

*NEPHAR 315*

*Pharmaceutical Chemistry  
Lab II*

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- *High Performance Liquid Chromatography (HPLC)*
- *Gas Chromatography (GC)*
- *Capillary Electrophoresis (CE)*





*Be determined in achieving your goals...*



# *High Performance Liquid Chromatography*

## *(HPLC)*



# HPLC

- HPLC is now *one of the most powerful tools in analytical chemistry*.
- It has the ability to **separate, identify, and quantitate** the compounds that are present in any sample that can be dissolved in a **liquid**.
- Today, compounds in trace concentrations as low as *parts per trillion* **[ppt]** may easily be identified.



**HPLC** can be applied to just about any sample, such as;

- *pharmaceuticals,*
- *food,*
- *nutraceuticals,*
- *cosmetics,*
- *environmental matrices,*
- *forensic samples,*
- *industrial chemicals.*



HPLC,  
provides analytical data that can be used;

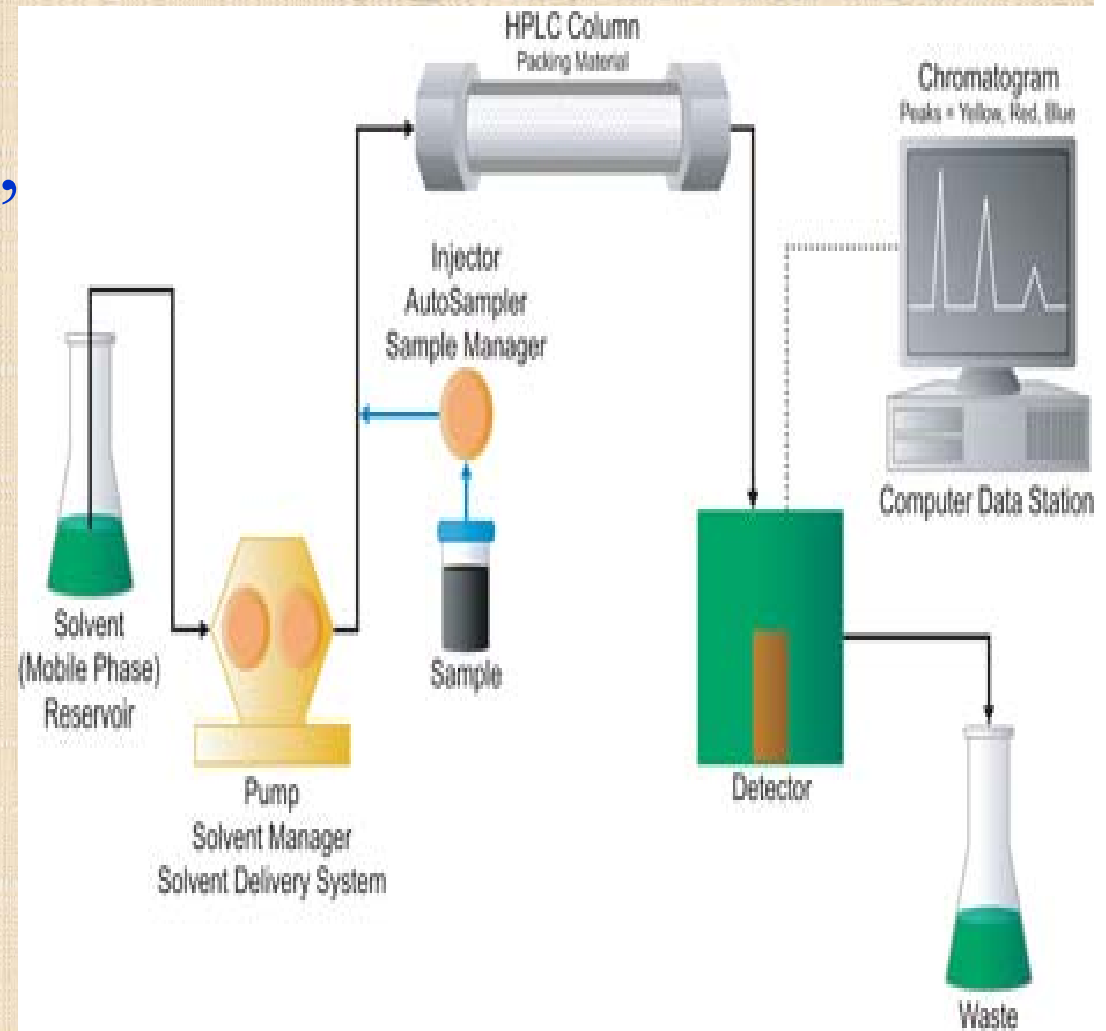
- to identify
- to quantify
- to separate

compounds present in a sample.



The basic components of an *HPLC* system include;

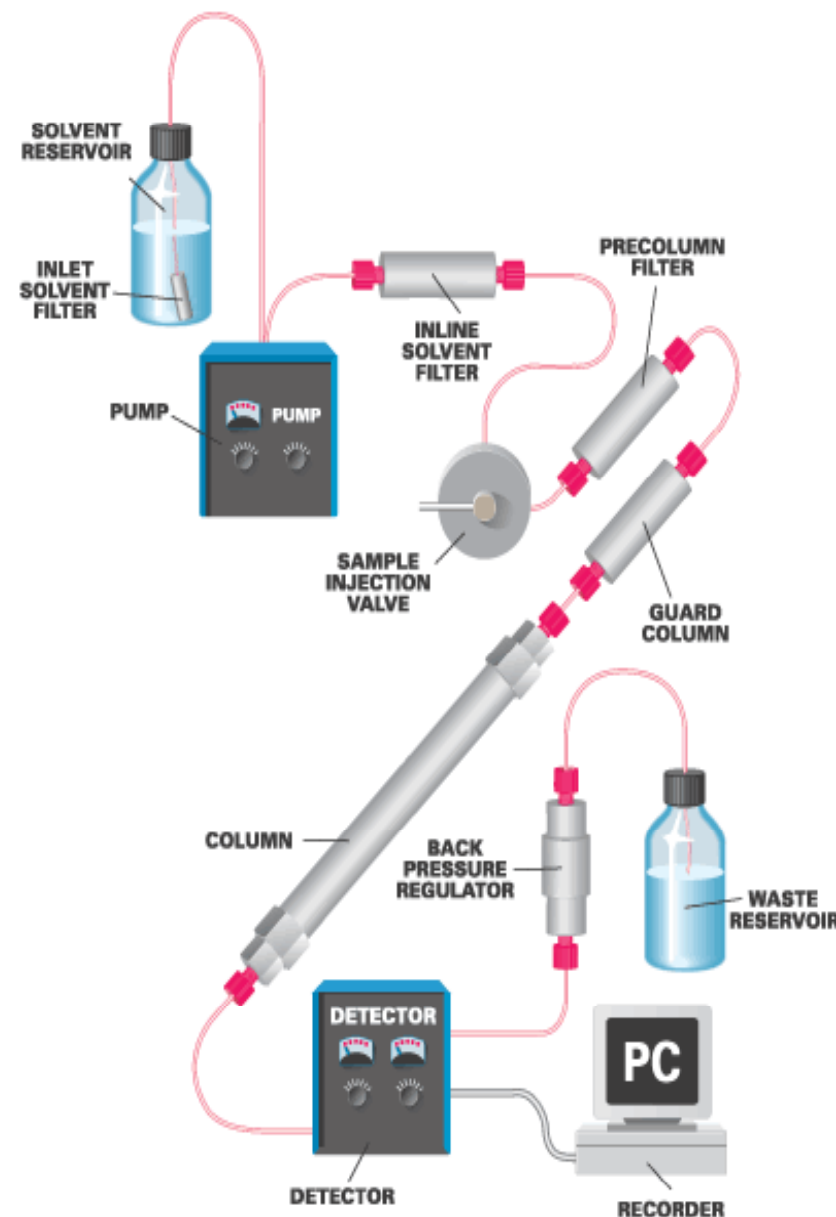
- a solvent reservoir,
- pump,
- injector,
- analytical column,
- detector,
- recorder,
- waste reservoir.





## *Other important elements are;*

- an inlet solvent filter,
- post-pump inline filter,
- sample filter,
- precolumn filter,
- guard column,
- back-pressure regulator
- solvent sparging system





## *HPLC system*

- A reservoir holds the solvent [mobile phase].
- A high-pressure pump [solvent delivery system or solvent manager] is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute.
- An injector [sample manager or autosampler] is able to introduce [inject] the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column.



- The column contains the chromatographic packing material needed to effect the separation. This packing material is called *the stationary phase* because it is held in place by the column hardware.

A guard column is often included just prior to the analytical column to *chemically remove components of the sample that would otherwise foul the main column.*



- A **detector** is needed to *see* the separated compound bands as they elute from the HPLC column. *The mobile phase* exits the detector and can be sent to waste, or collected, as desired.

*The detector* is wired to the computer data station, the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to **identify** and **quantitate** the concentration of the sample constituents.



Since sample compound characteristics can be very different, several types of detectors have been developed.

- UV-absorbance detector  
(DAD-diode array detector )
- fluorescence detector
- evaporative-light-scattering detector [ELSD].

(If the compound does not have either of these characteristics, a more universal type of detector is used)



- The most powerful approach is the use of multiple detectors in series.

*For example*, a **UV** and/or **ELSD detector** may be used in combination with a mass spectrometer **[MS]** to analyze the results of the chromatographic separation.

- This provides, from a **single injection**, **more comprehensive information** about an analyte. The practice of coupling a mass spectrometer to an HPLC system is called **LC/MS**.

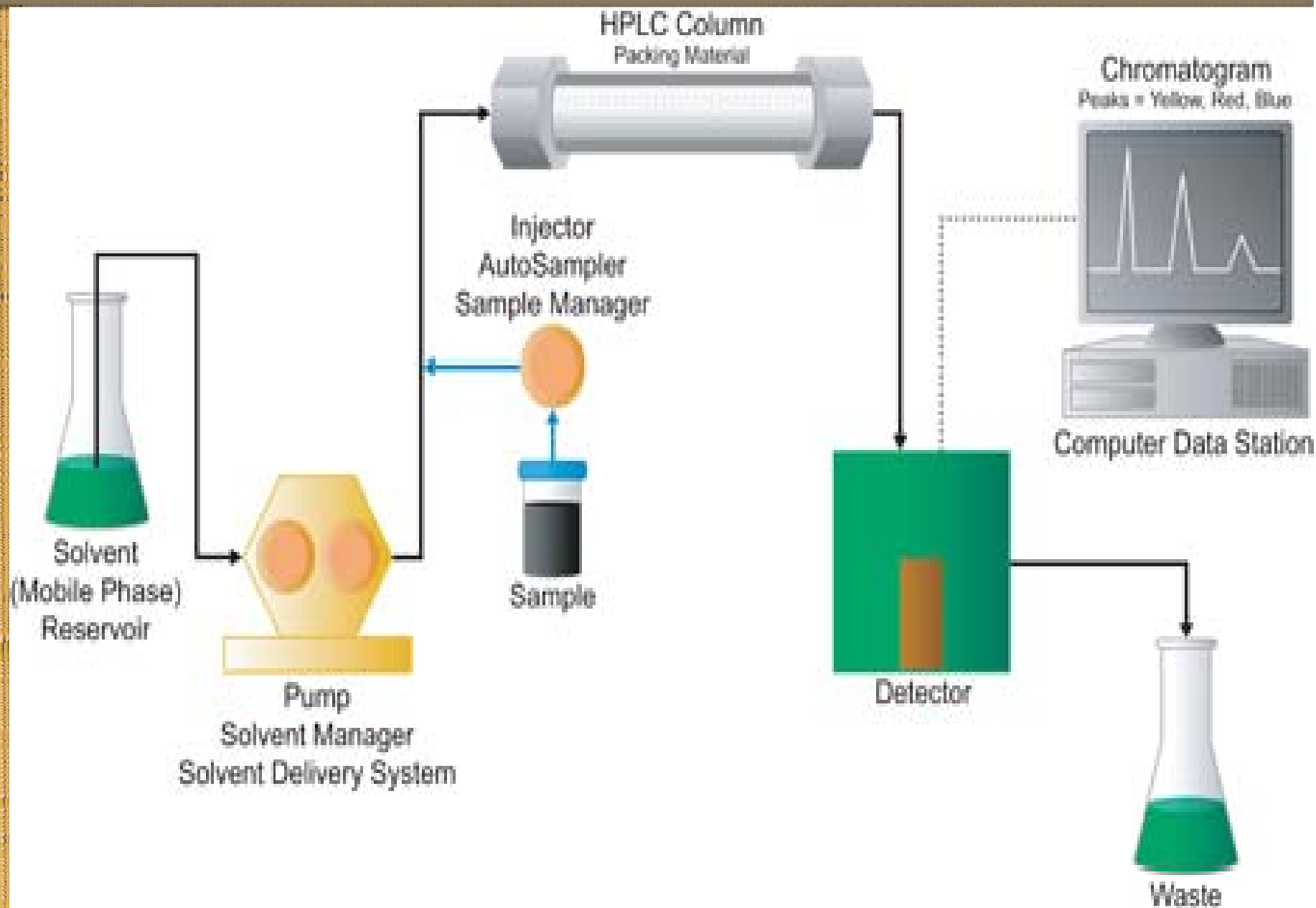


# *Isocratic and Gradient LC System Operation*

- Two basic elution modes are used in HPLC.
- The first is called *isocratic elution*. In this mode, the mobile phase, either a pure solvent or a mixture, *remains the same throughout the run*.
- The second type is called *gradient elution*, wherein, as its name implies, *the mobile phase composition changes during the separation*.



# *Isocratic LC System*





## *Gradient Elution*

This mode is useful for samples that contain compounds that span a **wide range** of chromatographic polarity.

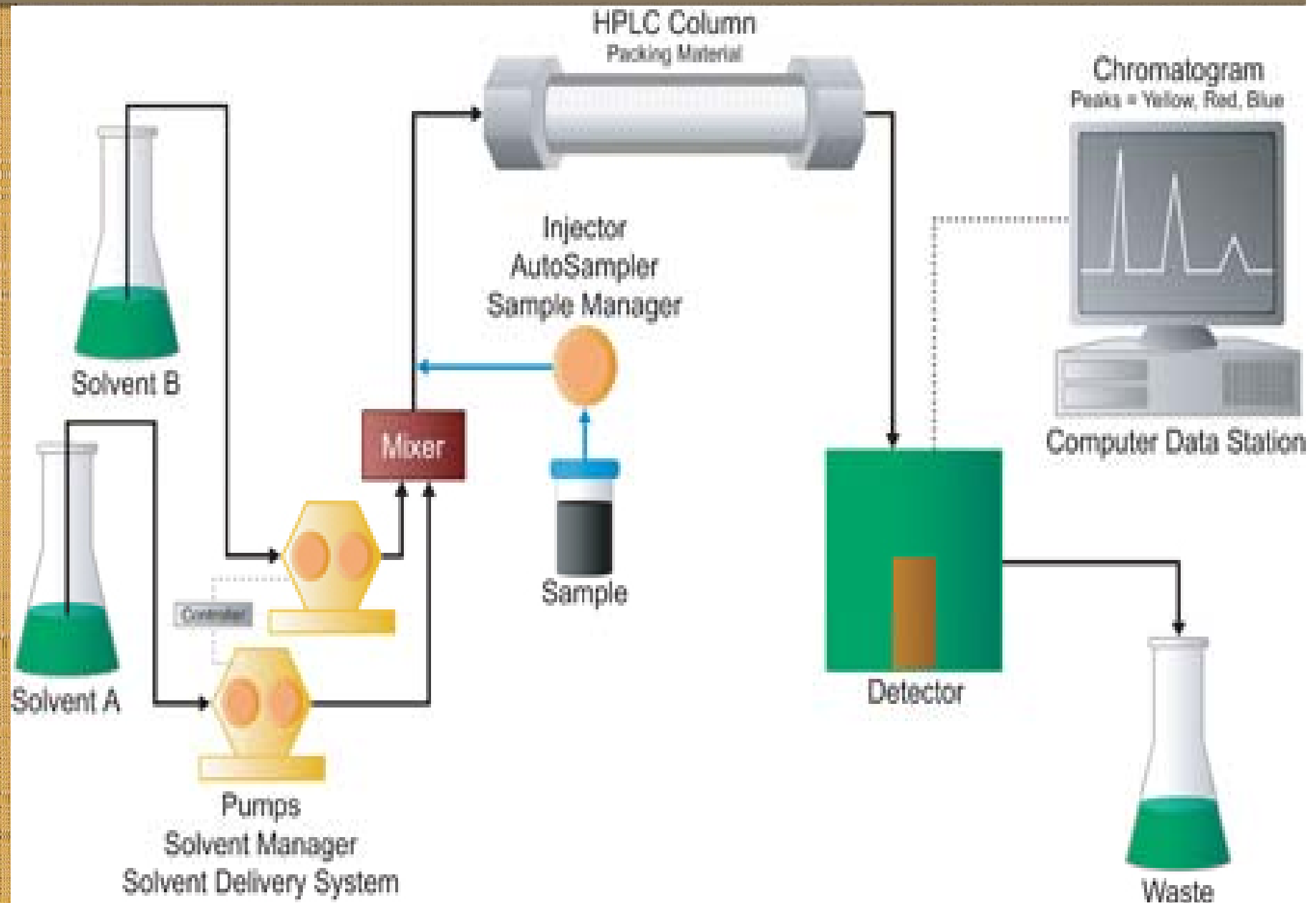
As the separation proceeds, the elution strength of the mobile phase is **increased** to elute the more strongly retained sample components.

*High-Pressure-Gradient System*

*Low-Pressure-Gradient System*

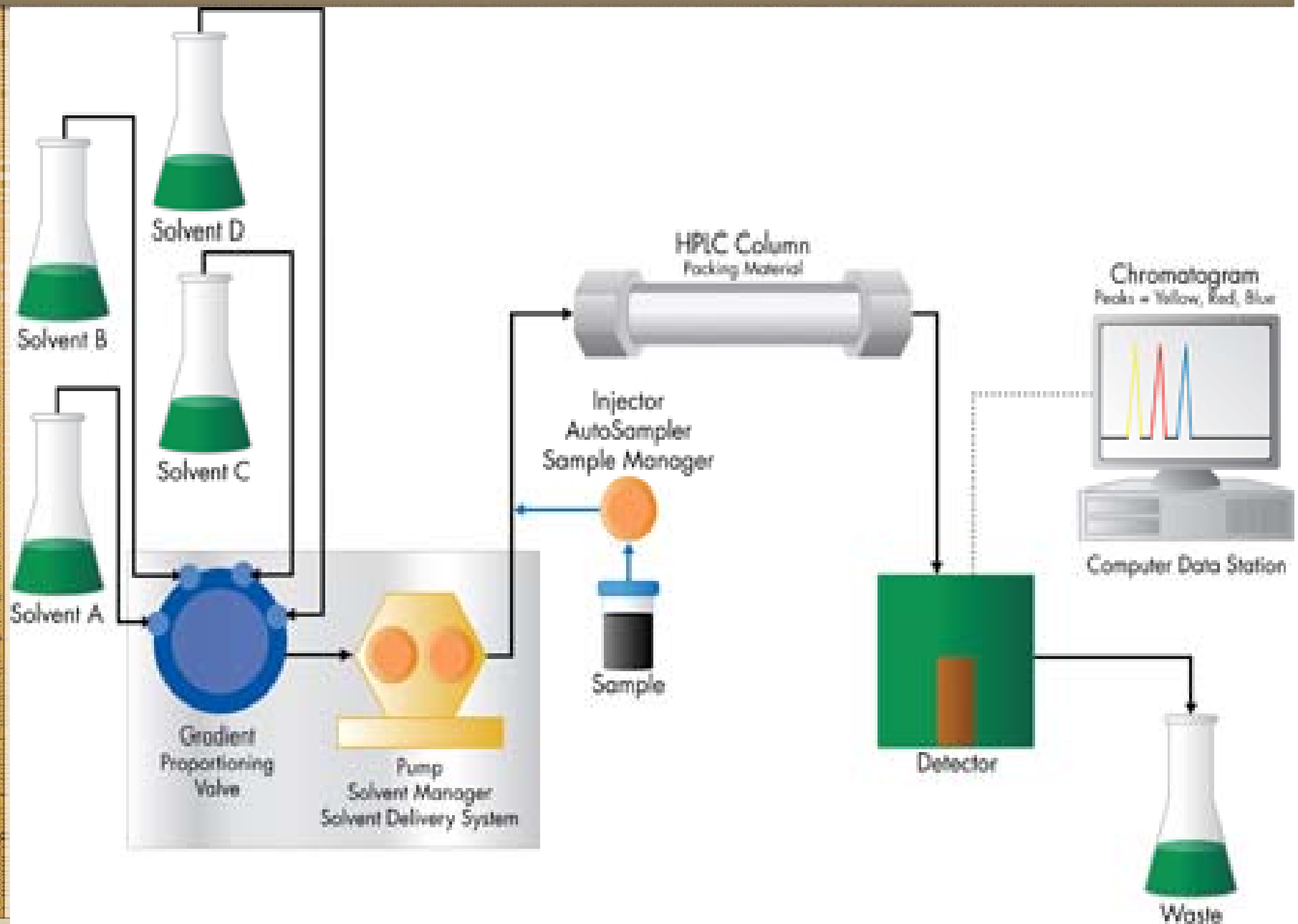


# *High-Pressure-Gradient System*





# *Low-Pressure-Gradient System*





## HPLC,

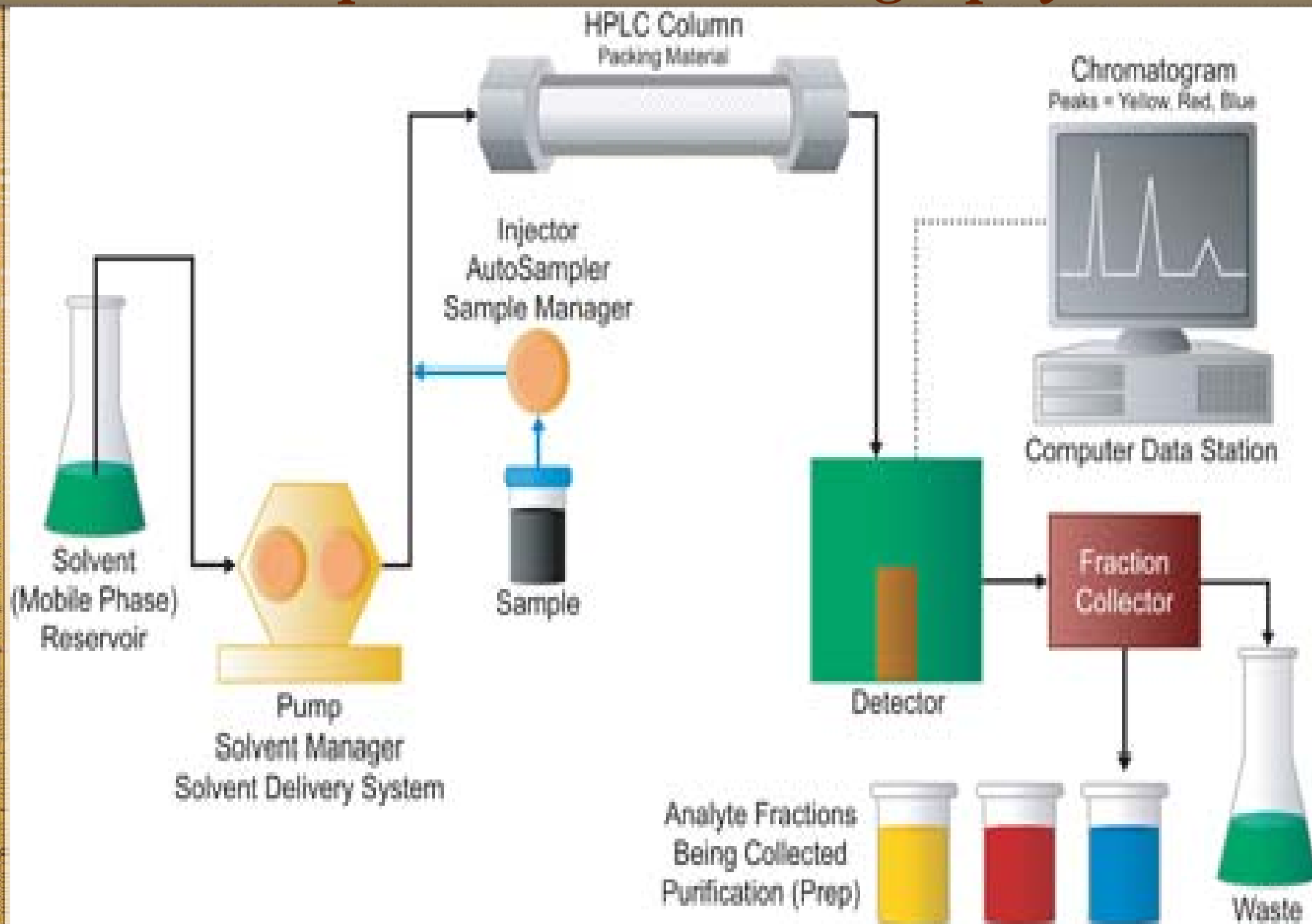
- can also be used to **purify** and
- **collect** desired amounts of each compound, using a **fraction collector** downstream of the detector flow cell.

This is called ***preparative chromatography***.

In ***preparative chromatography***, the scientist is able to **collect** the ***individual analytes*** as they elute from the column [e.g., in this example: **yellow**, then **red**, then **blue**].



# *HPLC System for Purification: Preparative Chromatography*

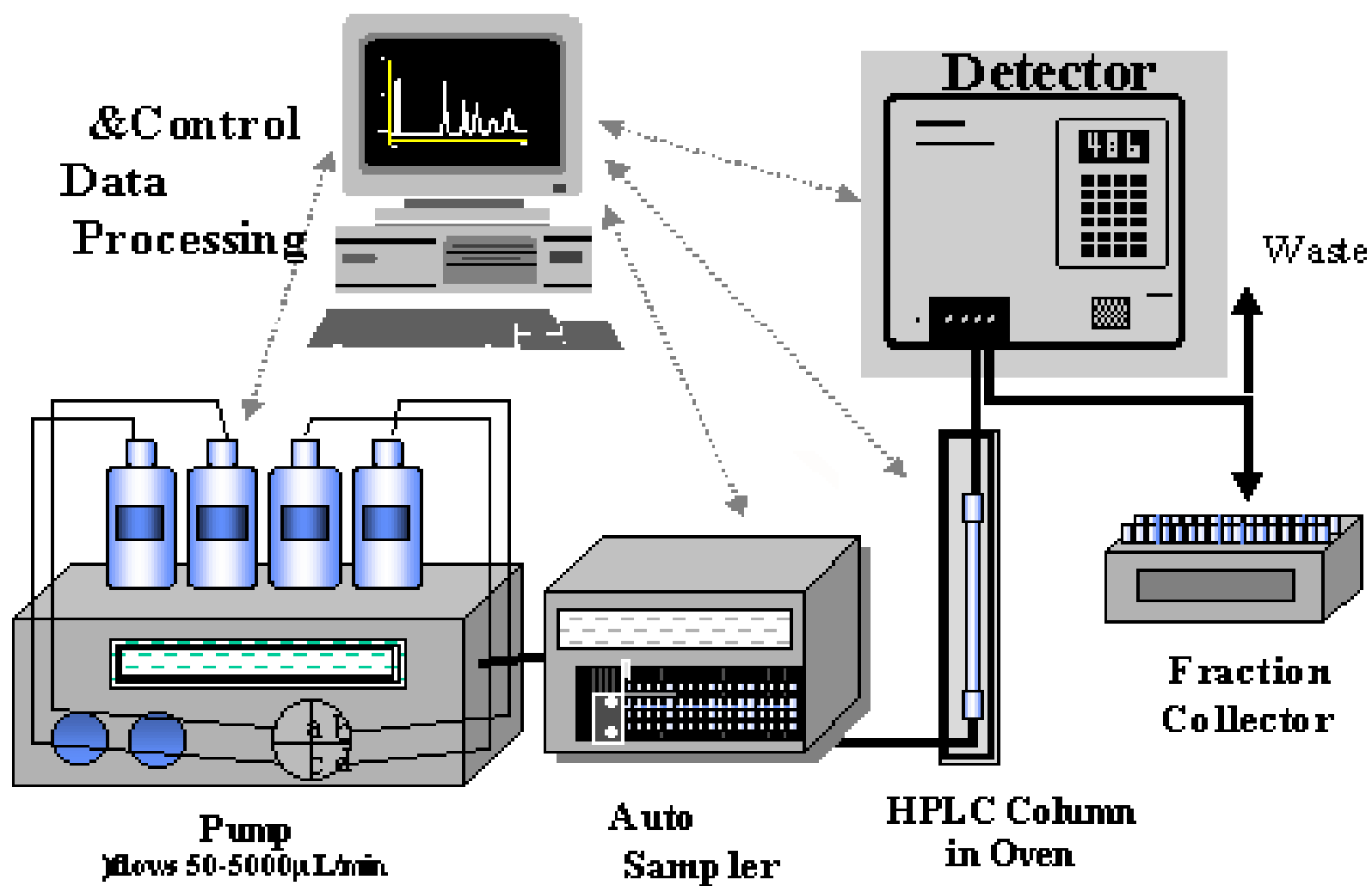




The fraction collector selectively collects the eluate that now contains a purified analyte, for a specified length of time.

The vessels are moved so that each collects only a single analyte peak. In general, as the sample size increases, the size of the HPLC column will become larger and the pump will need higher volume-flow-rate capacity.







## *HPLC columns*

- In general, **HPLC columns** range from 20 mm to 500 mm in length [L] and 1 mm to 100 mm in internal diameter [i.d.].
- As the scale of chromatography increases, so do column dimensions, especially the cross-sectional area. To optimize throughput, mobile phase flow rates must increase in proportion to cross-sectional area.
- If a *smaller particle size* is desirable for more separation power, pumps must then be designed to sustain **higher mobile-phase-volume flow rates** at **high backpressure**.



- **Table** presents some simple guidelines on selecting the **column i.d.** and **particle size range** recommended for each scale of chromatography.

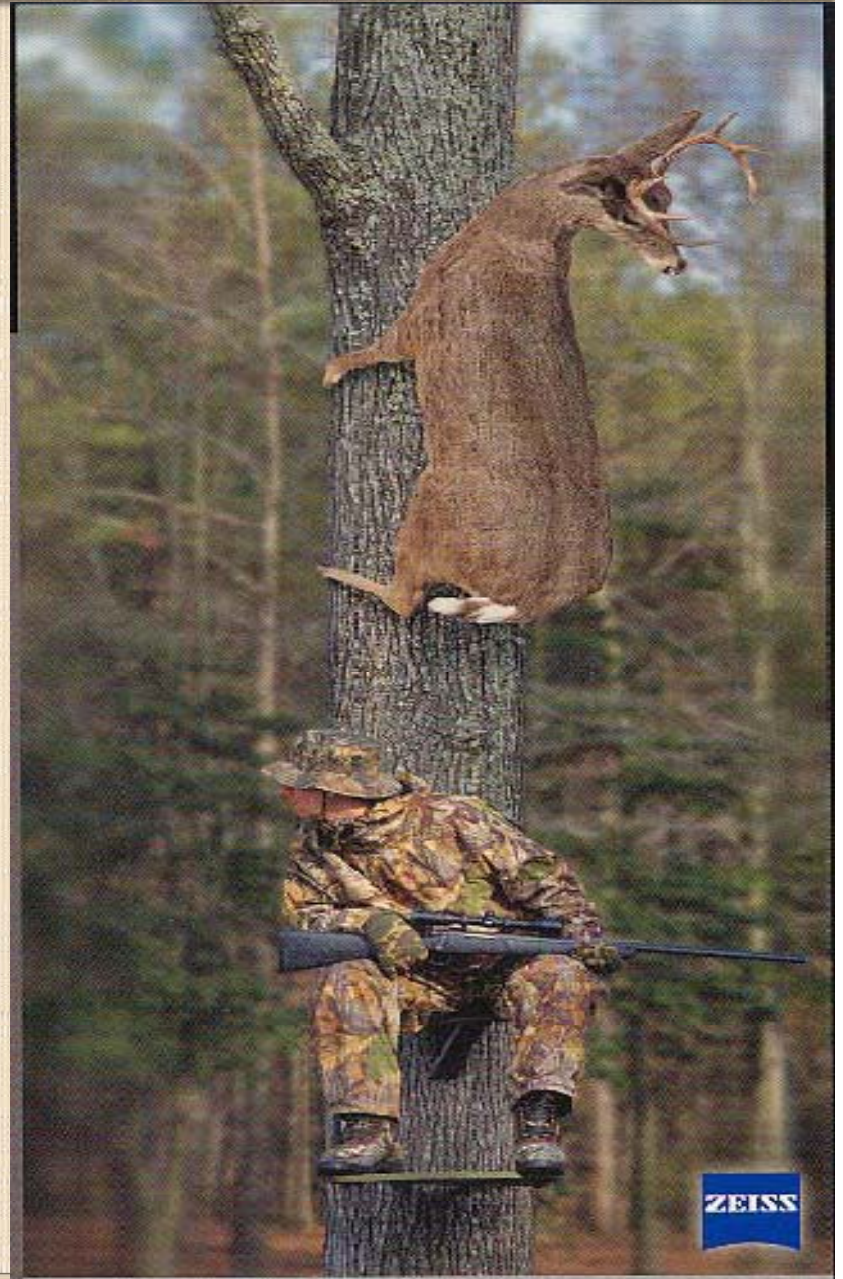
Scale	1- 8mm Column Diameter	10- 40mm Column Diameter	50-100mm Column Diameter	> 100mm Column Diameter	Particle Size micron
Analytical	X				1.7 -10
Semi-Prep		X			5 - 15
Prep			X		15 -100
Process				X	100+

For example, a semi-preparative-scale application [red X] would use a column with an internal **diameter** of 10–40 mm containing 5–15 **micron particles**.



*Always be alert and  
then wait.*

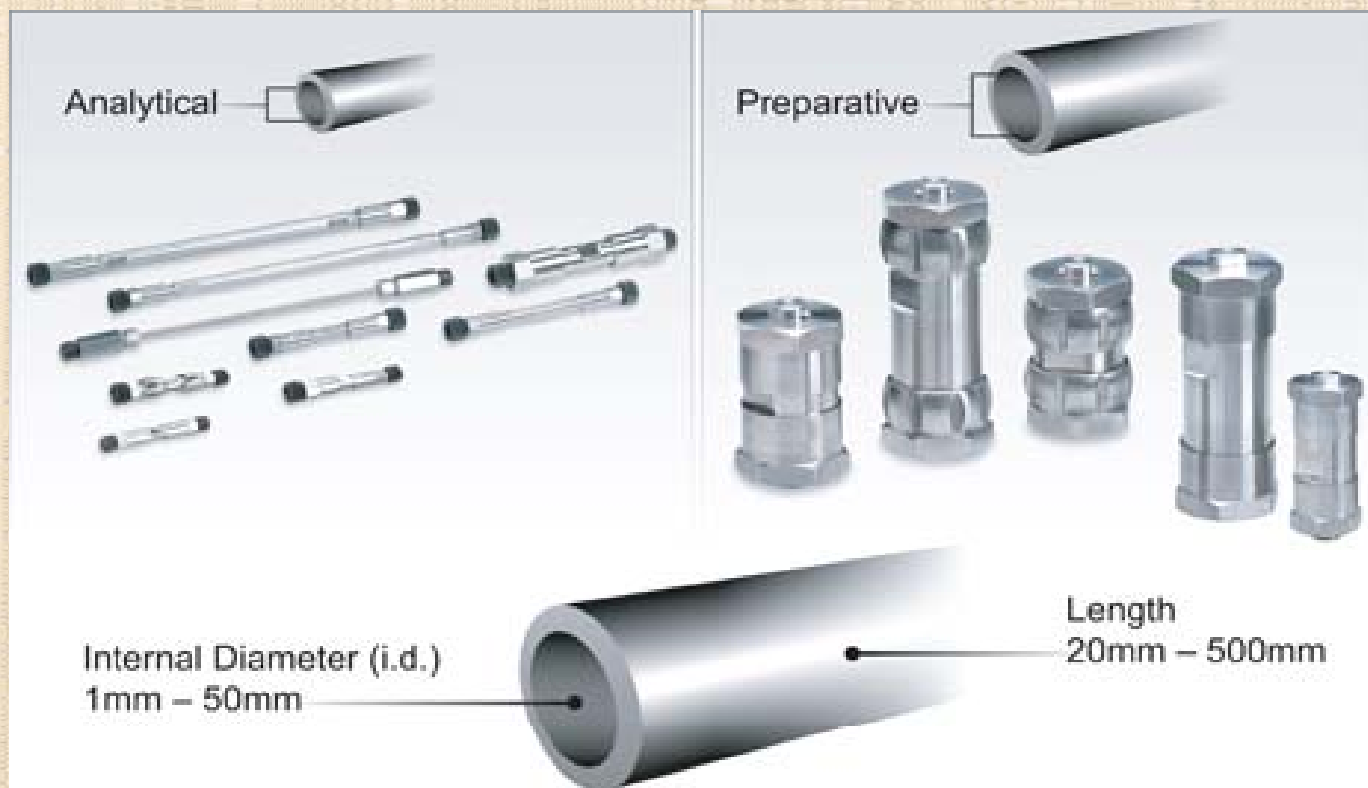
*Perhaps what you're  
looking for, will find  
you...*





- *Column length* could then be calculated based on how much purified compound needs to be processed during each run and on how much separation power is required.

### HPLC Column Dimensions





- A column tube and fittings must contain the chromatographic packing material [stationary phase] that is used to effect a separation.
- It must withstand backpressure created both during manufacture and in use.
- It must provide a well-controlled [leak-free, minimum-volume, and zero-dead-volume] flow path for the sample at its inlet, and analyte bands at its outlet, and be chemically inert relative to the separation system [sample, mobile, and stationary phases].



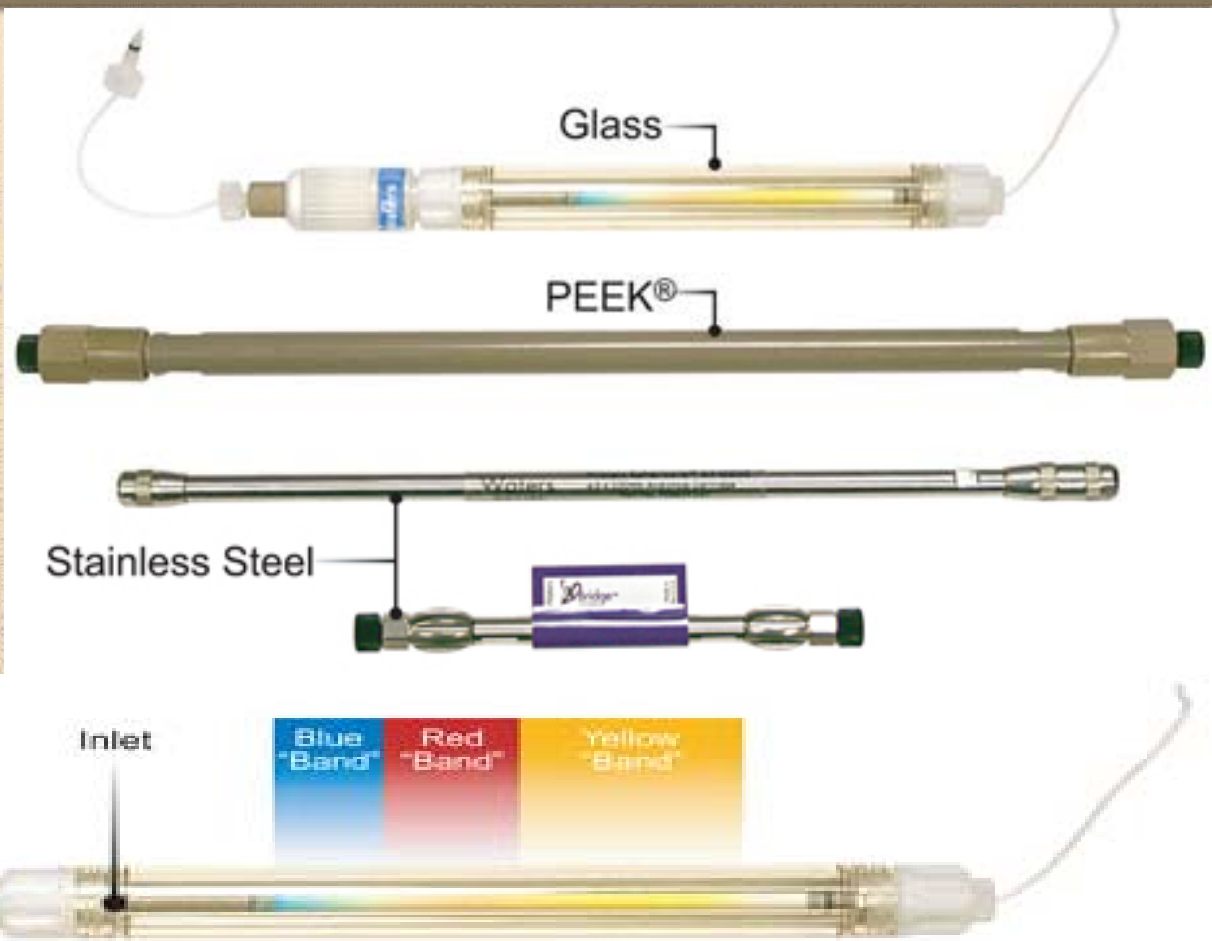
Most *columns* are constructed of

- stainless steel for *highest pressure resistance*.
- PEEK<sup>TM</sup> [an engineered plastic] and
- glass, while *less pressure tolerant*,  
may be used when *inert surfaces* are required for special chemical or biological applications.





## Column Hardware Examples

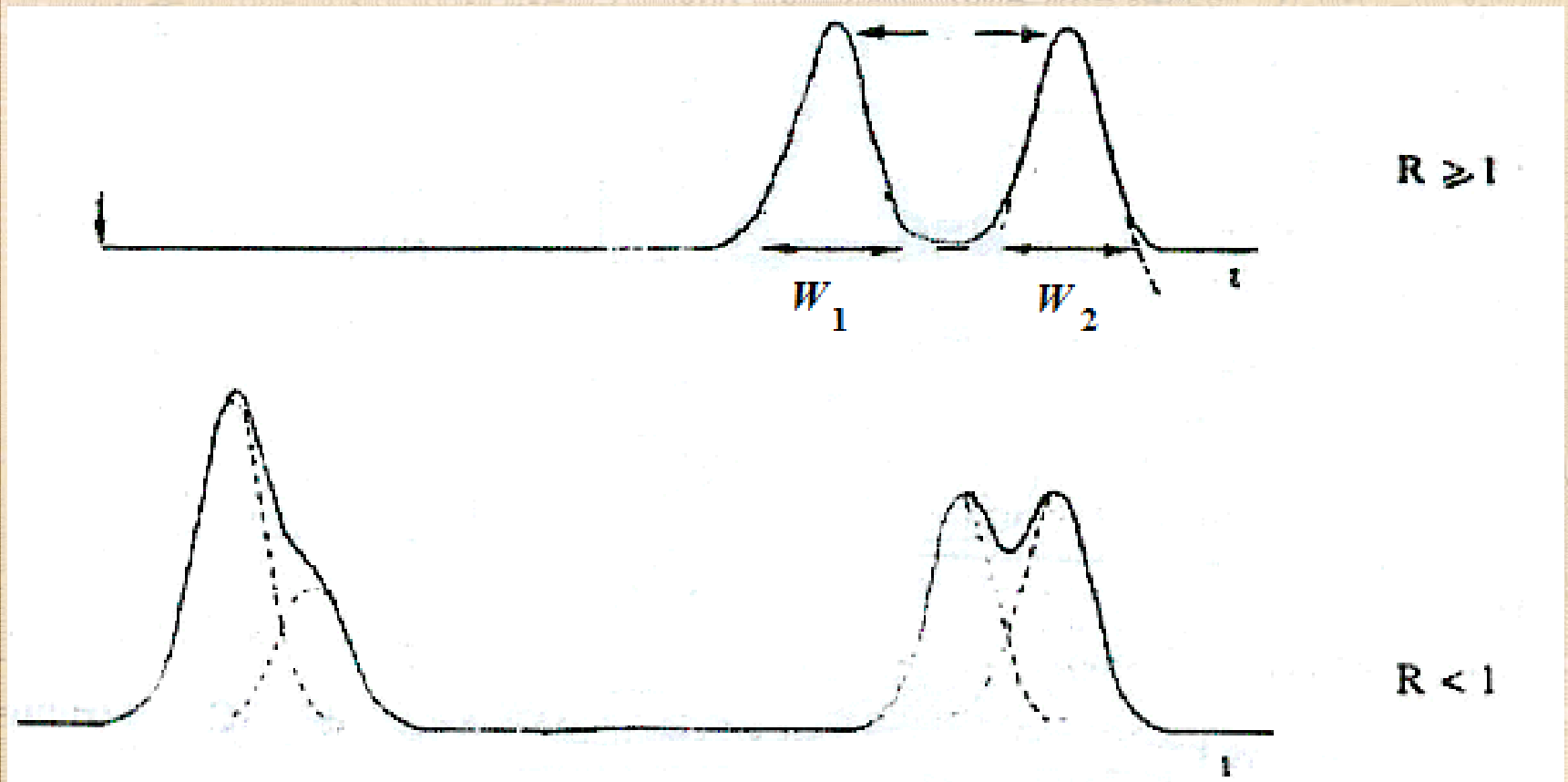


A **glass column** wall offers a **visual advantage**. The flow has been stopped while the sample bands are still in the column. You can see that the three dyes in the injected sample mixture have already separated in the bed; the yellow analyte, traveling *fastest*, is just about to exit the column.



# *Separation Performance – Resolution ( $R_s$ )*

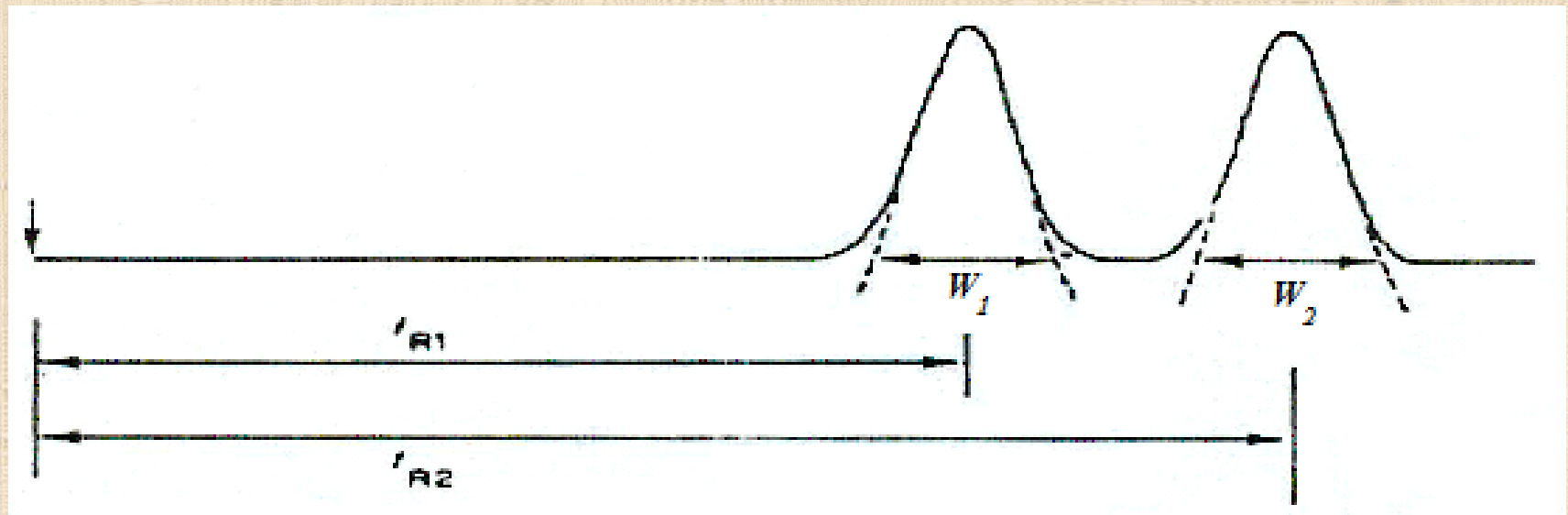
The degree to which two compounds are separated is called **chromatographic resolution** [ $R_s$ ].





- It can also be expressed in terms of the separation of the apex of two peaks divided by the tangential width average of the peaks:

$$R_s = \frac{(t_2 - t_1)}{0.5(W_1 + W_2)}$$





Resolution ( $R_s$ ); *Ability of a column to separate chromatographic peaks.*

*Resolution* can be improved by

- increasing column length,
- decreasing particle size,
- increasing temperature,
- changing the eluent or stationary phase.



Two principal factors that determine the overall *separation power* or *resolution* that can be achieved by an **HPLC column** are:

1- Mechanical separation power, created by

- the column length,
- particle size,
- packed-bed uniformity,

2- Chemical separation power, created by *the physicochemical competition* for compounds between the packing material and the mobile phase



# *1-Mechanical Separation Power – Efficiency*

*Efficiency* is a measure of mechanical separation power, while selectivity is a measure of chemical separation power.

If a **column** bed is stable and uniformly packed, its **mechanical separation power** is determined by *the column length* and *the particle size*.

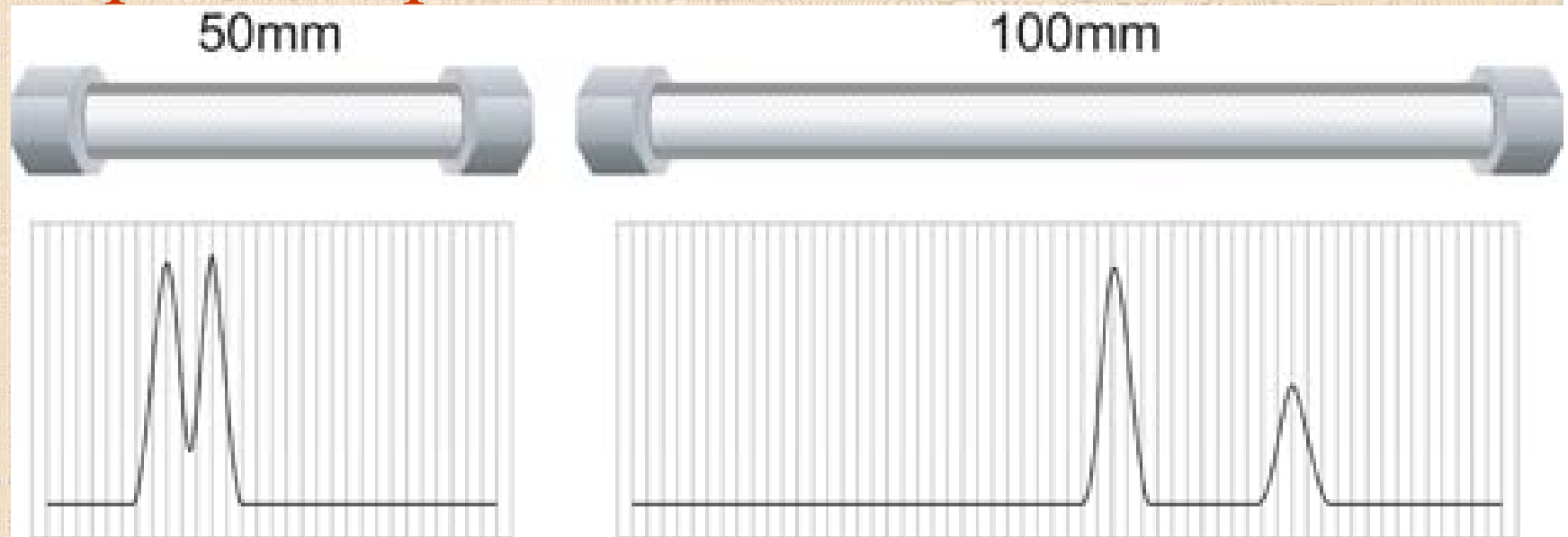
**Mechanical separation power**, also called *efficiency*, is often measured and compared by a **plate number** [symbol = **N**].



- Smaller-particle chromatographic beds have **higher efficiency** and **higher backpressure**.
- For a given **particle size**, more mechanical separation power is gained by **increasing column length**.
- However, the trade-offs are longer chromatographic run times, greater solvent consumption, and higher backpressure.



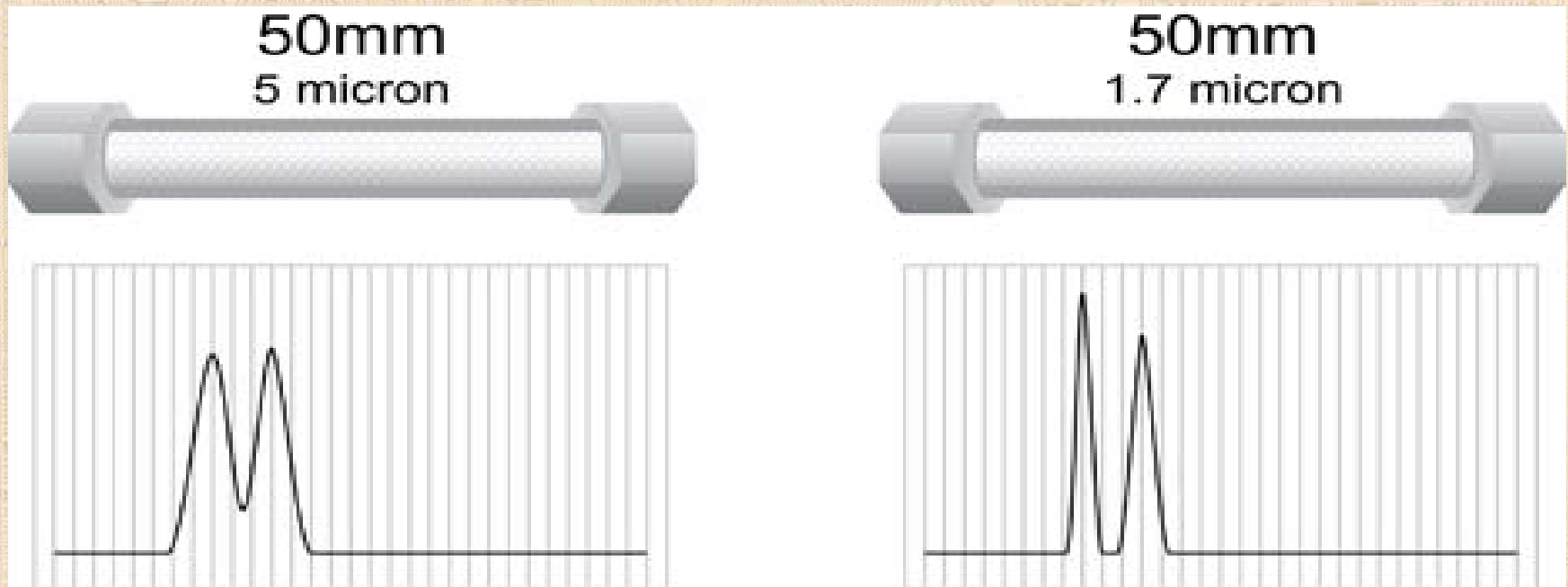
- *Shorter column lengths* minimize all these variables but also reduce **mechanical separation power**.



Column Length and Mechanical Separating  
Power [Same Particle Size]



A column of the same length but with *a smaller particle size*, will deliver **more mechanical separation power** in the same time. However, its backpressure will be **much higher**.





## *2-Chemical Separation Power – Selectivity*

The choice of a combination of particle chemistry [stationary phase] and mobile-phase composition

—*the separation system*—will determine the degree of chemical separation power.



*Optimizing selectivity( $\alpha$ )* is the most powerful means of creating a separation; this may obviate the need for the brute force of the highest possible mechanical efficiency.

To create a separation of any two specified compounds, a multiplicity of phase combinations [stationary phase and mobile phase] and retention mechanisms [modes of chromatography] could be chosen.



- There are **two factors**, effecting the *separation* in analytical chromatography:
- **Chemical factors:** They have an effect on *resolution*.
  - Capacity Factor ( $k'$ )
  - Selectivity ( $\alpha$ )
- **Physical factors:**
  - Efficiency ( $N$ )



## Capacity Factor ( $k'$ )

- Expression that measures the degree of retention of an analyte relative to an unretained peak, where  $t_R$  is the retention time for *the sample peak* and  $t_0$  is the retention time for *an unretained peak*.
- At a constant velocity of the mobile phase, the capacity factor  $k'$  (*ratio of retention time of the compounds in the stationary and mobile phases*) is a compound specific value.
- If  $k'$  is too low, it means that the sample eluted from the column *earlier* and not interact with solid phase.

$$k' = (t_R - t_0) / t_0$$



- A measurement of capacity will help determine whether retention shifts are due to the column (capacity factor is changing with retention time changes) or the system (capacity factor remains constant with retention time changes).
- It is a measure of retention. ( $k'$ : 1-5)

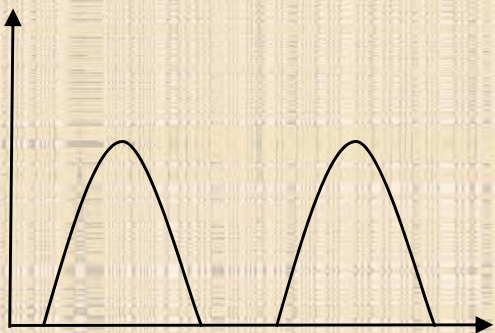


## *Selectivity ( $\alpha$ )*

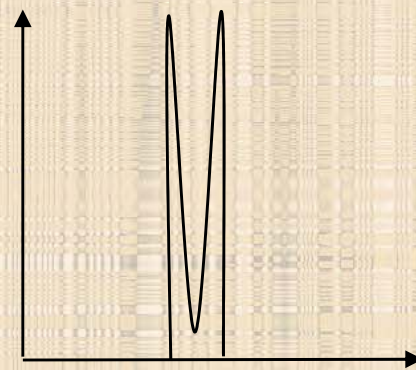
- It reflects how the peaks are separated from each other.

$$\alpha = k'_2 / k'_1$$

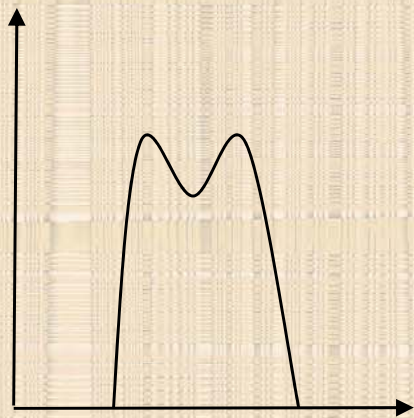




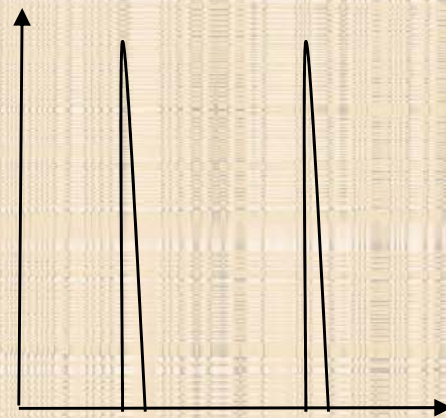
$\alpha \uparrow, N \downarrow$



$N \uparrow, \alpha \downarrow$



$\alpha \downarrow, N \downarrow$



$\alpha \uparrow, N \uparrow$



## *Efficiency (N)*

- Number of **theoretical plates**.
- A measure of peak band spreading determined by various methods, some of which are sensitive to peak asymmetry.



## *Theoretical Plate*

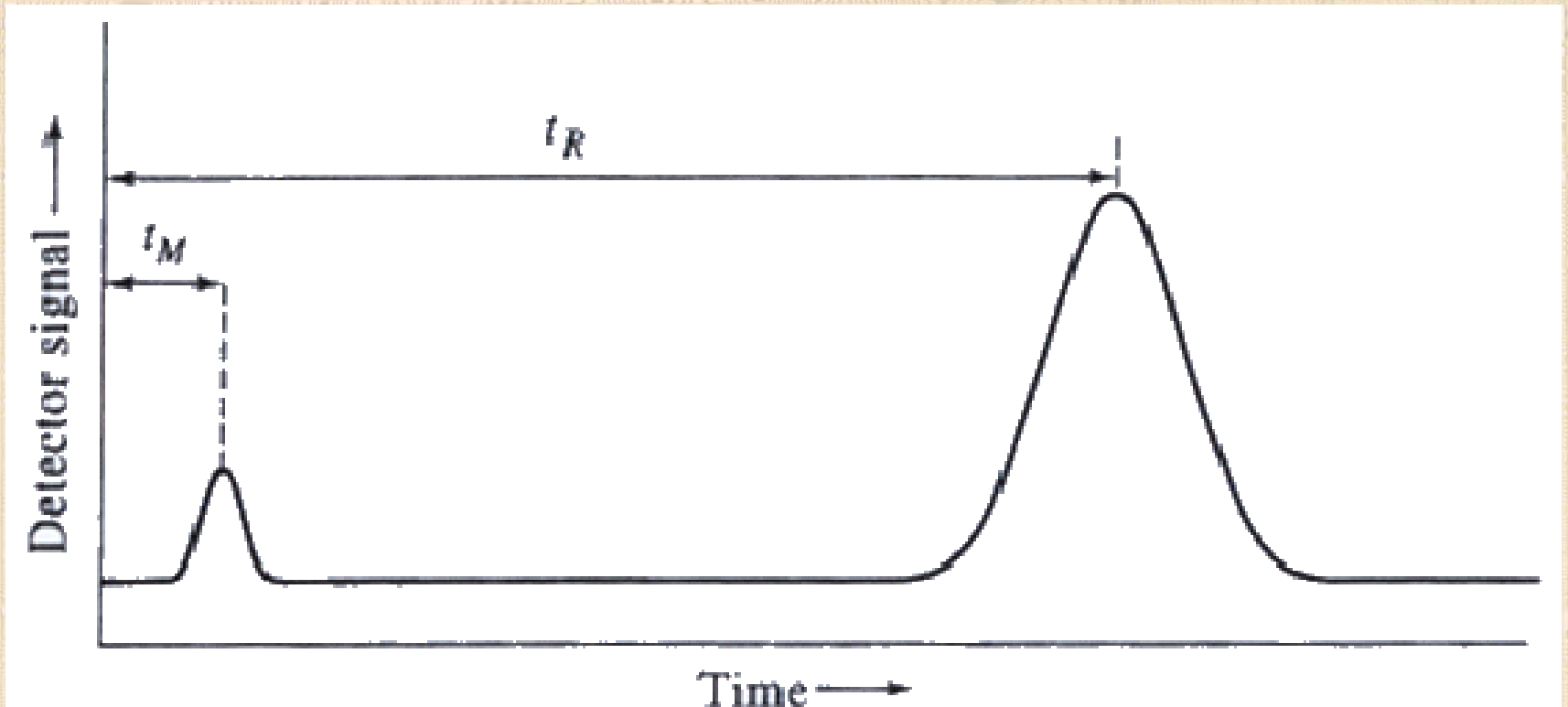
- Relates chromatographic separation to the theory of distillation.
- **Measure of column efficiency**. Length of column relating to this concept is called **height equivalent to a theoretical plate (HETP)**.
- For a typical well-packed HPLC column with 5  $\mu\text{m}$  particles, **HETP** (or **H**) values are usually between **0.01** and **0.03** mm. **L** is **column length** in millimeters and **N** is the **number of theoretical plates**.

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



# *Retention Time ( $t_R$ )*

*The time between injection and the appearance of the peak maximum.*



**This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.**



The time taken for a particular compound to travel through the column to the detector is known as its *retention time*.

Different compounds have *different* retention times.

Each solute has a *characteristic* retention time.

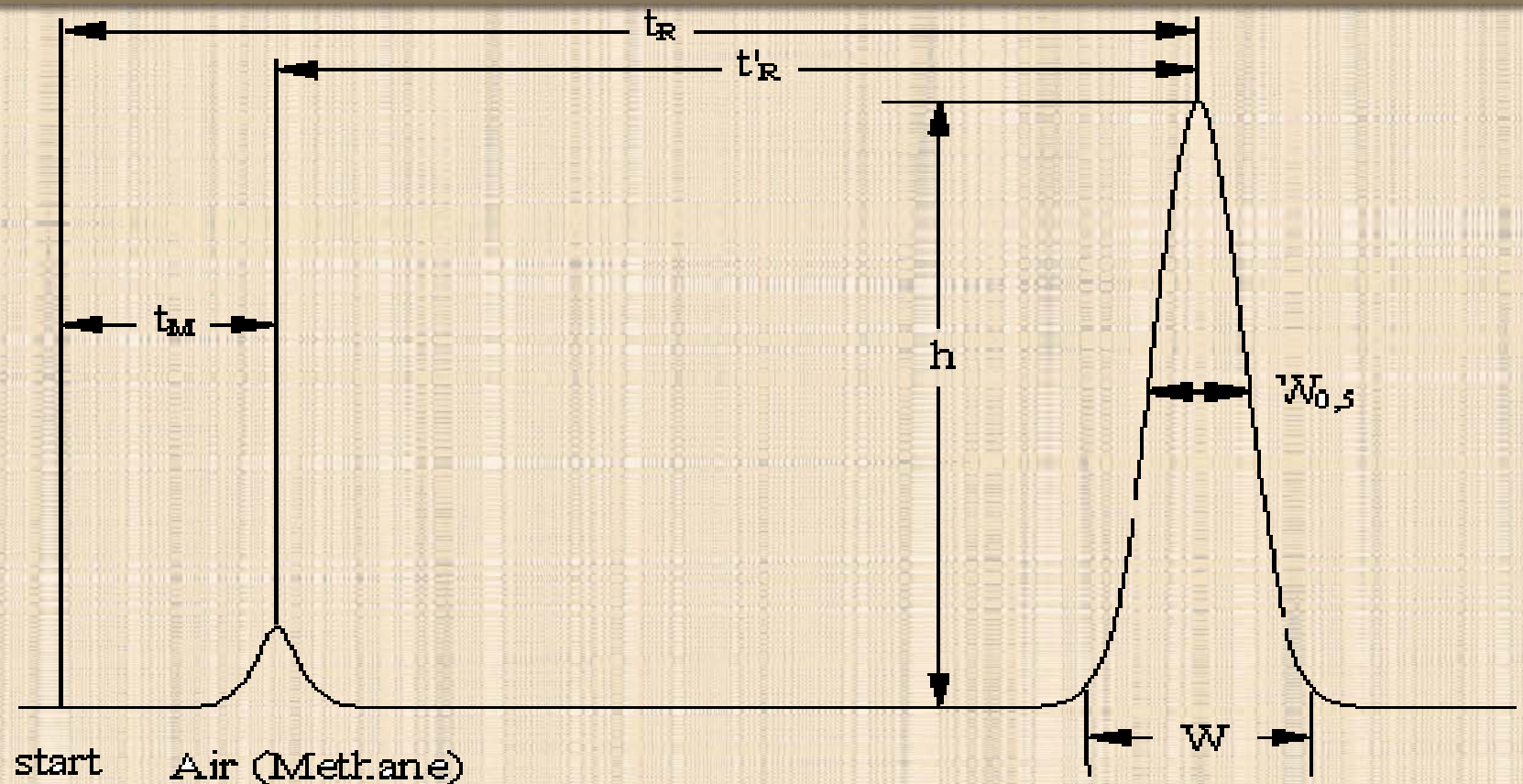
The conditions have to be carefully controlled if you are using retention times as a way of identifying compounds.



For a particular compound, the *retention time* will vary depending on:

- the pressure used (because that affects the flow rate of the solvent)
- the nature of the stationary phase (not only what material it is made of, but also particle size)
- the exact composition of the solvent
- the temperature of the column





- $t_R$  Total retention time of the compound
- $t'_R$  Corrected retention time of the compound (r.t. -stationary phase)
- $t_M$  "Dead time" (retention time-mobile phase)
- $W_{0,5}$  Peak width at half height
- $h$  Height of a signal
- $t/\text{min}$



- The *baseline* is any part of the chromatogram where only mobile phase is emerging from the column.
- The *peak maximum* is the highest point of the peak.
- The *injection point* is that point in time/position when/where the sample is placed on the column.
- The *dead point* is the position of the peak-maximum of an unretained solute.



- The *corrected retention time* ( $t'_R$ ) is the time elapsed between the *dead point* and the *peak maximum*.
- The *dead time* ( $t_M$ ) is the time elapsed between the *injection point* and the *dead point*.
- The *retention time* ( $t_R$ ) Total retention time of the compound in the whole chromatographic system

$$t_R = t'_R + t_M$$



- The *retention volume* ( $V_R$ ) is the volume of mobile phase passed through the column between the *injection point* and the *peak maximum*.

$$\text{Thus, } V_R = F \times t_R$$

where  $F$  is the flow rate in ml/min.

- The *band width* ( $t_w$ ) of the chromatographic band during elution from the column.

Small band widths usually represent *efficient separations*. Also referred to as peak width.



# *HPLC Separation Modes*

- In general, three primary characteristics of chemical compounds can be used to create HPLC separations.
  - *Polarity*
  - *Electrical Charge*
  - *Molecular Size*
- First, let's consider polarity and the two primary separation modes that exploit this characteristic:  
*normal phase* and *reversed-phase* chromatography.



## *Separations Based on Polarity*

- *A molecule's structure, activity, and physicochemical characteristics* are determined by the arrangement of its constituent atoms and the bonds between them.
- Within a molecule, a specific arrangement of certain atoms that is responsible for special properties and predictable chemical reactions is called a functional group.
- This structure often determines whether the molecule is *polar* or *non-polar*.



- Organic molecules are sorted into classes according to the principal functional group(s) each contains.
- Using a separation mode based on **polarity**, the relative chromatographic retention of different kinds of molecules is largely determined by the nature and location of these functional groups.



- *Water* [a small molecule with a high dipole moment] is a polar compound.
- *Benzene* [an aromatic hydrocarbon] is a non-polar compound.
- Molecules with similar chromatographic polarity tend to be attracted to each other; those with dissimilar polarity exhibit much weaker attraction, if any, and may even repel one another.
- This becomes the basis for chromatographic separation modes *based on polarity*.



- Another way to think of this is by the familiar analogy: oil [non-polar] and water [polar] don't mix.
- Unlike in magnetism where opposite poles attract each other, chromatographic separations based on polarity depend upon the stronger attraction between likes and the weaker attraction between opposites.  
Remember, “*Like attracts like*” in polarity-based chromatography.



- *Silica* has an active, hydrophilic [water-loving] surface containing acidic silanol [silicon-containing analog of alcohol] functional groups.
- The activity or polarity of the silica surface may be modified selectively by chemically bonding to it less polar functional groups [bonded phase].



In order of *decreasing polarity*,

- cyanopropylsilyl-[CN],
- n-octylsilyl-[C8],
- n-octadecylsilyl-[C18, ODS]

moieties on silica.

The latter is a hydrophobic [water-hating], very non-polar packing.



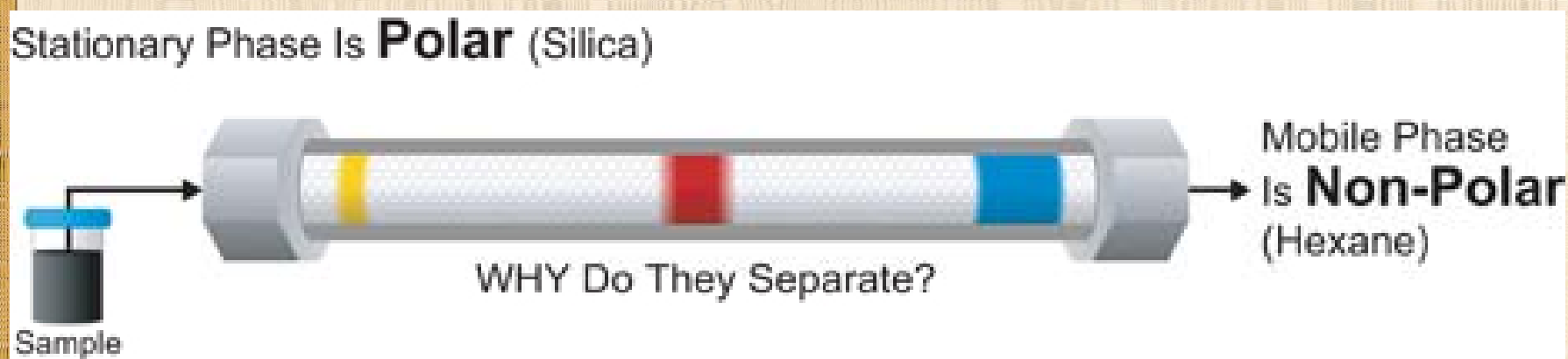
To summarize, the best combination of a mobile phase and particle stationary phase with appropriately opposite polarities must be chosen.

Then, as the sample analytes move through the column, the rule *like attracts like* will determine which analytes slow down and which proceed at a faster speed.



## Normal-Phase HPLC

- In his separations of plant extracts, Tswett was successful using a **polar stationary phase** [chalk in a **glass column**] with a much *less polar* [**non-polar**] *mobile phase*. This classical mode of chromatography became known as *normal phase*.



Normal-Phase Chromatography



- *The stationary phase is polar and retains the polar yellow dye most strongly.*
- The relatively non-polar blue dye is won in the retention competition by the mobile phase, a non-polar solvent, and *elutes quickly.*

Stationary Phase Is **Polar** (Silica)



A normal-phase chromatographic separation of our three-dye test mixture.



- Since the blue dye is most like the mobile phase [both are non-polar], it moves *faster*.

It is typical for *normal-phase chromatography on silica* that the mobile phase is 100% **organic**; no water is used.



# Reversed-Phase HPLC

- The term *reversed-phase* describes the chromatography mode that is just the opposite of normal phase, namely the use of a **polar mobile phase** and a **non-polar [hydrophobic] stationary phase**.

Stationary Phase Is **Non-Polar** (C<sub>18</sub>)

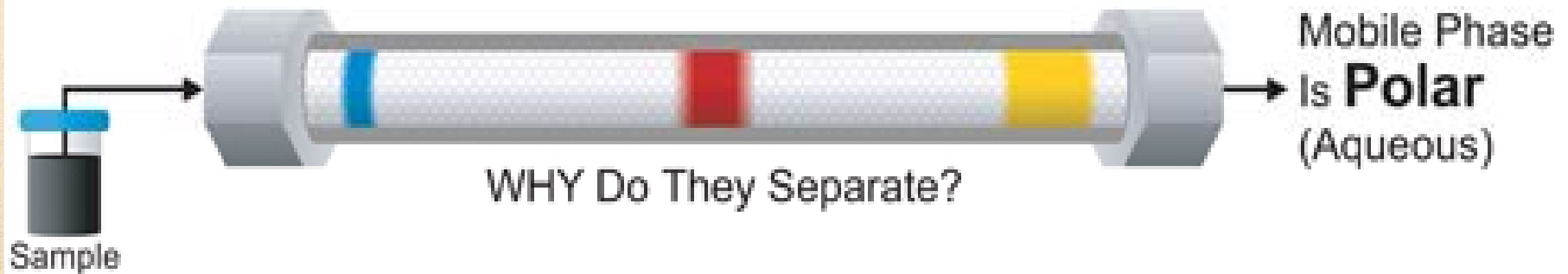


Figure illustrates the black three-dye mixture being separated using such a protocol.



Now the most strongly retained compound is the more *non-polar blue dye*, as its attraction to the *non-polar stationary phase* is *greatest*.

*The polar yellow dye*, being weakly retained, is won in competition by the polar, *aqueous mobile phase*, *moves the fastest* through the bed, and elutes *earliest*

Stationary Phase Is **Non-Polar** (C<sub>18</sub>)





- Today, because it is more reproducible and has broad applicability, *reversed-phase chromatography* is used for approximately 75% of all HPLC methods.
- Most of these protocols use as *the mobile phase* an aqueous blend of water with a miscible, *polar organic solvent*, such as *acetonitrile* or *methanol*. This typically ensures the proper interaction of analytes with the *non-polar*, hydrophobic particle surface.



- A C18—bonded silica [sometimes called ODS] is the most popular type of *reversed-phase HPLC* packing.
- Octadecylsilane phases are bonded to silica or polymeric supports. Both monomeric and polymeric phases are available.

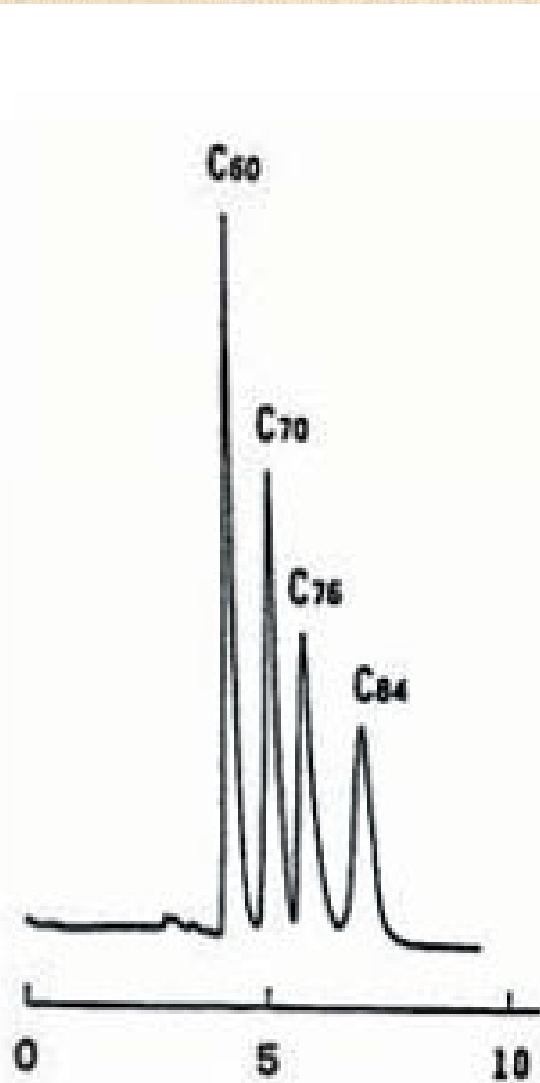


Table presents a summary of the phase characteristics for the two principal **HPLC separation** modes based upon **polarity**. Remember, for these polarity-based modes, *like attracts like*.

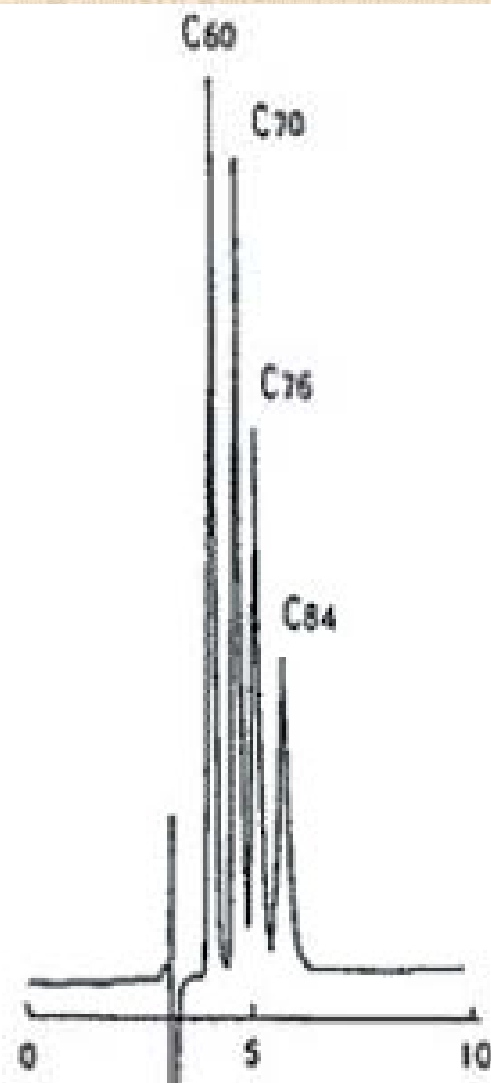
Separation Mode	Stationary Phase [particle]	Mobile Phase [solvent]
Normal phase	Polar	Non-polar
Reversed phase	Non-polar	Polar

*Phase Characteristics for Separations Based on Polarity*

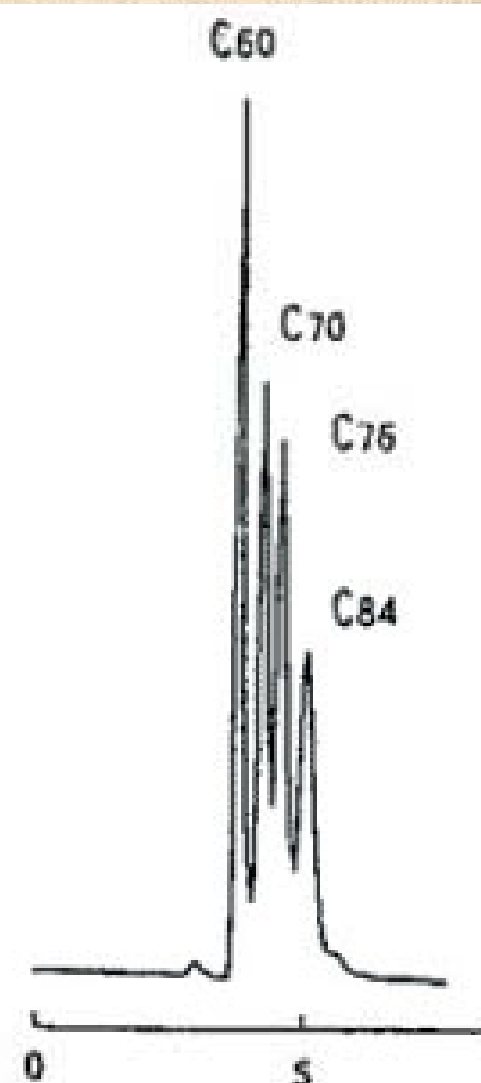




Mobile phase:  
o-Dichlorobenzene

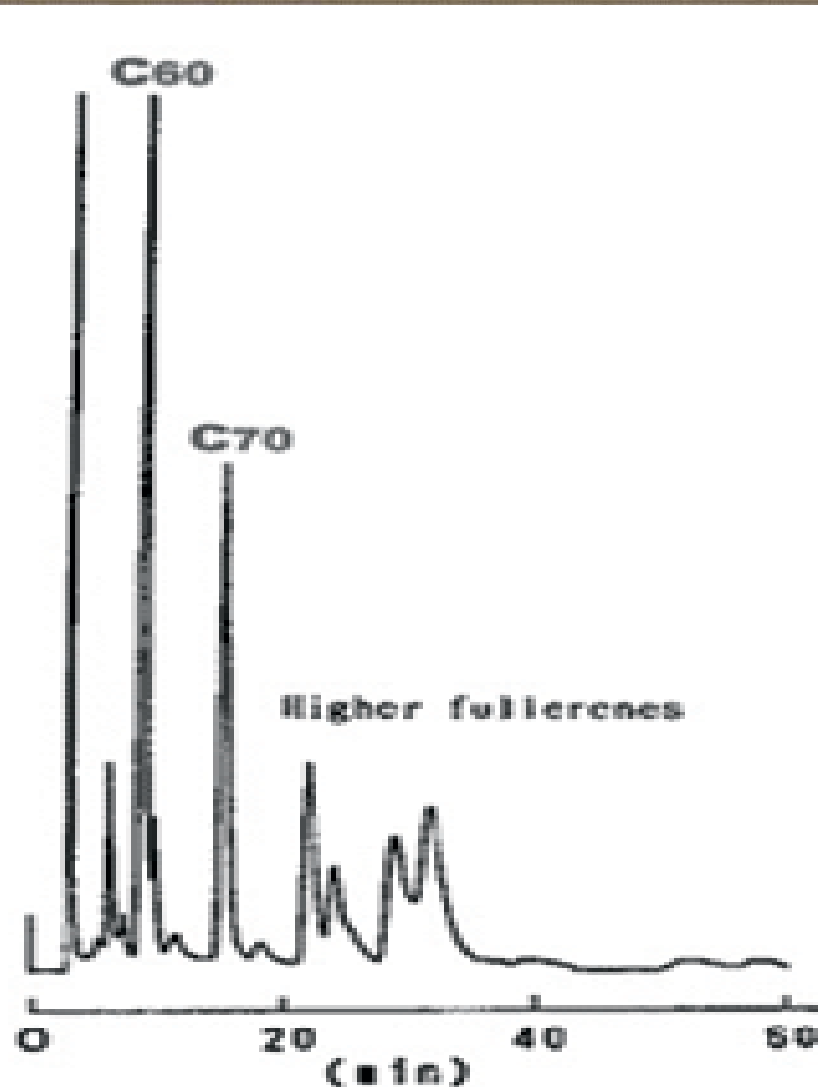


Mobile phase:  
Carbon Disulfide

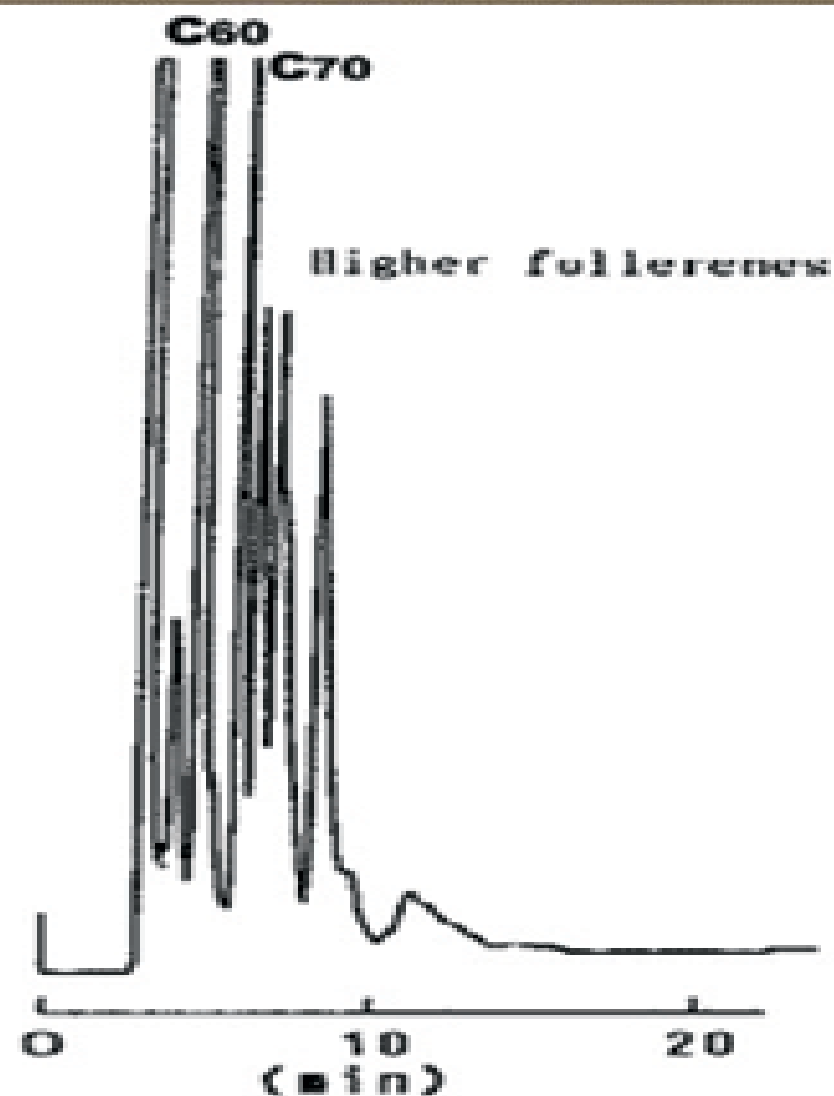


Mobile phase:  
1,2,4-Trichlorobenzene





Mobile phase: toluene  
 Column size 4.6mm I.D. × 250mm  
 Flow rate 1.0ml/min.  
 Temperature: 30°C

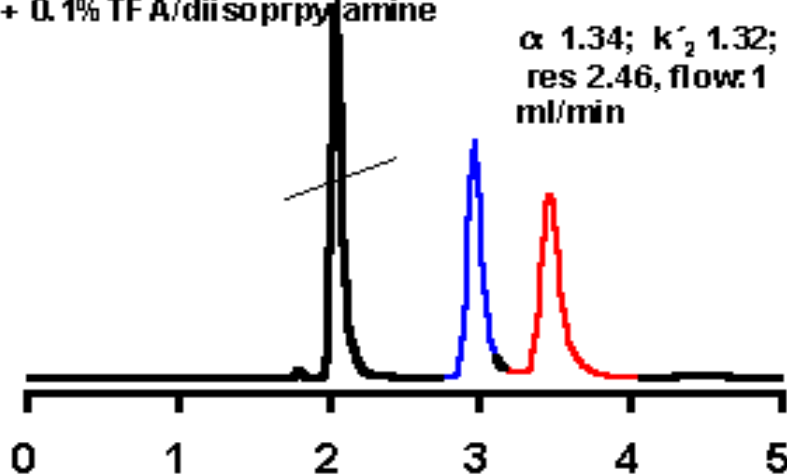


Mobile phase: chlorobenzene  
 Sample conc.: (2.5mg/ml)  
 Detection : UV 285nm

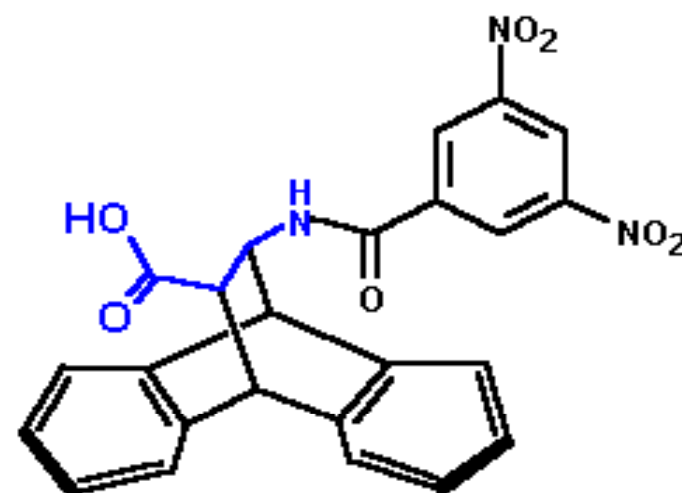
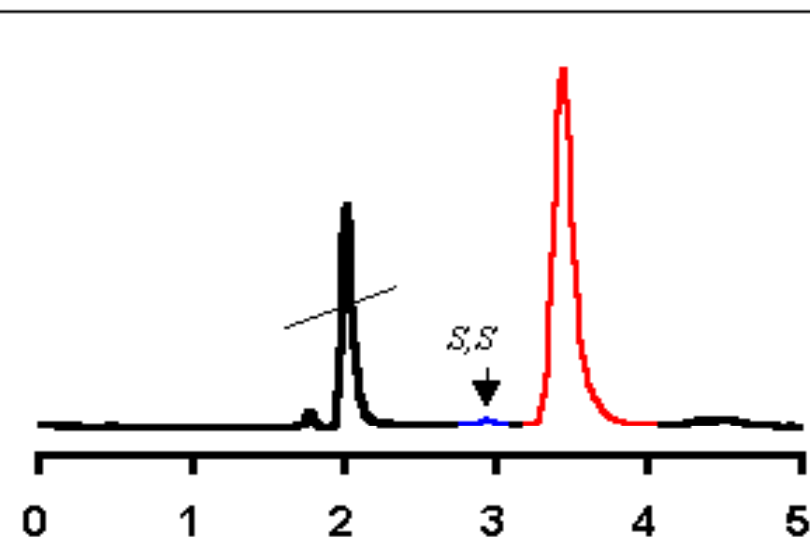


CSP IV DEA ZA ULMO [7]; heptane / 2-prop. 90 / 10  
+ 0.1% TFA / diisopropylamine

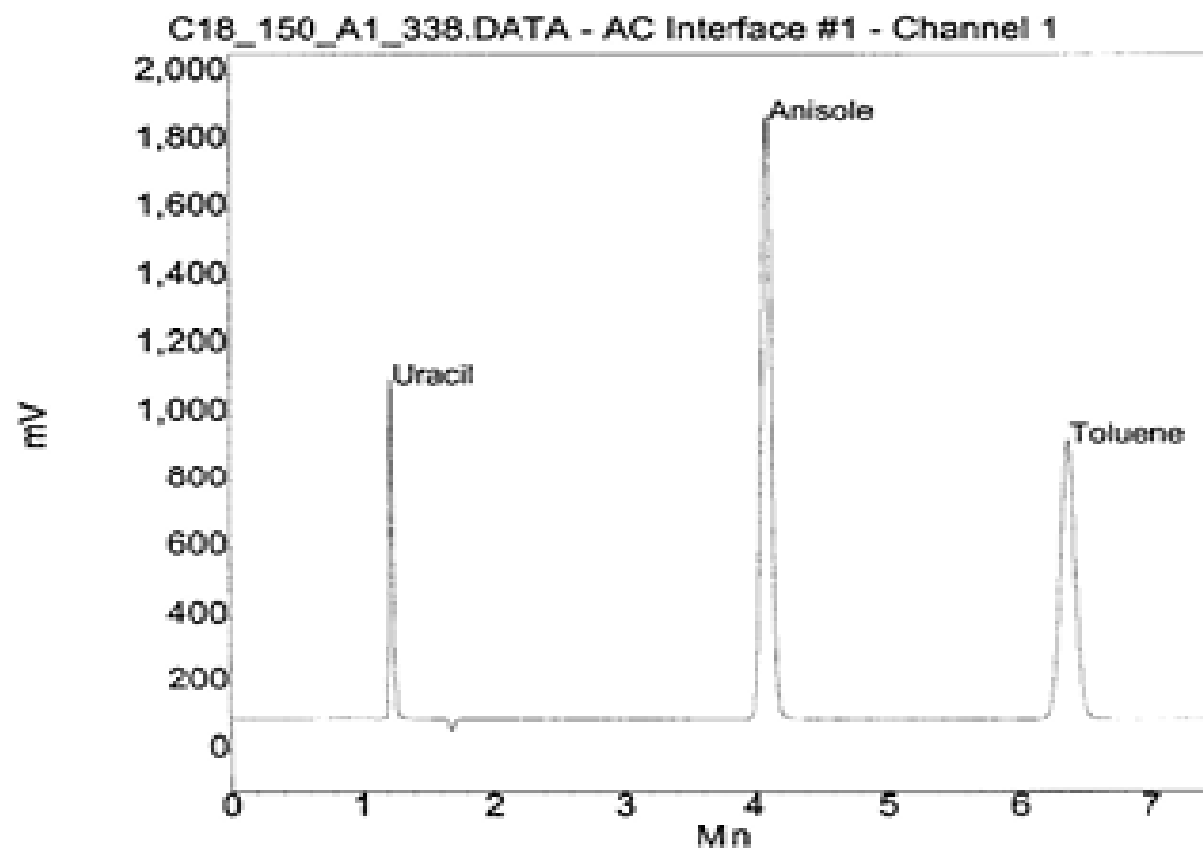
$\alpha$  1.34;  $k'_2$  1.32;  
res 2.46, flow: 1  
ml/min



HPLC-analysis of AMEAC as 3,5-dinitrobenzoylamide **5**. Above analyzed as racemate, and below as the purified (*R,R*)-enantiomer (99ee) after semipreparative chromatography on the deaza-ULMO CSP [7].







Index	Time	Name	Asymmetry (USP)	# Column Plates (HH)	# Plates/meter (HH)
	[Min]				
1	1.23	Uracil	1.25	8896	59306
2	4.10	Anisole	1.02	15375	102501
3	6.37	Toluene	1.02	15360	102402
Total					



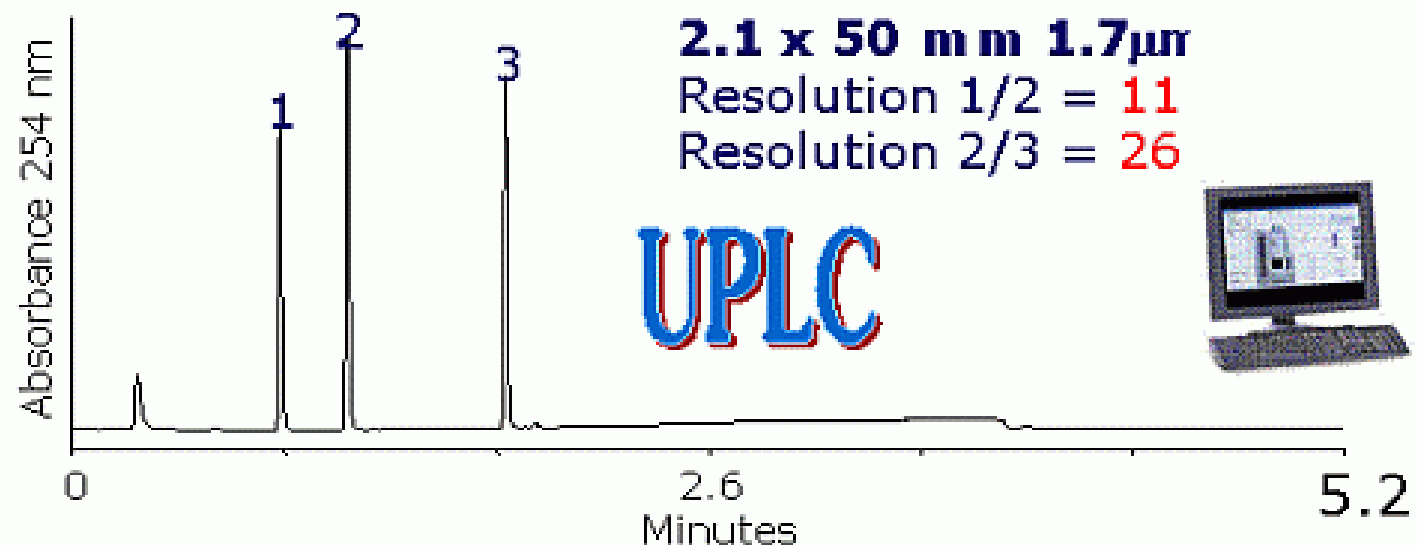
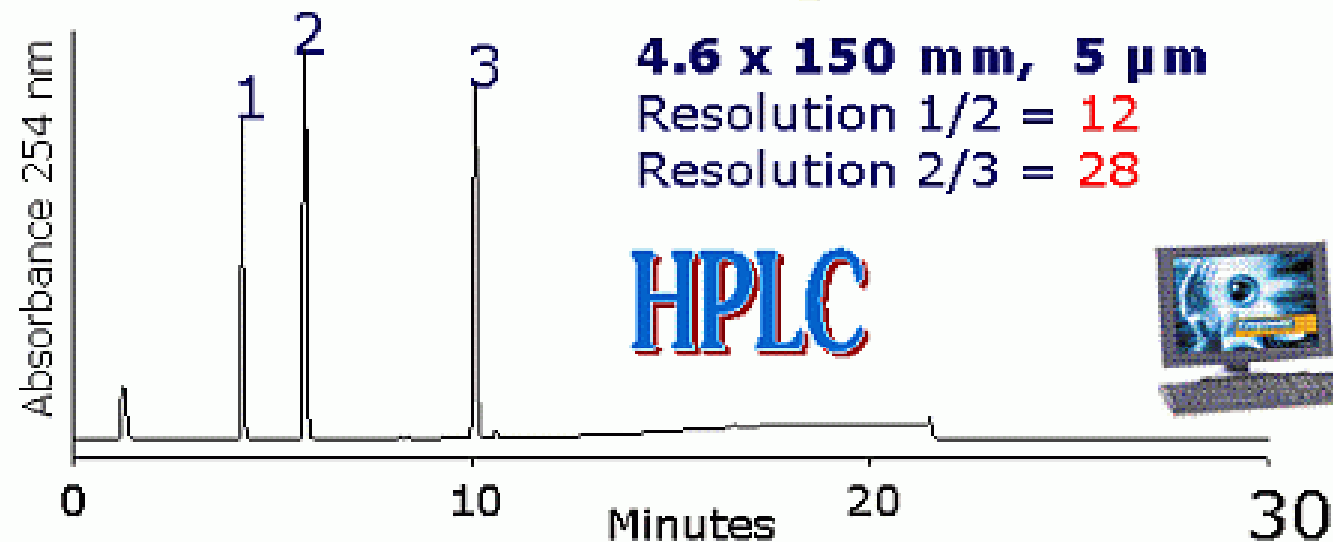
# *Ultra Performance Liquid Chromatography (UPLC)*

*UPLC increases in resolution, speed,  
and sensitivity* in liquid chromatography.

- Columns with smaller particles [1.7 micron]
- and instrumentation with specialized capabilities designed to deliver mobile phase at 15,000 psi [1,000 bar]



## Proportionally Magnified View of the Chromatograms





LC









# *Hydrophilic-Interaction Chromatography [HILIC]*

- **HILIC** may be viewed as a variant of normal-phase chromatography.
- In normal-phase chromatography, the mobile phase is 100% organic. Only traces of water are present in the mobile phase and in the pores of the polar packing particles.
- **HILIC** may be run in either isocratic or gradient elution modes.
- Polar compounds that are initially attracted to the polar packing material particles can be eluted as the polarity [strength] of the mobile phase is increased [by adding more water].



# Hydrophobic-Interaction Chromatography [HIC]

- **HIC** is a type of reversed-phase chromatography that is used to separate large biomolecules, such as proteins.
- It is usually desirable to maintain these molecules intact in an aqueous solution, avoiding contact with organic solvents or surfaces that might denature them.
- **HIC** takes advantage of the hydrophobic interaction of large molecules with a moderately hydrophobic stationary phase, *e.g.*, butyl-bonded [C4], rather than octadecyl-bonded [C18], silica. Gradient separations are typically run by decreasing salt concentration. In this way, biomolecules are eluted in order of increasing hydrophobicity.

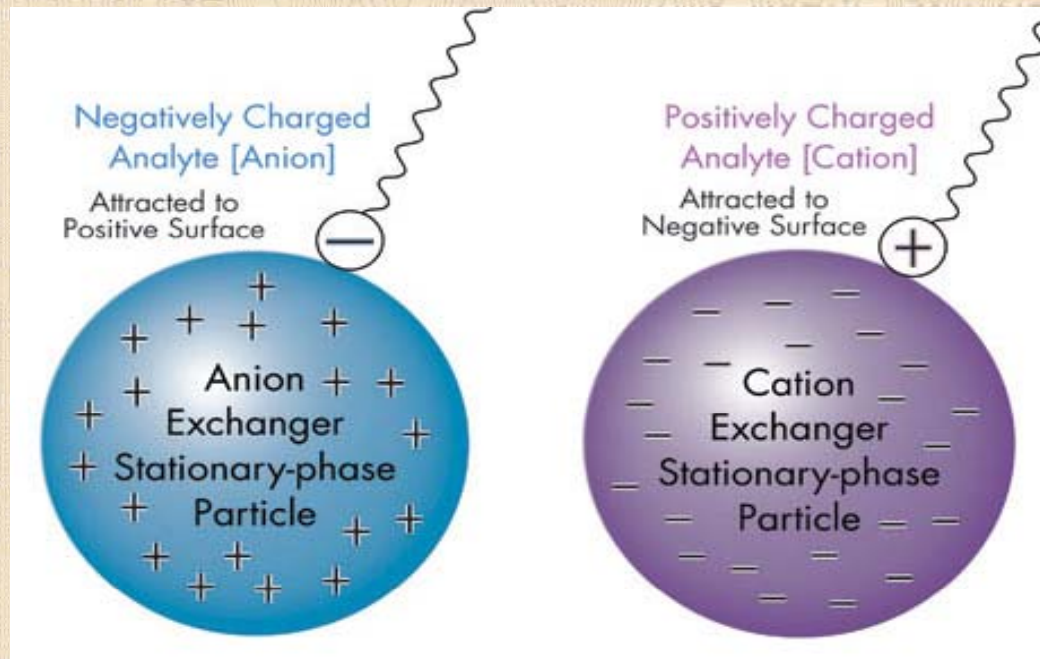


# *Separations Based on Charge: Ion-Exchange Chromatography [IEC]*

- For separations based on polarity, *like is attracted to like* and *opposites* may be repelled.
- In ion-exchange chromatography and other separations based upon electrical charge, the rule is reversed. *Likes may repel, while opposites are attracted to each other.*
- Stationary phases for ion-exchange separations are characterized by the nature and strength of the acidic or basic functions on their surfaces and the types of ions that they attract and retain.



- *Cation* exchange is used to retain and separate positively charged ions on a *negative* surface. Conversely, *anion* exchange is used to retain and separate negatively charged ions on a *positive* surface. With each type of ion exchange, there are at least two general approaches for separation and elution.





- *Strong* ion exchangers bear functional groups [e.g., quaternary amines or sulfonic acids] that are always ionized. They are typically used to retain and separate *weak* ions. These weak ions may be eluted by displacement with a mobile phase containing ions that are more strongly attracted to the stationary phase sites. Alternately, weak ions may be retained on the column, then *neutralized* by *in situ* changing the pH of the mobile phase, causing them to lose their attraction and elute.



# *Size-Exclusion Chromatography [SEC] – Gel-Permeation Chromatography [GPC]*

- All of these techniques are typically done on stationary phases that have been synthesized with a pore-size distribution over a range that permits the analysts of interest to enter, or to be excluded from, more or less of the pore volume of the packing.
- Smaller molecules penetrate more of the pores on their passage through the bed. Larger molecules may only penetrate pores above a certain size so they spend less time in the bed.
- The biggest molecules may be totally excluded from pores and pass only between the particles, eluting very quickly in a small volume.



# *Gas Chromatography*

## *(GC)*



# Gas Chromatography

- GC is a powerful means of performing **qualitative** and **quantitative** measurements of complex mixtures of volatile substances.
  - The organic compounds are separated due to differences in their partitioning behavior between **the mobile gas phase** and **the stationary phase** in the column.
- a) Gas-Liquid Chromatography-GLC
  - b) Gas-Solid Chromatography-GSC



# Gas-Liquid Chromatography

- *Mobile phases* are generally inert gases such as helium, argon, or nitrogen.
- Most columns contain *a liquid stationary phase* on a solid support.



- The *most common stationary phases* in GC columns are *polysiloxanes*, which contain various substituent groups to change the polarity of the phase.
- The **nonpolar** end of the spectrum is *polydimethyl siloxane*, which can be made more polar by increasing the percentage of phenyl groups on the polymer.



- For very **polar** analytes, *polyethylene glycol* (a.k.a. carbowax) is commonly used as **the stationary phase**.
- After the polymer coats the column wall or packing material, it is often cross-linked to **increase the thermal stability** of **the stationary phase** and prevent it from gradually bleeding out of the column.

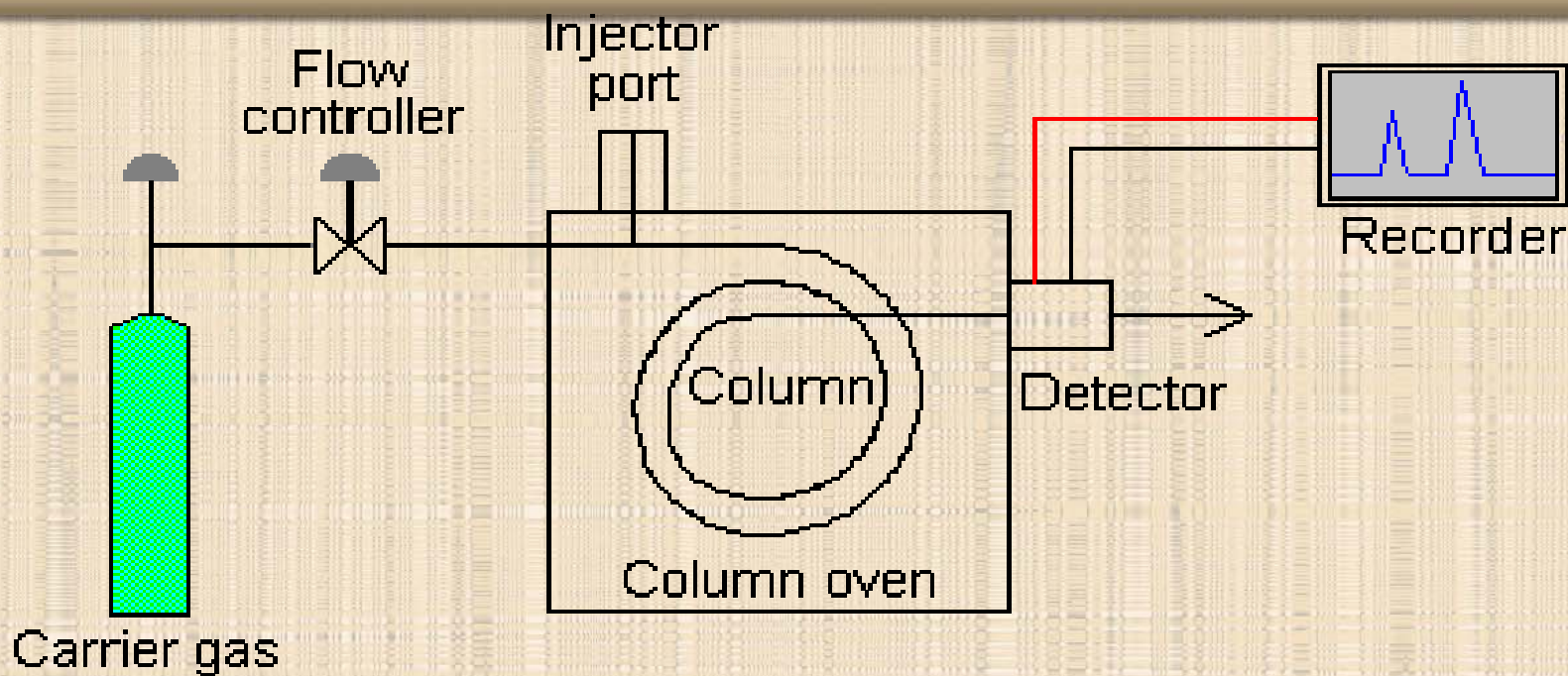


# *Gas-solid chromatography*

- Small gaseous species can be separated by gas-solid chromatography.
- *Gas-solid chromatography* uses **packed columns** containing high-surface-area inorganic or polymer packing.
- The gaseous species are separated by their size, and retention due to adsorption on the packing material.
- Separation of *low-molecular weight gases* is accomplished with solid adsorbents.

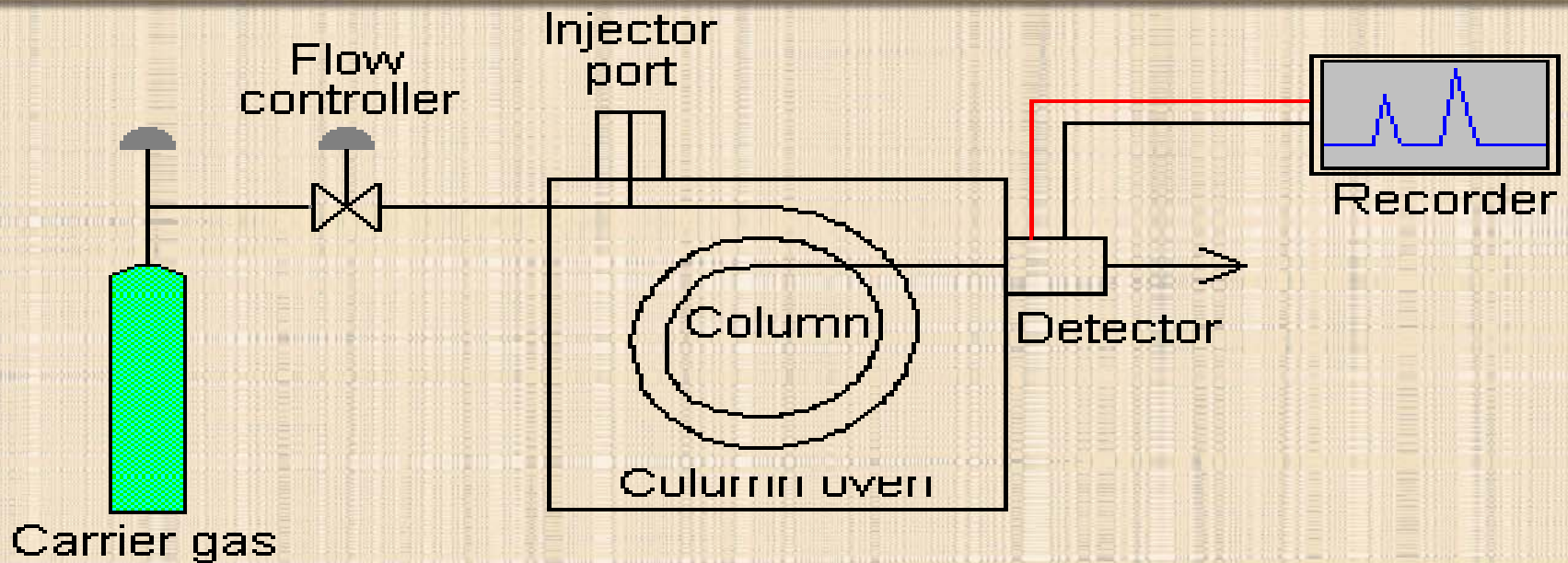


## *GC consists of;*



- a flowing mobile phase,
- an injection port,
- a separation column containing the stationary phase,
- a detector,
- a data recording system.





*The carrier* gas must be chemically **inert**. Commonly used gases include *nitrogen, helium, argon, and carbon dioxide*. The choice of carrier gas is often dependant upon the type of detector which is used. The carrier gas system also contains *a molecular sieve* to remove **water** and other impurities.



## *Sample injection port*

- *The injection port* consists of a rubber septum through which a syringe needle is inserted to inject the sample.
- *The injection port* is maintained at a higher temperature than the boiling point of the least volatile component in the sample mixture.
- For optimum column efficiency, the sample should not be **too large**, and should be introduced onto the column as a "plug" of vapour - slow injection of large samples causes band broadening and loss of resolution.

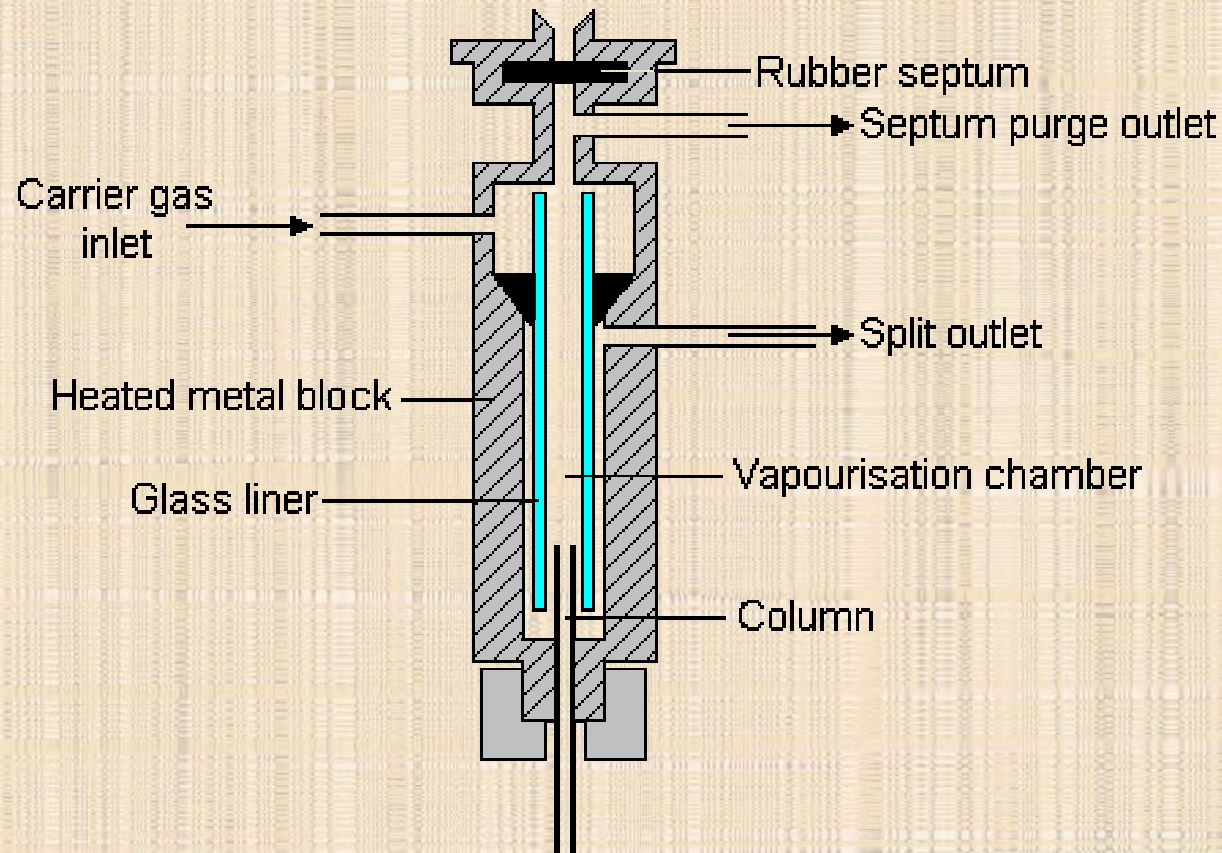


- The most common injection method is where a **microsyringe** is used to inject sample through a rubber septum into a flash vapouriser port at the head of the column.
- For **packed columns**, sample size ranges from tenths of a microliter up to 20 microliters.
- **Capillary columns**, on the other hand, need **much less sample**, typically around  $10^{-3} \mu\text{L}$ .



- For capillary GC, split/splitless injection is used. Have a look at this diagram of a split/splitless injector;

**The split / splitless injector**





## *Separation column*

- Since the partitioning behavior is dependant on temperature, the *separation column* is usually contained in a thermostat-controlled oven.
- Separating components with a wide range of boiling points is accomplished by starting at a low oven temperature and increasing the temperature over time to elute the high-boiling point components.



# Columns

GC columns are of two designs:

*packed or capillary*

- *Packed columns* are typically a glass or stainless steel coil that is filled with the stationary phase, or a packing coated with the stationary phase.
- *Packed columns* contain a finely divided, inert, solid support material (commonly based on diatomaceous earth) coated with liquid stationary phase.
- Most packed columns are 1.5 – 10 m in length and have an internal diameter of 2 – 4 mm.



- *Capillary columns* are a thin fused-silica (purified silicate glass) capillary that has the *stationary phase* coated on the inner surface.
- *Capillary columns* provide much higher separation efficiency than packed columns but are more easily overloaded by too much sample.
- They have an internal diameter of a few tenths of a millimeter.

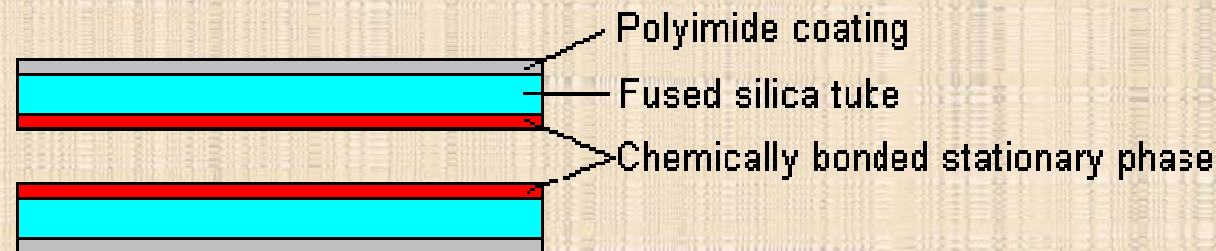


- *Capillary columns* can be one of two types; wall-coated open tubular (WCOT) support-coated open tubular (SCOT).
- Wall-coated columns consist of a capillary tube whose walls are coated with *liquid stationary phase*.
- SCOT columns are generally *less efficient* than WCOT columns. Both types of capillary column are *more efficient* than packed columns.



- In support-coated columns, the inner wall of the capillary is lined with a thin layer of support material such as diatomaceous earth, onto which the *stationary phase* has been adsorbed.
- In 1979, a new type of WCOT column was devised - the *Fused Silica Open Tubular* (FSOT) column;

**Cross section of a Fused Silica Open Tubular Column**





- These have much thinner walls than the glass capillary columns, and are given strength by the polyimide coating.
- These columns are flexible and can be wound into coils.
- They have the advantages of physical strength, flexibility and low reactivity.



# *Detectors*

- There are many detectors which can be used in GC.
- Different detectors will give different types of selectivity.
- A non-selective detector responds to all compounds except the carrier gas, a selective detector responds to a range of compounds with a common physical or chemical property and a specific detector responds to a single chemical compound.



- *Detectors* can also be grouped into *concentration dependant detectors* and *mass flow dependant detectors*.
- The signal from a **concentration dependant detector** is related to the concentration of solute in the detector, and does not usually destroy the sample dilution of with make-up gas will lower the detectors response.
  - Thermal conductivity (TCD)
  - Electron capture (ECD)
  - Photo-ionization (PID)



- Mass flow dependant detectors usually destroy the sample, and the signal is related to the rate at which solute molecules enter the detector. The response of a mass flow dependant detector is unaffected by make-up gas.

- Flame ionization (FID)
- Nitrogen-phosphorus
- Flame photometric (FPD)
- Hall electrolytic conductivity







# *Capillary Electrophoresis* (CE)



# Capillary Electrophoresis

- CE is relatively new separation technique compared to the traditional techniques such as HPLC or GC.
- It provides very attractive features which make it both competitive and a good alternative.
- One of the major advantages of CE over other separation technique is *the ability to separate both charged and non-charged molecules.*



The sample solution is introduced in the capillary as a small plug by

- difference in height (gravity inj.)
- applying pressure (hydrodynamic inj.)
- voltage (electrokinetic inj.)



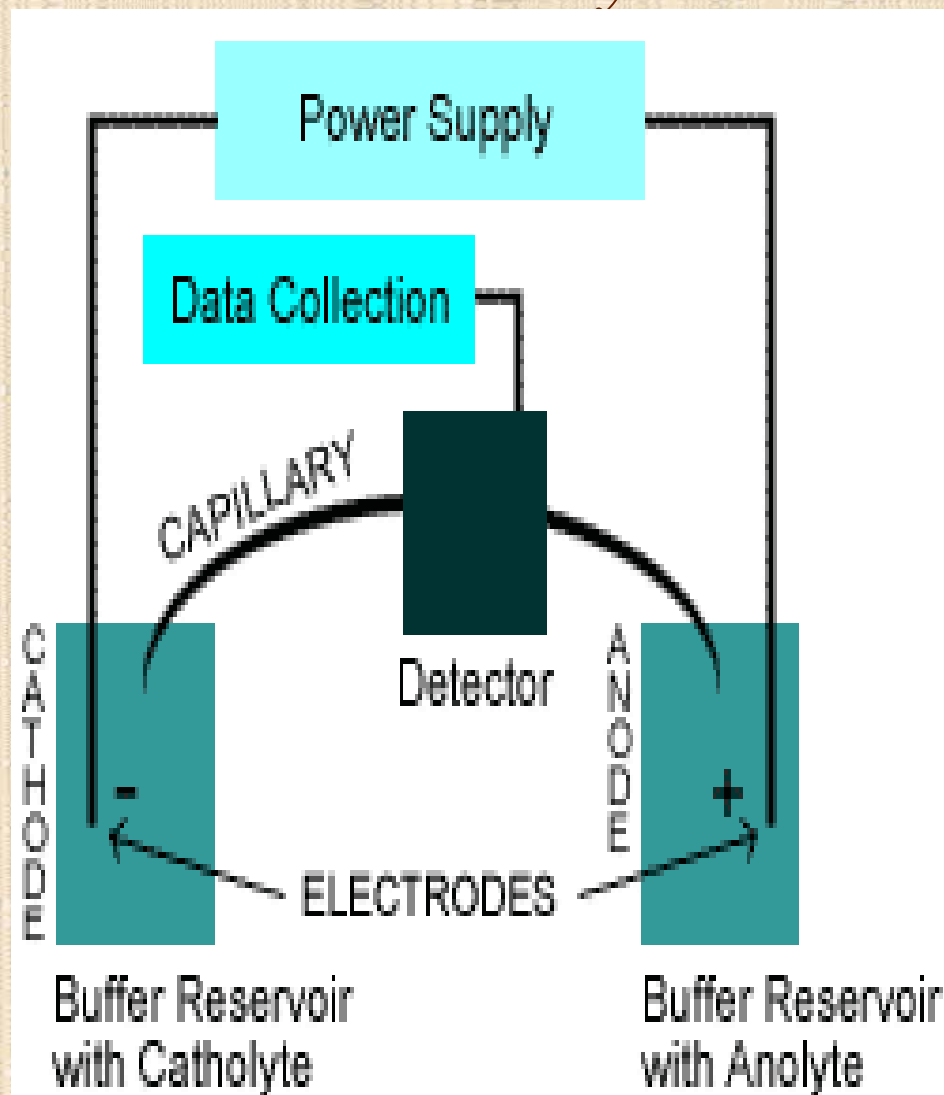
- *Gravity injection* is performed by **difference in height**. It causes sample to flow into capillary.
- *Hydrodynamic injection* is accomplished by the application of **a pressure difference** between the two ends of a capillary.
- *Electrokinetic injection* is performed by simply turning on **the voltage** for a certain period of time.

Then the buffer reservoir is replaced and voltage applied.



The basic components of an *CE system* include;

- *capillary,*
- *buffer reservoir,*
- *electrodes,*
- *a power supply,*
- *dedector.*





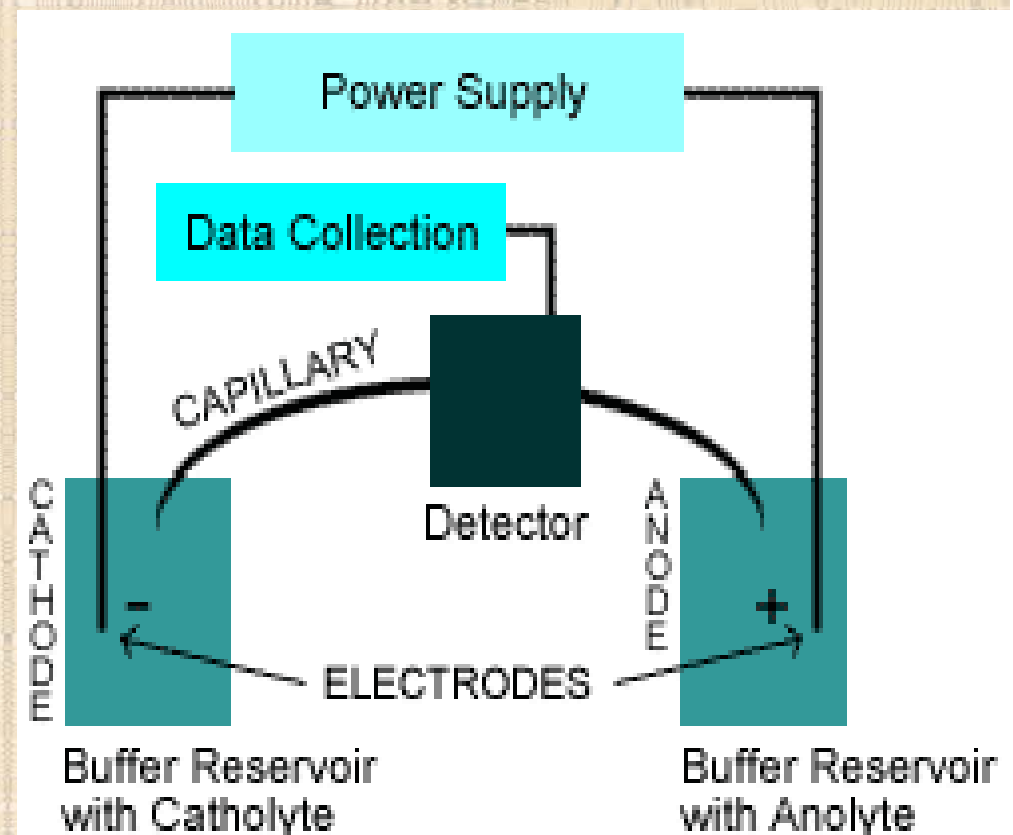
In *CE*, separation of analyte ions is performed in an electrolyte solution present in a narrow *fused-silica capillary*. The ends of the capillary are immersed into vials filled with *electrolyte solution*, which also contain electrodes connected to *a high voltage supply*.

-With the application of high voltage (5 – 30 kV) across the capillary, zones of analyte are formed due to different electrophoretic mobilities of ionic species and migrate toward the outlet side of the capillary.-



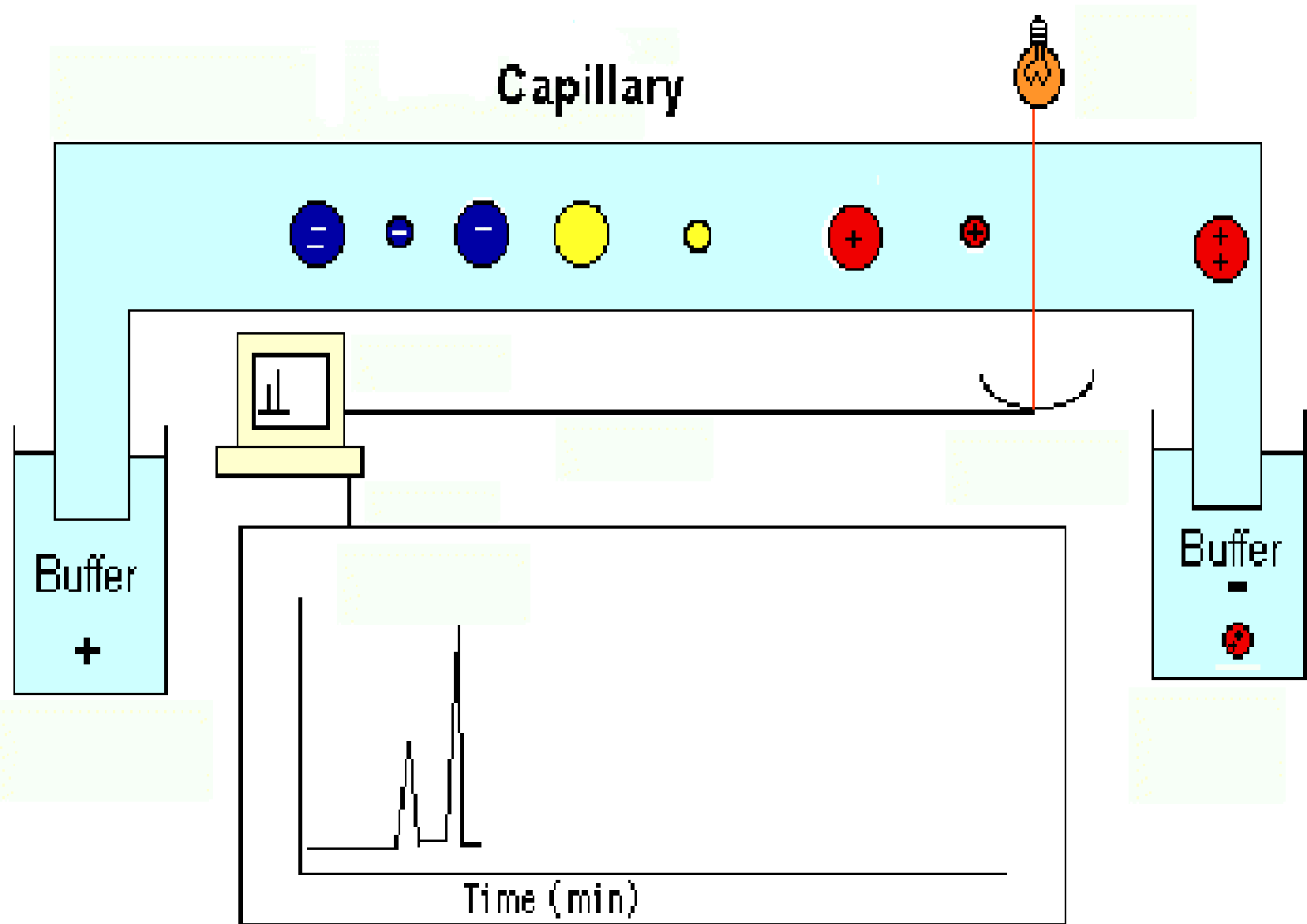
- In fact **different ions** can be separated when their **charge/size ratio** differs.

Before reaching the end of the capillary, the separated analyte bands are detected directly through the capillary wall.





Capillary

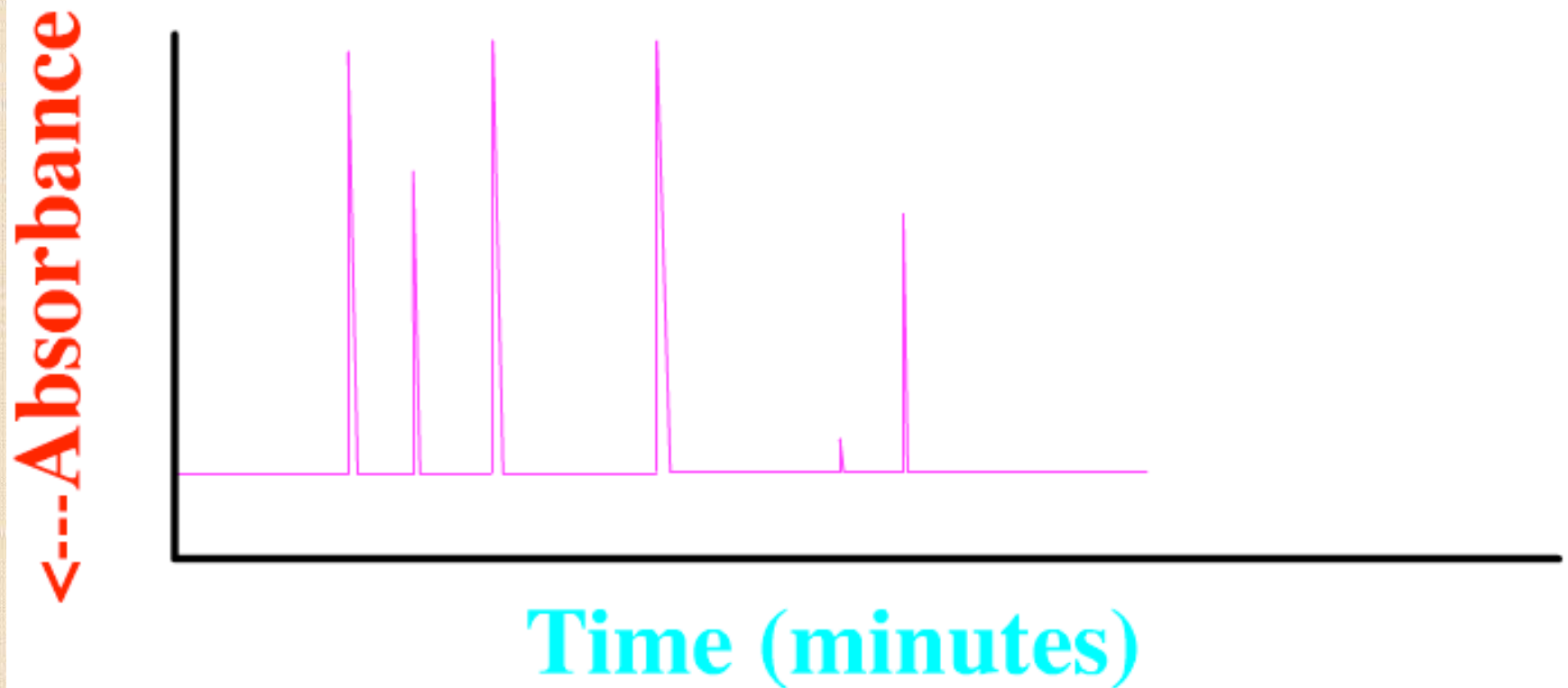




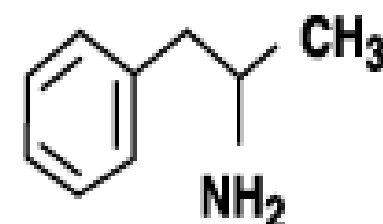
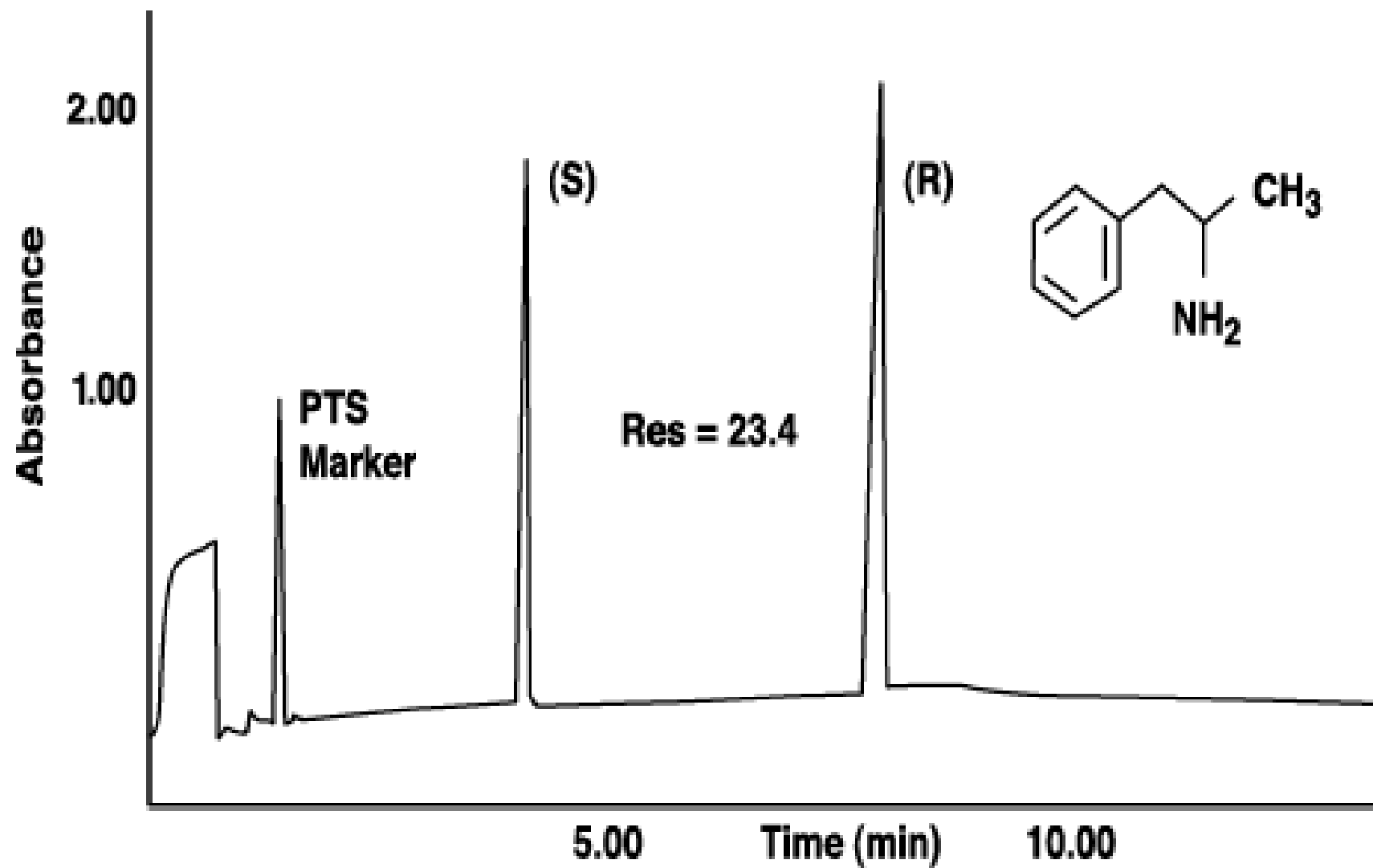
- An *electropherogram* is a plot of results from an analysis done by electrophoresis automatic sequencing.
- The CE electropherogram is a plot of *the time from injection* on the x axis, *the detector signal* on the y.



- *The electropherogram* example is shown below.







**5%  $\gamma$ -HSCD**







## *Electroosmotic Flow (EOF)*

- A vitally important feature of **CE** is the bulk flow of liquid through the capillary.
- This is called the **EOF** and is caused as follows. An uncoated fused-silica capillary tube is typically used for **CE**.
- The surface of the inside of the tube has ionisable **silanol groups**, which are in contact with the buffer during **CE**. These silanol groups readily dissociate, giving the capillary wall a **negative charge**.



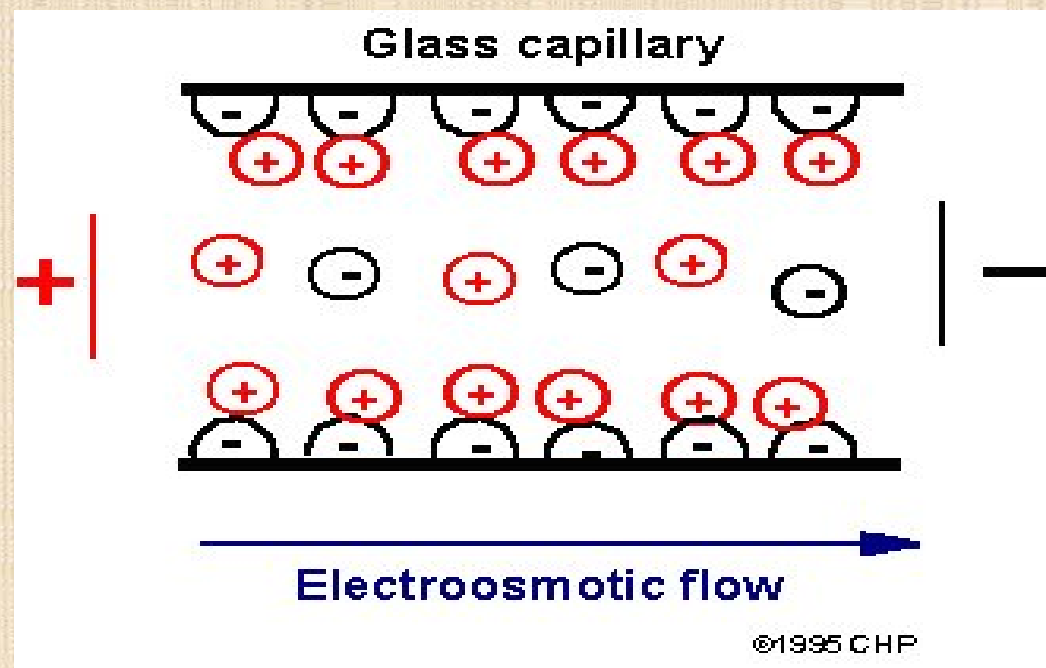
- Therefore, when the capillary is filled with buffer, the *negatively charged capillary wall* attracts *positively charged ions* from the *buffer solution*, creating an electrical double layer and a potential difference close to the capillary wall.



- Stern's model for an electrical double layer includes a rigid layer of adsorbed ions and a diffuse layer, in which ion diffusion may occur by thermal motion.
- The zeta potential is the potential at any given point in the double layer and decreases exponentially with increasing distance from the capillary wall surface.



When a voltage is applied across the capillary, cations in the diffuse layer are free to migrate towards the cathode, carrying the bulk solution with them. *The result is a net flow in the direction of the cathode,*





## *Based on the separation mechanism;*

- capillary zone electrophoresis (CZE)
- micellar electrokinetic chromatography (MEKC)
- micro-emulsion electrokinetic chromatography (MEECK)
- capillary gel electrophoresis (CGE)
- capillary isoelectric focusing (CIEF)
- capillary isotachopheresis (CITP)
- capillary electrochromatography (CEC)



## *Some of the advantages of the CE include:*

- high separation efficiency
  - short analysis time
  - low sample and electrolyte consumption
  - low waste generation
  - ease of operation
- 
- CE is *a rapidly growing separation technique*.
  - One of the main advantages of it, is its ability to inject *extremely small volumes* of sample.
  - The other greatest advantage is its *diverse application range*.



Some of its main application fields include:

- i) food analysis,
- ii) pharmaceutical analysis,
- iii) bioanalysis,
- iv) environmental pollutants analysis.

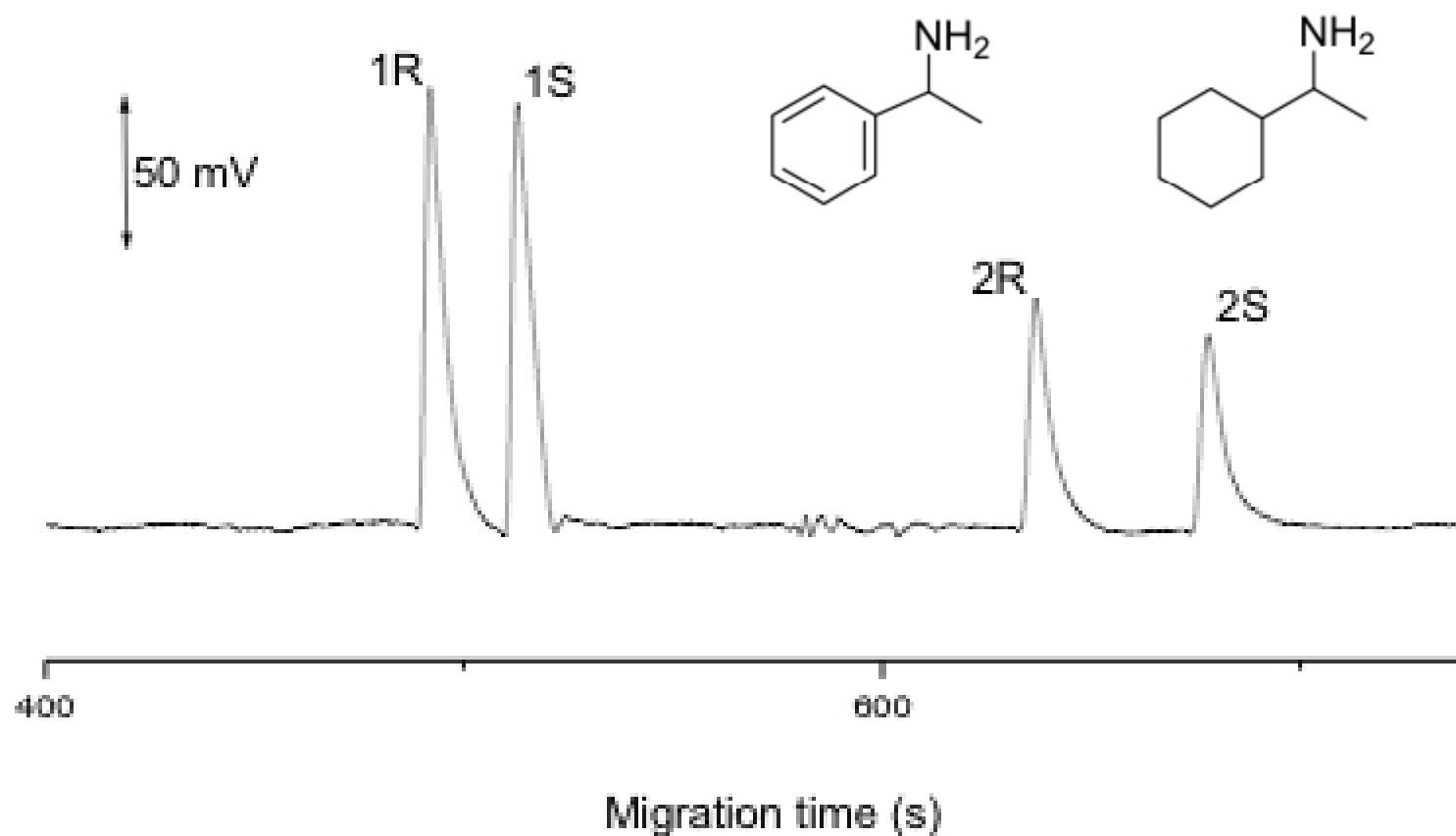


## Valuable applications of CE include:

- Genetic analysis
- Analysis of pharmaceuticals (containing nitrogenous bases)
- Pharmaceuticals with chiral centers (enantiomers)
- Counter-ion analysis in drug discovery
- Therapeutic Protein Characterization
- Protein characterization
- Carbohydrate analysis for the determination of post translational modifications



- Separation of the optical isomers of phenylethylamine and of cyclohexylethylamine.*



The determination of the enantiomers of chiral compounds is an important field of application of CE.



*Visit the link below!*

- [http://www.shsu.edu/~chm\\_tgc/sounds/flashfiles/CE.swf](http://www.shsu.edu/~chm_tgc/sounds/flashfiles/CE.swf)

