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A Brief Review on Recent Advances in HPLC Techniques

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Abstract
In the present time chromatography has proven to be a significant technique and it is conventionally used for analysis purpose. High-performance liquid chromatography (HPLC) is a principal technique of current pharmaceutical and biomedical analysis to separate molecules from heterogeneous solutions and for further characterization preferably used preceding to mass spectrometry in the identification of peptides, proteins and other molecules. HPLC grew popularity due to its reliability (usage of pressure driven liquid support) and versatility (capacity of maintaining the composition of both mobile and stationary phases). Though fast separation often results in very high operational pressure, which places a huge load on HPLC instrumentation. Owing to which in recent years, core-shell silica microspheres (with a solid core and a porous shell, also acknowledged as fused-core or superficially porous microspheres) and Zirconia packing's have been discovered. Designed for highly proficient and fast separation with reasonably low pressure for separation of small molecules, large molecules and complex samples. Ultra-high performance liquid chromatography (UPLC) can provide greater resolution, increased sensitivity and faster separation times compared with traditional HPLC, but sometimes it can also be less reproducible. The Rapid Resolution Liquid chromatography (RRLC) method is not only accurate but also sensitive; it effectively increases the sample analysis quality and quantity compared with conventional HPLC. The aim of this review is to highlight fundamental aspects on column technology and developments for Nano- bore, Micro- bore, RRLC, UPLC, UFLC, Nano LC. Also to focus on fundamentals of HPLC.

Keywords
Nano- bore, Micro- bore, RRLC, UPLC, UFLC and Nano LC.

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INTRODUCTION[1-10]
Chromatography is a physicochemical method for separation of complex mixtures, was discovered at the very beginning of twentieth century on 21st march 1903 by Mikhail S. Tswett (A Russian Italian Botanist, 1872 - 1919) in his paper "on the new form of adsorption phenomena and its applications in biochemical analysis" at the regular meeting of biology section of Warsaw Society of natural science. Which defined the fractional adsorption processes

with description of molecules of different analyses have different interactions with the adsorption surfaces[1].

After 10 years of this event L.S Palmer [U.S.] and C. Dhere [E.U.] were independently published the same theory of separation. Further in 1931, Lederer stated the relevance of L.S Palmer and M. S. Tswett and published a paper on purification of Xanthophyll's on CaCo3 adsorption column.

Discovery and development continued with the A. J. P Martin and R. L. M Synge has proposed theory on Partition Chromatography in 1941 at Cambridge University at UK for which they awarded with the noble prize on 1952. The practical aspects for separation is come in action with the Prof. Horvath from Yale University in 1970 who has defined the actual name for process of HPLC at 21st Pittsburgh conference in Cleveland[1].

IUPAC has defined chromatography as "A method used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary or stable while other moves". Chromatography as the method of mass transfer or separation for components of a mixture on an adsorbent column in a flowing system.

The fundamental of separation science are adsorption and partition with the basic rule *like attracts like.* The mobile phase may be gaseous or liquid and stationary phase may be a solid or a liquid supported on a solid or a gel, which is packed in a device called as 'column'. Modern Column is a stainless steel tube packed with a very small particle (1-5µm) of rigid porus material.

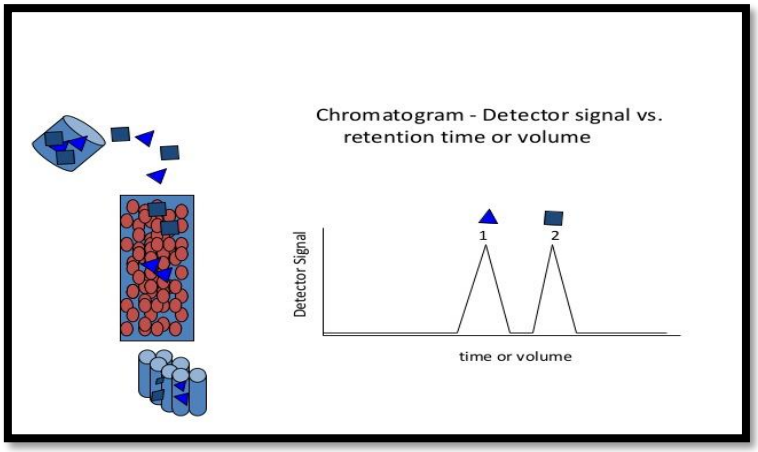
The substantial challenges in HPLC are fast and proficient separation for a wide range of samples. Fast separation probably results in very high operating pressure, which places a huge burden on HPLC instrumentation.

In recent years core-shell silica microspheres (with a solid core and a porous shell, also known as fused-core or superficially porous microspheres) and zirconia packaging have been investigated and used for highly proficient and fast separation with reasonably low pressure for separation of small molecules, large molecules and complex samples[2,3].

The perfect coupling of ultrahigh-pressure liquid chromatography (UHPLC) and MS instruments endures to be the megatrend in chromatography in recent years. With many second-generation UHPLCs systems introduced by all major manufacturers in prior years, currently analytical world facing mostly line extensions, application-specific systems, modules (particularly new MS or MS add-ons), and software products like Auto Chrome, LUMINATA, Meta Sense, Open lab CDS, sentinel, 1260 and 1220 infinity II etc[2, 3, 5, 6, 8].

HIGH-PERFORMANCE LIQUID-CHROMATOGRAPHY (HPLC) [4-15]

HPLC adores a steady increase in numbers of both instrumental sales and publications that describe new and innovative applications. Some recent development areas include reduction of HPLC systems, analysis of nucleic acids, intact proteins and protein digests, analysis of carbohydrates, and chiral analysis.



Principle: The principle of separation for normal phase mode and reverse phase mode is *adsorption*. When mixtures of components are fetching together in to a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more attraction towards the adsorbent moves slower. The component which has less attraction towards the stationary phase moves

faster. Since two components not have the identical affinity towards the stationary phase, the components are separated. It is a technique by which a mixture sample is separated into components for identification, quantification and purification of mixtures.

Novel Improvements in HPLC Technique:

HPLC is compared with the classical techniques characterized by:

- Rapid Resolution Liquid chromatography (RRLC)
- Ultra-Performance Liquid chromatography (UPLC)
- Ultra-Fast Liquid chromatography (UFLC)
- Nano Liquid chromatography (Nano LC)

Rapid Resolution Liquid Chromatography:

RRLC system was designed to provide highest analysis speed, resolution & pressure at a minimal bar. Rapid resolution analysis has become a routine method in the pharmaceutical industry. It holds outstanding peak shapes, enhanced reproducibility, high sensitivity, high-speed detection with reduced cost of analysis and is valuable for the quality control of herbal medicines. The separation resolution and reduction of analysis time radiantly improved in High Performance Liquid Chromatography (HPLC). For further improvement, column proficiency must be increased. The association among separation efficiency and the mobile phase linear velocity and particle size was investigated in detail in the early 1970s.

The use of a shorter length column leads to shortening in analysis time. However, loss of theoretical plates may cause due to a shorter column, hence a decrease in chromatographic resolution is required for a complex mixture of compounds. To balance the potential loss of resolution and due to use of smaller size particles has resulted in more efficient columns. Smaller particles packed with long length columns result in higher efficiency and higher resolution, with new RRLC technology, analysis time can be considerably reduced without losing chromatographic resolution.[9, 11, 12]

Table 1: Different characteristics of New Amendments in HPLC Technique

Characteristics	Particle size	Analytical column	Flow rate	Injection volume	Column dimensions (length x I.D)	Column temperature
HPLC	3 to 10 µ	XTerraC18, Alltima C18	0.01-5mL/min	5µL	150 X 3.2 mm	30^0 C
UPLC	Less than 2 µ	Acquity UPLCbeh C18, C8, rp	0.6 mL/min	2µL	150 X 2.1 mm	65^0 C
RRLC	1.8 µ	ZORBAX Eclipse XDB–C18 RRHT	0.2 – 20 µL/min	1.5 µL	2.1 - 4.6mm	Up to 100^0 C
UFLC	1.7 - 2.2 µ	Shim -Pack XR –ODS column	3.7 nL/min	0.1-100 µL	75mm X 3.0 mm	40^0 C
Nano LC	1.7 -3 µ	Capillary HPLC, Micro HPLC	20-200 nL/min	10 nL-125 µL	125 mm X 0.05mm -4.6mm	25^0-35^0C

Nano- bore and Micro- bore High Performance Liquid Chromatography: An analysis of very small amounts of samples has always posed a challenge. Nano bore High Performance Liquid Chromatography permit solutions to allow femtomole (10^{-15}) levels with good quality resolution for reliable identification and quantification. Recently Nano columns were identified as small as 75 µm and flow rates up to 300 nL/ minute used in Nano bore applications. Micro bore HPLC columns have around 1 mm and flow rates of 50- 75 µL per minute. Equally Nano bore and micro bore columns are used to their full potential with mass spectrometry detection systems particularly in analysis of peptides in biological matrices.

A refractive index gradient detector expressed as a universal detector in the micro-bore high-performance liquid chromatography analysis of carbohydrates. Concomitantly, low-ng and low-ppm injected amounts of carbohydrates are detected at the 3 x root-mean-square baseline noise level. A classic micro-bore high-performance liquid chromatography chromatogram separating fructose from sucrose followed by refractive index gradient

(RIG) detection was reported. Introduction of a position sensitive detector (PSD) in the RIG detector design and experimental considerations reported. Both are potential for the device in industrial and clinical applications.[13, 14, 15, 21, 22]

ULTRA FAST LIQUID CHROMATOGRAPHY

Ultra-Fast Liquid Chromatography (UFLC): Column efficiency can be increased with **r**eduction with particle size to 2µm particle was introduced in 2003 and this built a new way for fast separations. Recently it's possible to reduce column lengths and diameters to give faster separations with increased sensitivities. But small size particles may create problem of increased column back pressures which could be overcome by improvements in instrumentation to permit operation under high pressures. Allows ten time's higher speed and three times better resolution than other LC techniques and offers outstanding speed with excellent separation even at normal pressure levels. By enhancing the column and performance of the entire system UFLC minimizes the deviation from the van Demeter theory. The Distinction UFLC series provides ultrafast analysis, while maintaining high analytical precision and reliability.

Hyphenated LC techniques: A number of hyphenated techniques such as LC-MS, LC-FTIR, LC-AAS has been well known but amongst LC-MS has been vastly applied to a range of separations and quantification of complex molecules particularly in Bio-analytical separations.

Fast Protein liquid chromatography: FPLC mode has main role in purification of proteins. It is having potential for milligrams scale to kg scale separations. Separation and isolation has established charge separation between resin and a protein. Elution is based on variation of buffer composition.

Affinity chromatography: Affinity chromatography has potential in separation of several biochemical molecules such as a nucleic acids and protein purification in biological fluids such as blood. Affinity based on interactions between antigen-antibody, enzyme and substrate or receptor and ligand.

Chiral chromatography: Modern developments in stereo active column packing's typically polysaccharide derivatives like cellulose, amylase and cyclodextrin have been used for a wide range of optically active molecules. Primly separation of such molecules was very difficult using conventional separation modes.[21, 23]

Advances in stationary phases[13-25]

Stationary phase physical characteristics are the main determining factors contributing to efficient separation of mixture component by HPLC. Now day's Silica-based columns are predominantly used in majority of separations. However, recent developments in packaging materials have opened up new fields of applications. The key advantages offered by various packing's are listed below;

Core Shell Packing's: Several benefits offered by core shell packing's

- Greater performance on both HPLC and UHPLC systems
- Number of theoretical plates for such columns lie in the range of 200,000 to 300,000 so very high resolution and efficiency.
- Corresponding to sub2-µm size fully porous packing's efficiency is better but without high-back pressure problems that encountered with UHPLC systems
- High flow rates generate frictional heat. Core shell can withstand the excess of heat generated by mobile phase passing through the column at high velocities
- Van Demeter equation consist of three parameters which are responsible for band broadening, that are, eddy's diffusion, longitudinal diffusion and mass transfer. Separation efficiencies are enhanced by substantial reduction of all these parameters when using core shell packing's in comparison with fully porous particles
- Reduction in solvent compositions and faster analysis
- Detection and quantification at Low levels
- Exchange of 3 µm and 5µm analytical separation packing's with core shell packing's can outcome with significant improvements in resolution, sensitivity and speed of analysis.

Monolith packing's: Stationary phases for HPLC have evolved from irregular particles to spherical particles to sub- 2µm size particles and core shell technologies. Parallel improvements have been seen in development of monolithic packing's. A monolith can be imagined as a 'solid rock' like structure with no interstitial spaces. These may provide shorter diffusion distances and multiple pathways for solute dispersion. The porus rod-like arrangement is highly permeable and provides large surface area for separation in comparison with particle-based stationary phases. Monolithic columns are mainly silica-based or have organic polymer backbones.

Key application areas

Due to the multiple advantages monolith HPLC columns hold promising scope for applications in biotechnology covering specialized fields such as genomics, proteomics, metabolomics and

pharmacogenomics. High resolution and fast comeback of large molecules make possible chromatographic real-time monitoring of fermentation processes. Pharma, foods, forensics and are other areas where monolith columns will hold assurance in the upcoming time.

Nano HPLC is novel research area where monolith columns are having a future potential. Permeability of monolith packing circumvents the backpressure problem encountered with sub 2-µm packing's and it is possible to use very low ID columns of the order of 20 µm with long column lengths.

Zirconia packing's: Silica-based HPLC columns have remained pillars in HPLC laboratories. Over the years with advances in particle technologies size reduction has been possible from 5µm to sub- 2µm which led to development of UHPLC. Promote introduction of Core Shell Technologies and monolithic columns extended the range of applications and contributed to larger sample throughputs.

Properties of Zirconia supports

The key constraint of silica columns is availability of pH range between 2 and 8 and temperature to ambient. Alternatively, Zirconia supports exhibits operational stability over the entire pH range and working temperatures up to 200°C.

1) Lewis acid

Zirconia or 'ZrO2' (an oxide), in which the Zr atom acts as a Lewis acid. That is, it accepts electron pair due to presence of empty orbital. This feature provides enhanced selectivity over conventional silica columns.

2) Adsorption capacity

Adsorption capacity is larger in comparison to silica packing's

3) Chemical resistance

Packing's are highly resistance to acidic and basic solutions. Retention of basic and acidic analytes can be finely controlled by changing the pH of medium over the entire available range.

Types of phases

Four surface chemistries for based on the theory that this surface will maintain surface activities are available which greatly extend selectivity and retention of for improved separations

Polybutadiene coated Zirconia

The phase is parallel to reverse phase using C18 ($C_{18}H_{37}$) silica but has following advantages:

- Temperature stability up to 200°C
- Available pH ranges 1 - 13
- Less hydrophobic than C18 and is suitable for separations involving amines and bases

Zirconia-C18

- Can be used over the entire pH range (1 - 14) along with high temperature capabilities (up to 200°C)
- Porous carbon clad zirconia particles covalently improved with C18 groups.
- Selectivity is rather different from conventional C18 columns

Zirconia - polystyrene

- Valuable for separation of hydrophobic compounds and amines
- Operational pH ranges 1 - 13
- Porous Zirconia particles are improved with cross-linked polystyrene.
- Endure strongly aqueous solutions up (up to 100% pure water)
- No requirement for ion pairing requirement
- Affords mixed mode Reverse phase and Ion exchange separations
- Reduced tailing of basic compounds in comparison to silica-based packing's

Zirconia-Carbon

- Porous carbon clad Zirconia particles deliver enhanced retention of polar compounds
- Highly hydrophilic surface
- Useful for separation of isomers and diastereomers
- Good retention of fused – ring aromatic compounds

Column care

Contaminated columns can deteriorate the peak shape, effect retention times, lead to high backpressure and increase baseline noise. To overcome this concentration above 25 mM phosphate, acetate, carbonate/bicarbonate buffers and salts should be avoided. Anions like carboxylic acids, fluoride and phosphate strongly absorb on Zirconia columns. The recommended steps for column regeneration are;

1. Flush with a 20:80 mixtures of acetonitrile and pH 10 ammonium hydroxide for 50 column volumes followed by 10 column volumes of water
2. Flush with pure methanol, acetonitrile, isopropanol or tetrahydrofuran for 20 column volumes. Zirconia HPLC columns will involve the same solvents with at least 20% THF
3. Repeat with 20:80 mixture of acetonitrile and 0.1 M nitric acid for same column volumes

Storage

- Zirconia HPLC columns must not be put in storage in phosphate buffer. The suggested storage method is Overnight 10 volumes of organic modifier or water

- Long-time storage 10 volumes of pH 10 ammonium hydroxide followed by 10 volumes of 50: 50 organic modifier or water

It was observed that zirconia-based reverse phases offer a great choice of selectivity and retention. Additional benefits of column chemistry, pH and temperature extremes contribute to versatility of applications.

METHOD DEVELOPMENT AND SYSTEM SUITABILITY TESTS[15-29]

An existing HPLC system consists of a high-pressure solvent delivery system, a sample auto injector, a separation column, a detector (often an UV or a DAD) a computer to control the system and display results. Many systems include an oven for temperature control of the column and a pre-column that protects the analytical column from impurities. The actual separation takes place in the column, which is packed with chemically modified 3.5-10 µm (often silica) particles. A mobile phase is pumped through the column with the high-pressure pump and the analytes in the injected sample are separated depending on their degree of interaction with particles. An appropriate choice of stationary and mobile phase is essential to reach a desired separation. The practical application of HPLC is aided by an awareness of the concepts of chromatographic theory, in particular measurement of chromatography retention and the factors that influence resolution. The drug retention with a given packing material and eluent can be expressed as a retention time or retention volume, but both of these are dependent on various factors like flow rate, column length and column diameter. The retention is described as a column capacity ratio (k), which is independent of these factors. The column capacity ratio of a compound (A) is defined by equation

$$k_A = V_A - V_0 / V_0 = t_A - t_0 / t_0$$

Where, V_A is the elution time of A and V_0 is the elution volume of a non-retained compound (i.e. void volume). At constant flow rate, retention time (t_A and t_0) can be used instead of retention volumes.[13, 15, 17, 18]

Resolution: Describes the separation power of the complete chromatographic system relative to the particular components of the mixture. By convention, resolution (Rs) is expressed as the ratio of the distance between two peak maxima to the mean value of the peak width at the baseline, equation

$$Rs = 2(t_2 - t_1)/W_1 + W_2$$

Here, t_1 and t_2 are retention times and W_1 and W_2 are their baseline bandwidths. If we approximate peaks by triangles, then if Rs is equal to or more than 1, the components are completely separated.

Method Development in HPLC[19]: Before beginning the method development, we need to review what is known about the sample; also goal of the analysis should be defined at this point and concerns must be given regarding how many samples will be analyzed and what HPLC equipment are available. The nature of the sample (e.g., weather it is hydrophilic or hydrophobic, weather it contains protolytic functions etc.) determines the best approach to HPLC method development. The selection of suitable chromatographic method for organic compound depends on different strategies.

Strategy for HPLC Method Development[20-22]: First Reverse-phase should be tried and if not successful, Normal-phase should be taken into consideration. It is also important that before making experiments with Ion-exchange or Ion-pair chromatography, first ion suppression by pH control and Reversed-phase chromatography should be tried.

Reverse-Phase Chromatography: The stationary phase is non-polar (hydrophobic) in nature, while the mobile phase is a polar solvent, such as mixtures of water and methanol or acetonitrile. Here non-polar the material is more, the longer it will be retained.

Normal-phase chromatography: The stationary phase is intensely polar in nature (e.g., silica gel), and the mobile phase is non-polar (such as n-hexane or tetrahydrofuran). As a result, polar samples are retained on the polar surface of the column packing longer than less polar materials.

Ion forming organic compounds: Ion-pair chromatography should be preferred to ion-exchange chromatography. The analysis in HPLC is either qualitative or quantitative determination of different components present in the sample. The qualitative analysis determines the sample quality and quantitative analysis, involves comparison of standard and samples (their area or height). It is based on two requirements i.e. reproducible chromatogram and linear response of the detector for analytes of interest.

System suitability tests for chromatographic methods[20-28]: System suitability is the checking of a system to ensure system performance before or during the analysis of unknown samples. Factors such as plate count, tailing factors, resolution and reproducibility (% RSD retention time and area for six repetitions) are determined and compared against the specifications set for the method. These factors are measured throughout the analysis of a system suitability "sample" that is a mixture of main components and expected by-products.

The diligence of the system suitability test is to ensure that the complete testing system (together with instrument, reagents, column and analyst) is suitable for the intended application.

As defined in USP Chromatography General Chapter states "System suitability tests are an integral part of gas and liquid chromatographic methods. They are organized to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done." The tests are depending on the theory that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Various suitability parameters are shown in table 2.

Table 2: System suitability parameters in HPLC

Parameters	Recommendation
Capacity Factor (k')	The peak should be well resolved from other peaks and the void volume, generally k' > 2.0
Repeatability	RSD ≤ 1% for N ≥ 5 is desirable
Relative Retention	Not essential as long as the resolution is stated
Resolution (R_s)	R_s of > 2.0 between the peak of interest and the closest eluting potential interferents (impurity, excipient, degrade product etc.)
Tailing Factor (T)	T of ≤ 2
Theoretical Plates (N)	In general should be > 2000

The factors that are affected by the changes in chromatographic conditions are:

a. Resolution (R_s),
b. Capacity factor (k'),
c. Selectivity (α),
d. Column efficiency (N) and
e. Peak asymmetry factor (A_s).

a. Resolution (R_s):

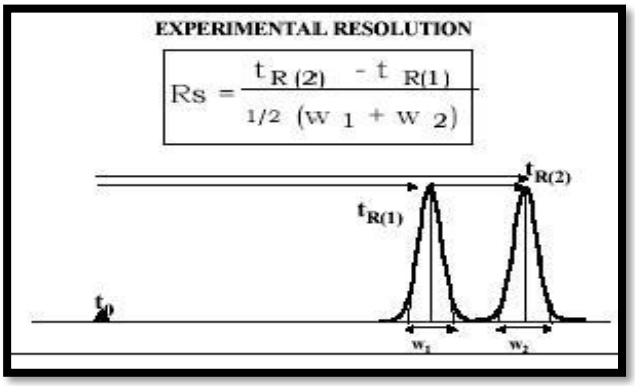


Figure 1: Experimental resolution

Resolution is the parameter relating the Separation Power of the complete chromatographic system relative to the particular components of the mixture. The resolution (R_s), of two adjacent peaks is defined as the ratio of the distance between two peak maxima. It is the dissimilarity between the retention times of two solutes divided by their average peak width. For baseline separation, the Ideal value of R_s is 1.5. [19-21]

It is calculated by using the formula,

$R_s = Rt_2 - Rt_1 / 0.5 (W_1 + W_2)$

Where, Rt_1 and Rt_2 are the retention times of components 1 and 2 and

W_1 and W_2 are peak width of components 1 and 2.

There are three fundamental considerations that influence the resolution of a chromatographic separation:

- Capacity factor
- Selectivity
- Column efficiency

b. Capacity Factor (k'):

Capacity factor is nothing but the ratio of the reduced retention volume to the dead volume.

Capacity factor (k') can be defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how sound the sample molecule is retained by a column during an isocratic separation. The Ideal value of k' ranges from 2-10. Capacity factor is determined by using the formula,

$k' = V_1 - V_0 / V_0$

Where, V_1 =Retention Volume at the apex of the peak (solute) and

V_0 = Void Volume of the system.

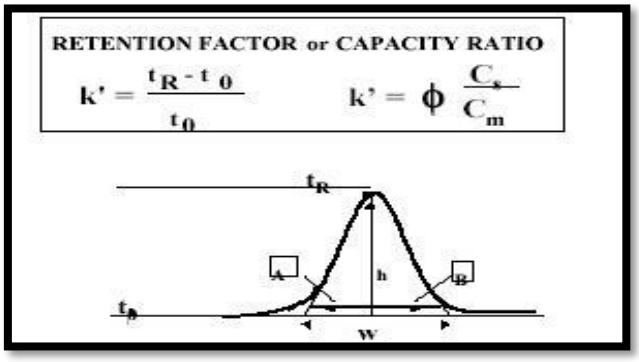


Figure 2: Retention factor or Capacity ratio

The values of k' for individual band is increase or decrease with change in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in water/ organic mobile phase.

Typically, an increase in percentage of the organic phase by 10% by volume will decrease k' of the bands by a factor of 2-3.

C. Adjusting capacity factor (k')

Good Isocratic method usually have a capacity factor (k') in the range of 2 -10 (typically 2 – 5). Lower values may give inadequate resolution. Higher values are usually associated with excessively broad peak and unacceptably long run time. If the shift in k' value is observed with both analyst and the column test solution, the problem is most likely due to change in column, temperature or mobile phase composition. This is true if the shift occurs gradually over series of run.

Capacity factor (k') values are sensitive to:

- Solvent strength
- Composition
- Purity
- Temperature
- Column chemistry
- Sample[21, 22]

c. Selectivity (α)

The selectivity factor (α) is a measure of relative retention of two components in a mixture.

It is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention time. Selectivity signifies the separation power of particular adsorbent to the mixture of these particular components.

This consideration is independent of the column efficiency; it only depends on the nature of the components, eluent type, and eluent composition and adsorbent surface chemistry. Usually, if the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency.

The ideal value of α is 2. It can be calculated by using formula,

$\alpha = V_2 - V_1 / V_1 - V_0 = k_1' / k_2'$

Where, V_0 = The Void Volume of the column,

V_1 and V_2 = The Retention Volumes of the second and the first peak respectively.

Adjusting selectivity (α)

When trouble shooting causes changes in selectivity (α), the approach is similar to the approach used in the capacity factor. When selectivity (α) is affected, the corrective action depends on whether the problem is mobile phase or column related. Be certain to compare results obtained with the test solution to those observed when the column was new.

Selectivity (α) values are sensitive to:

- Changes in mobile phase composition (pH ionic strength)
- Purity
- Temperature

d. Column Efficiency/ Band broadening:

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Similar the band spread, higher is the number of theoretical plates, indicating good column and system performance. Column with N ranging from 5,000 to 1, 00,000 plates/meter are ideal for a good system.[25-27]

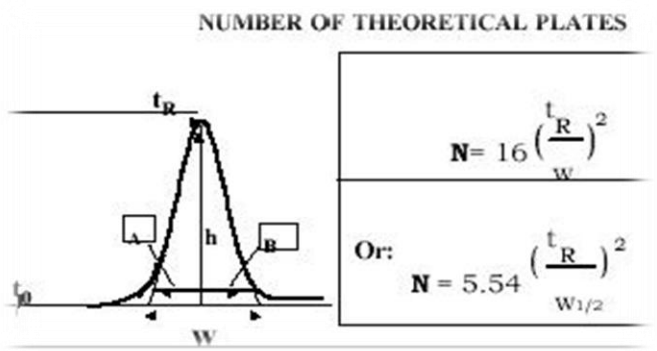


Figure 3: Number of Theoretical plates

Efficiency is calculated using the formula,

$N = 16\ R_t^2 / W^2$

Where, R_t is the retention time and

W is the peak width.

A decline in measured column efficiency may be due to:

- Age and history of the column
- Extra column band broadening (such as due to malfunctioning injector or improper tubing internal diameter)
- Inappropriate detector setting.
- Change in flow rate and solvent viscosity

You can recognize problems in your separation due to a loss of column efficiency when the width or shapes of all peaks are affected.

Methods of measuring column efficiency (N):

Method used for the measurement and calculation of column include (in order to sensitivity to abnormal peak shape):

- Asymmetry based (most sensitive to tailing or fronting)
- 5 sigma
- 4 sigma
- Tangent
- 3 sigma
- ½ height
- 2 sigma (Least sensitive to tailing or fronting).

Choose the method that best suits your operating requirements. It is critical that the same method always be used and executed reproducibility.[25-27]

e. Peak asymmetry factor (A_s):

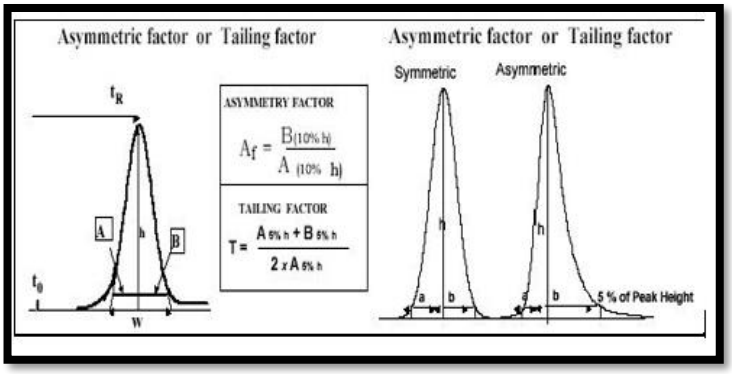


Figure 4: Peak asymmetry factor

Peak asymmetry factor, as, can be used as a criterion of column performance**.** The peak half width, b, of a peak at 10% of the peak height, divided by the corresponding front half width, a, gives the asymmetry factor.

$A_s = b / a$

For a well packed column, an asymmetry factor of 0.9 to 1.1 should be achievable.

CONCLUSION:

By the virtue of which HPLC studies relating to the development of new and significant advances in separation science. With the use of HPLC individual can produce extremely pure compounds. It is suitable to be used in both laboratory and clinical science. With the use of HPLC the accuracy, precision and specificity can be increased. The only disadvantage of HPLC is high cost. Advancement in core-shell silica microspheres (with a solid core and a porous shell, also known as fused-core or superficially porous microspheres) and Zirconia packing's have been broadly investigated and used for highly proficient and fast separation with reasonably low pressure for separation of small molecules, large molecules and complex samples. Ultra-high performance liquid chromatography (UPLC) can provide superior resolution, increased sensitivity and faster separation times compared with traditional HPLC. The RRLC method is a precise and sensitive; in addition, it effectively increases the sample analysis throughput compared with conventional HPLC.

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