UNIVERSITY OF TERAMO

Chromatography

Principles, techniques and detectors

Analytical Chemistry

BSc in Biotechnology · A.Y. 2026/2027

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01

Principles of chromatography

From Tsvett's coloured bands to modern instrumental separation

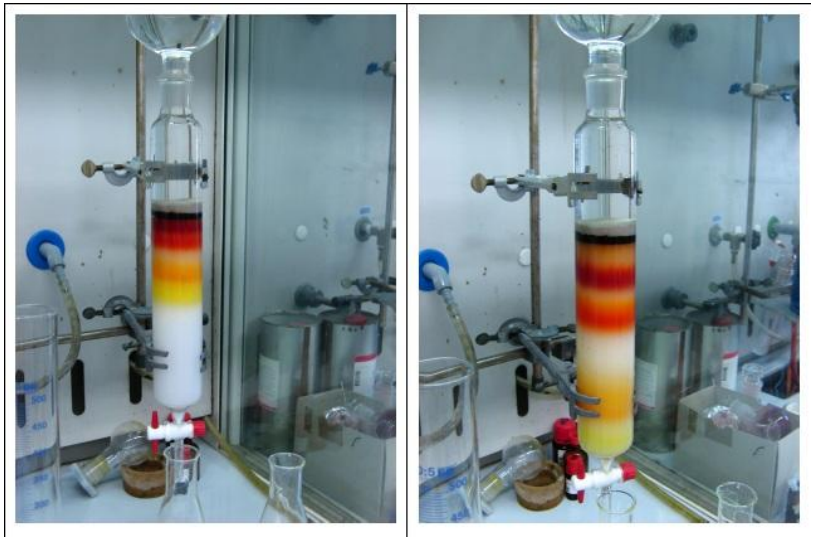
The origins: Tsvett and «colour writing»

The 1906 experiment

The Russian botanist Mikhail Tsvett packed a glass column with calcium carbonate and passed a leaf extract in petroleum ether through it. The pigments separated into distinct coloured bands along the column.

The name

The term chromatography comes from these bands, from the Greek chroma (colour) and graphein (to write). Colours are no longer needed: today analytes are detected by a detector at the column outlet.



Separation of plant pigments on a column: coloured bands before and after elution.

More than a century of chromatography

Each milestone either reduced band broadening or widened the range of separable analytes: the history of chromatography is a history of increasing efficiency.

1906 **Tsvett** Separation of leaf pigments on $CaCO_3$.

1941 **Martin and Synge** Partition chromatography and plate theory. Nobel Prize 1952.

1952 **James and Martin** Gas chromatography is born.

1956 **Van Deemter** Kinetic theory of band broadening.

1958 **Stahl** Standardisation of TLC.

1960s-70s **HPLC** High-pressure pumps and small particles.

2004 **UHPLC** Sub-2 µm particles and pressures up to 1000-1500 bar.

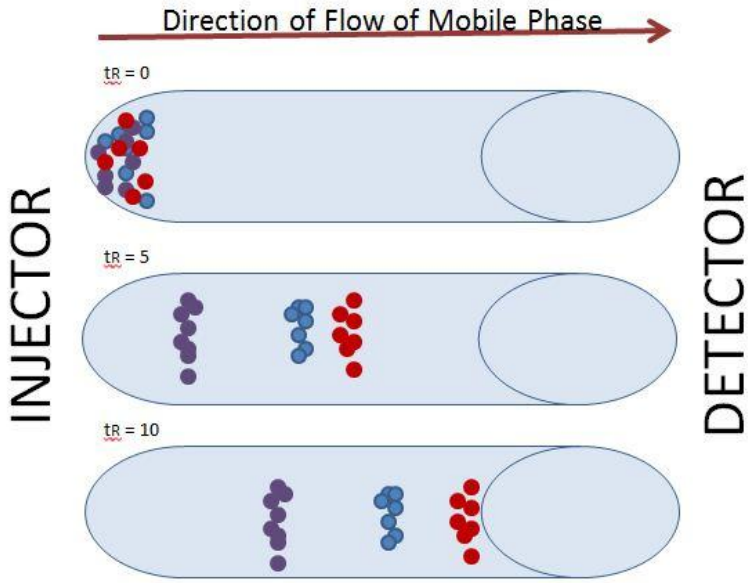
The principle of separation

Two phases

Every chromatographic method uses a stationary phase, fixed in a column or on a surface, and a mobile phase, liquid or gas, that flows through it carrying the sample. The movement of the mobile phase is called elution.

Partitioning

Each component distributes between the two phases according to its affinity. Molecules strongly retained by the stationary phase move more slowly, weakly retained ones leave first: the difference in speed produces the separation.



Progressive separation of a mixture along the column, from injector to detector.

The chromatogram

What it is

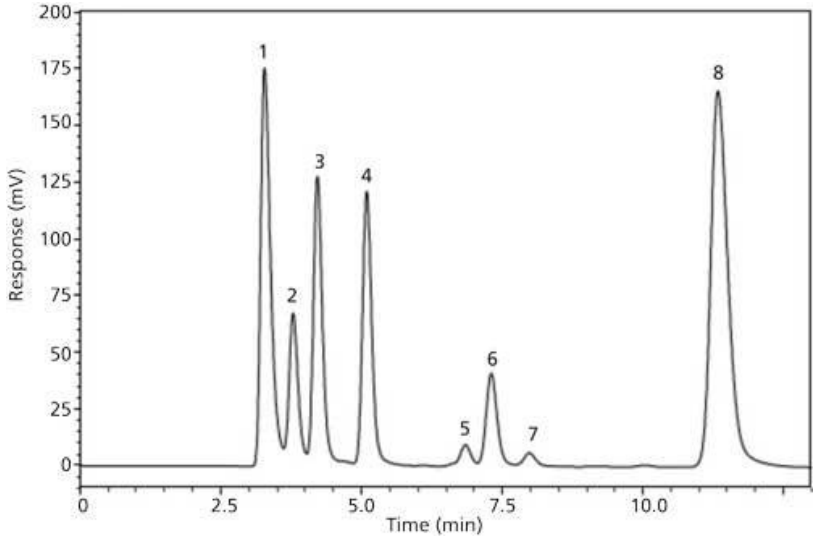
The chromatogram is the plot of the detector signal against time. Each separated component produces a peak, ideally Gaussian in shape.

Qualitative information

The position of the peak, the retention time, is characteristic of a compound under defined conditions and allows identification by comparison with standards.

Quantitative information

The peak area, or height, is proportional to the amount of analyte and is converted into concentration through the calibration curve. Well-defined, separated peaks are therefore required.



Example chromatogram with eight components.

02

Types of chromatography

Classification by geometry, nature of the phases and separation mechanism

TYPES

Planar and column chromatography

Planar

The stationary phase is spread on a flat surface, such as a silica gel plate, and the mobile phase rises through it by capillary action. Paper chromatography and TLC belong to this family.

Column

The stationary phase is held inside a tube and the mobile phase flows through it by gravity or, in instrumental techniques, driven by a pump or a pressurised gas. HPLC, UHPLC and GC are column techniques.



A pigment mixture before and after adding the eluent: the components separate into bands.

TYPES

Classification by mobile phase and mechanism

Mobile phase	Method	Stationary phase	Mechanism
Liquid (LC)	Liquid-solid	Solid adsorbent (silica, alumina)	Adsorption
	Bonded phase	Organic groups bonded to silica	Partition
	Ion exchange	Resin with charged groups	Ion exchange
	Size exclusion	Particles of controlled porosity	Hydrodynamic volume
	Affinity	Immobilised biospecific ligand	Molecular recognition
Gas (GC)	Gas-liquid	Liquid bonded to the wall	Partition
	Gas-solid	Solid adsorbent	Adsorption

The choice of technique depends on the property that distinguishes the analytes: polarity, charge, size, volatility or biological affinity.

Adsorption chromatography

Principle

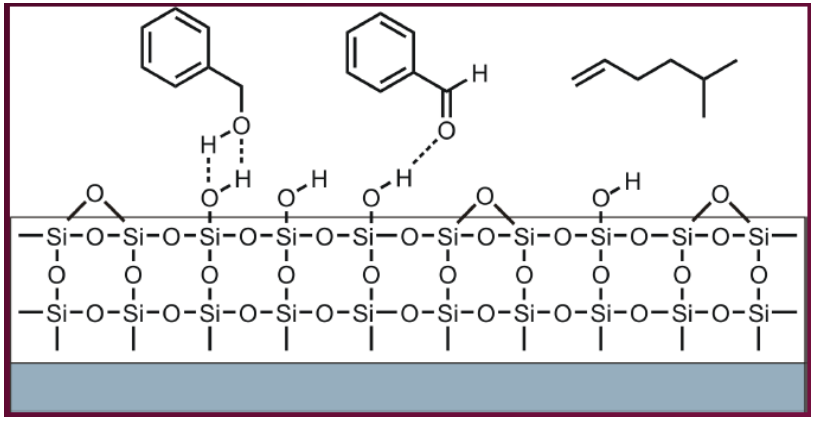
The stationary phase is a polar solid, typically silica or alumina, with active surface sites. Analytes compete with solvent molecules for these sites.

Interactions

Hydrogen bonds and dipole-dipole interactions with the Si-OH silanol groups dominate, together with dispersion forces. The more polar a compound, the longer it stays adsorbed.

Where it is used

It is the mechanism of TLC on silica and of normal-phase chromatography, suited to non-polar compounds and isomers.



Interaction of analytes with silanol groups on the silica surface.

Partition and bonded phases

Partition

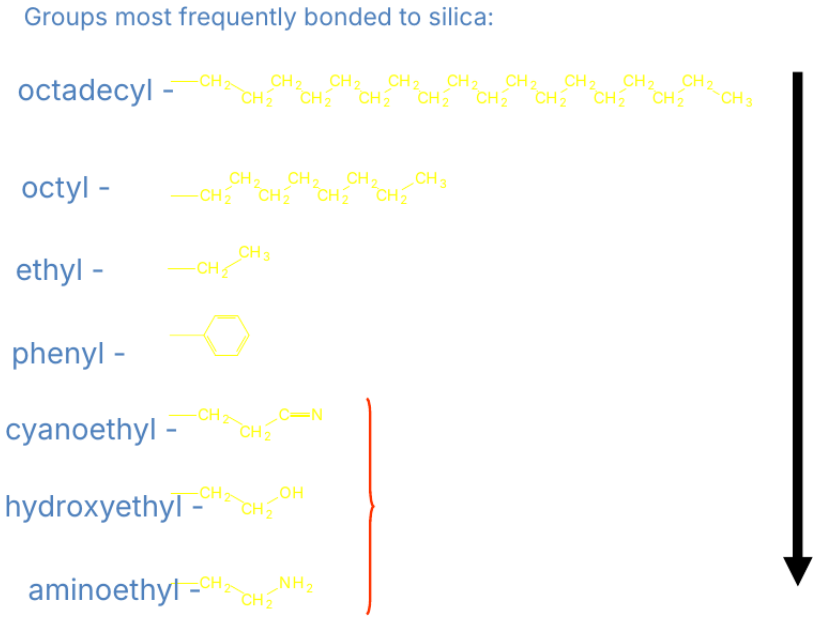
The analyte distributes between the mobile phase and a stationary liquid film, like a liquid-liquid extraction repeated thousands of times along the column.

Bonded phases

Today the stationary liquid is replaced by organic groups covalently bonded to silica, which behave as a pseudo-liquid. They are not washed off and allow gradient elution.

Increasing polarity

C18, C8, C4, phenyl, cyano, diol, amino: the choice of bonded group sets the selectivity of the column.



Groups most frequently bonded to silica, ordered by polarity.

TYPES

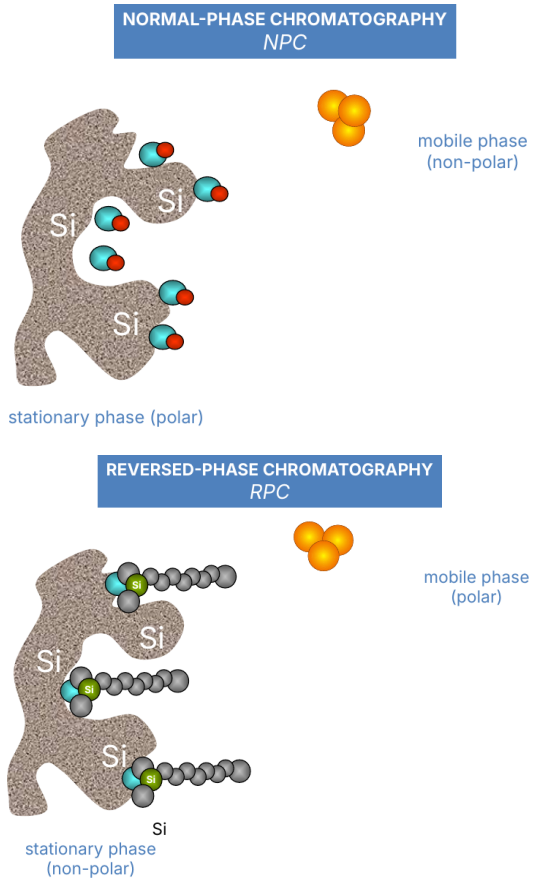
Reversed phase and normal phase

Reversed phase (RPC)

Non-polar stationary phase (C18, C8) and polar mobile phase (water with methanol or acetonitrile). Polar compounds elute first. It is the most common mode in HPLC and LC-MS.

Normal phase (NPC)

Polar stationary phase (silica, amino, diol) and low-polarity mobile phase (hexane, ethyl acetate). Non-polar compounds elute first; it is useful for isomers and compounds poorly soluble in water.



Normal phase (top) and reversed phase (bottom) on a silica support.

Ion-exchange chromatography

Principle

The stationary phase carries charged groups: sulfonic or carboxylic in cation exchangers, ammonium in anion exchangers. Analyte ions compete with mobile-phase ions for these sites.

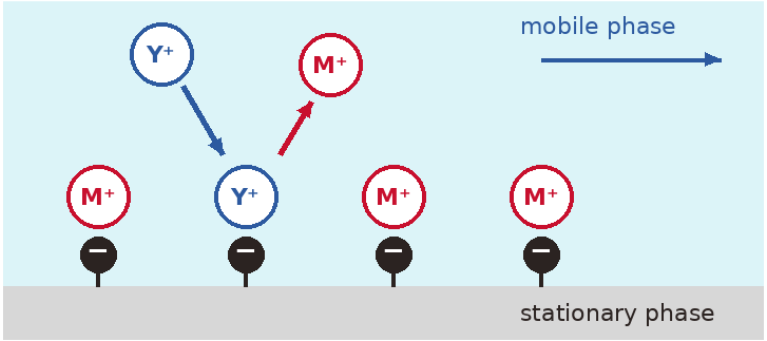
Retention and elution

Retention increases with the charge of the ion and decreases with its hydrated radius. Elution is achieved by increasing ionic strength or changing pH, for example with a salt gradient.

Applications

Purification of proteins and nucleic acids, amino acid analysis and ion chromatography of inorganic anions and cations.

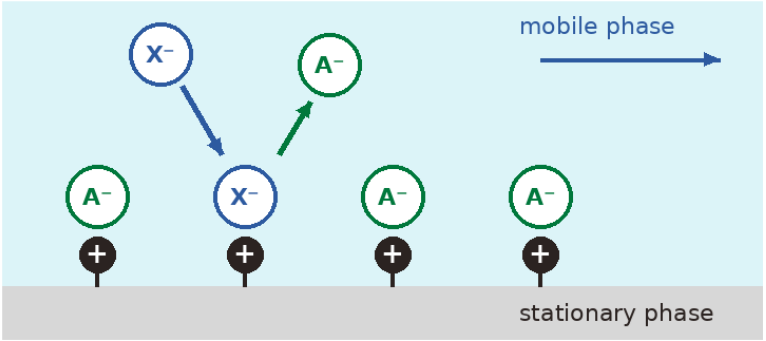
Cation exchange



$R\text{–}SO_3^- Y^+ + M^+ \rightleftharpoons R\text{–}SO_3^- M^+ + Y^+$

M^+, A^- = analyte ions Y^+, X^- = mobile-phase (eluent) ions

Anion exchange



$R\text{–}N(CH_3)_3^+ X^- + A^- \rightleftharpoons R\text{–}N(CH_3)_3^+ A^- + X^-$

Cation exchange (left) and anion exchange (right) at the stationary-phase surface.

Size-exclusion chromatography

Principle

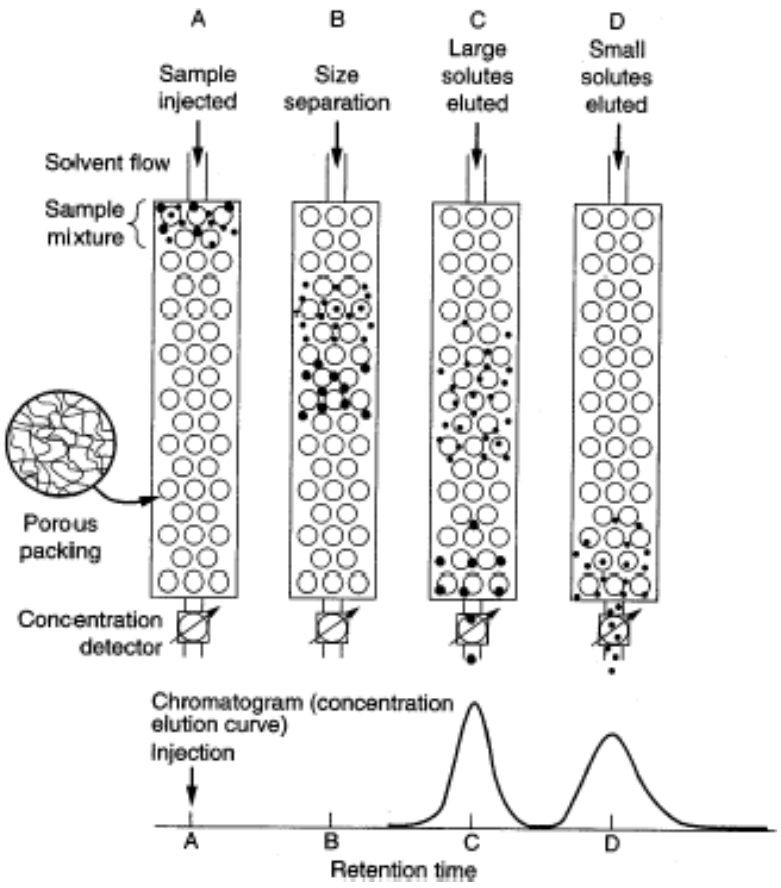
The stationary phase consists of particles with pores of controlled size. There is no chemical interaction: separation depends only on the hydrodynamic volume of the molecules.

Elution order

Molecules too large to enter the pores elute first, in the void volume. The smallest ones explore the whole pore volume and elute last.

Applications

Separation of proteins and their aggregates, desalting, estimation of the molar mass of polymers and biomolecules.



Large molecules are excluded from the pores and elute before small ones.

TYPES

Affinity chromatography

Separation exploits a specific biological recognition between the analyte and a ligand immobilised on the stationary phase: it is the most selective purification technique for biomolecules.

Principle

The sample is loaded under conditions that favour binding; contaminants are washed away and the target molecule is eluted by changing pH or ionic strength, or by adding a competitor.

IMAC

Ni^{2+} or Co^{2+} ions chelated on the resin bind recombinant proteins carrying a histidine tail (His-tag). Elution is performed with imidazole.

Protein A and G

They bind the Fc region of immunoglobulins: they are the industrial standard for purifying monoclonal antibodies.

TLC: thin-layer chromatography

What it is

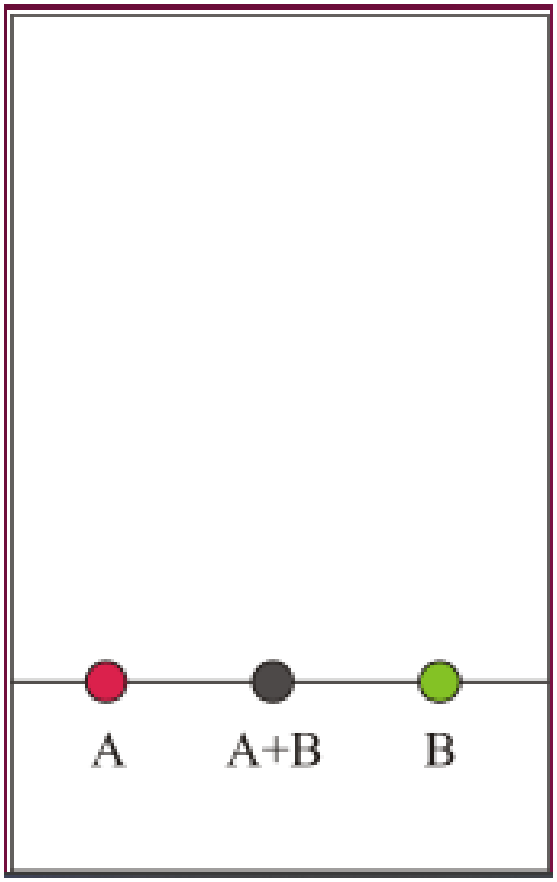
A glass, aluminium or plastic plate coated with a thin layer of stationary phase, usually silica gel. The sample is spotted at the bottom and the mobile phase rises by capillary action.

Normal and reversed phase

On silica the mechanism is adsorption and polar compounds migrate less. On modified silica (RP-TLC) the mobile phase is polar and partition dominates.

Current uses

Fast screening, monitoring of reactions and purity, choice of eluent before a column separation; HPTLC also allows quantitative analysis.



TLC plate with standard A, standard B and mixture A+B.

TLC: stationary and mobile phase

Stationary phase

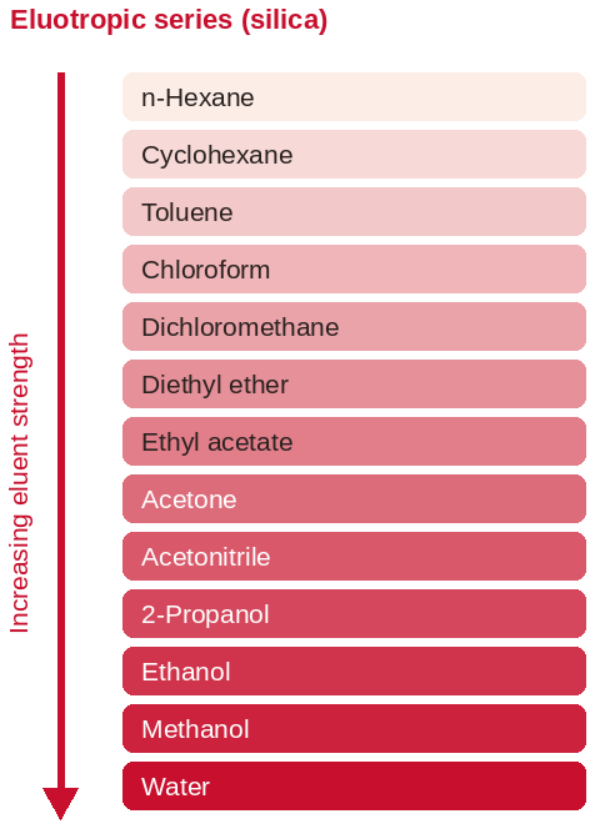
Silica gel is porous amorphous silica bearing silanol groups; alumina and cellulose are also used. The layer is about 250 µm thick on analytical plates and 0.5-2 mm on preparative ones.

Eluent strength

The ability of a solvent to move analytes depends on its polarity. The eluotropic series ranks pure solvents and mixtures by increasing eluent strength.

In practice

Start with low-polarity mixtures and progressively increase the polar component until R_f values fall between 0.2 and 0.8.



Eluotropic series of common solvents on silica.

TLC: spotting and development

Spotting

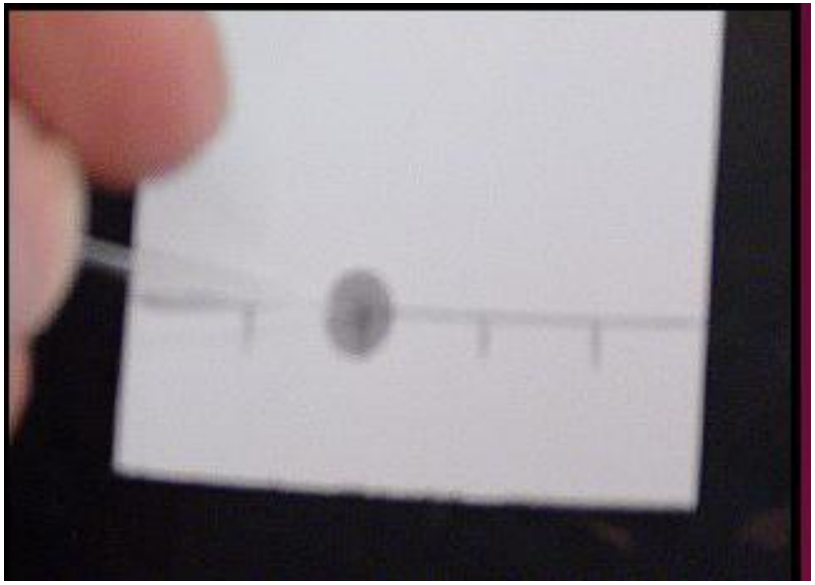
The sample is applied with a capillary about 1 cm from the bottom edge, as a spot or band, as compact as possible and without scratching the layer. Dilute samples are applied repeatedly, drying between applications.

Development

The plate is placed in a chamber saturated with eluent vapour, with the solvent level below the spotting line. When the front approaches the top edge the plate is removed and the front is marked.

Detection

UV lamp at 254 nm on fluorescent plates, or chromogenic reagents such as ninhydrin or iodine.



Spotting the sample with a capillary.

TLC: retardation factor R_f

$$R_f = d_{analyte} / d_{front}$$

Definition

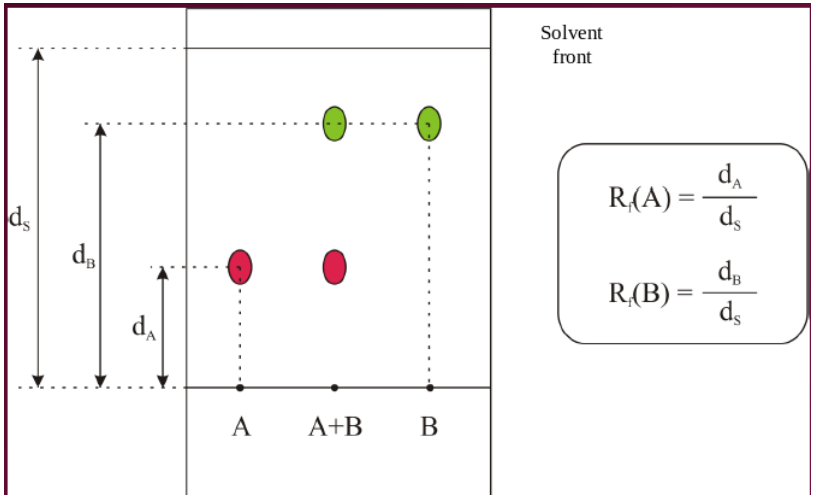
R_f is the ratio between the distance travelled by the spot and that travelled by the solvent front, and always lies between 0 and 1.

What it depends on

Activity and porosity of the stationary phase, layer thickness, eluent composition, temperature and chamber saturation.

Identification

Comparison with a standard is valid only on the same plate and in the same run: equal R_f values suggest identity, different ones exclude it.



Calculation of R_f for two components and their mixture.

03

Separation theory

Retention, efficiency and resolution

THEORY

Differential migration in a column

What happens

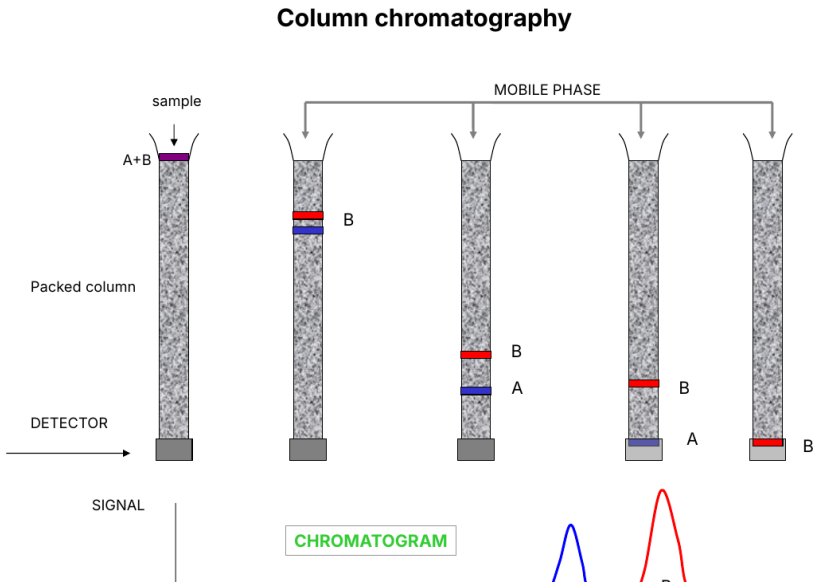
The A+B mixture is introduced at the head of the column. The mobile phase carries the components, which distribute between the two phases with different affinities.

Separation

B, more strongly retained, moves more slowly than A. At the outlet the two components reach the detector at different times and give two distinct peaks in the chromatogram.

The price

Every band broadens along the way: separation succeeds only if bands move apart faster than they spread.



Separation of A and B along the column and resulting chromatogram.

The solute moves only in the mobile phase

The mechanism

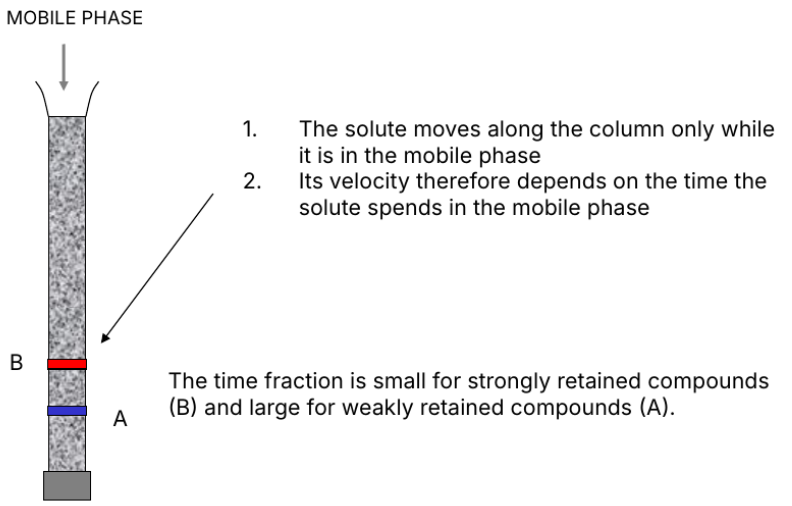
A molecule moves along the column only while it is in the mobile phase; while held by the stationary phase it does not move.

Velocity

The average velocity of a solute therefore depends on the fraction of time it spends in the mobile phase: small for strongly retained compounds (B), large for weakly retained ones (A).

Consequence

Partitioning between the phases determines elution times and therefore the ability to separate complex mixtures.



Partitioning between stationary and mobile phase therefore determines elution times and the ability to separate complex mixtures.

Fraction of time in the mobile phase for a weakly (A) and a strongly (B) retained compound.

THEORY

The distribution coefficient K

$$K = c_S / c_M$$

Definition

K is the ratio between the analyte concentration in the stationary phase and in the mobile phase at equilibrium. Each compound has its own K in a given chromatographic system.

Linear isotherm

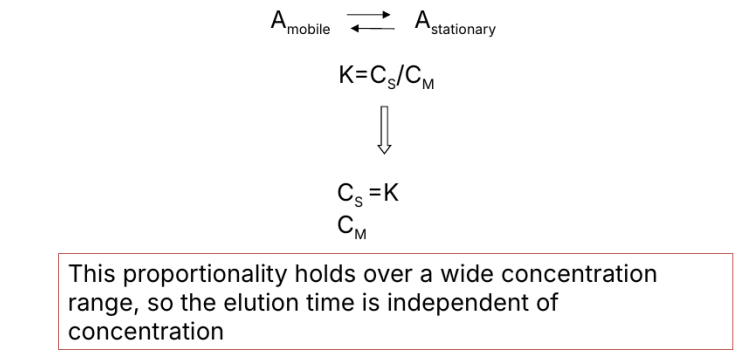
Over a wide concentration range K is constant: the elution time therefore does not depend on the amount injected and peaks remain symmetrical.

When it fails

At high concentrations the isotherm becomes non-linear and peaks distort: this is the origin of column overload.

Velocity depends on the partition ratio of solutes between MP and SP

Partition ratio or distribution coefficient



Distribution equilibrium between mobile and stationary phase.

Dead time and retention time

$$v = L / t_R \qquad u = L / t_M$$

t_M, dead time

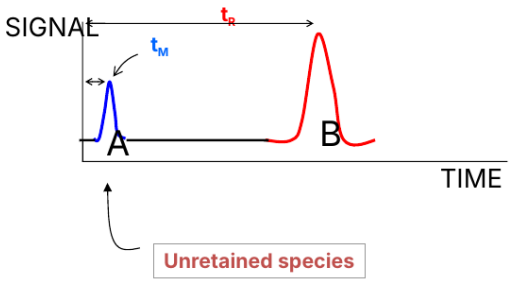
The time taken by an unretained species, which travels at the speed of the mobile phase. It corresponds to the void volume of the column.

t_R, retention time

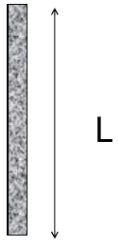
The time from injection to the peak maximum. The difference $t_R - t_M$ is the time spent in the stationary phase.

Linear velocities

v is the average velocity of the solute, u that of the mobile phase; L is the column length. Since the solute pauses in the stationary phase, v is always lower than u.



tR: time between sample injection and the appearance of the peak in the chromatogram



Average linear velocity of solute and mobile phase

$$v = L/T_R$$

$$u = L/T_M$$

Dead time, retention time and linear velocities.

THEORY

Retention factor k and selectivity α

$$k = (t_R - t_M) / t_M \qquad \alpha = k_2 / k_1 \quad (k_2 > k_1)$$

Retention factor k

It expresses how much longer an analyte stays in the stationary phase than in the mobile phase. Values between 2 and 10 are a good compromise: with k < 1 the analyte elutes near the dead time, with very high k the run becomes long and peaks broaden.

Selectivity α

The ratio of the retention factors of two adjacent peaks. With α = 1 the two compounds co-elute whatever the efficiency; to separate them the chemistry of the system must change.

How to change them

k is adjusted mainly through mobile-phase strength; α by changing stationary phase, organic solvent, pH or temperature.

Band broadening and the Gaussian peak

A random process

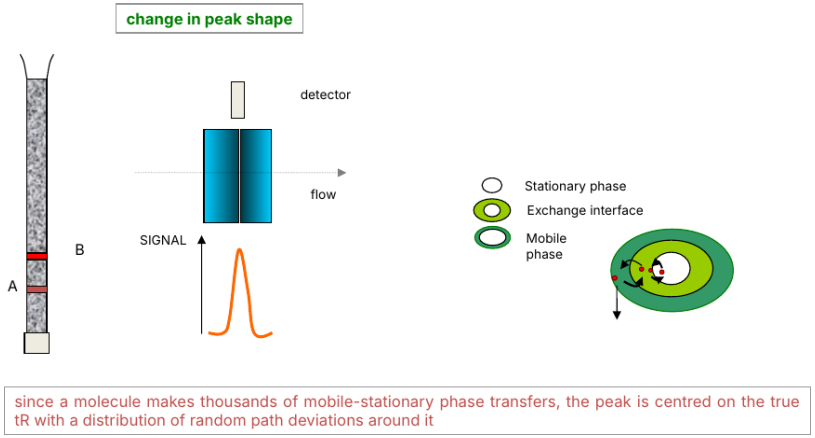
Each molecule makes thousands of transfers between mobile and stationary phase, with slightly different paths and residence times.

Peak shape

The sum of these random events gives a Gaussian distribution centred on the retention time. Its width, measured by the standard deviation σ, grows with the distance travelled.

Goal

An efficient column keeps bands narrow. Measuring and reducing this broadening is the purpose of plate theory and of the Van Deemter equation.



Transfers at the phase interface and resulting peak.

Efficiency: theoretical plates

$$H = \sigma^2 / L \qquad N = L / H$$

The model

Martin and Synge pictured the column as a series of stages, the theoretical plates, in each of which the analyte reaches equilibrium between the two phases.

Plate height H

The length of column corresponding to one plate; it measures broadening per unit length. The smaller H, the more efficient the column.

Plate number N

For the same length, a higher N means narrower peaks and therefore a better ability to separate closely eluting compounds.

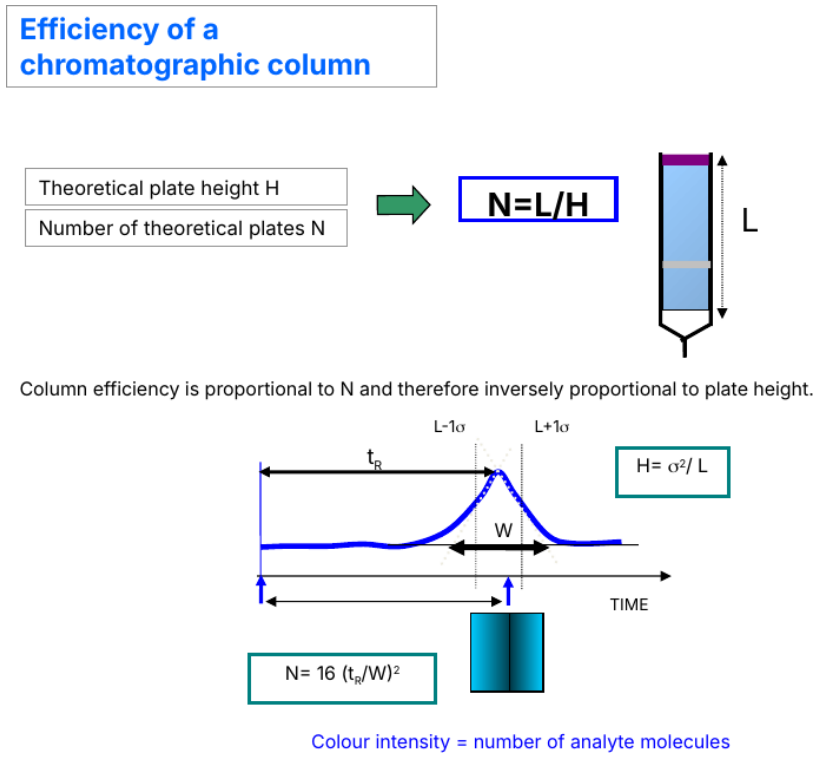


Plate height, plate number and measurement of N from the peak.

THEORY

Measuring N from the chromatogram

$$N = 16\,(t_R / w)^2 \qquad N = 5.54\,(t_R / w_{1/2})^2$$

Baseline width w

Obtained by drawing tangents at the inflection points of the peak down to the baseline: for a Gaussian $w = 4\sigma$. The method is sensitive to how the tangents are drawn.

Width at half height $w_{1/2}$

Simpler and more reproducible, it is the method used by integration software and pharmacopoeias: $w_{1/2} = 2.355\sigma$.

Typical values

A 15 cm HPLC column with 3-5 µm particles gives 10,000-20,000 plates; a 30 m GC capillary exceeds 100,000.

The Van Deemter equation: three terms

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

A · multiple paths

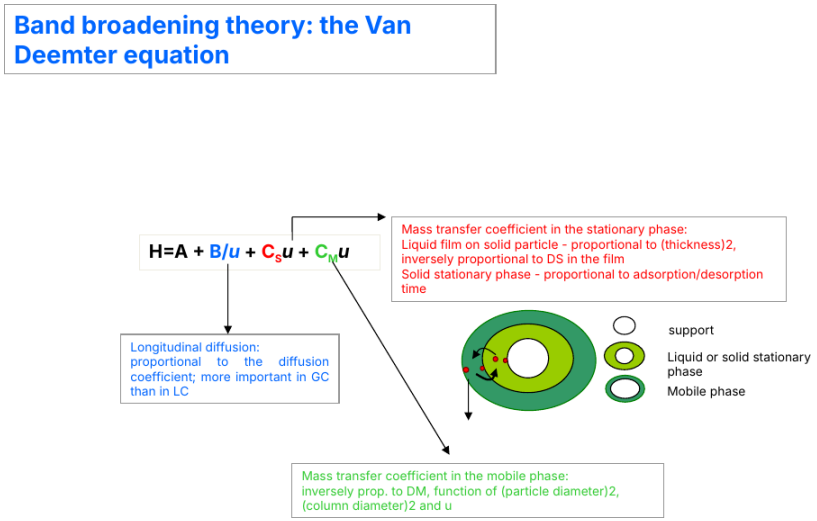
Molecules follow paths of different length between the particles. It depends on particle size and packing uniformity; it is absent in open capillary columns.

B/u · longitudinal diffusion

Molecules diffuse along the column axis; this term matters at low velocity and is far more important in GC than in LC.

C·u · mass transfer

Equilibrium between the phases is not instantaneous; this term grows with velocity, particle diameter and film thickness.



Contributions to band broadening in the Van Deemter equation.

The Van Deemter curve

$$u_{opt} = \sqrt{(B / (C_S + C_M))}$$

Shape of the curve

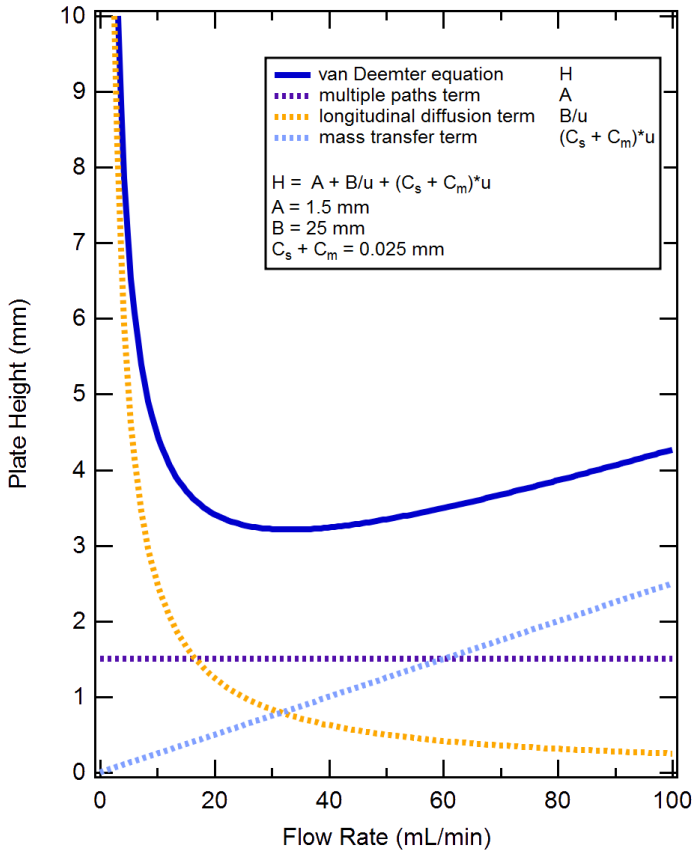
Adding the three terms, H as a function of linear velocity u gives a curve with a minimum: at low velocity longitudinal diffusion dominates, at high velocity mass transfer.

Optimum velocity

At the minimum the efficiency is highest. Working slightly above the optimum shortens the analysis with a limited loss of efficiency.

In practice

The curve is built by measuring N at different flow rates and is the criterion for choosing the working flow.



Van Deemter curve and contributions of the individual terms.

THEORY

Peak asymmetry: tailing and fronting

$$A_s = b / a \quad \text{(at 10\% of peak height)}$$

Tailing ($A_s > 1$)

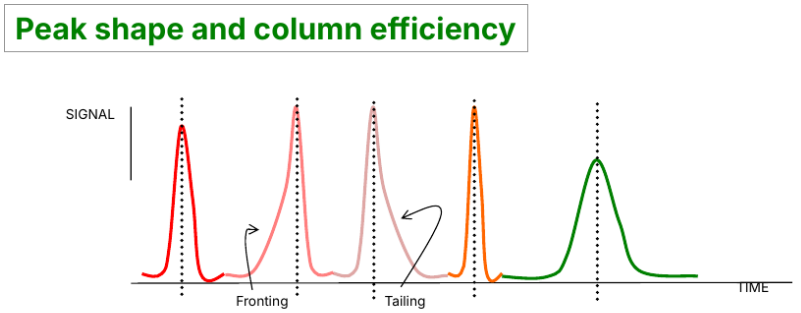
A tail towards longer times. In reversed phase it is often caused by secondary interactions with residual silanols, especially for bases; dead volumes and degraded columns also contribute.

Fronting ($A_s < 1$)

Broadening on the leading edge, typical of column overload or of an injection solvent stronger than the mobile phase.

Why it matters

Asymmetric peaks reduce resolution and make area integration less precise.



Tailing and fronting arise from a non-linear distribution coefficient

Symmetrical peak, fronting and tailing.

Resolution

$$R_s = 2\,[\,(t_R)_B - (t_R)_A\,] / (w_A + w_B)$$

Definition

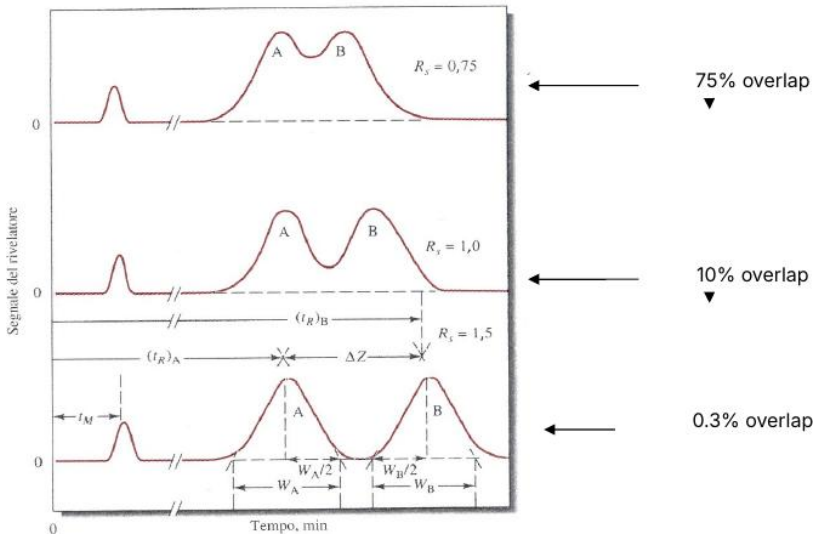
Resolution compares the distance between two peaks with their width: two distant but broad peaks may be separated worse than two close but narrow ones.

Reference values

With $R_s = 0.75$ peaks overlap heavily; with $R_s = 1$ the overlap is about 2%; with $R_s = 1.5$ baseline separation is reached, with 0.3% overlap.

Column resolution

$$R_s = \frac{2\Delta Z}{W_A + W_B} = \frac{2[(t_R)_B + (t_R)_A]}{W_A + W_B}$$



Overlap of two peaks as resolution increases.

Resolution and quantification

Overlapping peaks

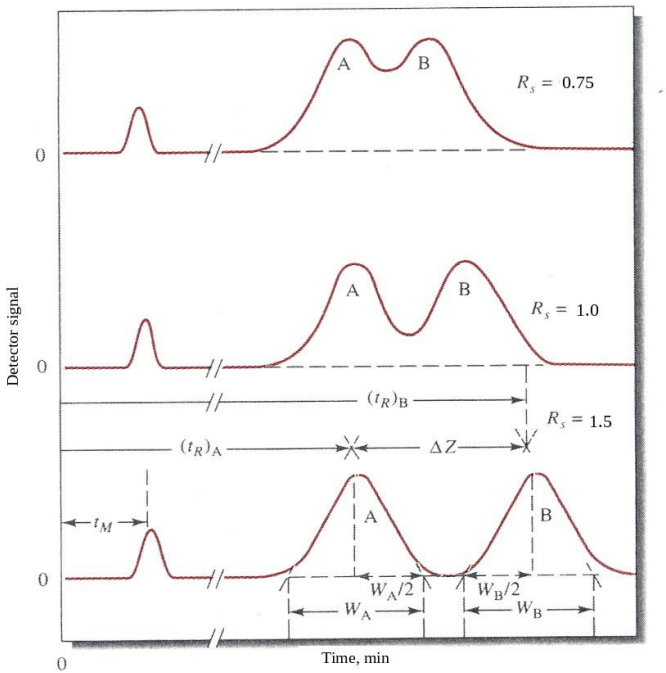
When two peaks are not resolved, integration must split the shared area arbitrarily: the quantification error grows rapidly as R_s decreases.

Peaks of different height

A small peak next to a large one requires higher resolution, because the tail of the main peak hides the minor one.

Practical goal

Separation is optimised for the most critical peak pair, the one with the lowest resolution.



Separations at R_s = 0.75, 1.0 and 1.5.

THEORY

Purnell equation: improving resolution

$$R_s = (\sqrt{N} / 4) \cdot ((\alpha - 1) / \alpha) \cdot (k / (1 + k))$$

Efficiency N

Longer columns or smaller particles increase N, but R_s grows only with √N: doubling the length improves resolution by 41% and doubles the analysis time.

Selectivity α

The most powerful term: small increases in α give large gains. It is changed through stationary phase, organic solvent, pH and temperature.

Retention k

Increasing k helps when it is low; above k ≈ 10 the factor k/(1+k) is almost 1 and the gain becomes negligible.

THEORY

Worked example: k, α, N and R_s

Data C18 column, 15 cm. Dead time t_M = 1.0 min. Peak A: t_R = 5.0 min, w = 0.40 min. Peak B: t_R = 5.6 min, w = 0.44 min.

k	$k_A = (5.0 - 1.0)/1.0 = 4.0 \quad k_B = (5.6 - 1.0)/1.0 = 4.6$
α	$\alpha = 4.6 / 4.0 = 1.15$
N	$N_A = 16\,(5.0/0.40)^2 = 2500 \quad H = 150\ \text{mm} / 2500 = 0.06\ \text{mm}$
R_s	$R_s = 2\,(5.6 - 5.0) / (0.40 + 0.44) = 1.43$

Conclusion R_s is just below 1.5. With k already optimal, it is better to increase N (longer column or smaller particles) or α (change organic solvent or temperature).

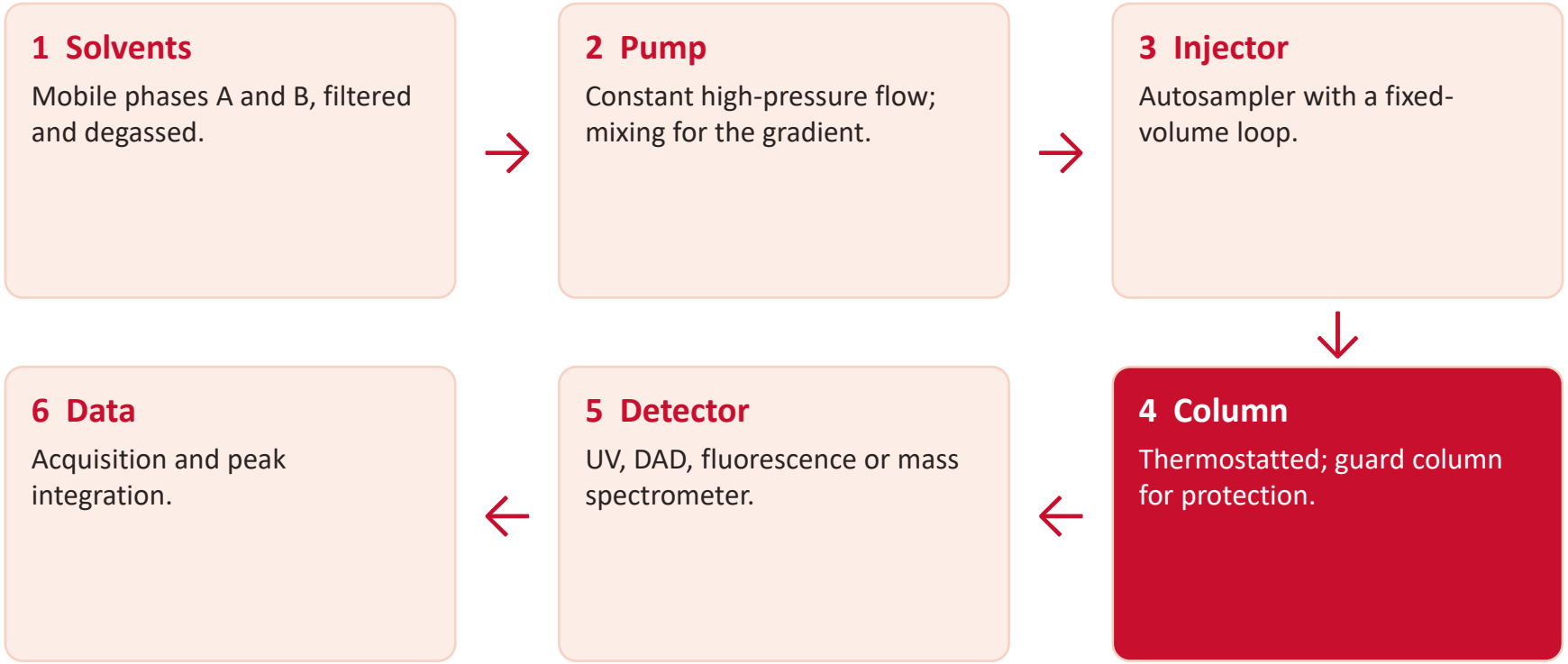
04

High-performance liquid chromatography

Instrumentation, columns, phases and optimisation

Layout of an HPLC system

Every component contributes to band broadening: long tubing, poorly made fittings and large detector cells degrade the efficiency achieved in the column.



Columns and guard columns

The column

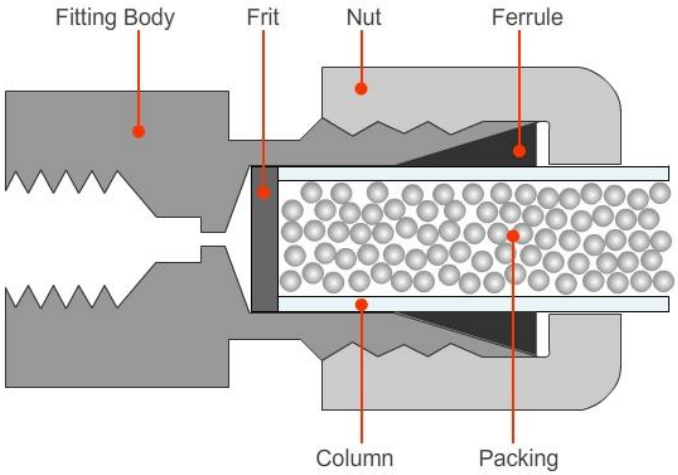
A stainless-steel or PEEK tube, typically 5-25 cm long with an internal diameter of 2.1 to 4.6 mm, packed with particles and closed by frits that distribute the flow.

The guard column

A short sacrificial column placed before the analytical column: it traps particles and matrix components that would bind irreversibly, extending column lifetime.

Thermostatting

Temperature affects retention, viscosity and pressure: it must be kept constant.



End fitting and packing of an HPLC column.

Back-pressure

$$\Delta P = \eta L u / (\theta d_p^2)$$

What it depends on

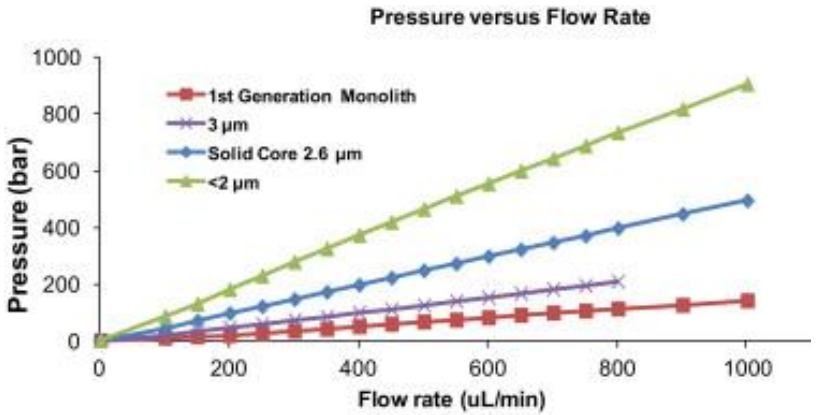
The pressure drop grows with mobile-phase viscosity η, column length L and linear velocity u, and is inversely proportional to the square of the particle diameter d_p.

The trade-off

Halving the particle diameter improves efficiency but quadruples the pressure. This is why sub-2 µm particles require UHPLC systems.

In practice

Water-methanol mixtures have a viscosity maximum around 50%: pressure changes during a gradient.



Pressure versus flow rate for different particle types.

Porous and sub-2 µm particles

Porous particles

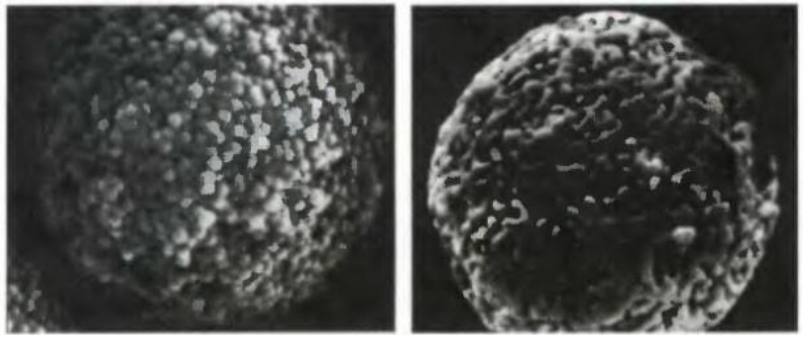
Silica spheres of 3-5 µm with 80-120 Å pores and a surface area of hundreds of m^2/g: they offer high capacity and are the standard for HPLC columns.

Sub-2 µm particles

They reduce the A and C terms of Van Deemter: H is lower and stays low even at high velocity. Separations become faster and more efficient, at the cost of pressures up to 1000-1500 bar (UHPLC).

Wide pores

For peptides and proteins 300 Å pores are used, allowing access to macromolecules.



Porous silica particles under the electron microscope.

Core-shell particles

Structure

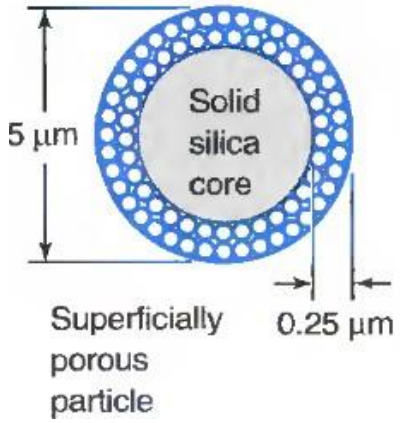
A non-porous silica core surrounded by a thin porous shell, for example a 1.7 µm core and a 0.5 µm shell.

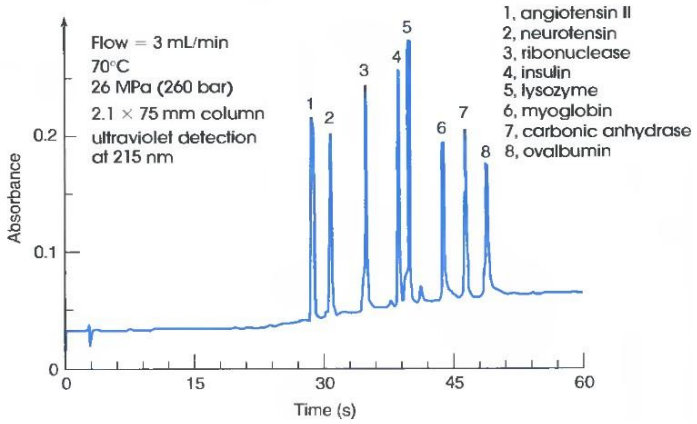
Why they are efficient

The short diffusion path in the shell reduces the C term; the very narrow size distribution reduces the A term. Efficiencies close to sub-2 µm particles are obtained at lower pressure.

Applications

Fast separations of small molecules and peptides, compatible with conventional HPLC systems.





Superficially porous particle and fast separation of peptides and proteins.

Van Deemter curves of modern columns

Comparison

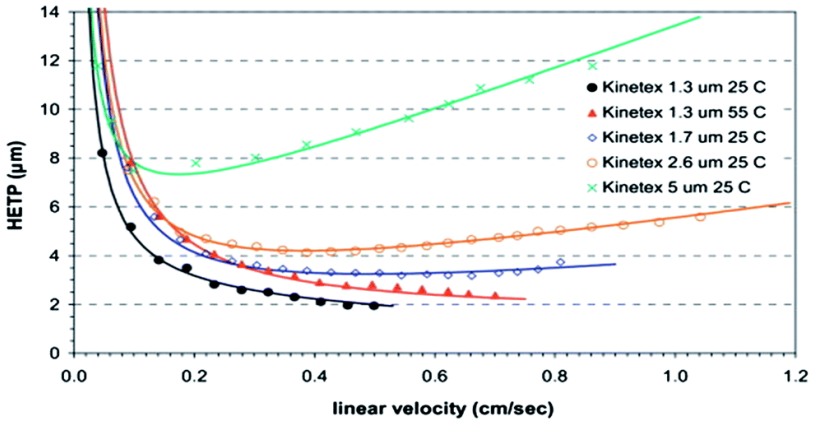
The curves show plate height against linear velocity for fully porous 5 µm, core-shell and sub-2 µm particles.

Reading the plot

With small or core-shell particles the minimum is lower and the curve rises less as velocity increases: flow can be raised, shortening the analysis, with almost no loss of efficiency.

Consequence

This flattening of the C term is what made UHPLC analyses of a few minutes possible.



Van Deemter curves for different particle types and sizes.

Monolithic columns

Structure

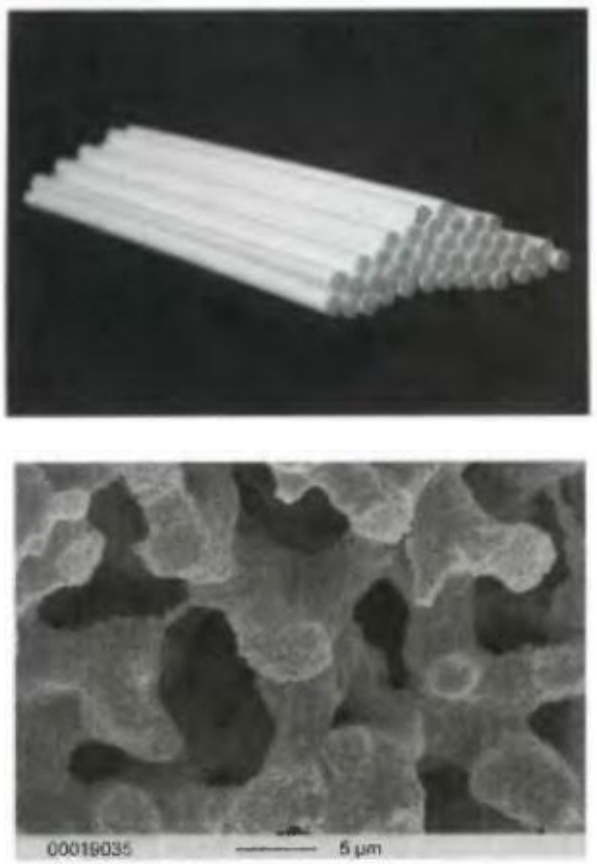
A continuous rod of porous silica obtained by in-situ polymerisation. About 80% of the volume is empty, with macropores of about 2 µm for flow and mesopores of about 13 nm for surface area.

Advantages

The open structure cuts back-pressure and allows high flow rates with good efficiency.

Limitations

Lower capacity and fewer available chemistries than packed columns: they are used mainly for fast separations and for large biomolecules.



Monolithic rods and porous structure under the electron microscope.

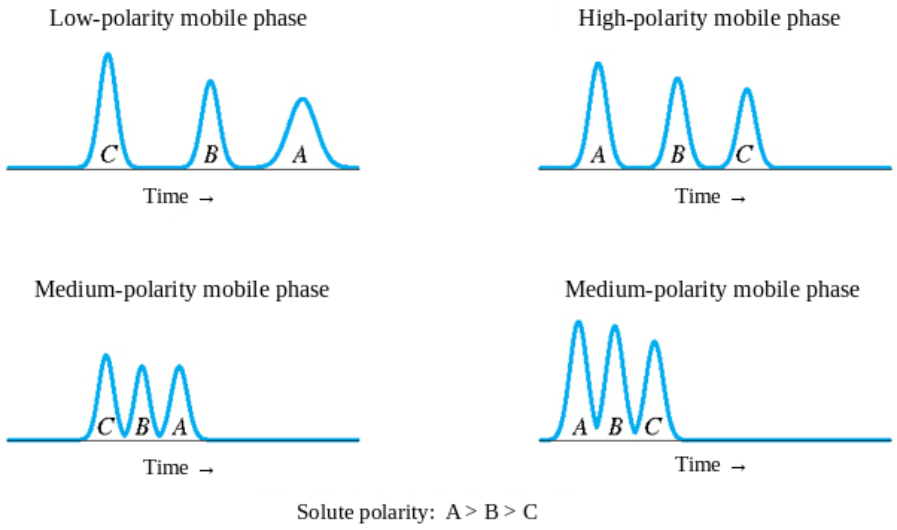
Normal and reversed phase: elution order

In normal phase (NPC)

More polar compounds elute later. To shorten retention times the polarity of the mobile phase is increased.

In reversed phase (RPC)

Less polar compounds elute later. To shorten retention times the polarity of the mobile phase is decreased, i.e. the fraction of organic solvent is increased.



Effect of mobile-phase polarity in NPC (left) and RPC (right); solute polarity A > B > C.

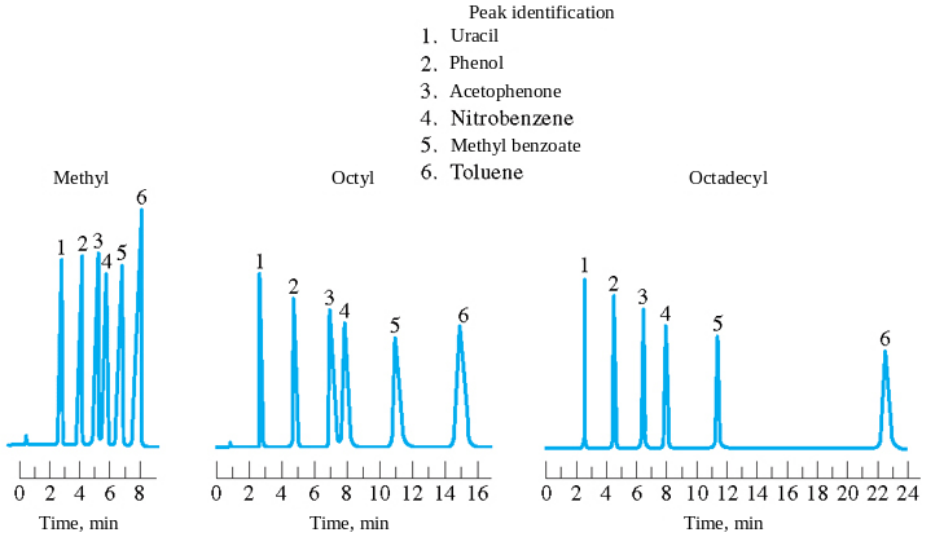
Effect of the stationary phase in reversed phase

Observation

Separating the same mixture on C1, C8 and C18 phases, retention of the less polar compounds increases with the length of the alkyl chain.

Interpretation

A longer chain is more hydrophobic and interacts more strongly with non-polar analytes. Separation improves at the expense of analysis time.



Separation of uracil, phenol, acetophenone, nitrobenzene, methyl benzoate and toluene on C1, C8 and C18.

Effect of the organic modifier

Experiment

The same mixture is separated on C18 with water-methanol at 24, 43, 63 and 82% methanol.

Result

As methanol increases the mobile phase becomes less polar, its eluent strength grows and all retention times decrease. With too much methanol peaks crowd together and separation is lost.

Optimum

43% methanol gives the best compromise between resolution and analysis time.

% Methanol	Retention time (min)				
	A	B	C	D	E
24	1.6	10.0	22.0	34.0	88.0
43	1.3	4.2	6.8	10.1	17.2
63	1.4	2.3	3.0	3.8	4.8
82	1.4	1.7	1.85	2.0	2.17

Retention times of five analytes at different methanol percentages.

Isocratic and gradient elution

Isocratic

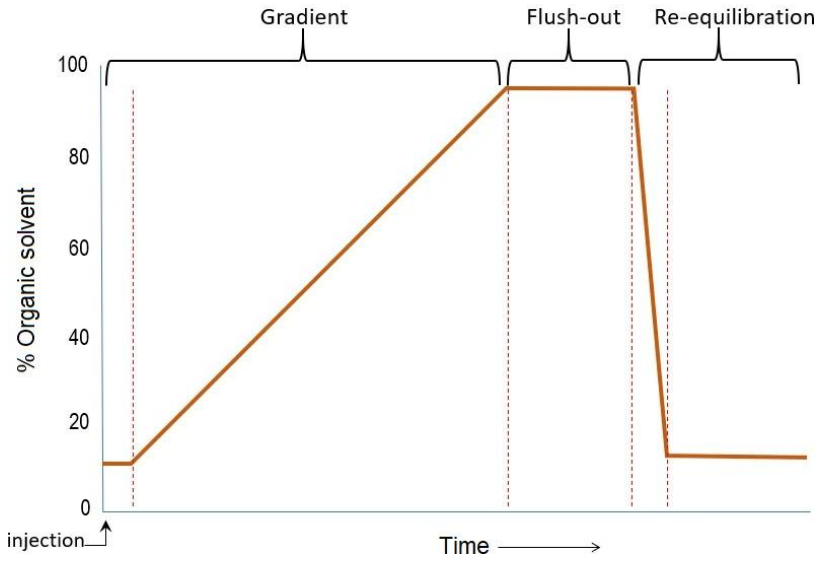
Mobile-phase composition stays constant throughout the run: simple and reproducible, suited to a few analytes of similar polarity.

Gradient

Composition changes during the run: in reversed phase the fraction of organic solvent is progressively increased. Polar compounds separate early, non-polar ones elute in reasonable time with narrow peaks.

Re-equilibration

At the end the system returns to the initial conditions and the column is equilibrated before the next injection.



Gradient profile with final flush and re-equilibration.

Ionisable analytes and pH

In reversed phase the neutral form of an analyte is retained more than the ionic form: controlling mobile-phase pH controls retention and peak shape.

Weak acids

$HA \rightleftharpoons A^- + H^+$. At least 2 pH units below the pK_a the neutral form HA dominates and is more retained. For carboxylic acids 0.1% formic acid is often used.

Weak bases

$BH^+ \rightleftharpoons B + H^+$. At least 2 pH units above the pK_a of the conjugate acid BH^+ the neutral base B dominates.

Column limits

Silica-based bonded phases are stable between pH 2 and 8: below, the bonded phase hydrolyses; above, silica dissolves. Hybrid phases reach pH 1-12.

HILIC and LC-MS mobile phases

HILIC

Polar stationary phase (silica, amide, zwitterionic) with an acetonitrile-rich mobile phase. A water layer on the surface retains polar analytes that would elute at the dead time in reversed phase.

When to use it

Sugars, amino acids, nucleotides, polar metabolites and organic acids: it is complementary to reversed phase in metabolomics.

Mobile phases for LC-MS

Volatile additives are required: formic acid, ammonium formate and ammonium acetate. Phosphate buffers are not compatible with the mass spectrometer source.

Biomolecules: SEC and ion exchange

SEC calibration

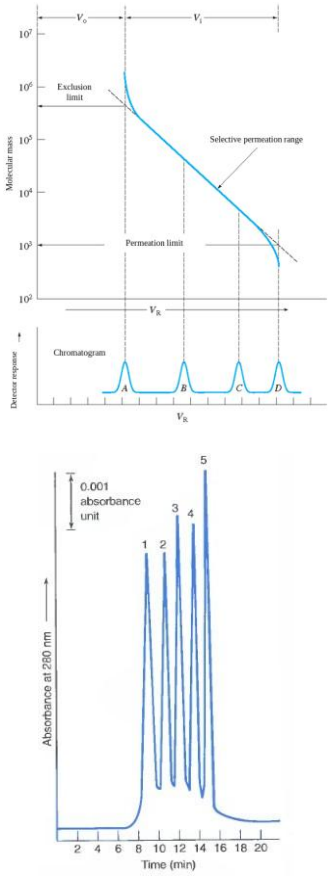
The logarithm of molar mass is roughly linear with elution volume between the exclusion limit and the permeation limit. With known standards the mass of an unknown protein can be estimated.

Protein separation

Enzymes between 12 and 290 kDa are separated by size; ion exchange instead separates them by net charge at the mobile-phase pH.

Combined use

Affinity, ion exchange and SEC are used in sequence to purify a recombinant protein.



SEC calibration curve and separation of five enzymes (glutamate dehydrogenase, lactate dehydrogenase, enolase, adenylate kinase, cytochrome c).

05

Gas chromatography

Separation of volatile and thermally stable compounds

Principle and instrument

Scope

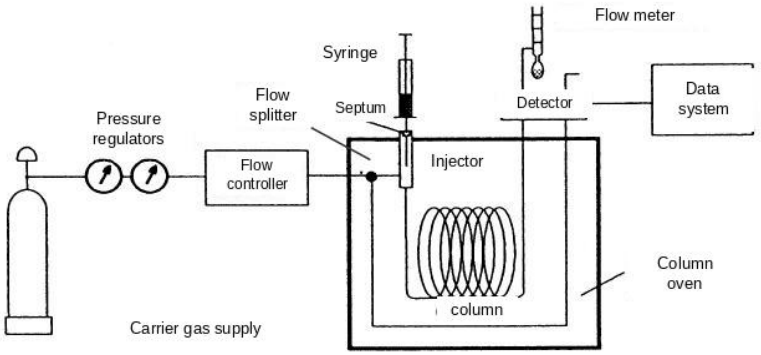
Analytes that are volatile and stable at injector and oven temperatures: solvents, aromas, pesticides, fatty acids, sterols and derivatised metabolites.

Gas-liquid and gas-solid

In gas-liquid GC the stationary phase is a liquid bonded to the wall and the mechanism is partition. In gas-solid GC an adsorbent separates permanent gases and light hydrocarbons.

The instrument

Carrier-gas cylinder and regulators, heated injector, column in a thermostatted oven, detector and data system.



Layout of a gas chromatograph.

Carrier gas and injection

Carrier gas

Helium, hydrogen or nitrogen. Hydrogen has the flattest Van Deemter curve: it keeps efficiency at high velocity and shortens analyses. Nitrogen has the lowest minimum but at low velocity. The B term weighs far more than in LC.

Split

Only a fraction of the vaporised sample enters the column; the rest is vented. Used for concentrated samples, it avoids overload.

Splitless

Almost all the sample enters the column: it is the mode for trace analysis. On-column and PTV injection are used for thermolabile compounds.

Capillary and packed columns

Capillary

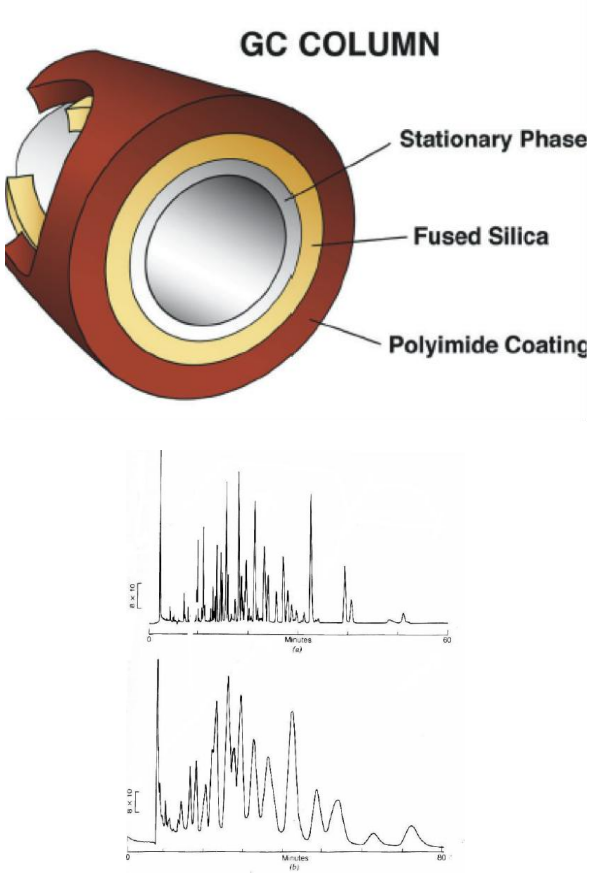
Fused-silica tubes with an external polyimide coating, 15-60 m long with an internal diameter of 0.1-0.53 mm. In WCOT columns the liquid phase is a 0.1-5 µm film on the wall; in PLOT columns it is a porous adsorbent layer.

Packed

Inert support coated with liquid phase: higher capacity but much lower efficiency. Today they are limited to gas analysis.

The comparison

The absence of the A term and the great length give capillaries a vastly higher plate number.



Cross-section of a capillary column; the same mixture on capillary (top) and packed (bottom) columns.

Stationary phases and temperature programming

Stationary phases

Polydimethylsiloxane (non-polar), 5% phenyl-polysiloxane (the most widely used), cyanopropyl-polysiloxanes (polar, for fatty-acid isomers), polyethylene glycol or WAX (very polar). The rule is «like dissolves like».

Thermal limits

Each phase has a maximum temperature: above it the phase degrades and releases fragments (column bleed) that raise the baseline.

Temperature programming

Raising the oven temperature during the run is the GC equivalent of an LC gradient: volatile compounds separate at low temperature and high-boiling ones elute as narrow peaks.

Identification and derivatisation

$$I = 100 \left[n + (\log t'_{R,x} - \log t'_{R,n}) / (\log t'_{R,n+1} - \log t'_{R,n}) \right]$$

Kováts index

It expresses the retention of an analyte relative to a series of n-alkanes, each assigned 100 times its number of carbon atoms. It is far more transferable between instruments than the retention time alone.

Why derivatise

-OH, -COOH and -NH_2 groups make analytes poorly volatile and cause tailing peaks. Derivatisation masks them.

Typical reactions

Silylation with BSTFA or MSTFA (sterols, sugars), methylation of fatty acids to FAMEs, derivatisation of short-chain fatty acids. Headspace and SPME: extraction module.

Worked example: Kováts index

Data 5% phenyl-polysiloxane column, isothermal. t_M = 1.0 min. Decane (C10): t_R = 9.0 min. Undecane (C11): t_R = 15.0 min. Analyte X: t_R = 12.0 min.

t'_R	Decane 8.0 min Undecane 14.0 min X 11.0 min
log	$\log 8.0 = 0.903$ $\log 14.0 = 1.146$ $\log 11.0 = 1.041$
I	$I = 100\,[10 + (1.041 - 0.903) / (1.146 - 0.903)] = 100\,(10 + 0.57) \approx 1057$

Conclusion The index 1057 is compared with literature values for the same stationary phase: together with the mass spectrum it makes identification much more reliable.

Detectors: an overview

Detector	Technique	Responds to	Notes
FID	GC	Compounds with C-H bonds	Most widely used; destructive
TCD	GC	All compounds	Universal, low sensitivity
ECD	GC	Halogenated, nitro compounds	Very sensitive; ^{63}Ni source
UV-Vis / DAD	LC	Compounds with chromophores	Most used in HPLC; spectra with DAD
Fluorescence	LC	Fluorescent compounds	Very sensitive; derivatisation
Refractive index	LC	All (sugars)	Low sensitivity; no gradient
Mass spectrometry	GC and LC	Ions by m/z	Structural information

Detectors will be covered in detail in the following lectures; mass spectrometry will have a dedicated lecture.

SUMMARY

Which chromatographic technique?

Volatile and thermally stable?	→	**GC (possibly after derivatisation)**
Small molecule, non-polar to moderately polar?	→	**Reversed-phase HPLC (C18)**
Very polar (sugars, amino acids, metabolites)?	→	**HILIC or ion exchange**
Ion or charged molecule?	→	**Ion exchange**
Macromolecule to separate by size?	→	**Size exclusion**
Tagged protein or antibody?	→	**Affinity (IMAC, Protein A)**
Fast, low-cost screening?	→	**TLC**

SUMMARY

GC and HPLC compared

	GC	HPLC
Mobile phase	Inert gas, no interaction	Liquid, contributes to selectivity
Analytes	Volatile, thermally stable	Non-volatile, also thermolabile and macromolecules
Typical efficiency	> 100,000 plates (capillary)	10,000-50,000 plates
Main lever on k	Oven temperature	Mobile-phase composition
Sample preparation	Often derivatisation	Often direct
Coupling to MS	EI, library spectra	ESI, ideal for biomolecules

The two techniques are complementary: the choice depends on the volatility and stability of the analyte, not on a preference for an instrument.