Theory Of Chromatography

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Separation of Sand and NaCl using water

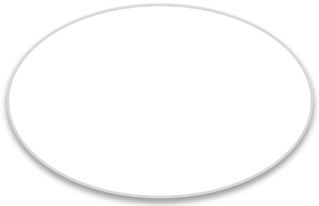
Petri plate with salt

Beaker with water

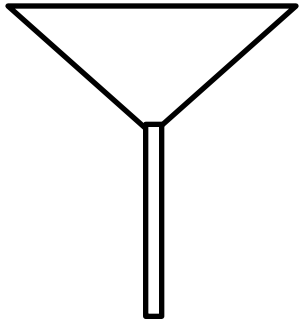
Glass rod

Water bath

Petri plate with sand

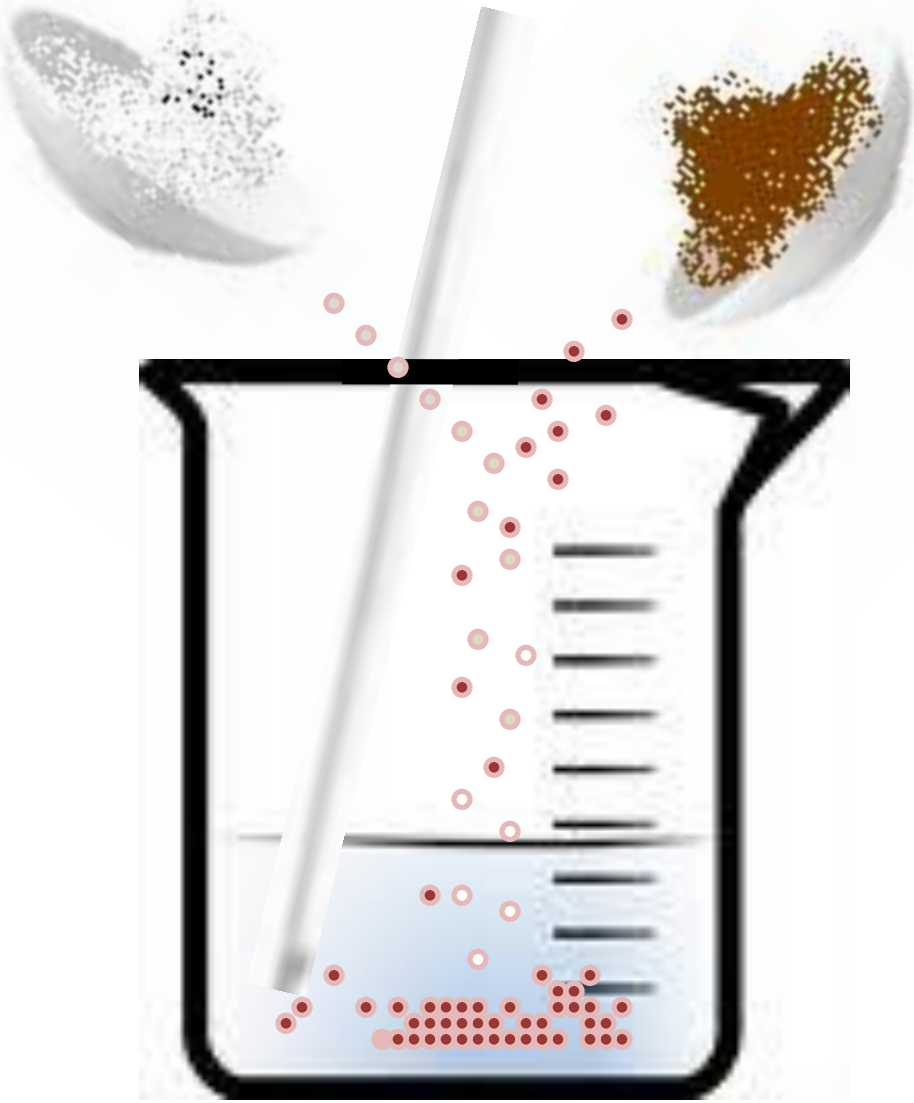


Filter paper



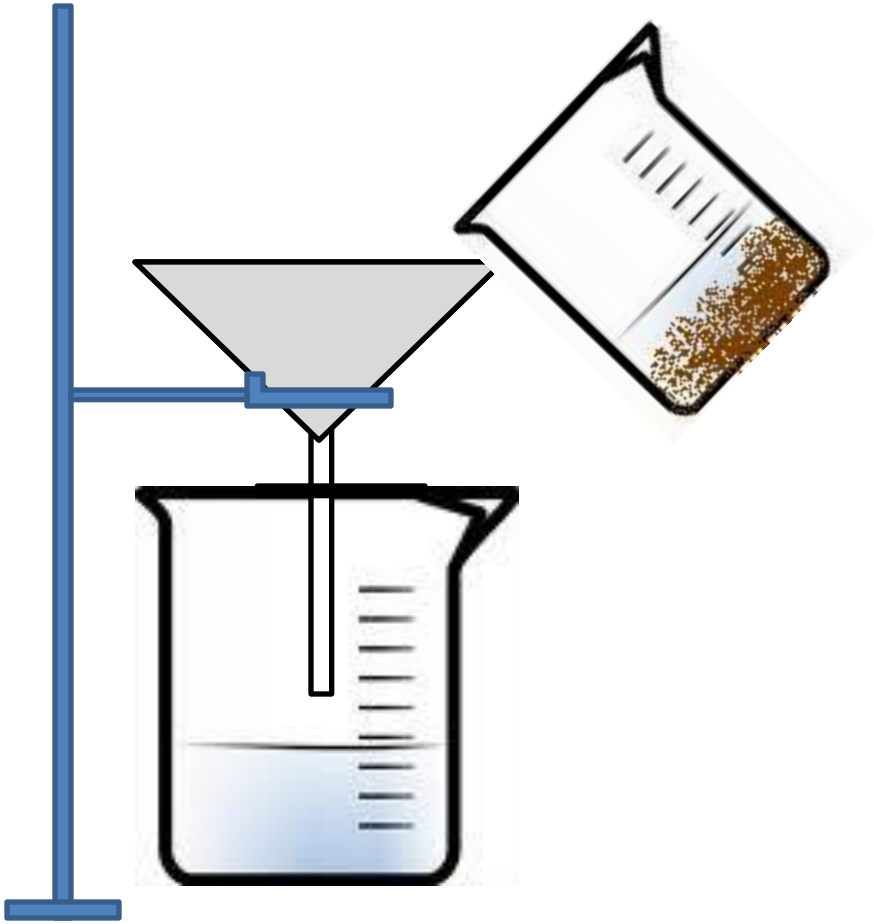
Funnel

Adding Sand and NaCl to water



Beaker containing mixture of Sand and NaCl

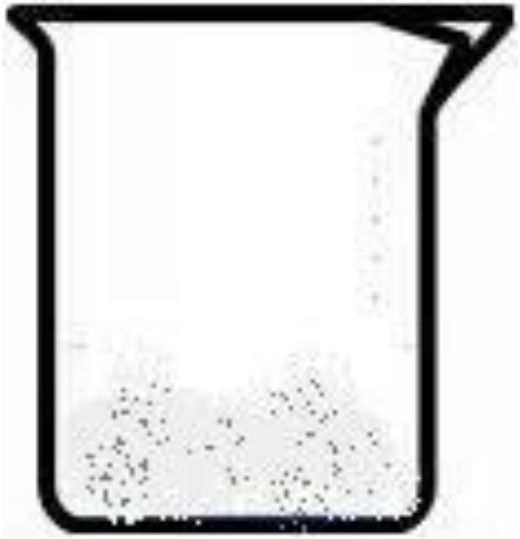
Filtration



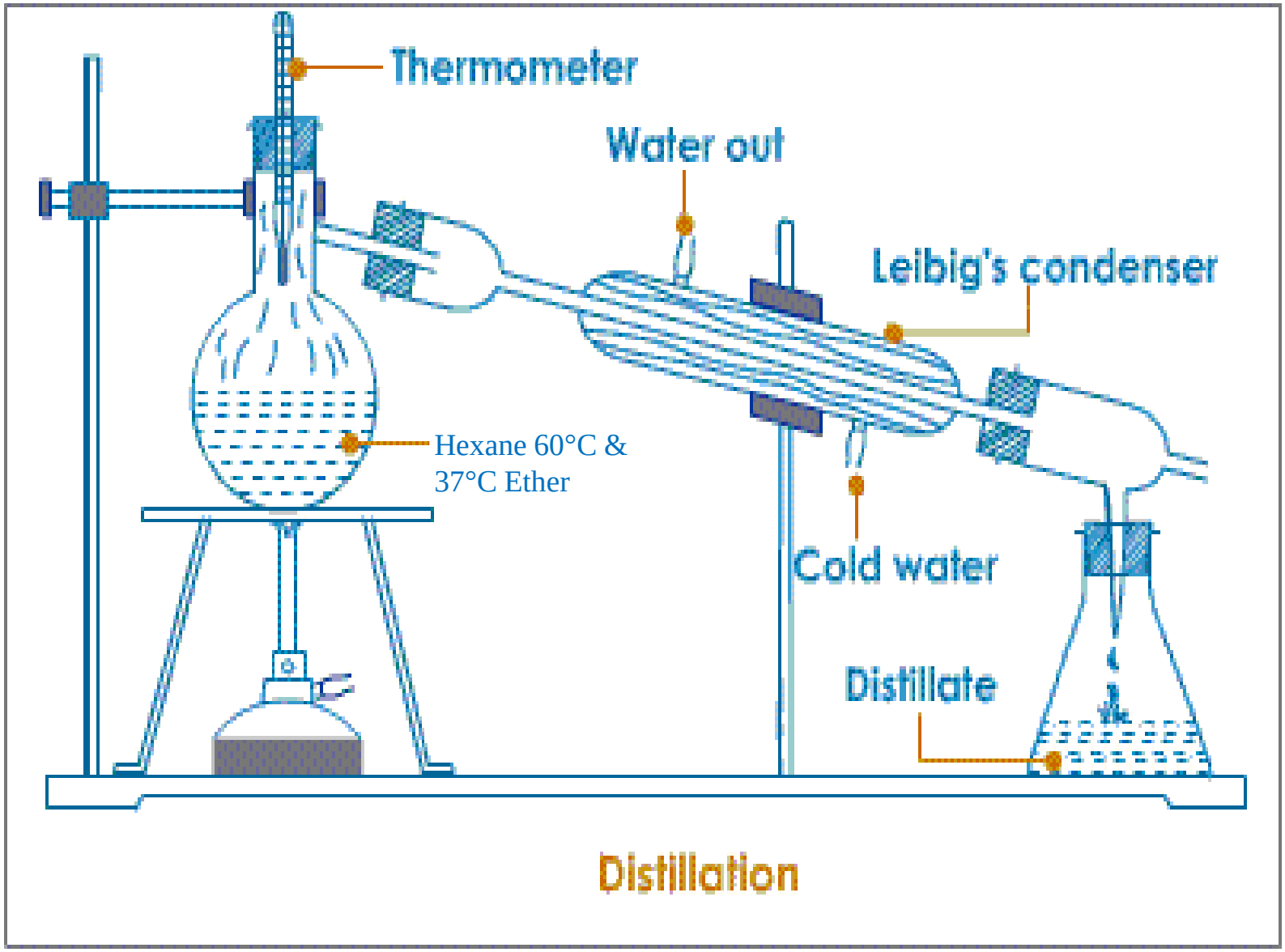
Filter paper with sand

Beaker containing NaCl dissolved in water

Heating the NaCl solution



Beaker containting the NaCl


Thermometer
Water out
Leibig's condenser
Hexane 60°C &
37°C Ether
Cold water
Distillate
Distillation

- Michael Tswett decided to conduct one experiment

- To the great surprise of Michael Tswett, five distinct coloured bands were developed on the column.

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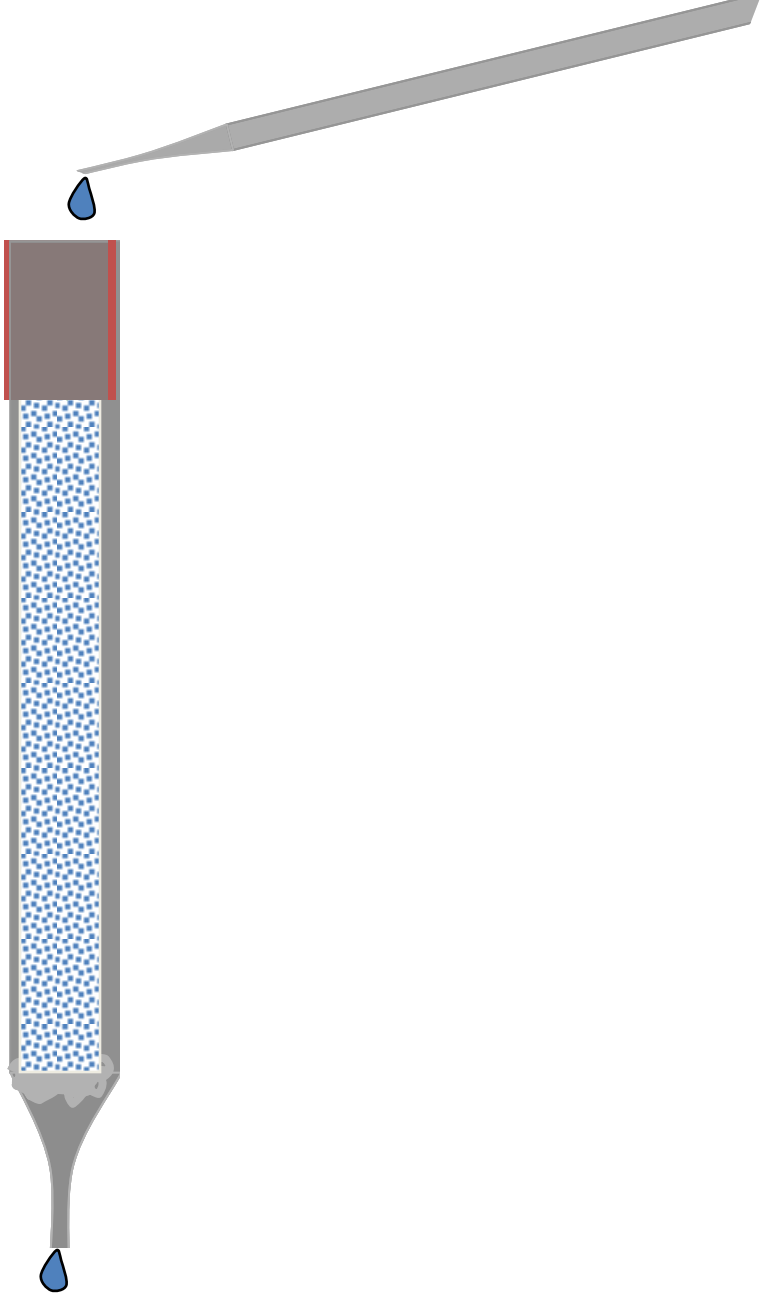
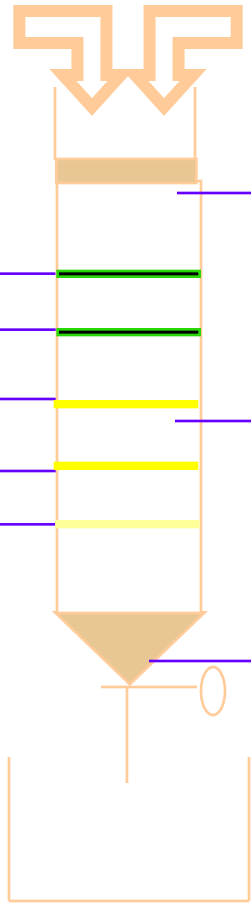


WASHING/ ELUTION WITH ETHANOL

CONCENTRATED SOLUTION OF CHLOROPHYLL IN PETROLEUM ETHER



COTTON PLUG

β CHLOROPHYLL

α CHLOROPHYLL

XANTHOPHYLL

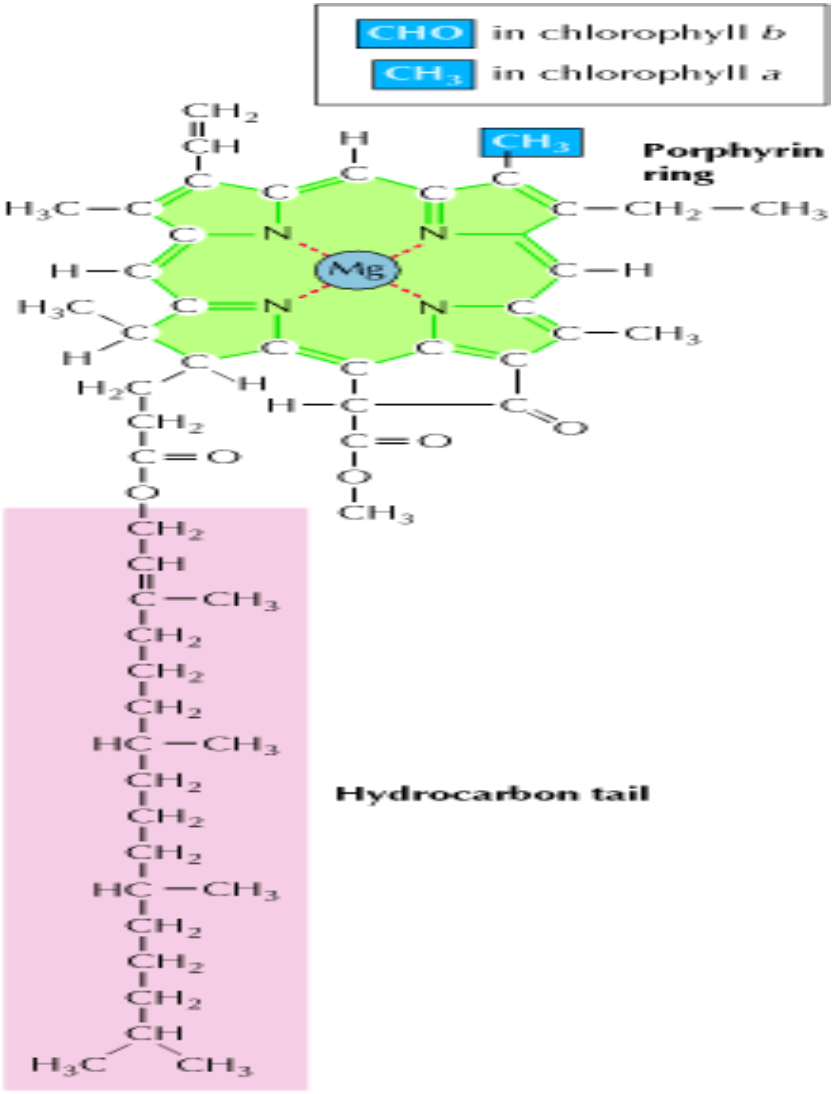
VIOLA XANTHINE

CAROTENE

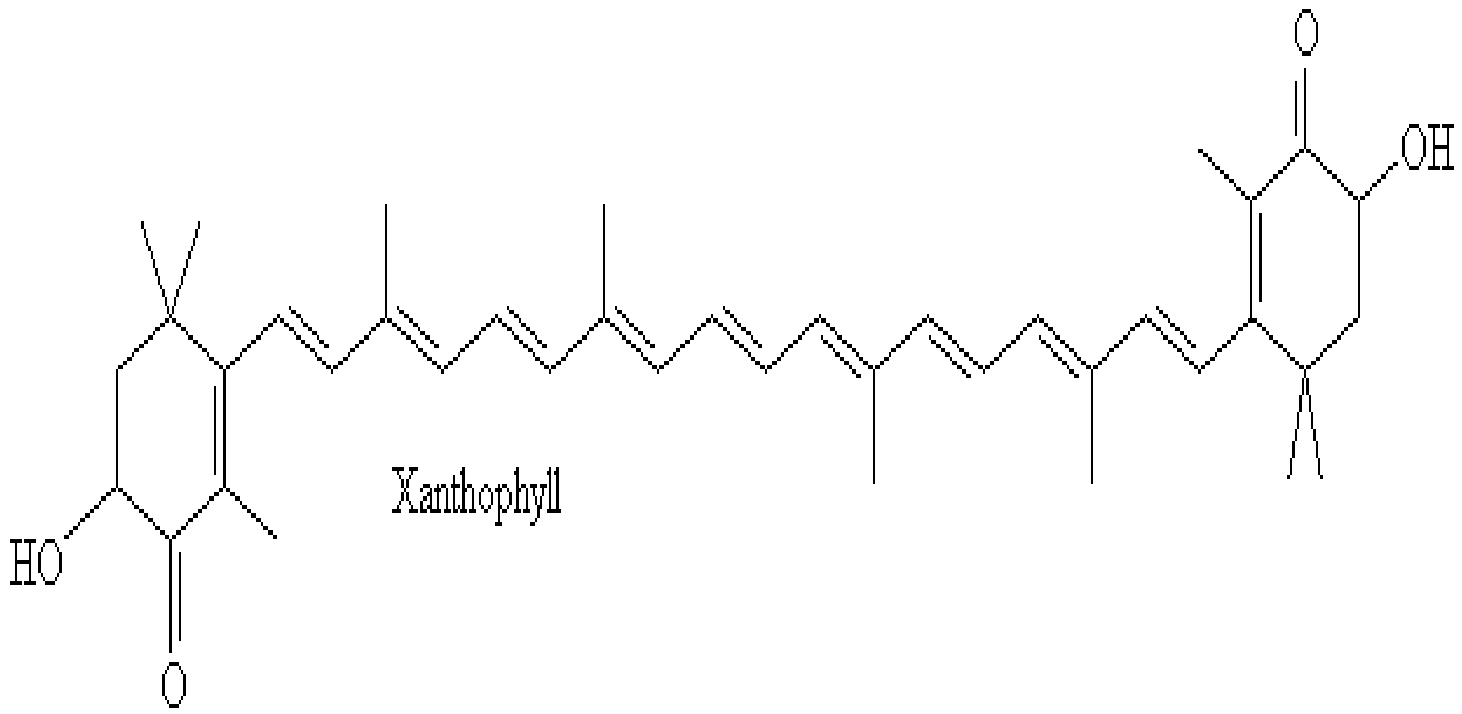
SLURRY OF $CaCO_3$ POWDER IN PETROLEUM ETHER

COTTON PLUG

α CHLOROPHYLL and β CHLOROPHYLL

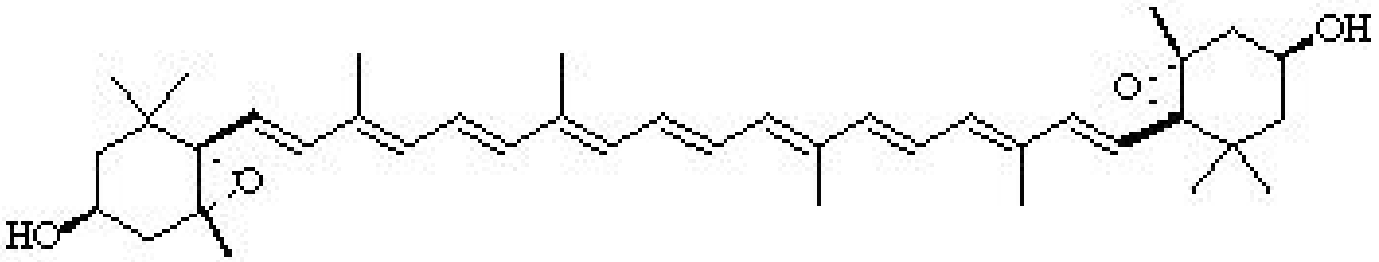


XANTHOPHYLL

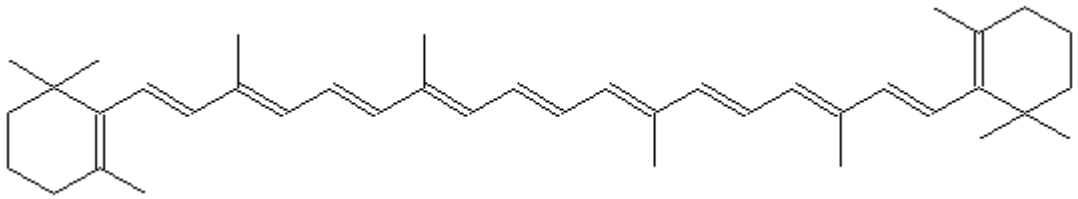


Xanthophyll

VIOLA XANTHENE



CAROTENE



- For example α – chlorophyll (MW 893) differ from β – chlorophyll by only one methyl group yet these two solutes are easily separated in the simplest apparatus.

- Michael Tswett's main problem was resolved and in the process he discovered a simple, time saving and relatively less laborious method of separating complex solutes from a mixture.

- He visualized the coloured bands from chlorophyll as coloured writing.

- Today the term chromatography is used for separations involving colourless solutes also.This is done to honour the discoverer of the technique.

- Michael Tswett coined the term “Chromatography for this new technique of separation. (Chromatus = colour, graphein = to write)

- His paper has been translated to English and republished [Strain.H.H and Sherma.J.J.Chem.Educ.,44,338(1967)] because of its importance to the field.

- Tswett is credited as being the "Father of Chromatography" principally because he coined the term chromatography (literally colour writing) and scientifically described the process.

- In the scientific discoveries, one who publishes his work first will always get the credit for his discovery.

- D.T. Day from England and some others were working in separating complex mixtures simultaneously. However Michael Tswett published his work in 1906 before others. The credit of discovery of chromatography therefore rightly goes to him.

Publishing Vs Patenting

Whenever one discovers something new or novel he/she has two alternatives to claim the credit of the discovery.

1. To publish in a well referred international journal.
2. To file a patent for the novelty of the discovery.

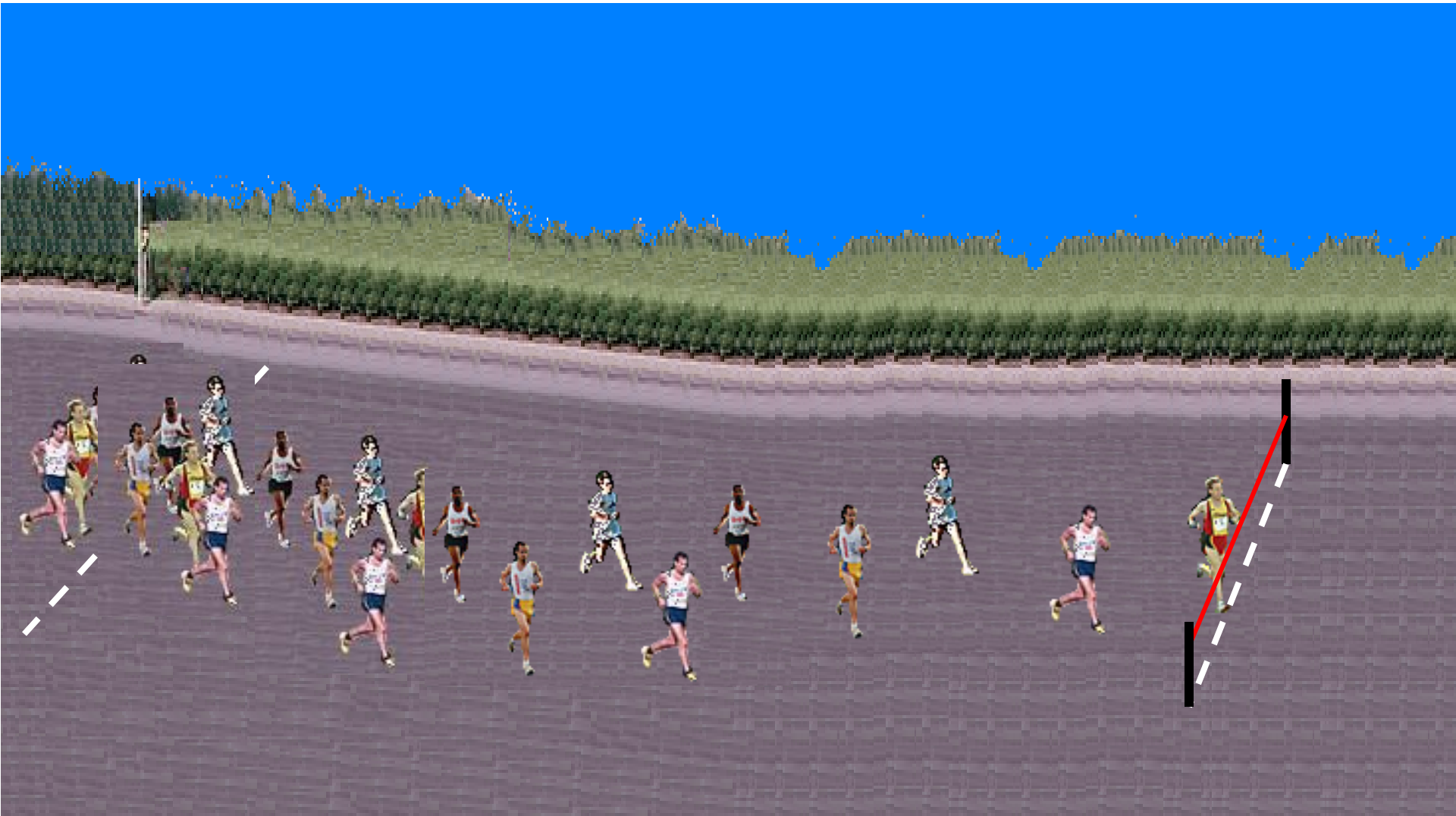
As a chemist, a researcher or simply as an inquisitive person, two questions come to our mind on the results of Tswett's experiment.

1. Why do the five solutes separate on the column?

2. How do the five solutes separate on the column?

Let us imagine a hundred meter running competition being viewed in slow motion.

Chromatographic separation as understood from runners running a race



Can this concept be applied to the five solutes in Tswett's experiment?

Yes of course!

On the other hand β - chlorophyll is the last solute to come out of the column because of its slowest migration velocity of the five solutes.

The five solutes have different migration velocities. Since,carotene has the highest migration velocity it reaches the other end of the column first.

So, we have the answer to the first question.

“The five solutes are separated on the column because of the differences in their migration velocities”

Migration velocities increase from β- chlorophyll to carotene.

Although the experimental conditions are similar, the five solutes travel across a good adsorbent- $CaCO_3$.

But why does carotene travel the fastest and β- chlorophyll the slowest when all the five solute travel across the column under similar experimental conditions?

Let me give you a hint at this stage.

We are quite familiar with the phenomena of adsorption. It is a surface phenomenon in which some solutes (adsorbates) adsorb on the surface of another solute (adsorbent).

Adsorption process depends on

1. the properties of surface of an adsorbent.
2. the properties of adsorbates.

Again the same adsorbates may not adsorb to the same degree on the surface of another adsorbent, let us say, a cellulose paper.

For example,

If we consider our palm as a surface of an adsorbent, then the degree of adsorption of water and oil (adsorbates) on the same surface is different.

- **“The five solutes have different migration velocities across the column of $CaCO_3$, because they have different degrees of adsorption on the surface of $CaCO_3$”**

Carotene has the least degree of adsorption on $CaCO_3$ surface and hence the highest migration velocity while β- chlorophyll has the highest degree of adsorption on $CaCO_3$ surface and hence the lowest migration velocity ofthe five solutes.

We can therefore say that the degree of adsorption on $CaCO_3$ surface decreases from β- chlorophyll to carotene and the migration velocity decreases from carotene to β- chlorophyll.

Adsorbents

- While there can be a legion of adsorbents which can be used in chromatography, it may be emphasized that comparatively few will suffice to work out a large variety of chromatograms by appropriate variation in the solvents used for elution.

By far the most important agent in chromatography is the adsorbent.

- Therefore, utmost care is to be bestowed in the selection of proper adsorption material and also the process of building up a column with it and the maintenance of the column.

Essential requirements to be satisfied by every adsorbent

- It must be insoluble in the solution under analysis and the solvent that is used for elution

- It should not chemically react with the mixture under analysis.

- It should generally be colourless, but this is not a rigid rule as there are instances where coloured adsorbents have been used successfully.

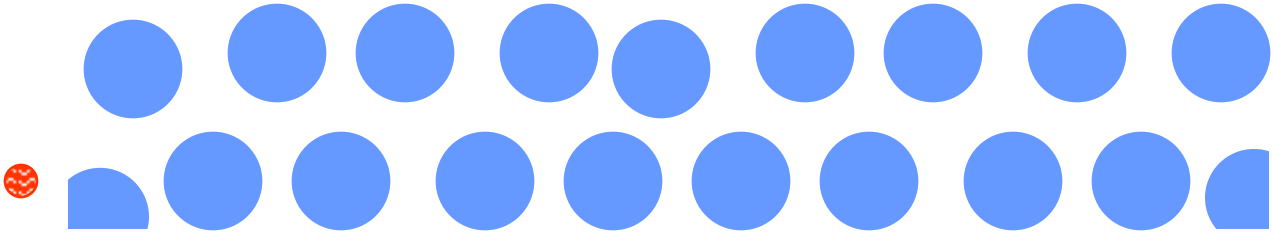
- There are different grades of alumina for adsorption, prepared by strongly heating the alumina and allowing it to absorb moisture to different degrees.

- Bigger particles will short-circuit the solvent, while too fine size will clog the passage of the liquid.

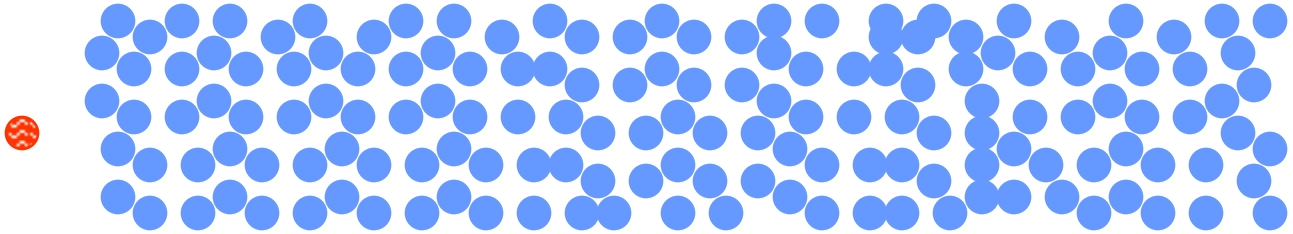
- Adsorbent properties of different materials can be standardized by means of dyes by the method of Brockmann and Schodder.

- The method consists in testing columns of adsorbents with a series of dye-stuffs ; and the grade of the adsorbent is decided by the dye-stuff which is adsorbed most strongly.

Too loose , large paricles.



Too tight , small paricles.



Adsorbents may be classified as follows according to the force with which they hold the ions.

Type	Examples
Weak	Sucrose and starch
Intermediate	Calcium carbonate, calcium phosphate, calcium hydroxide and magnesium hydroxide
Strong	Alumina, charcoal and Fuller's earth.

- The above classification of adsorbents into weak, intermediate & strong is made on the basis of adsorption of certain standard coloured dyes on different adsorbents.

- It should be emphasized here that on the same adsorbent different ions may evince different coefficients of adsorption, as for example, on alumina the trivalent ions are held more firmly than the others.

From Tswett's experiment,

If we change slurry of $CaCO_3$ and replace it with slurry of Al_2O_3, will the five solutes form chlorophyll separate and will the sequence of separation remain the same?

and

If we replace chlorophyll extract with a mixture of A+B+C+D+E solutes, will the solutes separate and what will be the sequence of their separation?

- In the first instance, we have changed the adsorbent, other things remaining unchanged.

- The sequence of separation will depend on the relative degrees of adsorption of the five solutes on the surface of alumina(Al_2O_3).

- The five adsorbates have a new surface of Al_2O_3.

- If they exhibit differences in the degree of their adsorption on the surface of Al_2O_3, they will get separated.

- In the second instance, we have changed the five solutes, other things remaining unchanged.

- The sequence of their separation will depend on the relative degrees of adsorption of the five solutes on the surface of $CaCO_3$.
- They will get separated, if they exhibit differences in the degree of their adsorption on the surface of CaCO3.
- Although the surface of CaCO3 is the same as that in Tswett's experiment, the five solutes are different.

- Let us consider a situation where in the glass column we put a liquid which remains stationary even if the tap is opened.

- If we allow three solutes A, B and C to pass through the chromatographic column and realize their separation after some time, we can ask again the same two questions which we did in adsorption chromatography.

- Another liquid which is immiscible with the stationary liquid moves through it.

- How do the three solutes separate?

- Why do the three solutes separate?

- Solute ‘C’ has the lowest migration velocity of the three and reaches the end of the column last.

- The three solutes separate because they have different migration velocities.

- Solute ‘A’ has the highest migration velocity of the three and reaches the end of the column first.

- Solute ‘C’ on the other hand is more soluble in the stationary phase and hence lags behind.

- The migration velocity increases from solute ‘C’ to solute ‘A’.

- Solute ‘A’ travels the fastest because it is more soluble in the liquid which moves across the stationary liquid.

- So the principle of separation here is differences in solubility or partitioning of solutesbetween the stationary and mobile liquid phases.

- It is called as “Partition Column Chromatography”.

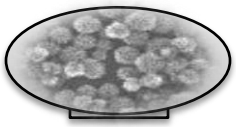
- The solution is spread uniformly on the surface of the inert solid support.

- A fixed weight (say 100g) of an inert solid support, normally silica, of certain particle size is taken in a round bottom flask.

- A ‘suitable liquid’ is weighed (say 5g) and dissolved in minimum volume of a volatile solvent.

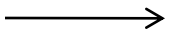
Liquid Phase Loading

Round bottom flask

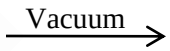


Petri plate with silica particals

Test tube containing concentrated solution of a suitable liquid (5g) in minimum volume of volatile solvent



Pouring solution



5g of suitable liquid coated on the surface of 100g inert solid support (5% liquid Phase Loading)

- The ‘suitable liquid’ is coated on the surface of the inert solidsupport and we get a liquid stationary phase with 5% liquid phase loading.

- The round bottom flask is rotated and connected to vacuum.The volatile solvent is removed.

- In practice, generally liquid phase loadings of 2% to 20% are used in partition column chromatography.

- Fractional crystallization is used to separate solid solutes from a mixture taking advantage of the differences of solubility of solutes in a given solvent or in a mixture of solvents.

- In fractional distillation liquid solutes in a mixture having very close boiling points are separated based on the differences in the boiling points of the liquid solutes.

- In comparison, column chromatographic separations are relatively simple, clean and time saving.

- Both, fractional crystallization and fractional distillation process are tedious, laborious and time consuming.

- Here one point must be emphasized.

- For solutes to get separated from a mixture, they must exhibit some differences with reference to some property like solubility, boiling point, adsorption, partitioning etc.,

- If they do not show differences with reference to any property they cannot be separated.

- Let us now understand some common terms used in chromatographic separations.

Mobile phase: A phase which moves across the stationary phase. It could be a gas or a liquid.

Stationary phase: Phase which remains stationary. It could be a solid or a liquid bonded to an inert solid support.

Elution: The process of washing a chromatographic column with a liquid or a gas.

Liquid phase loading: Amount of a liquid coated per 100g of an inert solid support.

Solutes: This term is used in place of compounds or substances.

Reverse phase mode of separation: Here the stationary phase is relatively less polar than the mobile phase.

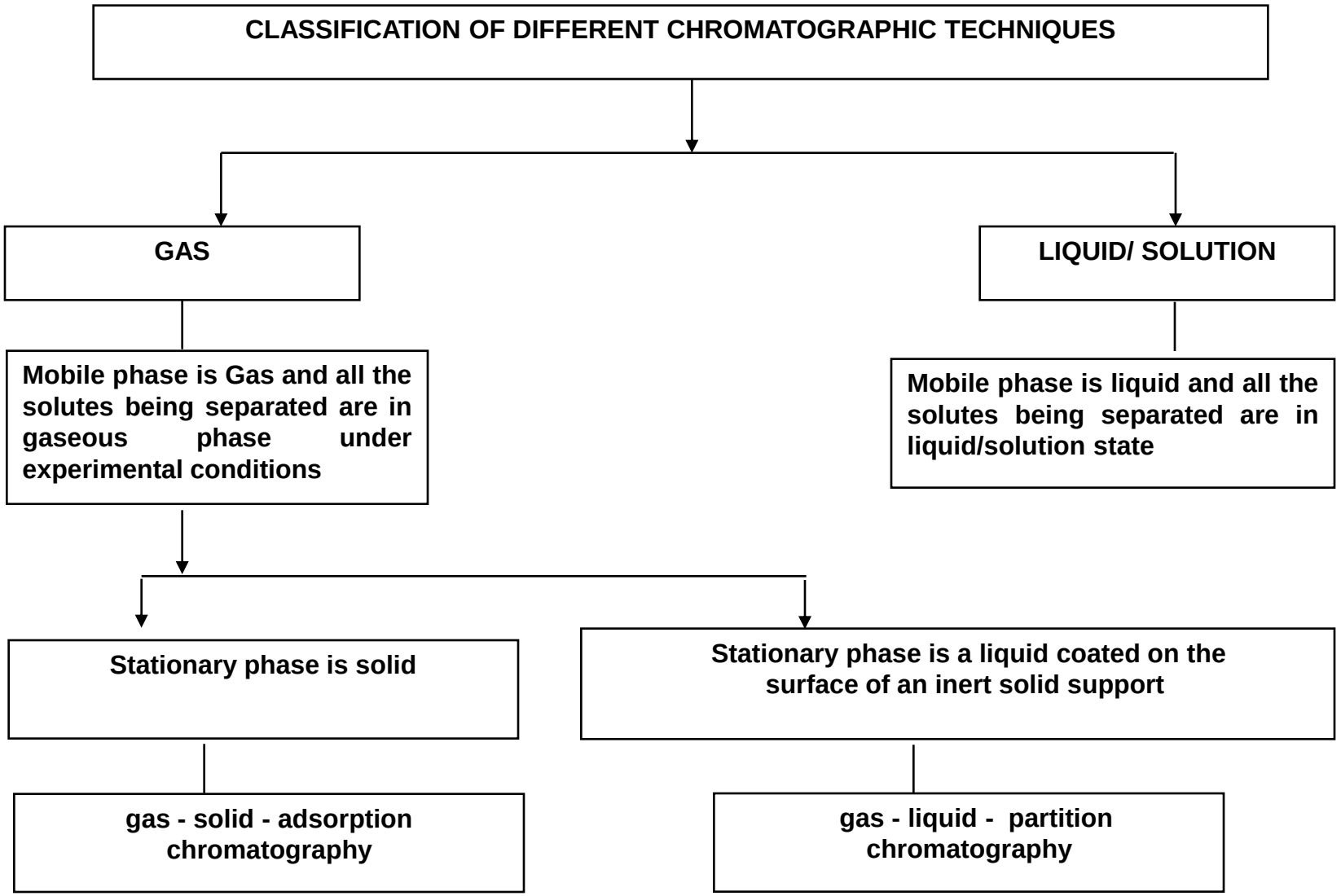
Normal phase mode of separation: Here the stationary phase is relatively more polar than the mobile phase.

- Let us now see the common nomenclature used to describe different chromatographic techniques.

- Nearly 85%-90% practical separations are carried out by reverse phase mode of separation because of the large possible variations in the composition of mobile phases.

MOBILE PHASE	STATIONARY PHASE	PRINCIPLE OF SEPARATION	CONTAINER	NAME OF THE CHROMATOGRAPHIC TECHNIQUE
LIQUID	SOLID	DIFFERENCES IN ADSORPTION	COLUMN	LIQUID-SOLID ADSORPTION COLUMN CHROMATOGRAPHY
LIQUID	SOLID	DIFFERENCES IN ADSORPTION	THIN LAYER	LIQUID-SOLID ADSORPTION THIN LAYER CHROMATOGRAPHY
LIQUID	LIQUID	DIFFERENCES IN PARTITIONING	COLUMN	LIQUID-LIQUID PARTITION COLUMN CHROMATOGRAPHY
LIQUID	LIQUID	DIFFERENCES IN PARTITIONING	THIN LAYER	LIQUID-LIQUID PARTITION THIN LAYER CHROMATOGRAPHY
GAS	LIQUID	DIFFERENCES IN PARTITIONING	COLUMN	GAS-LIQUID-PARTITION COLUMN CHROMATIGRAPHY
GAS	SOLID	DIFFERENCES IN ADSORPTION	COLUMN	GAS-SOLID ADSORPTION COLUMN CHROMATOGRAPHY

- There are many ways of classification of different chromatographic techniques, however the simplest way is shown ahead…


CLASSIFICATION OF DIFFERENT CHROMATOGRAPHIC TECHNIQUES
GAS
LIQUID/ SOLUTION
Mobile phase is Gas and all the solutes being separated are in gaseous phase under experimental conditions
Mobile phase is liquid and all the solutes being separated are in liquid/solution state
Stationary phase is solid
Stationary phase is a liquid coated on the surface of an inert solid support
gas - solid - adsorption chromatography
gas - liquid - partition chromatography

Liquid / Solution Chromatography

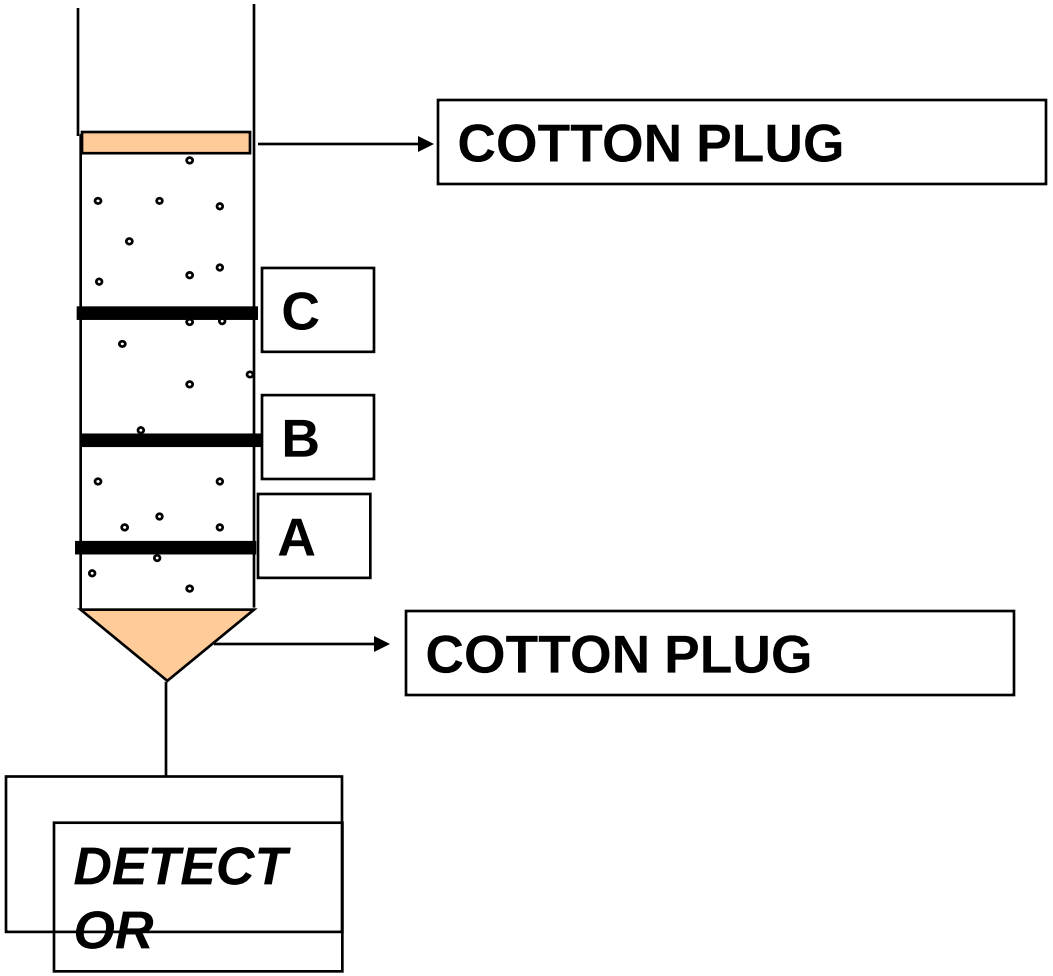
- **ADSORPTION** : Separation of solutes on the basis of their degree of adsorption on the surface of an adsorbent.
- **PARTITION** : Separation of solutes on the basis of their differences in partition coefficient of solutes in two mutually immiscible solvents.
- **ION EXCHANGE** : Separation of similarly charged ions based on the differences in the degree of their affinity towards the ion exchange resin.
- **SIZE EXCLUSION** : Separation of solutes based on the differences in their molecular weights or sizes.
- **ELECTROPHORETIC** : Separation based on the movement of charged solutes to opposite poles under the applied electrical field.
- **AFFINITY** : Separation of solutes based on specific affinity of solutes on the specific sites of the stationary phase.

- The detector does not sense and count the number of molecules of mobile phase entering into it.

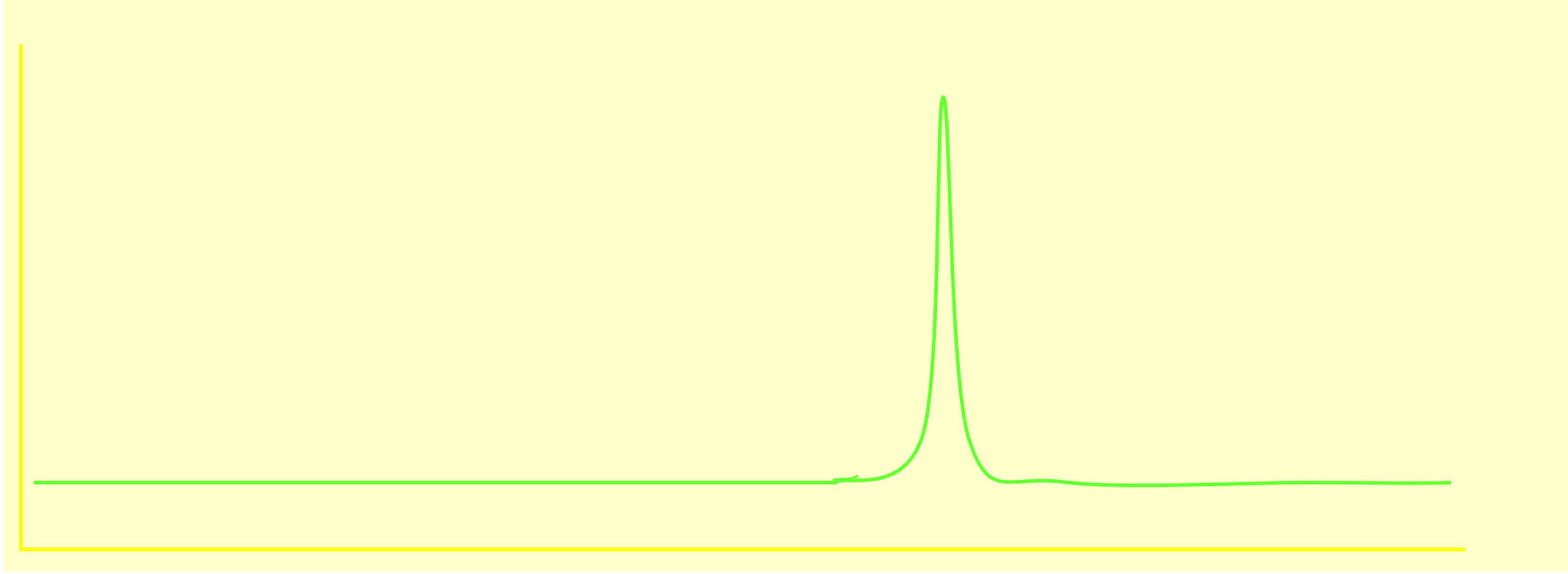
- Let us consider a chromatographic column on which three solutes A, B and C are separated in that order.

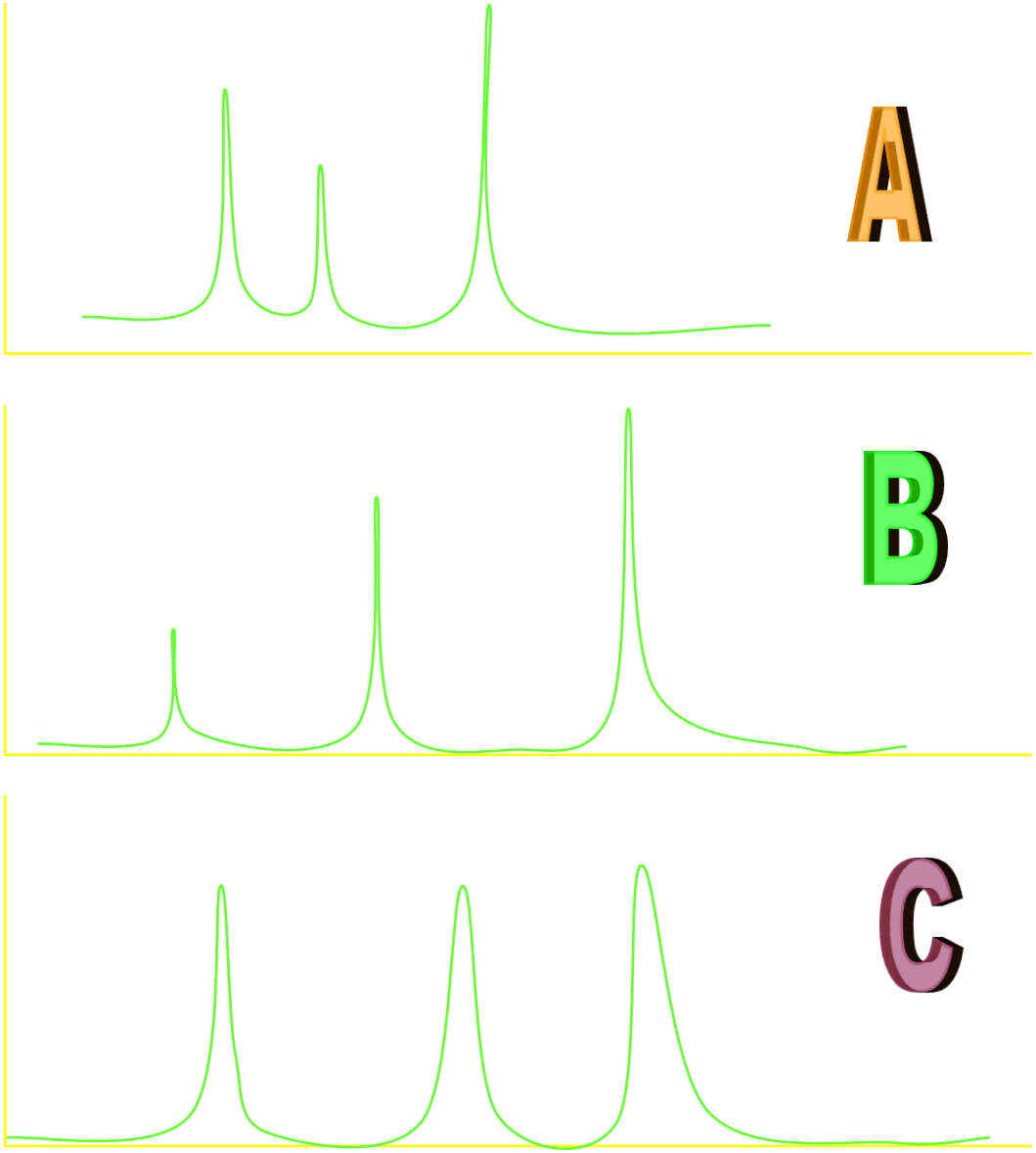
- Let there be sensor or a detector fixed at the other end of the column.

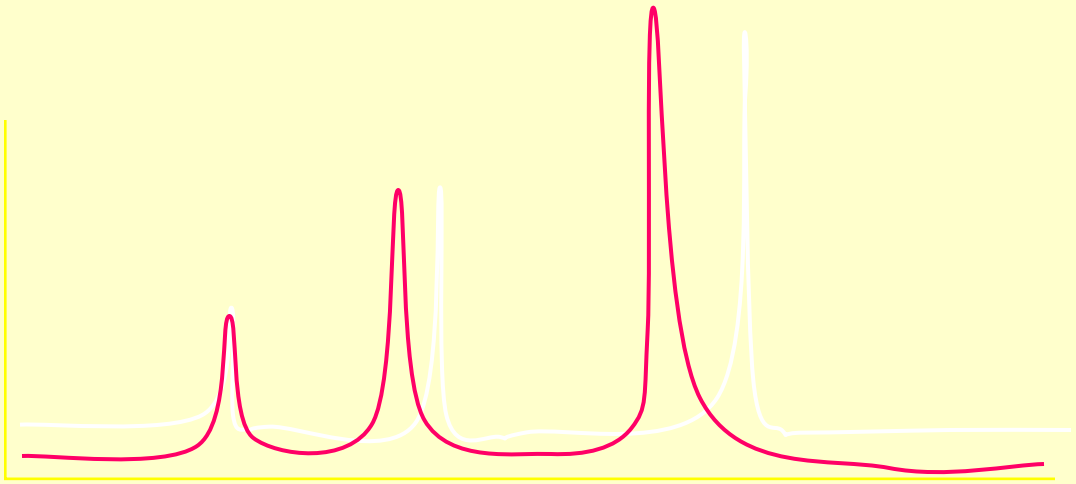
- The detector can sense and count the number of molecules of solute entering into it.

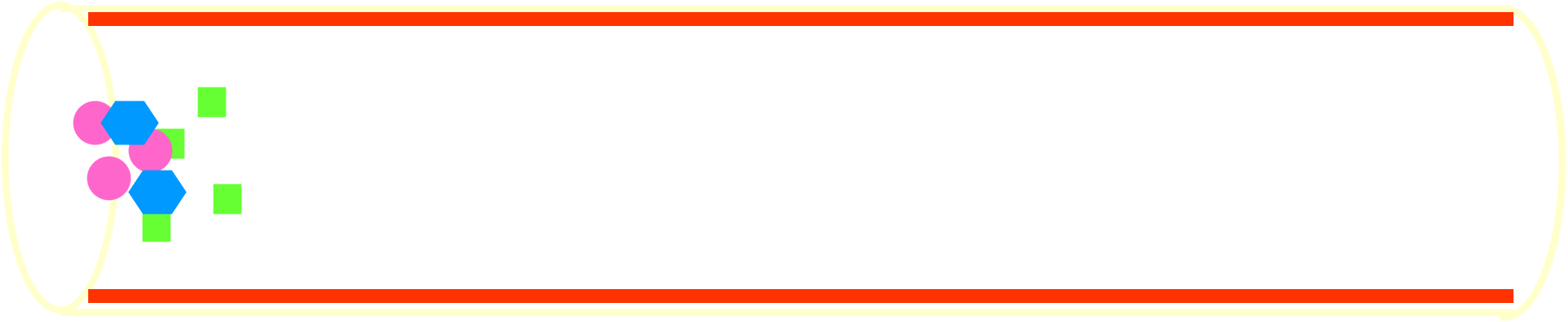


- As we can see from the figure, first the mobile phase will enter into the detector and no response will be generated.




A
B
C



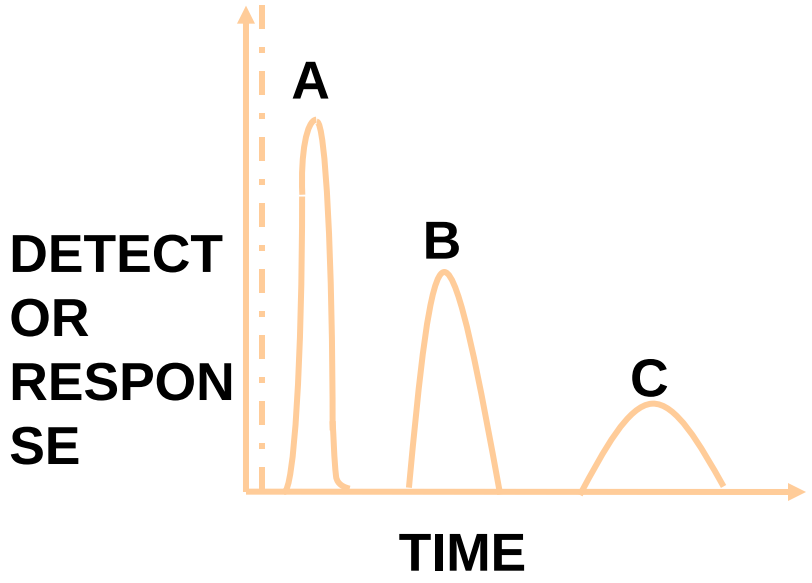


- Heights of these 3 peaks in the last slide are proportional to number of solute molecules present in each solute.
- *e.g.* No. of Blue molecules = 2

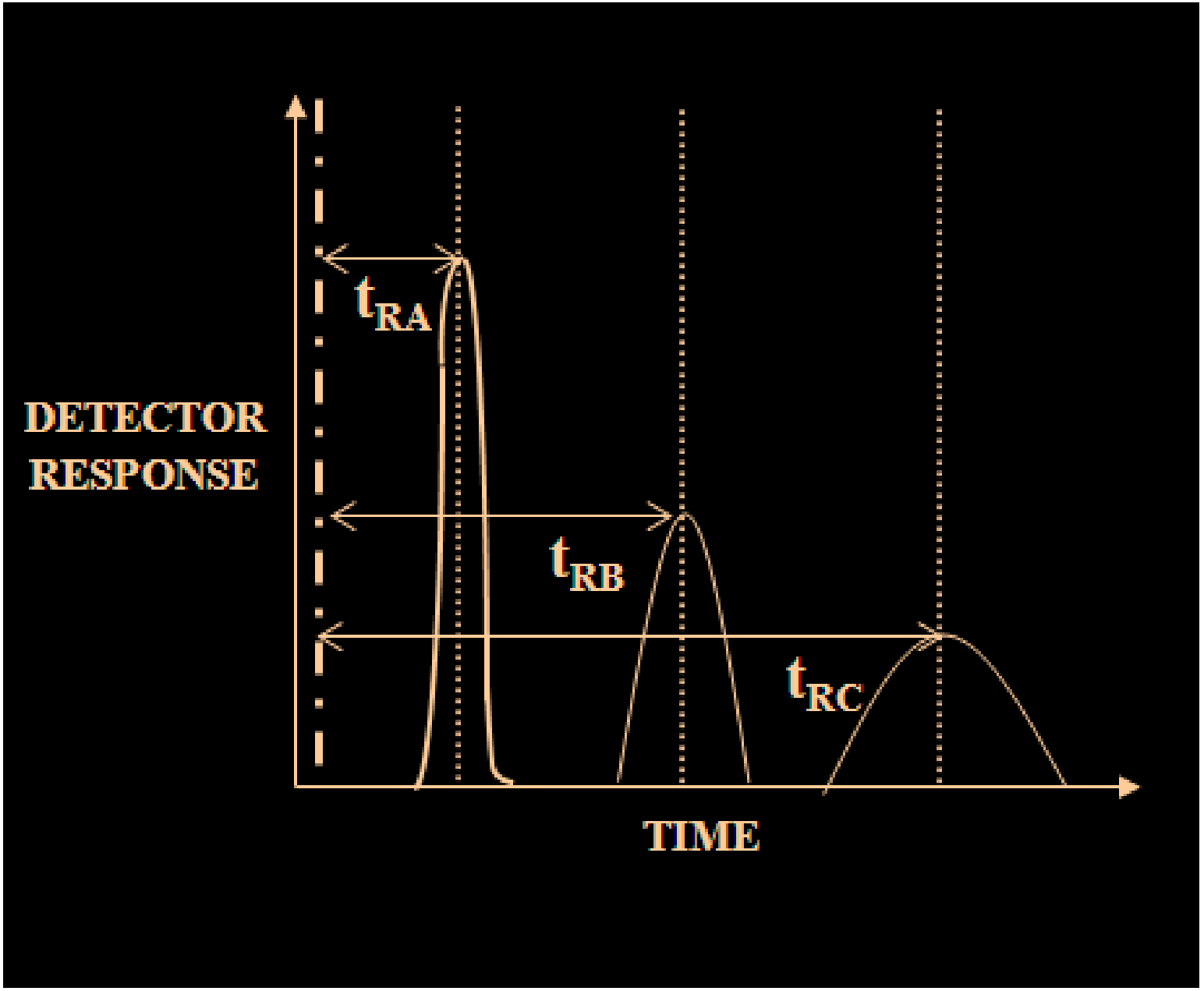
  No. of Pink molecules = 3

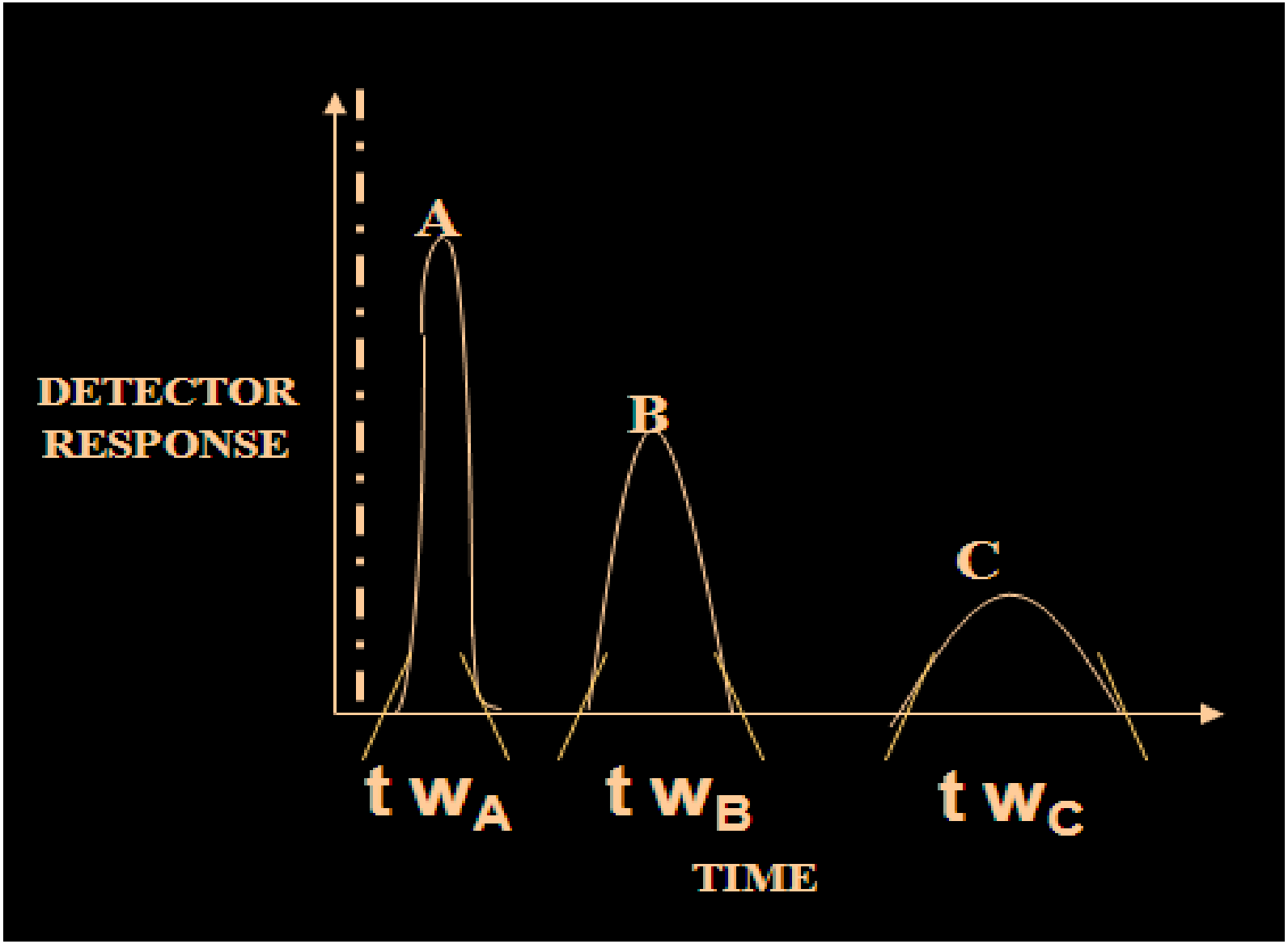
  No. of Green molecules = 4

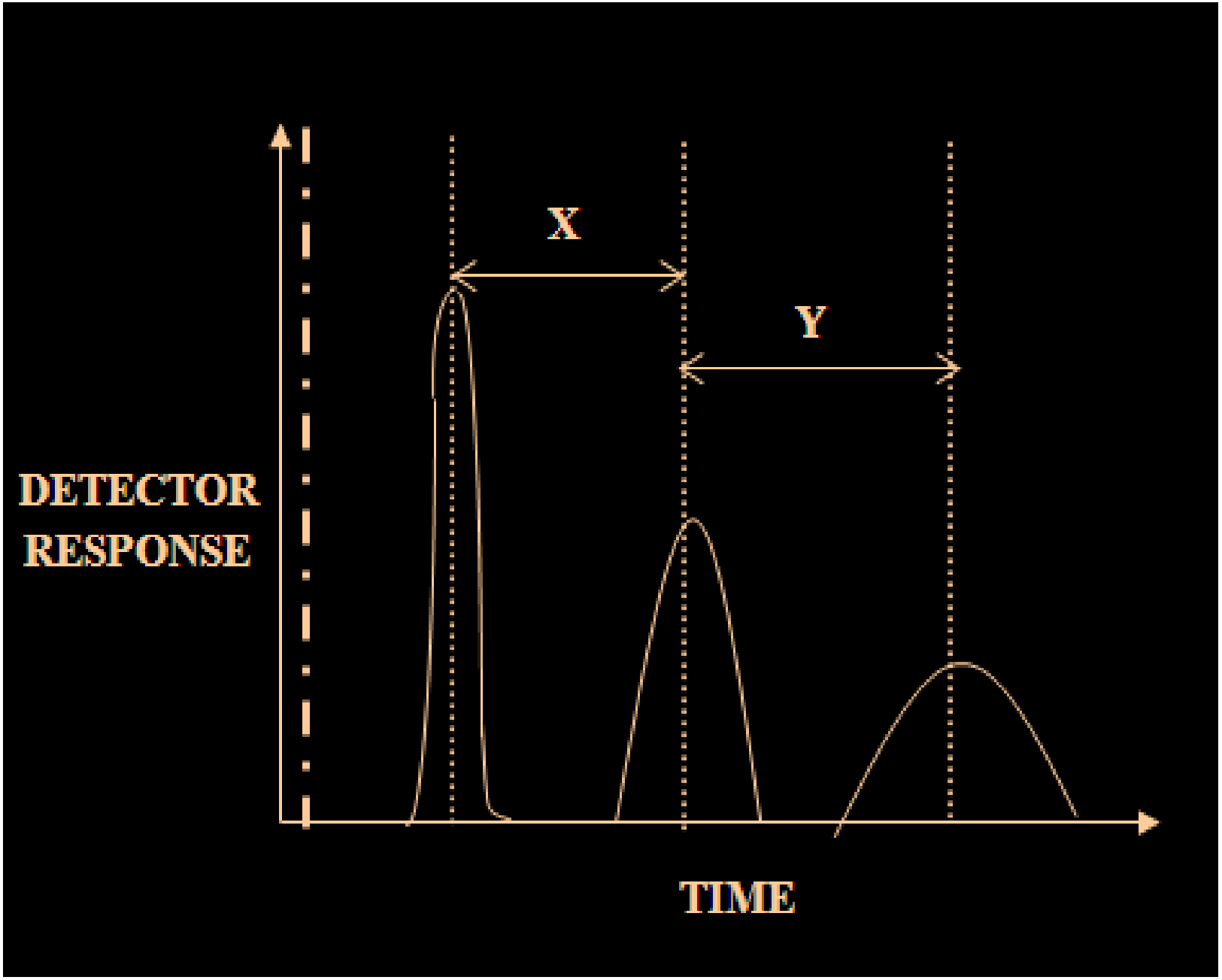
- If we plot a graph of graph of detector response as a function of time, the above description can be converted into the following picture.

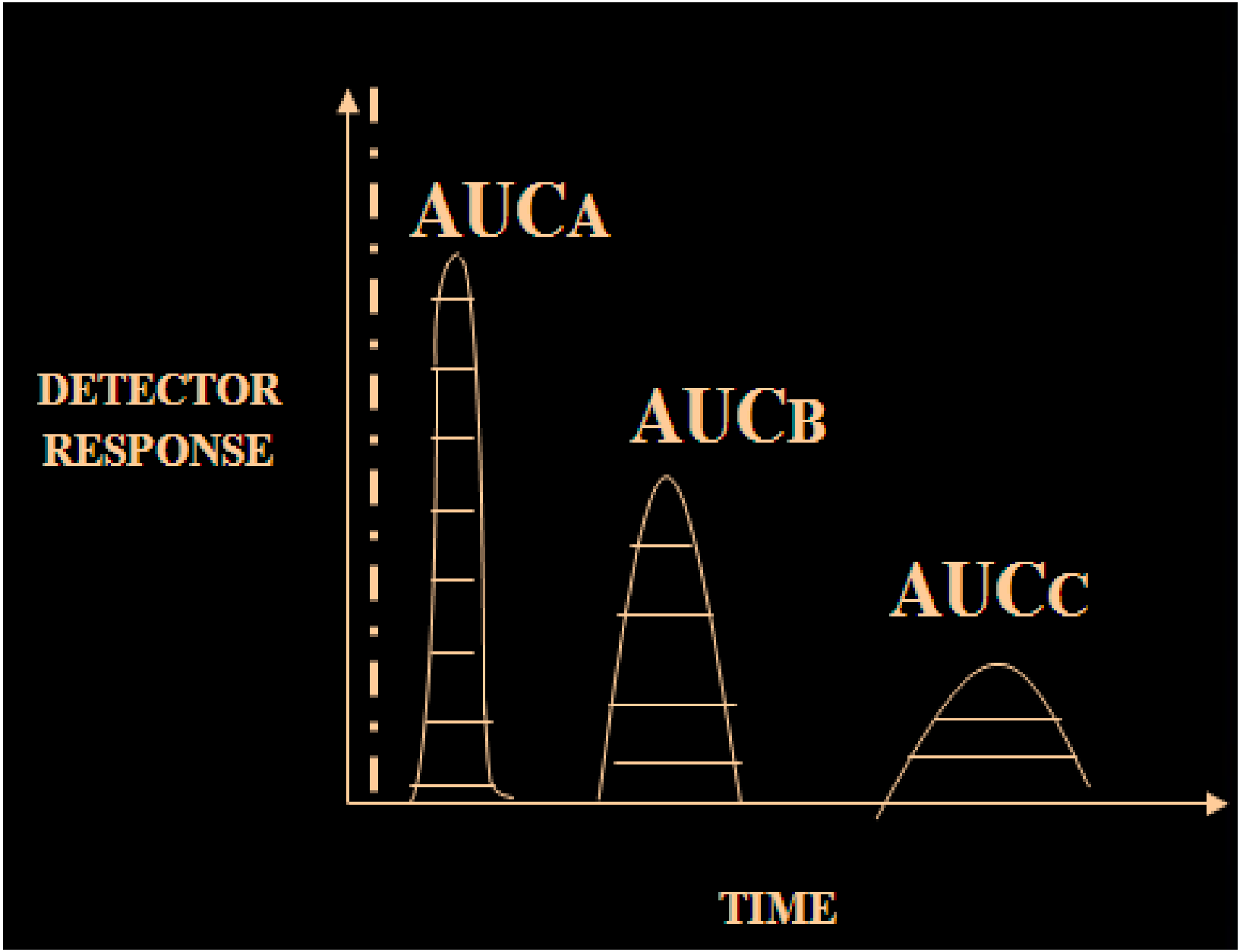


- This is called a chromatogram.


DETECTOR
RESPONSE
t_{RA}
t_{RB}
t_{RC}
TIME


DETECTOR
RESPONSE
A
B
C
t w_A
t w_B
t w_C
TIME


X
Y
DETECTOR
RESPONSE
TIME


AUCA
AUCB
AUCC
DETECTOR RESPONSE
TIME

- A normal chromatogram has certain characteristic features.

✓ Each peak has a characteristic retention time (distance between center of the peak and thereference point) under given experimental conditions.This is useful in qualitative Chromatographic analysis.

✓All the peaks are bell shaped symmetrical peaks.

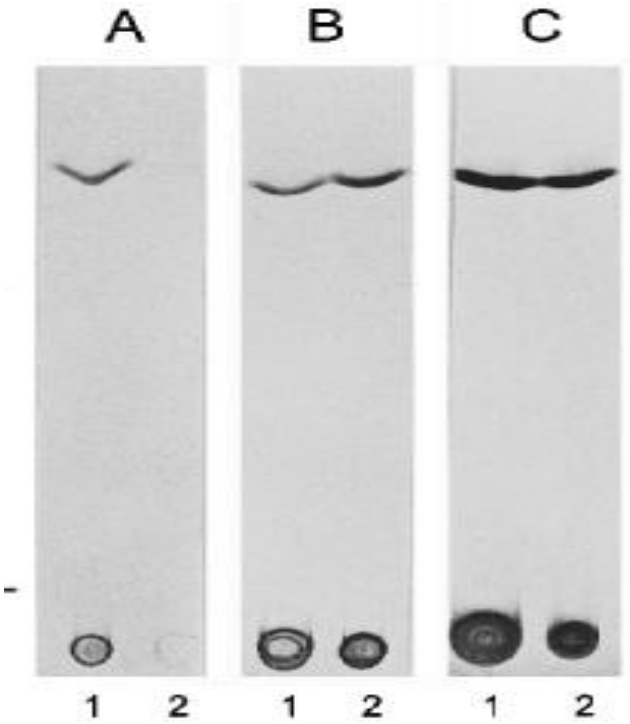
✓ Neighbouring peaks are separated by some distance.

✓ Each peak has certain area under curve (AUC) and AUC α Concentration of Solute. This is useful in quantitative chromatographic analysis. A separate calibration graph is needed for each peak in a chromatogram.

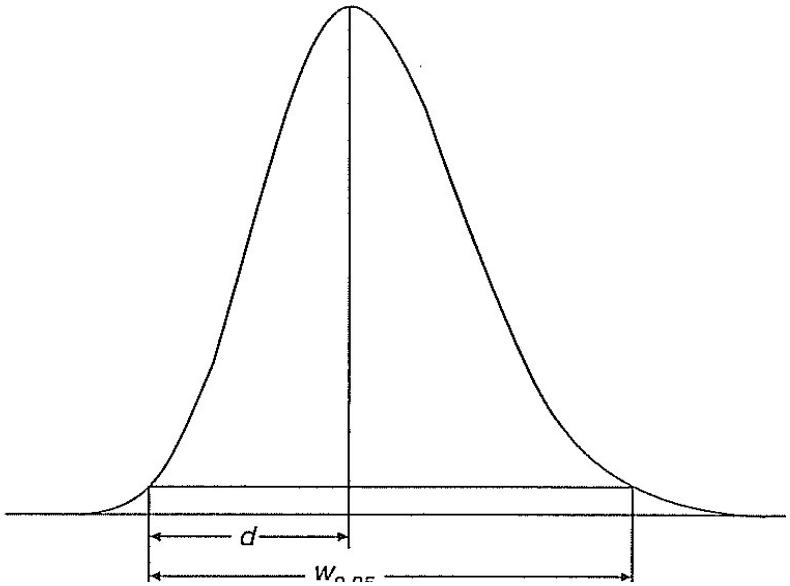
✓ Each peak has certain width at base which increases with increase in retention time.

✓ Volumetric and Gravimetric methods, on the other hand, are the examples of absolute methods of quantitative analysis.

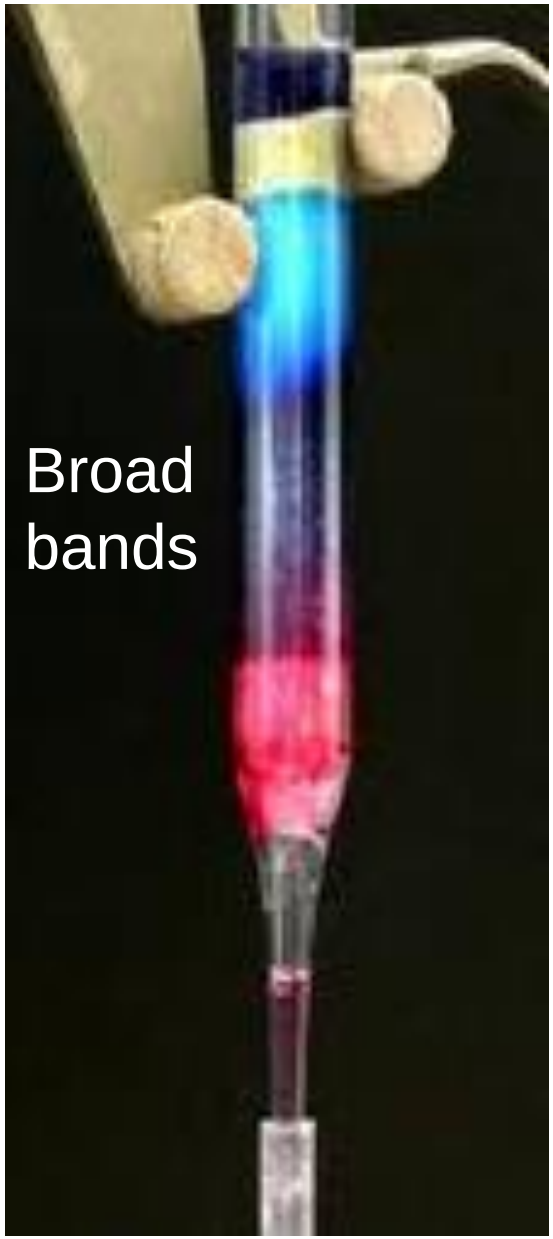
✓ Both qualitative and quantitative chromatographic analysis are relative methods of analysis in which the standard solutes and the sample solutes are chromatographed under the same experimental conditions.



Broad spots



Broad peak

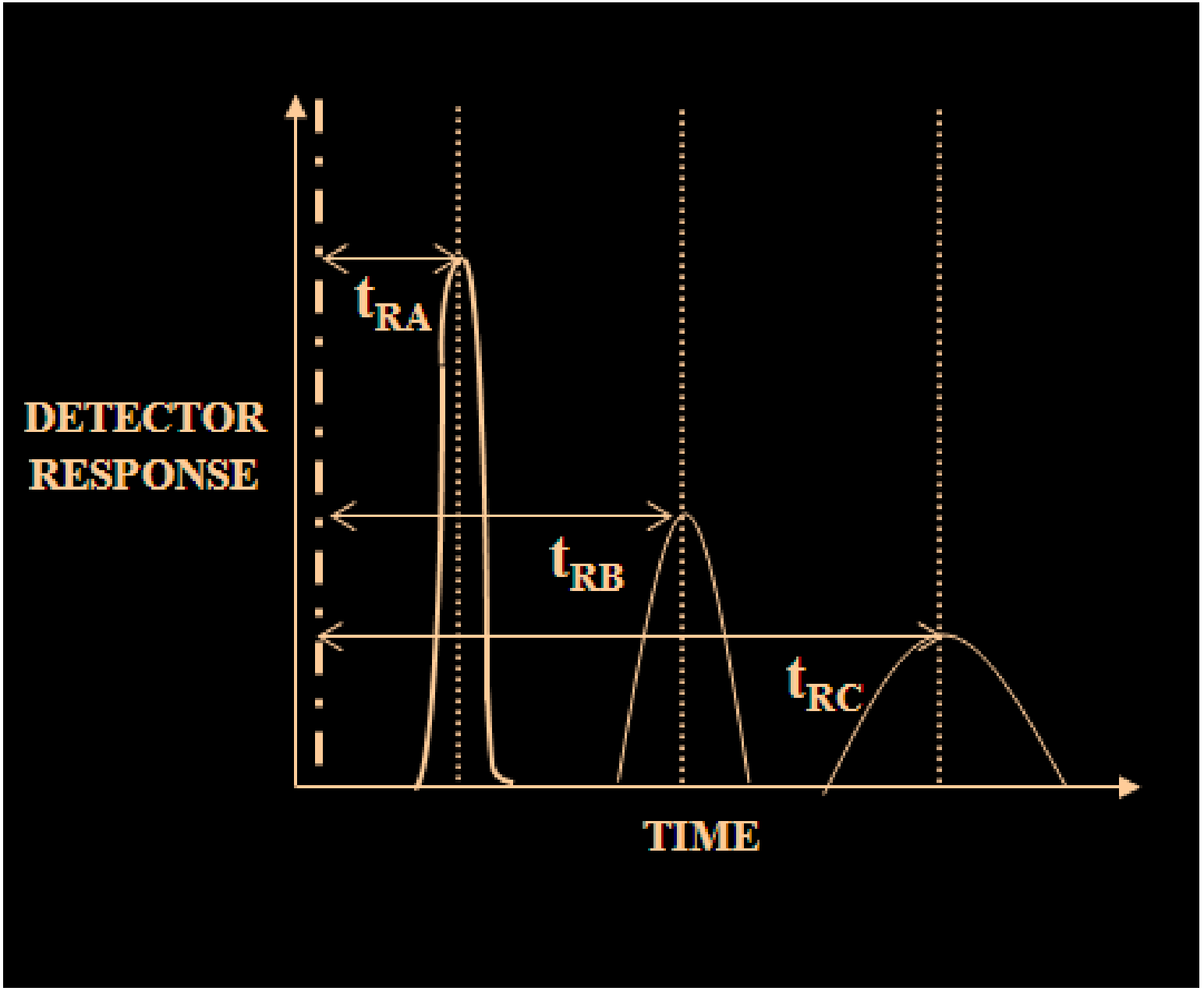


Broad bands

➢ What could be the reasons for this?

- If we observe our earlier Chromatogram carefully, we see that for each solute, solute molecules start eluting at a particular time and the elution is complete after a few seconds.

- It is observed in practical Chromatographic separations that the spots or bands or peaks are always broad. They are never very sharp.


DETECTOR
RESPONSE
t_{RA}
t_{RB}
t_{RC}
TIME

- It means that all the molecules of the same solute do not travel across the stationary phase with the same speed. Some travel faster than others. Therefore some reach the detector first and others latter.

- In other words molecules of the same solute spread across the stationary phase which results in **band broadening.**

- We must find out at least some important causes for molecular spreading and band broadening.

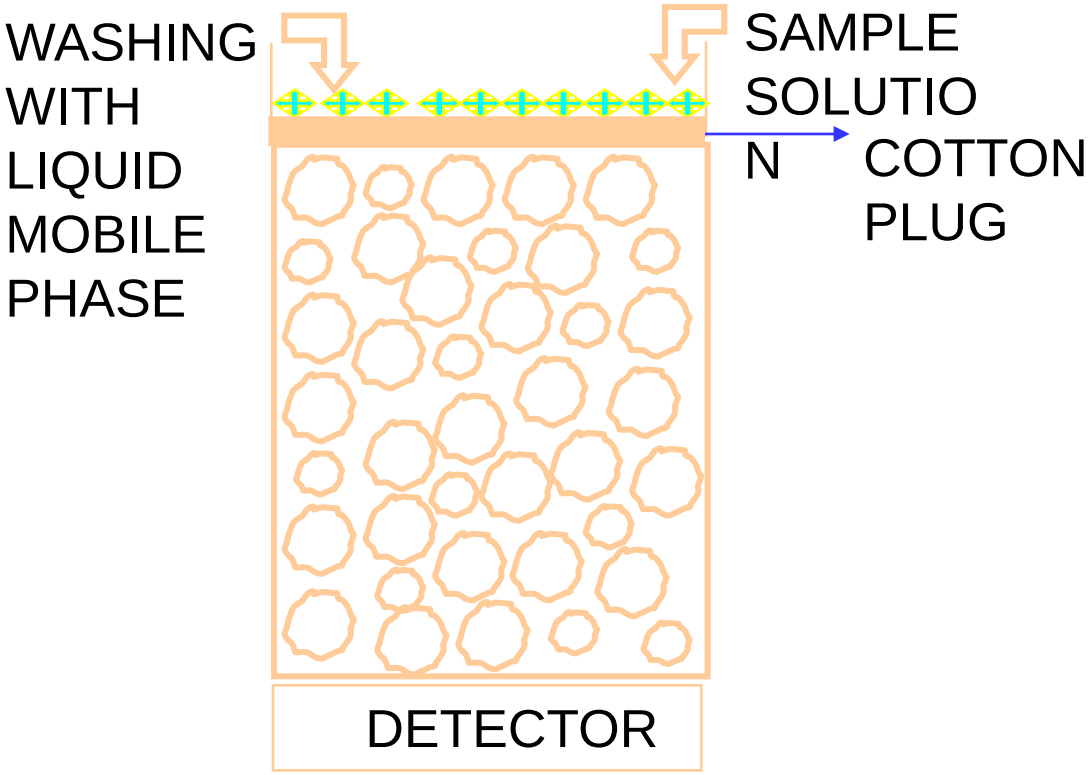
- We can visualize four important causes leading to molecular spreading and band broadening.

- Secondly, the spherical particles are always porous in nature. That is, the surface is not smooth.

- We should know in the beginning that the particles of stationary phases are approximately spherical in nature and the particles are not exactly of the same size.

- When we say that the particle size is 100μ, it really means that it is the average particle size and that every particle is not of exact 100μ diameter.

Let us now imagine a magnified picture of a chromatographic column



- We can see that when the solute molecules travel across the stationary phase, they have different paths to flow down the column. Each path has different path length.

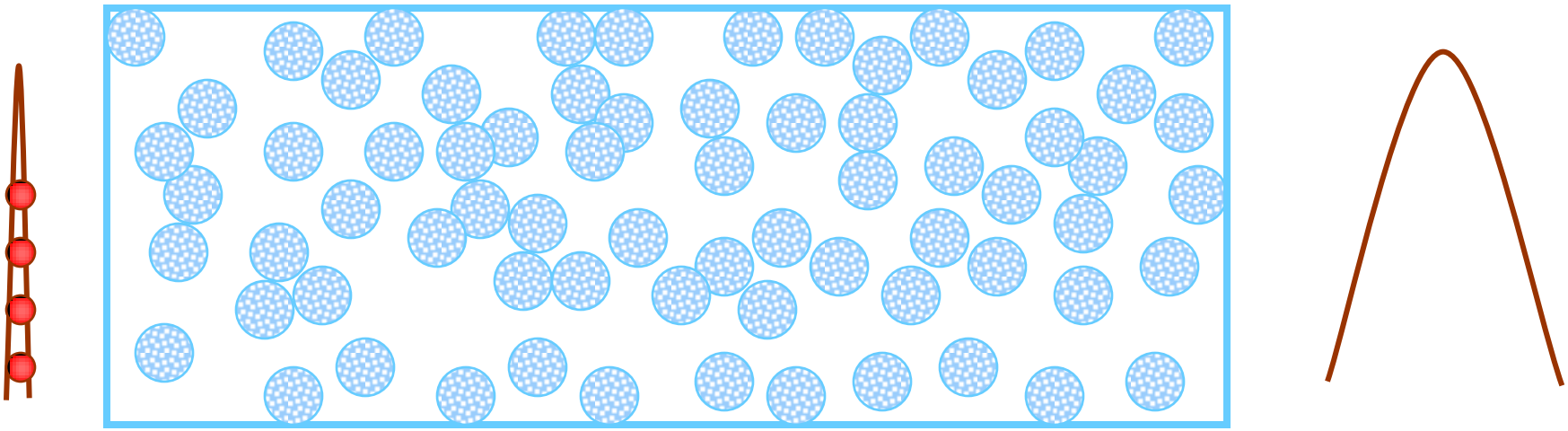
- As a result of multiple path flows, molecules of the same solute spread across the stationary phase .

- This cause of molecular spreading leading to band broadening is known as, “Eddy Diffusion” or “Multiple path flows Effect”.

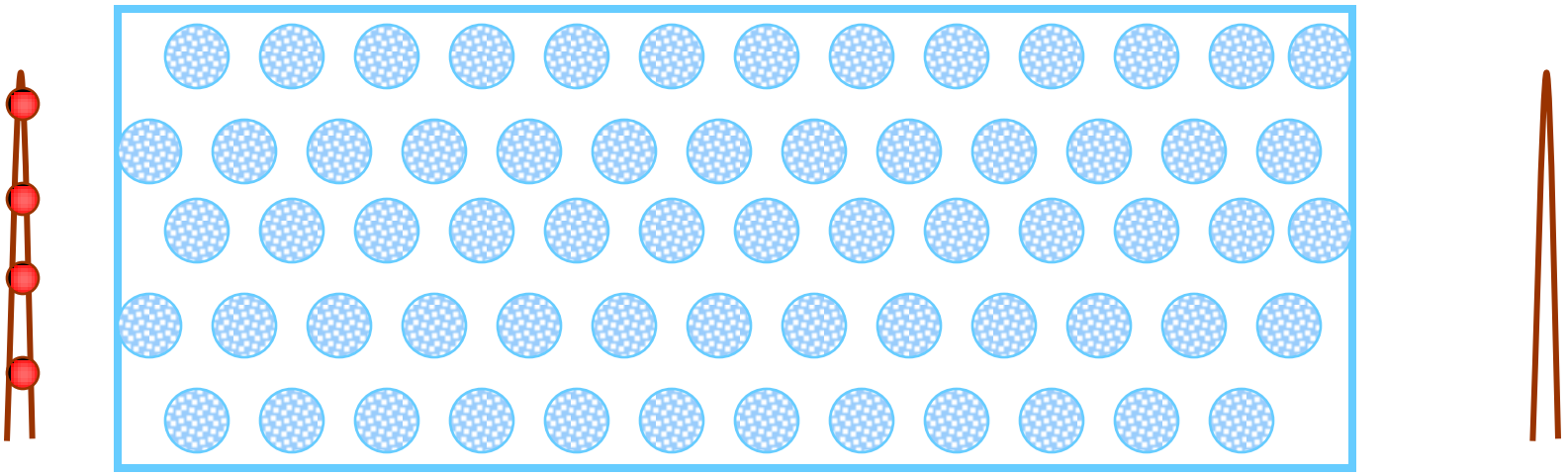
- The molecular spreading results in band broadening.

- Solute molecules travelling in longer paths reach the detector late.
- Solute molecules travelling in shorter paths reach the detector early.

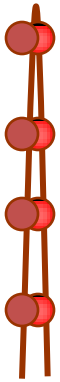
1. Eddy diffusion. (difference in the mobile phase stream flow due to non homogeneity of stationary phase) in packed columns.

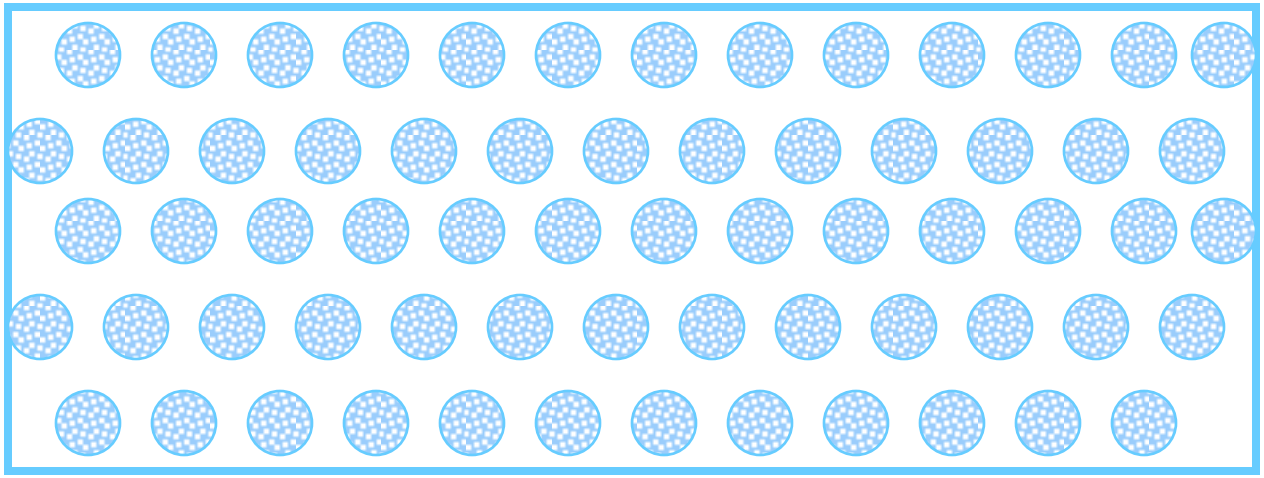


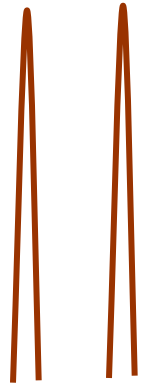
1. No or minimum Eddy diffusion. (difference in the mobile phase stream flow due to non homogeneity of stationary phase) in homogeneously packed columns.



1. No or minimum Eddy diffusion. (difference in the mobile phase stream flow due to non homogeneity of stationary phase) in packed columns.



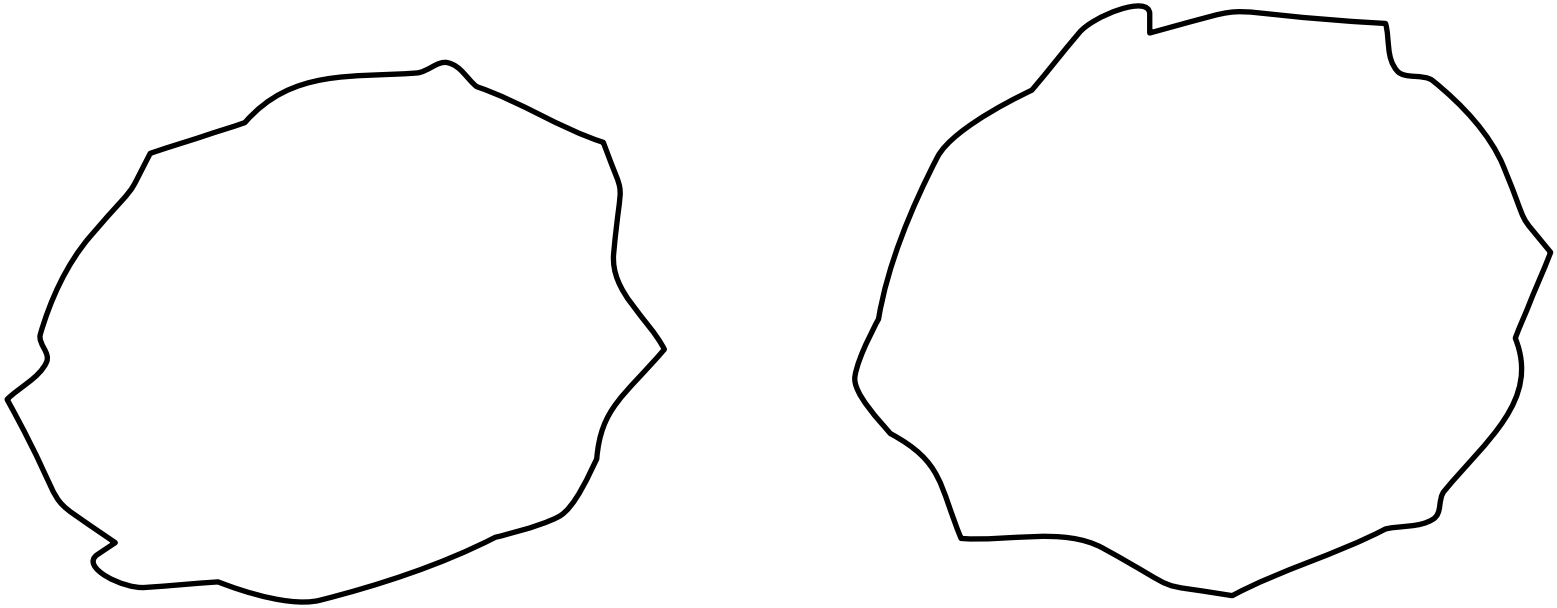




➢ Can Eddy diffusion be avoided?

- What is achieved by compact packing of stationary phase particles in the column probably is that the numbers of multiple path flows are minimized and the extent of molecular spreading leading to band broadening is reduced.
- Eddy diffusion, therefore, cannot be avoided, it can be optimized.
- Even if we bring the stationary phase particles closer together than what they are in the above picture, there will always be some distance between the neighbouring particles.

- Let us now have a magnified picture of two neighbouring stationary phase particles.



DETECTOR

- In the above picture we have three streams in which solute molecules are moving between two stationary phase particles.
- Two streams flow closer the surface of the stationary phase particles.

- This cause known as mobile phase mass transfer cannot be eliminated.

- Solute molecules in these streams experience resistance to flow from the surface of the stationary phase particles.

- Their migration velocity will be relatively smaller than those solute molecules traveling in the middle.

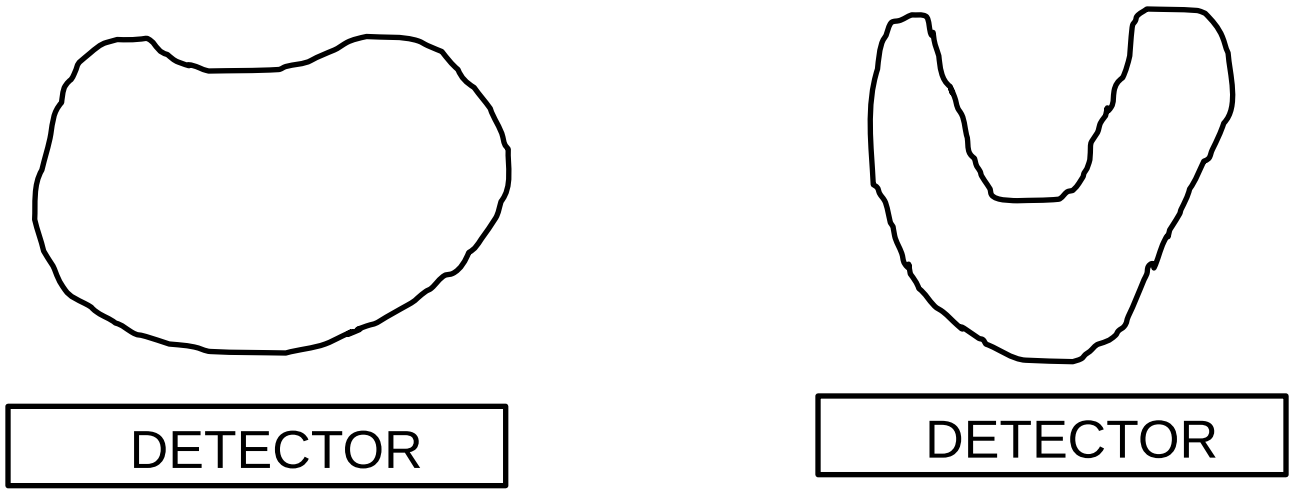
- This obviously results in molecular spreading adding to band broadening.

- But in a loosely packed column the contribution of Eddy diffusion will be more.

- In fact in closely packed column the contribution of this cause called "Mobile phase mass transfer" will be more than in a loosely packed column.

- In practice we have to strike a balance.

Let us now consider two pictures of two magnified porous particles.



A B

- In both the above pictures A and B, while solute molecules travel across the stationary phase , some of them enter inside the pores and remain stagnant for sometime before they are flushed out by the mobile phase which is continuously flowing across the chromatographic column.

- There is a small difference however.

- In either case, the solute molecules which enter into the pores have relatively smaller migration velocity as when compared to those which do not enter into the pores.
- In picture A the pore is shallow and the solute molecules remain stagnant along with the mobile phase for short time. This cause is known as “Stagnant Mobile Phase Mass Transfer”.
- This cause is known as “Stationary Phase Mass Transfer”.
- This results in molecular spreading and band broadening.
- In picture B, solute molecules enter into a deep pore. Inside the deep pore they have enough time to interact with the liquid stationary phase coated on the surface of the stationary phase particles.They remain in the deep pore for a longer period of time as that compared to solute molecules in a shallow pore.

➢ Can the last two causes be eliminated?

- The answer is ‘No’.
- If our pre-requisite of stationary phase is “porous particles” we can not eliminate.
- We can certainly minimize the last two causes by having stationary phase particles with shallow pores.

- In summary, it can be said that broad spots or broad bands in chromatographic separations are caused by molecules spreading across the stationary phase which results in band broadening.

- The longitudinal diffusion of solute molecules which is predominant in gas chromatographic separations does not have significant contribution in liquid chromatographic separations.

- Eddy diffusion, mobile phase mass transfer, stagnant mobile phase mass transfer, and stationary phase mass transfer are the four main causes for molecular spreading and band broadening.

- They can be optimized by choosing stationary phase particles with uniform size having shallow pores and packing compactly in a chromatographic column.
- The causes for molecular spreading leading to band broadening cannot be eliminated.

- Let us now consider in quantitative terms what we have discussed qualitatively about molecular spreading and band broadening in chromatographic separations.

- In order to do that let us make the following assumptions:

t_R - retention time of a solute

t_O - retention time of the mobile phase

u_X - migration velocity of the solute

u - migration velocity of the mobile phase

Lcm - Length of chromatographic column

n_s - number of moles of solute in the stationary phase

n_m - number of moles of solute in the mobile phase

R - fraction of moles of solute in the mobile phase

$$R = n_m / n_s + n_m$$

- Solute molecules move across the stationary phase only when they are in the mobile phase, therefore t_R depends on u_X .

So now, $u_x \alpha u$

$u_x \alpha R$

So,

$u_x = uR$ ---- after eliminating proportionality constants

So,

$$u_x / u = R = n_m / n_s + n_m$$

- Let us consider the ratio $n_s : n_m$, if $n_s > n_m$ then solute molecules will stay for longer period of time on stationary phase.

- If $n_m > n_s$ then solute molecules will stay for shorter period of time on stationary phase.

- It means that the ratio n_s / n_m is a deciding factor about how long solutes molecules will remain on the stationary phase under given experimental conditions.

- This ratio is called as capacity factor & denoted as k'

- k' is defined as the ratio of number of moles of solute in the stationary phase to that in the mobile phase.

  Therefore, $k' = n_s / n_m$

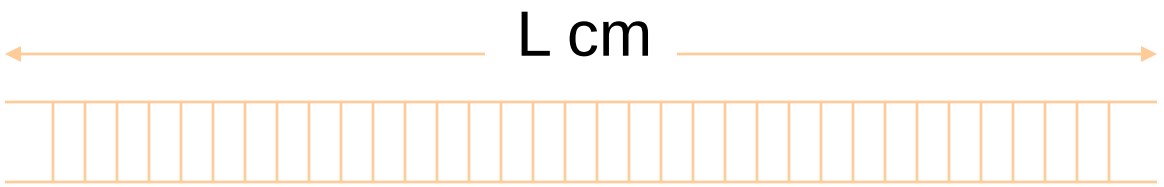
- Let us now consider one more important chromatographic parameter k' called as capacity factor or retention parameter.

- In other words larger the magnitude of k' longer will be the retention time of a solute and smaller the magnitude of k' shorter will be the retention time of a solute.

- If $n_s > n_m$ then k' value will be >1 and a solute will be retained on the stationary phase for a longer period of time.

- If $n_s < n_m$ then k' value will be < 1 and a solute will be retained on the stationary phase for a shorter period of time.

- Let us now try to understand the correlation between k‘ and t_R.



- For a solute with migration velocity u_x cm/min

$$t_R = L/u_x$$

And for mobile phase with migration velocity u cm/min

$$t_o = L/u \text{ therefore }, L = t_o u$$

We can rewrite the equation

$$t_R = L / u_x$$

$$= t_o u / u_x$$

But, $u / u_x = 1/R$

$$= k'+1$$

Therefore, $t_R = t_o (k'+1)$ or $k' = \frac{t_R - t_o}{t_o}$

- This is a very important equation to calculate k' values of solute in a chromatogram.

- Under given experimental conditions, therefore,each peak in a chromatogram has a characteristic k' value and characteristic retention time.

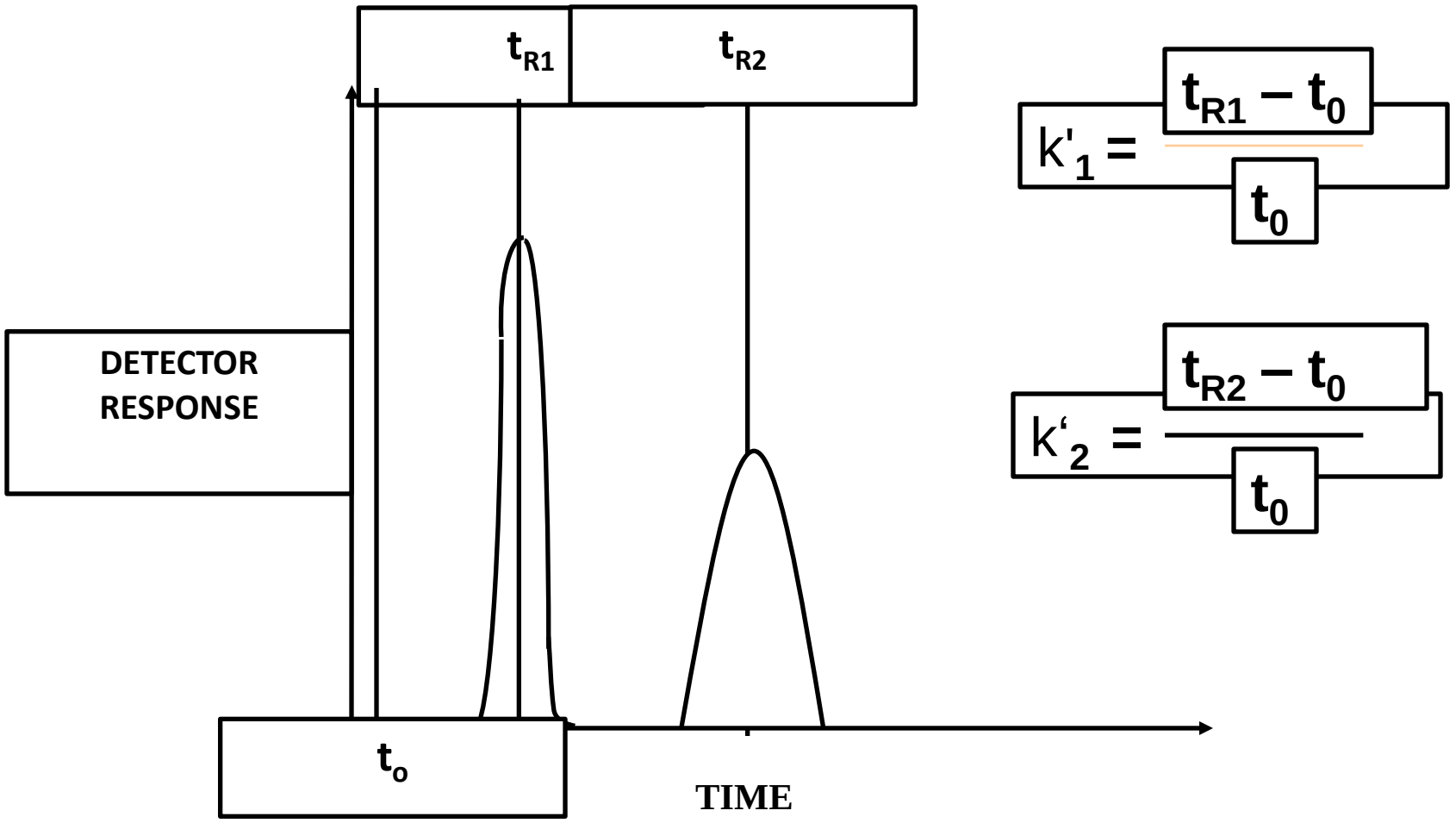
- Now we have the equation

$k' = n_s / n_m$ .Therefore, $k'+1 = n_s / n_m + n_m / n_m$

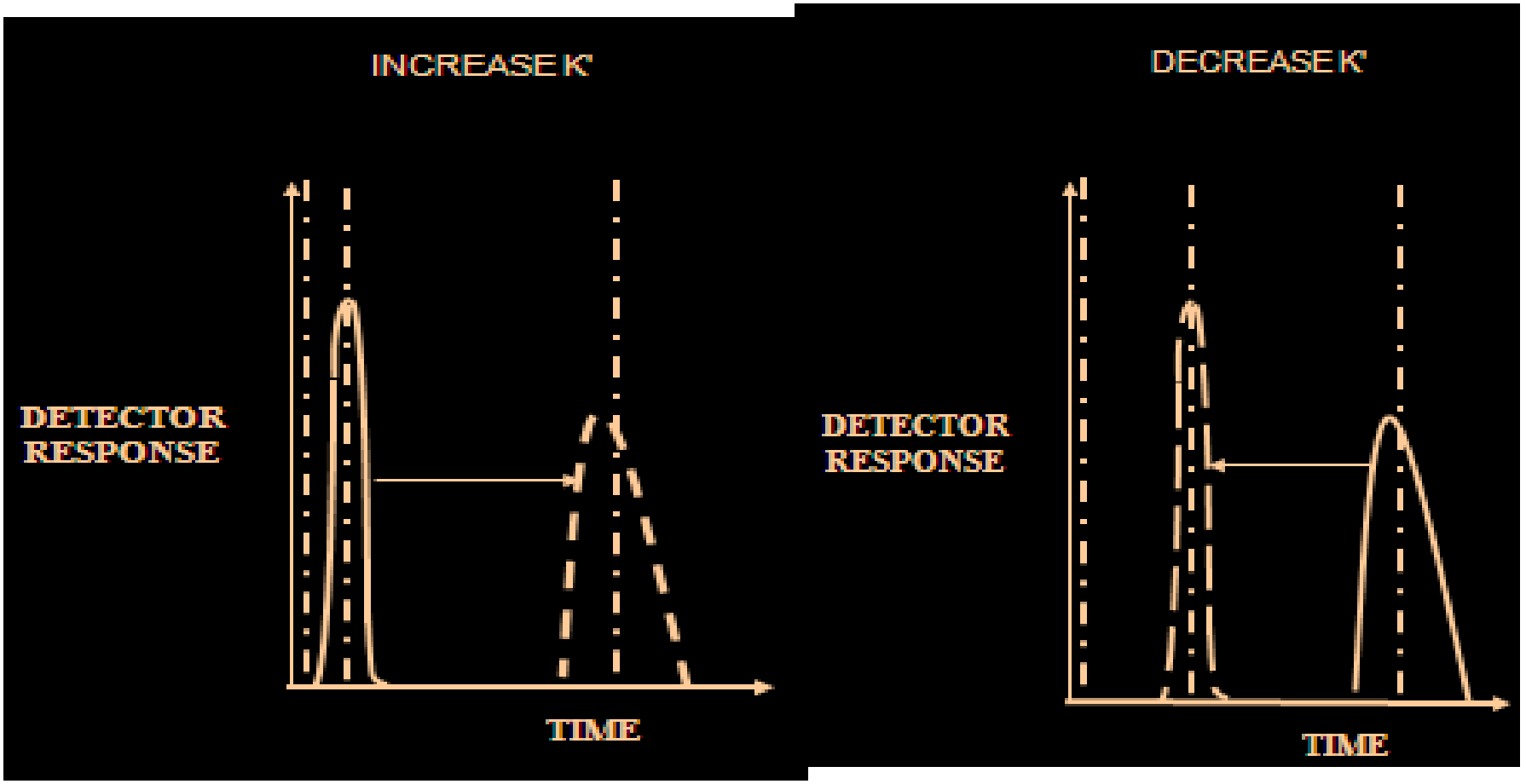
$$k'+1 = \frac{n_s + n_m}{n_m} \quad \text{but, } \frac{n_s + n_m}{n_m} = 1/R$$

Therefore, $k'+1 = 1/R$

➢ How do we calculate k' values from a chromatogram?



Let us imagine two different examples.



- In the first example we will have to increase k' while in the second example we have to decrease k' to achieve our goal.
- In the first example a solute has very short retention time and we would like to increase it.
- In the second example solute has very long retention time and we want to decrease it.

➢ **But how do we increase or decrease k' values?**

- For that we must know the factors on which k' depends. We will then be able to maneuver the factors to get the desired values of k'

- Larger the liquid phase loading, more will be the interactions of a solute with the stationary phase and longer will be its retention time or higher will be its k' value.

- The first factor is the liquid phase loading of the stationary phase.

- There are two important factors on which the magnitude of k' depends.

- Conversely by lowering the liquid phase loading both retention time and k' value of a solute can be reduced.

- The second factor which governs the magnitude of k' is the eluting strength or solvent strength of the liquid mobile phase.

- For a mixture of non-polar solutes, for example, hexane will have high eluting strength while water will have low eluting strength.

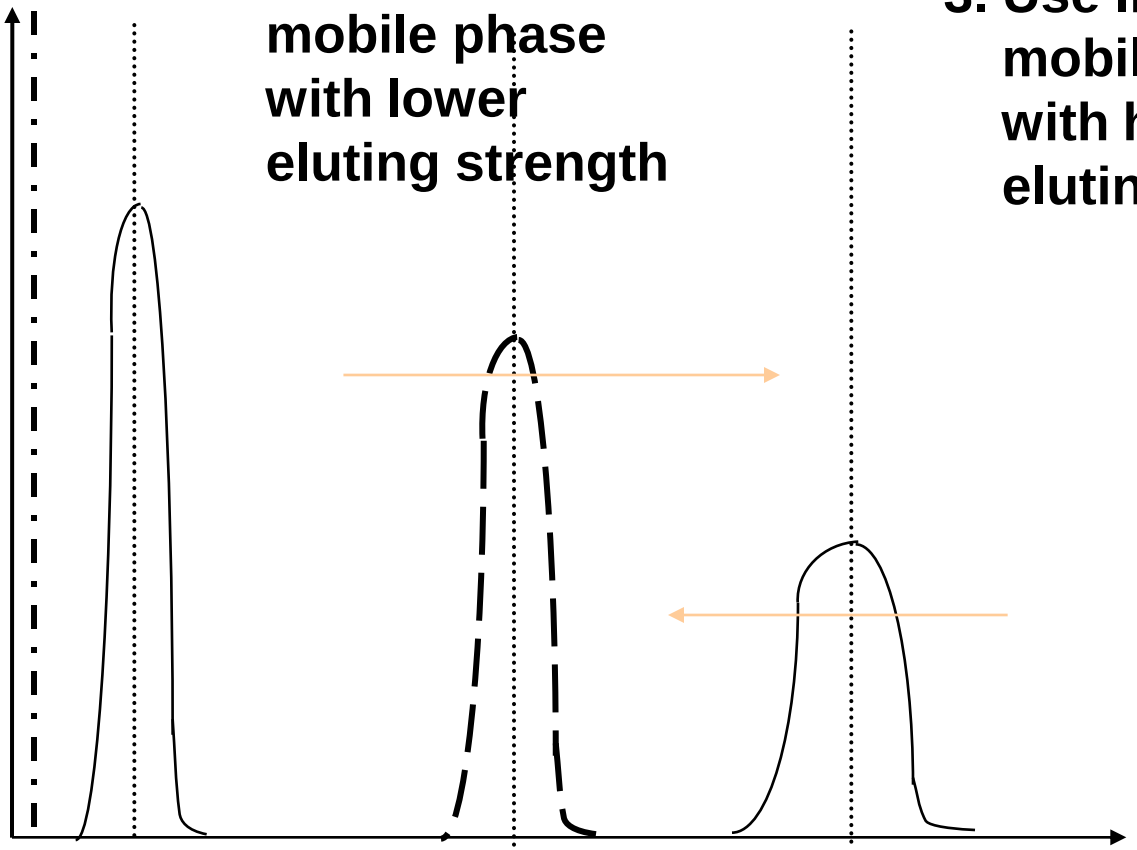
- Higher the eluting strength of the liquid mobile phase shorter will be the retention times and lower will be the magnitudes of k'.

- Conversely liquid phase with lower eluting strength will result in longer retention times and higher k' values.

- The eluting strength of a liquid mobile phase will depend on the nature of solutes being separated.

1. Increase k‘
2. Increase liquid phase loading
3. Use liquid mobile phase with lower eluting strength

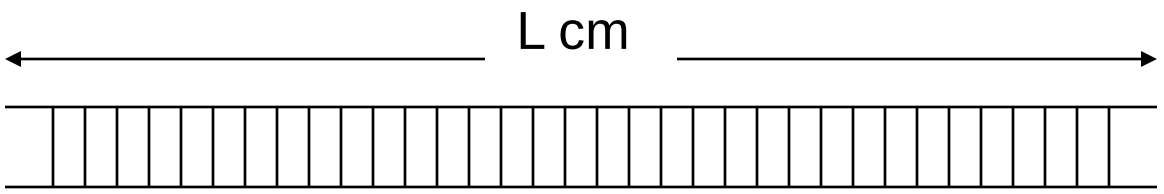
1. Decrease k‘
2. Decrease liquid phase loading
3. Use liquid mobile phase with higher eluting strength



DETECTOR RESPONSE

TIME

- Let us imagine that a chromatographic column of Lcm length is divided into N small sections of equal length or height.



- Each small section is called a “Theoretical Plate” because it is a theoretical concept.

- No such sections or plates exist in reality.

- The length or height of each theoretical plate is such that when solute molecules travel that distance a new equilibrium is established.
- The solute molecules constantly get exchanged between the stationary phase and the mobile phase when they travel across the column.
- The solute molecules travel in the forward direction along with the mobile phase only when they are in the mobile phase . They remain stagnant when they are in the stationary phase.
- There will be, therefore ‘N’ number of such equilibria across the length of the column.

- It is then easy to imagine that more the number of theoretical plates associated with the given length of chromatographic column, more will be such equilibria and solute molecules will have less time and chances to spread across the stationary phase.

- It means that more the N (number of theoretical plates), lesser is the molecular spreading and sharper will be the resulting bands or peaks.

- We also know that L / N = H, height equivalent to a theoretical plate. N and H are inversely proportional.

- In other words we can say that higher the N associated with a given length of chromatographic column higher is

“ column separation efficiency.”

- It can, therefore, be concluded that higher the N or lower the H associated with a given length of chromatographic column, higher is the column separation efficiency.

- How do we get a chromatographic column with high N or low H?

- We must know the factors on which N or H depends. Once we know them, we can maneuver them to get the desired separation efficiency.
- A chromatographer needs a chromatographic column with high separation efficiency. It means he wants a column with high N or low H.
- We have, therefore, introduced a very Important chromatographic parameter, which can be described in terms of the magnitude of N or H.

- The following three factors govern the column separation efficiency.

1. Particle size of stationary phase.

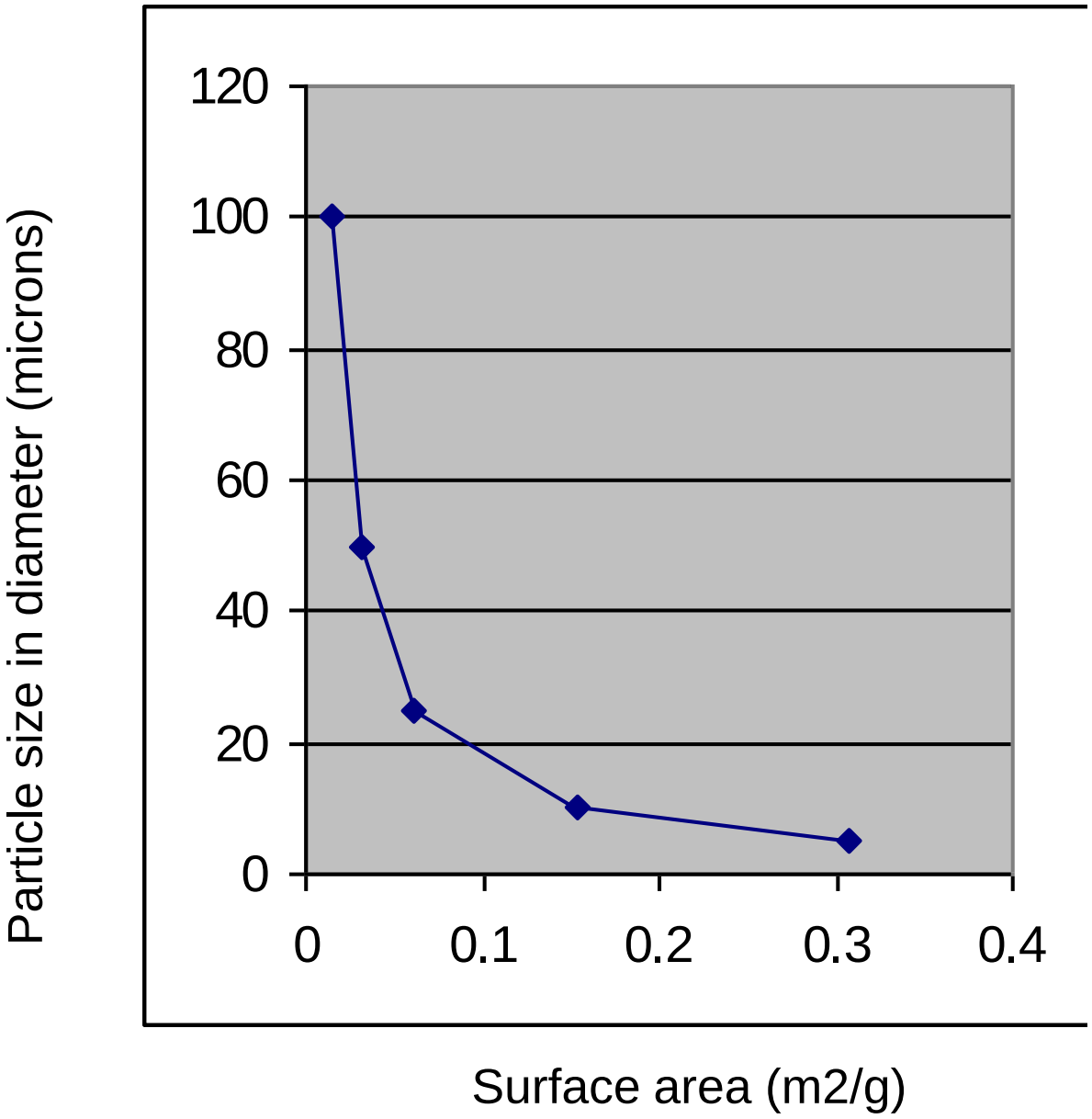
2. Length of chromatographic column.

3. Mobile phase velocity.

Particle size of stationary phase

- If we reduce the particle size diameter from 100µ to 50µ, 25µ, 10µ, 05µ, the surface area will increase as follows.
- We normally assume stationary phase particles to be spherical in shape.

Particle size in diameter (microns)	Surface area (m^2/g)
100	0.01538
50	0.0307
25	0.0615
10	0.153
5	0.307


Particle size in diameter (microns)
120
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
Surface area (m2/g)

- When the surface area increases, the amount of the liquid phase coated also increases proportionately. More the liquid phase loading more will be the interactions of solute molecules with the liquid stationery phase, less will be molecular spreading and band broadening.

- We can see that as the particle size of spherical particles is reduced, the surface area increases exponentially.

- Then we can say that lower the particle size higher will be N and higher will be the column separation efficiency.

- We have therefore to optimize the particle size without sacrificing analytical times. Because to achieve good separation with reasonable analytical time is practically important and cannot be overlooked.
- However we have also to remember that as the stationary phase particles become smaller there is more resistant to mobile phase flow.

- With longer columns ,however, retention time of solutes will increase and peaks will become broader and analytical time will increase.

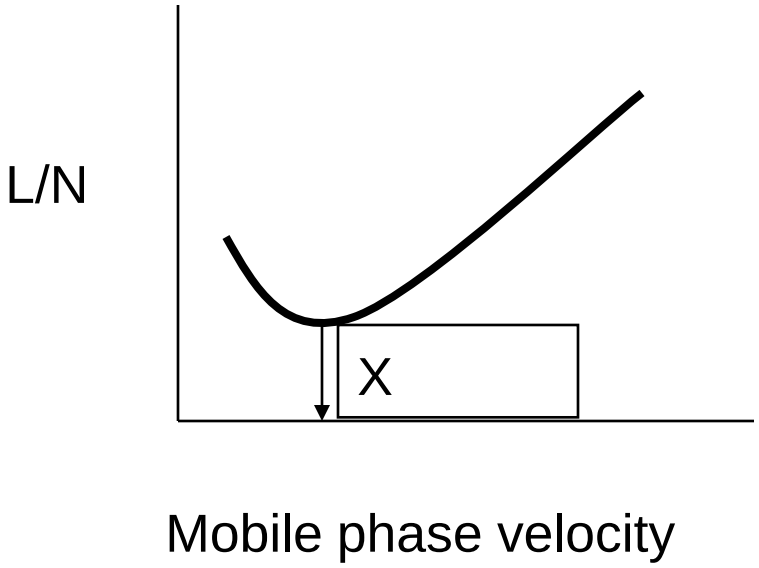
- If with a given length of say 100cm long column, N = 10,000 then with L = 200cm, N= 20,000, keeping particle size of stationary phase the same.

- Thus, longer the chromatographic column, higher will be N and column separation efficiency.

- Unfortunately, there is no direct correlation between the mobile phase velocity and N or H. For each type of stationary phase we have to construct a plot of L/N or H vs. mobile phase velocity and then optimize the same.

- We have to, therefore, optimize the column length just as we optimized the particle size. Our main aim being good separation with reasonable analytical time.

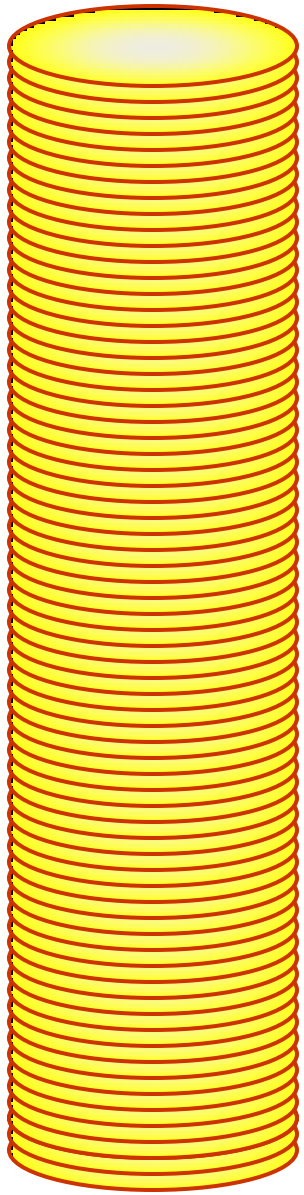
For example for 10µ diameter porous stationary phase particles we get a graph as shown below.



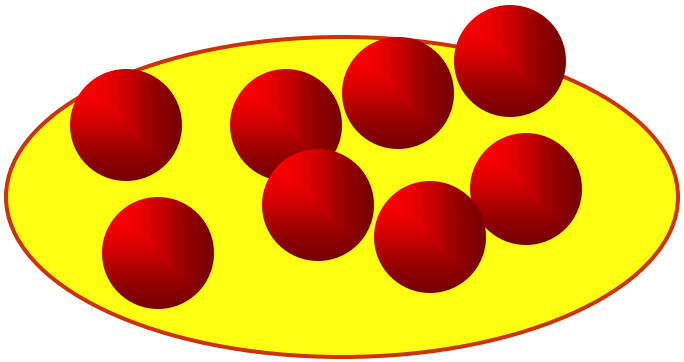
- It can be seen that initially as the mobile phase velocity increases, H decreases i.e. separation efficiency increases.
- It reaches at the maximum with mobile phase velocity ‘x’.

- From the above discussion it becomes clear that for getting good separation efficiency with reasonable separation time we have to optimize particle size, column length and mobile phase velocity.
- We may, therefore, be tempted to work at the mobile phase velocity ‘x’ because at this point we get the maximum separation efficiency.
- However at this mobile phase velocity if the separation times are long, then we will have to work at higher mobile phase velocity at the cost of lower separation efficiency to achieve reasonable analytical time.

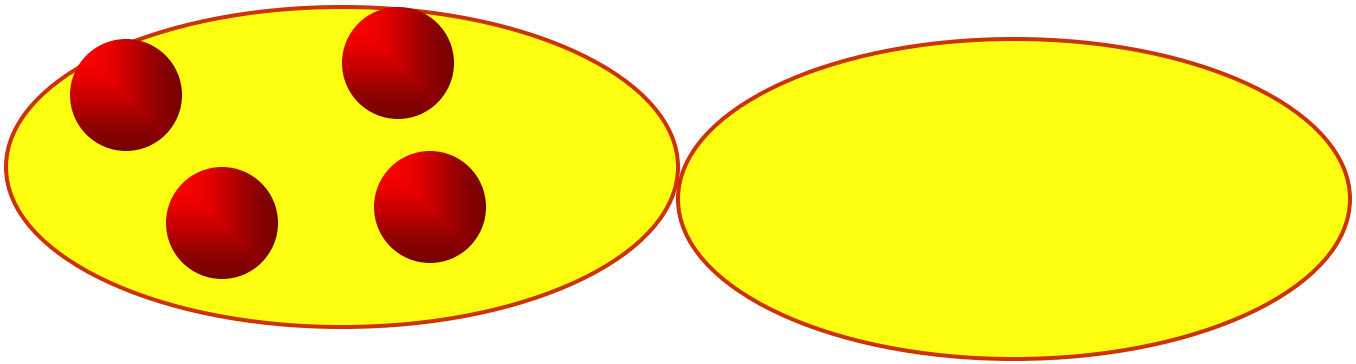
The plate theory



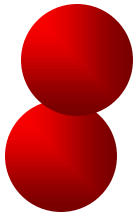
$$K_c = \frac{[A]_S}{[A]_M} = \frac{1}{1} = 1$$

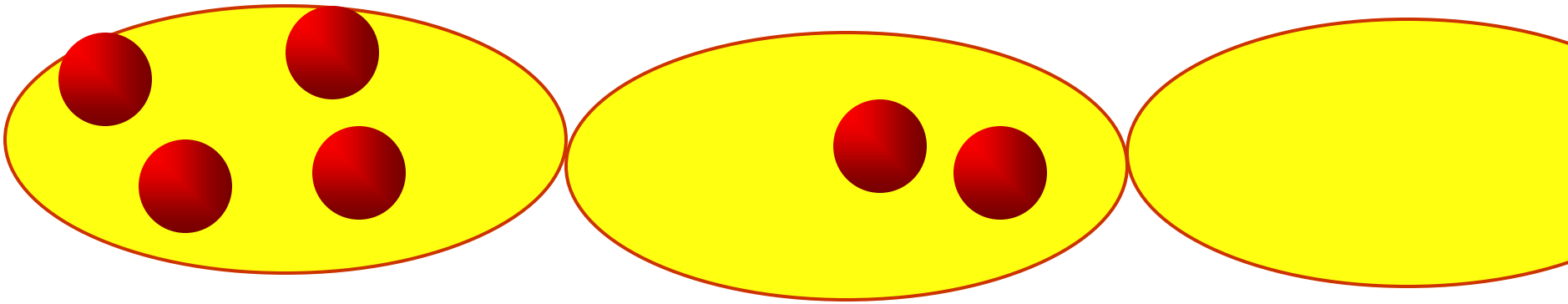


$$K_c = \frac{[A]_S}{[A]_M} = \frac{1}{1} = 1$$

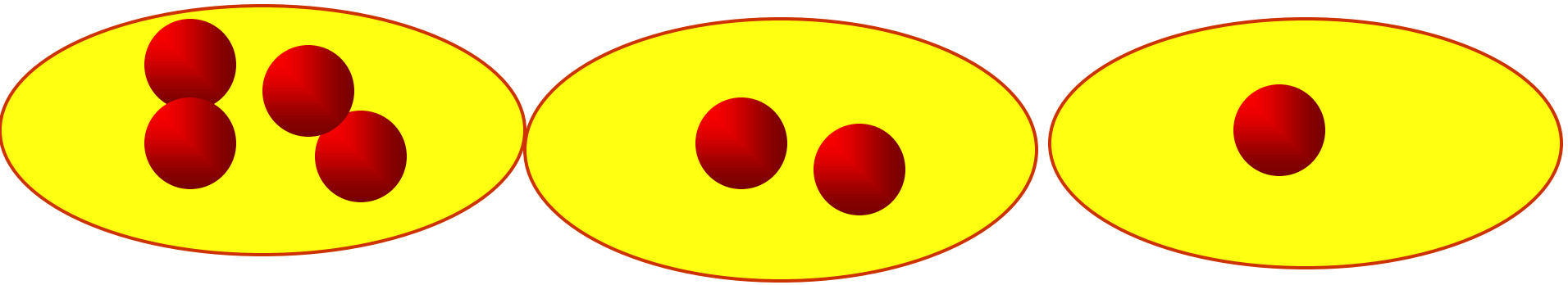


$$K_c = \frac{[A]_S}{[A]_M} = \frac{1}{1} = 1$$

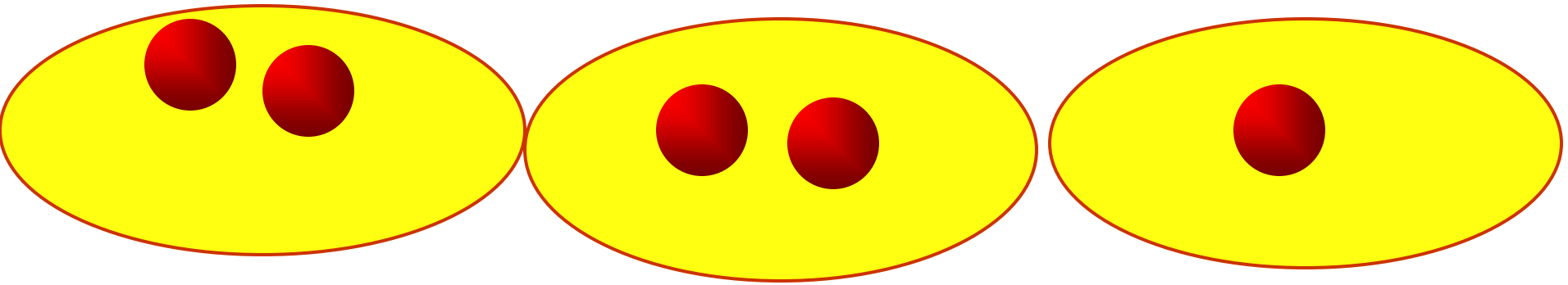




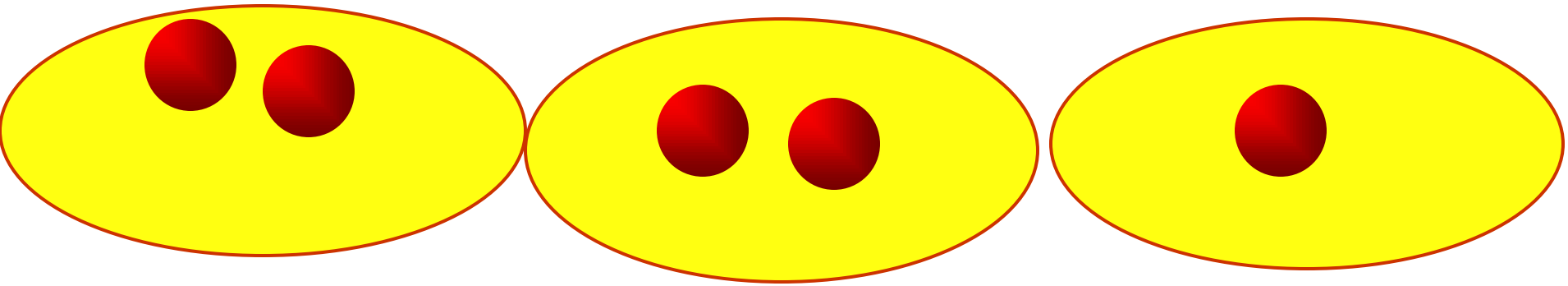
$$K_c = \frac{[A]_S}{[A]_M} = \frac{1}{1} = 1$$



$$K_c = \frac{[A]_S}{[A]_M} = \frac{1}{1} = 1$$



$$K_c = \frac{[A]_S}{[A]_M} = \frac{1}{1} = 1$$

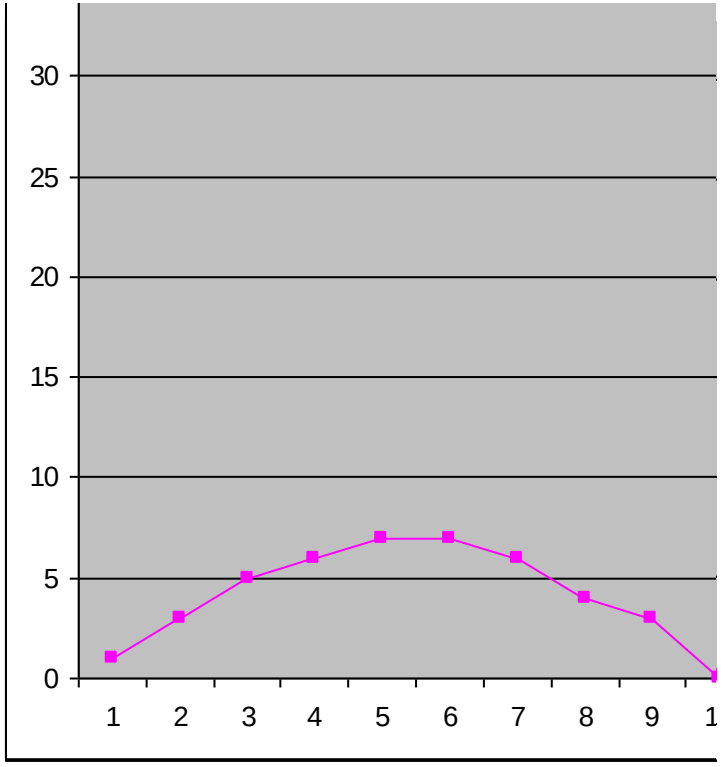


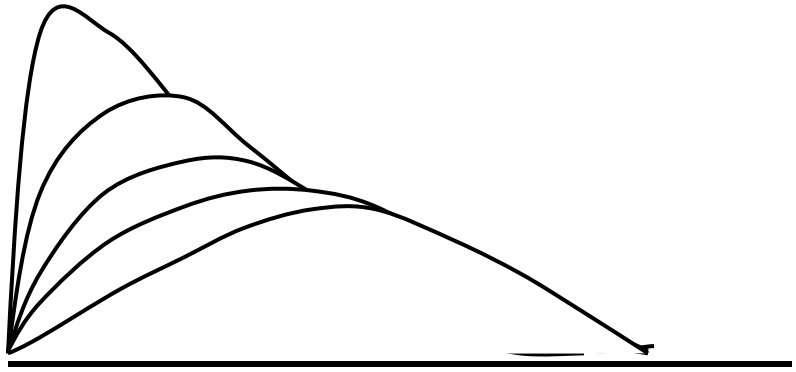
Lets take an example where 32 molecules of analyte are injected to a column. let the Distribution constant Kc be 1.

$$K_c = \frac{[A]_S}{[A]_M} = \frac{1}{1} = 1$$

$$\text{Retardatio n factor , R} = \frac{1}{1+\mathrm{K_c}} = \frac{1}{1+1} = 0.5 \quad \text{or } 50\%$$

32	16	8	4	2	1			
16	16	12	8	5	3	1		
8	12	12	10	7	5	3	1	
4	8	10	10	8	6	4	2	1
2	5	7	8	8	7	5	3	2
1	3	5	6	7	7	6	4	3





Note that

1. The curve assumes the Gaussian shape as it progresses.

2. The inflection point shifts along the X axis

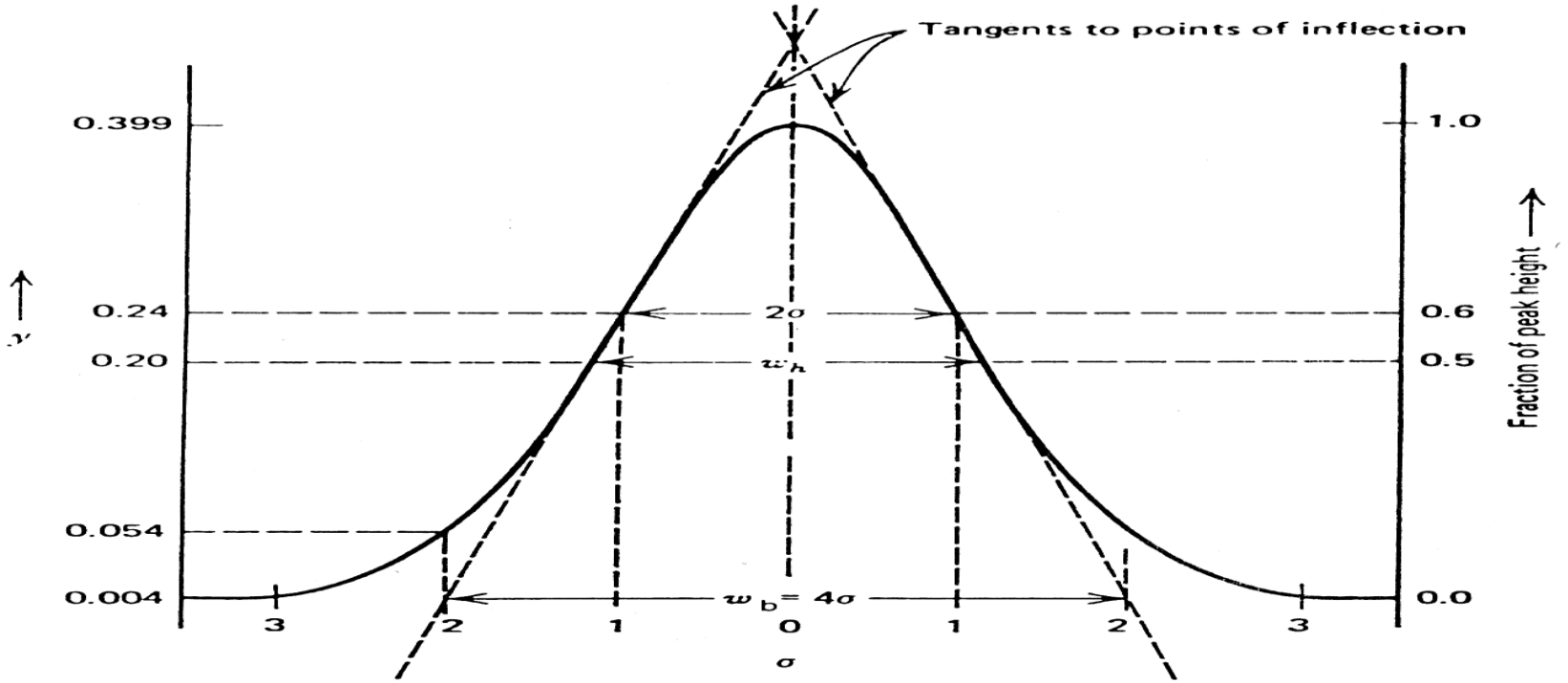
3. The area under the curve remains the same even its shape changes, till eluted out.

4.fronting and tailing is lesser as plate no N increases.

- How do we calculate N or H from a chromatogram?

- The most common measure of the efficiency of a chromatographic system is the plate number, N.

  $N = (t_R/\sigma)^2 = 16(t_R/w_b)^2 = 5.54(t_R/w_h)^2$



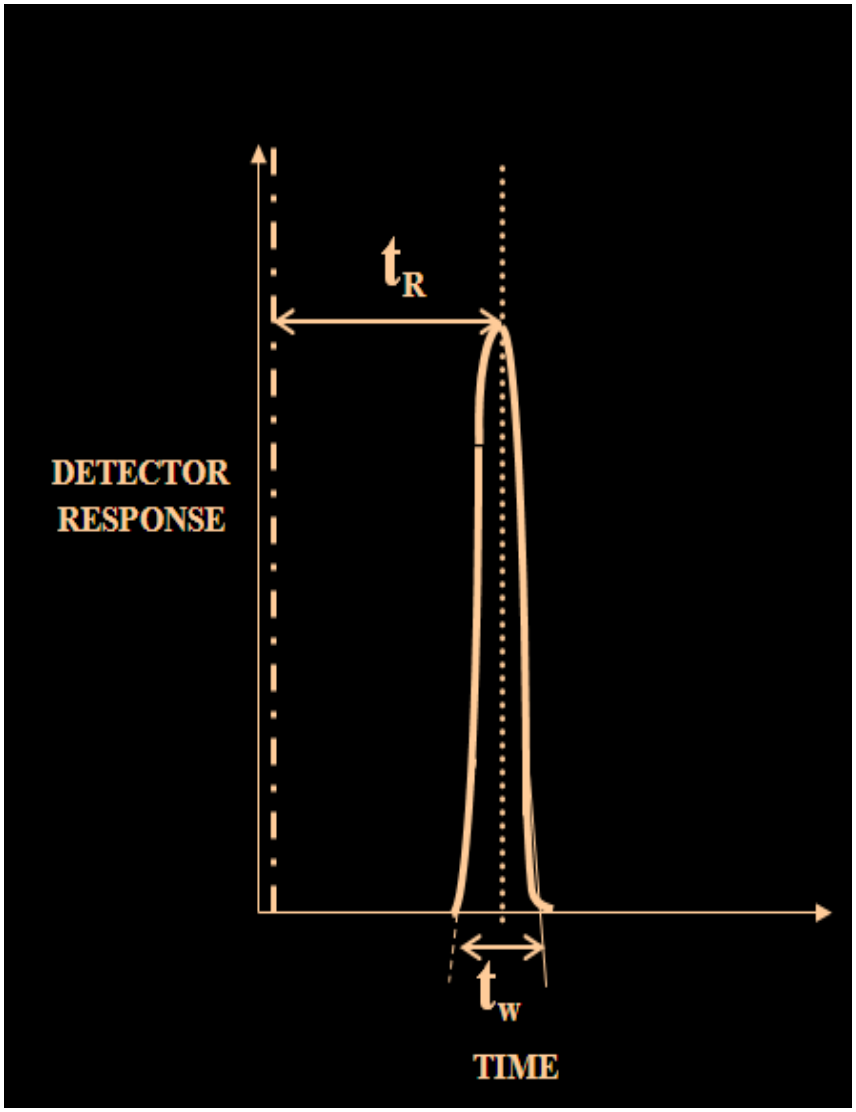
• The above figure shows the measurements needed to make this calculation.

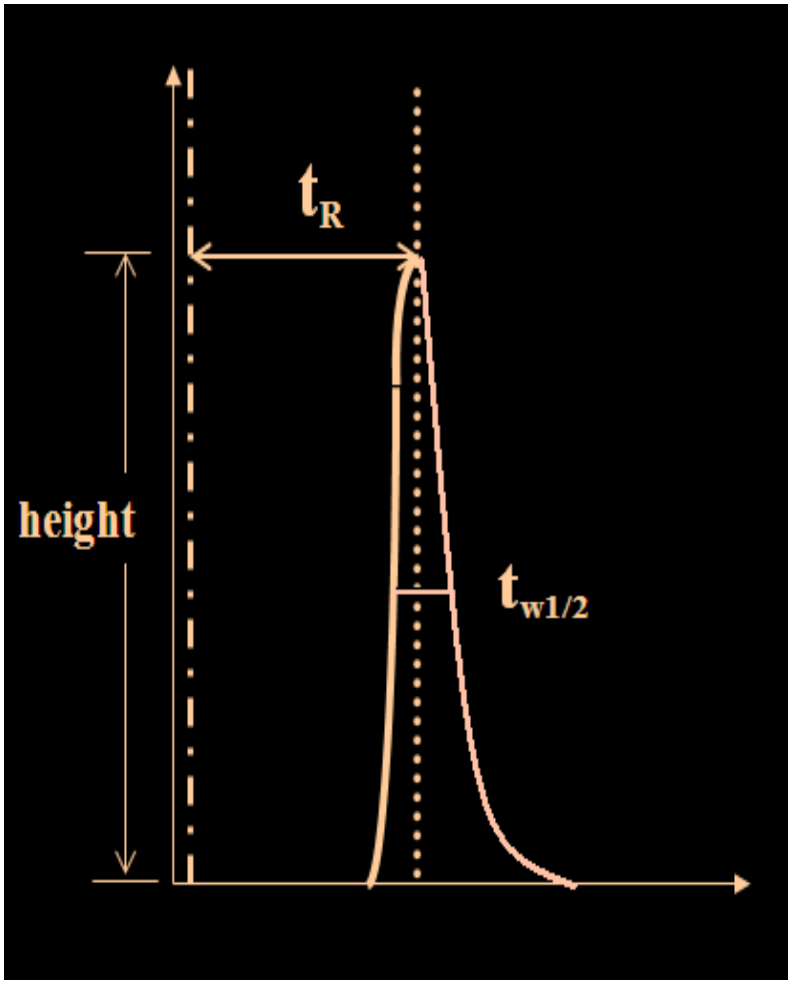
• Different terms arise because the measurement of σ can be made at different heights on the peak.

• At the base of the peak, w_b is 4σ , so the numerical constant is 4^2 .

- Independent of the symbols used, both the numerator and the denominator must be given in the same units, and therefore N is unitless.

- At half height, w_h is 2.354σ and the constant becomes 5.54.





$$N = 16 (t_R / t_W)^2 \qquad \text{or} \qquad N = 5.56 (t_R / t_{W1/2})^2$$

- It is observed that ‘N’ for a given chromatographic column and under given experimental conditions is approximately constant.

- Where for a symmetrical peak, peak width is taken at the base (t_W); for an unsymmetrical peak, peak width is taken at half the peak height, h/2 ($t_{W1/2}$).

- In other words if there are ‘n’ peaks in a chromatogram, each peak will have approximately the same N.

Let us carry out some calculations from the following data

Retention time Sec.	t_W Sec.
100	4
200	
400	
1200	
1600	
2400	

$$N = 16 (t_{R1} / t_{W1})^2$$
$$= 16 (100/4)^2$$

.

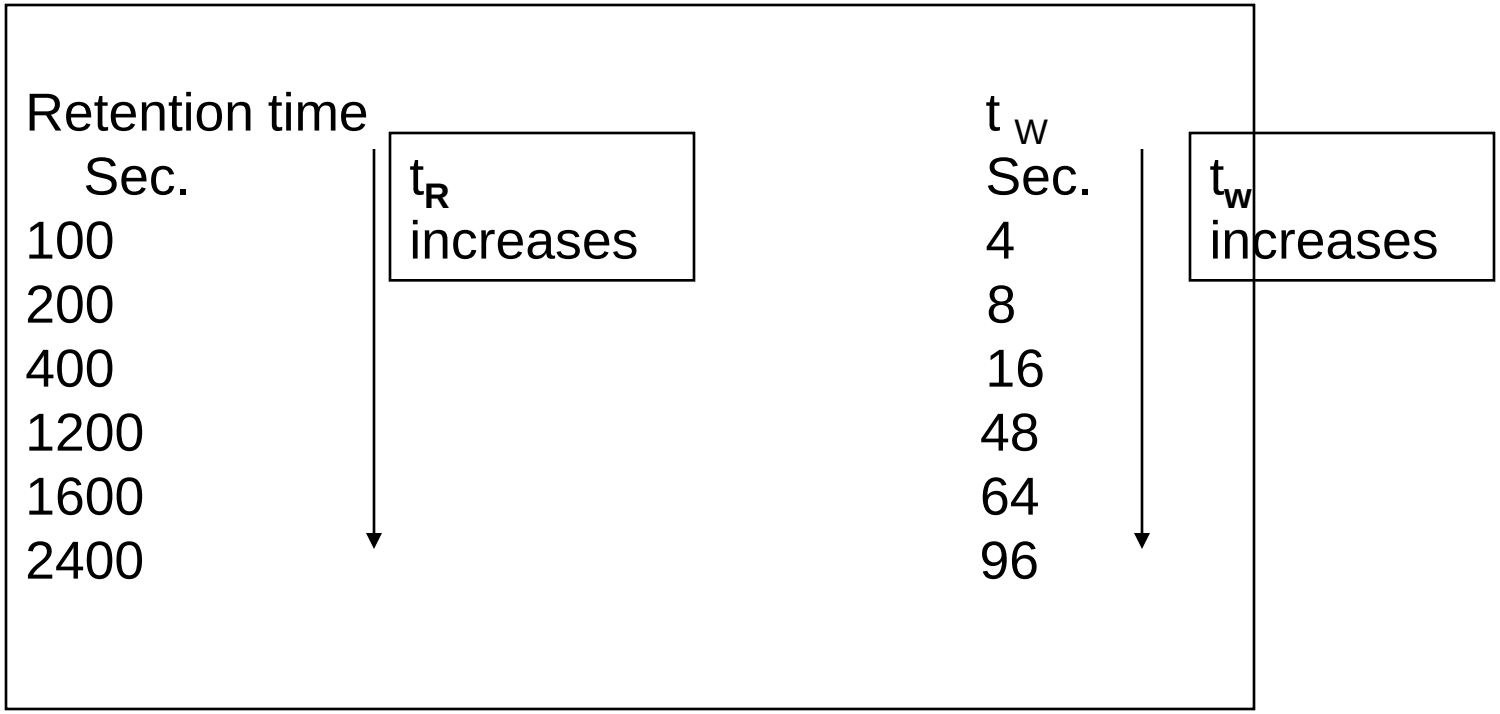
. . N = 10,000.

N will be approximately 10,000 for all other five peaks. Let us therefore calculate the respective t_W values.

$$10{,}000 = 16\,(t_{R2}/\,t_{W2})^2$$
$$= 16\,(200/\,t_{W2})^2$$
$$100 = \frac{4 \times 200}{t_{W2}}$$
$$t_{W2} = 8 \text{ Sec}$$

Similarly we can see that

$t_{W3} = 16$ sec
$t_{W4} = 48$ sec
$t_{W5} = 64$ sec
$t_{W6} = 96$ sec


Retention time
Sec.
100
200
400
1200
1600
2400
t_R increases
t_w
Sec.
4
8
16
48
64
96
t_w increases

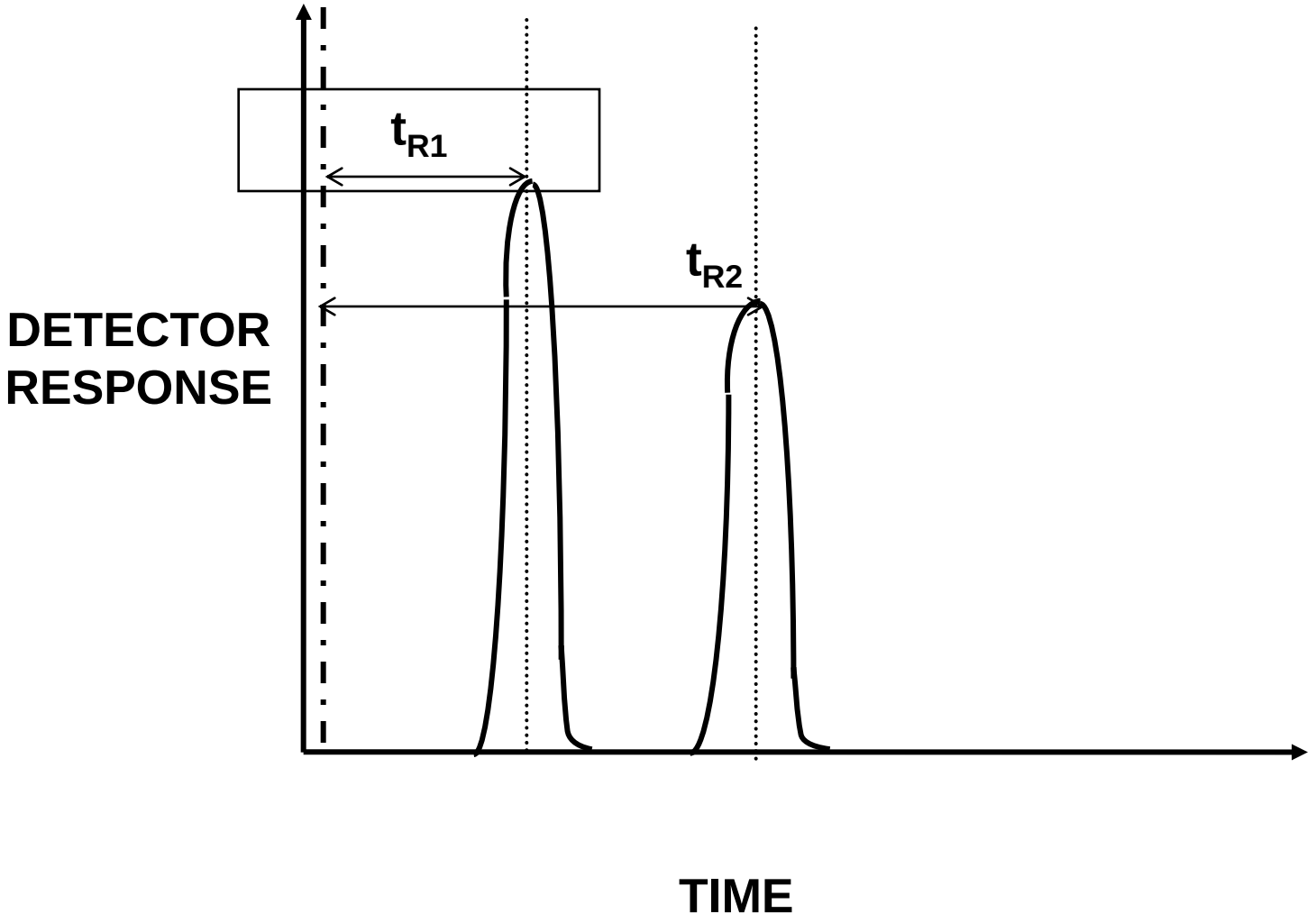
- From the above discussion, it is clear that N is calculated using the equation.

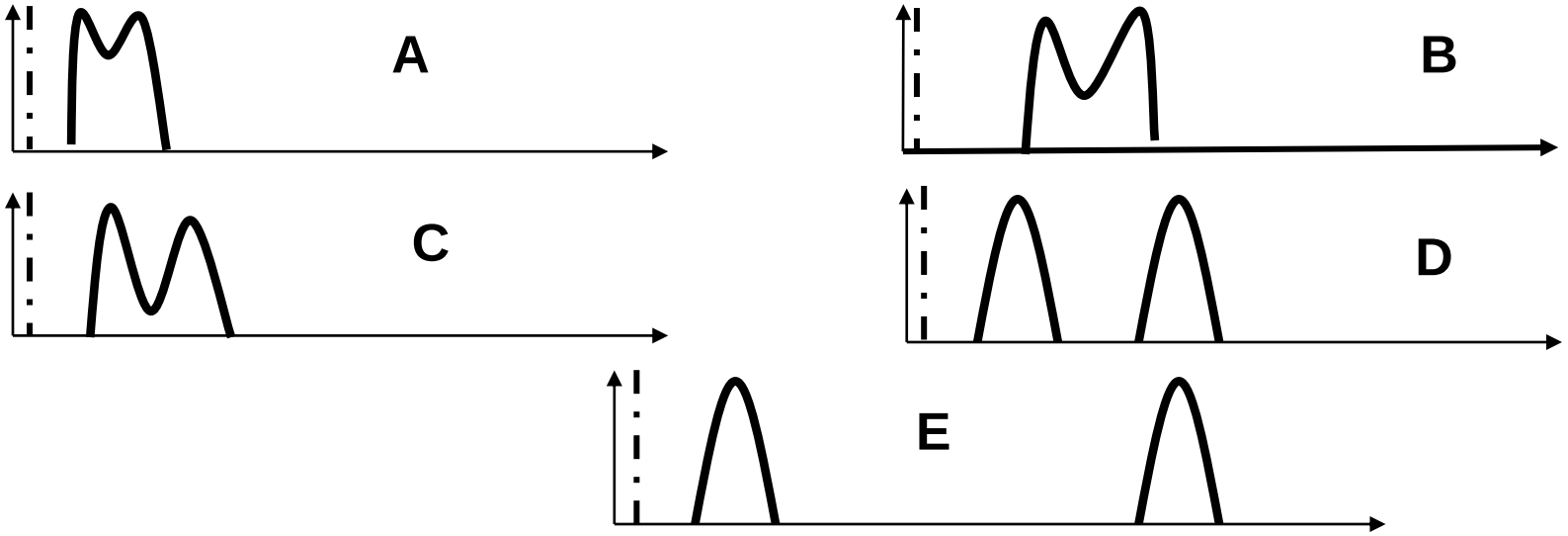
$$N = 16 (t_R / t_W)^2$$

- Now if N is approximately constant for all the peaks then it follows that as t_R increases, t_W proportionately increases.
- This explains our earlier statement that peaks become broader with increasing retention time.

- The basic need of any chromatographer is base line separated neighbouring peaks. Because only with such peaks he is able to do precise and accurate measurements of retention times and AUC’s for precise and accurate qualitative and quantitative analysis.

- Base line separated neighbouring peaks



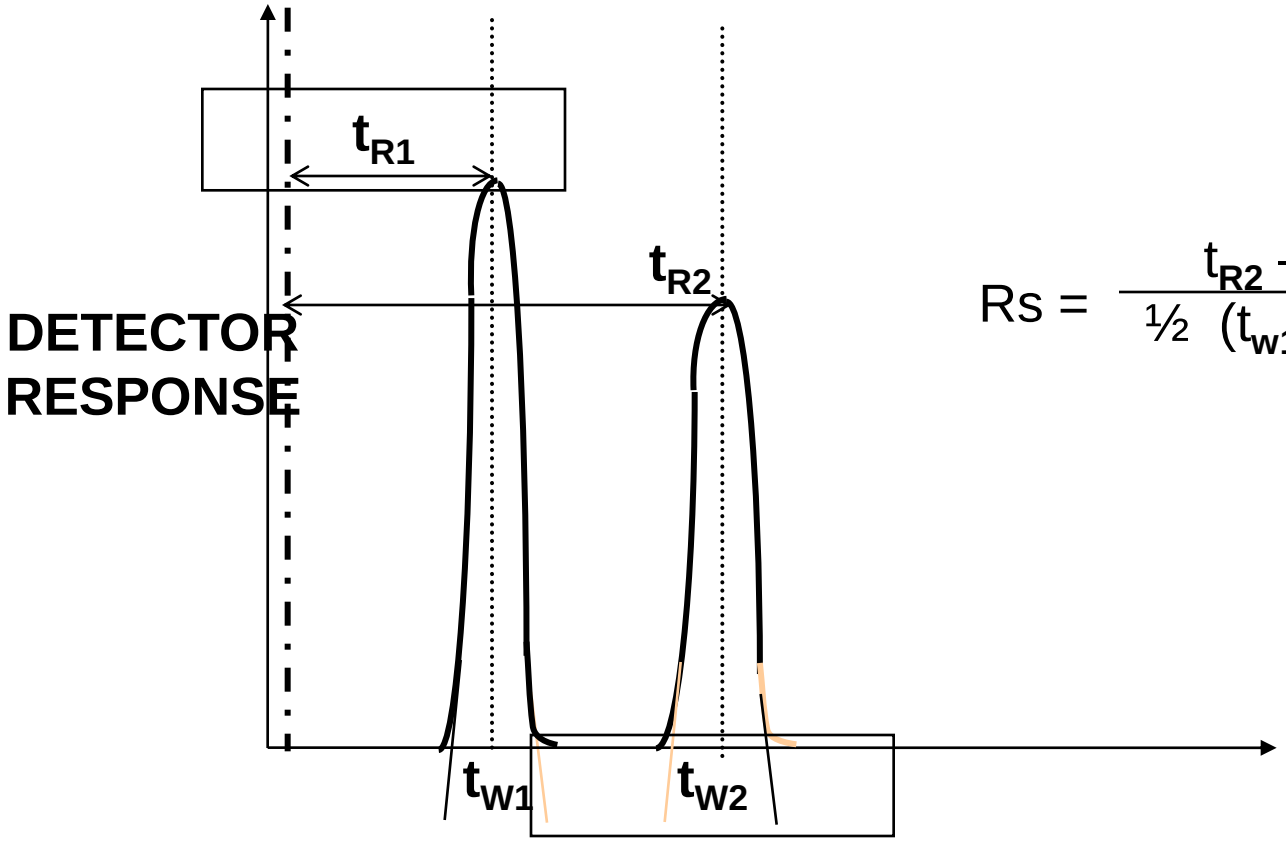


- In the process of extrapolation we lose some degree of accuracy in qualitative and quantitative analysis
- For calculations of retention times and AUC's, we need to extrapolate both the overlapping peaks
- In Fig. A, B & C above the neighbouring peaks are overlapping.
- The extent of overlapping is decreasing from Fig. A to C.
- Fig. D, is a satisfactory base line separation
- Therefore Fig. D, is practically acceptable one.
- In Fig. E, also there is a base line separation but unnecessary long analytical time.

➢How do we achieve base line separation of neighbouring chromatographic peaks if we get overlapping peaks instead?

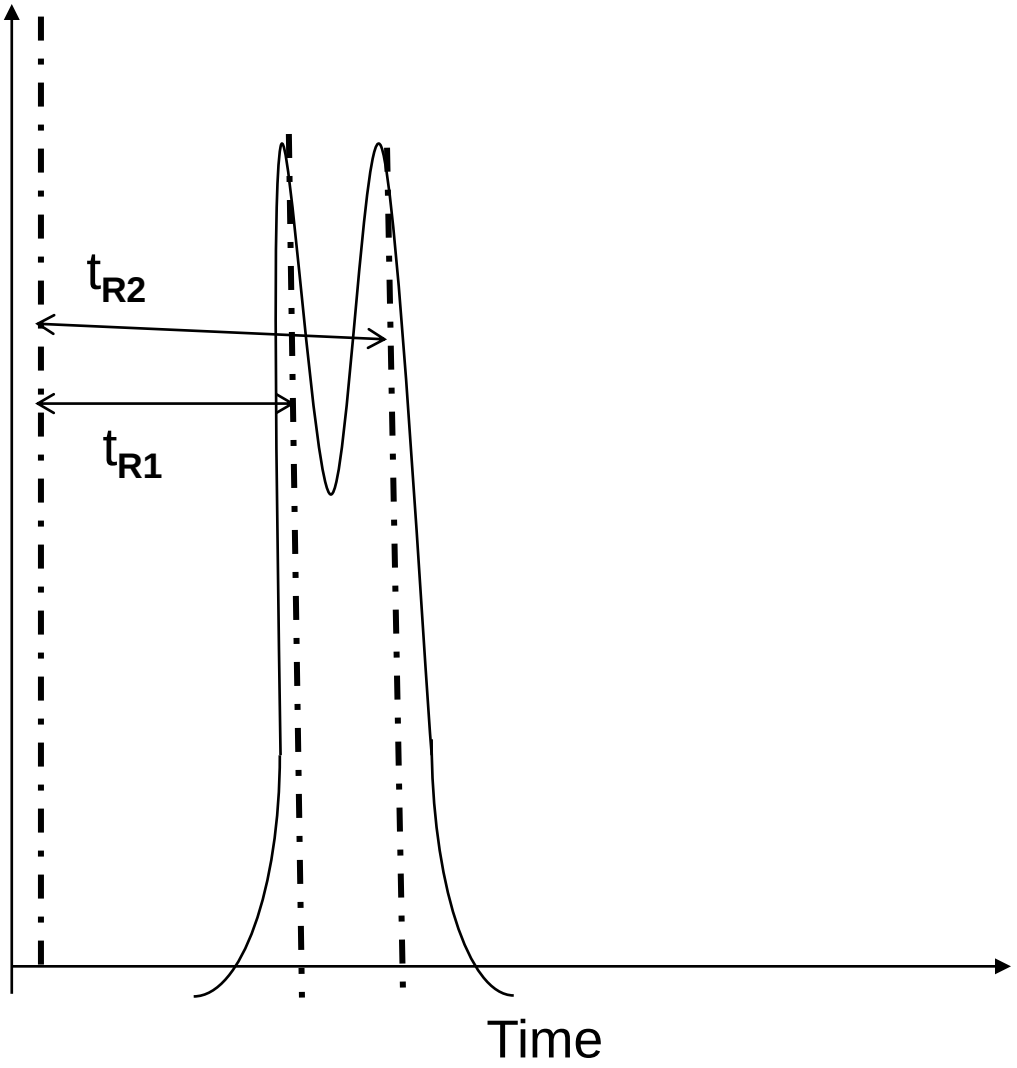
- Now the question is how do we improve the situation from Fig.A to Fig.D?

Let us first define the term "Resolution" between two neighbouring chromatographic peaks.



$$Rs = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(t_{w1} + t_{w2})}$$

- Looking at the equation, for resolution it is difficult to imagine how we can improve the resolution of two overlapping peaks.
- For simplification , let us imagine two almost overlapping peaks.



- For the overlapping peaks we will assume that,

$t_{R1} \approx t_{R2}$ & $t_{w1} \approx t_{w2}$

Therefore, $t_{w1}+t_{w2} = 2t_{w1}$

Therefore

$$Rs = \frac{t_{R2} - t_{R1}}{1/2 \; 2t_{w1}}$$

$$= \frac{t_{R2} - t_{R1}}{t_{w1}}$$

We have the equation,

$$t_R = t_0(k'+1)$$

$$t_{R2} = t_0(k'_2 + 1)$$

$$t_{R1} = t_0(k'_1+1)$$

$$t_{R2} - t_{R1} = t_0 k'_2 + t_0 - t_0 k'_1 - t_0$$

$$t_{R2} - t_{R1} = t_0(k'_2 - k'_1)$$

- A careful consideration of the resolution equation immediately reveals that we will be able to substitute the retention time and tw in terms of the chromatographic parameters with which we are familiar. e.g. , N and k'

- Similarly we have,

$$N = 16(t_R/t_w)^2$$

Therefore, $N = 16\,(t_{R1}/\,t_{w1})^2$

$$\sqrt{N} = 4t_{R1}/t_{w1}$$

Therefore, $t_{w1} = 4t_{R1}/\sqrt{N}$

$$= 4\,t_0\,(k'_1 + 1)/\sqrt{N}$$

- Substituting t_{w1} and t_{R2}- t_{R1} in the equation for resolution we get,

$$Rs = t_{R2} - t_{R1} / t_{w1}$$

$$= \frac{t_0 (k'_2 - k'_1)}{4\, t_0 (k'_1 + 1)} \sqrt{N}$$

$$Rs = \tfrac{1}{4} \sqrt{N} \frac{(k'_2 - k'_1)}{(k'_1 + 1)}$$

$$= 1/4 \sqrt{N} (k'_2/k'_1 - k'_1/k'_1) (k'_1/k'_1 + 1)$$

$k'_2 / k'_1 = \alpha$ –The Separation factor

Therefore the general equation will be,

$Rs = ¼ \sqrt{N} (\alpha-1) (k' / k'+1)$

Where,

$\sqrt{N}$ = Separation efficiency parameter.

α = Separation factor.

(k’ / k' +1) = Capacity factor.

- k'& α are thermodynamic factors & N is kinetic factor.

$$Rs = ¼ \sqrt{N} (\alpha-1) (k' / k'+1)$$

- For the most practical purposes the factors governing N, α, & k' are approximately independent.
- It is therefore possible to optimize one parameter at a time, keeping other two constant
- From the above equation it is clear that resolution between two neighbouring chromatographic peaks is governed by N, α, & k'.

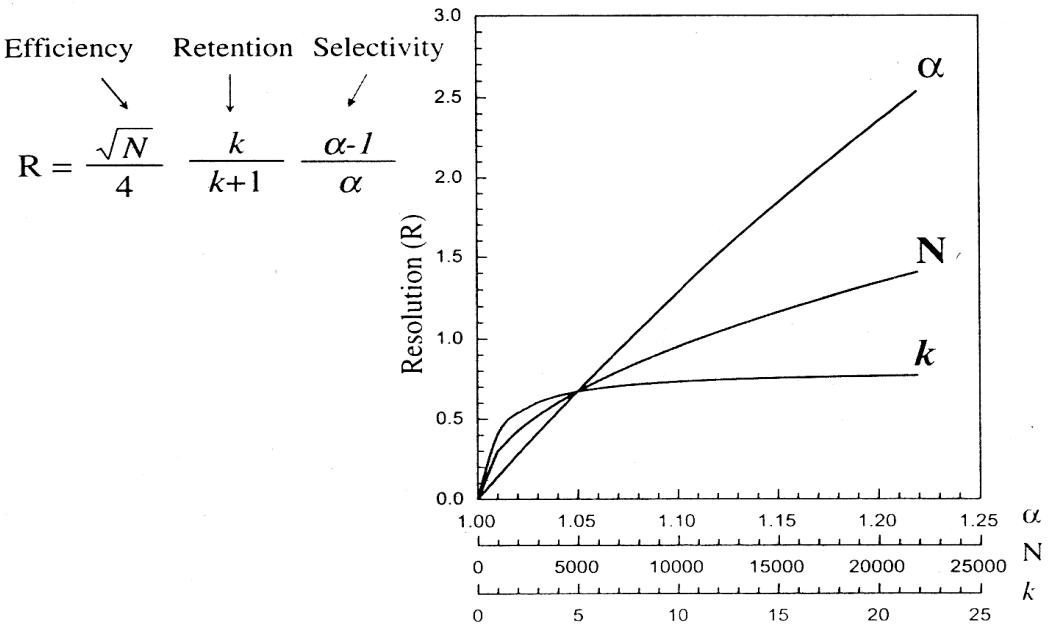


Figure 2.15. Graph illustrating the effects of α, k, and N on resolution. Diagram Courtesy of Supelco Inc.

- To maximize Rs, k' should be relatively large, though any k' values >10 will drive the retention term of (k'/ k'+1) to approach to unity. Selectivity (α) is typically between 1.01 & 1.50 for closely eluting solutes. Where α =1, R=0 and co-elution of the solutes occurs. Selectivity is maximized by optimizing column and mobile phase conditions during method development.

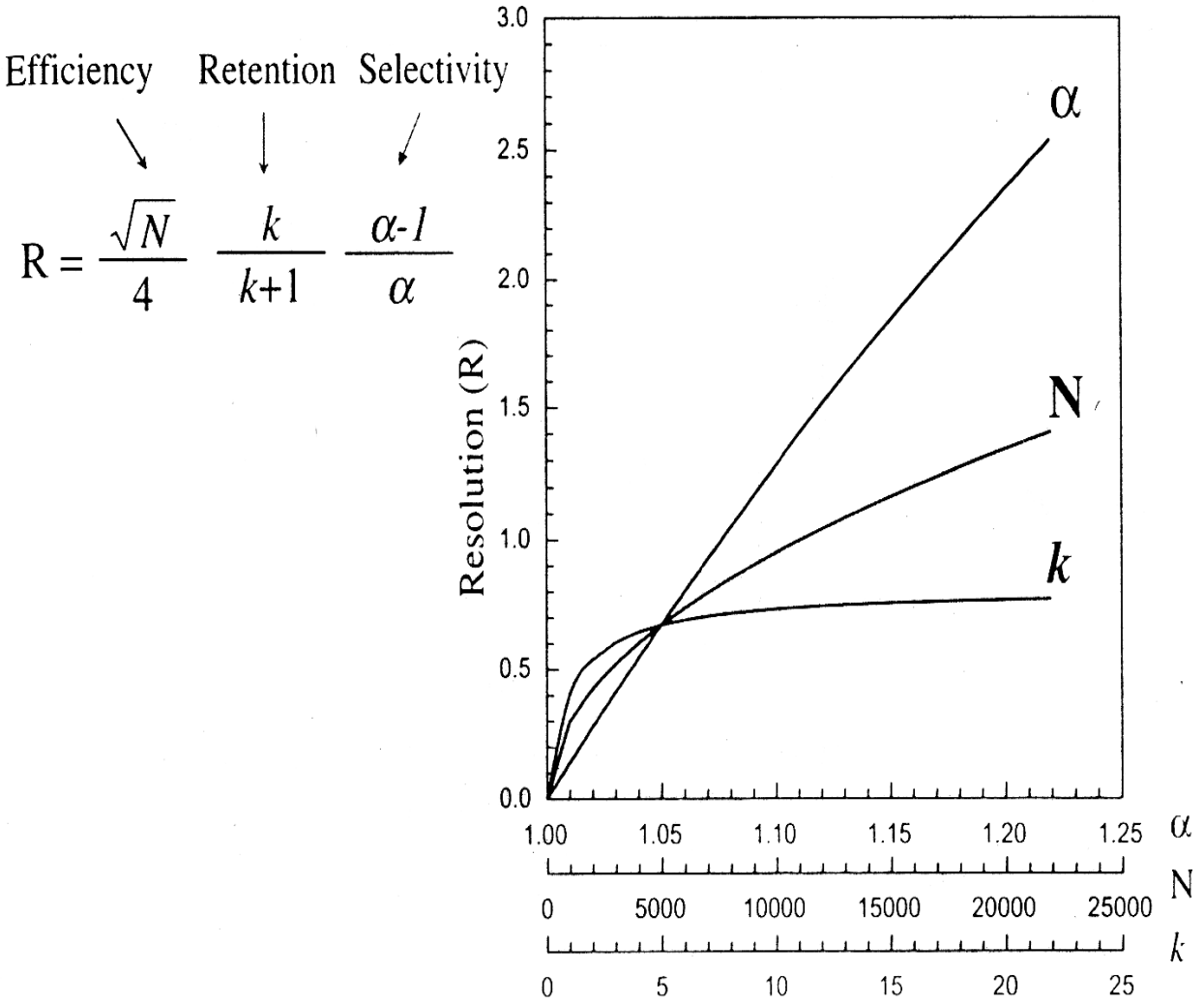
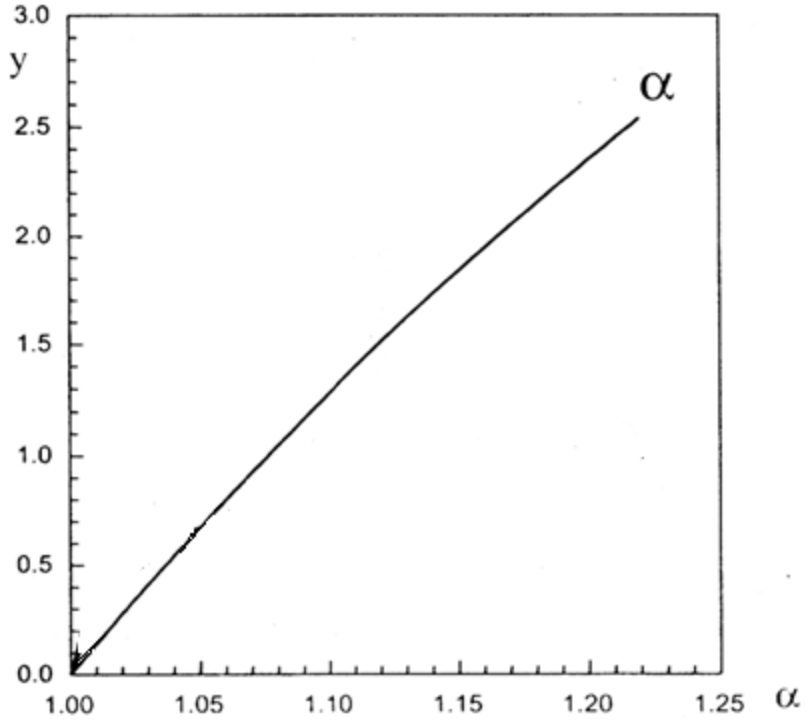
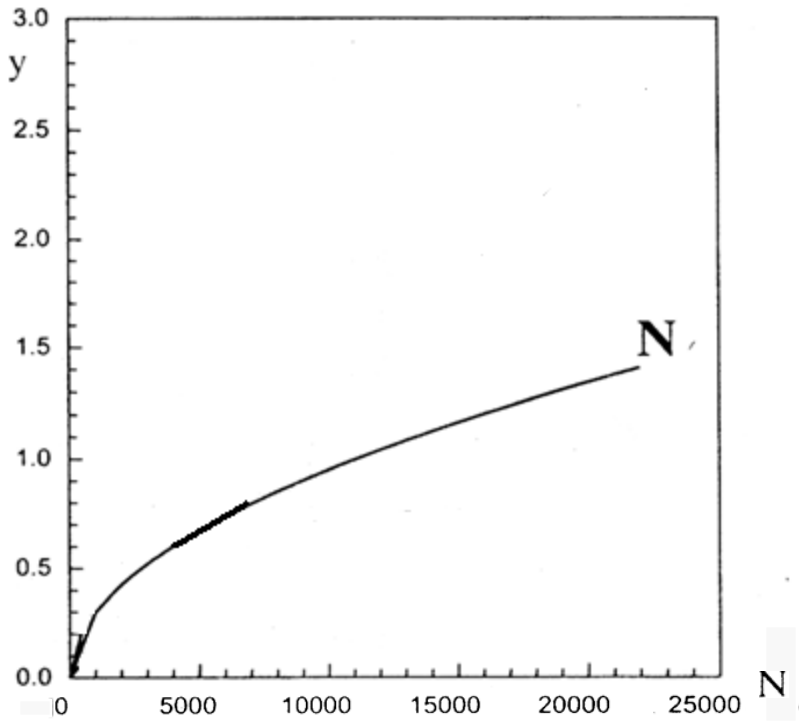
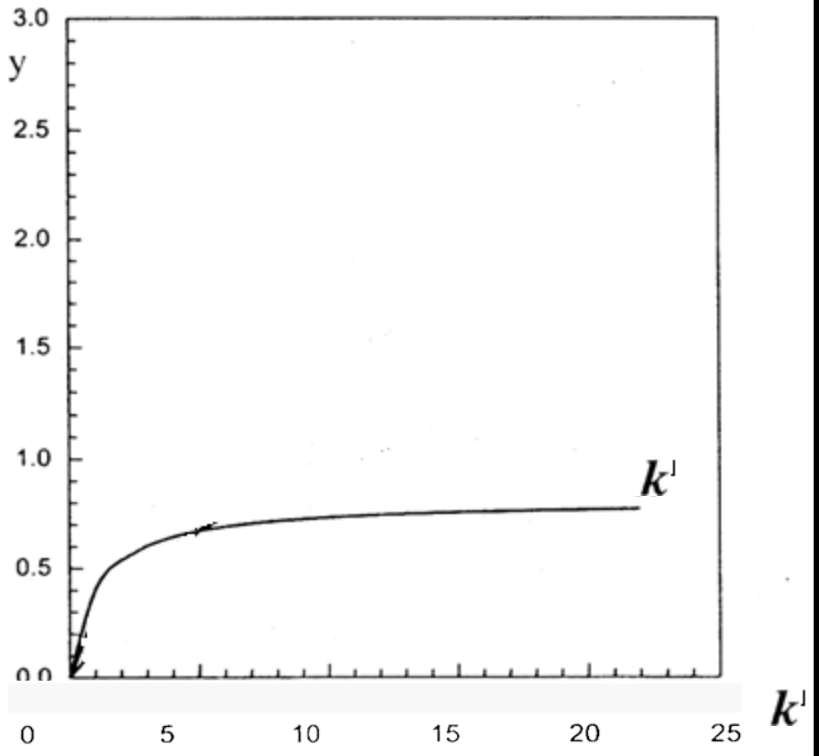


Figure 2.15. Graph illustrating the effects of α, k, and N on resolution. Diagram Courtesy of Supelco Inc.


3.0
y
2.5
2.0
1.5
1.0
0.5
0.0
α
1.00
1.05
1.10
1.15
1.20
1.25
α


3.0
y
2.5
2.0
1.5
1.0
0.5
0.0
N
0
5000
10000
15000
20000
25000
N


3.0
y
2.5
2.0
1.5
1.0
0.5
0.0
k^1
0
5
10
15
20
25
k^1

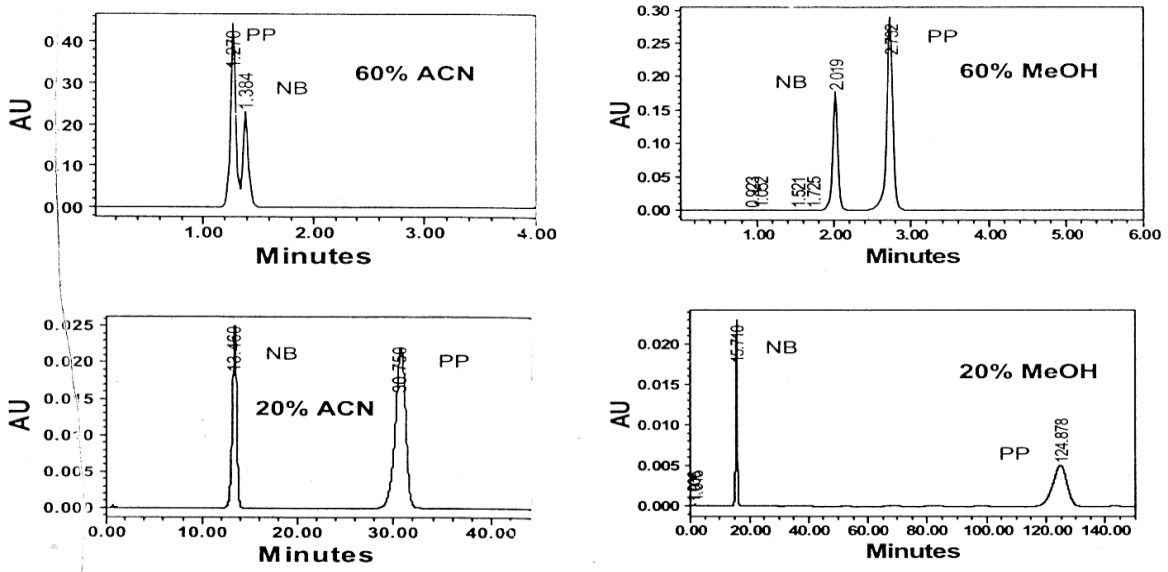
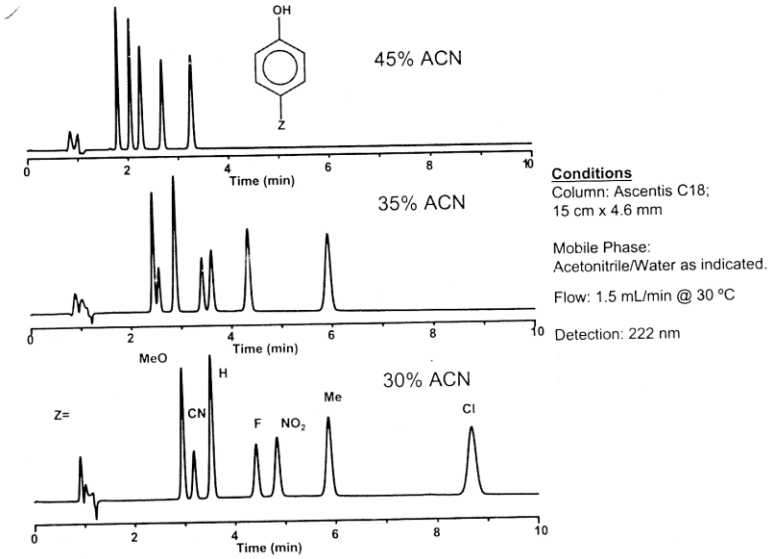
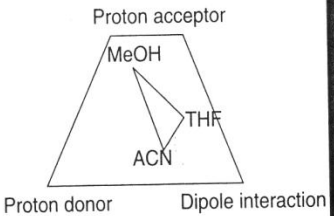
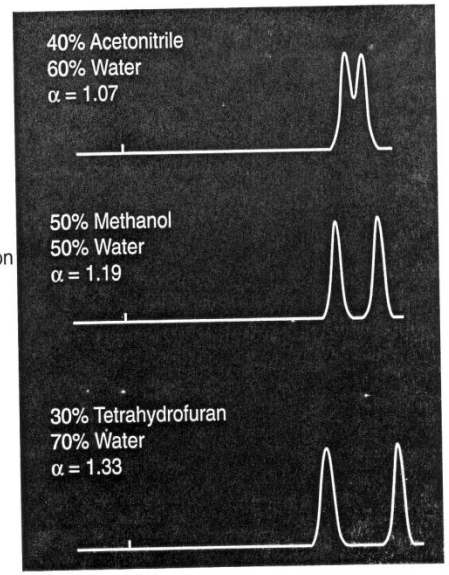


Figure 2.12. Four RPLC chromatograms illustrating the effect of mobile phase strength and selectivity of acetonitrile (ACN) and methanol (MeOH). See Figure 2.10 for LC conditions.

- The above Fig. illustrates how resolution can be enhanced by exploiting the selectivity effect of the mobile phase (i.e, by switching from 60% ACN to 60% MeOH). It is important to note that small change of selectivity can have a major effect on resolution as resolution is proportional to (α -1). Columns of different bonded phases (i.e,C_8, phenyl, CN ,polar- embedded etc) can also provide different selectivity effects.







- Finally, the plate number of the column (N) should be maximized by using a longer column or a more efficient column. Increasing N is not an efficient way to achieve resolution since Rs is proportional to √N. Doubling N by doubling the column length increases analysis time by 2 but only increases resolution by √2 or by 41%. In contrast, increasing α from 1.05 to 1.1 will almost double the resolution. For complex samples with many peaks, increasing N is a viable and the most direct approach.

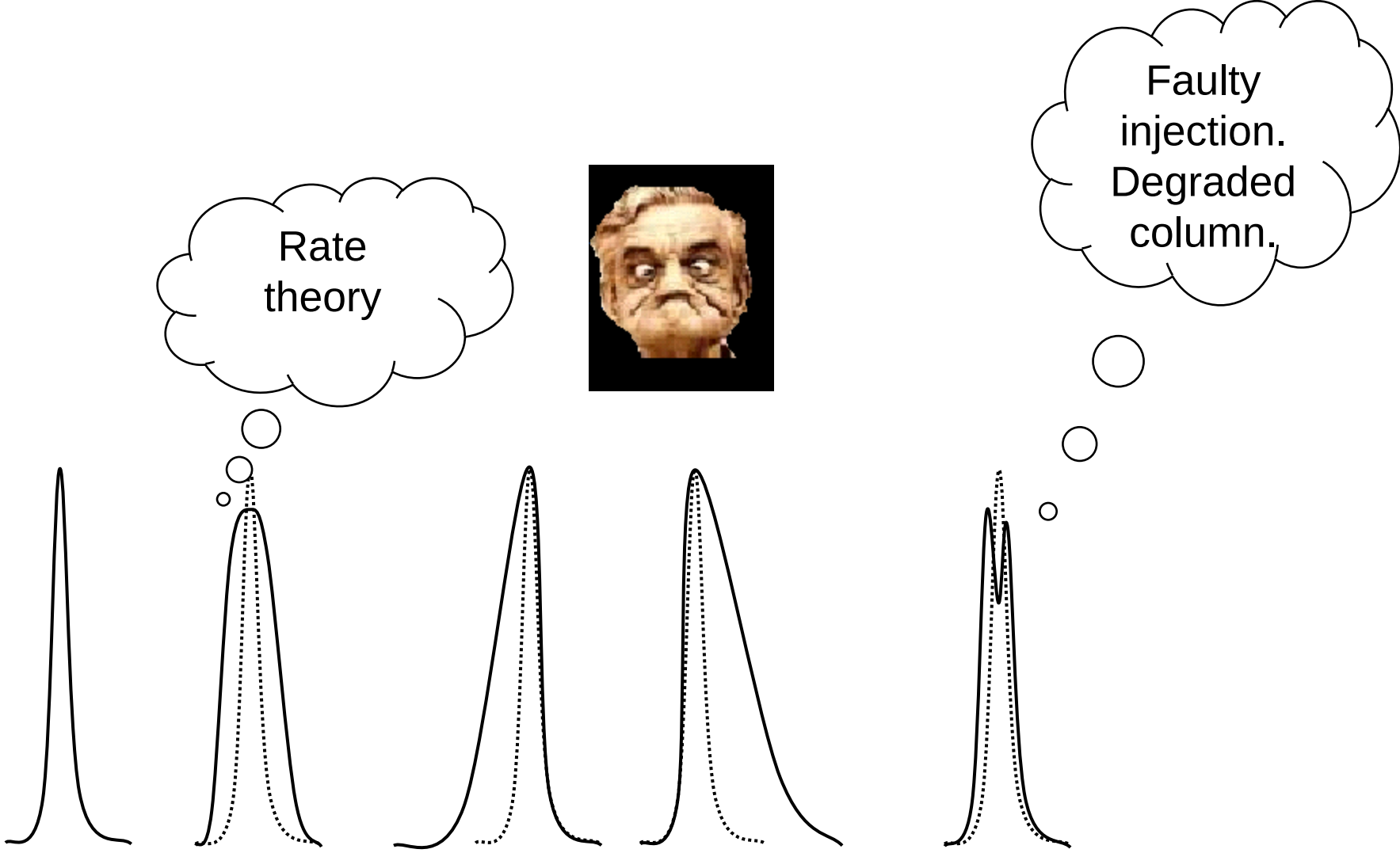
Concept of tailing and fronting

Nonsymmetrical peaks usually indicate that some undesirable interaction has taken place during the chromatographic process.

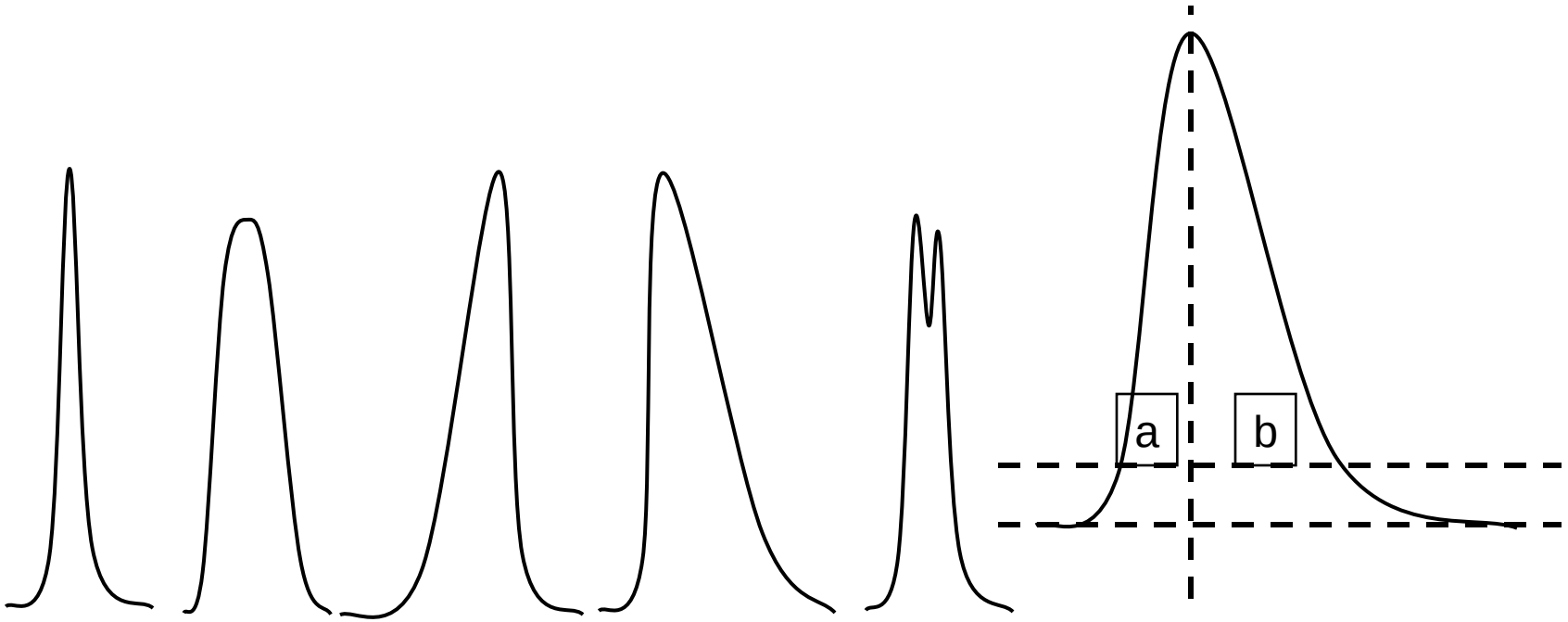
Individual solute molecules act independently of one another during the chromatographic process.

As, a result they produce a randomized aggregation of retention times after repeated sorptions and desorptions.

The result of a given solute is a distribution, or peak, whose shape can be approximated as being normal or Gaussian.



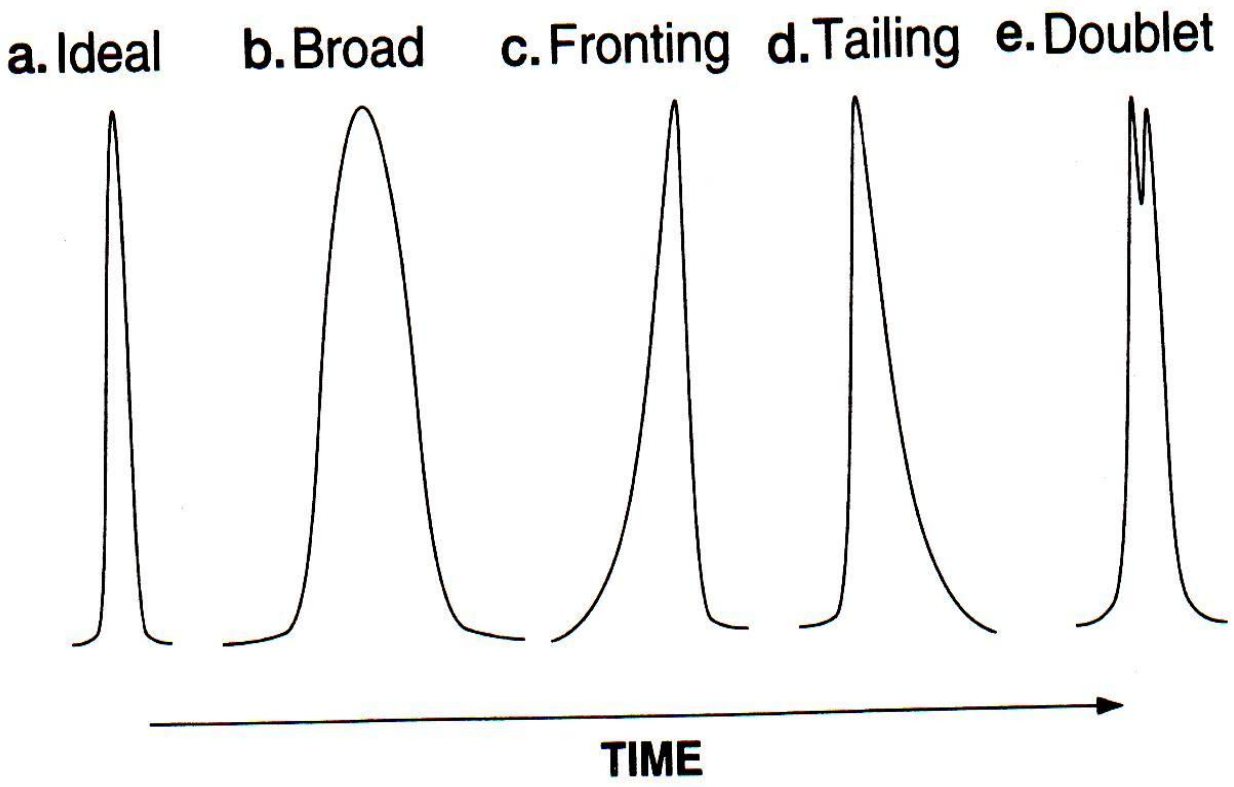
•Ideal Guassian Broad Fronting Tailing Doublet

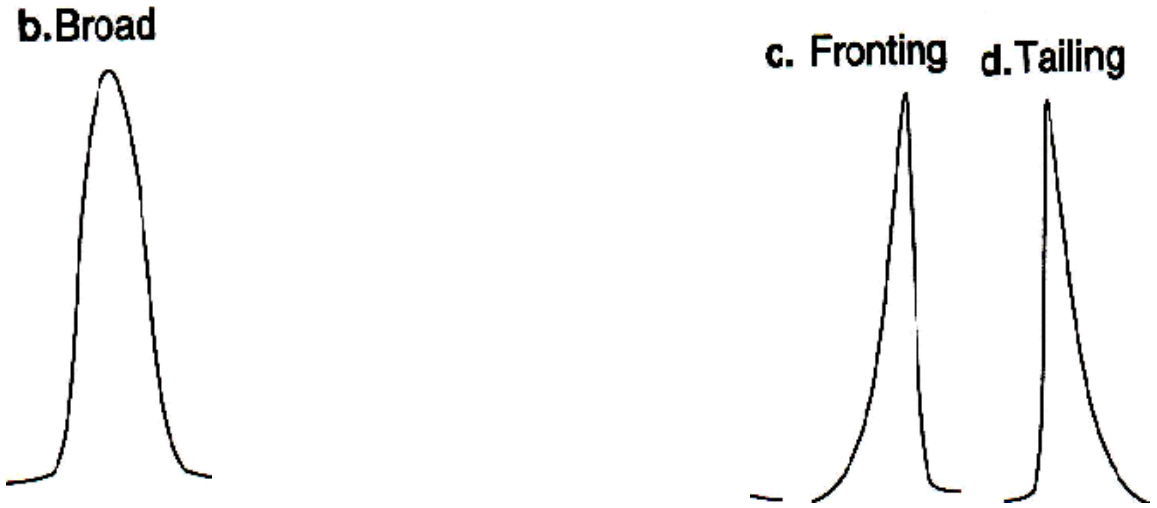


Tailing factor TF = a / b (at 10% of the peak height)

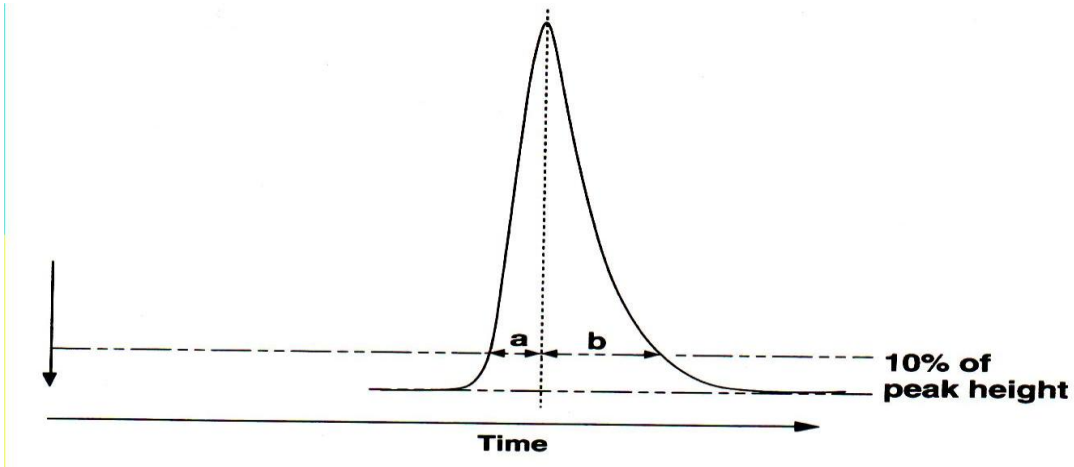
TF > 1 Tailing **TF < 1 Fronting**

The figure below shows some shapes that can occur in actual samples.





- The extent of asymmetry is defined as the tailing factor (TF).
- Broad peaks like (b) are more common in packed columns and usually indicate that the kinetics of mass transfer is too slow.
- Asymmetric peaks can be classified as fronting (c) or tailing (d) depending on the location of asymmetry.



- While the definition was designed to provide a measure of the extent of tailing and is so named, it also measures fronting.

TF = b/a

- Both a and b are measured at 10% of the peak height shown.

- As can be seen from the equation, a tailing peak will have a TF greater than one.

- The opposite symmetry, fronting, will yield a TF less than one.

- The doublet peak like (e) can represent a pair of solutes that are not adequately separated.

- Repeatability of a doublet peak should be verified because such peak shape can also result from faulty injection technique, too much sample, or degraded columns

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