HPLC – Theoretical aspects

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Origins of Liquid Chromatography

The Russian botanist Mikhail Tswett first used the term ‘Chromatography’ (Latin for ‘colored drawing’) in 1906, to describe the separation that occurred when solutions of plant pigments were passed through columns of calcium carbonate or alumina, using petroleum ether.

Why Choose Liquid Chromatography

The two main chromatographic techniques used in modern analytical chemistry are Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC).

HPLC uses a liquid mobile phase to transport the analytes (sample) through the column, which is packed with a stationary phase material.

In contrast, Gas Chromatography uses a gaseous mobile phase to transport sample components through either packed columns or hollow capillary columns.

In most cases, GC columns have smaller internal diameter and are longer than HPLC columns.

GC

Samples analyzed by GC must be volatile (have a significant vapor pressure below 250 °C)

Derivatization to increase analyte volatility is possible but cumbersome and introduces possible quantitative errors .

Most GC analytes are under 500 Da Molecular Weight for volatility purposes

HPLC

HPLC analysis has no volatility issues, however the analyte must be soluble in the mobile phase.

HPLC can analyse samples over a wide polarity range and is able to analyse ionic samples. Mobile phase components are selected to ensure sample solubility.

HPLC has no real upper molecular weight limit and large proteins of many thousands of Daltons may be analysed.

Solubility in the mobile phase may preclude the analysis of very large molecules.

Chromatographic Separation Mechanisms

HPLC separations involve both the mobile phase (a liquid) and the stationary phase (usually materials of varying hydrophobicity chemically bonded to a solid support).

In contrast, GC uses the mobile phase only to carry the analyte through the column.

As an illustration, the amount of water in an HPLC mobile phase will determine how strongly a hydrophobic analyte is repelled into the stationary phase, and how well it is retained.

The chemical nature of the stationary phase will also govern how strongly the analyte is retained. For this reason, HPLC is a technique that is driven by the ‘selectivity’ achieved using two interacting phases

The Chromatogram

As the components elute from the column they pass into a detector – where some physicochemical property of the analyte produces a response from the detector. This response is amplified and plotted against time – giving rise to a ‘chromatogram’.

Gaussian – following the shape of a ‘normal’ distribution as described by ‘Gauss’ – used to describe a ‘symmetrical’ bell shaped peak.

Theory of HPLC

Theoretical principles of HPLC separation technique is explained with two theories

- Plate theory
- Rate theory

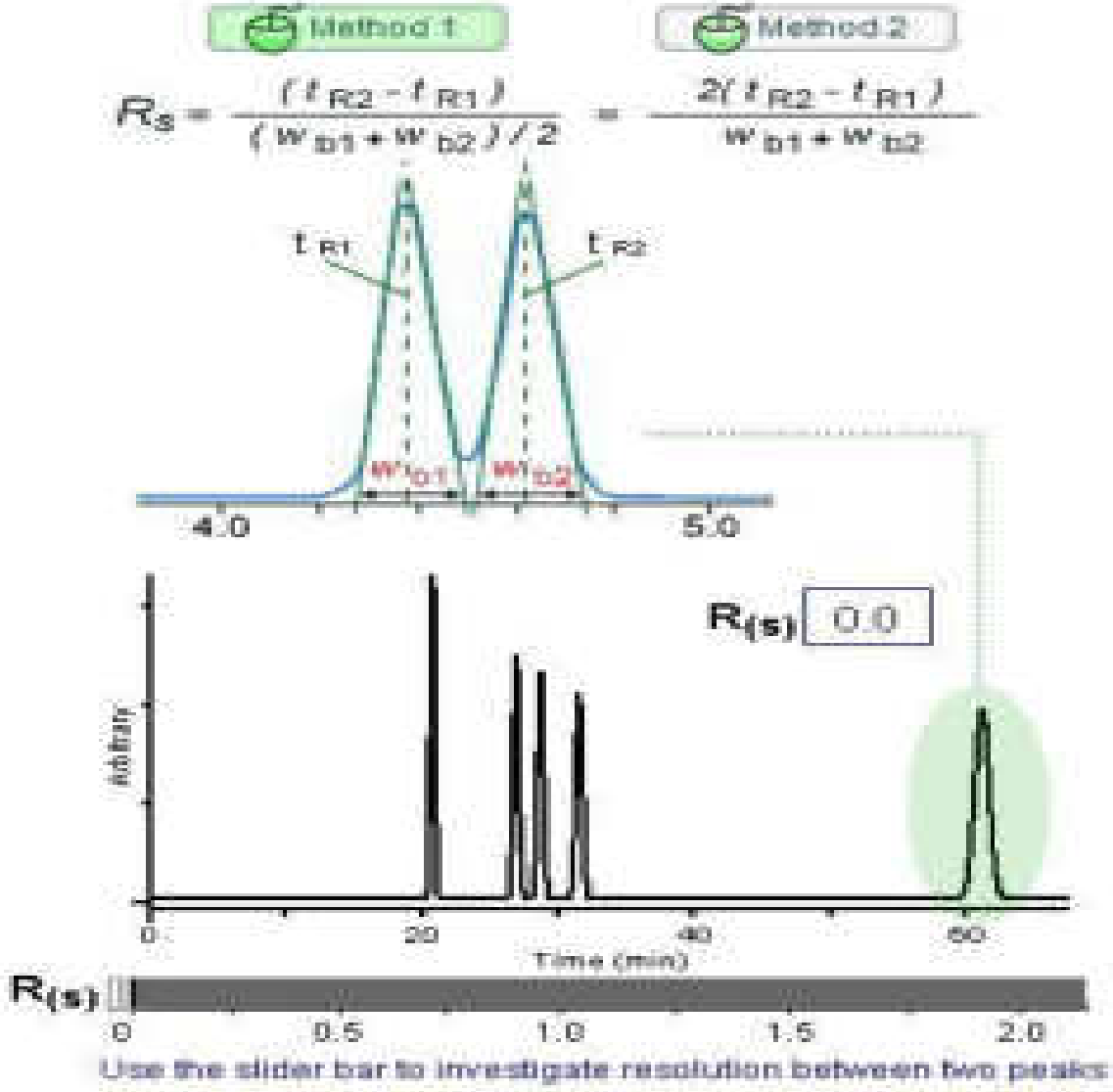
Chromatographic Parameters

The following are some chromatographic parameters explained as part of theoretical aspects.

- Resolution
- Capacity factor
- Selectivity factor
- Column efficiency
- Peak symmetry

Resolution (Rs)

- The most important thing in HPLC is to obtain the optimum resolution in the minimum time.
- A resolution value of 1.5 or greater between two peaks will ensure that the sample components are well (baseline) separated to a degree at which the area or height of each peak may be accurately measured.



Calculation of Chromatographic Resolution (RS)

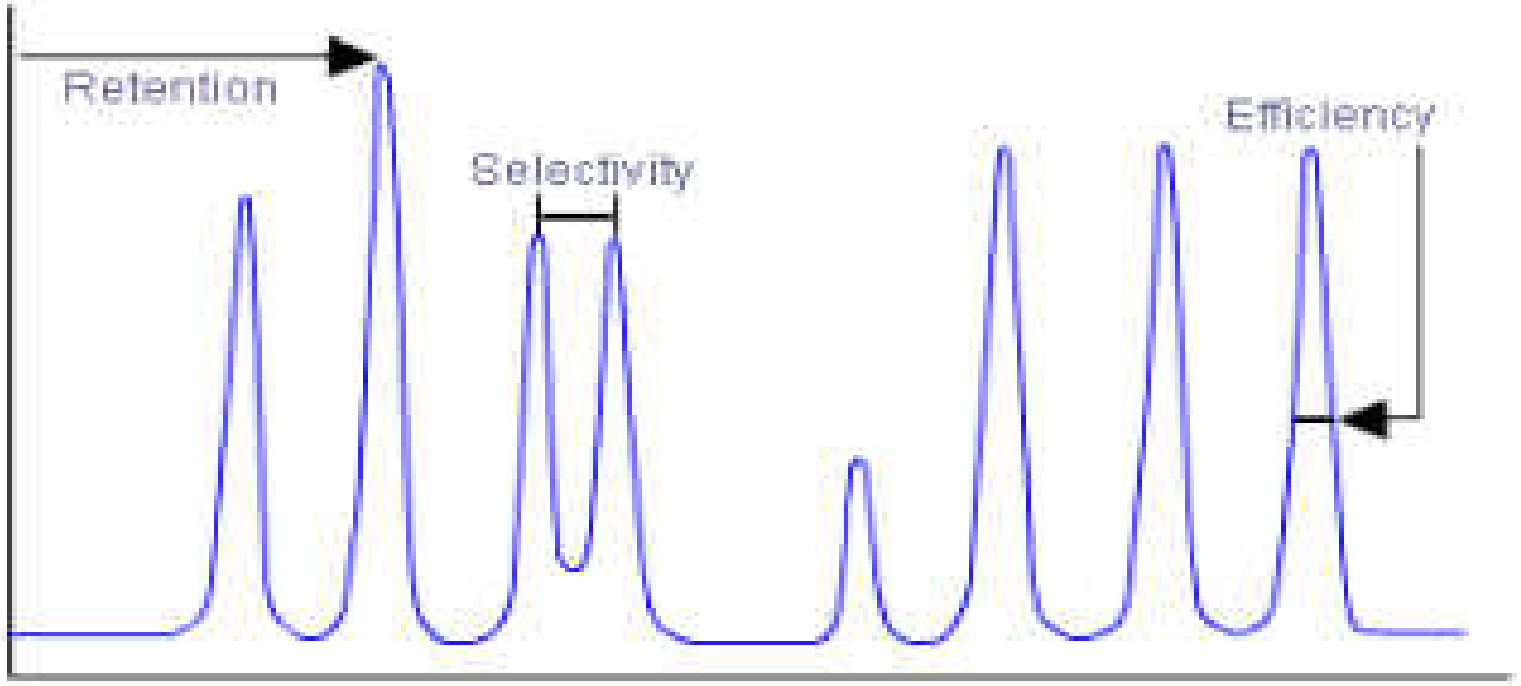
The Resolution Equation

The Fundamental Resolution Equation (shown) indicates that resolution is affected by three important parameters:

1. Selectivity (Separation factor)
2. Efficiency
3. Retention (Capacity factor)

$$R_S = \underbrace{1/4\sqrt{N}}_{\text{Efficiency}} \times \underbrace{\frac{\alpha - 1}{\alpha}}_{\text{Selectivity}} \times \underbrace{\frac{k}{1+k}}_{\text{Retention}}$$

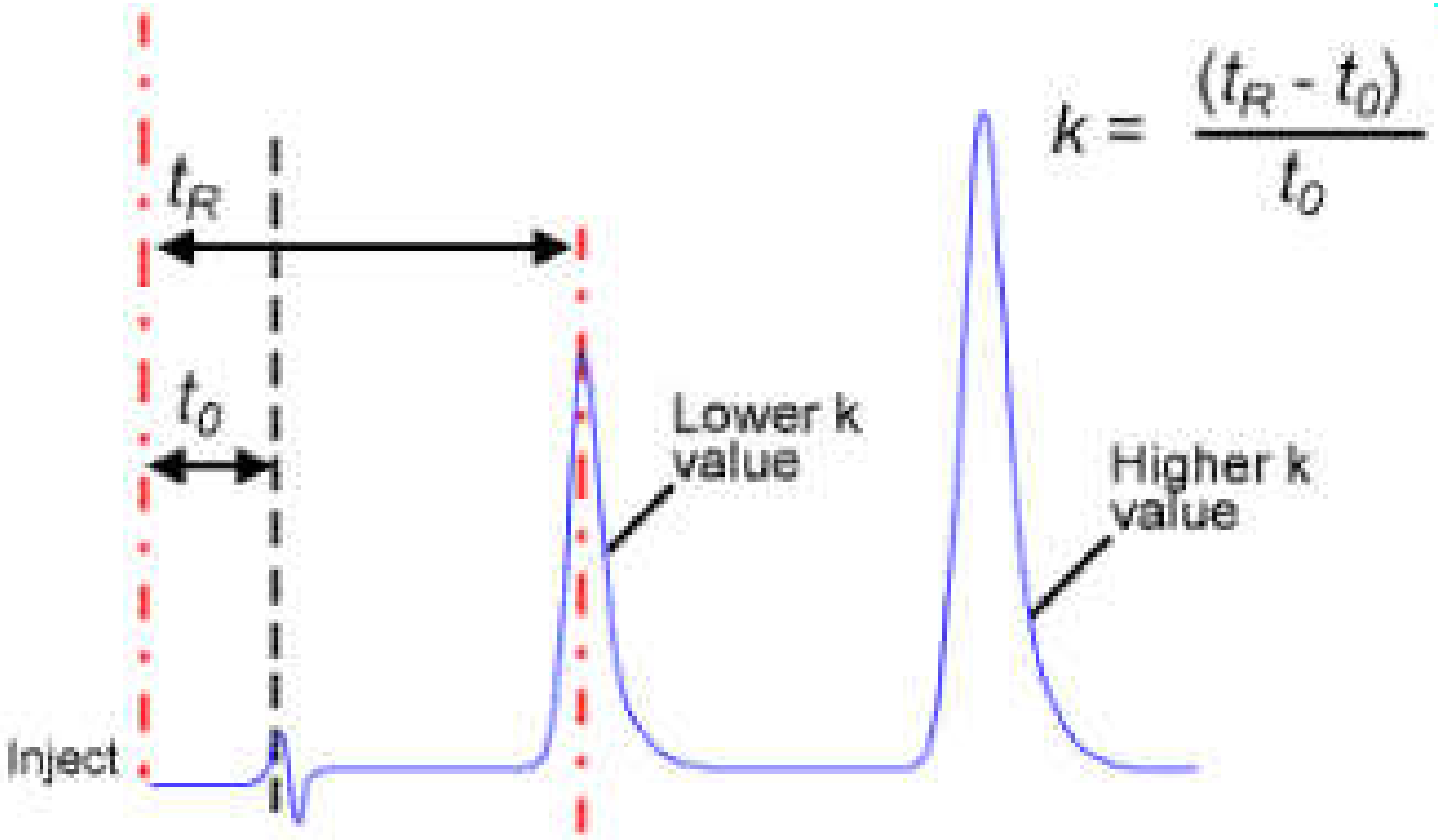
The Fundamental Resolution Equation



The Fundamental Resolution Equation and contributing parameters

- **Retention (Capacity) Factor (k)**

The retention (or capacity) factor (k) is a means of measuring the retention of an analyte on the chromatographic column.



Determination of Retention Factor (k)

- A high k value indicates that the sample is highly retained and has spent a significant amount of time interacting with the stationary phase.
- The retention factor is equal to the ratio of retention time of the analyte on the column to the retention time of a non-retained compound. The non-retained compound has no affinity for the stationary phase and elutes with the solvent front at a time t0, which is also known as the ‘hold-up time’ or ‘dead time’.

There are several ways to determine t0 including:

- The time at the baseline disturbance seen due to differences in absorbance or refractive index as the injection solvent passes through the detector
- Retention time of uracil (reversed phase HPLC)
- Retention time of hexane (normal phase HPLC)

- Retention factor is independent of some key variable factors including small flow rate variations and column dimensions.
- Therefore, it is a useful parameter when comparing retention of chromatographic peaks obtained using different HPLC systems.

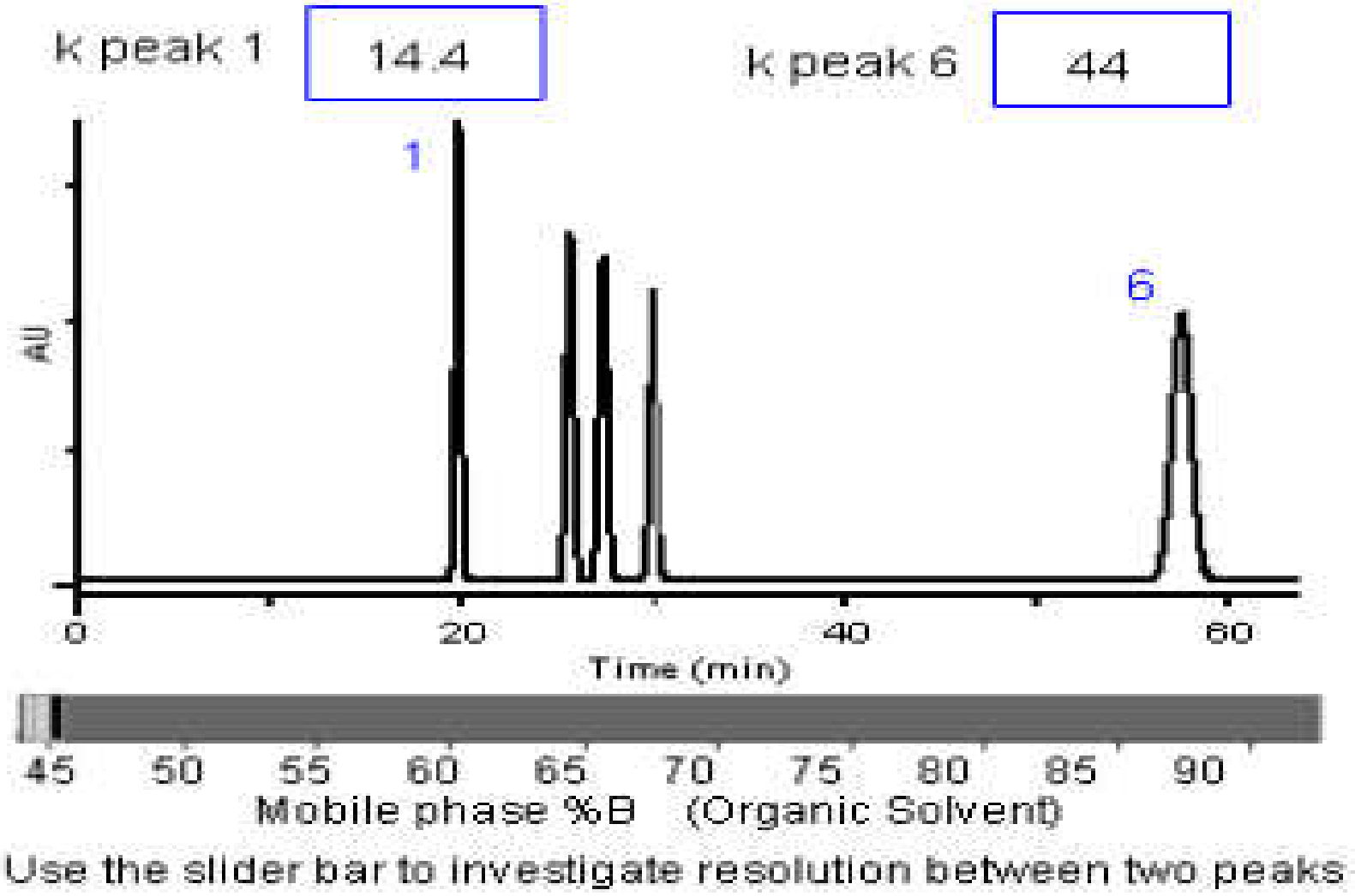
Calculation of Typical Retention Factor (k) values

Chromatographers like to keep k values between 1 and 10 for good separations. If the t_0 time of the system was 1.0 minute, this would equate to a retention time range of:

$$t_R = (k \times t_0) + t_0 = \underset{(k=1)}{2.0\,\text{min}} \quad \text{to} \quad \underset{(k=10)}{11.0\,\text{min}}$$

How to change Retention (Capacity) Factor

- The most effective and convenient way to alter the retention factor of a peak is to adjust the ‘solvent strength’ of the chromatographic mobile phase. This is usually achieved in reversed phase chromatography by changing the amount of organic solvent (modifier) in the mobile phase mixture.
- Characteristically reversed phase HPLC has a non-polar stationary phase, therefore, increasing the polarity of the mobile phase will increasingly repel the hydrophobic (non-polar) sections of the analyte molecules into the stationary phase and the analyte will be retained for longer on the column. The converse is also true as can be seen in the interactive example.
- An increase in the organic content of the mobile phase of 10% decreases k for each component by a factor of 2 to 3. Altering the mobile phase composition is a very powerful separation tool to use when optimizing HPLC separations.



Effect on Retention Factor (k) values of changing mobile phase organic composition

Example

Analytical conditions:

Column: C18, 15 cm × 0.46 mm × 5 µm

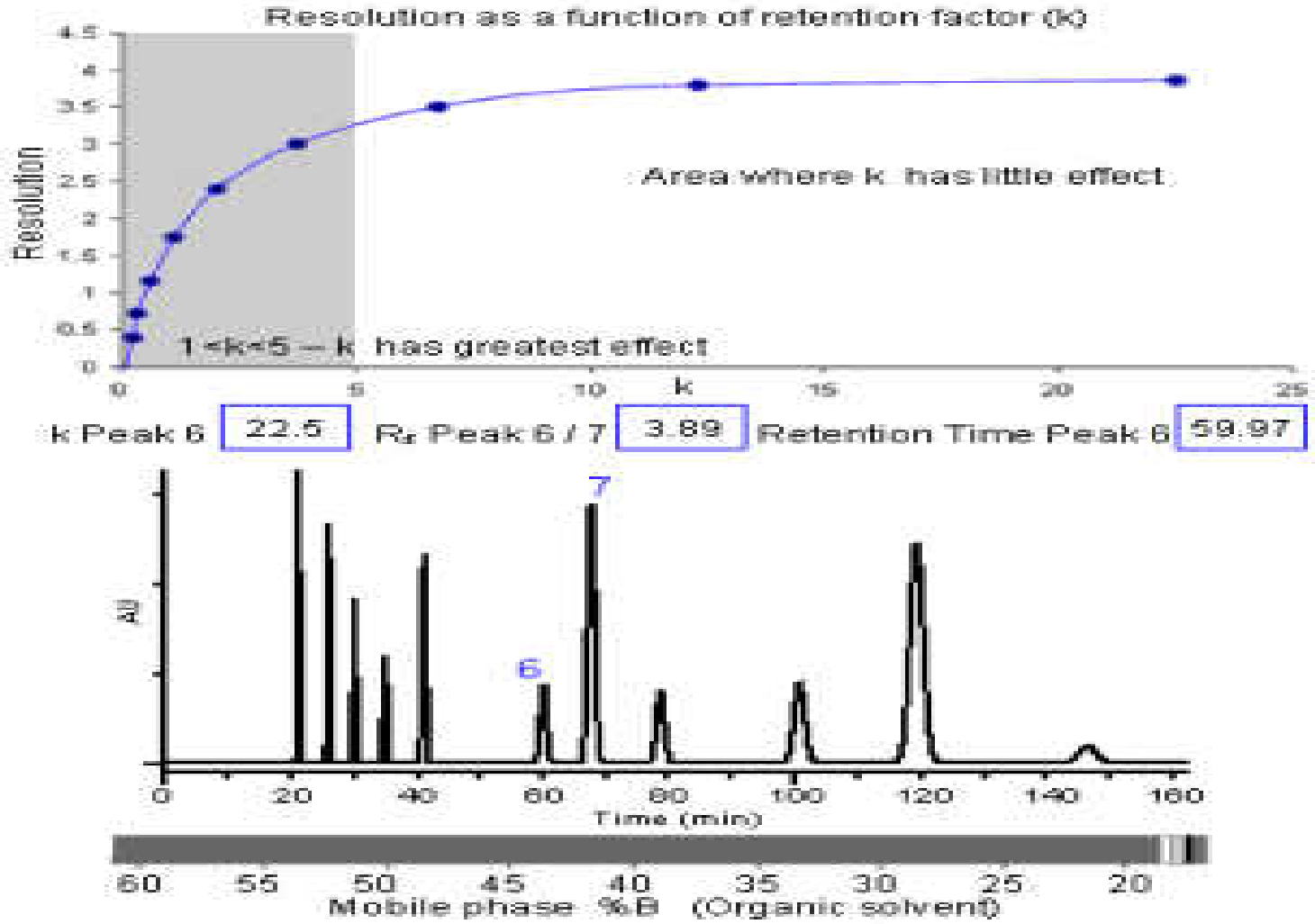
Flow: 2.0 mL/min

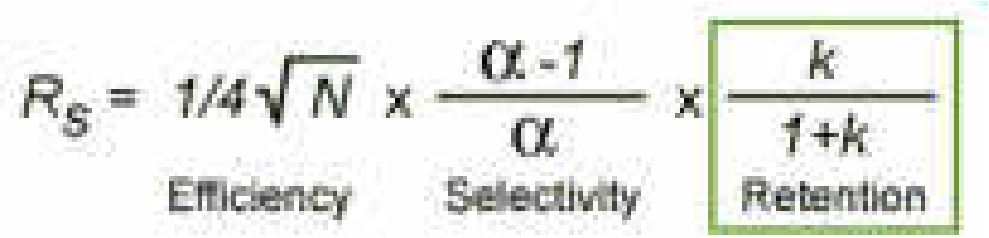
t0: 1.28 min

- At low mobile phase organic solvent composition, the retention factor is high – the analytes are interacting strongly with the stationary phase
- Increasing the organic composition by 10%B brings about a 2-3 fold reduction in retention factor

When retention factors are very high or very low, the quality of the separation is reduced. Retention factors below 1, for any of the analytes, generally indicate that the separation will be poor

Effect of Altering Retention Factor (K) (by altering mobile phase composition) on Resolution





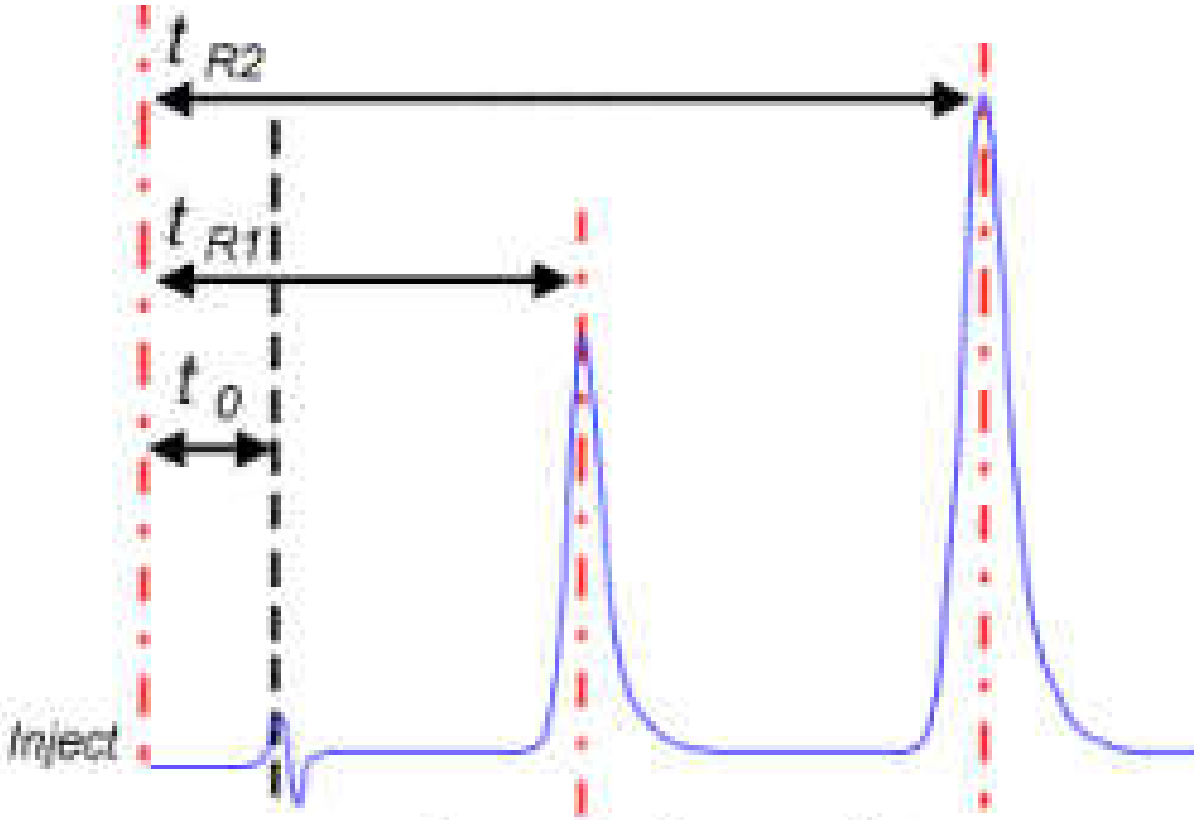
- The largest gain in resolution is achieved when the k value is between 1 and 5.
- As k is very simple to change it is often worthwhile adjusting the k range of peaks within the chromatogram to obtain an optimum resolution
- k values less than 1 are unreliable as analytes may be eluting with other sample components or solvent.

- Above a k value of approximately 5, increasing retention only provides minimal increases in resolution.
- Too much retention wastes valuable analysis time and the chromatographic peak height will decrease as the bandwidth of the peaks increases.
- For more complex sample mixtures, the useful k range may be extended to $2 < k < 10$.
- If you have not achieved the desired resolution and the k values of your sample components are above 10, you will find that increasing the selectivity or efficiency of your separation will be more useful.

Selectivity (Separation) Factor (α)

- The selectivity (or separation) factor (α) is the ability of the chromatographic system to ‘chemically’ distinguish between sample components.
- It is usually measured as a ratio of the retention (capacity) factors (k) of the two peaks in question and can be visualized as the distance between the apices of the two peaks.

Determination of Selectivity (α)



$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

- High α values indicate good separating power and a good separation between the APEX of each peak. However, the alpha value is NOT directly indicative of the resolution.
- By definition, the selectivity is always greater than one – as when α is equal to one, the two peaks are co-eluting (i.e. their retention factor values are identical).
- The greater the selectivity value, the further apart the apices of the two peaks become.
- As the selectivity of a separation is dependent upon the chemistry of the analyte, mobile, and stationary phases all of these factors may be altered in order to change or optimize the selectivity of an HPLC separation.

How to change Selectivity (Separation) Factor (α)

Some of the many factors that can be used to manipulate the selectivity of HPLC separations are shown in Table 1.

Table 1. Parameters affecting selectivity in reversed phase HPLC

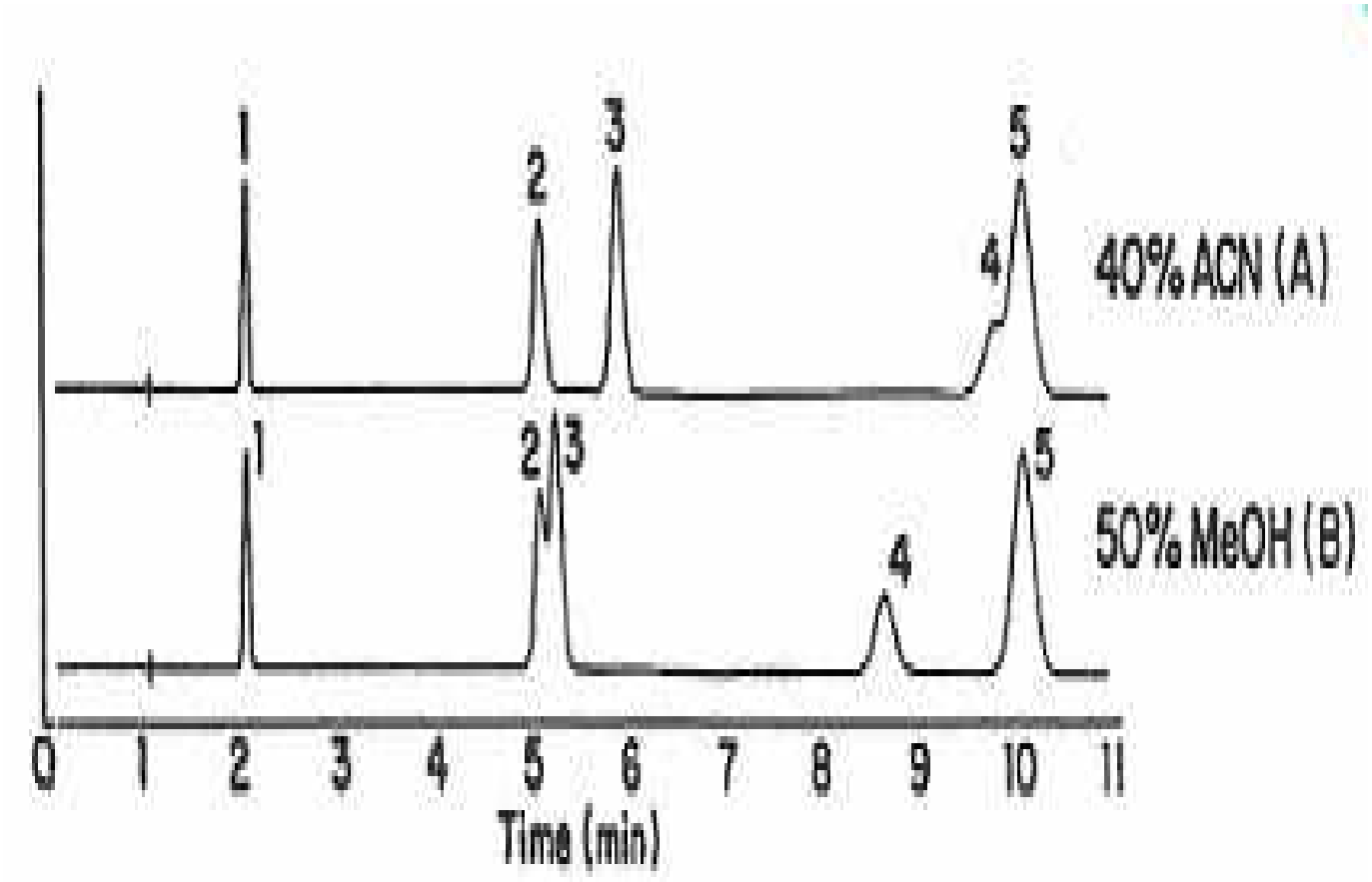
Parameter	Usage
Sub 2: Organic solvent	Changing to a different solvent (e.g. methanol to acetonitrile in reversed phase HPLC) will alter the selectivity
Sub 3: Mobile phase pH	Can alter the degree of ionization of some analytes – affecting their hydrophobicity
Sub 4: Solvent strength and additives	Can be adjusted to affect selectivity as well as retention (capacity) factor
Sub 5: Stationary phase	One of the most popular ways to alter the selectivity of a separation
Sub 6: Temperature	Can have an effect with certain analytes in reversed phase and Chiral HPLC

Organic Solvent Type

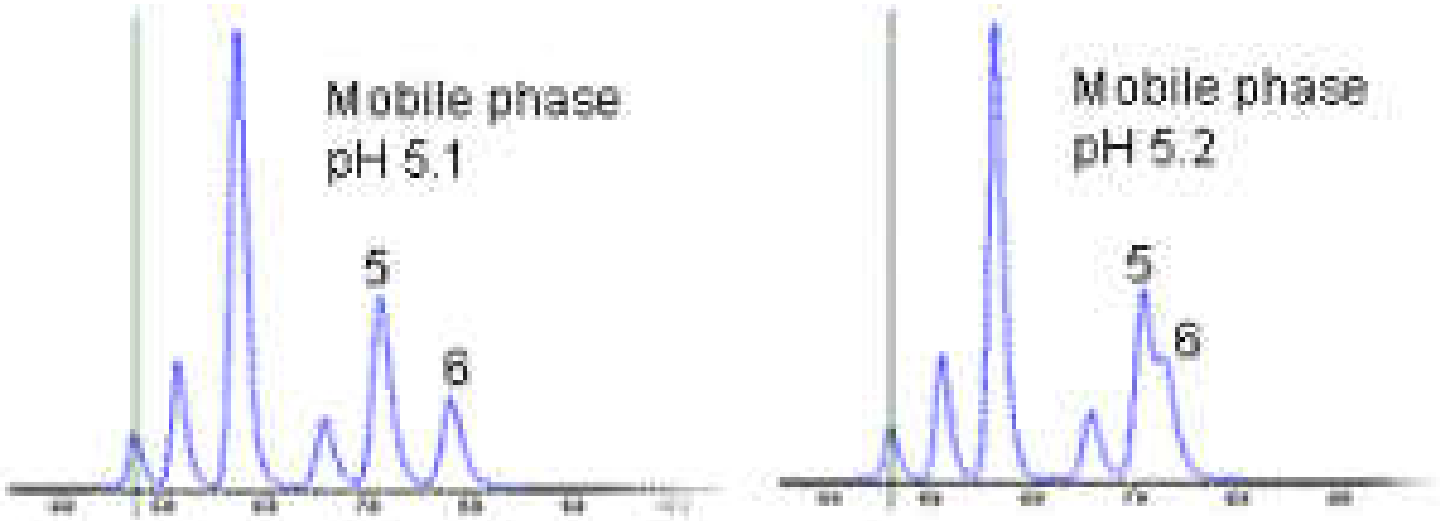
In this example, the organic solvent has been changed, and the solvent strength altered to make the eluent systems isoeluotropic (i.e. the k value of the last peak is the same in both cases).

It can be clearly seen that although the overall analysis time is the same, the selectivity of the separation between peaks 2 and 3 and peaks 4 and 5 is different when methanol is substituted for acetonitrile.

Effect of changing organic modifier type on chromatographic selectivity in Reversed Phase HPLC



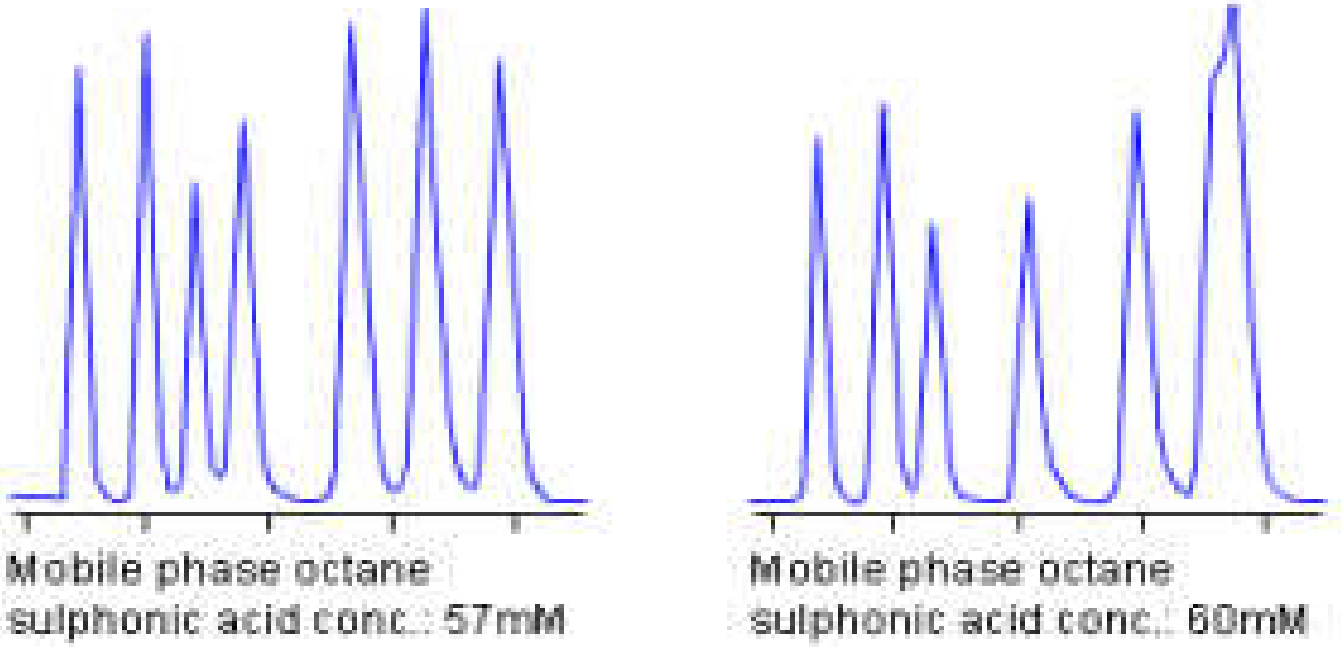
- **Mobile Phase pH**



Effect of changing mobile phase pH on chromatographic selectivity in Reversed Phase HPLC

- In this reversed phase separation of acidic analytes on a C8 column it is clear that a change in pH of just 0.1 units can bring about a substantial change in selectivity between peaks 5 and 6.
- The pH of the mobile phase is usually a key parameter for selectivity optimization when dealing with analyte molecules that have ionizable groups.
- Changes in mobile phase pH should be undertaken carefully as not all silica based HPLC columns are resistant to extremes of pH.

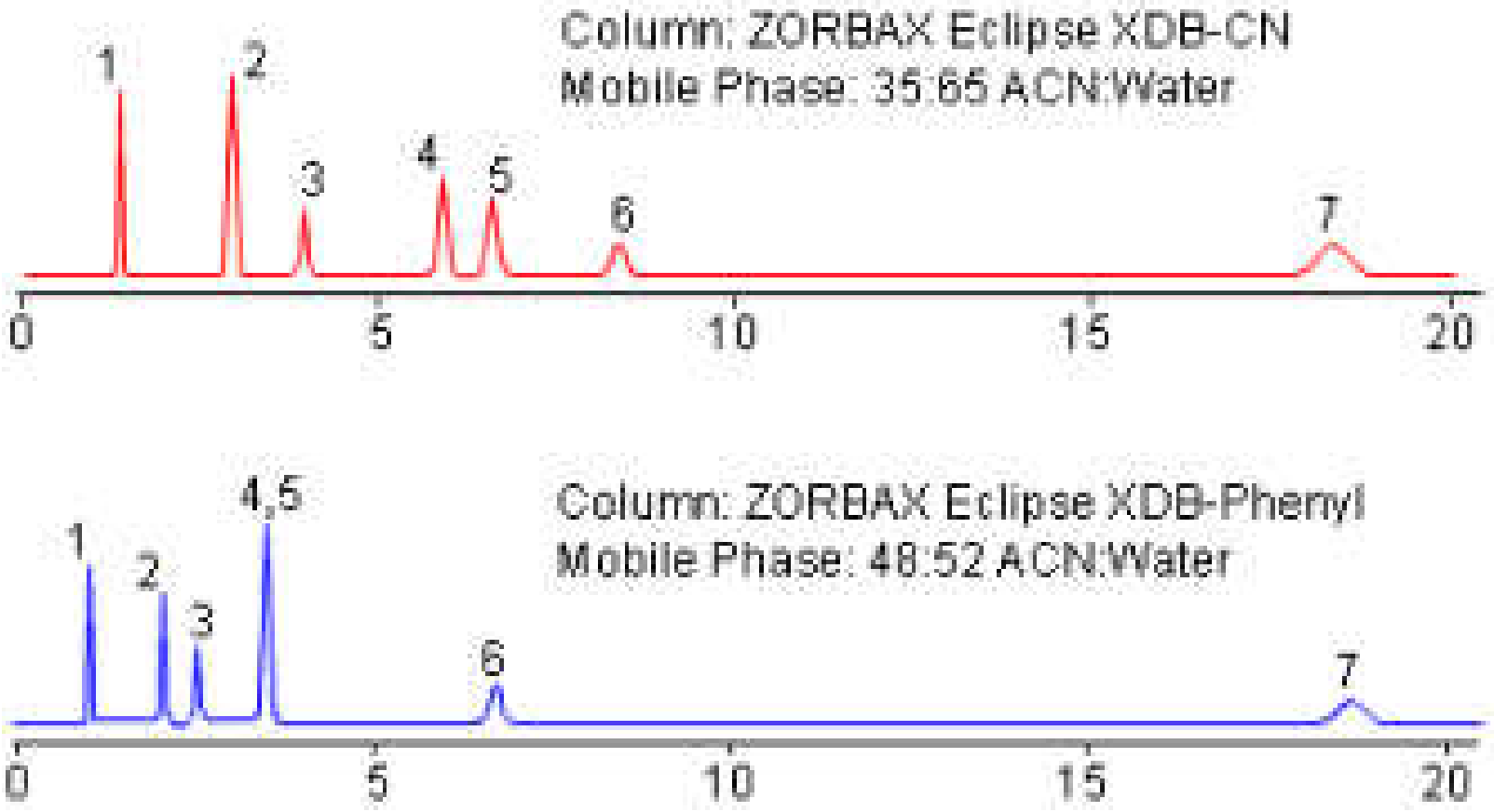
Solvent Strength and Additives



Changes in separation selectivity caused by small alterations to mobile phase ion-pair concentration in reversed phase HPLC

- In this example of changing selectivity, octanesulfonic acid (an ion-pairing reagent), has been added to the mobile phase in differing concentrations.
- As can be seen, even small adjustments in ion-pair concentration can produce large differences in the chemical separating power of the chromatographic system.
- The analytes in this case are basic pharmaceutical compounds, separated on a C18 column.

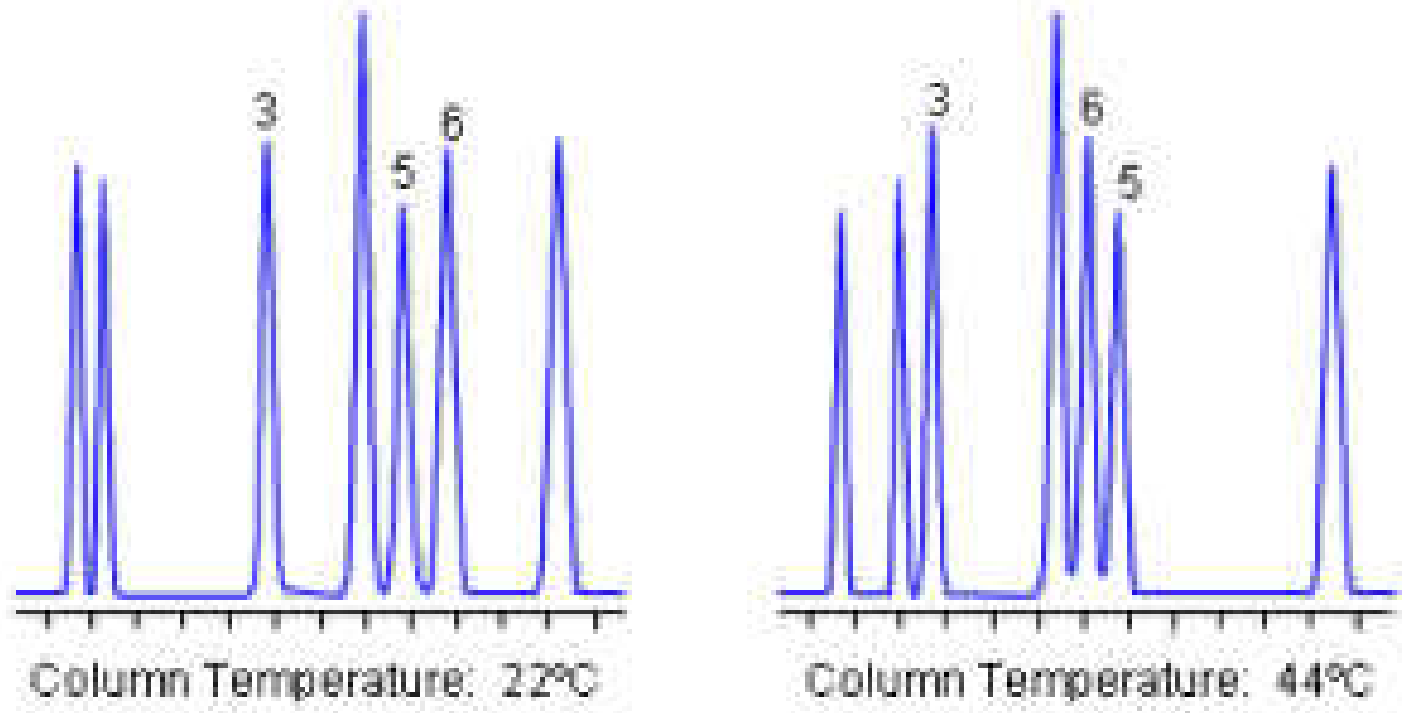
Stationary Phase Type



Changes in separation selectivity caused by altering the chemical nature of the stationary phase in reversed phase HPLC

- Here we can see how changing the column stationary phase can affect the selectivity of a separation. The hydrophobicity, polarity, and nature of the base silica all play a critical role in the physicochemical interaction with the analyte.
- As a chromatographer one of the most powerful options to change a separation will come from changing the stationary phase.
- Picking the correct stationary phase is amongst the most important choices to be made when developing methods.

Column temperature

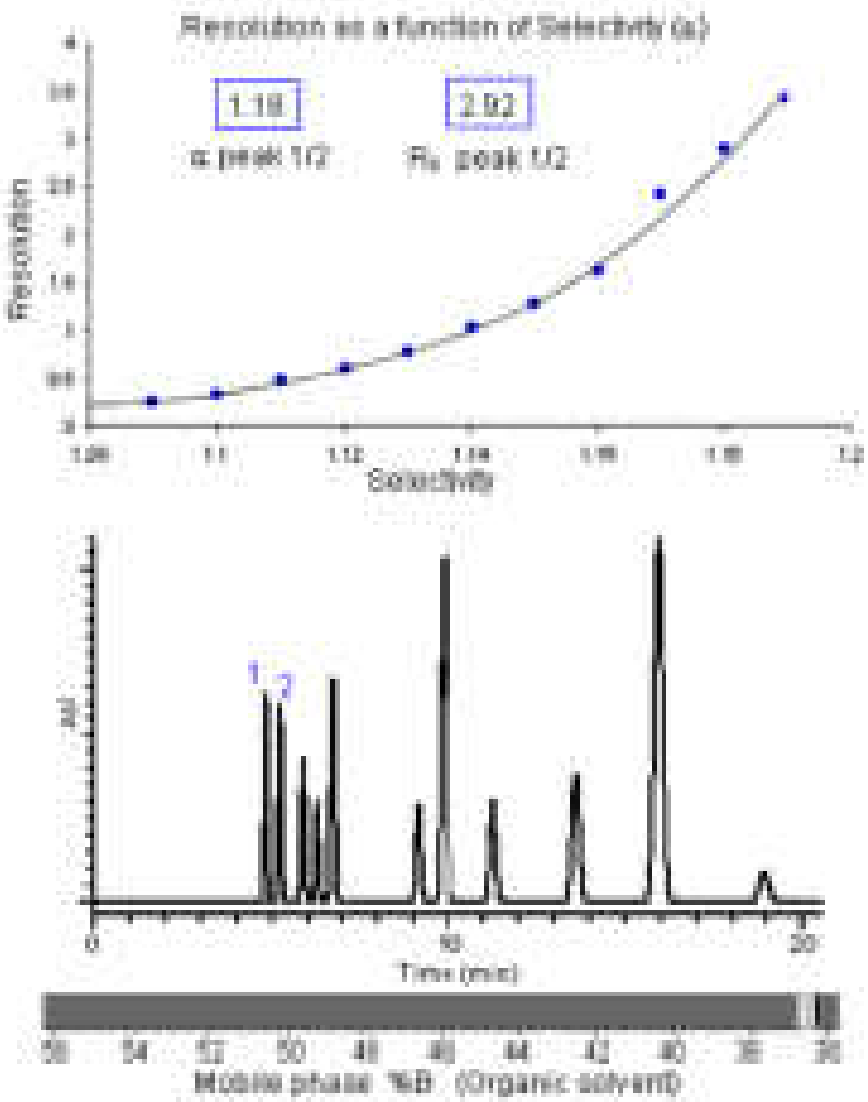


Changes in separation selectivity caused by altering the column temperature in reversed phase HPLC

- Temperature of the column (and hence the mobile phase) is used in this example to alter the selectivity of the separation.
- It is interesting to note that peaks 5 and 6 have swapped as the temperature is adjusted.
- Temperature produces the smallest changes in selectivity; however, it can be usefully used to optimize separations, especially when the analytes involved are ionizable.

- **Effects of Selectivity (α) on Resolution**
- The graph shown illustrates the effect of changing the selectivity (separation factor) on the resolution between two early eluting peaks in the chromatogram.

Effect of Altering Selectivity (α) (by altering mobile phase composition) on Resolution (RS)



- Changing selectivity can have a dramatic effect on the chromatographic resolution
- Selectivity is relatively simple to alter, with mobile phase constituents (solvent type, ion pair reagents etc.) and pH being the most frequently used methods of adjustment
- If suitable resolution cannot be achieved by altering the mobile phase constituents, an alternative column chemistry should be investigated as a means of altering the selectivity of the separation

Column Efficiency

Efficiency of column is evaluated using plate number and HETP values.

What is theoretical plate?

It is the state of equilibrium between the solute in the stationary phase and mobile phase.

HETP: Height equivalent to theoretical plate – the length of the column required for one equilibration is called HETP.

- The efficiency of a chromatographic peak is a measure of the dispersion of the analyte band as it travels through the HPLC system and column.
- In an ideal world, chromatographic peaks would be pencil thin lines, however, due to dispersion effects the peaks take on their familiar ‘Guassian’ shape.
- The plate number (N) is a measure of the peak dispersion on the HPLC column, which reflects the column performance.

- Efficiency is derived from an analogy of Martyn and Synge who likened column efficiency to fractional distillation, where the column is divided into Theoretical Plates.
- Each plate is the distance over which the sample components achieve one equilibration between the stationary and mobile phase in the column.
- Therefore, the more theoretical plates available within a column, the more equilibrations are possible, and the better quality the separation.

***Theoretical Plates* (*N*):**

The number of theoretical plates characterizes the quality or efficiency of a column.

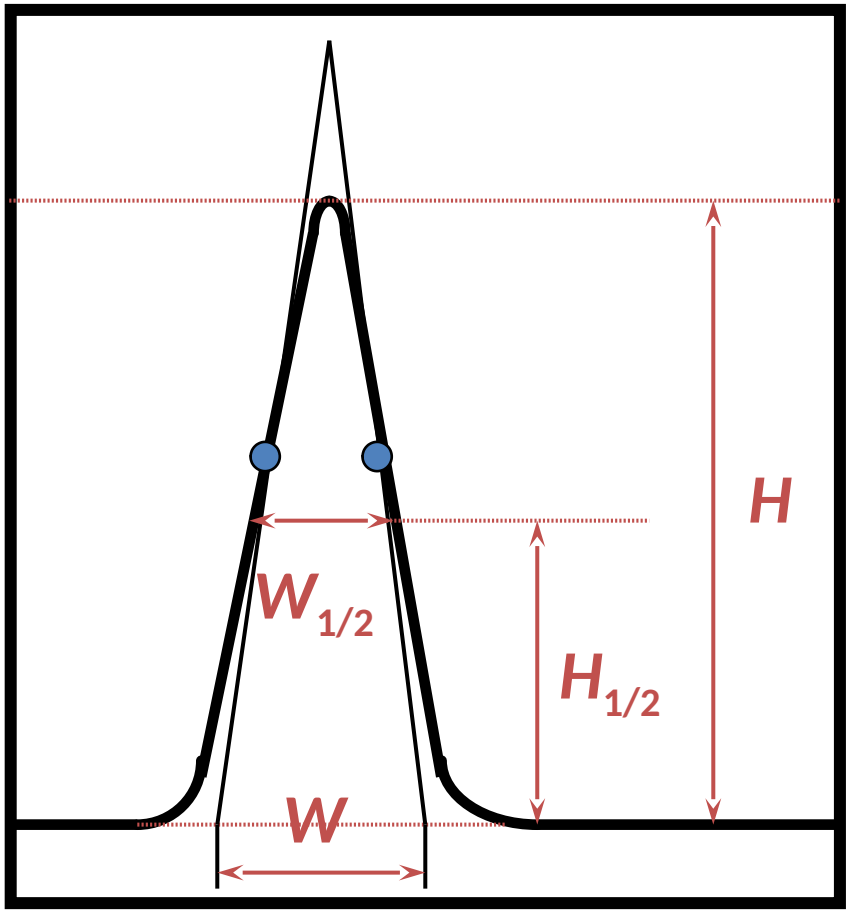
$$N = 5.54 [(tR) / w1/2]2$$

or

$$N = 16 (tR/W)2$$

Ideal N value is 50,000 plate per meter

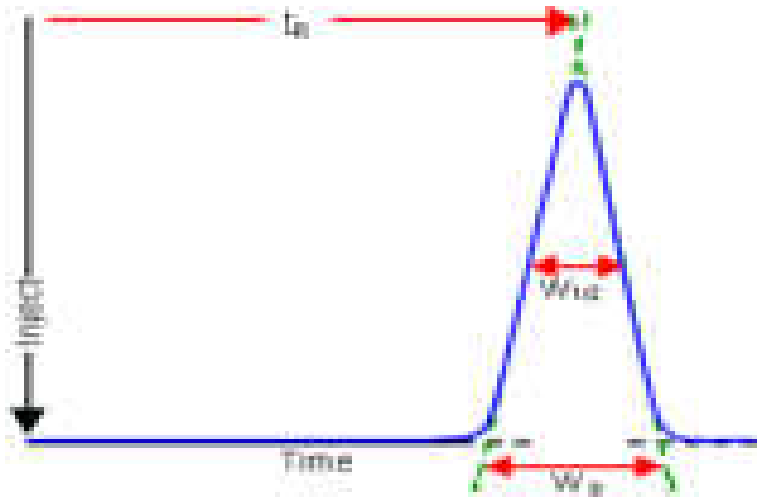
Theoretical Plate Number, *N*



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

$$= 5.54\left(\frac{t_R}{W_{1/2}}\right)^2$$

$$= 2\pi\left(\frac{t_R \bullet H}{Area}\right)^2$$



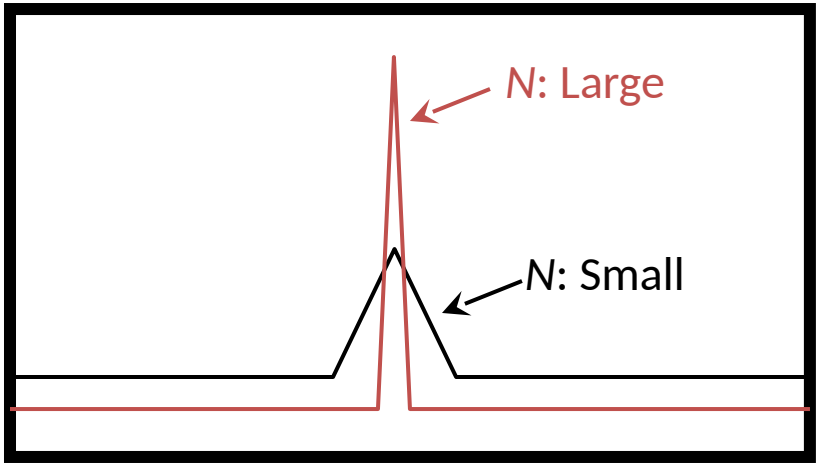
✓Higher values for the Plate Number (N) are expected for subsequent peaks within a chromatogram.

✓Later eluting peaks that look broad in comparison to early eluting peaks may have a higher plate count.

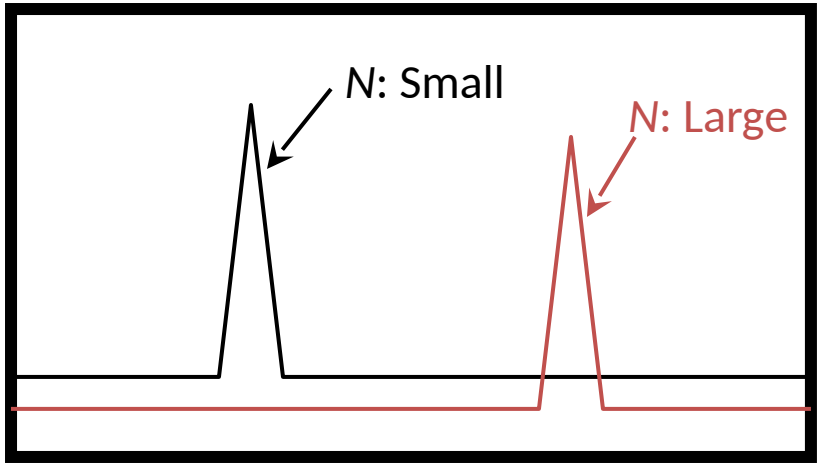
✓If this is not the case then your system contains a large extra-column dead volume!

Evaluation of Column Efficiency Based on Theoretical Plate Number

- If the retention times are the same, the peak width is smaller for the one with the larger theoretical plate number.



- If the peak width is the same, the retention time is longer for the one with the larger theoretical plate number.



- The method for calculating column efficiency is shown.
- A typical plate number for a 4.6 × 100 mm column with 5 µm particles is between 5000 and 8000.
- Of course, for a given column length, more plates means less dispersion of chromatographic bands, narrower peaks, and a better quality separation.
- This links directly to the concept of ‘Plate Height’, which is explained in the following sections.

- Similarly, for a fractionating tower of a given length (L), the higher the number of plates, the lower the distance between each plate, shown as plate height in the diagram.
- Therefore, for high efficiency separations, the plate number (N) will be high and the plate height (H) low. Note that plate height is often called ‘Height Equivalent to a Theoretical Plate (HETP)’

These two terms are related through the expression:

$$H = L/N$$

- The number of theoretical plates is often used to establish the efficacy of a column for a given method. The method developer may decide that a given method is no longer valid when the plate number falls below a predetermined value. At that time, the column would be replaced with a new one.

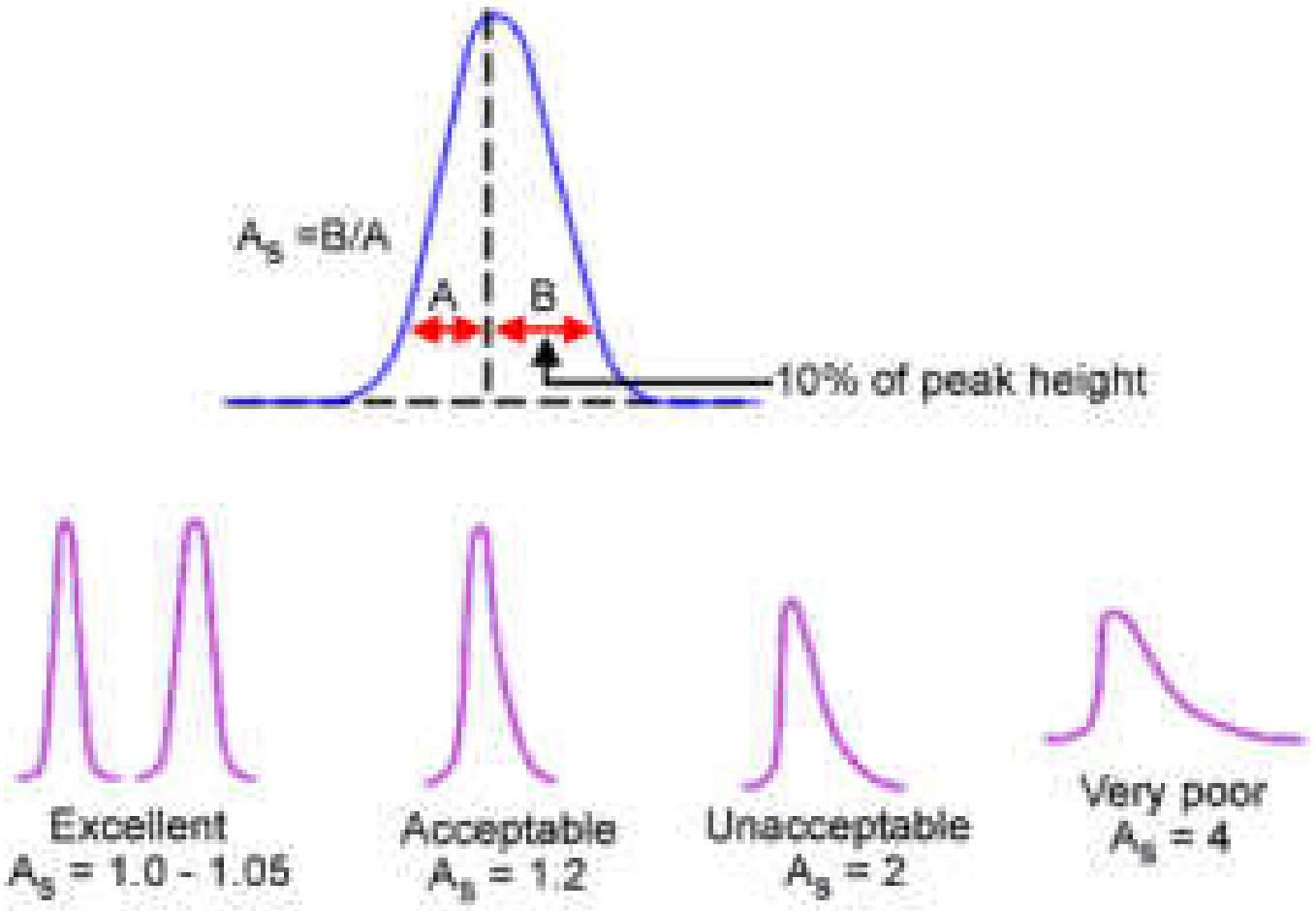
- As column length increases the peaks become narrower (more efficient)
- As the peak efficiency increases the separation quality increases
- As the column length is increased, the analysis time increases significantly (this should be intuitive)
- Increasing the column length by an order of magnitude (2.5 to 25 cm) the efficiency of the peaks also increases by about one order of magnitude
- 10,000 - 20,000 plates can be generated by a well-packed 15 × 0.46 cm column with 5 µm packing material

Peak shapes

Peak Asymmetry

- In the ideal world all chromatographic peaks would be symmetrical (or Gaussian).
- However, due to the effects of instrument dead-volume, adsorptive effects of the stationary phase and the quality of the column packing, peaks may often show a tailing behavior.
- Tailing describes a peak whose tail portion (distance ‘B’ in the diagram) is wider than the front portion (distance ‘A’ in the diagram).
- Also, if the sample concentration is too high or if the column is damaged and contains ‘channels’ then a fronting peak shape may occur.

Determination of Peak Asymmetry (AS) and examples of good and poor peak shape



- Asymmetric peaks often present problems with resolution and quantitation of the peaks within the chromatogram.
- Asymmetrical peaks are more difficult to resolve, therefore, integration of the peak to provide a peak area for quantitation will also be much less reproducible.
- Often, chromatographers will set limits for peak asymmetry beyond which chromatography will be deemed unsuitable. Some examples are shown for reference.

HPLC Column packing materials

Types of particles available for HPLC separations

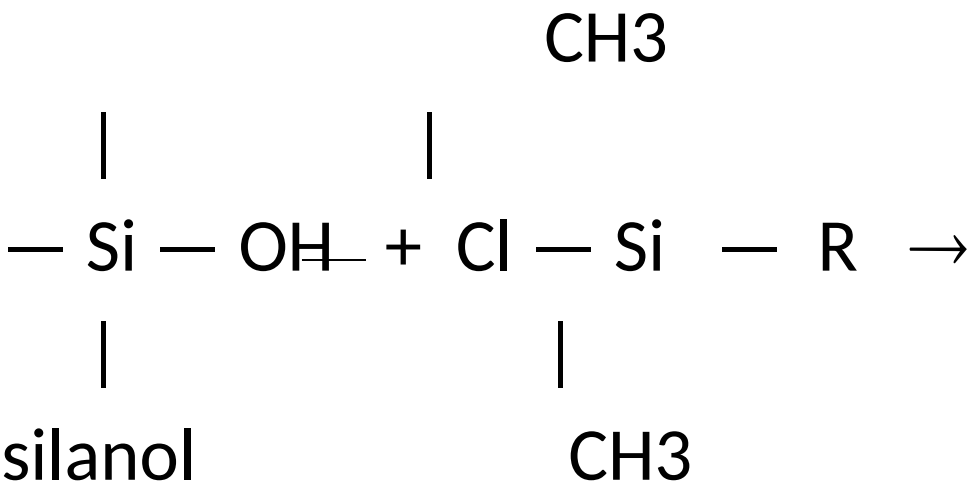
- Totally porous microsphreres 5 µm
- Micro pellicular 1.5 to 2.5 µm
- Perfusion particles 0.4 to 0.8 µm

- HPLC columns are usually packed with pellicular, or porous particles.
- Pellicular particles are made from polymer, or glass beads. Pellicular particles are surrounded by a thin uniform layer of silica, polystyrene-divinyl-benzene synthetic resin, alumina, or other type of ion-exchange resin.
- Porous particles are more commonly used and have diameters between 3 to 10 µm. Porous particles are made up silica, polystyrene-divinyl-benzene synthetic resin, alumina, or other type of ion-exchange resin.
- Silica is the most common type of porous particle packing material.

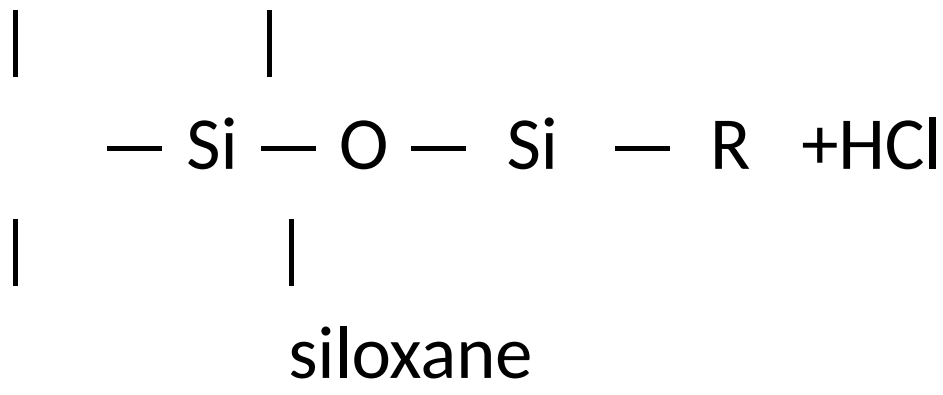
Bonded phases

- Partition HPLC uses liquid bonded phase columns, where the liquid stationary phase is chemically bonded to the packing material.
- The packing material is usually hydrolyzed silica which reacts with the bond-phase coating. Common bond phase coatings are siloxanes
- Silica particles with diameters 3, 5 and 10 µm are used as supports for preparation of bonded phases.
- Hydrolyzed by heating with 0.1M HCl for a day or two.
- Fully hydrolyzed silica is made up of chemical reactive silanol groups.
- Bonded phase coatings are siloxanes formed by reaction of the fully hydrolyzed silica surface with organochloro silane (silylation)

Silylation……..

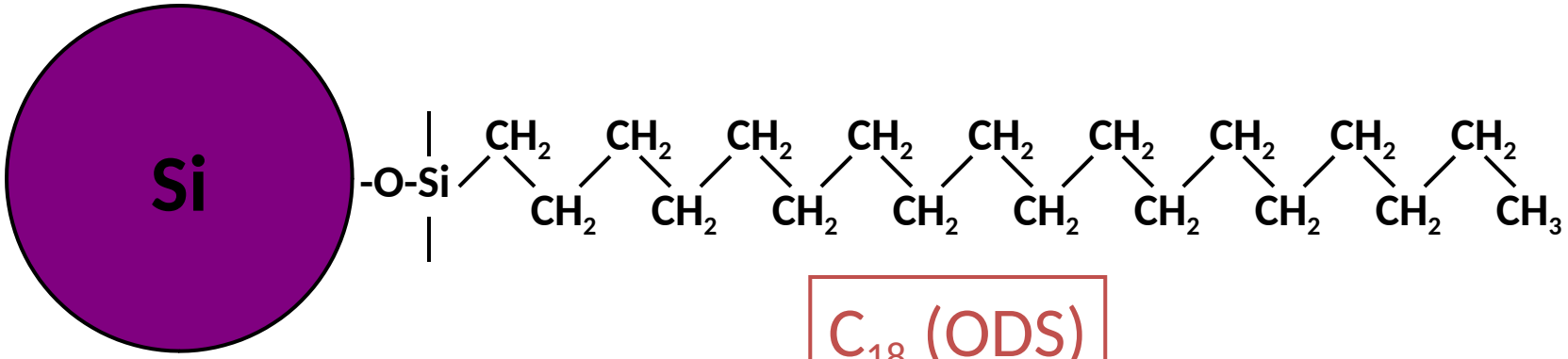


Where
R = an aklyl group or substituted alkyl group



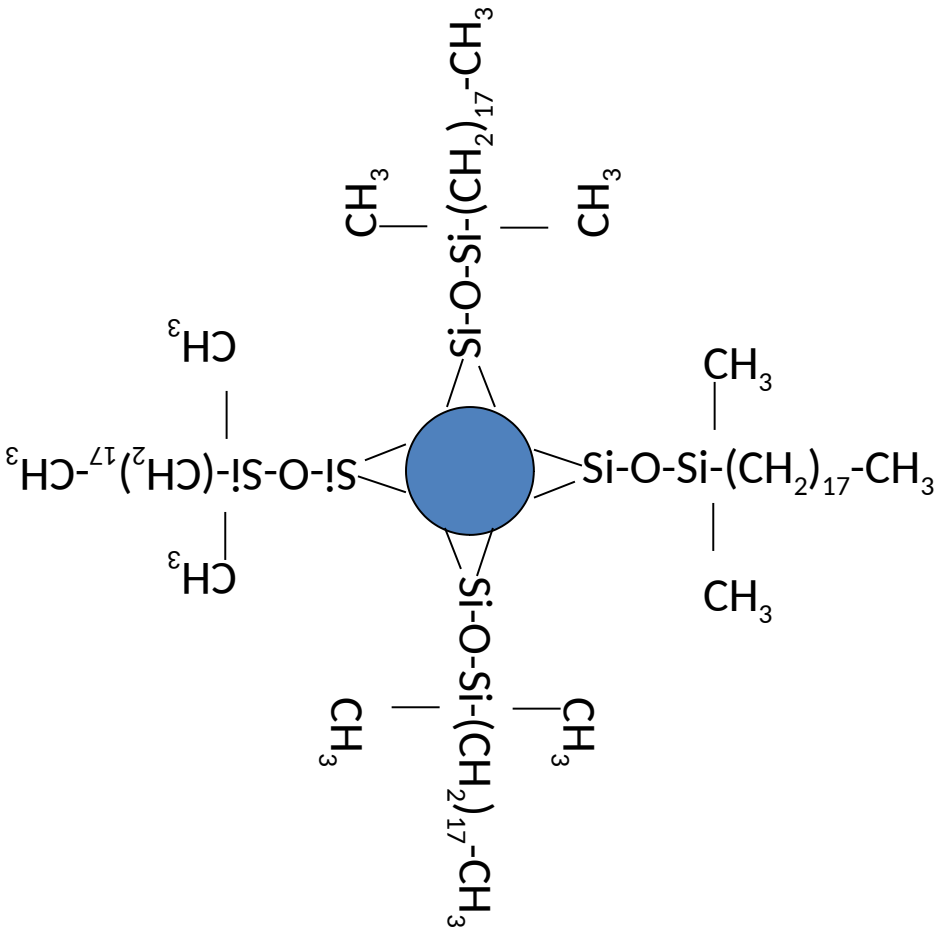
Separation Column for Reversed Phase Chromatography

- Phenyl type
- C_{18} (ODS) type
- TMS type
- C_8 (octyl) type
- Cyano type
- C_4 (butyl) type

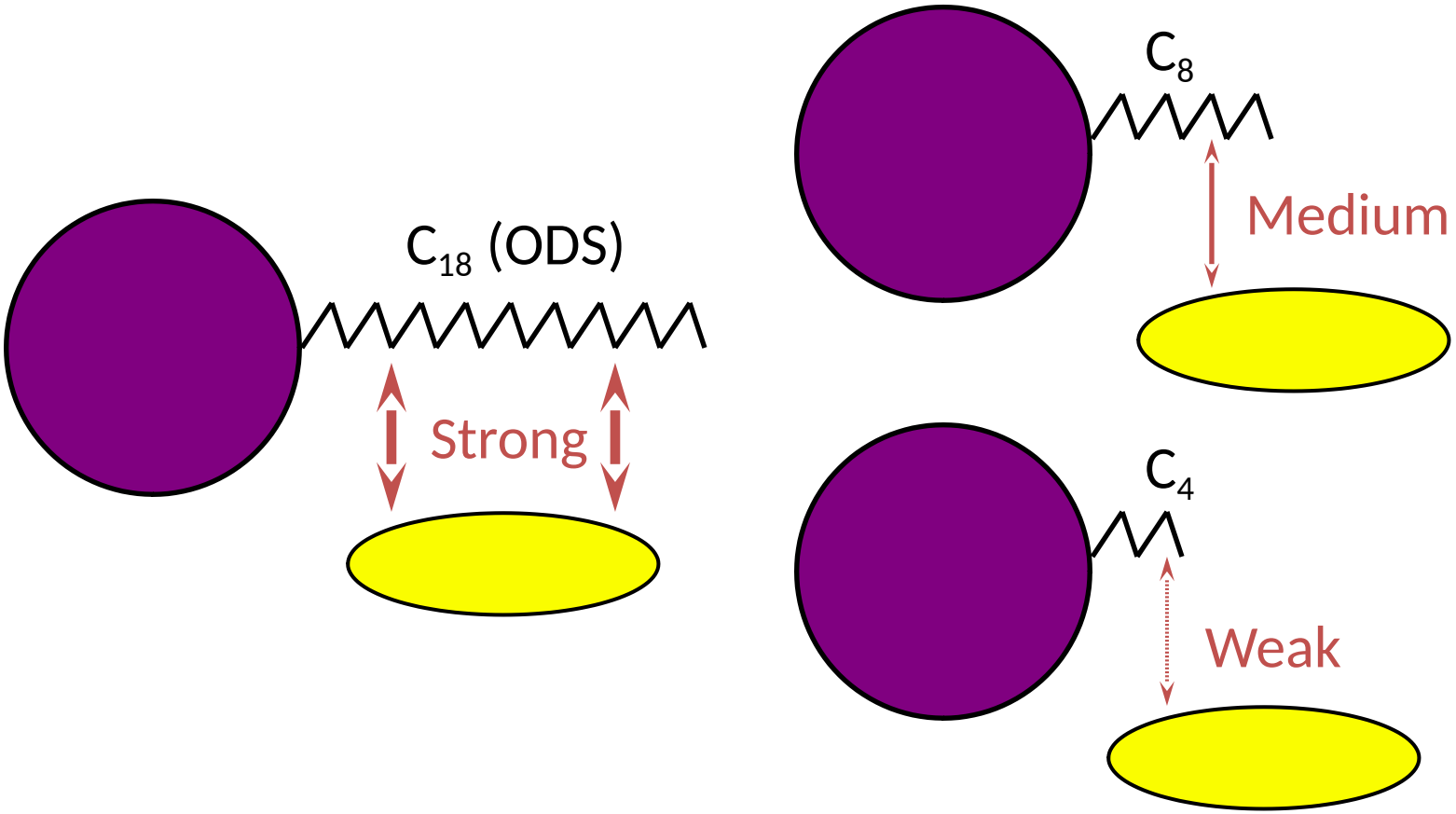


C_{18} (ODS)

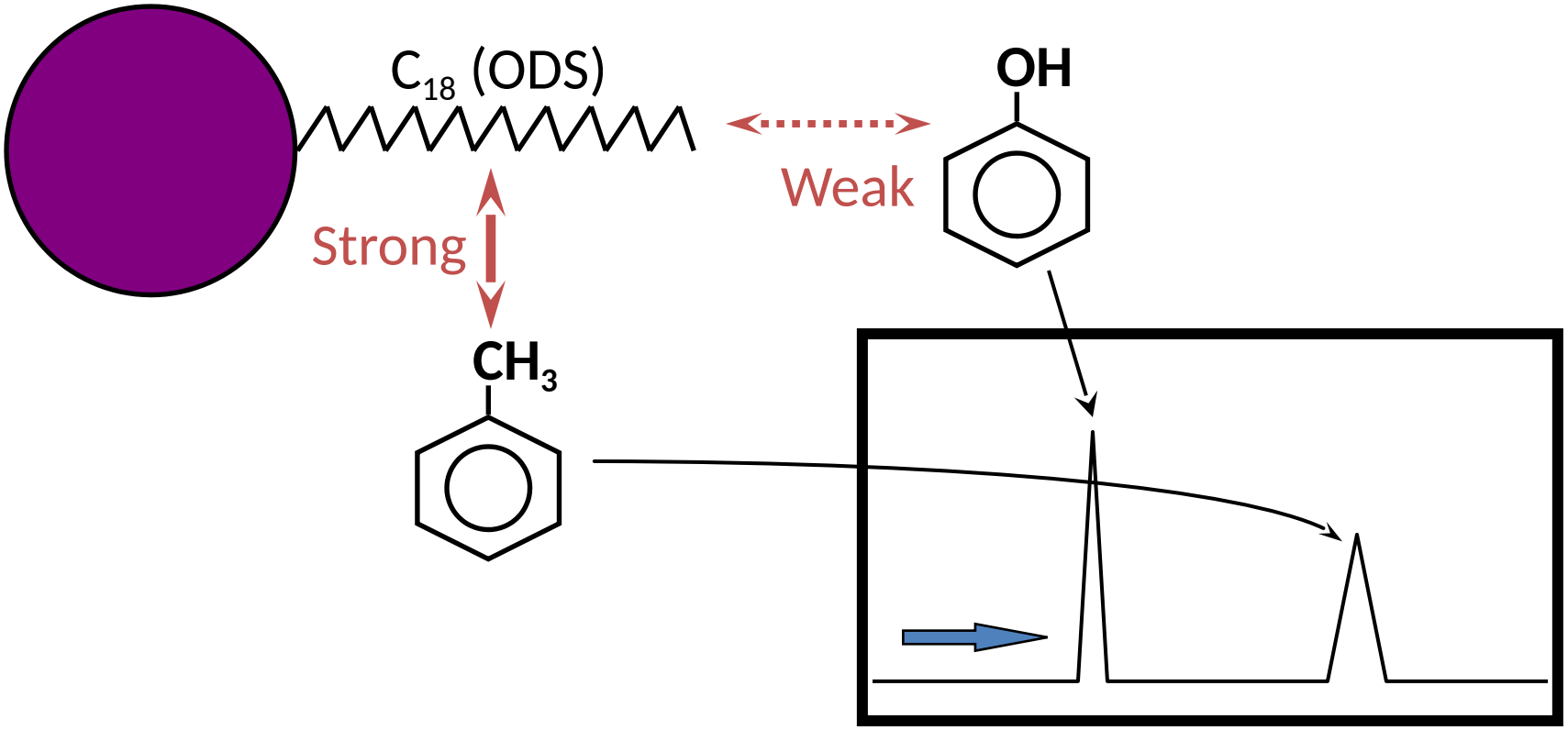
- Most common; n-octyldecyl, C_{18}



Effect of Chain Length of Stationary Phase



Relationship Between Retention Time and Polarity



HPLC Analysis

HPLC analysis can be carried out in

Isocratic mode

Gradient mode

Isocratic analysis

When the composition of the mobile phase does not change during an analysis (i.e. the composition is constant), the method is said to be isocratic.

Several potential problems are associated with isocratic analysis. These are listed below.

- When the range of analyte polarities is broad, some analytes may be poorly retained and resolution is lost with peaks eluting at or near the void volume (t0)
- Alternatively, other analyte components may be significantly more hydrophobic and show unacceptably long retention times
- Due to the various band broadening processes, these late eluting peaks will be broad and show reduced sensitivity due to reduced peak height
- It is possible that some components will be irreversibly adsorbed on the column and cause contamination

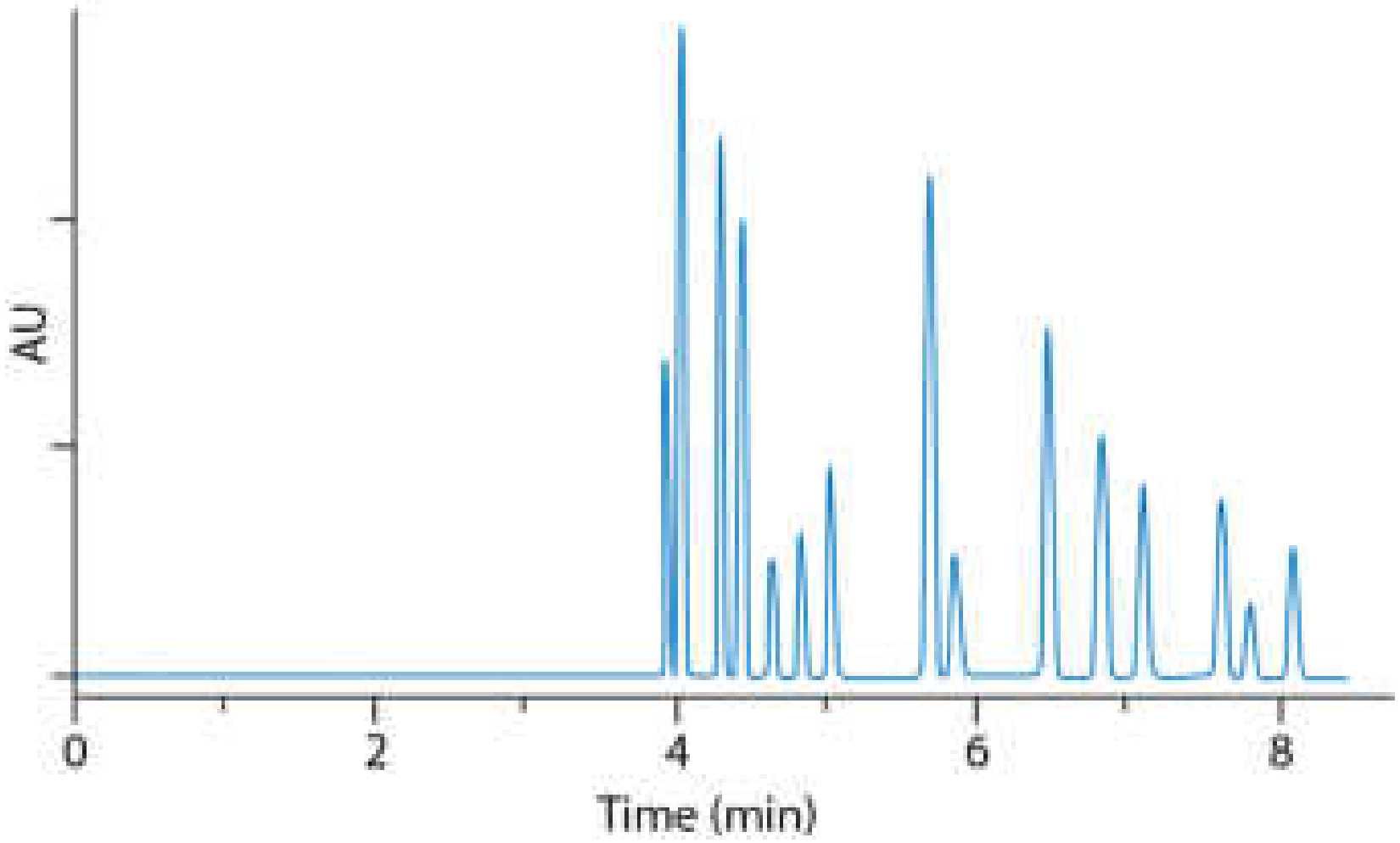
Gradient HPLC Analysis

- Many of the problems associated with Isocratic HPLC analysis can be overcome using gradient HPLC. In this mode of analysis, the mobile phase composition is altered during the analysis – normally by increasing the amount of organic modifier.
- The initial composition is chosen so that the strength is appropriate to retain and resolve early eluting analytes
- The elution strength is then increased in a predetermined way to elute compounds with optimum resolution
- The final mobile phase composition is chosen to ensure elution of all compounds of interest from the column within a reasonable time
- It is possible to increase the organic modifier concentration to wash strongly retained, potentially contaminating components from the column

- Gradient elution is best suited to analyses carried out using reversed phase, normal phase separations using bonded stationary phases, and for ion exchange chromatography.
- Particular pumps are required to carry out gradient HPLC analysis, which allow on-line mixing of the mobile phase components.

Gradient HPLC analysis - the mobile phase progressively changes to a stronger composition during the run

Figure 1: Chromatogram generated from a gradient HPLC analysis.



Advantages of Gradient Elution:

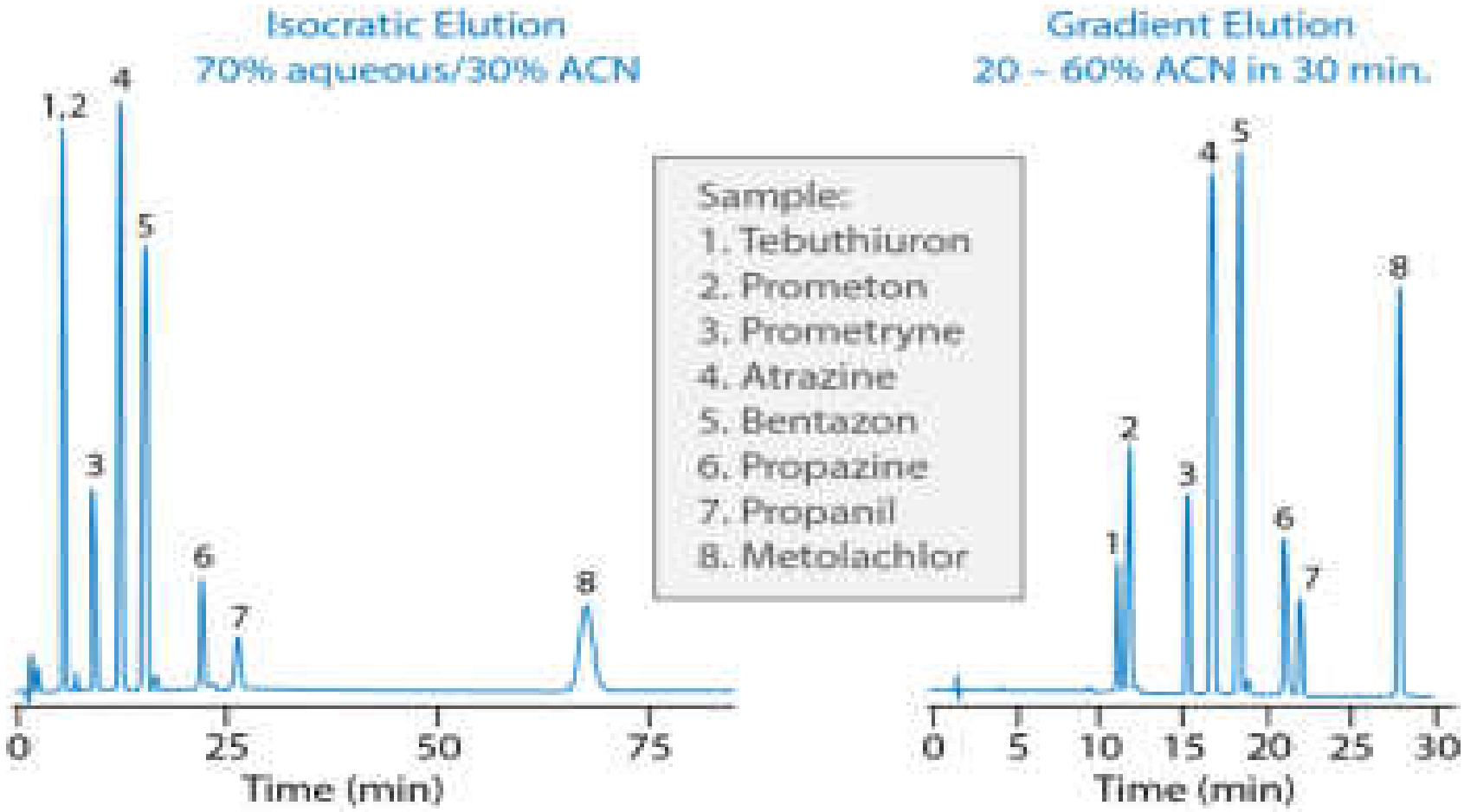
- Improved resolution
- Increased sensitivity
- Ability to separate complex samples
- Shorter analysis times
- Decrease in column deterioration due to strongly retained components

Disadvantages of Gradient Elution:

- More expensive instrumentation
- Possible precipitation at solvent interfaces when using multiple proportioning (mixing) valves
- Re-equilibration time adds to analysis time
- Instruments vary in their dwell volume (VD), which can cause method transfer problems

Use of gradient elution to separate samples having components that vary widely in polarity.

Separation of Herbicides on Zorbax C18 column.



Optimizing Gradient Parameters

Several parameters are required to be specified and optimized in a gradient HPLC method (Figure 1).

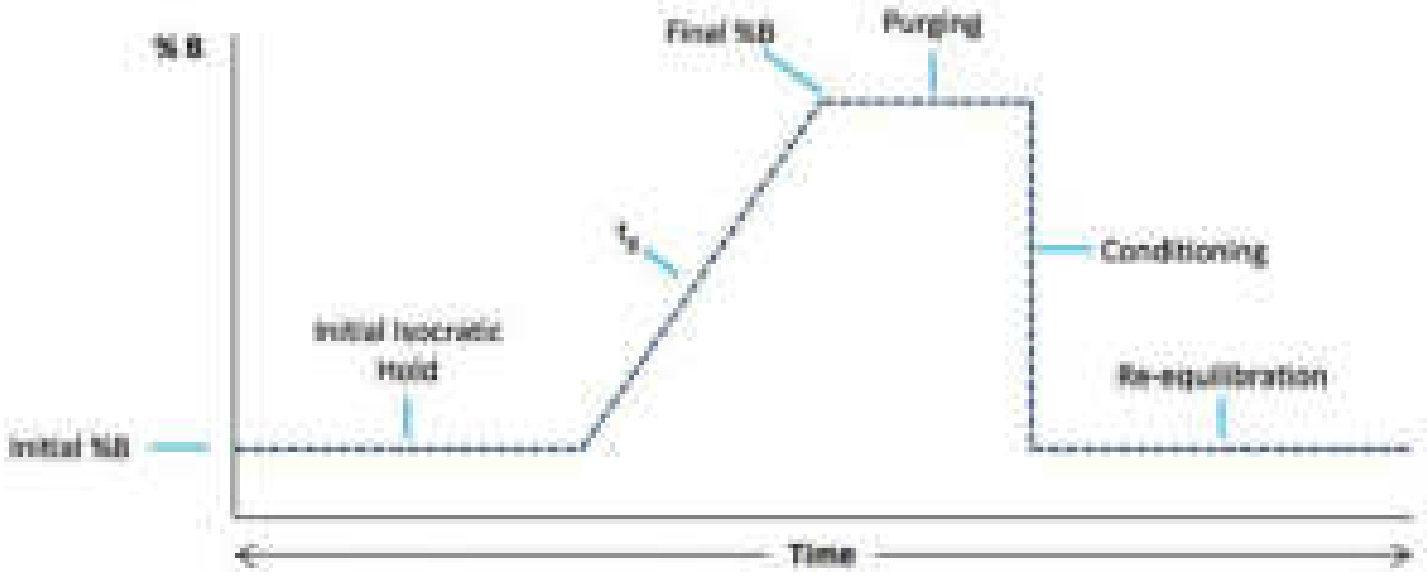


Figure 1: Typical gradient profile.

- Initial %B - starting mobile phase composition (described in terms of the % of the strong solvent B).
- Isocratic hold - a period within the gradient in which the eluent composition is held at the initial %B. This achieves a degree of analyte focusing but also crucially enables easy transfer of gradients between different instruments based on the specific instrument gradient dwell volume (VD).
- Gradient time (tG) - the time during which the eluent composition is changing.
- Final %B - final mobile phase composition.
- Purging - usually achieved using a short ballistic gradient ramp to high %B in order to elute highly retained components (of no analytical interest) from the column. There may be an isocratic hold at this composition to ensure elution of all analytes and strongly absorbed components of no analytical interest.

- Conditioning - returning the system (specifically the column) to the initial gradient composition. In practice, with modern instruments, this step is programmed to occur very rapidly.
- Equilibration - the time taken to ensure the whole of the analytical column is returned to initial gradient composition. This is an important step and if not properly considered can lead to retention time and quantitative variability.
- The equilibration time used is dependent on the column dimensions and flow rate. It is recommend that 10 column volumes (Vm) of eluent at the initial composition are used (calculate the time for the method flow rate and remember to add VD).
- The re-equilibration time can be reduced empirically; the method should be monitored for any retention time irreproducibility. The eluent flow rate can be increased during re-equilibration BUT ensure stabilization prior to injection of the subsequent sample.

Peak Shape in Gradient HPLC

In isocratic elution the peaks are relatively broad, the peak width increasing with retention time. In gradient elution, the peaks are narrow with almost equal peak widths.

- The main reason for the narrow peak shape is the velocity of the peak as it leaves the column. During gradient elution, all compounds accelerate through the column and thus elute at a high velocity. The retention time difference between compounds is a consequence of the percent organic modifier at which each starts to accelerate. All compounds should have approximately the same speed when they leave the column.
- Another reason for peak focusing is the fact that the front and tail of a peak are residing in different concentrations of organic modifier. The tail will experience a higher percentage of organic modifier than the heart of a peak. The velocity of the tail will thus be slightly higher than the heart of the peak and vice versa for the front. This results in peak focusing. Asymmetric peaks are less frequently a problem in gradient elution. In practicality, the narrow peaks obtained in gradient elution provide better detection limits and higher loading capacities.

HPLC solvents

Reversed Phase Mobile Phase Solvents

- The mobile phase in reversed phase HPLC usually consists of water/aqueous solution (commonly an aqueous buffer) and an organic modifier. When ionizable compounds are analyzed, buffers and other additives may be present in the aqueous phase to control retention and peak shape.
- Chromatographically, in reversed phase HPLC water is the ‘weakest’ solvent. As water is most polar, it repels the hydrophobic analytes into the stationary phase more than any other solvent, and hence retention times are long – this makes it chromatographically ‘weak’. The organic modifier is added (usually only one modifier type at a time for modern chromatography), and as these are less polar, the (hydrophobic) analyte is no longer as strongly repelled into the stationary phase, will spend less time in the stationary phase, and therefore elute earlier. This makes the modifier chromatographically ‘strong’ as it speeds up elution/reduces retention.

- As progressively more organic modifier is added to the mobile phase, the analyte retention time will continue to decrease.
- The common organic modifiers are detailed below (Figure 1).
- The Snyder polarity index value is shown which gives a measure of the polarity of the solvents. The ε° values are also shown which give a measure of relative elution strength.

Water 10.2

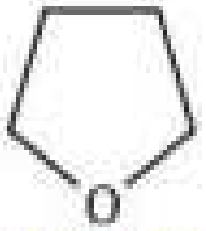
ε^{0} (C_{18}) = N/A

Methanol 5.1

ε^{0} (C_{18}) = 1.0

Acetonitrile 5.8

ε^{0} (C_{18}) = 3.1



Tetrahydrofuran (THF) 4.0

ε^{0} (C_{18}) = 3.7

Properties of the ideal solvent

- Water-miscible
- Low viscosity
- Low UV detection
- Good solubility properties
- Chemically unreactive

First of all, the chosen organic solvent must be miscible with water. All of the solvents listed on the previous page are miscible with water.

Second, a low viscosity mobile phase is favored to reduce dispersion and keep system backpressure low. The use of a low viscosity solvent is preferable (Table 1) due to the lower pressure drop produced at a specific flow rate. It also allows for faster chromatography due to the increased rate of mass transfer.

Solvent Properties

- Listed below are definitions of the properties that will often be reported for solvents. Knowledge of what these properties describe can be useful when selecting solvents for HPLC applications. The values for many of these solvent properties for individual solvents are reported in the tables throughout this module.
- Viscosity – η is given in mPa s at 20 °C. Solvents with a higher viscosity than water (η = 1.00) are less suitable for HPLC due to the high pressure drop that will be produced.
- UV Cutoff – The wavelength at which the absorbance of the pure solvent is 1.0, measured in a 1 cm cell with air as the reference (10% transmittance).

- Boiling point – Solvents with low boiling points are less suitable for HPLC. Produce a danger of vapor bubbles in the HPLC system. Solvent loss can occur during degassing.
- Purity - HPLC grade solvents and reagents should always be used. Water should be a free solvent; however, high purity water is required for all sample and mobile phase preparation protocols in HPLC. Poor quality solvents, reagents, and water can produce a multitude of chromatographic errors including; altered resolution, ghost peaks, changes in stationary phase chemistry and baseline issues.
- Possible sources of organic contaminants are from feed water (i.e. tap water), leached from purification media (filters), tubing and containers, bacterial contamination, and potentially, absorption from the atmosphere. For example, alkaline mobile phases are known to absorb polar organics such as formaldehyde, amines, and atmospheric carbon dioxide. Organic contaminants with UV active chromophores can interfere with quantification if they are present in high enough levels. Reactive contaminants may also produce unwanted side reactions with analyte molecules.

- The solvent must be stable for long periods of time. This disfavors tetrahydrofuran which, after exposure to air, degrades rapidly, often forming explosive peroxides. Tetrahydrofuran is an interesting solvent in that it is one of the strongest chromatographically and can produce separations in very short times, whilst still being fully miscible with water. However, it does have a relatively high UV cut off (215 nm). Column equilibration can also be slower with THF than with MeOH or MeCN. In the presence of air or oxidizers THF will also form hazardous, explosive peroxide species, which pose both a safety risk and can be reactive towards analytes. Recently THF has also been recently upgraded to carcinogen status by some bodies.

- The two remaining mobile phase solvents are methanol and acetonitrile. The use of both solvents usually gives excellent retention characteristics.
- When mixing MeOH and aqueous mixtures, each solvent should be weighed or volumetrically measured due to the solvent contraction that occurs upon mixing i.e. 500 mL of water topped up to 1000 mL with MeOH will result in a solution with a MeOH content in excess of 50% by volume.
- Care should also be taken if reactive analytes, such as alcohols, aldehydes, or carboxylic acids are being analyzed, as in the presence of MeOH methyl esters can be formed giving rise to erroneous peaks in the chromatogram and quantification errors.
- If a UV detector is being used it is important to consider the UV cut off of the mobile phase (organic modifier, buffers, additives etc. Table 1) to ensure that there is no interference with the λmax of the analyte.
- Once prepared, HPLC mobile phases will have a given shelf life (Table 3). The values shown in Table 3 are a conservative estimate of the usable time period for given mobile phases, therefore, it is important to monitor mobile phase stability periodically and local policies or procedures should take precedence.