

LECTURE # 6

Theory of HPLC

INTRODUCTION

High performance liquid chromatography is defined as a *separation of mixtures* of compounds due to differences in their distribution equilibrium between two phases, the stationary phase packed inside columns and the mobile phase, delivered through the columns by high pressure pumps.

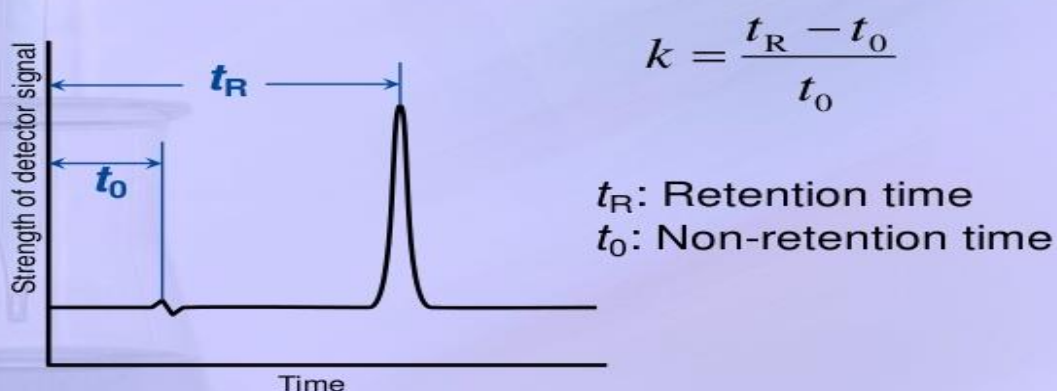
The theory of chromatography has been used as the basis for *System Suitability tests*, which *are set of quantitative criteria that test the suitability of the chromatographic system to identify and quantify drug related samples by HPLC at any step of the pharmaceutical analysis.*

RETENTION FACTOR OR CAPACITY FACTOR K

- In a chromatogram, different peaks correspond to different components of the separated mixture.
- Retention time (**RT**) is the difference in time between the point of injection and appearance of peak maxima.
- It is also defined as time required for 50% of a component to be eluted from a column.
- It is measured in minutes and seconds.
- *The retention time is longer when the solute has higher affinity to the stationary phase due to its chemical nature.*

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A high **k** value indicates that *the sample is highly retained and has spent a significant amount of time interacting with the stationary phase.*

It is suggested that the value of k should be between 2-10

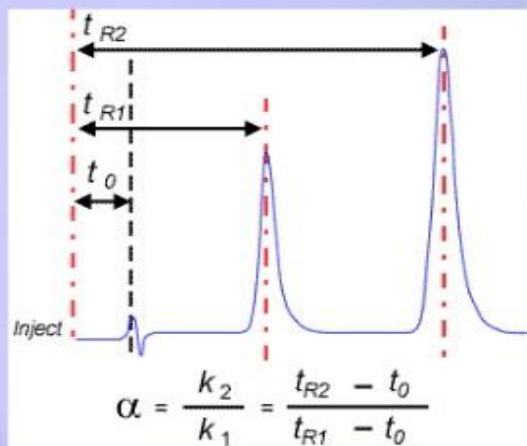
The retention factor is equal to *the ratio of retention time of the analyte on the column to the retention time of a non-retained compound.*

The non-retained compound has no affinity for the stationary phase and elutes with the solvent front at a time t_0 , which is also known as **the 'hold up time' or 'dead time'.**

SELECTIVITY(SEPARATION FACTOR)

- 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.**

- **SEPARATION FACTOR: RATIO OF k 'S OF TWO PEAKS**



$$\alpha = \frac{k_2}{k_1}$$

$$(k_2 > k_1)$$

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Higher the α indicates good separating power and a good separation between the apex of each peak.

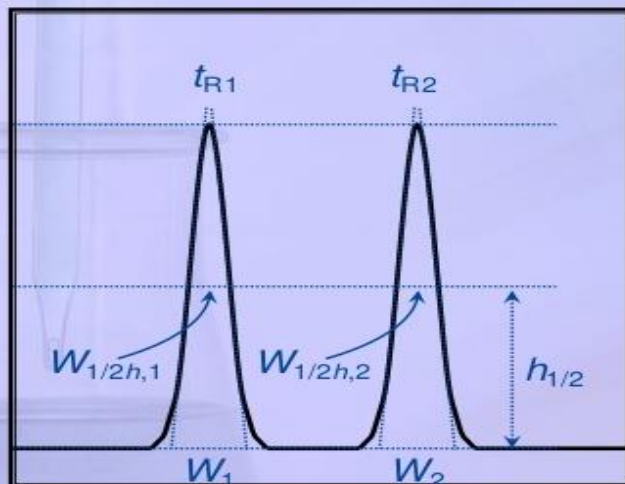
Selectivity values	Separation
≥ 2	Easy separation
1.5-2	Possible separation
1.2-1.5	Difficult separation
≤ 1.2	Very difficult separation

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Resolution(R_s)

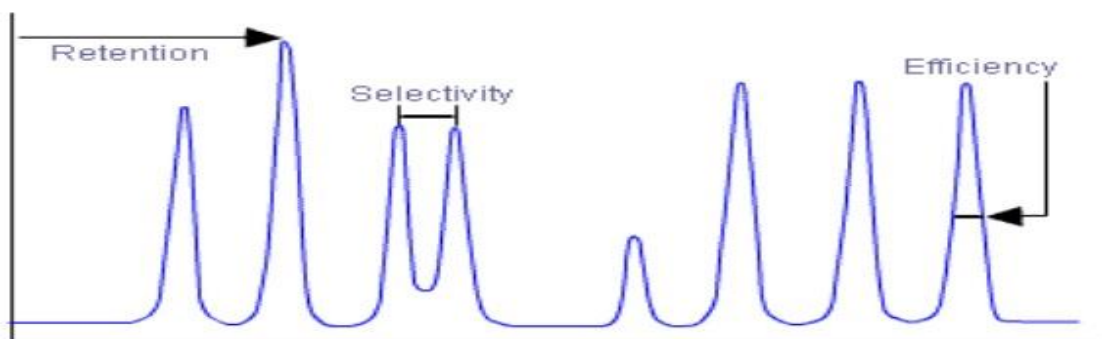
- The most important thing in HPLC is to obtain the optimum resolution in the minimum time.
- A resolution value of 1.5 or greater between two peaks will ensure that the sample components are well ('baseline') separated - to a degree at which the area or height of each peak may be accurately measured.
- Resolution is calculated using the separation of two peaks in terms of their average peak width at the base ($t_{R2} > t_{R1}$).



$$R_s = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(W_1 + W_2)}$$
$$= 1.18 \times \frac{t_{R2} - t_{R1}}{W_{1/2h,1} + W_{1/2h,2}}$$

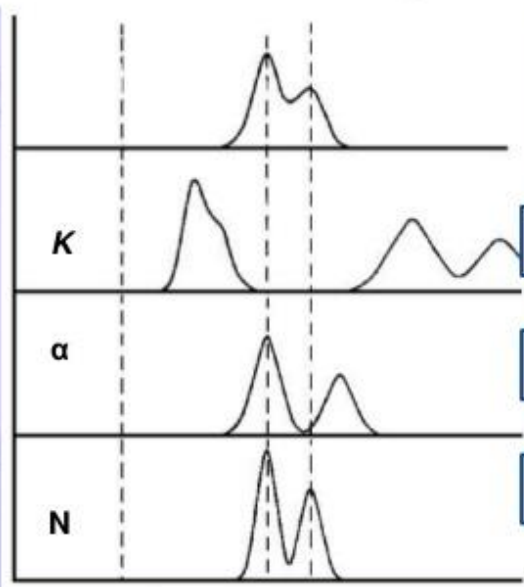
$$R_s = \underbrace{1/4\sqrt{N}}_{\text{Efficiency}} \times \underbrace{\frac{\alpha-1}{\alpha}}_{\text{Selectivity}} \times \underbrace{\frac{k}{1+k}}_{\text{Retention}}$$

The Fundamental Resolution Equation



FACTORS THAT IMPROVE RESOLUTION

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



INCREASE
RESOLUTION

CHANGE RELATIVE
PEAK POSITION

REDUCE PEAK
WIDTH

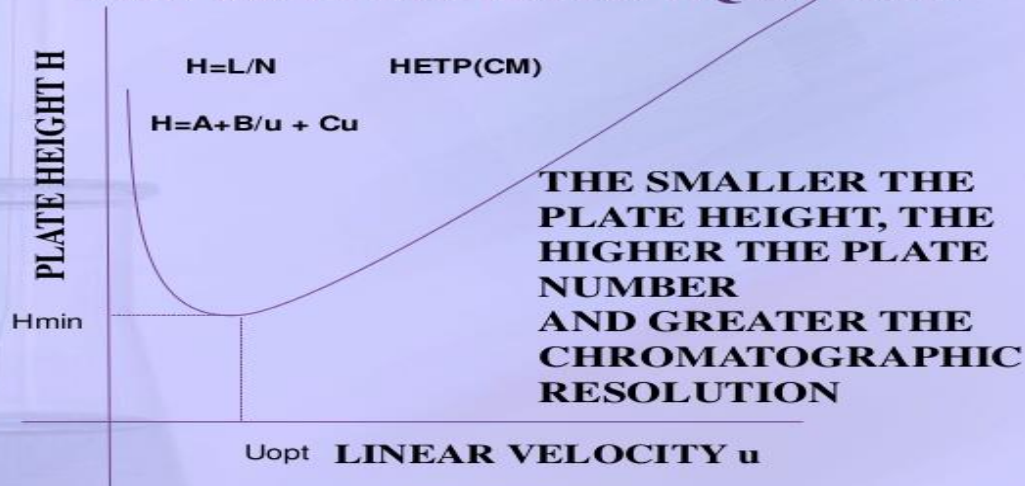
HEIGHT EQUIVALENT OF A THEORETICAL PLATE (HETP)

- A theoretical plate is an imaginary or hypothetical unit of a column where distribution of solute between stationary phase and mobile phase has attained equilibrium. It can also be called as a functional unit of the column.
- The height of one theoretical plate is referred to as the '*Height Equivalent of a Theoretical Plate*'
- A theoretical plate can be of any height, which describes the efficiency of separation. If HETP is less, the column is more efficient. If HETP is more, the column is less efficient.
- $\text{HETP} = \text{length of the column} / \text{no. of theoretical plates}$
- HETP is given by Van Deemeter equation

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THE VAN DEEMETER EQUATION



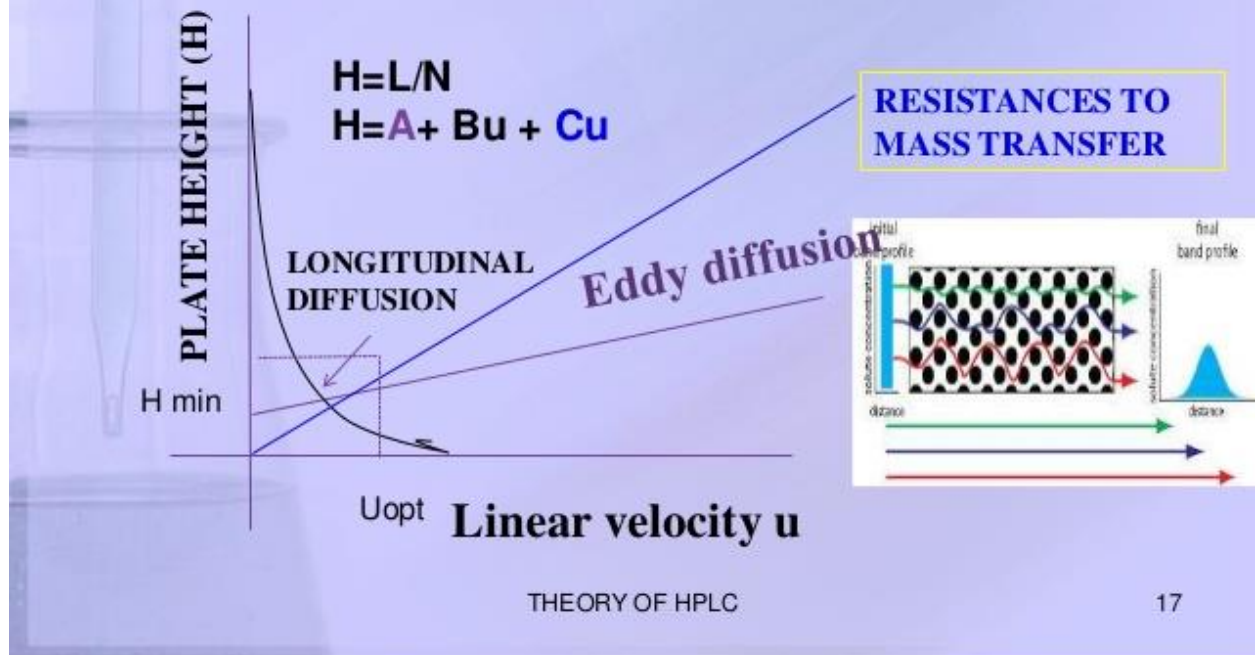
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EDDY DIFFUSION

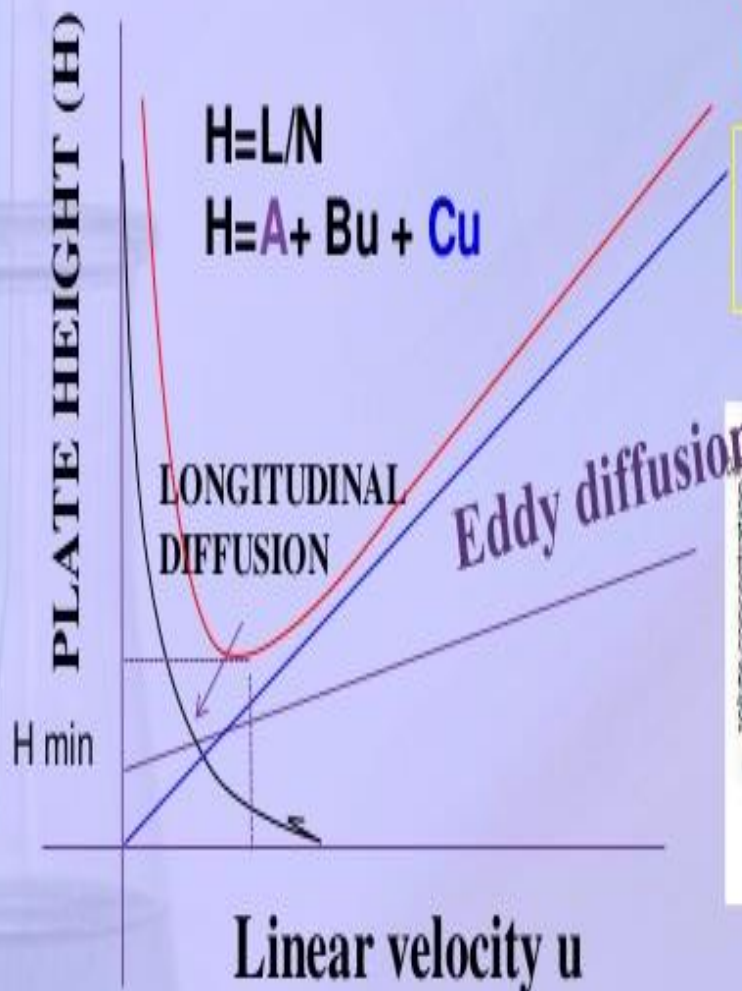


RESISTANCE TO MASS TRANSFER



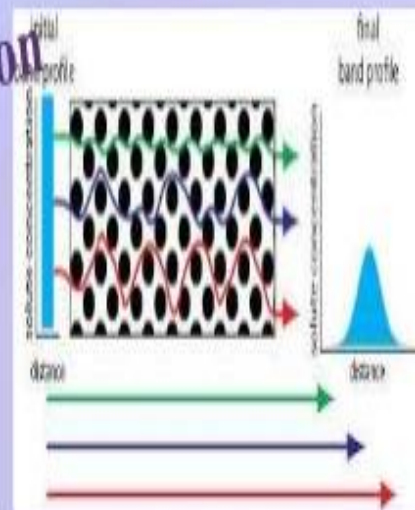
PUTTING IT ALL TOGETHER

THE VAN DEEMETER EQUATION



SUM CURVE: VAN DEEMETER

RESISTANCES TO MASS TRANSFER



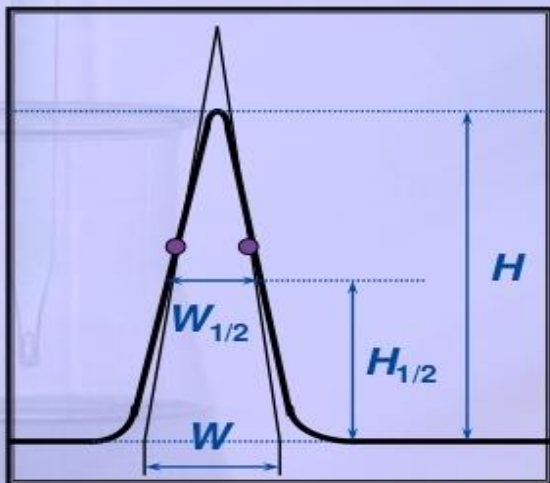
Efficiency

- The efficiency of a chromatographic peak is a measure of the dispersion of the analyte band as it travelled through the HPLC system and column.
- The plate number (N) is a measure of the peak dispersion on the HPLC column, reflecting the column performance.
- 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 possible and the better quality the separation.

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Theoretical Plate Number, N



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$$N = 16 \left[\frac{t_R}{W} \right]^2$$

Where n = no of theoretical plates

t_R = retention time

w = peak width at base

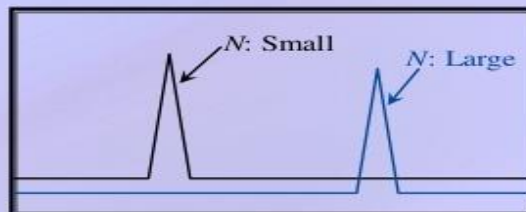
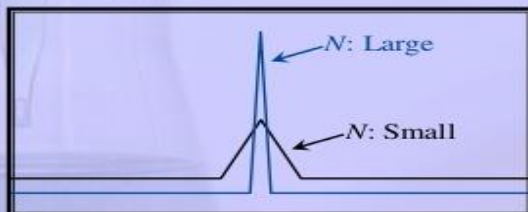
t_R and w are measured in common units (cm or mm, min or sec). No of theoretical plates

Is high, the column is said to be highly efficient. For GLC, a value of 600/ meter is sufficient. But in HPLC, high values like 40,000 to 70,000/ meter are recommended.

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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.



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- In order to optimize separation efficiency, it is necessary to maximize the number of theoretical plates, which requires reducing the plate height.
- The plate height is related to the flow rate of the mobile phase, so for a fixed set of mobile phase, stationary phase, and analyte ; separation efficiency can be maximized by optimizing flow rate
- Efficiency can be increased by increasing the column length, reducing the column internal diameter, or decreasing the particle size. It is better to use a smaller diameter packing than increase the column length, which will increase analysis time. However, a decrease in particle size will result in an increase in system backpressure. The use of smaller particles and narrower column internal diameter both require minimized extra column dead volume in order to avoid efficiency losses.
- The FDA stipulates a value for $N > 2000$ which is typically easily achieved with modern HPLC columns


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ASYMMETRIC FACTORS

- A chromatographic peak should be symmetrical about its centre and said to follow Gaussian distribution. But in practice due to some factors, the peak is not symmetrical and shows tailing or fronting.
- Fronting is due to saturation of stationary phase and can be avoided by using less quantity of sample. Tailing is due to more active adsorption sites and can be eliminated by support pretreatment.

- Asymmetry factor (0.95 to 1.05) can be calculated by $AF = b/a$ (b, a calculated by 5% or 10% of the peak height).
- Broad peaks occur due to the more conc. of sample, large injection volume, column deterioration.
- Ghost peaks occur due to the contamination of the column, compound from earlier injections.

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- Negative peaks occur if mobile phase absorbance is larger than sample absorbance.
 - Peak doubling occurs due to the co-elution of interfering compound, column over load, channeling in column.
 - Base line spikes occur due to the air bubbles in the mobile phase and/or detector, column deterioration.



Tailing peaks create issues with resolution, quantization (integration), and reproducibility. Peak shape is often the controlling factor when optimizing complex separations, especially when components are present in very different concentrations.

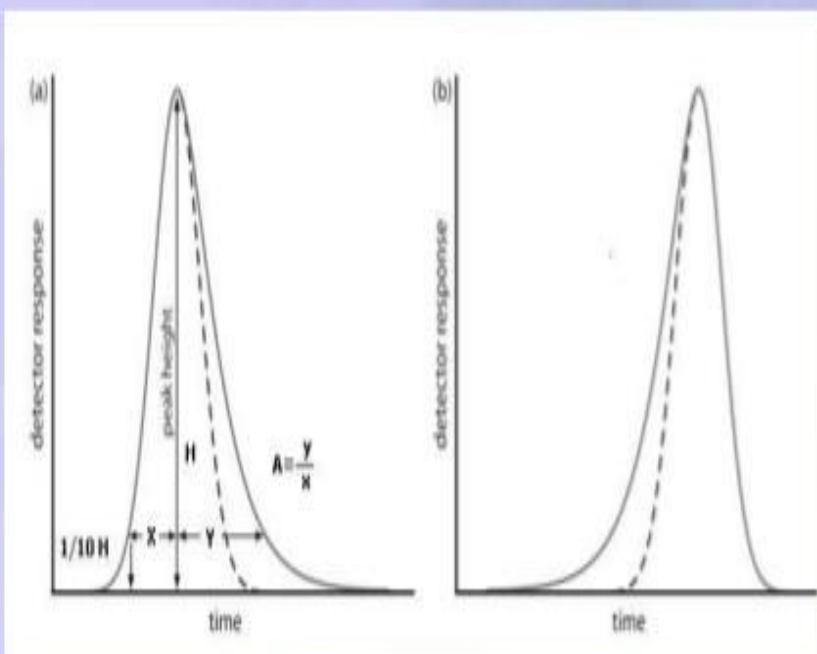
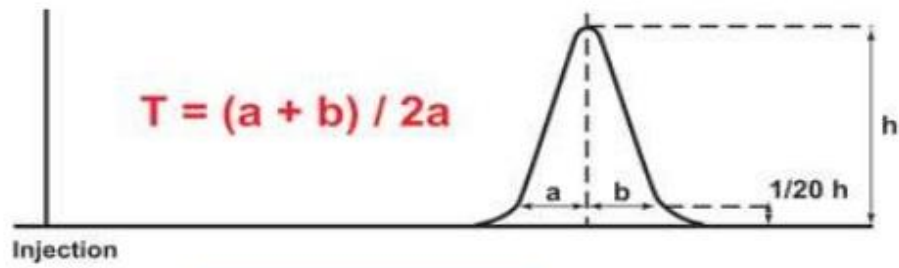


Figure 1: Examples of asymmetric chromatographic peaks showing (a) peak tailing and (b) peak fronting. For both (a) and (b) the un dotted chromatogram is the asymmetric peak and the dotted chromatogram shows the ideal, Gaussian peak shape.



$$T_f = (A+B)/2A$$

$$T \leq 2$$

Where:

T = tailing factor (measured at 5% of peak height)

b = distance from the point at peak midpoint to the trailing edge

a = distance from the leading edge of the peak to the midpoint

TAILING IS CAUSED BY

- 1) DEAD VOLUME
- 2) ADSORPTIVE EFFECTS
- 3) COLUMN PACKAING
- 4) INJECTION SOLVENT EFFECTS