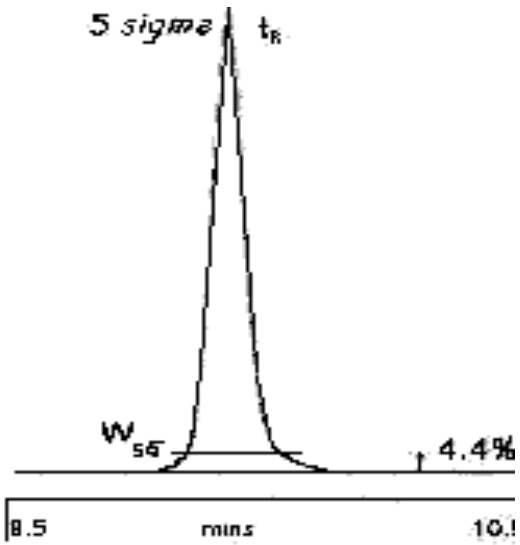
(DIN 55 672)



$$N = 16 \left(\frac{t_R}{W} \right)^2 \Bigg/ L$$

N = 71100 plates/m

(ASTM D5296)



$$N = 25 \left(\frac{t_R}{W_{5\sigma}} \right)^2 \Bigg/ L$$

N = 59500 plates/m

Chromatographic parameters

Chromatographic resolution is influenced by the following terms:

- Selectivity: $\alpha - 1$

Small changes in a result in large changes in resolution R_s. The value of α is determined by the phase system

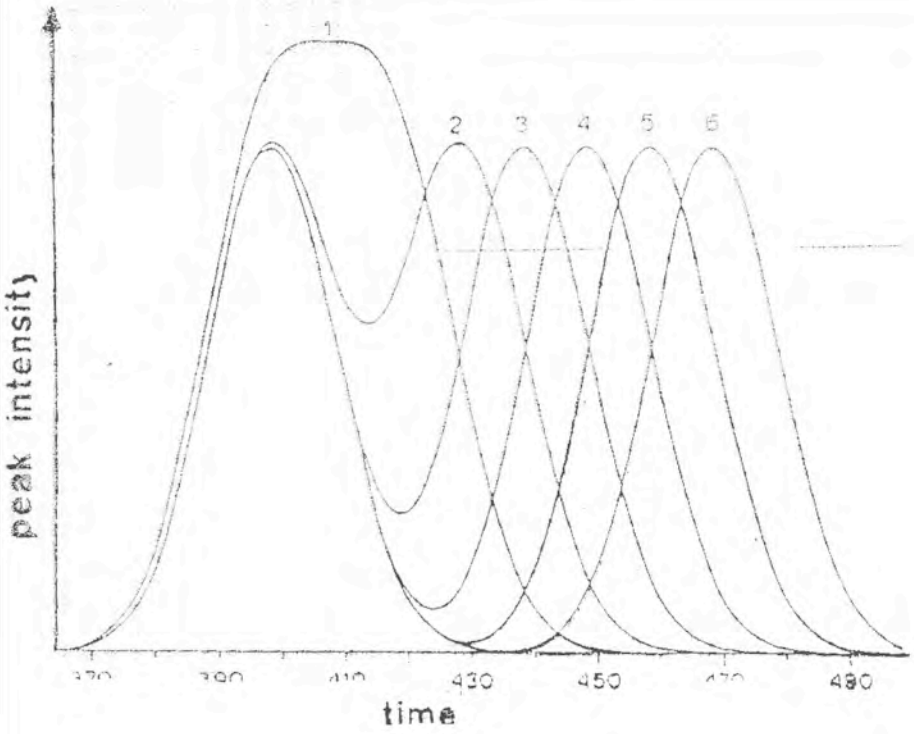
- Retention: $k_1/(1+k_1)$

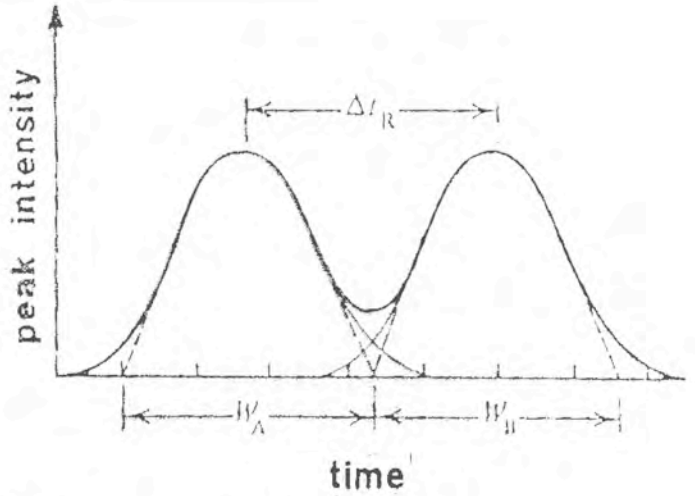
This term influences resolution for small values of k. When k becomes large, this term approaches the value 1.

- Dispersion: $\sqrt{N_1} = \sqrt{(L/H_1)}$

This term describes the separation efficiency of the column.

Chromatographic parameters





1: Rs=0.50, 2: Rs=0.75,
3: Rs=1.00, 4: Rs=1.25,
5: Rs=1.50, 6: Rs=1.75.

$$Rs = 2\,\frac{ta - tb}{Wa + Wb}$$

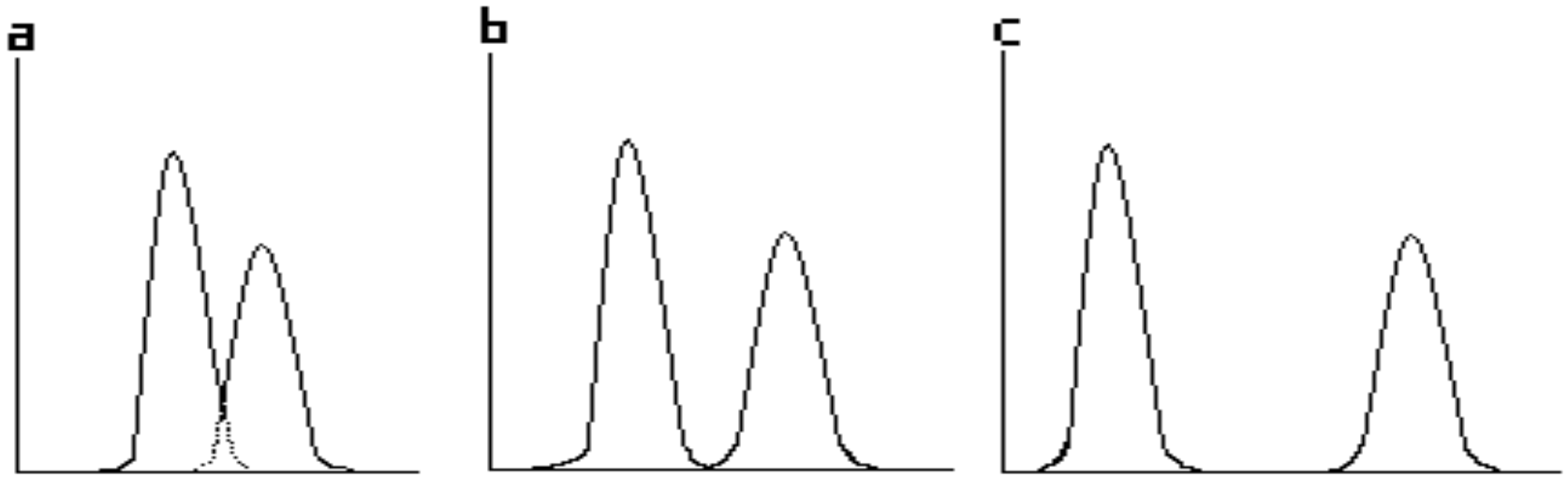
Rs : resolution of two peaks,
Wa : peak width of peak a,
Wb : peak width of peak b,
ta : retention time of peak a,
tb : retention time of peak b,

Chromatographic parameters

Conclusions:

- Separation is most strongly affected by the selectivity term which depends on the appropriate choice of the phase system
- Improvement in resolution can be attained by increasing the retention factor. However, improvement in resolution levels off for increasing k values and for practical purposes, little improvement in resolution is obtained for k values > 10.
- When the column length is increased by a factor of two, resolution increases only by a factor of $\sqrt{2} = 1.4$
- It is important to keep the theoretical plate height to a minimum. This can be achieved by using well-packed columns and materials with small particle diameter

Chromatographic parameters



Chromatographic resolution:

a) bad resolution b) optimal resolution c) waste of time

Thermodynamics of separation

Distribution of solute I between two immiscible phases x and y

$$I^y \Leftrightarrow I^x$$

Equilibrium coefficients K^x and K^c

$$K^x = x^x/x^y \qquad K^c = c^x/c^y \qquad K^c = K^x\,(V^y/V^x)$$

x^x, x^y mole fraction of I in phase x and y

c^x, c^y molar concentration of I in phase x and y

V^y,V^x molar volume of compounds

Thermodynamics of separation

Thermodynamic distribution constant K^0

$$K^0 = a^x / a^y$$

a^x , a^y activity of solute I in phase x and y

$$RT \ln K^0 = - \Delta\mu^0 = \mu^{0,y} - \mu^{0,x}$$

$\mu^{0,x}$, $\mu^{0,y}$ chemical potential of solute I in phase x and y (standard state)

Thermodynamics of separation

Relationship between K^x and K^0

K^0 is a function of the chosen state

$$K^0 = \gamma^x x^x / \gamma^y x^y \qquad K^x = (\gamma^y / \gamma^x) K^0$$

γ^y , γ^x activity coefficient of I in phase x and y

For the infinite dilution reference state of each phase one obtains $\gamma^x = \gamma^y = 1$ and $K^x = K^0$

Thermodynamics of separation

$$\Delta G^0 = - RT \ln K^0 = \Delta \mu^0$$

ΔG^0 change of standard free energy

$$\Delta \mu^0 = \Delta h^0 - T\Delta s^0$$

Δh^0 , Δs^0 change of partial molar enthalpy and entropy at standard state

Selectivity in separation processes

Selectivity coefficient α

$$\alpha = K^I_j / K^I_i = K^C_j / K^C_i$$

K^I_j / K^I_i capacity factor of solute j and i

K^C_j / K^C_i distribution coefficient of solute j and i

$$RT \ln \alpha = - (\Delta\mu^0_i - \Delta\mu^0_j)$$

$\Delta\mu^0_i - \Delta\mu^0_j$ difference of the standard chemical potential between phases x and y for solutes i and j

Distribution coefficients

Methods to estimate distribution coefficients

- solubility parameter model

$$\ln K^{C}_{i} = (v_i [(\delta_i - \delta_m)^2 - (\delta_i - \delta_s)^2])/ RT$$

- Snyder equation

$$\ln K^{C}_{i} = \ln v_a + \alpha' (S^0 - A_s \varepsilon)$$

- Huber (liquid – liquid partition)

$$\ln K^{C}_{i} = \sum_{10} a_{ip} + v_{jp}$$

Distribution coefficients

Relationship between chromatographic retention expressed as K^i and the distribution coefficient K^c

$$K^i = (v_s / v_m) K^c = \Phi K^c$$

v_s , v_m volume of stationary and mobile phase

$$\ln K^i = - (\Delta H^0 / RT) + (\Delta S^0 / R) + \ln \Phi$$

enthalpic term

entropic term

Quantitative models

- Hildebrand's solubility parameter theory
- Martin equation

  (additively of group interactions)
- Flory – Huggins equation

  (mixing of components of different sizes)

Displacement without localization

$$X_n + nM_a \Leftrightarrow X_a + nM_n$$

The subscripts n and a refer to molecules in the non-adsorbed and adsorbed phases

The dimensionless standard state free energy ΔE of X becomes

$$\Delta E = E_{X_a} + nE_{M_n} - E_{X_n} - nE_{M_a}$$

$$\Delta E \sim E_{X_a} - nE_{M_a}$$

Gibbs formalism

In adsorption chromatography the adsorption of solute i can be defined in terms of the Gibbs formalism of surface excess quantities

capacity factor of solute i is defined as

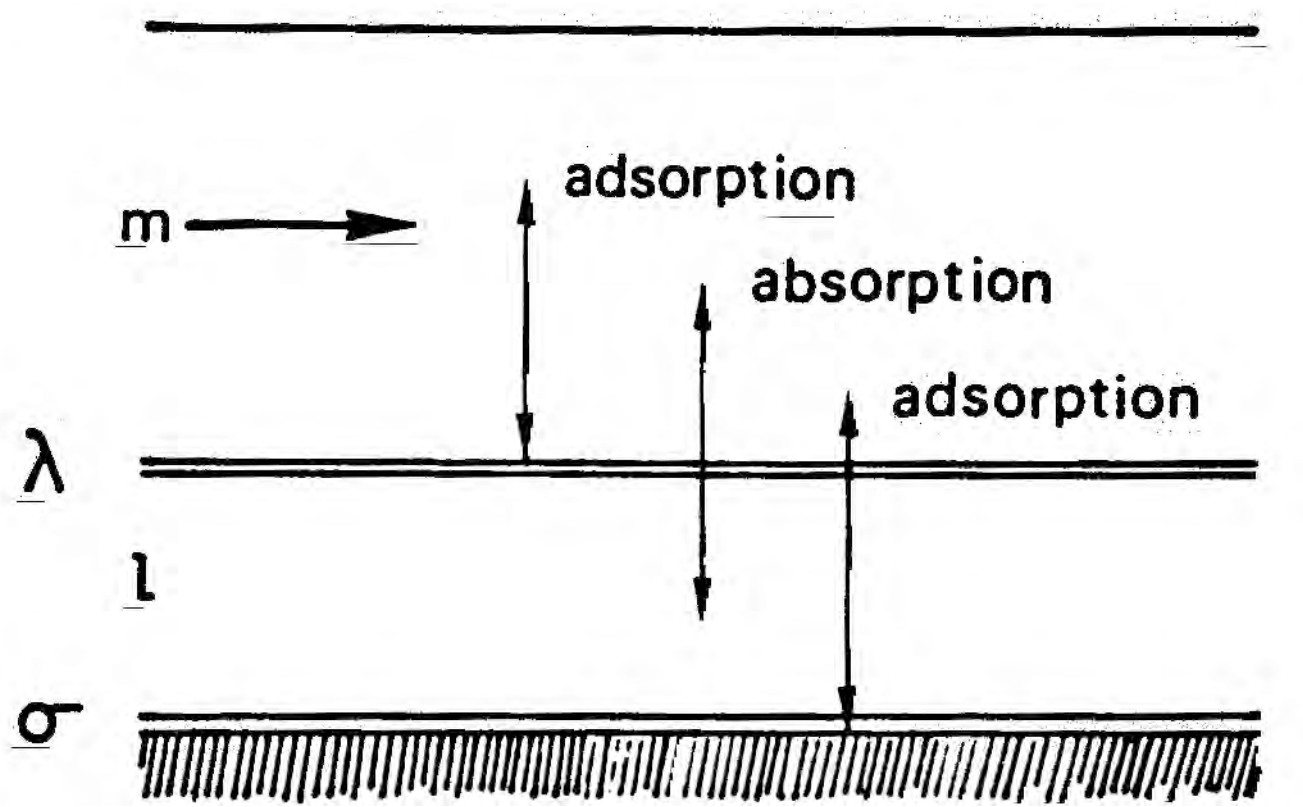
$$K^{I}_{i} = n^{\delta}_{i} / n^{e}_{i}$$

n^{δ}_{i} , n^{e}_{i} amount of solute i in the adsorbed and liquid phase

The volume reduced surface excess amount is given by

$$n^{\delta}_{i}{}^{(v)} = n_{i} - c_{i} v_{m}$$

Chromatographic parameters

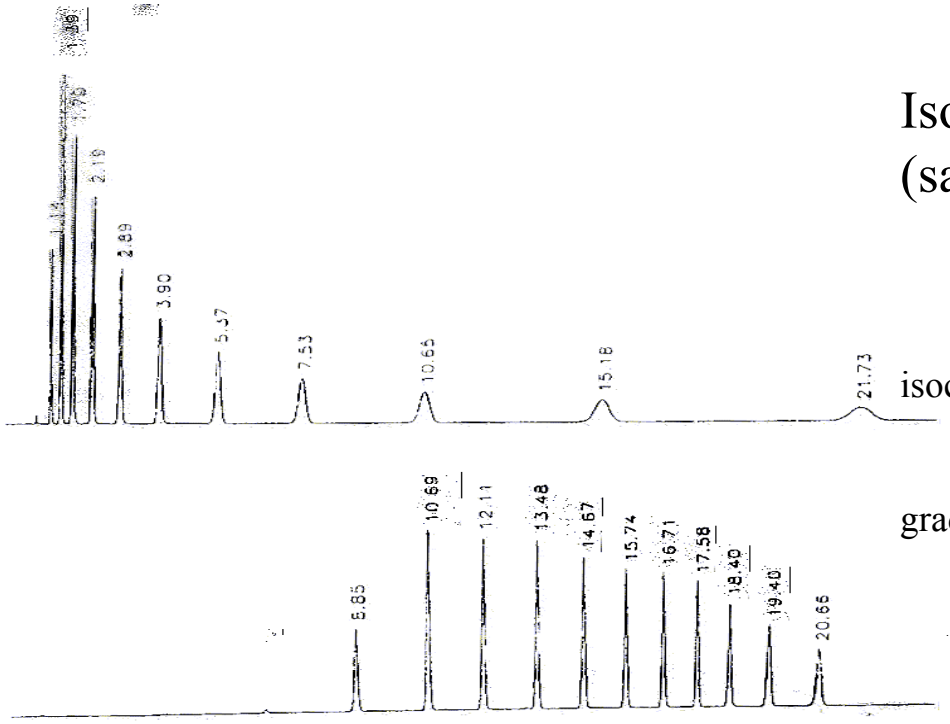


Elution modes in HPLC

Elution can be achieved by two different modes:

✓Isocratic elution, without changing the mobile phase composition

✓Gradient elution, where the mobile phase composition is changed during the course of the separation



Isocratic elution vs. gradient elution
(sample detected at equal attenuation)

isocratic: water/acetonitrile

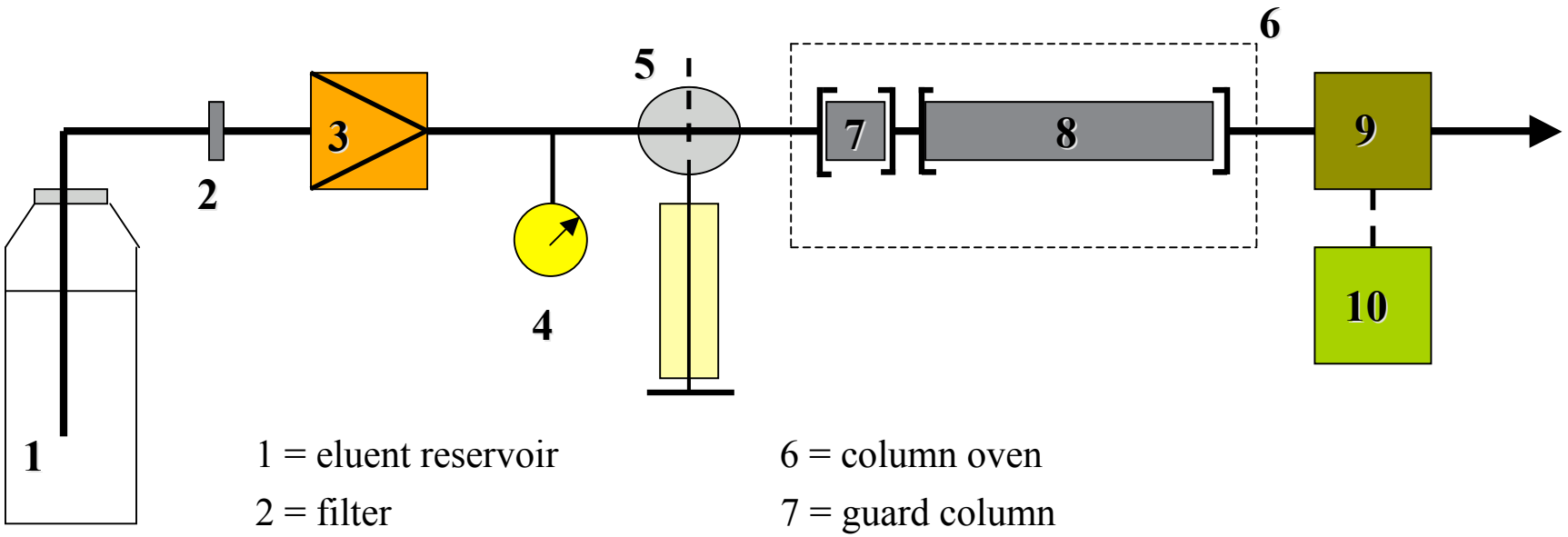
25/75 (V/V) continuous

gradient: water/acetonitrile

from 90/10 (1min), at 5%/min, to 0/90 (5 min)

Methodology and instrumentation

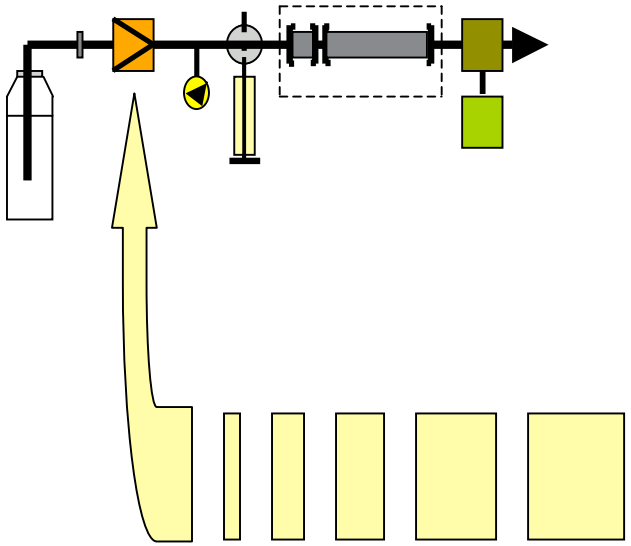
Schematic diagram of an HPLC system for isocratic operation



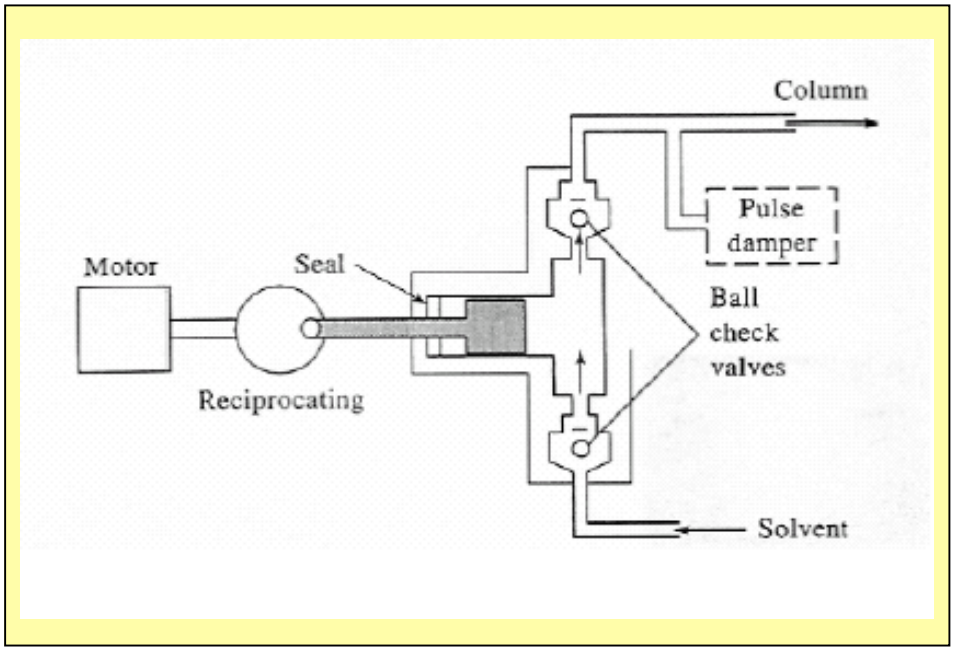
1 = eluent reservoir
2 = filter
3 = high pressure pump with pulse dampener
4 = pressure gauge
5 = sample injection valve with syringe
6 = column oven
7 = guard column
8 = column
9 = detector
10 = recorder (integrator, PC etc.)

Methodology and instrumentation

Pumps



Schematic drawing of a serial short-stroke dual-head reciprocating pump

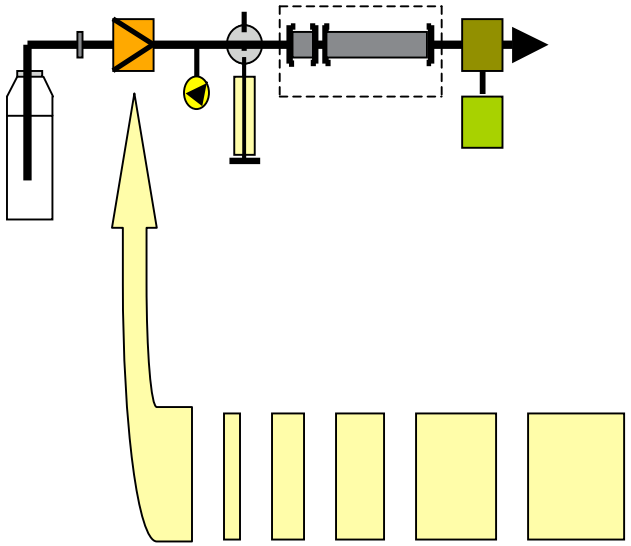


An analytical HPLC pump must comply with following specifications:

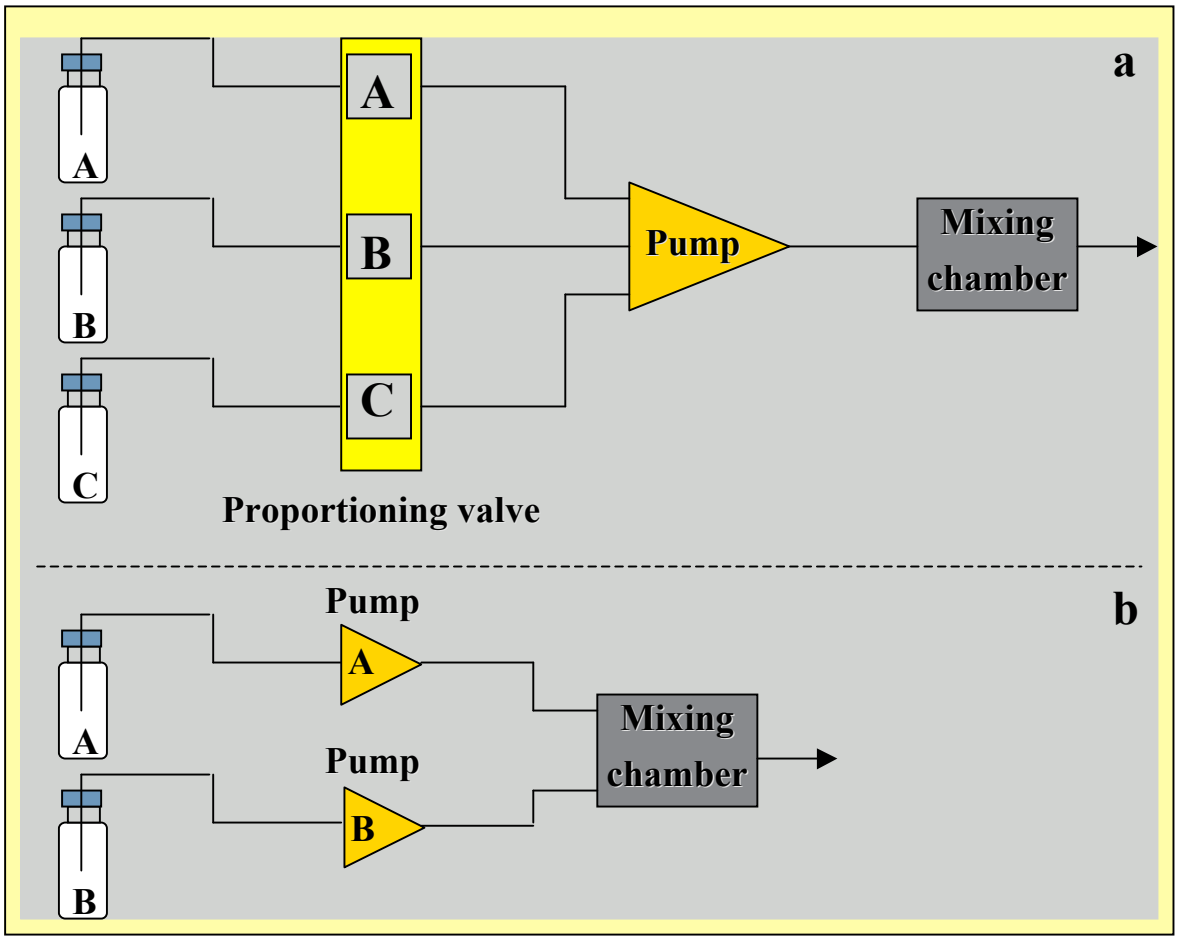
- variable flow rates between 0.1 and 10 ml/min
- constant flow of 2% or better at back pressures up to 40 Mpa (400 bar)

Methodology and instrumentation

Pumps



Mixing of mobile phases for HPLC



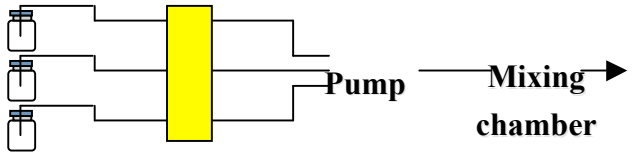
a) Low-pressure gradient system;

b) High-pressure gradient system

Methodology and instrumentation

Pumps

- The advantages of a low-pressure gradient system are:



  - *More than two solvents can be mixed;*
  - *Only one high-pressure pump is needed;*
  - *Reproducibility of gradient formation is higher compared to a high pressure gradient system;*
  - *When the mobile phase composition is changed, it is easier to obtain a constant flow (after changing the mobile phase composition)*

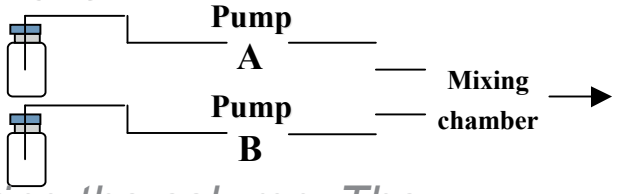
- Disadvantages of the low-pressure gradient system are:

  - *Proportioning valves are susceptible to contamination and can cause inaccuracies in eluent composition*
  - *The additional mixing chamber increases the internal volume of the system and causes a delay in the formation of the gradient (the gradient which is actually delivered to the column) compared to the programmed value.*

Methodology and instrumentation

Pumps

- Advantages of a high-pressure gradient system are:



  - *The two solvents are combined just before entering the column. The effective mobile phase composition is therefore consistent with the programmed mobile phase composition*
  - *The internal volume of the high-pressure system is very small and it is therefore possible to run steep gradient profiles and to perform quick mobile phase changes.*
  - *Degassing becomes unnecessary when mobile phase is composed at high pressure*

- Disadvantages of a high-pressure gradient system are:
  - *High costs, as two pumps are needed*
  - *Lower precision of most pumps, when operated at lower flow rates, limits the precision and reproducibility of gradient formation at extreme mobile phase compositions*
  - *A third pump is needed to create a gradient based on a ternary solvent composition*

Methodology and instrumentation

Pumps

- When using mobile phase gradients it is important to consider the following points:

  – *The solvents must be miscible over the entire gradient composition range.*

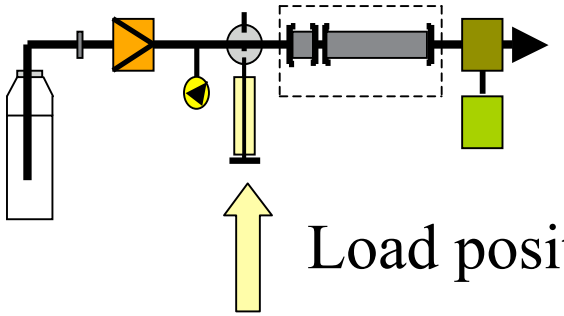
  – *The viscosity of the resulting mobile phase can change considerably when the solvents are mixed resulting in an increased column back pressure.*

  – *The solvents must be thoroughly degassed otherwise bubbles can form during mixing. Water/methanol and water/acetonitrile phases are very susceptible. The solvents can be degassed by vacuum, sonication or helium sparging. Flow-through on-line degassers can also be used.*

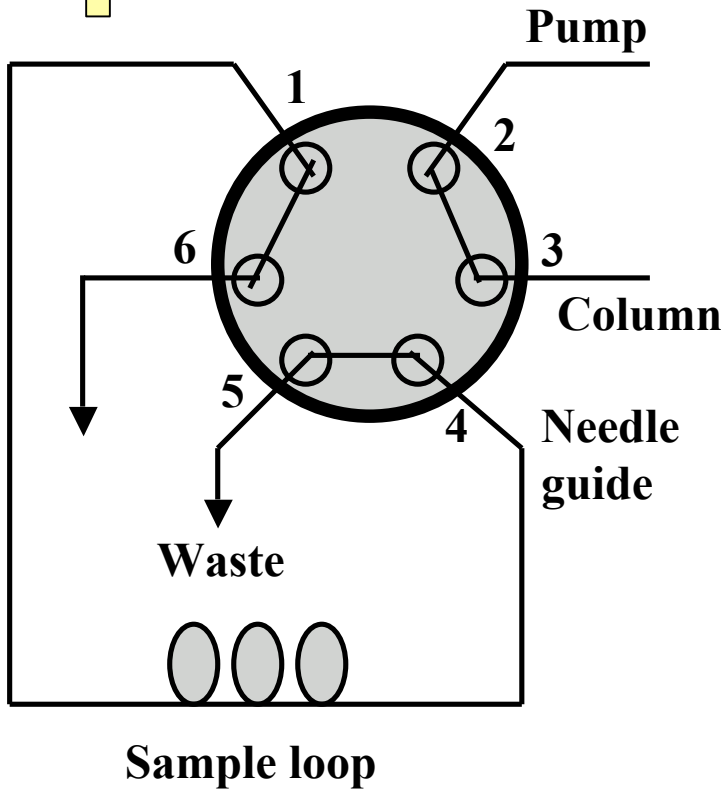
  – *The detector must be able to handle gradients. The baseline should remain stable over the entire mobile phase composition range. UV and fluorescence detectors are suitable for gradient elution whereas a reflective index and an electrochemical detector cannot be used.*

Methodology and instrumentation

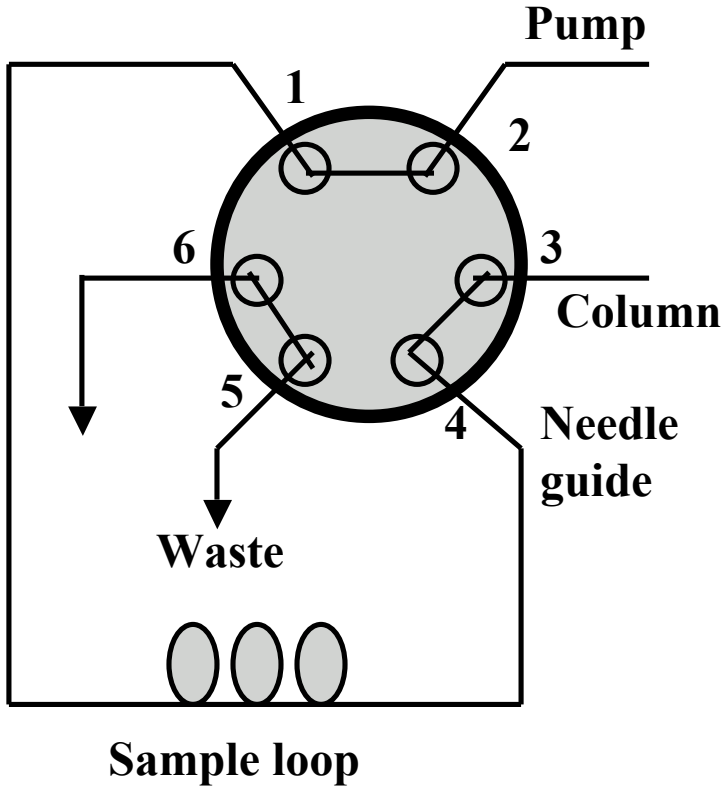
Injection valves



Load position



Injection position



Methodology and instrumentation

Packing Materials

Packing materials consists of porous or nonporous particles. The particles are spherical, pellicular or irregularly formed. They are available in different sizes.

Average particle diameter dp (μm)	**Application in chromatography**
3 - 5	fast separation in short columns
5 - 10	analytical applications
10 – 100	preparative separations

Packing materials can be divided into three groups:

- Inorganic packing materials such as silica and alumina (IP)
- Organic polymers such as crosslinked agarose, copolymers of styrene-divinylbenzene, polymethylmethacrylate (OP)
- Bonded packing materials (BP)

Methodology and instrumentation

Packing Materials

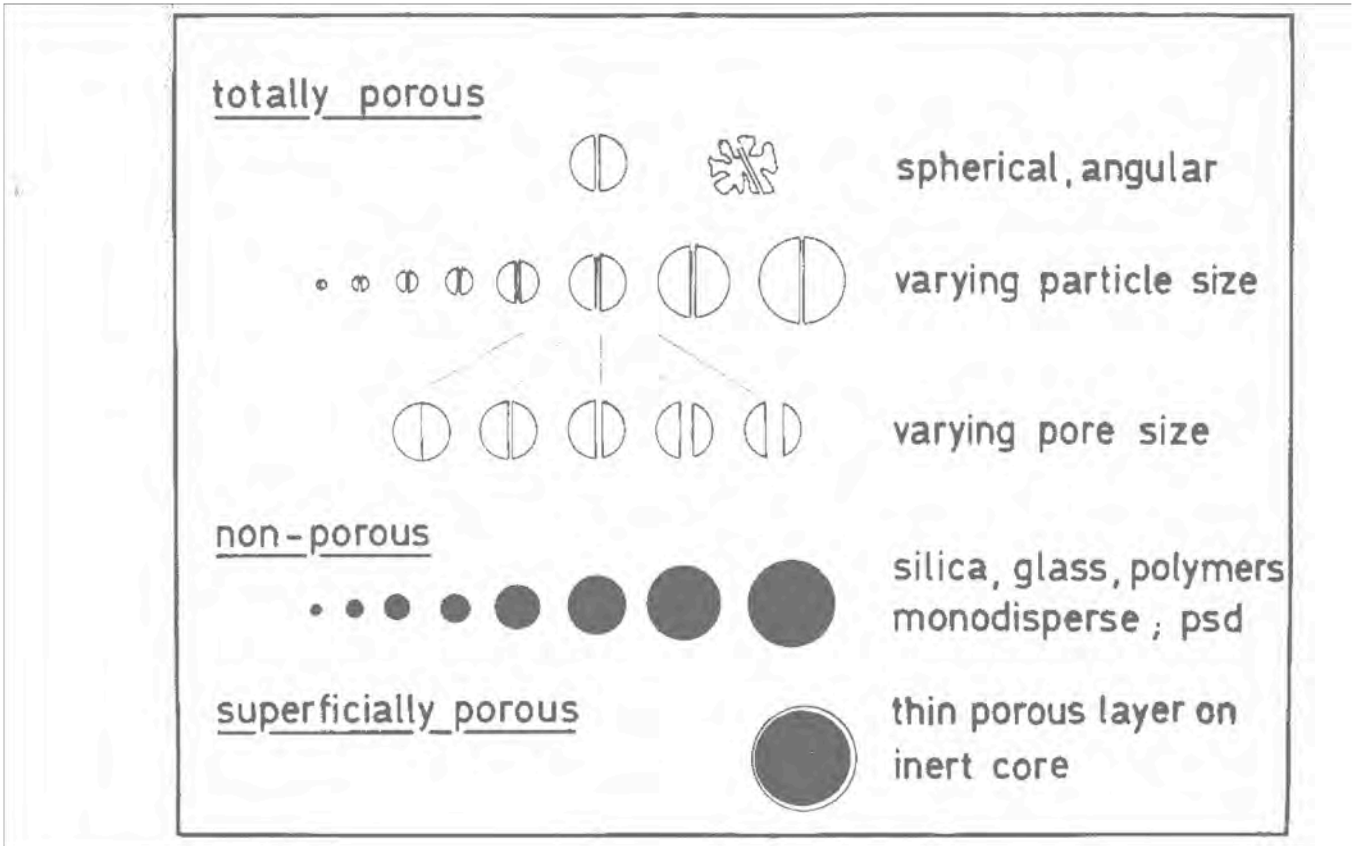
Criteria of packings, stationary phases and columns:

- *high selectivity and specificity,*
- *high flexibility,*
- *preservation of biological activity and high mass recovery,*
- *high bed stability and low flow resistance,*
- *high chemical stability during use and storage,*
- *adequate efficiency,*
- *fast and complete regeneration,*
- *no fouling,*
- *lot-to-lot and column-to-column consistency,*
- *available at graduated particle sizes.*

Methodology and instrumentation

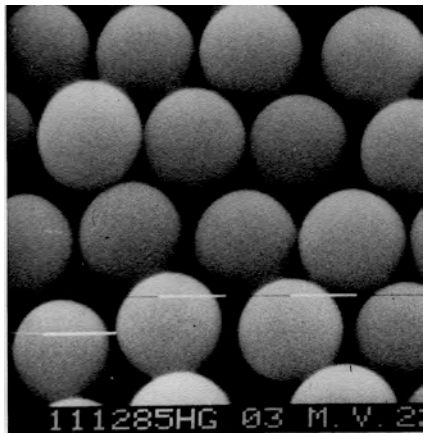
Packing Materials

Inorganic packing materials such as silica and alumina, according to their porosity can be grouped to:

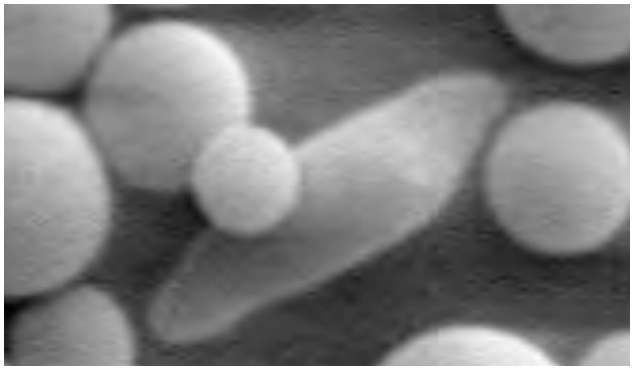


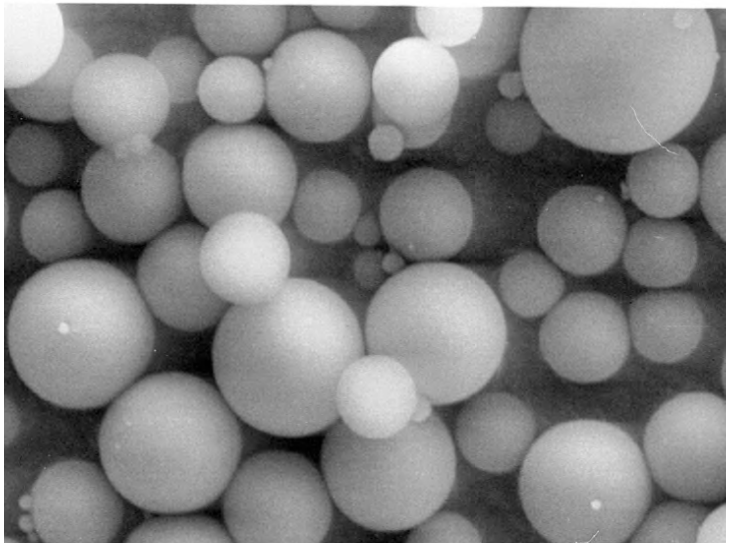
Methodology and instrumentation

Packing Materials



Transmission electron micrographs of silica





Methodology and instrumentation

Packing Materials

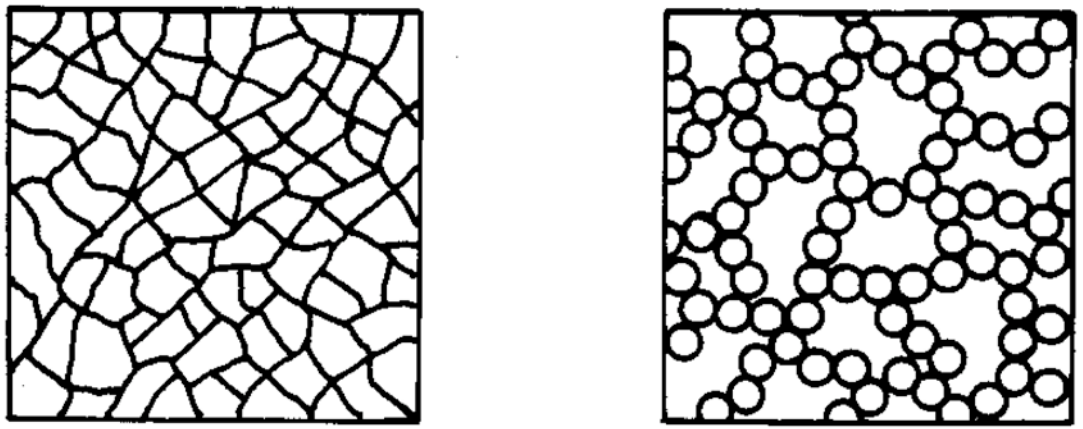
Table 1. Characteristics of bodies of packed spheres of radius R (with permission by Avery and Ramsay[4])

Type of packing	n	ε	Pore volume per cm^3 of spheres (cm^3/cm^3)	Radius of sphere inscribed in the cavities, R''	Radius of circle inscribed in the throats connecting cavities, R'
hexagonal close packed	12	0.260	0.350	0.225 R octahedral 0.414R tetrahedral	0.155R
body-centered tetragonal	10	0.302	0.432	0.291R	0.265R 0.155R
primitive hexagonal	8	0.395	0.654	0.527R	0.414R 0.155R
primitive cubic	6	0.476	0.910	0.732R	0.414R
tetrahedral	4	0.660	1.94	1.00R	0.732R

Methodology and instrumentation

Packing Materials

Schematic comparison of organic and inorganic packing materials



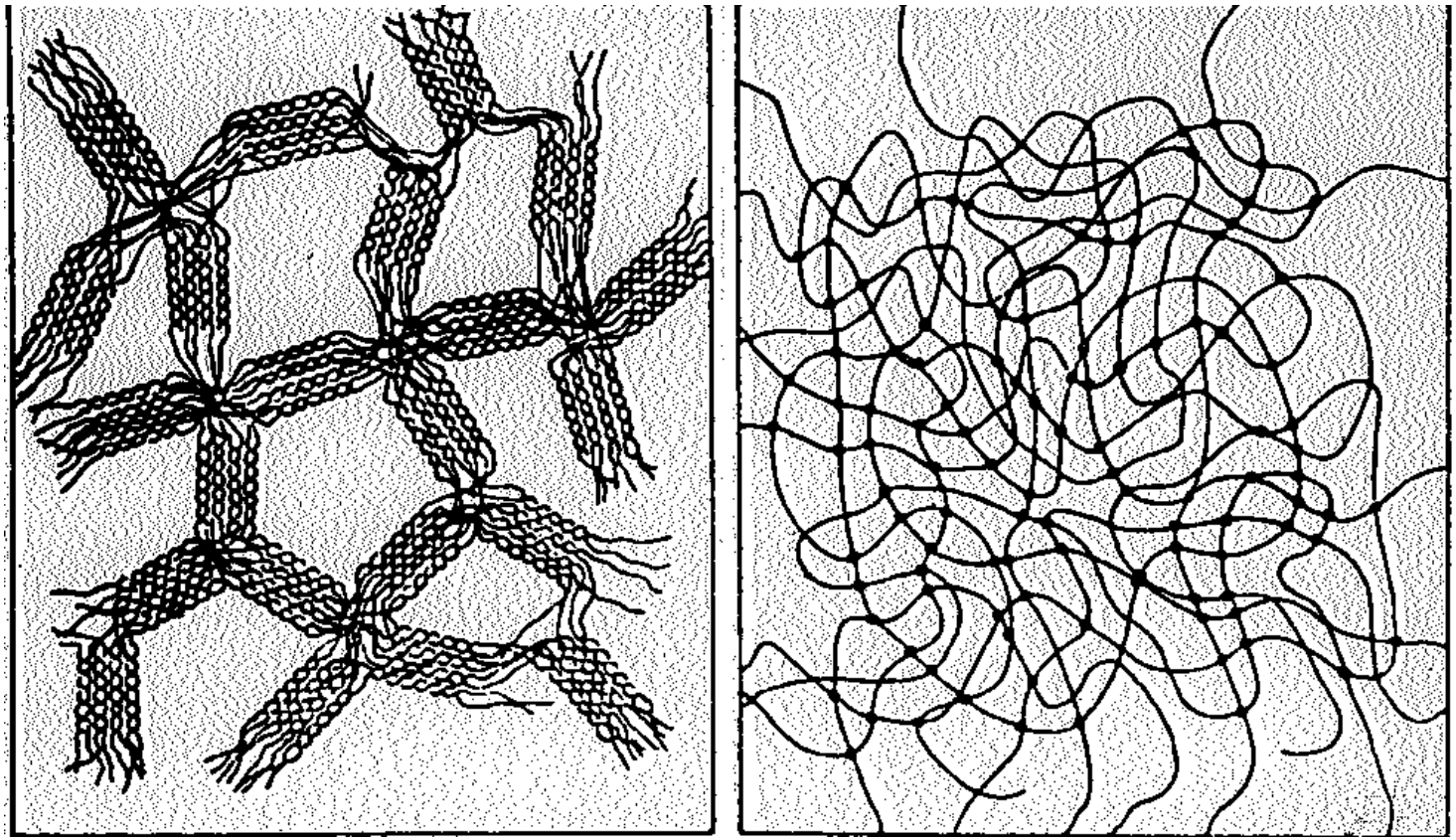
cross-linked organic gel

inorganic oxide

Methodology and instrumentation

Packing Materials

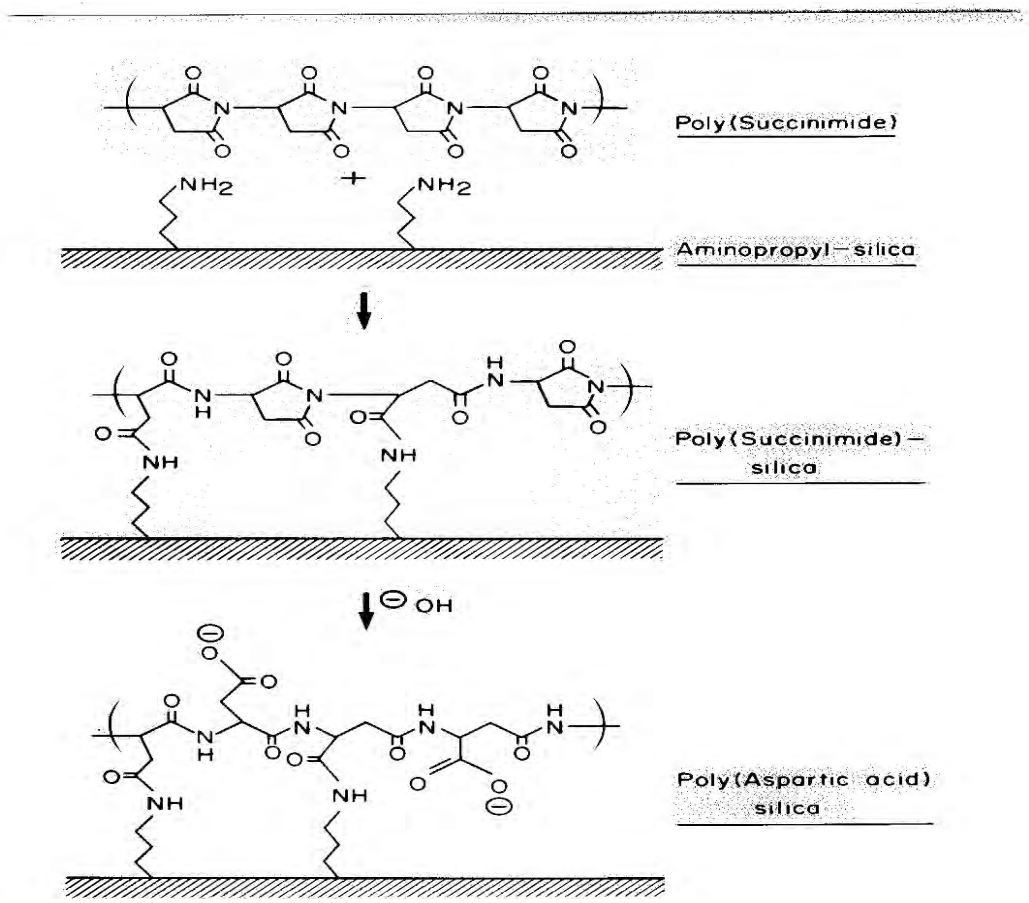
Schematic view of organic packing material



Methodology and instrumentation

Packing Materials

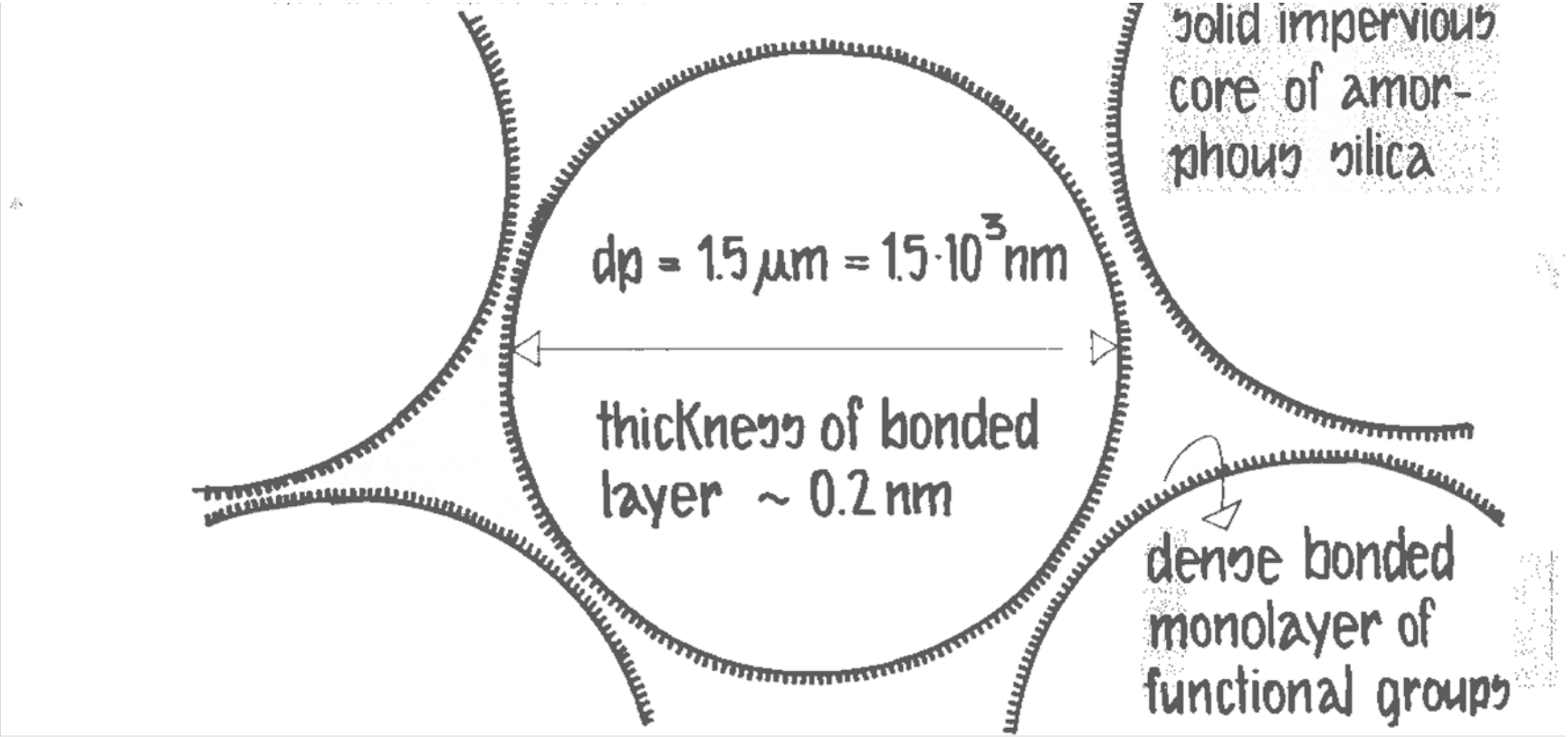
Various organic packing materials



Methodology and instrumentation

Packing Materials

Structure of non-porous monodisperse bonded silica packings

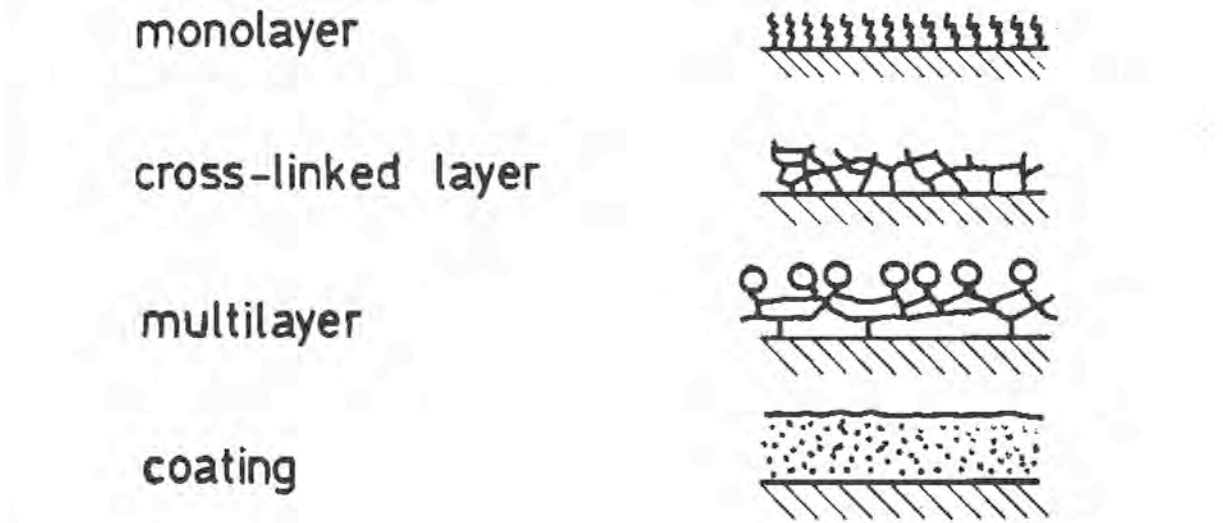


Methodology and instrumentation

Packing Materials

Bonded packing materials can be divided by:

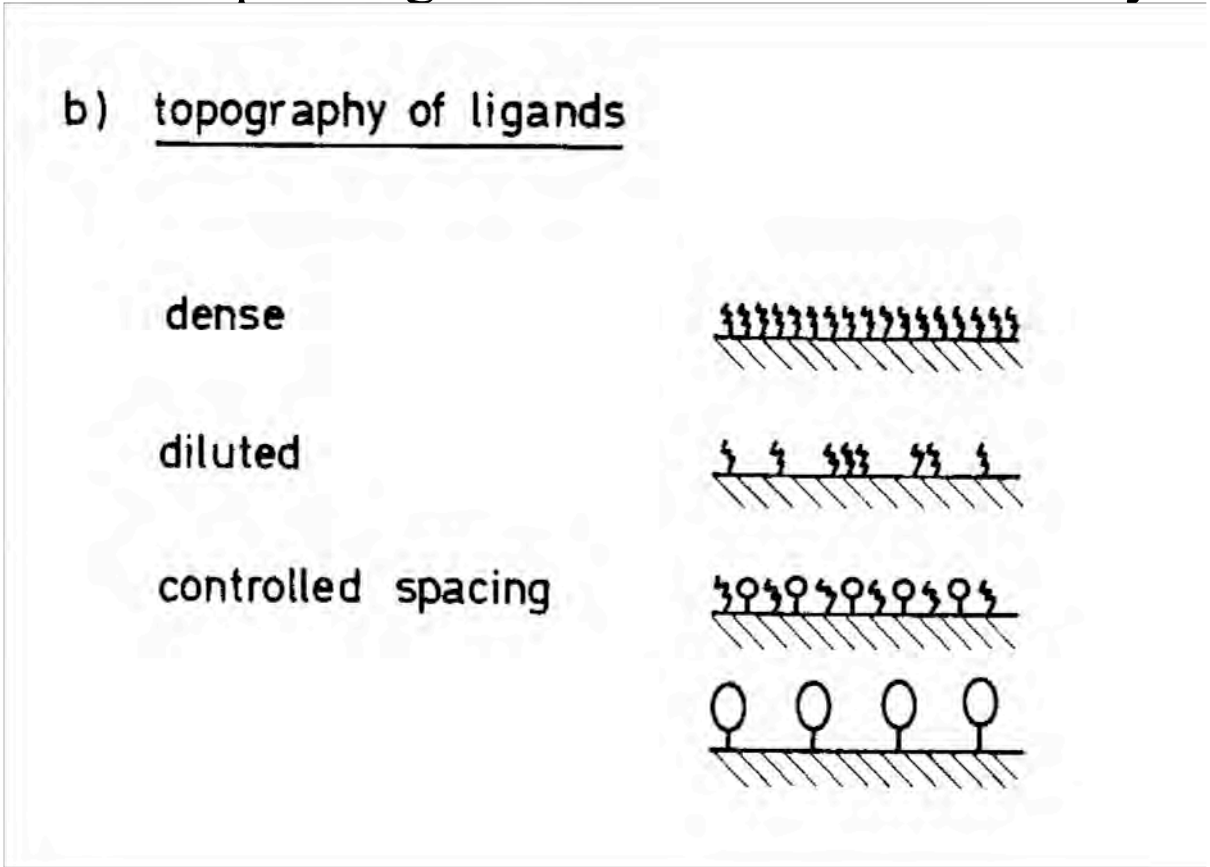
a) types of bonded phases



Methodology and instrumentation

Packing Materials

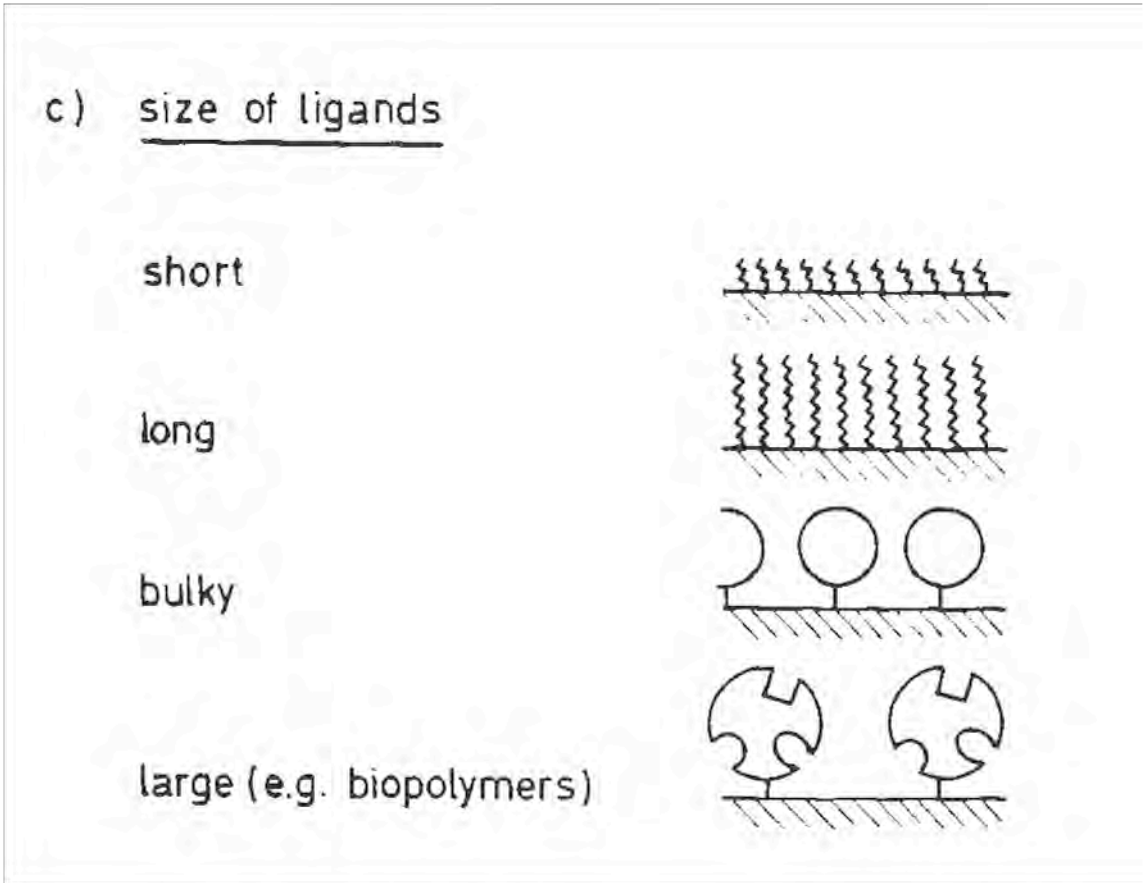
Bonded packing materials can be divided by:



Methodology and instrumentation

Packing Materials

Bonded packing materials can be divided by:

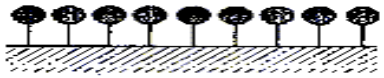


Methodology and instrumentation

Packing Materials

Bonded packing materials can be divided by structure into:

monolayer

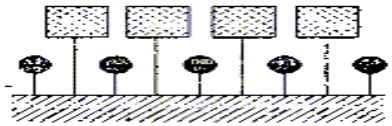


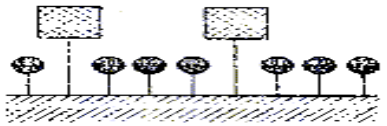
polymer layer, coating

sandwich structure

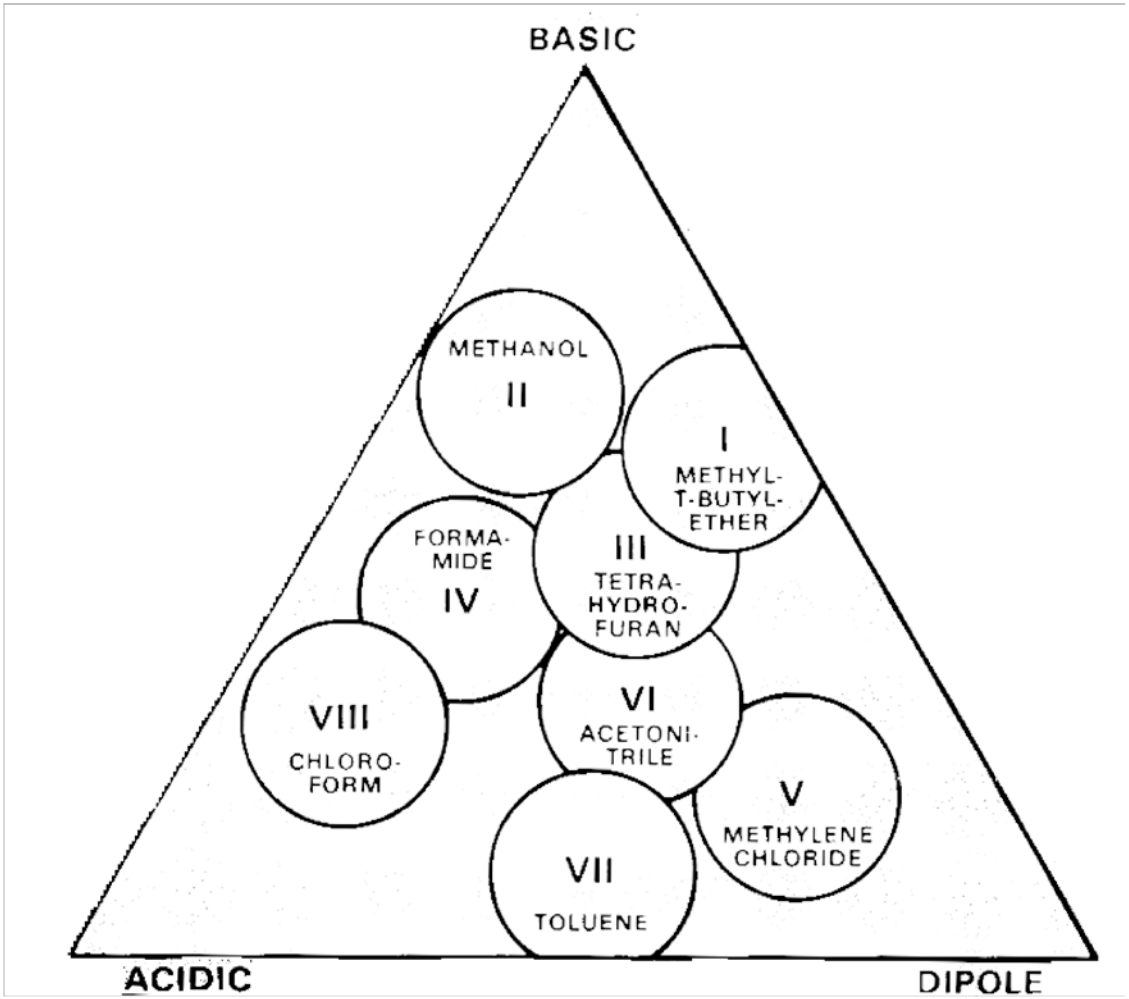
structure with
diffusion barrier

mixed mode structure





Methodology and instrumentation



Methodology and instrumentation

Types of HPLC

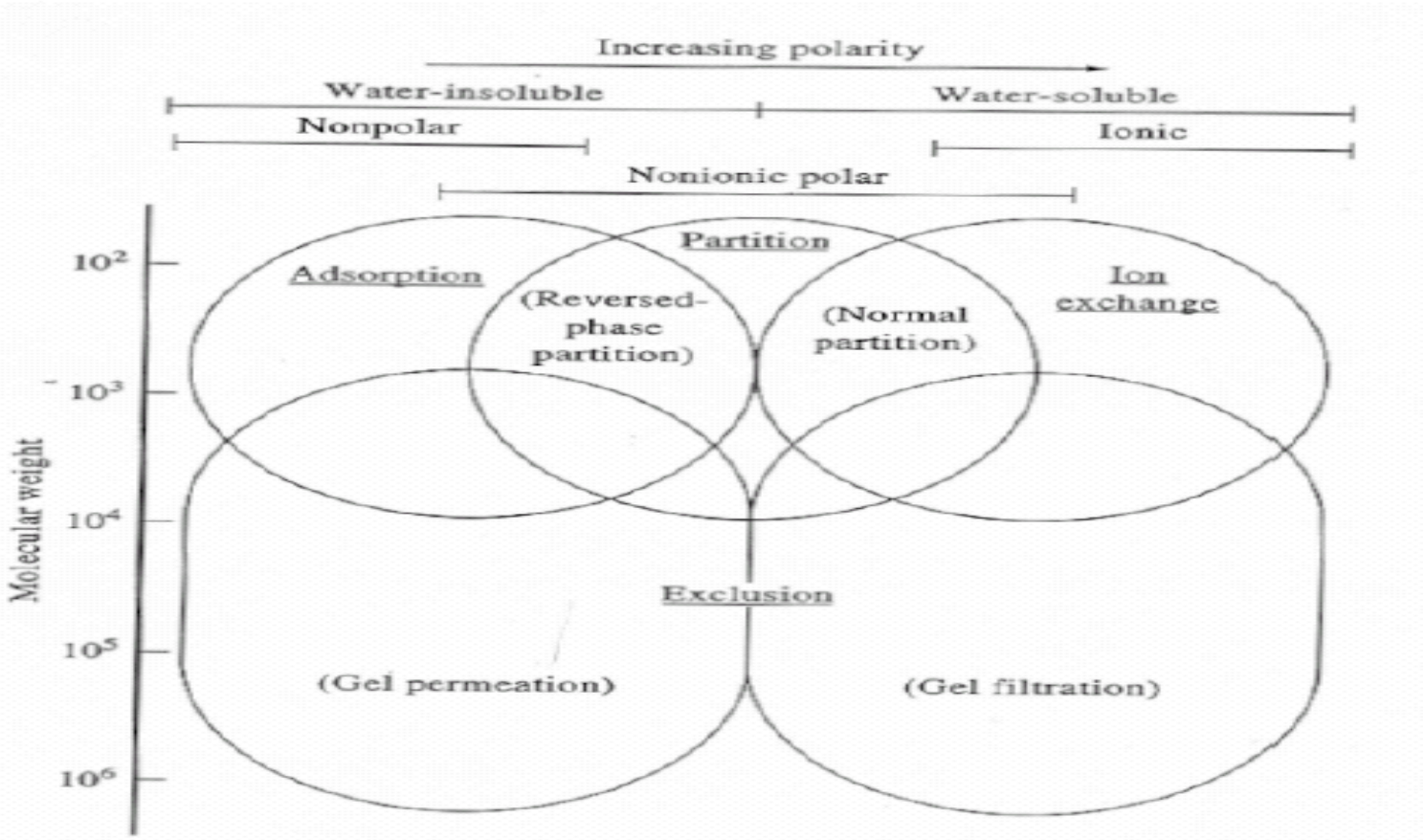
Major types of HPLC include:

- Partition chromatography (normal and reverse phase chromatography)
  - o Adsorption chromatography
  - o Ion exchange chromatography
  - o Size exclusion chromatography

Methodology and instrumentation

Types of HPLC separations

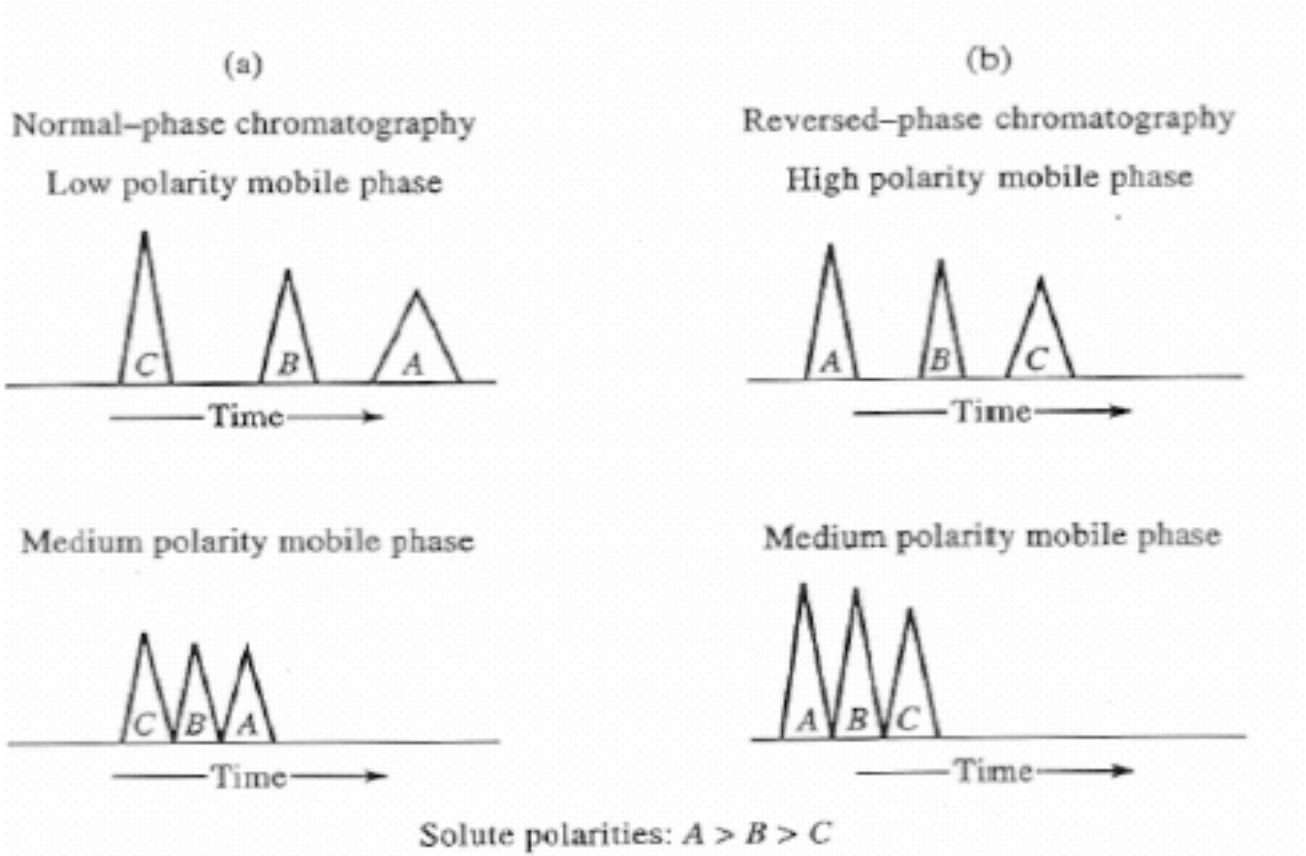
Selection of HPLC mode according to sample properties



Methodology and instrumentation

Types of HPLC separations

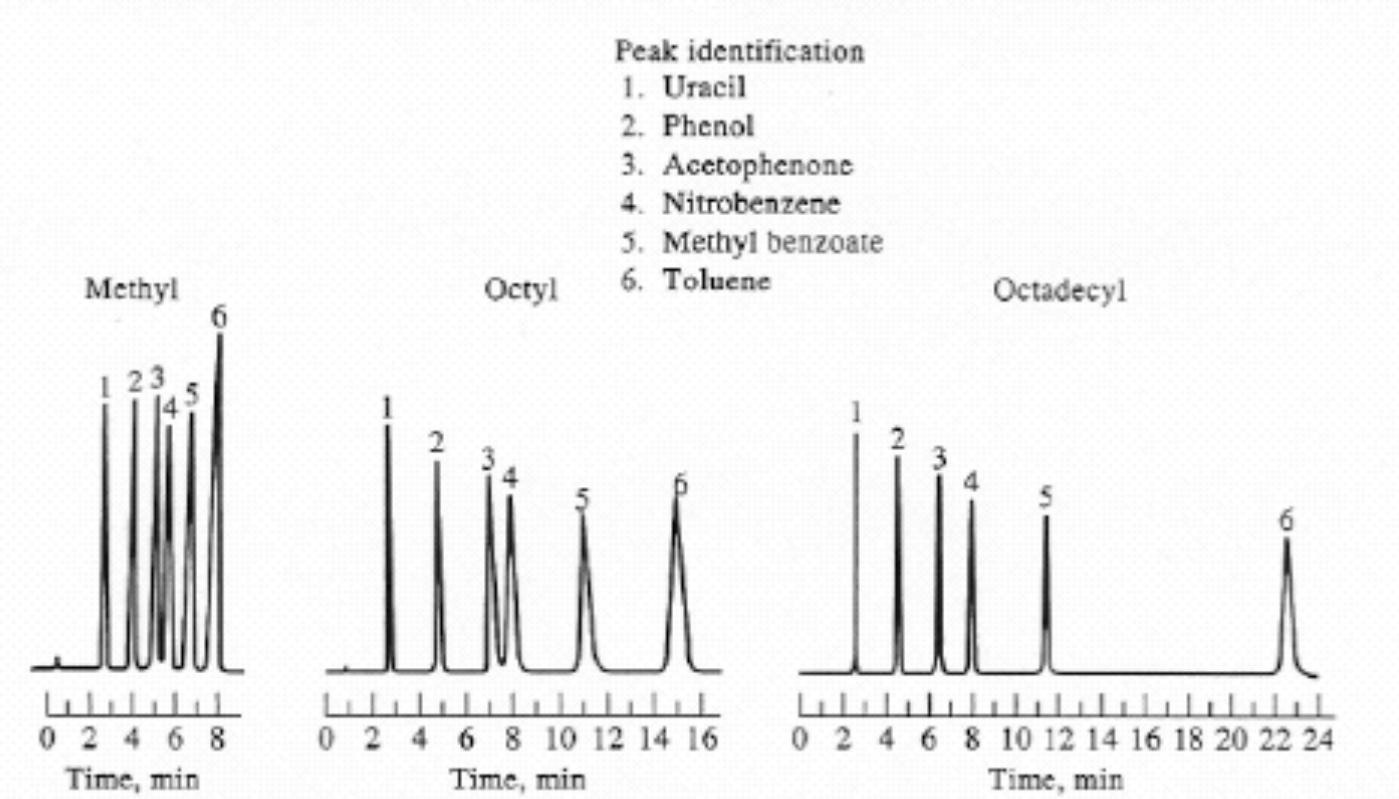
Relative elution times for polar and non-polar analytes in normal and reversed-phase HPLC



Methodology and instrumentation

Types of HPLC separations

Effect of chain length on separation efficiency for common reversed-phase HPLC stationary phases

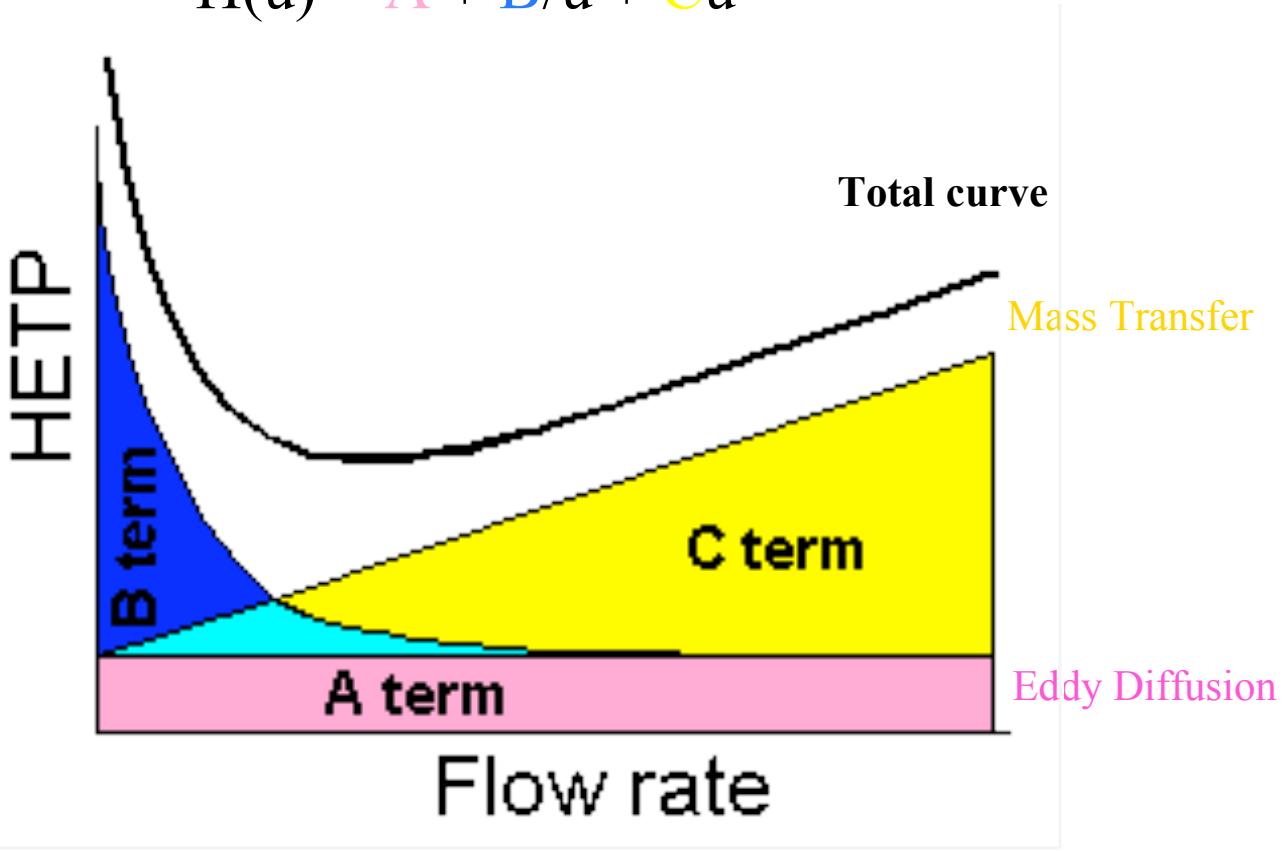


Methodology and instrumentation

Selection of optimal conditions

Relationship between theoretical plate height H and the linear flow velocity u of the eluent

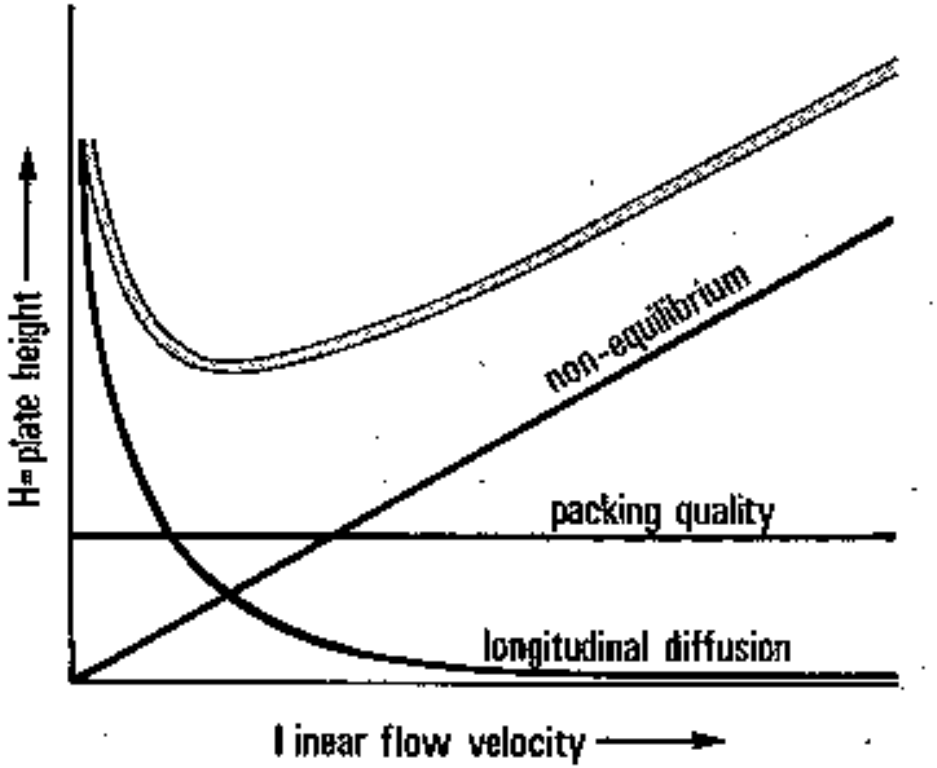
$$H(u) = A + B/u + Cu$$



Methodology and instrumentation

Selection of optimal conditions

Relationship between theoretical plate height H and the linear flow velocity u of the eluent



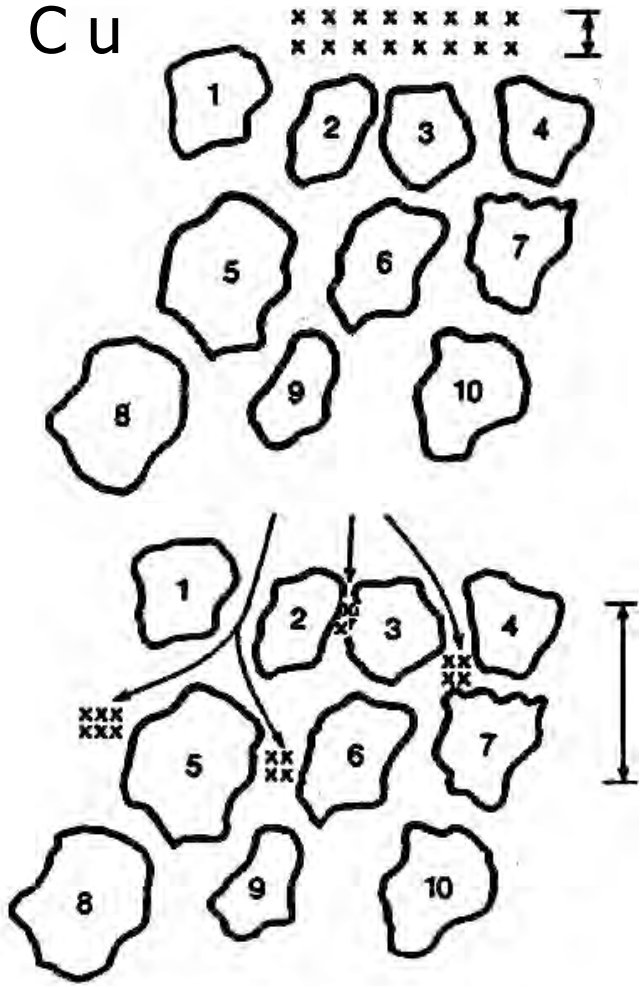
Methodology and instrumentation

Selection of optimal conditions

van-Deemter-Gleichung $H(u) = A + B/u + C\,u$

Eddy-Diffusion (convection):

- independant of u
- caused by different path ways
- dependant from mean particle diameter



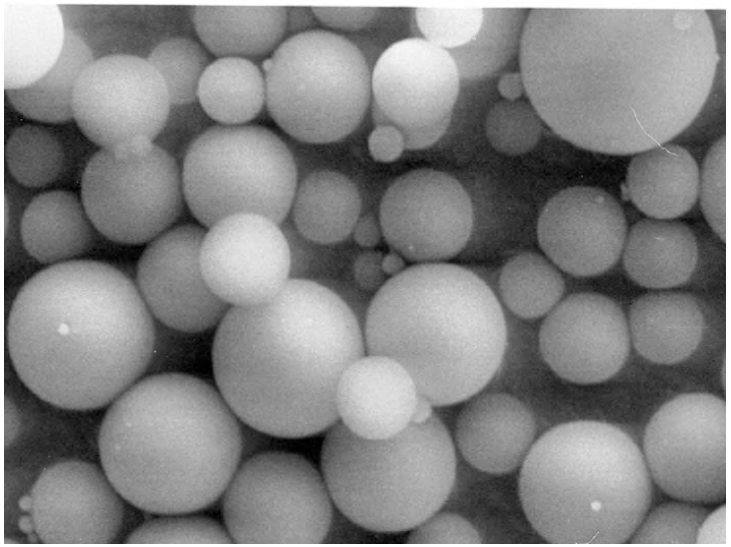
Methodology and instrumentation

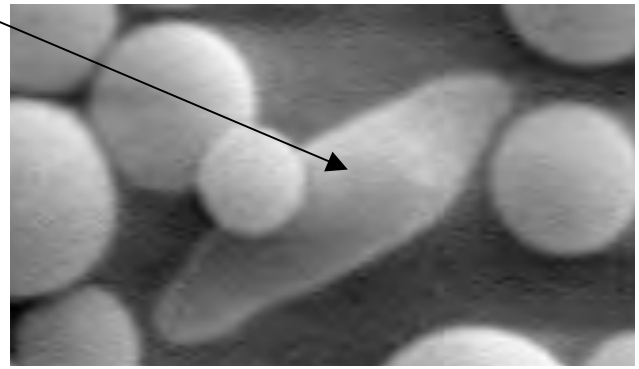
Selection of optimal conditions

$$H(u) = A + B/u + C\,u$$

Eddy diffusion

- dependant on particle size distribution
- morphology of packing material





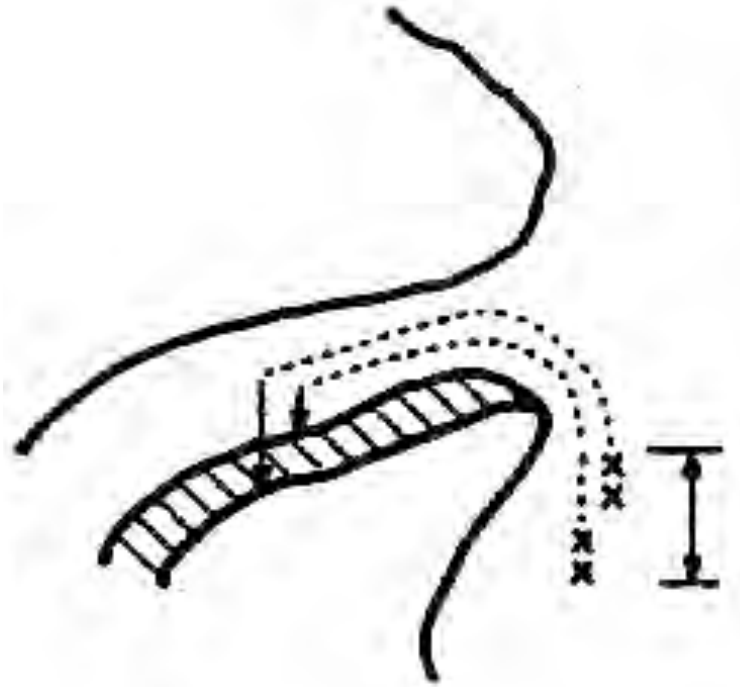
Methodology and instrumentation

Selection of optimal conditions

$$H(u) = A + B/u + C\,u$$

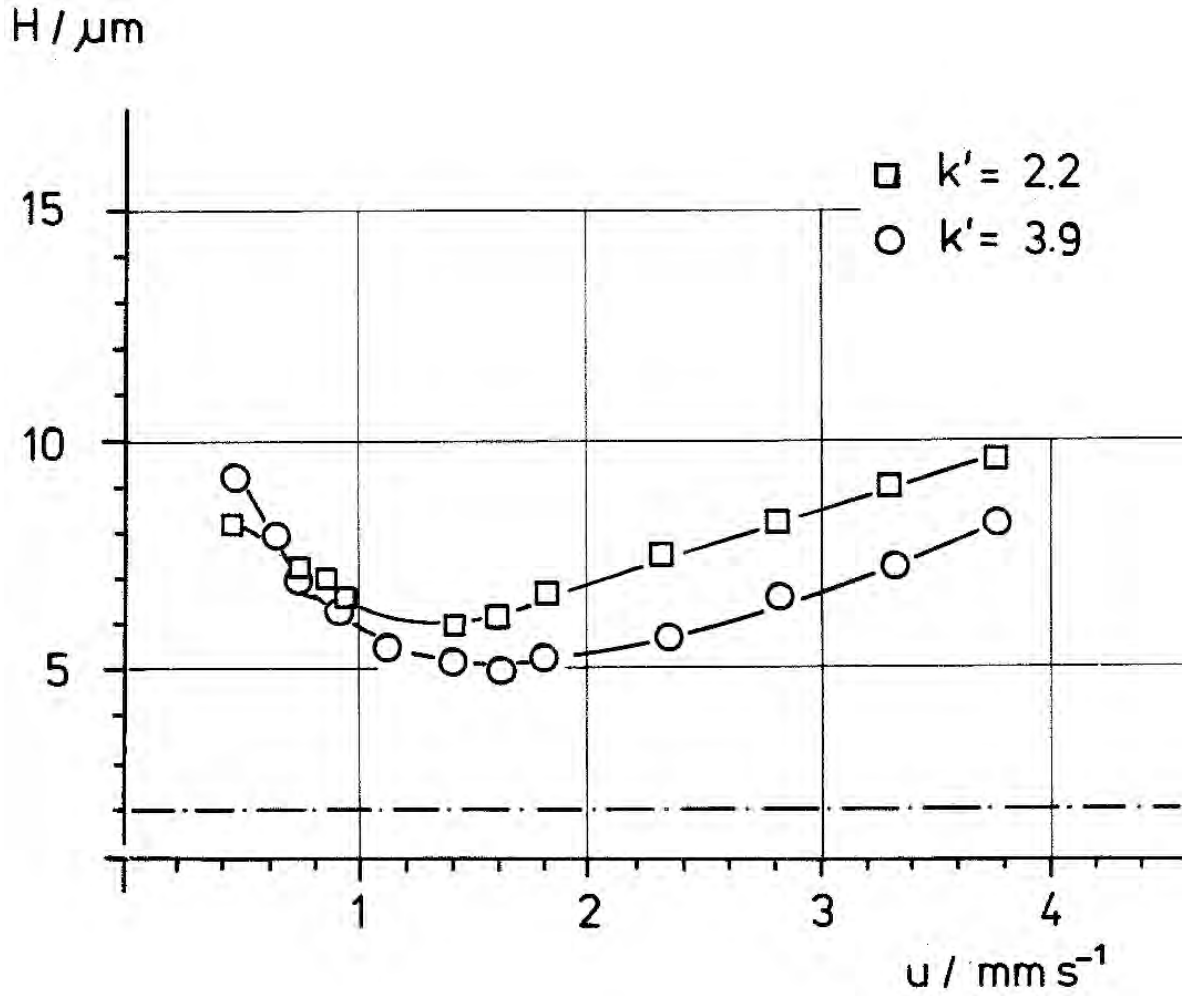
Mass transfer between mobile and stationary phase

- proportional to flow rate
- caused by interaction between analyte in the mobile phase and the adsorbent
- proportional to the surface area of the adsorbent



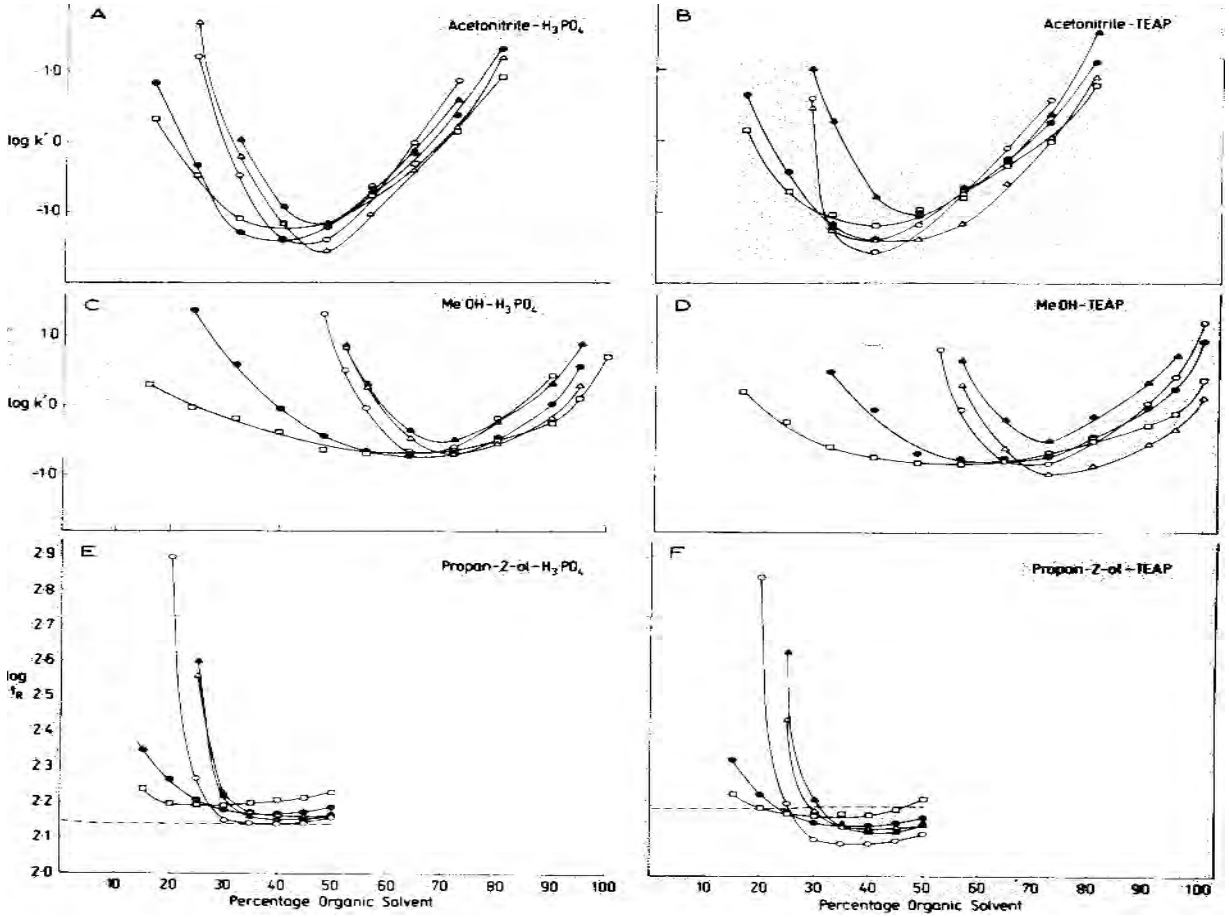
Methodology and instrumentation

Selection of optimal conditions



Methodology and instrumentation

Selection of optimal conditions



Methodology and instrumentation

Detection

Types of Detectors

1) Bulk property detectors ⇒ respond to mobile phase property such as refractive index, dielectric constant or density which is altered by the presence of analytes

2) Solute property detectors ⇒ respond to properties of solute via UV-Vis absorbance, fluorescence, or current

Methodology and instrumentation

Detection

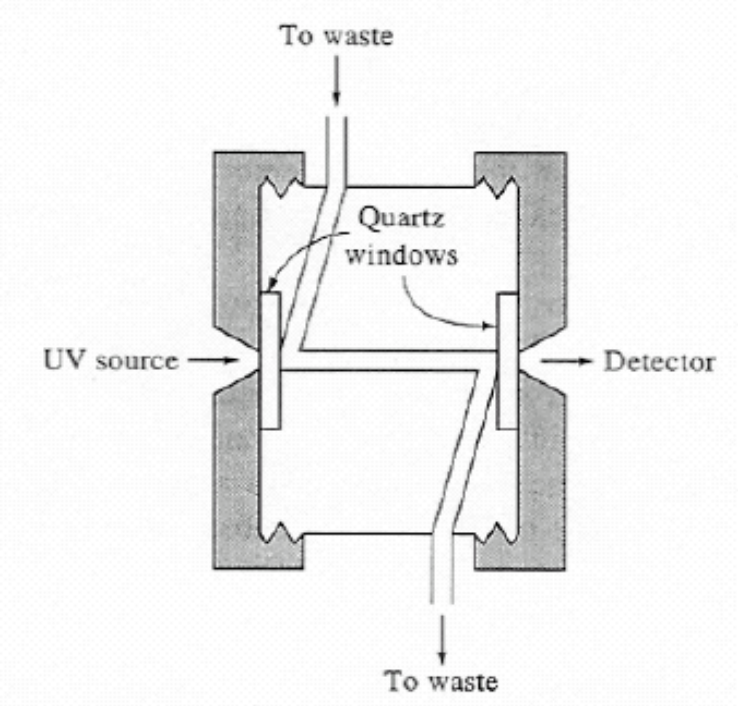
Performances of LC detectors

LC Detector	Commercially Available	Mass LOD (commercial detectors)[a]	Mass LOD (state of the art)[b]
Absorbance	Yes[c]	100 pg–1 ng	1 pg
Fluorescence	Yes[c]	1–10 pg	10 fg
Electrochemical	Yes[c]	10 pg–1 ng	100 fg
Refractive index	Yes	100 ng–1 μg	10 ng
Conductivity	Yes	500 pg–1 ng	500 pg
Mass spectrometry	Yes[d]	100 pg–1 ng	1 pg
FT–IR	Yes[d]	1 μg	100 ng
Light scattering[e]	Yes	10 μg	500 ng
Optical activity	No	—	1 ng
Element selective	No	—	10 ng
Photoionization	No	—	1 pg–1 ng

Methodology and instrumentation

Detection

Absorbance Detectors



- Based on measurement of the absorbance of eluents as they come off the end of the column

- Typically use z-folded flowcell to generate useful pathlengths while minimizing volume to reduce extra-column broadening

- Often based on double-beam designs to allowreal-time correction of background

Methodology and instrumentation

Detection

Filter-based Detectors

• Simple detectors that use Hg lamp (usually at 254 nm) in conjunction with filters

• 254 nm is extremely useful for detection of biological compounds such as proteins (amide bond absorption) or DNA (nucleotide absorption)

• Can also operate with D2 or tungsten lamps in conjunction with interference filters to allow operation at other wavelengths where aromatic groups absorb

Methodology and instrumentation

Detection

Monochromator Based Detectors

- Can use grating based scanning monochromators for UV or UV-Vis detection

- Usually operate in either single or dual wavelength mode

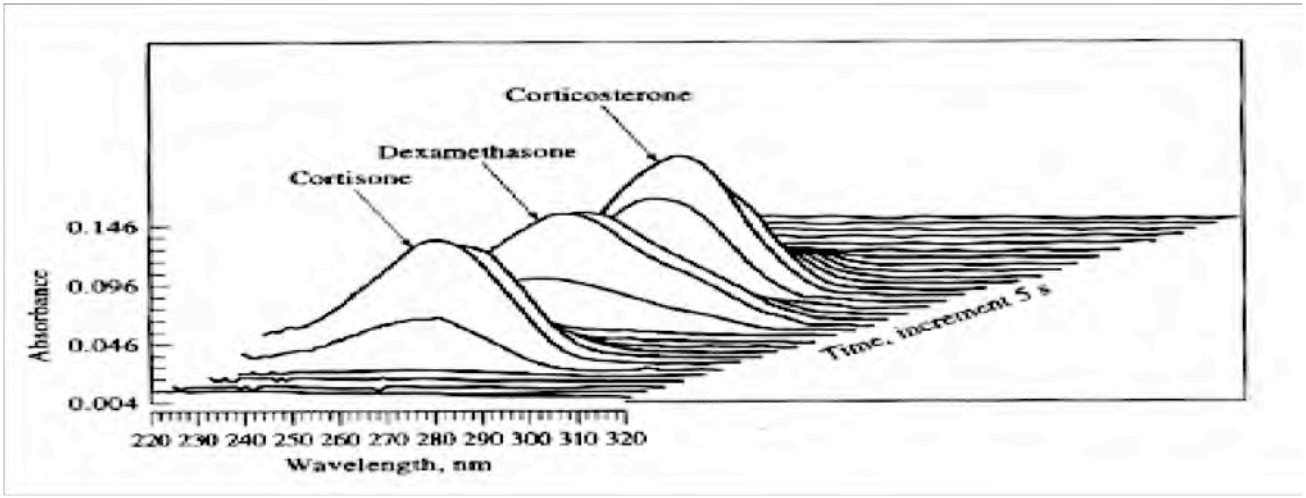
- Full spectra can't be obtained unless flow is stopped owing to slow slewing time

Methodology and instrumentation

Detection

Photodiode Array Detectors

- Most useful UV-Vis absorbance detector
- Now common in most instruments
- Can obtain whole spectrum in 1 s
- Useful diagnostic tool for identification



Methodology and instrumentation

Detection

IR absorbance

- Cell is similar to that used in UV-Vis absorbance except that a glow-bar is used as an IR source and a thermistor is used as a IR detector
- Can use conventional scanning monochromator and monitor single or dual wavelengths
- Alternatively, can use FTIR detector and obtain full IR spectrum as peaks elute
- Useful for selective detection of functional groups (i.e., C=O, CN, etc) or for determination of species identification using IR libraries for comparison
- Main drawbacks are poor detection limit and potential interference from mobile phase solvents that absorb in the IR

Methodology and instrumentation

Detection

Fluorescence

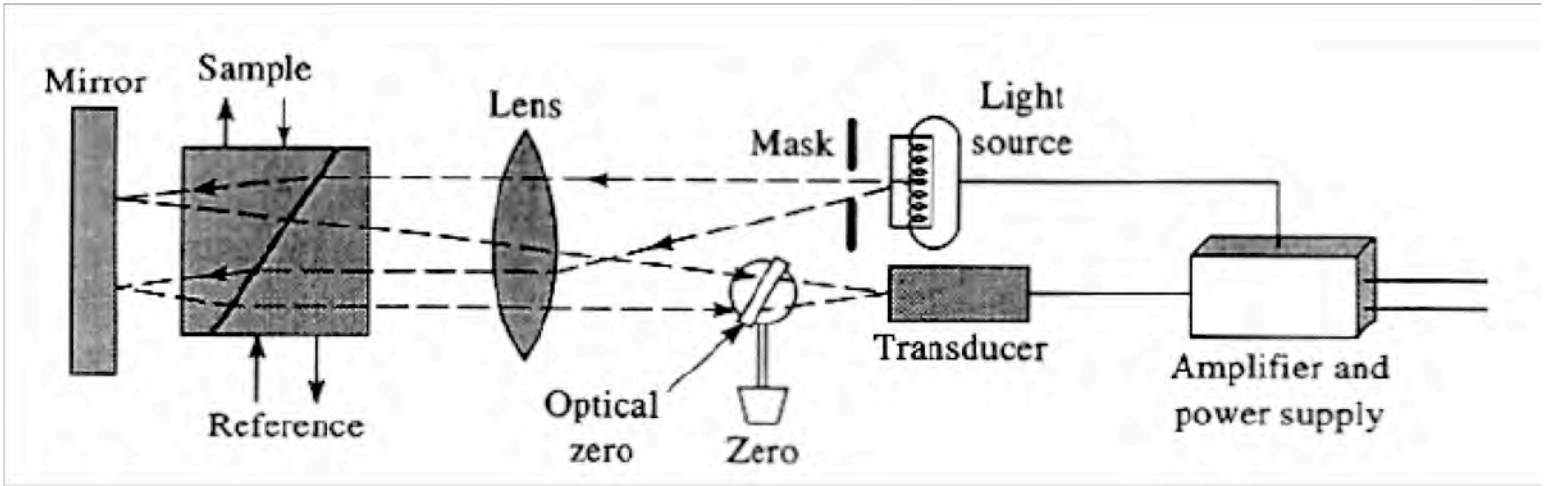
- Most sensitive of all HPLC detectors with ultimate detection limits as low as 10 fg
- Single molecule detection has been demonstrated with capillary LC in conjunction with laser excitation
- Similar in design to fluorimeters and spectrofluorimeters described earlier
- Simple systems use Hg lamp in conjunction with filters
- More sophisticated systems use Xe arc lamps and monochromators
- Useful for analysis of pharmaceuticals, natural products, petroleum products and clinical samples, but not as versatile as absorbance or RI detectors
- Range of use can be extended by sample pre-treatment to produce fluorescent derivatives (particularly useful for protein and DNA samples)
    - Fluorescent chlorides (dansyl chloride) react with 1° and 2° amines
    - Isothiocyanates react with primary amines
    - Iodoacetoxy or maleimide species react with thiols

Methodology and instrumentation

Detection

Refractive Index Detector

- Relatively versatile detector based on differences in refractive index for mobile phase with an without analyte present
- Figure below shows a differential RI detector which compares RI of sample and reference streams
- Presence of analyte in sample stream will lead to a deflection of the light beam on the photodetector, and a corresponding change in signal which is amplified and recorded
- Main disadvantages are temperature sensitivity and relatively poor detection limits



Methodology and instrumentation

Detection

Evaporative Light Scattering

- Based on nebulizing sample into a drift tube where controlled evaporation of solvent takes place
- Presence of analyte leads to higher boiling point and thus less evaporation and hence bigger droplets
- Droplets pass through a laser beam and light is scattered more by the bigger droplets, leading to an analyte dependent signal
- Major advantage is versatility ⇒ shows similar response to all non-volatile solutes
- Another advantage is better sensitivity than RI detector (2-20x better)

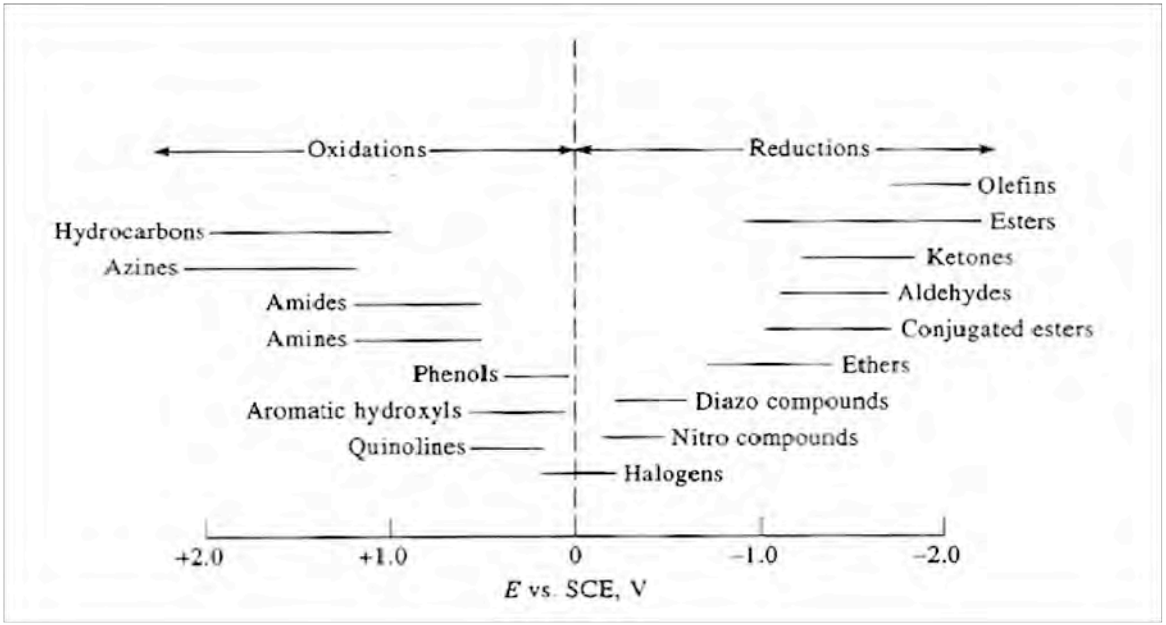
Methodology and instrumentation

Detection

Electrochemical

• Based on amperometry, polarography, coulometry or conductometry

• Very versatile detectors with very good detection limits (~100 fg), which can be used to detect a wide variety of organic functional groups

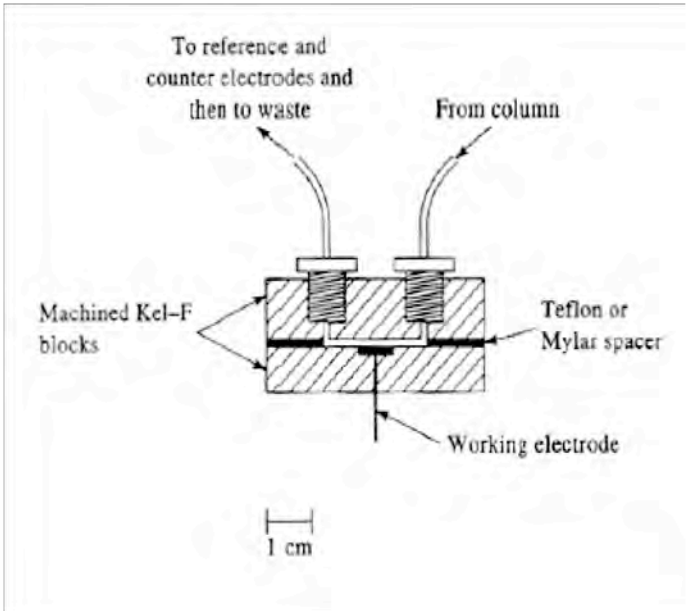


Methodology and instrumentation

Detection

Typical Configuration

- Based on thin-cell layout with a cell volume of 1 – 5 μL ⇒
- Eluent passes through insulating flow chamber past a working electrode
- Redox reaction at electrode produced a current that is recorded when analyte is present



Novel developments and approaches

Focus on the following areas:

- adsorbents and stationary phases
- modelling of processes, simulation and optimization
- engineering aspects: process design, control and automation

Novel developments and approaches

Adsorbents and stationary phases

- Manufacture of improved silica packings as well as other oxides
- Search for novel packings with controlled pore structure, particle texture and particle morphology with respect to high stability and utilization of capacity
- Design of surfaces with improved selectivity

Novel developments and approaches

Modelling

- Assessment of composite isotherms based on individual isotherms determined under analytical conditions by means of frontal analysis; calculation of elution profiles
- Modelling of mass transfer processes in columns and calculation of reliable kinetic parameters
- Simulation of processes (displacement, closed loop recycling, simulated moving bed, etc.)
- Optimization of process parameters, eg. cycle time, number of cycles, optimum flow rate

Novel developments and approaches

Process design and engineering aspects

Processes

- ✓ flip-flop concept
- ✓ closed loop recycling
- ✓ counter current processes (true and simulated moving bed)
- ✓ expanded bed technology
- ✓ utilization of stationary phase selectivity

Engineering tasks

- ❖ adjustment and control of operational parameters
- ❖ automation
- ❖ analytical control of product composition