Separation methods: Chromatography

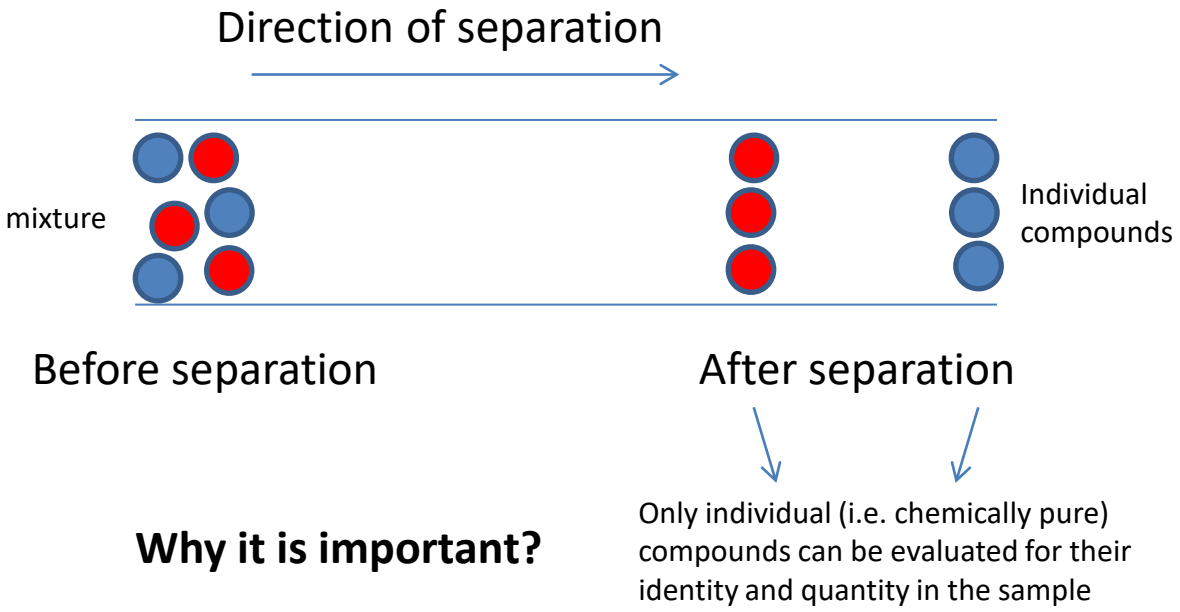
basic aspects in theory, instrumental schemes, and utilization + new trends

Analytical Chemistry: Instrumental Analysis

Peter Mikus, Faculty of Pharmacy Comenius University in Bratislava

Analytical separation methods

- techniques in analytical chemistry enabling to separate multicomponent mixtures to their individual compounds



Why it is important?

Main gropus of analytical separation methods used in pharmacy

- Analytical separation methods can be broken down into two main groups depending on which separation mechanism is employed:
- Chromatography (chromatographic mechanism)
- Electrophoresis (electrophoretic or electromigration mechanism)

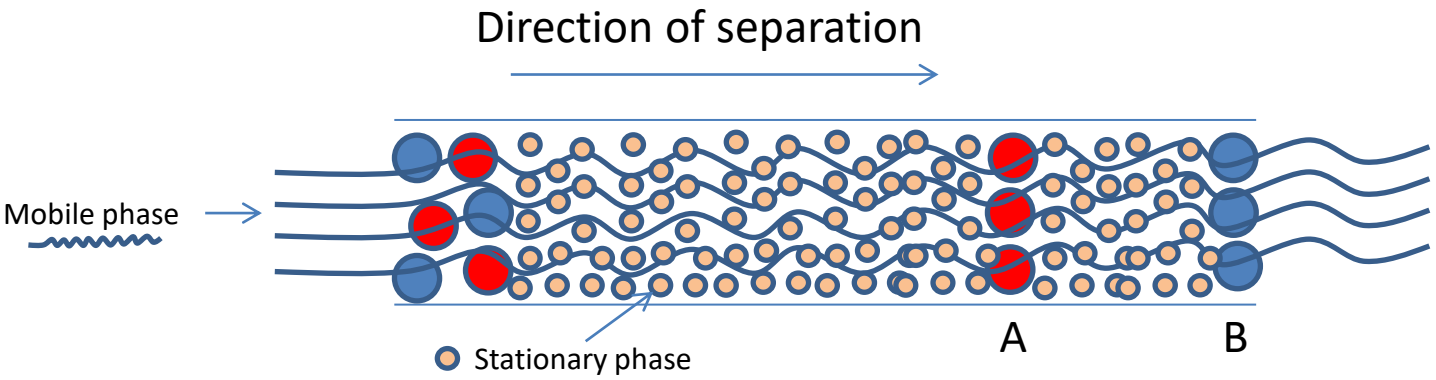
these methods are frequently used in pharmacopoeia for drug control as well as for the monitoring of drugs and their metabolites in biological systems

Chromatography

Chromatography

(principle)

- **Chromatography** is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the *mobile phase*, which carries it through a structure holding another material called the *stationary phase*. The various constituents of the mixture travel at different speeds (velocities), causing them to separate. The separation is based on differential partitioning of these constituents (analytes) between the mobile and stationary phases (i.e. different interactions of the analytes with the mobile and stationary phases). Subtle differences in a compound's partition coefficient (distribution coefficient) result in differential retention on the stationary phase and thus affect the separation.



Different interactions ($K_{DA}>K_{DB}$) = different velocities ($v_A<v_B$) = different retention time ($t_A>t_B$) = separation A from B

Distribution coefficient of compound X

$$K_{DX}=\frac{c_{sX}}{c_{mX}}$$

c_{sX} — Concentration of the analyte X in stationary phase

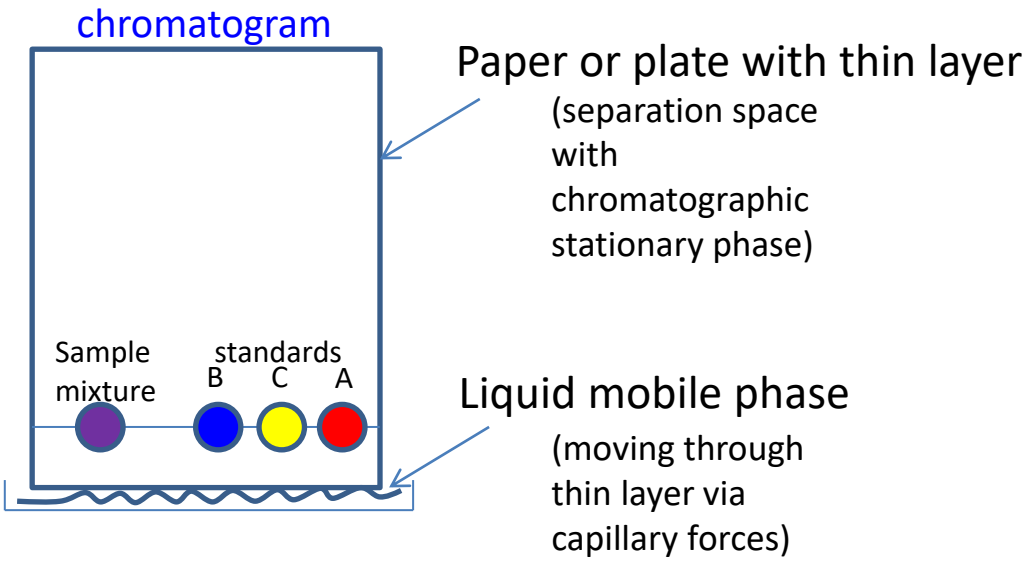
c_{mX} — Concentration of the analyte X in mobile phase

Methods in chromatography

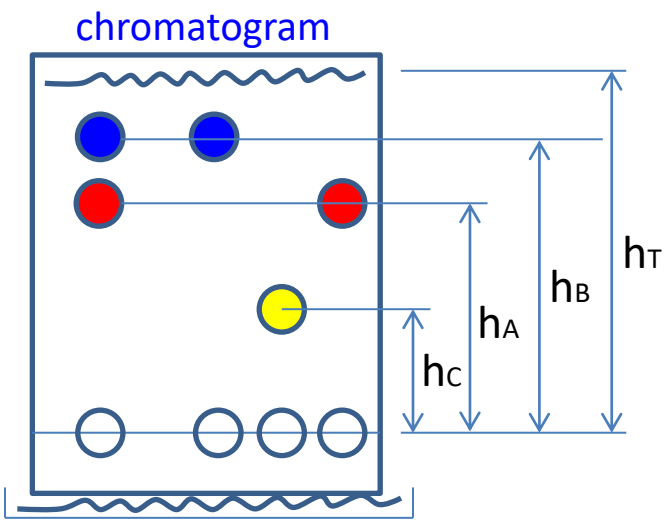
- Methods in analytical chromatography can be classified according to two main aspects:
- Instrumental arrangement
  - planar chromatography (on paper or plate with thin chromatographic layer)
  - column chromatography (in tubes or capillaries)
- Type of mobile phase
  - liquid = liquid chromatography LC
  - gaseous = gas chromatography GC

Planar chromatography

Before separation



After separation



Preliminary confirmation of identity of compounds in sample mixture

- Distance h reflects velocity of movement in given time (extrapolated to total time t_T)
- Preliminary proof of identity based on comparing h of compounds eluted from the sample spot with those eluted from the spots of standards (the same h = the same identity)
- In this sample mixture were present compounds A and B

Utilization of paper (PC) or thin layer (TLC) chromatography:

Useful for preliminary control/proof of •identity of drugs, •purity of drugs, •new compounds (potential drugs) from organic synthesis reaction mixtures

Not useful for quantification of compounds in the sample

Column chromatography

tube — LC

I.D. usually 5 mm
More sample can be injected but higher consumption of mobile phase

capillary — GC, LC

I.D. less than 1 mm
Less sample can be injected but lower consumption of mobile phase



GC columns

Length: usually more than 10 m

https://www.chemeurope.com/en/whitepapers/126475/columns-for-gas-chromatography.html

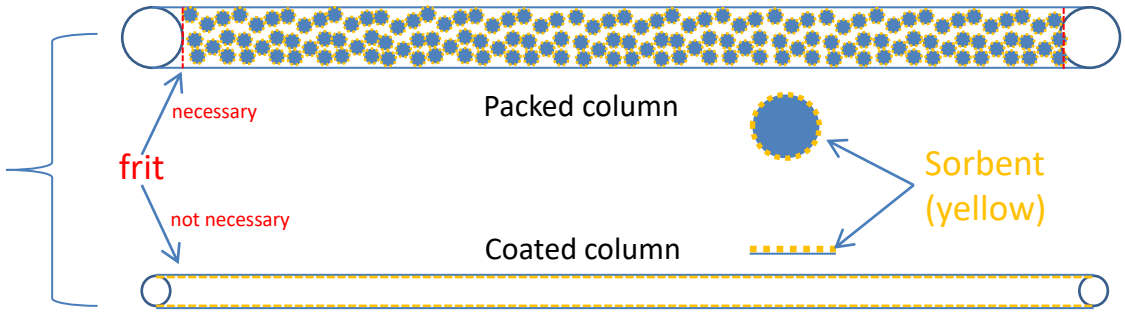


LC columns

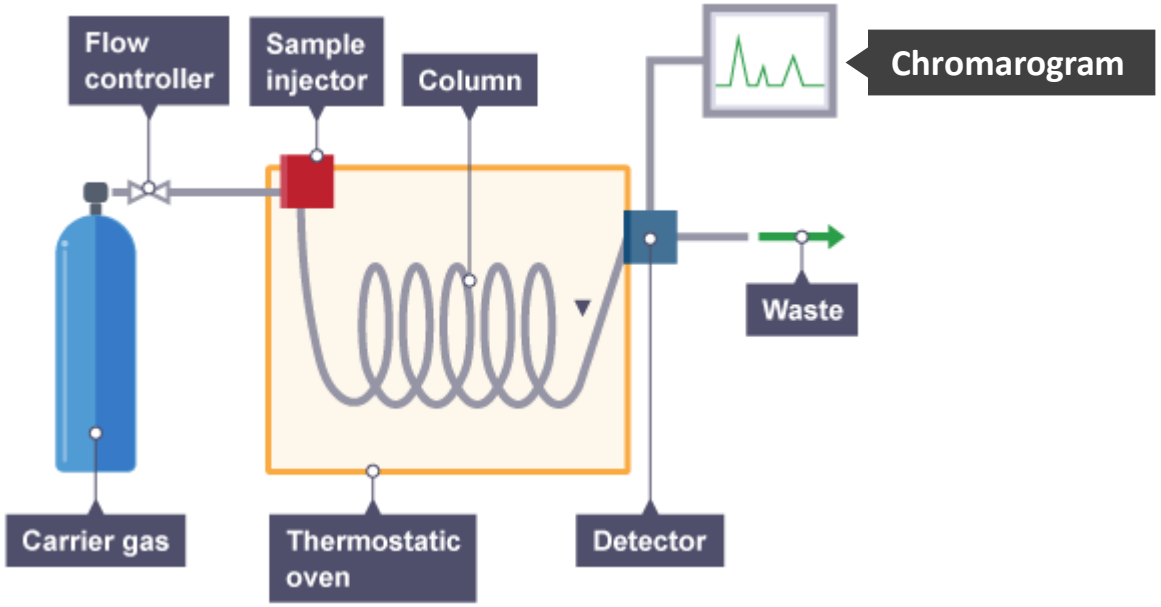
Length: usually less than 0.3 m

https://www.indiamart.com/proddetail/high-performance-liquid-chromatography-column-14612548591.html

Column represents the separation space where a separation proces is carried out. Inside the column is a proper chromatographic stationary phase (sorbent) fixed (immobilized) on particles or inner wall of the column (used mainly in capillary formates).



Gas chromatography



Instrumental scheme of Gas Chromatography (GC)

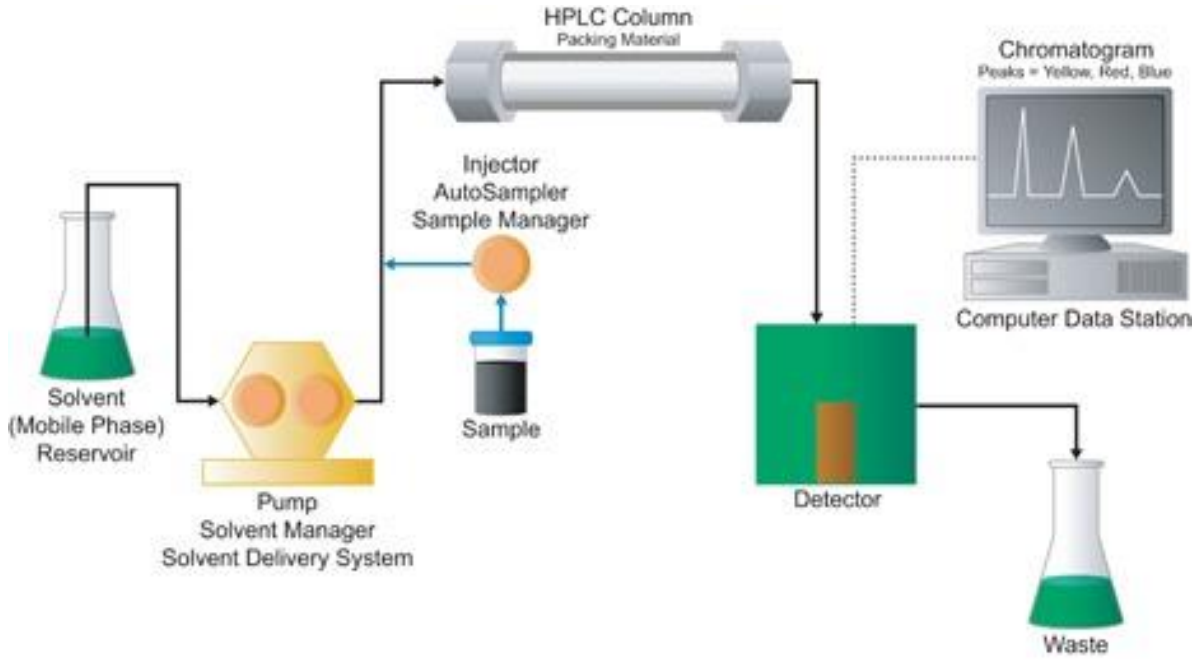
http://delloyd.50megs.com/moreinfo/gaschrom.html

Utilization of GC in pharmacy:

Useful for identification and quantification of volatile compounds (drugs, essential oils) in multicomponent samples such as pharmaceuticals, plants, plant extracts, etc.

Non-volatile compounds can be analyzed only after their proper derivatization (i.e. implementation of functional group or moiety enhancing volatility of the derivative)

Liquid chromatography



Instrumental scheme of High-Performance Liquid Chromatography (HPLC)

https://www.waters.com/waters/en_AU/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055&locale=en_AU

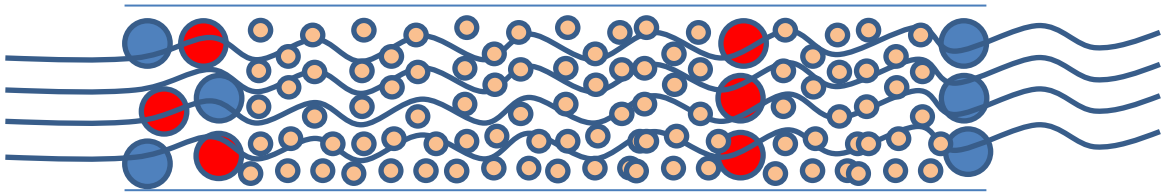
Utilization of HPLC in pharmacy:

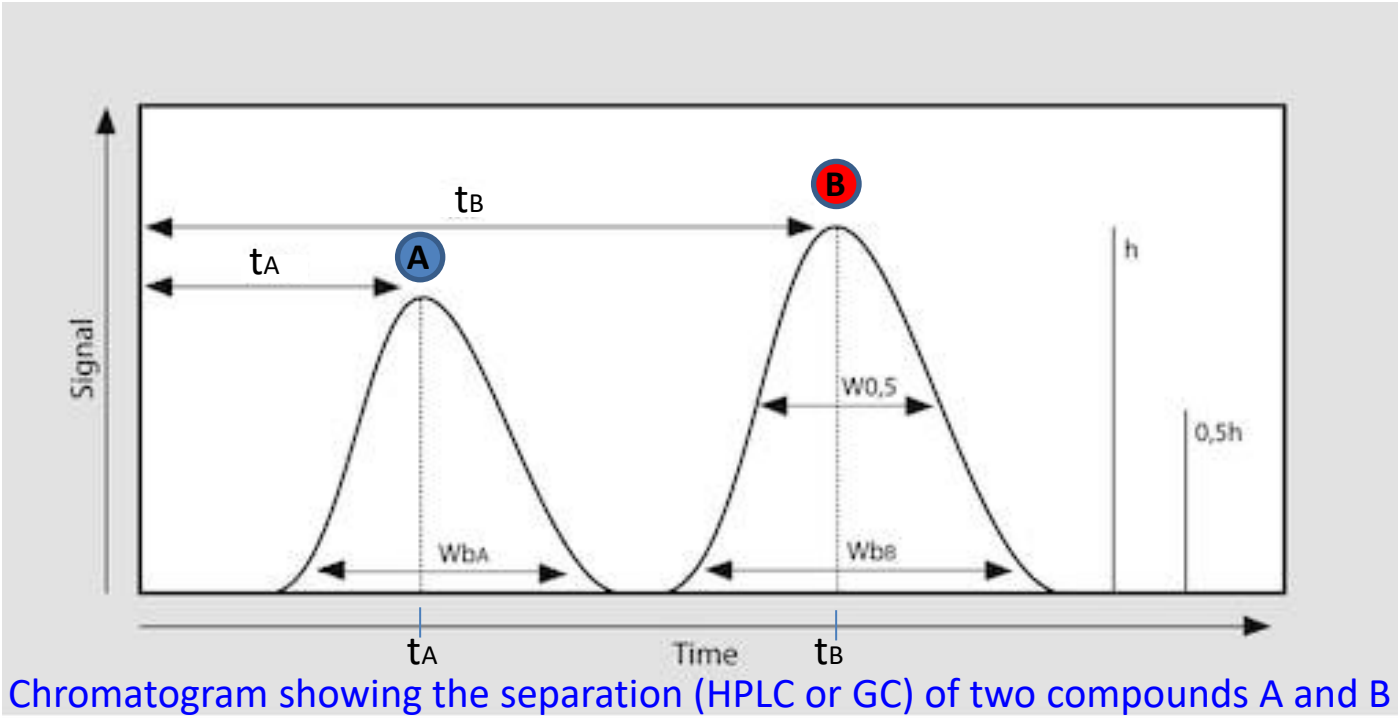
Useful for identification and quantification of a wide scale of compounds of various nature (polar, non-polar, acidic, basic, neutral, low-molecular, polymeric), it is the most universal analytical separation technique and, therefore, the most frequently used. It is applied for a majority of drugs, their metabolites as well as various endogenous biomarkers in a wide scale of multicomponent matrices (pharmaceuticals, biological and environmental material, etc.)

Specific pharmaceutical and biomedical applications include: quality/purity drug control, clinical testing, various research studies (diagnostic analyses, pharmacokinetic study, drug biodistribution monitoring, drug-drug or drug-carier interaction examining, metabolic profiling for therapy optimization, etc.)

Chromatogram

how to identify and quantify from chromatogram?





Chromatogram showing the separation (HPLC or GC) of two compounds A and B

https://www.shutterstock.com/cs/search/chromatogram

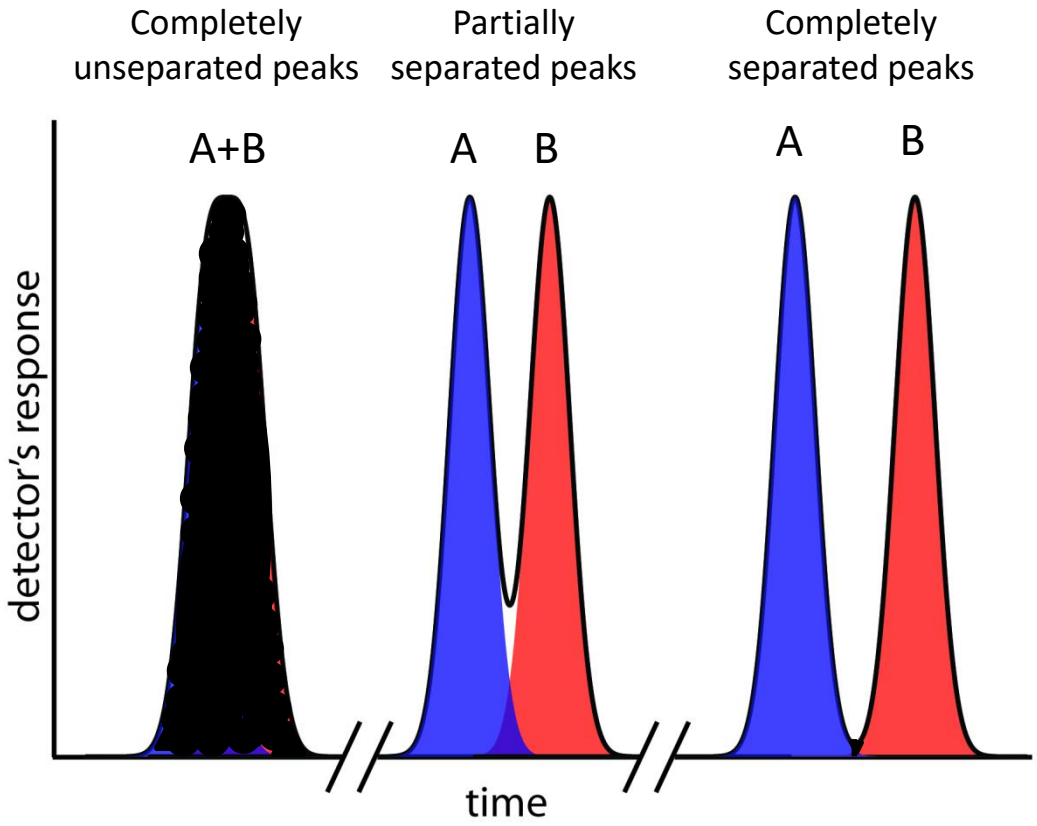
Qualitative parameter: position of peak in chromatogram expressed via its **elution time t** (characteristic and constant parameter for given compound under given separation conditions)

Quantitative parameter: **height or area of the peak** (changed with the concentration of the analyte, height and area increase with increased concentration of analyte and vice-versa for decreased concentration)

Optimization of chromatographic method

how to achieve best separation?

Chromatographic method with the optimum separation conditions provides completely separated peaks in chromatogram:



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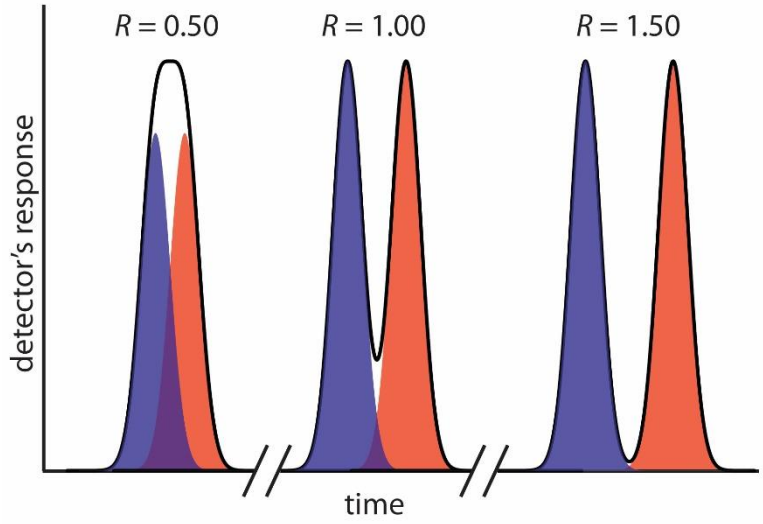
Optimization of chromatographic method

what is resolution of chromatographic peaks?

The goal of chromatography is to separate a mixture into a series of chromatographic peaks, each representing a single component of the mixture. The **resolution** between two chromatographic peaks, R_{AB}, is a quantitative measure of their separation, and is defined as:

$$R_{AB} = \frac{t_{r,B} - t_{r,A}}{0.5(w_B + w_A)} = \frac{2\Delta t_r}{w_B + w_A}$$

where B is the later eluting of the two solutes, t_r is the retention (elution) time, w is the baseline width. The separation of two chromatographic peaks improves with an increase in R_{AB}. Because resolution is a quantitative measure of a separation's success, it is a useful way to determine if a change in experimental conditions leads to a better separation.



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Three examples showing the relationship between resolution and the separation of a two component mixture. The blue and red peaks are the elution profiles for the two components. The chromatographic peak—which is the sum of the two elution profiles—is shown by the solid black line.

Optimization of chromatographic method

how to calculate resolution of chromatographic peaks?

Example

In a chromatographic analysis of lemon oil a peak for limonene has a retention time of 8.36 min with a baseline width of 0.96 min. γ-Terpinene elutes at 9.54 min with a baseline width of 1.64 min. What is the resolution between the two peaks?

Solution

Using equation R_{AB} we find that the resolution is

$$R_{AB} = \frac{2\Delta t_r}{w_B + w_A} = \frac{2(9.54\ \text{min} - 8.36\ \text{min})}{1.64\ \text{min} + 0.96\ \text{min}} = 1.48$$

Answer

The chromatographic resolution between limonene and γ-Terpinene is 1.48, so that the peaks are almost completely (i.e. baseline) resolved.

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Optimization of chromatographic method

what parameters is resolution depend on?

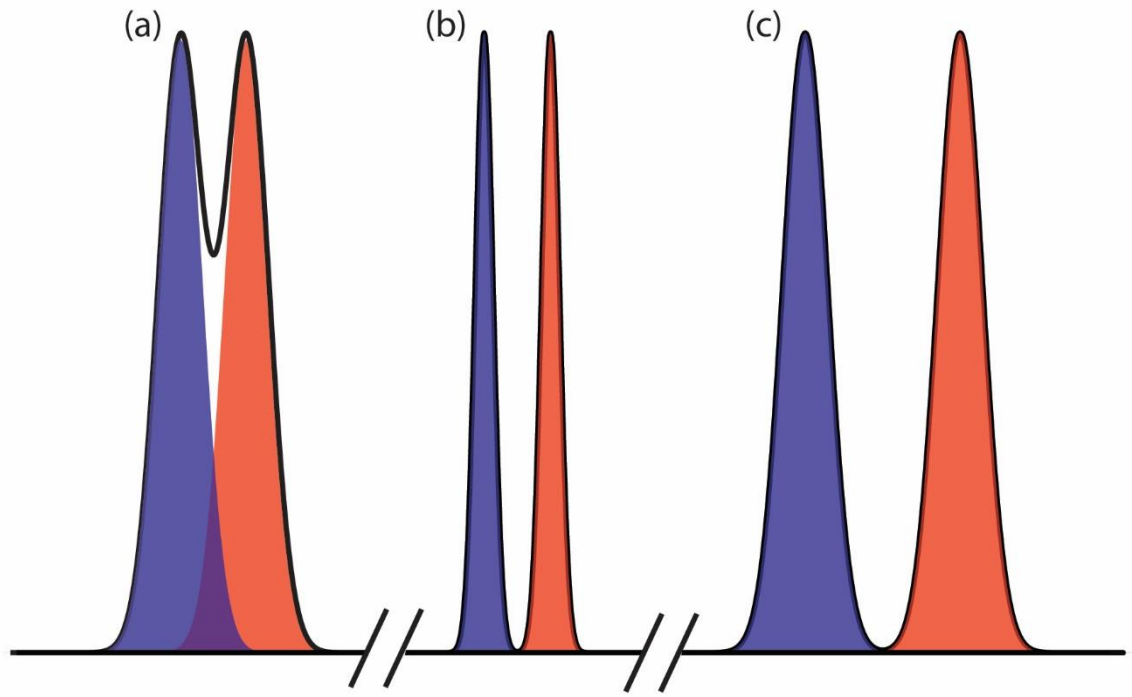


***Figure**: Two methods for improving chromatographic resolution: (a) original chromatogram; (b) chromatogram after decreasing w_A and w_B by 4×; (c) chromatogram after increasing Δt_r by 2×.*

Equation R_{AB} suggests that we can improve resolution by increasing Δt_r, or by decreasing w_A and w_B (see Figure). To increase Δt_r we can use one of two strategies. One approach is to adjust the separation conditions so that both solutes spend less time in the mobile phase—that is, we increase each *solute's retention factor*—which provides more time to effect a separation. A second approach is to increase *selectivity* by adjusting conditions so that only one solute experiences a significant change in its retention time. The baseline width of a solute's peak depends on the solutes movement within and between the mobile phase and the stationary phase, and is governed by several factors that we collectively call *column efficiency*. We should consider each of these approaches for improving resolution.

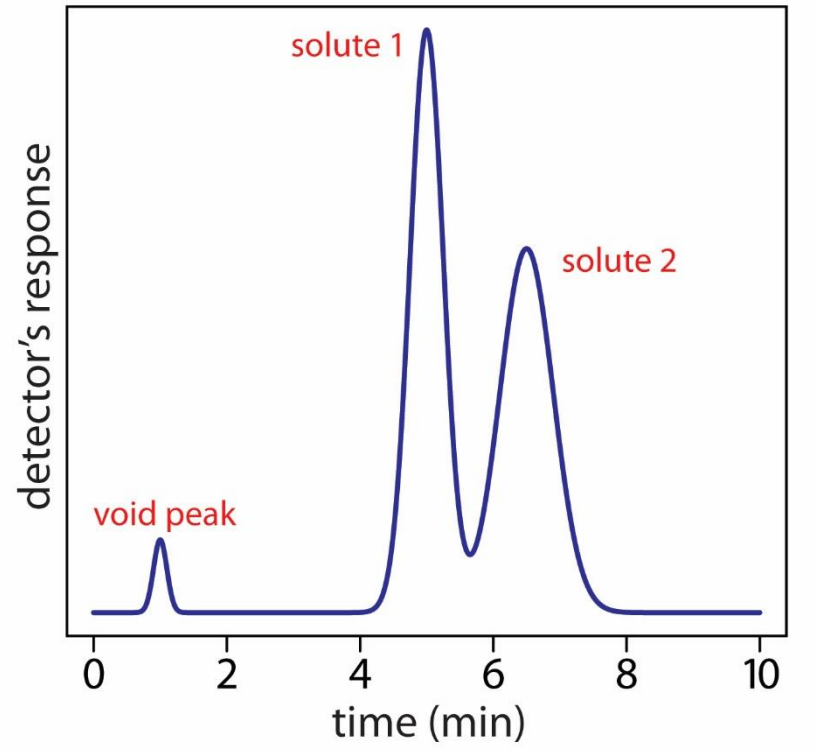
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Optimization of chromatographic method

what is retention factor?

We can determine a solute's retention factor (or capacity factor) from a chromatogram by measuring the column's void time (time for passing the mobile phase through a given column from the injection point to the detection point), t_m, and the solute's retention time, t_r (see Figure). We are solving equation for *k:*

$$k = \frac{t_r - t_m}{t_m}$$



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Optimization of chromatographic method

how to calculate retention factor?

Example

In a chromatographic analysis of low molecular weight acids, butyric acid elutes with a retention time of 7.63 min. The column's void time is 0.31 min. Calculate the retention factor for butyric acid.

Solution

Using equation for *k* we find that the retention factor for butyric acid is

$$k_{\text{but}} = \frac{t_{\text{r}} - t_{\text{m}}}{t_{\text{m}}} = \frac{7.63\ \text{min} - 0.31\ \text{min}}{0.31\ \text{min}} = 23.6$$

Answer

The retention factor for butyric acid is 23.6.

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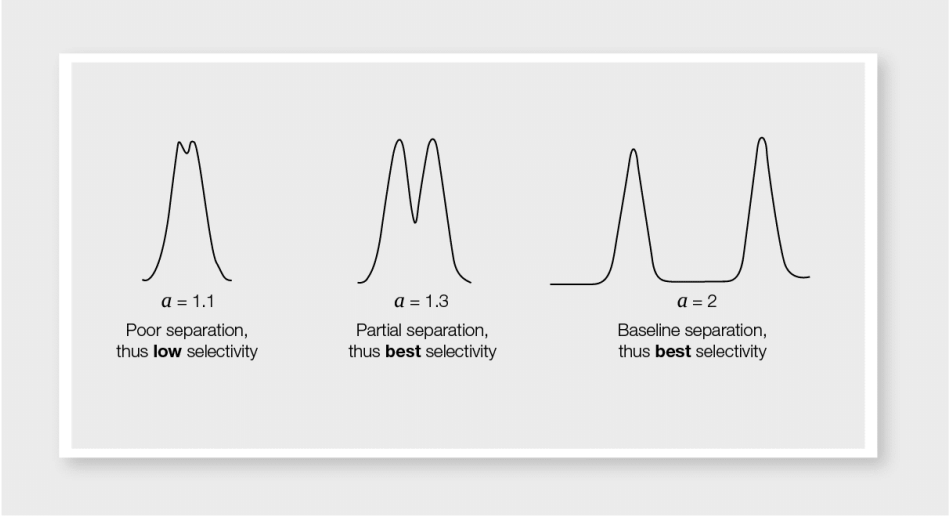
Optimization of chromatographic method

what is selectivity?

Selectivity is a relative measure of the retention of two solutes, which we define using a selectivity factor, α

$$\alpha = \frac{k_B}{k_A} = \frac{t_{r,B} - t_m}{t_{r,A} - t_m}$$

where solute A always has the smaller retention time. When two solutes elute with identical retention time, α = 1.00; for all other conditions α > 1.00.



https://www.barts-blog.net/how-to-choose-a-stationary-phase-optimize-selectivity-and-get-better-resolution-in-chromatography/
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Optimization of chromatographic method

how to calculate selectivity?

Example

In a chromatographic analysis of low molecular weight acids, butyric acid elutes with a retention time of 7.63 min, while the retention time for isobutyric acid is 5.98 min. The column's void time is 0.31 min. What is the selectivity factor for isobutyric acid and butyric acid?

Solution

Using equation for *k* we calculate the retention factors for butyric and isobutyric acid.

$$k_{\text{but}} = \frac{t_{\text{r}} - t_{\text{m}}}{t_{\text{m}}} = \frac{7.63\ \text{min} - 0.31\ \text{min}}{0.31\ \text{min}} = 23.6$$

$$k_{\text{iso}} = \frac{t_{\text{r}} - t_{\text{m}}}{t_{\text{m}}} = \frac{5.98\ \text{min} - 0.31\ \text{min}}{0.31\ \text{min}} = 18.3$$

The selectivity factor, using the equation for α, is

$$\alpha = \frac{k_{\text{but}}}{k_{\text{iso}}} = \frac{23.6}{18.3} = 1.29$$

Answer

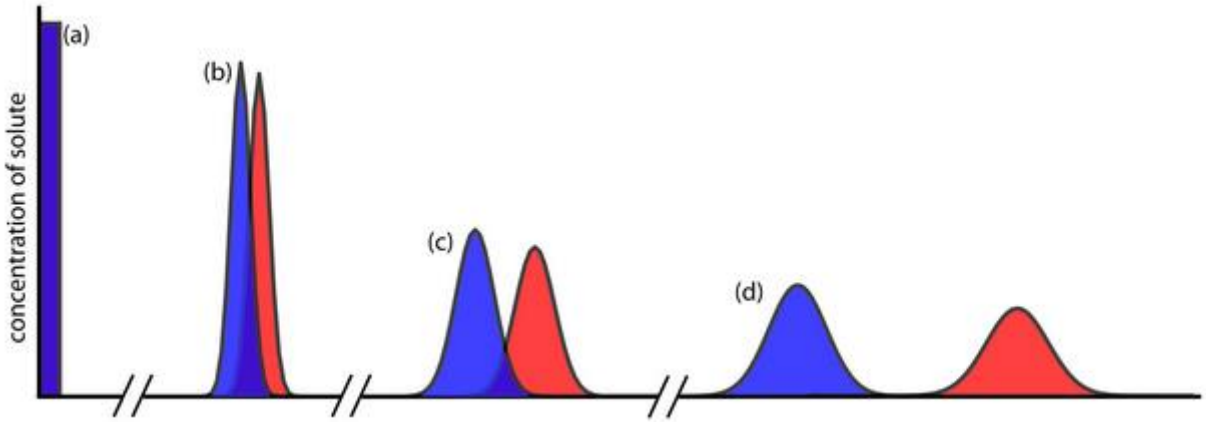
The selectivity factor for isobutyric and butyric acid is 1.29.

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Optimization of chromatographic method

what is column efficiency?

Suppose we inject a sample consisting of a single component. At the moment of injection the sample occupies a narrow band (zone) of finite width. As the sample passes through the column, the width of this band continually increases (due to various diffusion processes) in a process we call band broadening. Column efficiency provides a quantitative measure of the extent of band broadening. This situation is illustrated for two injected compounds in following figure:



dwelling time/passing distance in the column (from the injection point to the detection point)

The concentration of two solutes as a function of their dwelling time or passing distance *in the column.*

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Optimization of chromatographic method

what is column efficiency?

In their original theoretical model of chromatography, Martin and Synge divided the chromatographic column into discrete sections—what they called theoretical plates—in which there is an equilibrium partitioning of the solute between the stationary phase and the mobile phase. They described column efficiency in terms of the number of theoretical plates, *N*,

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

where *L* is the column's length and *H* is the height of a theoretical plate. Column efficiency improves—and chromatographic peaks become narrower—when there are more theoretical plates.

The column efficiency can be calculated from a chromatogram using the following equation:

$$N = 16\frac{t_r^2}{w^2}$$

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Optimization of chromatographic method

how to calculate column efficiency?

Example

A chromatographic analysis for the chlorinated pesticide Dieldrin gives a peak with a retention time of 8.68 min and a baseline width of 0.29 min. What is the number of theoretical plates? Given that the column is 2.0 m long, what is the height of a theoretical plate in mm?

Solution

Using a proper equation for *N*, the number of theoretical plates is

$$N = 16\frac{t_r^2}{w^2} = N = 16\frac{(8.68\ \text{min})^2}{(0.29\ \text{min})^2} = 14300\ \text{plates}$$

Solving equation for *H* gives the average height of a theoretical plate as

$$H = \frac{L}{N} = \frac{2.0\ \text{m}}{14300\ \text{plates}} \times \frac{1000\ \text{mm}}{\text{m}} = 0.14\ \text{mm/plate}$$

Answer

The column efficiency for the analyzed compound is 14300 theoretical plates and the height of a theoretical plate in this case is 0.14 mm/plate.

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Optimization of chromatographic method

what are the parameters influencing resolution?

For the optimization of the chromatographic separation, it is important to know how the solute retention factor, selectivity, and column efficiency affect the resolution of two closely eluting peaks A and B:

$$R_{AB} = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_B}{1 + k_B}$$

It is clear from this equation that the efficiency, selectivity as well as retention have to be optimized in order to achieve required (baseline) separation of the analyzed compounds.

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Optimization of chromatographic method

how to enhance column efficiency in chromatography effectively?

An approach to improving resolution is to adjust the column's efficiency by increasing the number of theoretical plates, *N*.

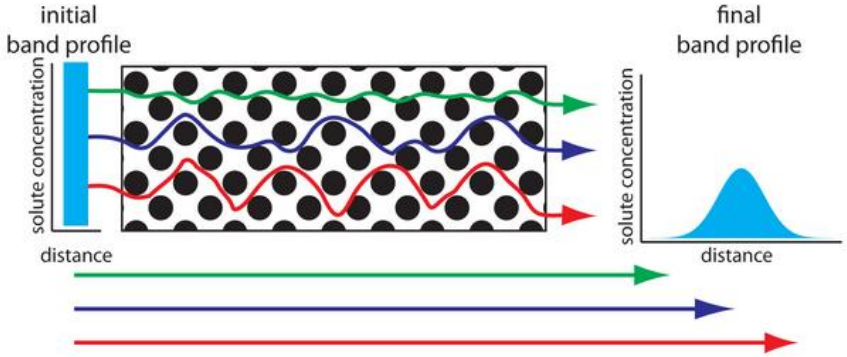
- The easiest way is to increase the length of the column, however, this also prolong the analysis time.
- A better approach is to decrease the height of a theoretical plate, *H*, providing the desired resolution without changing the analysis time.

To decrease the height of a theoretical plate we need to understand the experimental factors that affect band broadening. We can consider four contributions: variations in paths length, longitudinal diffusion, mass transfer in the stationary phase, and mass transfer in the mobile phase.

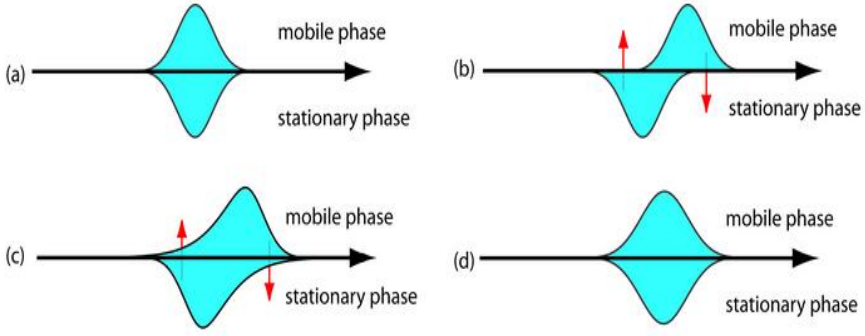
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Optimization of chromatographic method

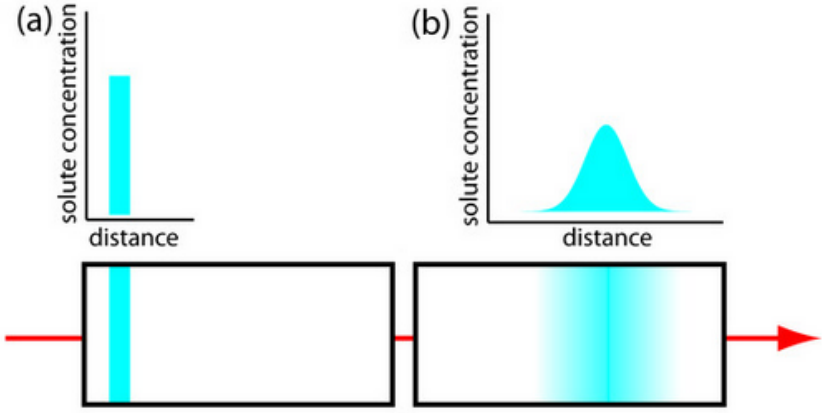
how to effectively decrease band broadening in chromatography?



The effect of multiple paths on a solute's band broadening. The solute's initial band profile is rectangular. As this band travels through the column, individual solute molecules travel different paths. As a result, the solute's band profile at the end of the column is broader and Gaussian in shape. **Solution:** A smaller range of particle sizes and a more consistent packing suppress band broadening. For a column without packing material, there is no contribution to band broadening from multiple paths (therefore capillary chromatography provides significant enhancement in efficiency).



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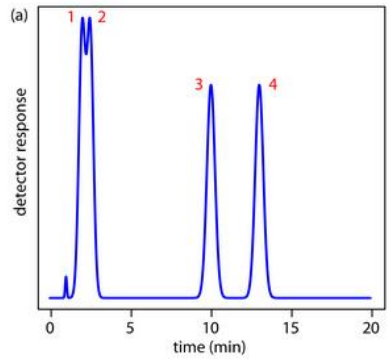
The effect of longitudinal diffusion on a solute's band broadening. Solute molecules are constantly in motion, diffusing from regions of higher solute concentration to regions where the concentration of solute is smaller. Two horizontal crosssections through the column and the corresponding concentration versus distance profiles are shown, with (a) being earlier in time. The red arrow shows the direction in which the mobile phase is moving. Because a solute's diffusion coefficient is larger in the gas phase than in a liquid phase, longitudinal diffusion is a more serious problem in gas chromatography. **Solution:** a higher velocity (of mobile phase) provides less time for longitudinal diffusion.

Effect of mass transfer on band broadening: (a) Ideal equilibrium Gaussian profiles for the solute in the mobile phase and in the stationary phase. (b, c) If we allow the solute's band to move a small distance down the column, an equilibrium between the two phases no longer exits. The red arrows show the movement of solute—what we call the mass transfer of solute—from the stationary phase to the mobile phase, and from the mobile phase to the stationary phase. (d) Once equilibrium is reestablished, the solute's band is now broader. **Solution:** band broadening is directly proportional to the mobile phase velocity—smaller velocities provide more time for mass transfer.

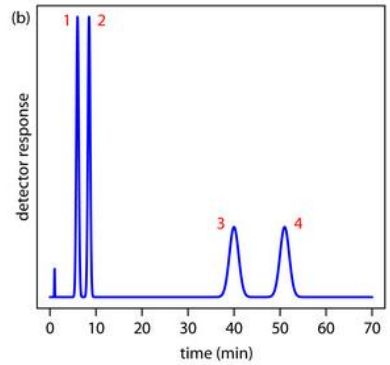
Optimization of *N* as a compromise in the mobile phase velocity

Optimization of chromatographic method

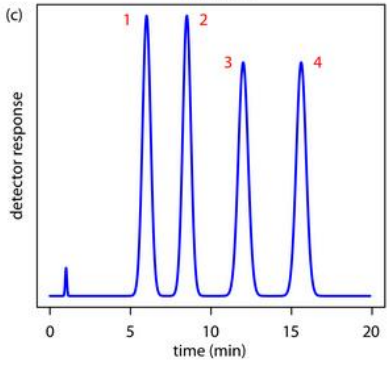
how to effectively solve the general elution problem in chromatography?



Fast but not complete separation



Complete but long separation



Optimum separation: complete and fast

Example showing the general elution problem in chromatography.

Solved via k (less effective)

- Changing the temperature in GC
- Changing the solvent's eluting strength in LC

Solved via α (more effective)

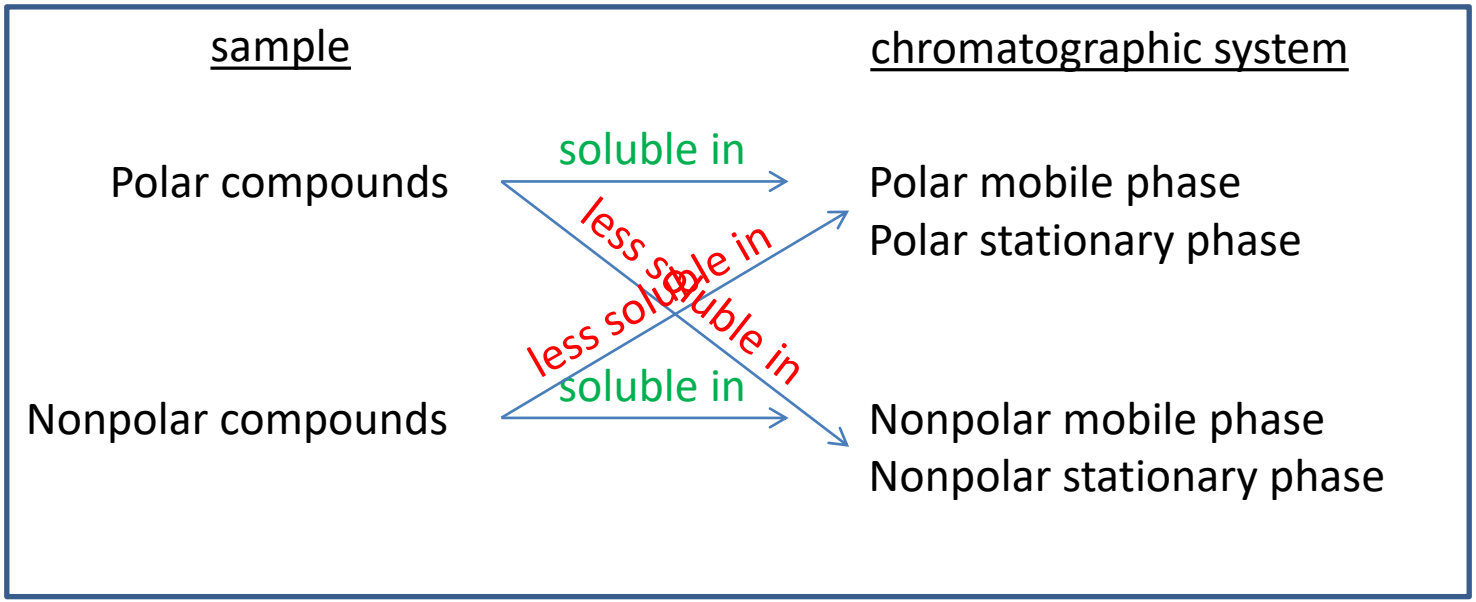
- Changing the stationary phase in GC
- Changing the composition of the mobile phase (pH, type of solvent) and type of the stationary phase in LC

https://chem.libretexts.org/Under_Construction/Purgatory/Book%3A_Analytical_Chemistry_2.0_(Harvey)/12_Chromatographic_and_Electrophoretic_Methods/12.2%3A_General_Theory_of_Column_Chromatography

Optimization of chromatographic method

what is the role of solubility in chromatography?

Basic principle of solubility: similar is soluble in similar



Compounds less soluble in mobile phase = higher retention, lower velocity, higher retention time
Compounds more soluble in stationary phase = higher retention, lower velocity, higher retention time
Compounds more soluble in mobile phase = lower retention, higher velocity, lower retention time
Compounds less soluble in stationary phase = lower retention, higher velocity, lower retention time

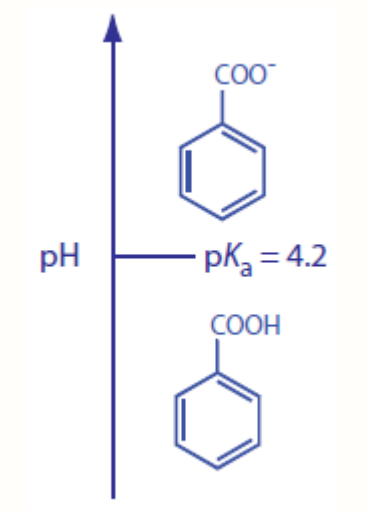
Optimization of chromatographic method

what is the role of pH in chromatography?

An example

why pH can affect a solute's retention time?

Let's use benzoic acid, C_6H_5COOH, to explain why pH can affect a solute's retention time. The separation uses an aqueous mobile phase and a nonpolar stationary phase. At lower pHs, benzoic acid is predominately in its weak acid form, C_6H_5COOH, and easily partitions into the nonpolar stationary phase. At more basic pHs, however, benzoic acid is in its weak base form, $C_6H_5COO^-$. Because it now carries a charge, its solubility in the mobile phase increases and its solubility in the nonpolar stationary phase decreases. As a result, it spends more time in the mobile phase and has a shorter retention time.



https://chem.libretexts.org/Under_Construction/Purgatory/Book%3A_Analytical_Chemistry_2.0_(Harvey)/12_Chromatographic_and_Electrophoretic_Methods/12.2%3A_General_Theory_of_Column_Chromatography

Optimization of chromatographic method

what is the role of the mobile phase in chromatography?
what is the solvent's eluting strength?
what is isocratic and gradient elution?

solvent's eluting strength

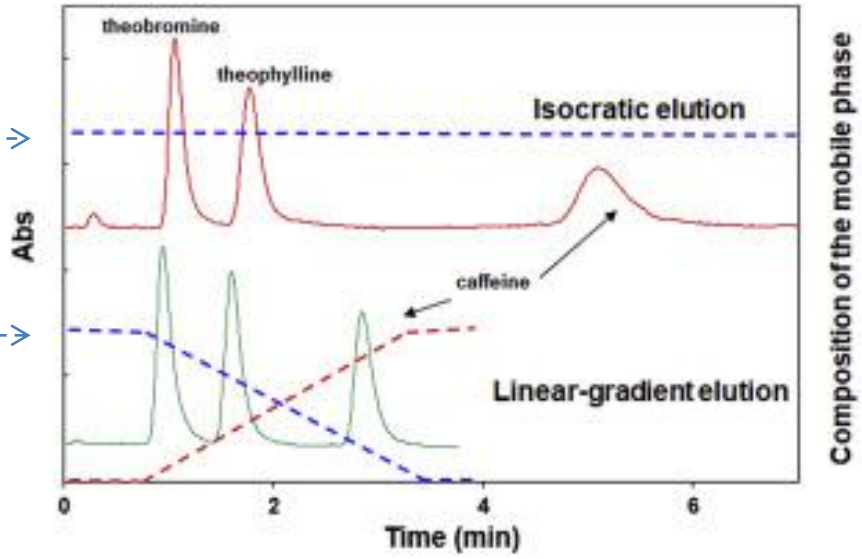
- **ability of the mobile phase to provide elution of the separated compound**
- **depend on an ability of the solvent to solubilize the separated compound**

Example:

- for polar compounds the solvent's eluting strength is increased with its polarity (and vice versa)
- In this way we can influence the retention of the separated compounds in the chromatographic column

Less effective isocratic elution
(composition of the mobile phase is constant in time, i.e. elution strength is constant in time)

More effective gradient elution
(composition of the mobile phase is changed in time, i.e. elution strength is changing in time, e.g. by increasing or decreasing polarity, increasing or decreasing pH, etc.)



https://www.sciencedirect.com/science/article/abs/pii/S0039914019304357

Optimization of chromatographic method

what is the role of interactions and stationary phase in chromatography?

Interactions in chromatography are prerequisite for a recognition of the separated compounds according to their chemical structures.

Compounds with different chemical structures provide different interactions with the stationary phase of the chromatographic system.

Example:

- polar compounds interact stronger with polar stationary phase (and vice versa)
- In this way we can influence the retention and selectivity of the separated compounds in the chromatographic column

Optimization of chromatographic method

how to select a proper stationary phase in chromatography?
what are basic chromatographic modes according to the type of stationary phase and what is their application?

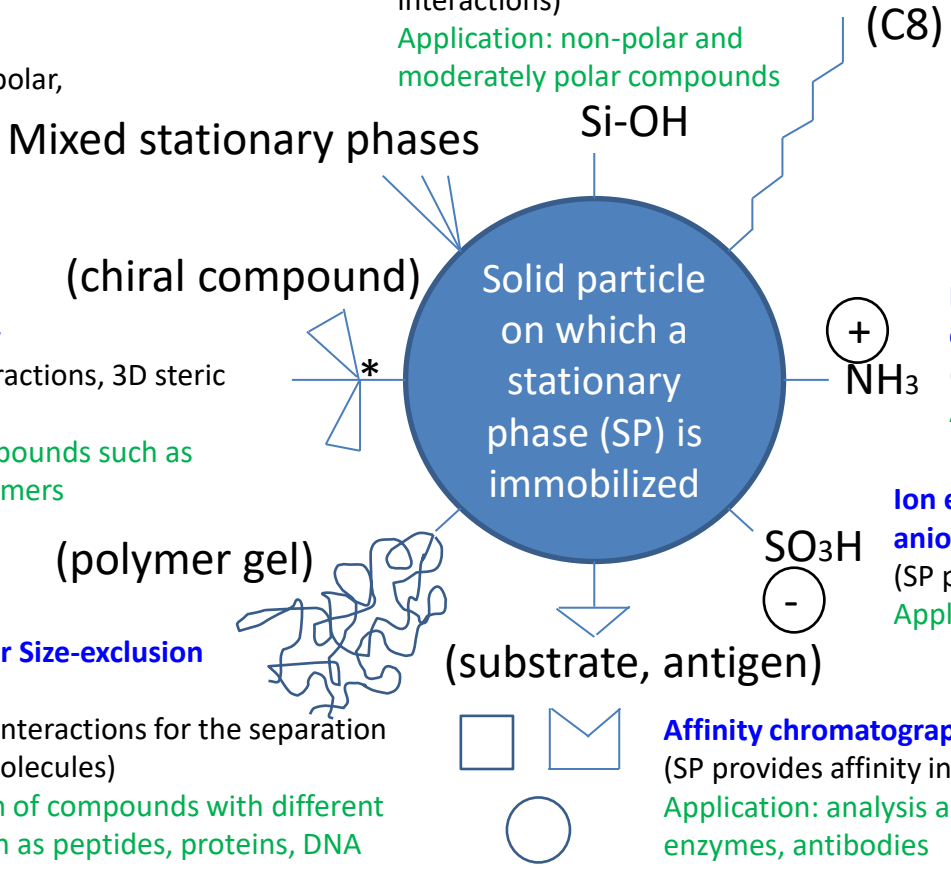
Type of the stationary phase is selected according to the chemical properties/structures of the separated compounds to obtain the best overal selectivity.

To achieve the best separation, a selected stationary phase must be properly combined with other optimized parameters such as mobile phase, temperature, length of the chromatographic column, etc.

Mixed mode chromatography
(SP provides mixed interactions (polar, non-polar, ionic)
Application: majority of compounds with various polarity

Normal phase (NP) chromatography (SP provides polar interactions)
Application: non-polar and moderately polar compounds

Reversed phase (RP) chromatography
(SP provides non-polar interactions)
Application: majority of compounds with various polarity, mainly polar and moderately polar compounds



Chiral chromatography
(SP provides chiral interactions, 3D steric interactions)
Application: chiral compounds such as enantiomers, diastereomers

Ion exchange chromatography (IEC) - cationic
(SP provides ionic interactions)
Application: negatively charged compounds

Ion exchange chromatography (IEC) - anionic
(SP provides ionic interactions)
Application: positively charged compounds

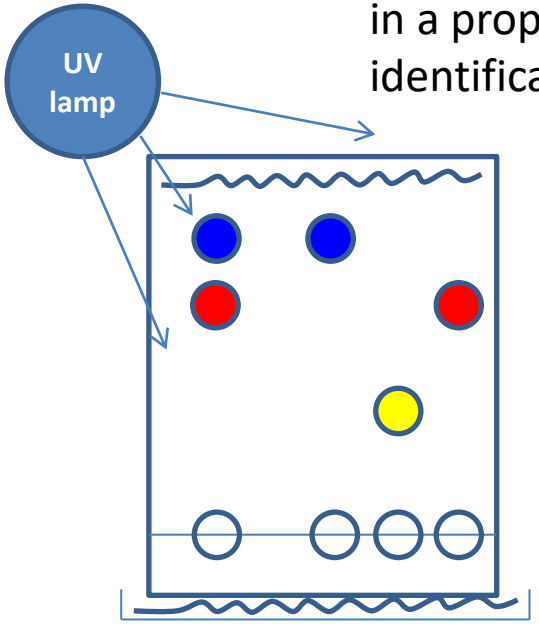
Gel chromatography or Size-exclusion chromatography (SEC)
(SP provides 3D steric interactions for the separation according to sizes of molecules)
Application: separation of compounds with different molecular weigths such as peptides, proteins, DNA

Affinity chromatography
(SP provides affinity interactions, 3D steric interactions key-lock)
Application: analysis and purification of biopolymers such as enzymes, antibodies

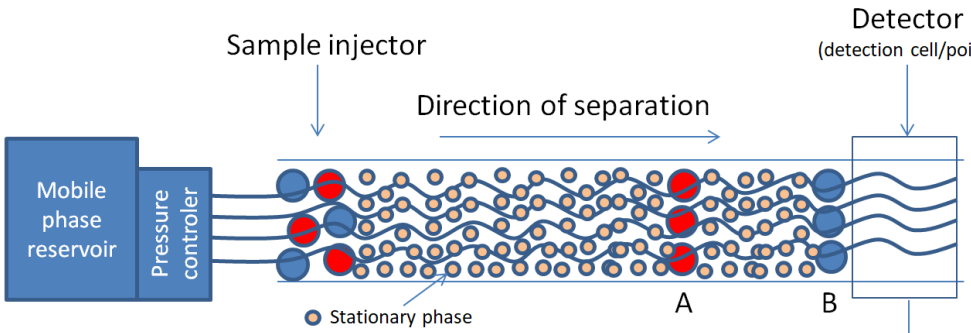
Detection in chromatography

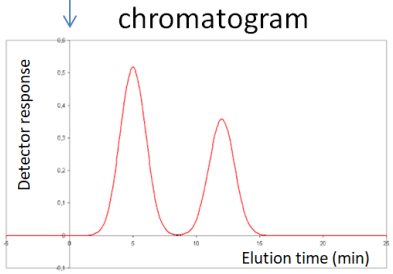
what is a purpose of detection in chromatography?

Detection is necessary to visualize separated compounds in a proper form (spot, peak) in order to provide their identification and quantification.



In planar chromatography we can detect separated compounds as colored spots (for VIS absorbing compounds) or using UV lamp (we can observe UV absorbing compounds)





In column chromatography we can detect zones of separated compounds passing through the detection cell as corresponding chromatographic peaks. The detector should give characteristic detection response on given compound. Hence, detectors are selected according to the characteristic measurable attributes of the separated compounds, such as conductivity, refractive index, UV-absorbace, fluorescence, electrochemical activity, molecular mass, etc.

Notice: These facts (except from instrumental specifics) are applicable for both chromatography and electrophoresis as well.

Detection in chromatography

what is universal or selective detection in chromatography?
what is specificity of detection signal?

With respect to the application range of the detector, the detector can be universal or selective.

Universal detector

- no specific attribute of the analyzed compound is required, e.g. mass spectrometer can measure each compound that was previously ionized, so that a wide scale of compounds can be detected
- Examples: conductivity detectors, light scattering detectors, refractive index detectors, mass spectrometry detectors, etc.

Selective detector

- response of the detector is due to the characteristic feature of the analyzed compound, such as fluorescence, electrochemical activity, etc., so that only a narrow scale of compounds can be detected
- Examples: fluorescence detector, electrochemical detectors, etc.

Detection signal of the detector can be non-specific or specific.

Non-specific detection signal

- the detection signal does not refer to a specific feature of given (only one) compound (e.g conductivity, light scattering, etc.) and many other compounds give similar responses
- Examples: conductivity detectors, light scattering detectors, refractive index detectors, etc.

Specific detection signal

- the detection signal refers to a specific feature of given (only one) compound (e.g molecular weigth, UV or mass spectrum, etc.) and no or very limited number of other compounds can give similar responses
- Examples: spectral detectors (UV, fluorescence, mass spectrometry)

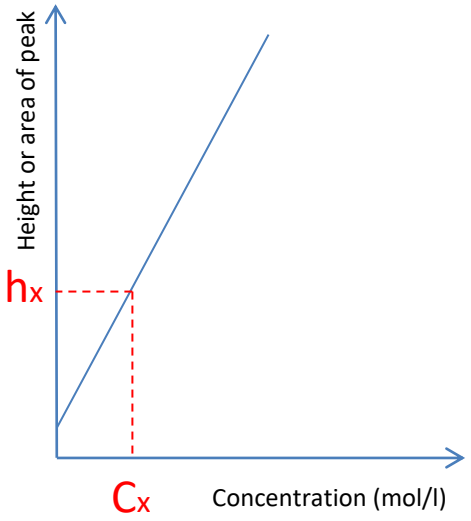
Notice 1: Universal MS detector can provide highly specific detection response.

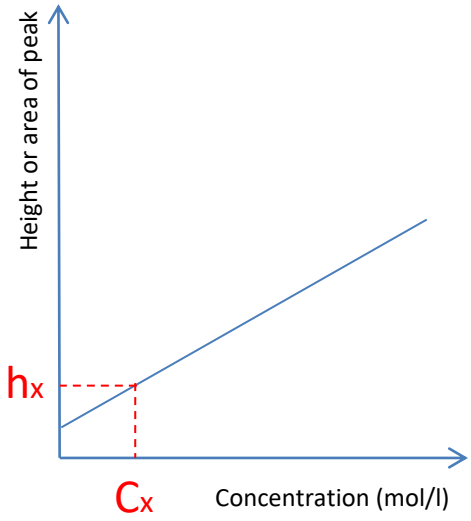
Notice 2: These facts are applicable for both chromatography and electrophoresis as well.

Detection in chromatography

what is detection sensitivity?
what is calibration curve?

Sensitivity of the detector is a measure of its ability to discriminate between small differences in analyte concentration (i.e. when response of the detector increases more/less for a given concentration of the analyte, the detector is more/less sensitive). So, it is actually the slope of the calibration curve (i.e. the higher the slope of a calibration curve the higher the sensitivity of a detector for that particular component). Highly sensitive detectors are prefered.





Calibration curve is a dependence of height or area of the peak on concentration of given analyte. This dependence is prepared from measuring a series of standard solutions of given analyte with known concentrations. Mathematical expression of calibration line is following: $y=ax+b$, where *y* is height or area of the peak, *x* is concentration of given compound, *a* is slope of calibration line, *b* is intercept of calibration line. Given calibration line is described by *a* and *b*, so that measuring height or area of the peak of given compound we can easily recalculate its unknown concentration.

Calibration curves obtained with a more sensitive detector (left dependence) or a less sensitive detector (right dependence). For a given concentration c_x of the analyte X was obtained higher (left dependence) or lower (right dependence) detection signal (peak).

Notice 1: Sensitivity of a detector is not the minimum amount that can be detected, this is the Limit of detection, LOD (see later). This value (LOD) is influenced by the chromatographic conditions , namely a noise of the baseline.
Notice 2: These facts are applicable for both chromatography and electrophoresis as well.

Detection in chromatography

what detection techniques are used in chromatography?

A chromatography detector is a device used in gas chromatography (GC) or liquid chromatography (LC) to detect components of the mixture being eluted off the chromatography column. There are two general types of detectors:

- Destructive
  - The destructive detectors perform continuous transformation of the column effluent (burning, evaporation or mixing with reagents) with subsequent measurement of some physical property of the resulting material (plasma, aerosol or reaction mixture).
- Non-destructive

The non-destructive detectors are directly measuring some property of the column eluent (for example UV absorption, Fluorescence, conductivity, refractive index) and thus affords greater analyte recovery.

Notice: These facts (with a proper terminology) are applicable for both chromatography and electrophoresis as well.

Detection in chromatography

what are destructive detection techniques used in chromatography?

Destructive detectors

In liquid chromatography:

- Charged aerosol detector (CAD)
  - CAD is used to measure the amount of chemicals in a sample by creating charged aerosol particles which are detected using an *electrometer*. It is commonly used for the analysis of compounds that cannot be detected using traditional UV/Vis approaches due to their lack of a chromophore. The CAD can measure all non-volatile and many semi-volatile analytes including, but not limited to, antibiotics, excipients, ions, lipids, natural products, biofuels, sugars and surfactants.
- Evaporative light scattering detector (ELSD)
  - ELSD, like CAD, belongs to the group of aerosol detectors, providing universal detection. In the detection region, a beam of light crosses the column of analyte (after its evaporation from aerosol) and the scattering of light is measured by a photodiode or photomultiplier tube. ELSD is commonly used for analysis of compounds do not efficiently absorb UV radiation, such as sugars, antivirals, antibiotics, fatty acids, lipids, oils, phospholipids, polymers, surfactants, terpenoids and triglycerides.

In gas chromatography:

- Flame ionization detector (FID)
  - The operation of the FID is based on the detection of ions formed during combustion of organic compounds in a hydrogen flame. The generation of these ions is proportional to the concentration of organic species in the sample gas stream.
- Flame photometric detector (FPD)
  - FPD uses a photomultiplier tube to detect spectral lines of the compounds as they are burned in a flame.
- Nitrogen Phosphorus Detector (NPD)
  - NPD, a form of *thermionic* detector, where nitrogen and phosphorus alter the *work function* on a specially coated bead and a resulting current is measured.
- Atomic-emission detector (AED)
  - In AED, a sample eluting from a column enters a chamber which is energized by microwaves that induce a plasma. The plasma causes the analyte sample to decompose and certain elements generate an atomic emission spectra. The atomic emission spectra is diffracted by a diffraction grating and detected by a series of photomultiplier tubes or photo diodes.

In all types of chromatography:

- Mass spectrometer (MS)*
  - highly effective and sensitive, even in a small quantity of sample. MS detector can be used to identify the analytes in chromatograms by their mass spectra.

*the most important destructive detection technique for GC and LC

Detection in chromatography

what are non-destructive detection techniques used in chromatography?

Non-destructive detectors

In liquid chromatography:

- UV detectors*
  - fixed or variable wavelength, which includes diode array detector (DAD or PDA). The UV absorption of the effluent is continuously measured at single or multiple wavelengths. These are by far most popular detectors for LC.
- Fluorescence detector
  - Irradiates the effluent with a light of set wavelength and measure the fluorescence of the effluent at a single or multiple wavelength.
- Refractive index detector (RI or RID)
  - Continuously measures the refractive index of the effluent. The lowest sensitivity of all detectors. Often used in *size exclusion chromatography* for polymer analysis.
- Radio flow detector
  - Measures radioactivity of the effluent. This detector can be destructive if a *scintillation cocktail* is continuously added to the effluent.
- Chiral detector
  - continuously measures the *optical angle of rotation* of the effluent. It is used only when chiral compounds are being analyzed.
- Conductivity monitor
  - Continuously measures the conductivity of the effluent. Used only when conductive eluents (water or alcohols) are used.

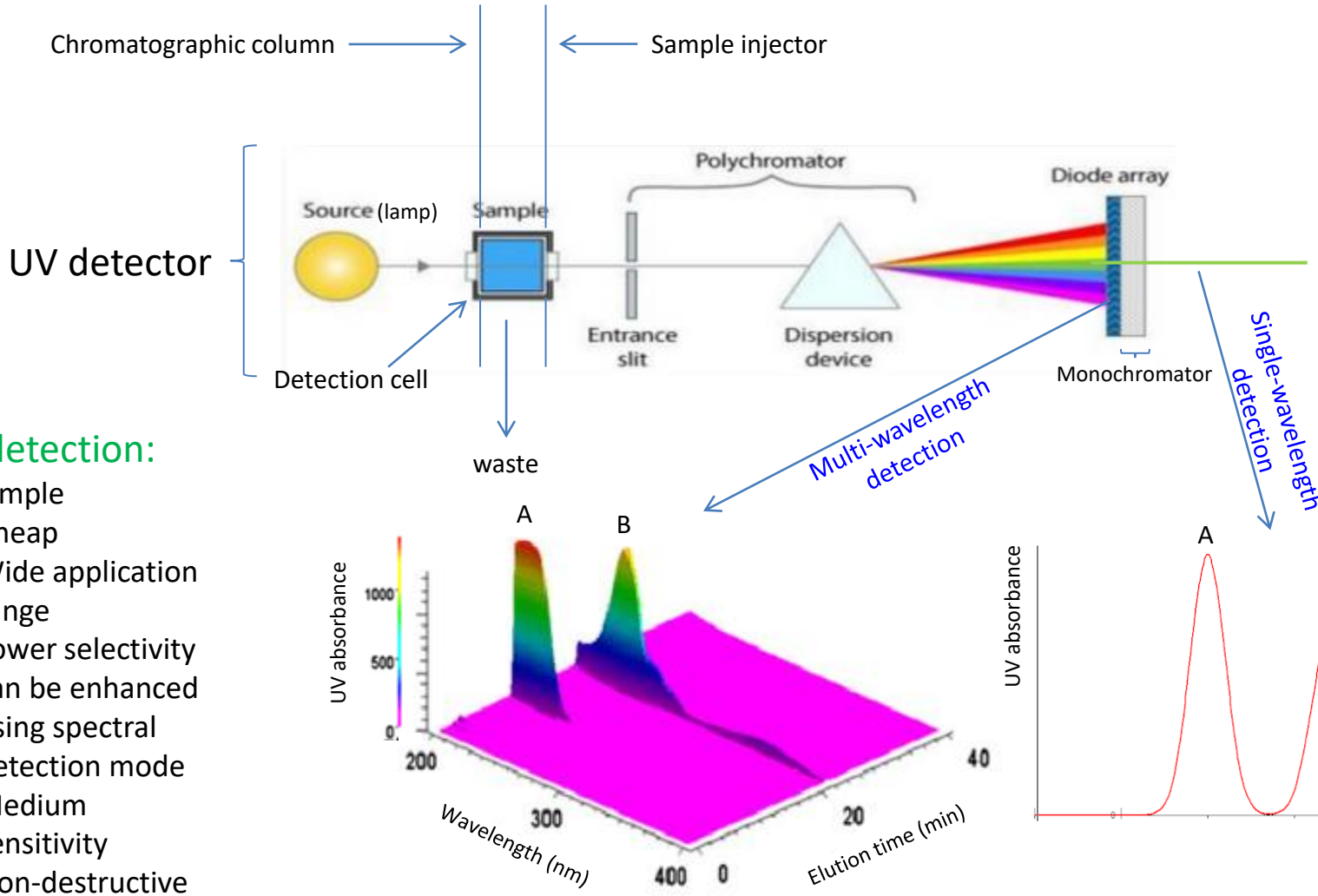
In gas chromatography:

- Thermal conductivity detector (TCD)
  - Measures the thermal conductivity of the eluent.
- Electron capture detector (ECD)
  - The most sensitive detector known. Allows for the detection of organic molecules containing halogen, nitro groups etc.
- Photoionization detector (PID)
  - Measures the increase in conductivity achieved by ionizing the effluent gas with UV radiation.

*the most frequently used non-destructive detection technique for LC

Detection in chromatography

UV detection



Notice: These detection scheme is applicable for both chromatography and electrophoresis as well.

UV detection:

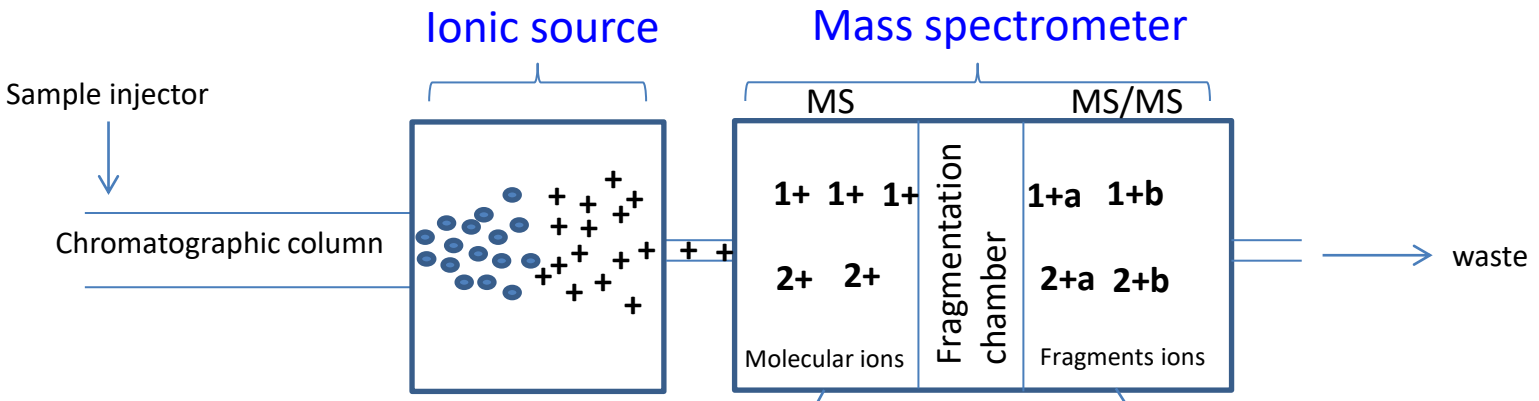
- Simple
- Cheap
- Wide application range
- Lower selectivity can be enhanced using spectral detection mode
- Medium sensitivity
- Non-destructive
- Fast detection response

Spectral UV detection. Advanced 3D chromatogram measured at multi-wavelength (here, interval 200-400 nm).
At each elution time is showed whole absorption spectrum of given compound.

UV detection at one wavelength. Common 2D chromatogram measured at one selected wavelength.

Detection in chromatography

MS detection

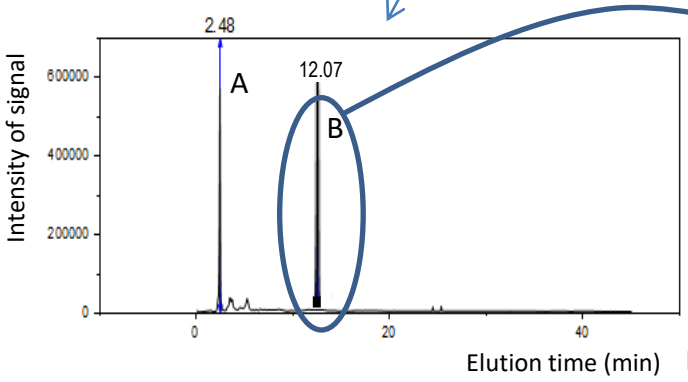


nebulization of eluate, evaporation of solvent, ionization of the analytes, only ionic analytes can be subsequently analyzed and detected in mass spectrometer

Separation of the ionic analytes according to the ratio mass-to-charge (m/z), fragmentation (cleavage) of the separated ions to their fragments, producing of fragmentation spectra (set of fragments with characteristic m/z values) for the separated analytes (fragmentation or mass spectrum is characteristic for a given analyte structure)

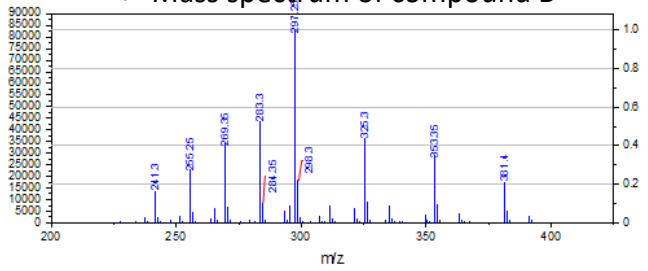
MS detection:

- High selectivity, can be further enhanced using spectral detection mode
- Provides structural information
- High sensitivity
- Wide application range
- Fast detection response
- Destructive
- Expensive



MS identification according to the molecular weigth of the separated compounds: m/z (A) and m/z (B), eluting with different elution times (two identifiers: elution time and m/z).

Mass spectrum of compound B



Enhanced (unequivocal) MS/MS identification according to the characteristic mass (fragmentation) spectra of the separated compounds (two identifiers: elution time and m/z spectrum). At each elution time is recorded whole absorption spectrum of given compound. Mass spectra of compounds with different structures are differing from each other.

Notice: These detection scheme is applicable for both chromatography and electrophoresis as well.

New trends in chromatography

P. Mikus et al. / „Kvapalinová chromatografia, hmotnostná spektrometria a ich kombinácie vo farmaceutickej a biomedicínskej analýze“, and references cited therein

- **novel stationary phases**
  - low-size particles of the sorbents
  - core–shell particles
  - monolithic stationary phases
  - other sorbent/modes: e.g. porous graphitic carbon (PGC), hydrophilic interaction liquid chromatography (HILIC)
- **innovative chromatographic techniques**
  - Ultra-high-performance liquid chromatography (UHPLC)
  - High-temperature liquid chromatography (UTLC)
  - Supercritical fluid chromatography (SFC)
  - Hybrid: UHTLC, UHPSFC
- **miniaturization**
  - micro-LC
  - LC on chip
- **two-dimensional systems**
  - two-dimensional LC systems
    - LCxLC (comprehensive two-dimensional liquid chromatography)
    - LC-LC (fraction or „heart-cut“ two-dimensional liquid chromatography)
    - mLC-LC (multiple „heart-cut“ two-dimensional liquid chromatography)
  - two-dimensional SPE-LC system

New trends in chromatography

novel stationary phases

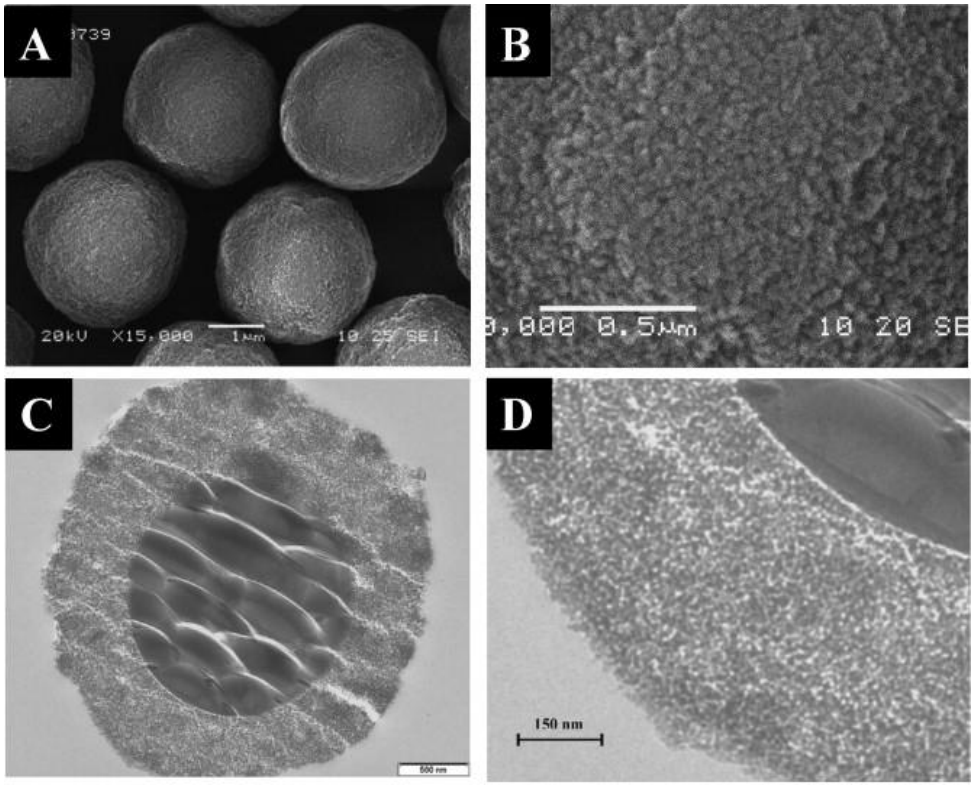
Conventional/low-size particles of the sorbents: 3-5 µm size / lower than 2 µm size

Enhance the separation efficiency with negligible back pressure
(but decreasing pore layer decreases also sorption capacity)

Enhance the separation efficiency and sorption capacity
(but, unfavorably, increase back pressure)

Core–shell silica particles (*fused-core*, *solid core* or *superficially porous* particles)
- have been increasingly used for highly efficient separation with fast flow rate and relatively low back pressure

Structure of core–shell silica particles

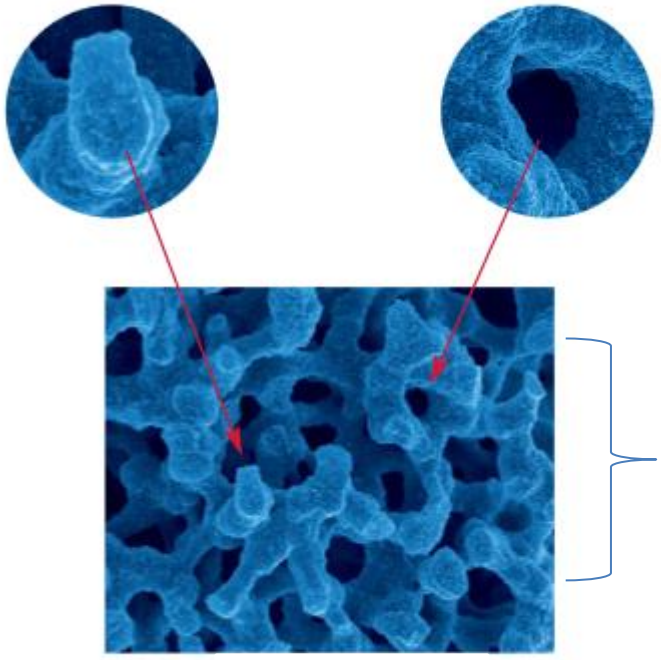


New trends in chromatography

novel stationary phases

Monolithic stationary phases: not spheric particles but one compact structure (silicagel or organic polymers)

Enhance the separation efficiency
(but, unfavorably, also back pressure)



Structure of silicagel monolith

macropores (1 - 2µm), mezopores (2 - 50nm) a micropores (< 2 nm)

Favorable for column efficiency and sorption capacity

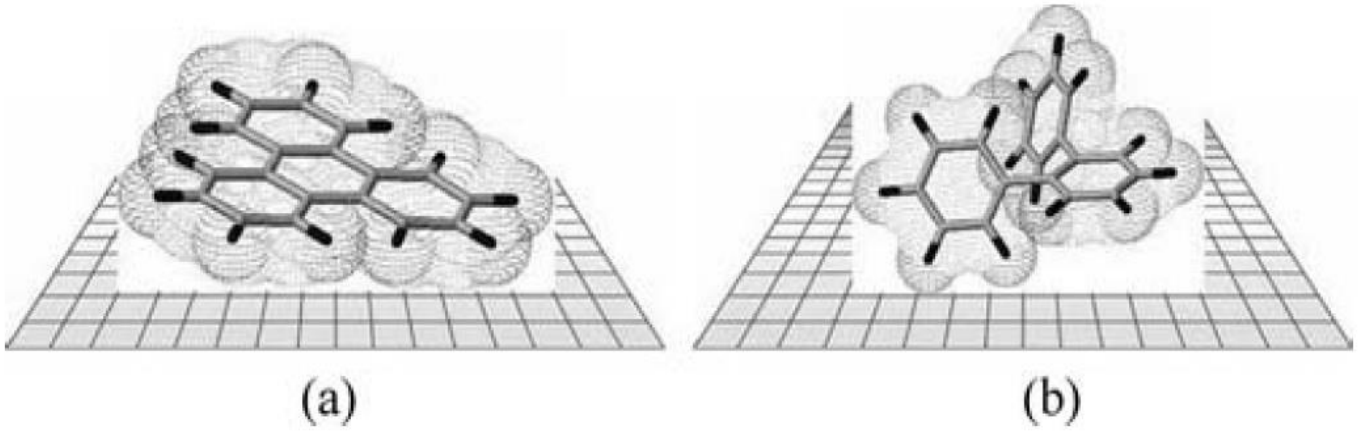
Favorable for column permeability, lower column pressure, higher flow rate

New trends in chromatography

novel stationary phases

Other sorbents: porous graphitic carbon (PGC)

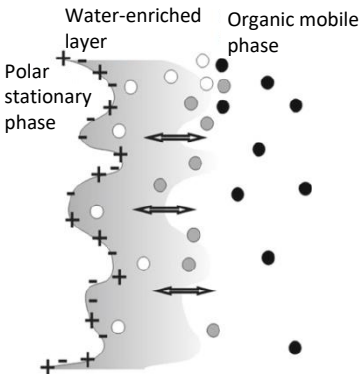
- **Provides affinity towards both polar and nonpolar compounds (RP silica sorbents, e.g. C18, only for nonpolar)**
- **High chemical stability: pH 1-14**
- **High termal stability: up to 200°C**



Schematic illustration of interaction of the analyte with the surface of PGC stationary phase. (a) favorable steric arrangement of the analyte providing a strong interaction with the surface of the stationary phase, (b) unfavorable steric arrangement of the analyte providing a weak interaction with the surface of the stationary phase.

New trends in chromatography

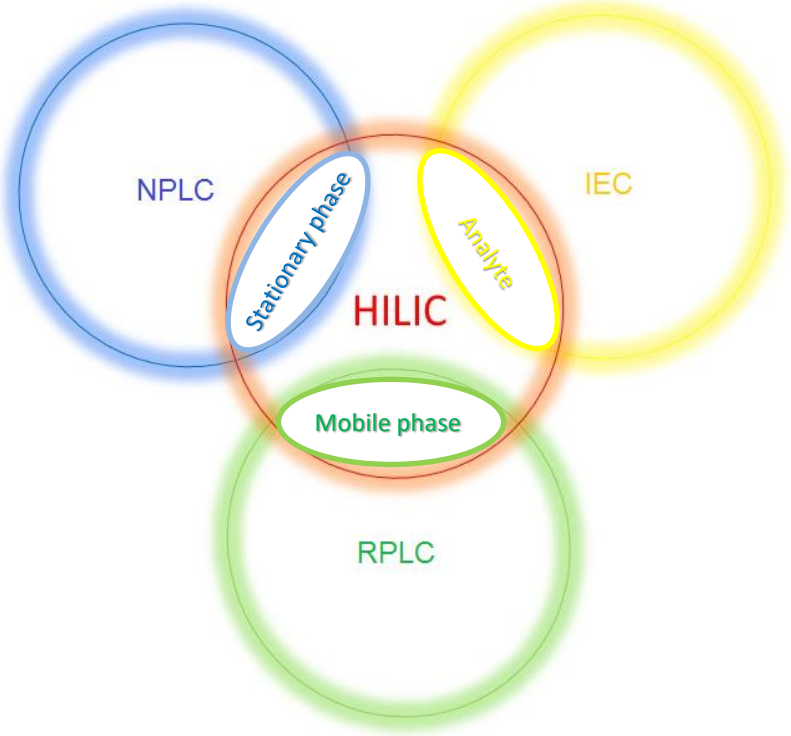
novel stationary phases/modes



HILIC principle

Other sorbents/modes: hydrophilic interaction liquid chromatography (HILIC)

- **Favorable solution for analysis of polar analytes that have very strong affinity to the stationary phase in NPLC (cannot elute from the column) on one side and have very weak or no affinity to the stationary phase in RPLC (elute in void time) on the other side**
- **Organic volatile mobile phases are favorable for coupling HILIC to MS detection**

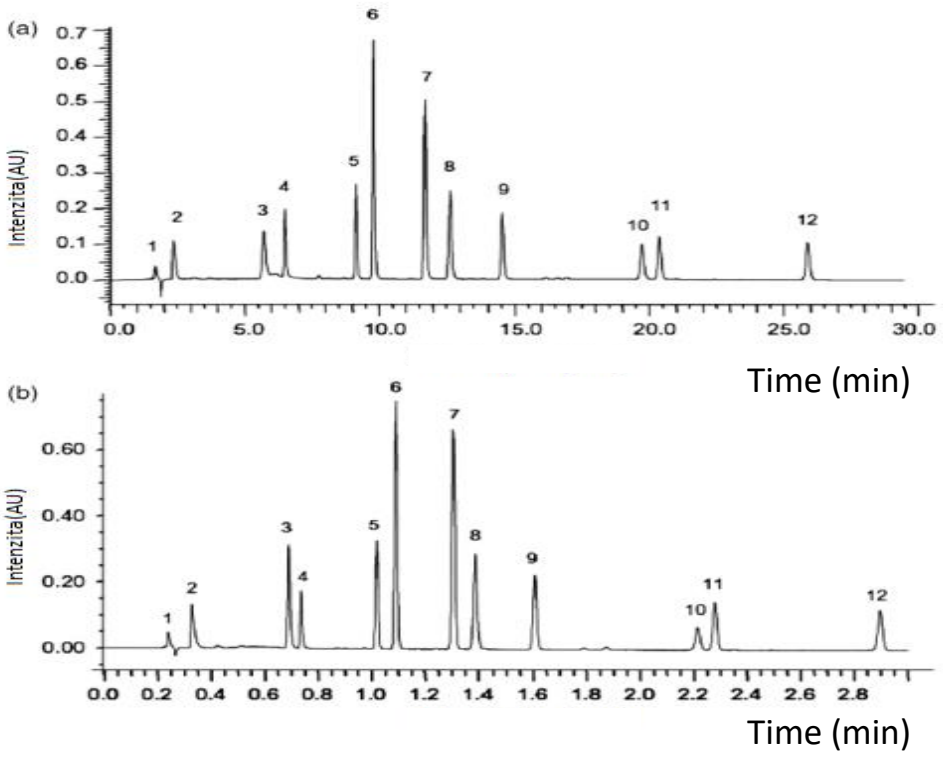


HILIC is a variant of normal phase liquid chromatography that partly overlaps with other chromatographic applications such as ion chromatography and reversed phase liquid chromatography. HILIC uses hydrophilic stationary phases with reversed-phase type eluents. The chromatographic mechanism for it is as liquid-liquid partition chromatography where analytes elute in order of increasing polarity.

New trends in chromatography

innovative chromatographic techniques

- *Ultra-high-performance liquid chromatography (UHPLC): for fast separations*



HPLC

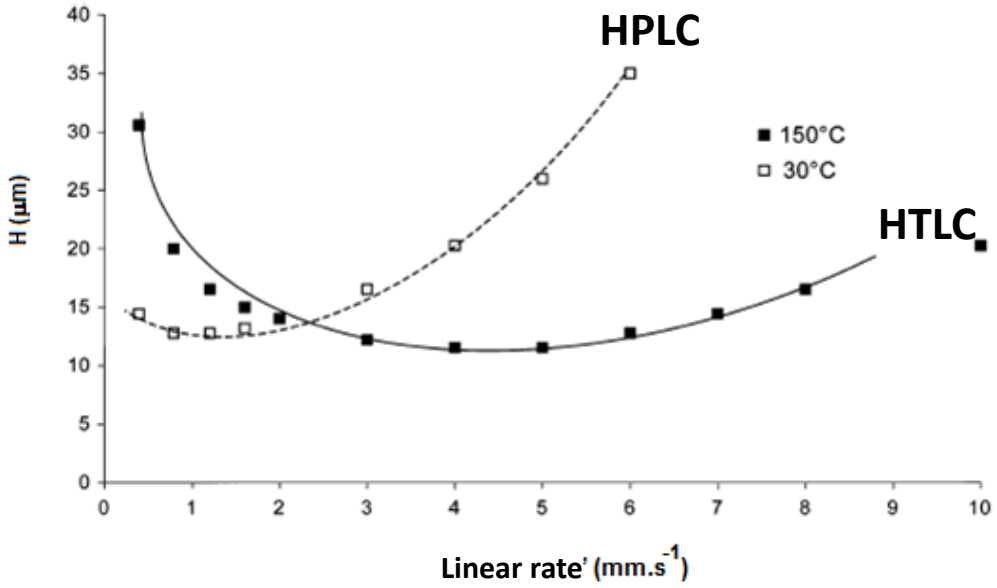
UHPLC

Comparison of UHPLC and HPLC separation. Chromatografic profiles from the analysis of a dosage form. Main active drug (6) and impurities.

New trends in chromatography

innovative chromatographic techniques

- *High-temperature liquid chromatography (UTLC): for shortening analysis time, increasing column efficiency, increasing intensity od interaction, i.e. selectivity, and lower consumption of organic solvents (possible thanks to proper instrumental modifications and more temperature stable stationary phases)*

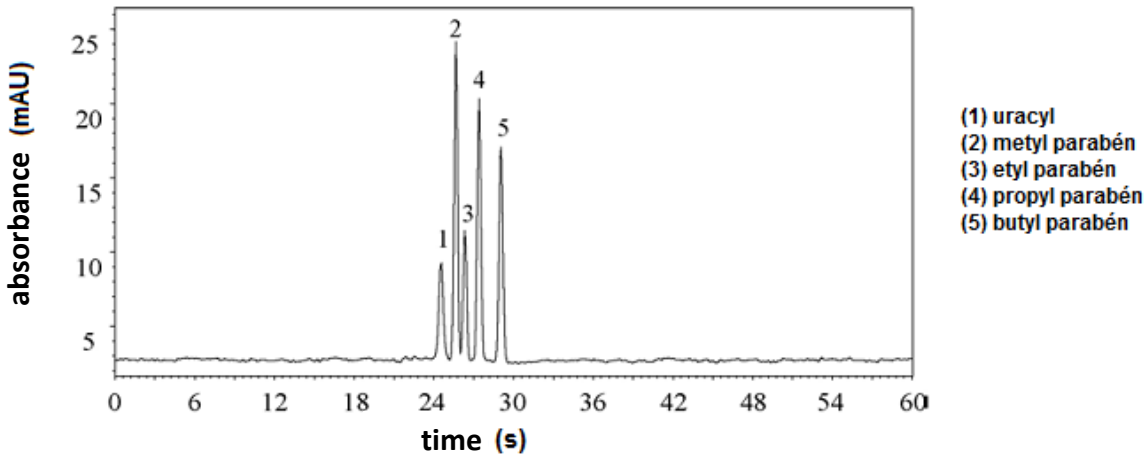


Comparison of HTLC and HPLC separation: effect of temperature to H (height equivalent to a theoretical plate) for given linear rate

New trends in chromatography

innovative chromatographic techniques

- *UHTLC: benefits of UHPLC and HTLC*

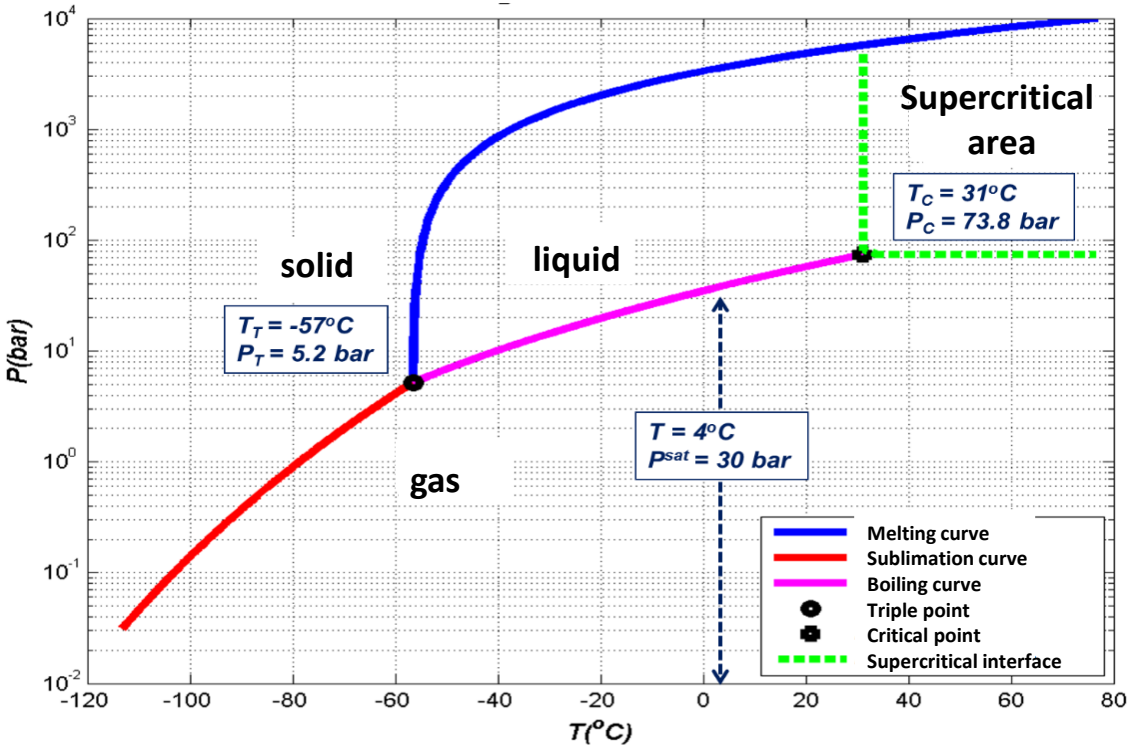


UHTLC: temperature 80°C, pressure 2068 bar, capillary column 15 cm x 50 µm, 1 µm particles Kovasil MS-H, mobile phase: water(20 mM NH_4Ac, pH 3,5)/acetonitril (60/40, v/v).

New trends in chromatography

innovative chromatographic techniques

- *Supercritical fluid chromatography (SFC): lower viscosity of mobile phase - higher flow rate – faster analyses, increased column efficiency, increased intensity od interaction, i.e. selectivity, useful for temperature labile compounds, useful for detection techniques requiring volatile mobile phases such as MS (possible thanks to proper instrumental modifications and use of mobile phase under supercritical conditions)*



Phase diagram of CO_2

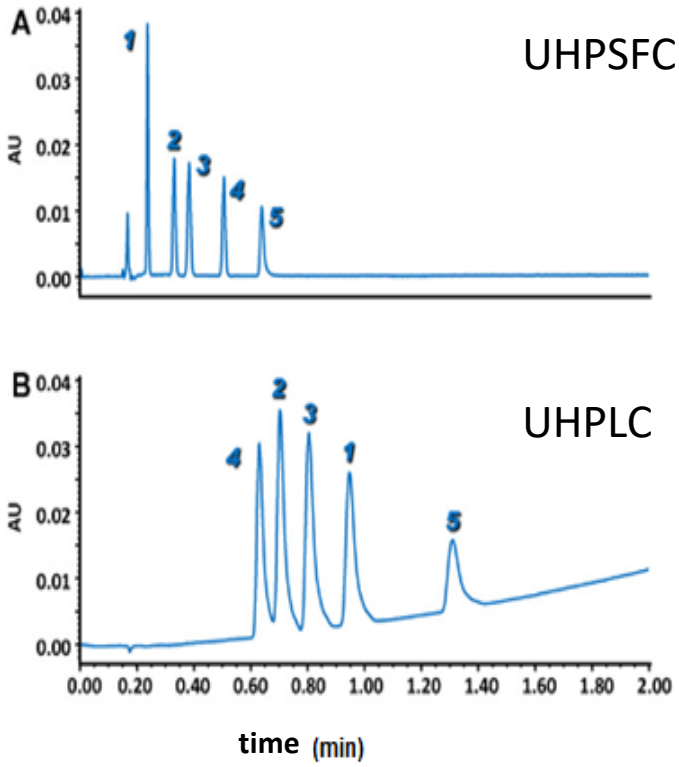
Mobile phases in SFC:

CO_2
N_2O
NH_3
SO_2
halogen carbohydrates

New trends in chromatography

innovative chromatographic techniques

- UHPSFC: *benefits of UHPLC and SFC*



UHPSFC

UHPLC

1. 17-metyltestosterón
2. 4-androzen-3,17-dión
3. testosterón
4. boldenón
5. stanozolol

(A) Podmienky SFC:
Acquity UPC^2 BEH 100 mm x 3.0 mm, 1.7 µm,
40 °C, 145 bar, dávkovaný objem: 0.5µm, 220nm,
gradient 10-12 % MeOH v CO_2, 3,25ml/min za 1 min

(B) Podmienky LC:
Acquity BEH RP18 50 mm x 2,1mm, 1,7 µm,
40 °C, dávkovaný objem: 0.5µm, 220nm,
gradient 55-70% MeOH v H_2O, 0,7ml/min za 1,5min,
max. tlak 932 bar

Comparison of UHPSFC and UHPLC separation. Separation of steroids using UHPSFC (A) a UHPLC (B) methods.

New trends in chromatography

miniaturization

- Micro-LC, LC on chip*: faster analysis, higher efficiency and sensitivity (due to a lower dilution of the sample with the mobile phase), lower consumption of organic solvents and samples (thanks to miniaturization of a separation column size)*

New trends in chromatography

miniaturization

- Micro-LC

Table: Comparison of parameters of miniaturized and conventional LC techniques

Type of LC	Internal diameter of the column	Flow rate of the mobile phase
conventional	< 10 mm; > 1 mm	> 1 ml.min^{-1}
micro	1 mm ~ 500 µm	100 ~ 500 µl.min^{-1}
capillary	500 ~ 100 µm	1 ~ 25 µl.min^{-1}
nano	< 100 µm	< 1 µl.min^{-1}



Nano, capillary and micro UHPLC columns

New trends in chromatography

miniaturization

- LC on chip

best in miniaturization and automatization

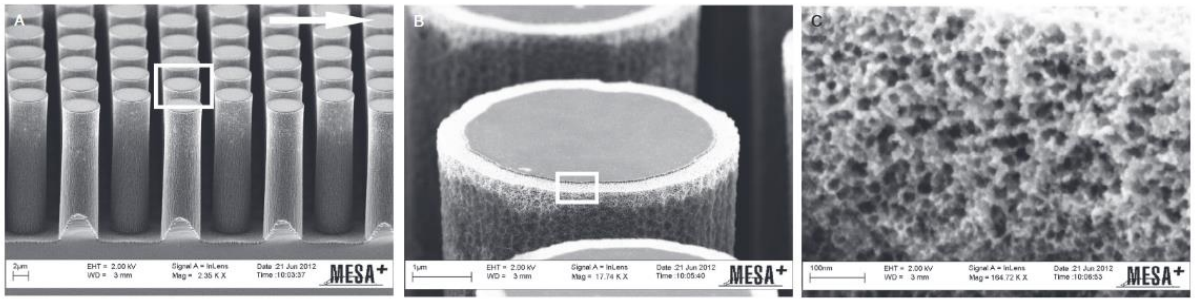


Photo of „pillar arrays“ columns (PAC) for LC on chip. A high reproducibility in arrangement of pillar arrays results in a higher reproducibility of data and higher separation efficiency (due to elimination of the effect of multiple paths on a solute’s band broadening) obtainable in comparison to package or monolithic columns.

New trends in chromatography

two-dimensional LC systems

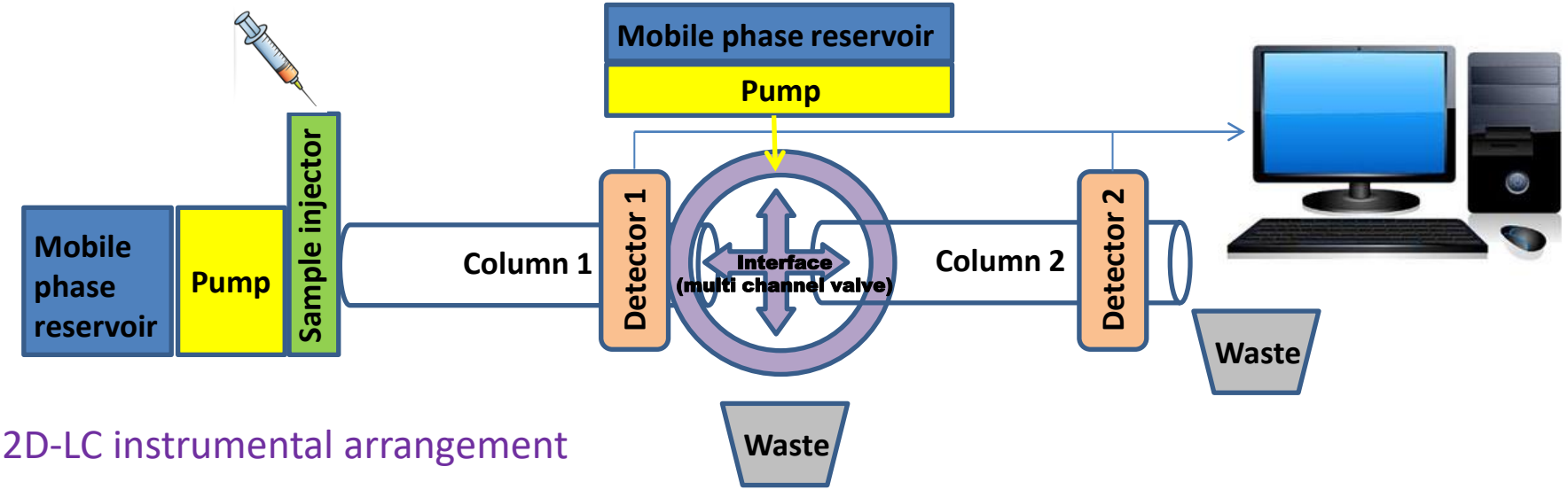
2D-LC (two-dimensional liquid chromatography):

- LCxLC (comprehensive two-dimensional liquid chromatography)
- LC-LC (fraction or „heart-cut“ two-dimensional liquid chromatography)
- mLC-LC (multiple „heart-cut“ two-dimensional liquid chromatography)

2D-LC enables:

to enhance separation capacity and selectivity – prerequisite to achieve sufficient resolution of analytes present in multicomponent matrices, e.g.:

- Metabolites in biological samples, metabolic profiling (**metabolomics**)
- Proteins in biological samples, identification of protein structure (**proteomics**)
- DNA or RNA in biological samples, identification of polynucleotide structure (**genomics**)

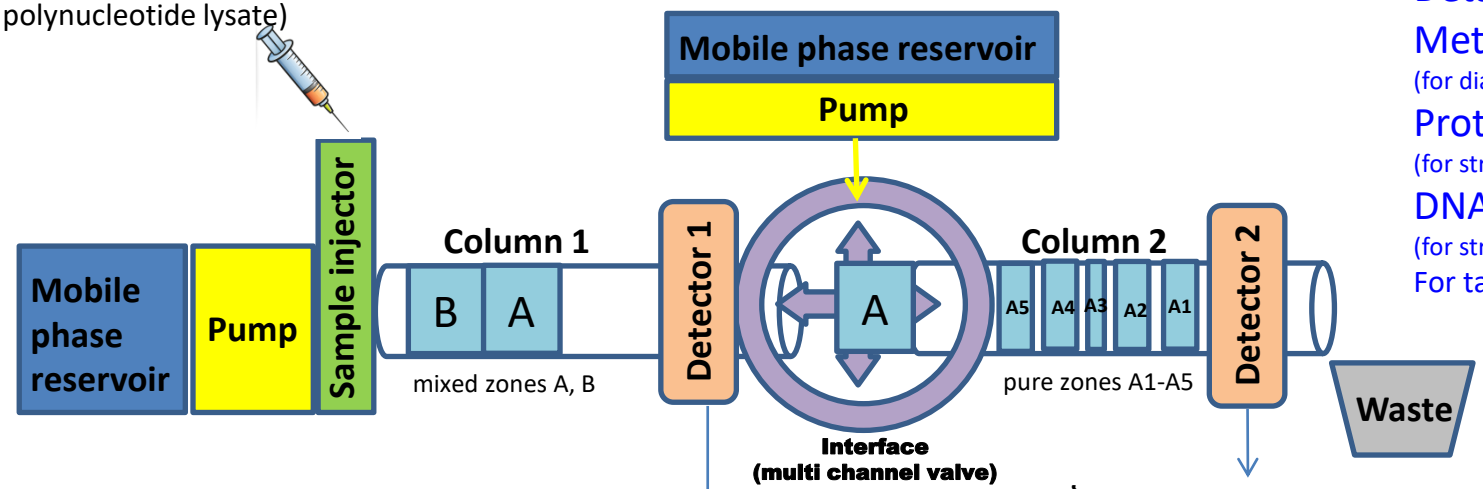


2D-LC instrumental arrangement

New trends in chromatography

two-dimensional LC systems - LCxLC

Sample mixture (biological sample, protein lysate, polynucleotide lysate)



Practical use

Detail profiling of:

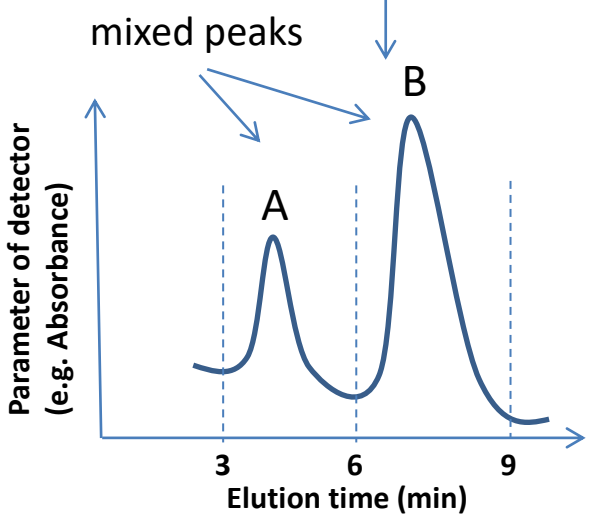
Metabolites
(for diagnostics)

Protein fragments
(for structure identification)

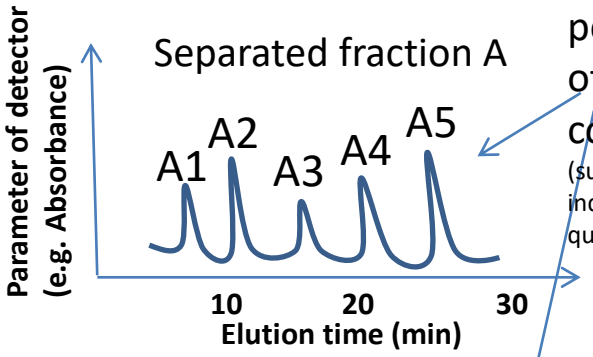
DNA/RNA fragments
(for structure identification)

For target and untarget analysis

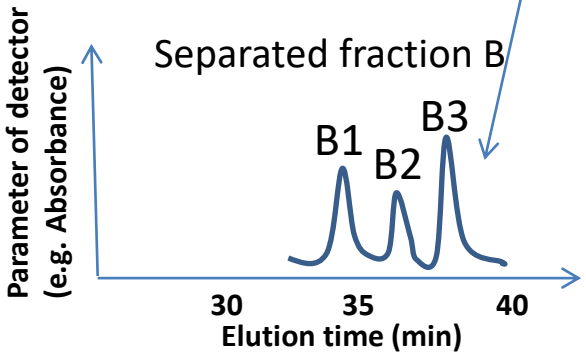
Sample fractions A, B defined in the 1st column are separated to their individual constituents (A1-A5, B1-B3) under different separating conditions in the 2nd column



LCxLC: All sample fractions are analyzed



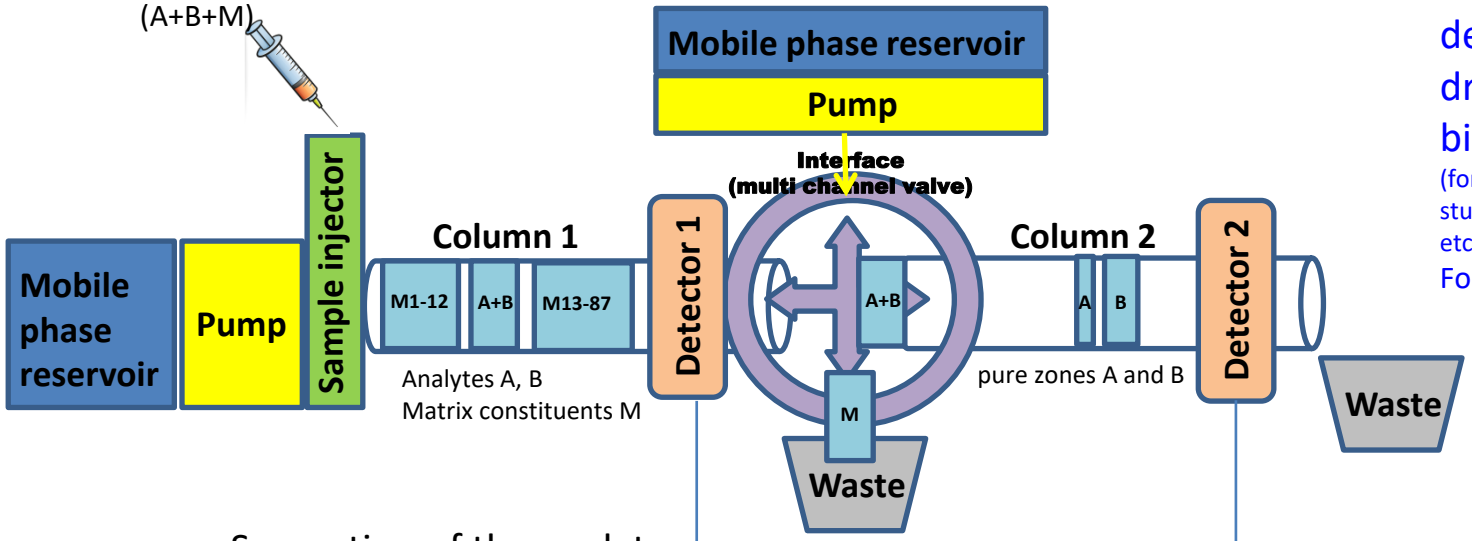
peaks of pure compounds
(suitable for indentification and quantification)



New trends in chromatography

two-dimensional LC systems – **(m)LC-LC**

Sample mixture containing significant amount of matrix (A+B+M)



Practical use

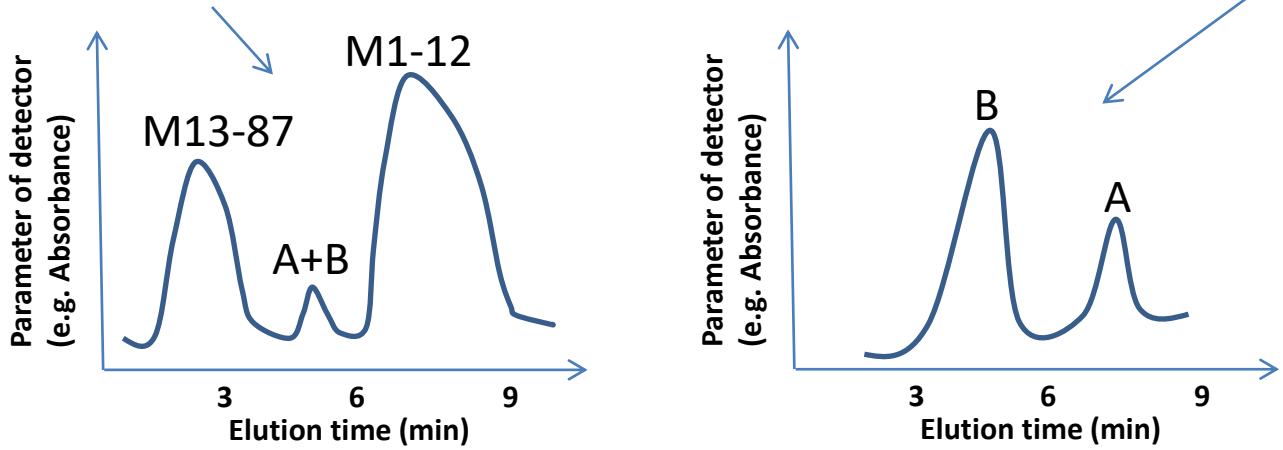
Sensitive determination of: drugs, metabolites, biomarkers

(for diagnostics, pharmacokinetic studies, biodistribution studies, etc.)

For target analysis

Separation of the analytes from the sample matrix

peaks of pure compounds (suitable for indentification and quantification)



(m)LC-LC: selected sample fraction(s) is(are) analyzed

Sample fraction containing analytes (A+B) separated from the sample matrix constituents (M1-87) in the 1st column is cut, transferred and separated to its individual constituents (A and B) under different separating conditions in the 2nd column; M is not entering the 2nd column

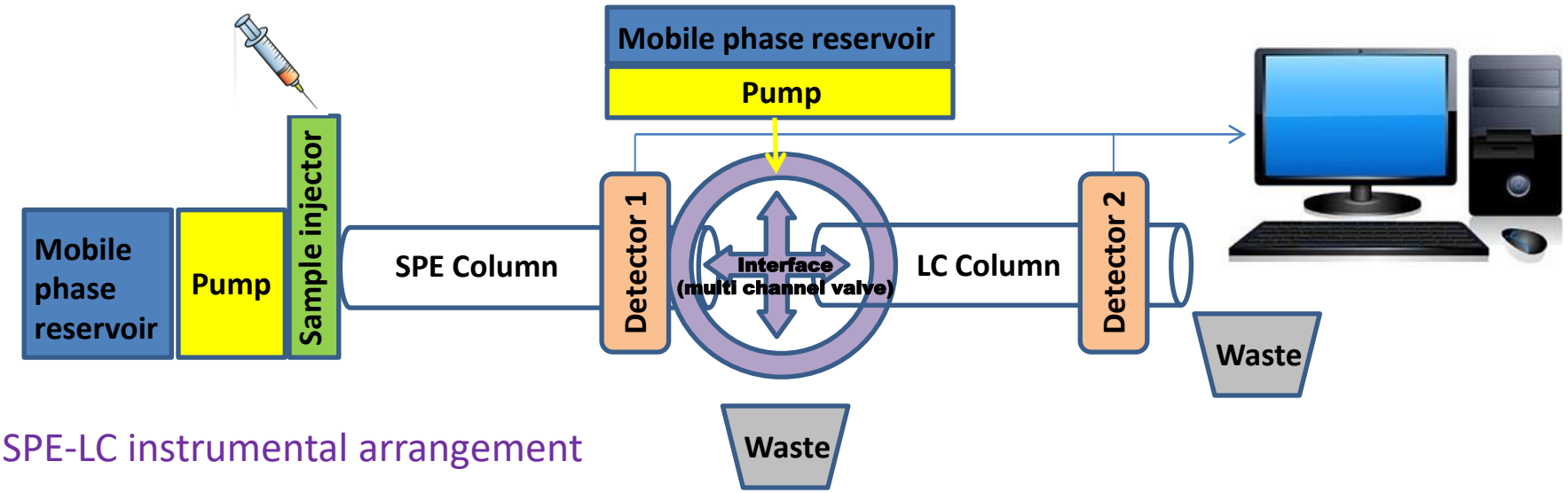
Notice: LC-LC = one analyte fraction = one cut; mLC-LC = more than one analyte fraction = more than one cut

New trends in chromatography

two-dimensional SPE-LC systems

SPE-LC enables:
to on-line purify and preconcentrate the sample – prerequisite to achieve sufficient resolution of analytes present in multicomponent matrices and to achieve higher intensity of the analytical signal (higher sensitivity, lower LOD and LOQ concentration levels), e.g.:

- Target analyte(s) (drug, metabolite, biomarker) in various multicomponent matrices (biological material – body fluids, tisues, dosage forms - pharmaceuticals, environmental material – water, soil, etc.)



SPE-LC instrumental arrangement

New trends in chromatography

two-dimensional SPE-LC systems

Practical use

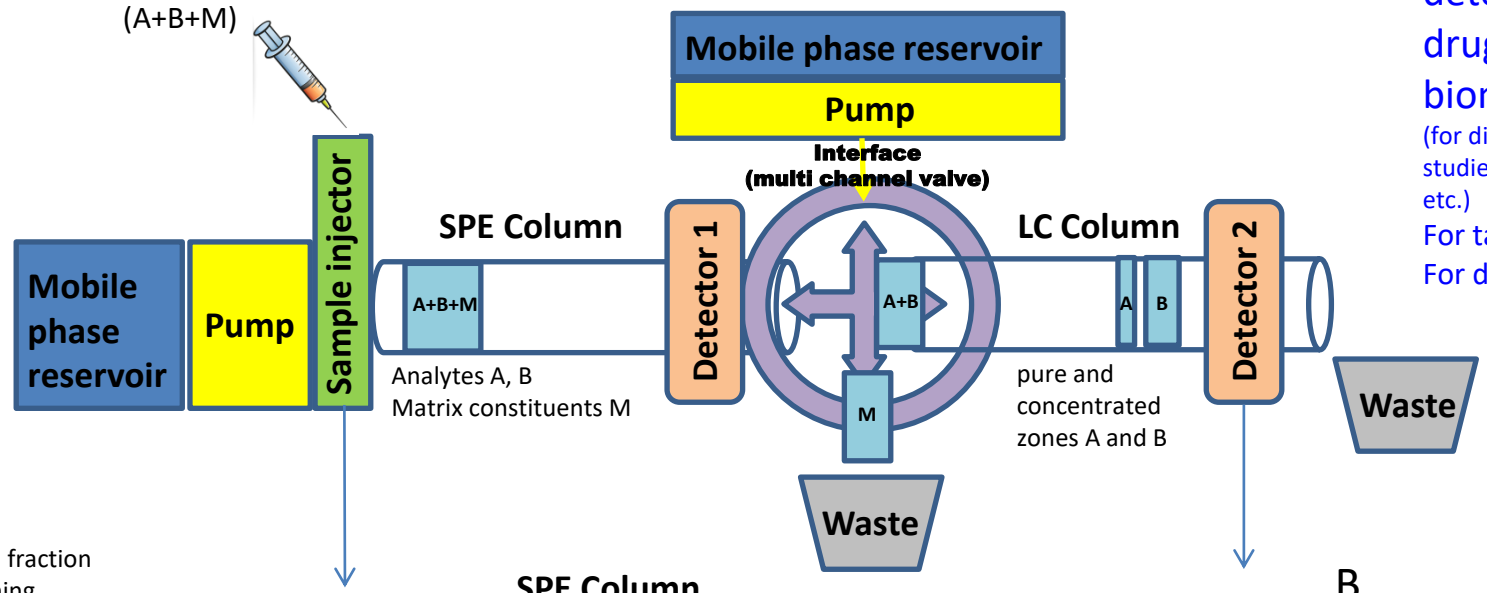
Sensitive determination of: drugs, metabolites, biomarkers

(for diagnostics, pharmacokinetic studies, biodistribution studies, etc.)

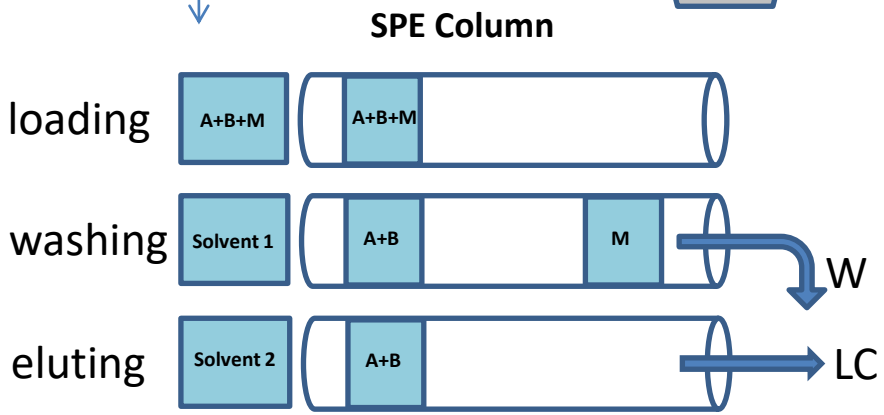
For target analysis

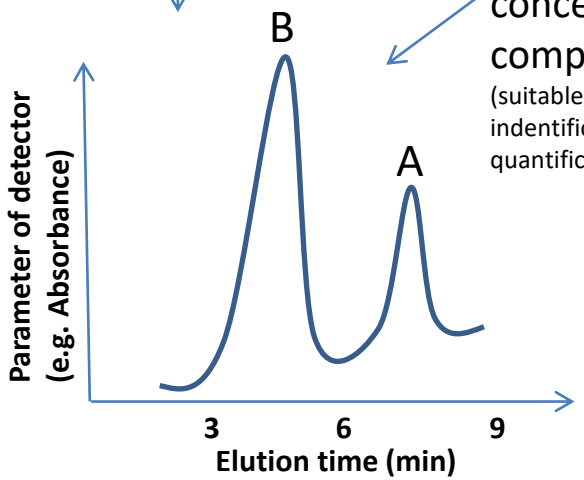
For diluted samples

Sample mixture containing significant amount of matrix and diluted analytes (A+B+M)



Sample fraction containing analytes (A+B) is cleaned/izolated from the sample matrix constituents (M) in the SPE column, and transferred as a concentrated plug into the LC column for the separation to its individual constituents (A and B); M is not entering the LC column





peaks of pure and concentrated compounds (suitable for indentification and quantification)

SPE-LC: selected sample fraction is concentrated and analyzed

Electrophoresis

Recommended literature:

Mikuš, Peter; Maráková, Katarína:
HYPHENATED ELECTROPHORETIC TECHNIQUES IN ADVANCED ANALYSIS, KARTPRINT, Bratislava, 2012