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PREPARING COLUMNS FOR HPLC AND THEIR APPLICATIONS IN DIFFERENT COMPOUND ANALYSIS.

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Abstract :

High-Performance Liquid Chromatography (HPLC) is a widely utilized analytical technique in pharmaceutical, environmental, and biochemical analysis due to its high precision, sensitivity, and versatility. The efficiency of HPLC largely depends on the proper selection and preparation of chromatographic columns, which play a critical role in achieving accurate separation and quantification of compounds. This review focuses on the various types of HPLC columns, including normal-phase, reversed-phase, ion-exchange, and size-exclusion columns, along with their preparation procedures such as column packing, conditioning, and equilibration.

The paper also highlights the physicochemical properties of stationary phases, particle size, pore size, and surface chemistry, which influence chromatographic performance. Special emphasis is given to reversed-phase columns (e.g., C18), which are most commonly used in pharmaceutical analysis. Furthermore, the applications of HPLC columns in the analysis of diverse compounds such as drugs, metabolites, proteins, and environmental pollutants are discussed. The role of column selection in method development, resolution optimization, and analysis of complex mixtures is also examined.

Overall, this review provides a comprehensive understanding of HPLC column preparation techniques and their applications in different fields, serving as a valuable resource for researchers and analysts involved in chromatographic studies.

I. Introduction :

High-Performance Liquid Chromatography (HPLC) is a cornerstone analytical technique that has revolutionized the analysis of complex mixtures in diverse scientific disciplines. The precision and efficiency of HPLC are critically dependent on the design and preparation of the chromatographic column, which serves as the pivotal element for separating target compounds based on their chemical interactions with the stationary phase. As the demand for more sensitive, selective, and efficient analyses grows across industries ranging from pharmaceuticals to environmental monitoring, the preparation and optimization of HPLC columns have become a subject of substantial research and technological advancement.[1]

The stationary phase of the HPLC column is central to its functionality, influencing parameters such as separation efficiency, retention time, resolution, and sensitivity. Columns are typically prepared with carefully selected materials, such as silica-based particles or polymeric substrates, which are then functionalized to provide specific interactions (e.g., hydrophobic, polar, or ionic) with the analytes of interest. The choice of stationary phase—ranging from reversed-phase, normal-phase, ion-exchange, size-exclusion, to chiral columns—largely determines the types of compounds that can be effectively separated.[2]

Given the vast array of potential applications, the preparation of HPLC columns is often tailored to the specific needs of the analysis. For instance, reversed-phase columns are predominantly used in pharmaceutical and biochemical research for the analysis of hydrophobic compounds, while ion-exchange and chiral columns are applied in specialized fields such as protein purification and enantiomeric separations. In environmental science, size-exclusion chromatography plays a crucial role in the analysis of complex environmental matrices, such as soil and water, for pollutants and contaminants.[3]

In addition to the conventional uses, the ongoing innovation in column technology, including the development of high-capacity, high-stability materials, has broadened the scope of HPLC applications. New materials and techniques continue to enhance column performance, particularly for the analysis of complex biological samples, food safety testing, clinical diagnostics, and forensic toxicology.

This article reviews the preparation and optimization of HPLC columns, with a focus on their diverse applications in the analysis of various compounds. It explores the principles behind column selection and preparation, offering insights into the latest advancements in column chemistry, as well as their impact on improving the efficiency and accuracy of HPLC-based analyses in research and industry. Through understanding the intricacies of column preparation and its applications, researchers can optimize HPLC methodologies for a wide range of analytical challenges.[4]

II. HPLC :

High-Performance Liquid Chromatography (HPLC) remains a keystone analytical method in varied scientific fields due to its high-quality for flexibility, precision, and asset in extrication, classifying, and measuring compounds in multifarious mixtures. Current research remains to enhance HPLC approaches, magnify its application scope, and benchmark its performance against other analytical devices. This review summarizes key findings from recent studies, focusing on the principles, applications, and comparative advantages of HPLC, alongside an overview of the current research landscape

Core Principles of HPLC: HPLC works on the principle of separating analytes based on their differential partitioning between a mobile phase and a stationary phase packed within a cylindrical tube called column. A pump of a high-pressure forces the mobile phase containing the sample through the column. Different constituents in the sample interact with the stationary phase to variable points based on their physicochemical properties such as size, polarity, and charge, etc. This results in different movement rates, accordingly, a separation of the components occurred according to their elution from the column

and detected by a suitable detector (e.g., UV, fluorescence, electrochemical and mass spectrometry). Significant separation modes, include reversed-phase (most common), normal-phase, ion-exchange, size-exclusion, and affinity chromatography, are selected according to specific types of analysts.

Briefly, HPLC operates on the principle of separating components in a mixture based on their interactions with a stationary phase and a mobile phase. Recent studies have emphasized the efficiency of HPLC in separating complex mixtures, particularly in pharmaceutical analysis. In contrast, GC is limited to volatile compounds, while Capillary Electrophoresis (CE) offers advantages in speed and resolution for ionic species. [5]

Types of HPLC Columns :

1) Normal Phase Columns :

Introduction:

Normal-phase liquid chromatography is defined as a separation technique that utilizes a non-polar mobile phase and a polar stationary phase to separate target compounds based on their polarity, with polar compounds exhibiting a higher affinity for the stationary phase.

Normal phase LC involves a combination of a polar stationary phase and a less polar (or even nonpolar) mobile phase [4]. Normal phase was one of the first developed separation methods and, for this reason, reversed phase LC was labeled just that, as it involves stationary and mobile phases with the reversed polarities. Today, a polar functional group is bonded to silica, which is advantageous when analytes that are insoluble in polar solvents need to be separated [4]. In regard to elemental speciation analysis by ICP spectrometry, normal phase LC has very limited use. This is partially due to the incompatibility of the mobile phase with the conventional ICP but also because reversed phase LC can do many of the separations as well [4]. Often, normal phase LC is used in combination with size exclusion chromatography. Although not as popular today, normal phase LC is still a corner stone of LC.[6]

Normal-phase liquid chromatography (NPLC) is the oldest chromatographic mode, discovered by M. S. Tswett more than 100 years ago. It has been the predominant mode in thin-layer chromatography (TLC) and low-pressure dry-column liquid chromatography before the introduction of reversed-phase technique, which is a preferred mode in high-performance liquid chromatog-raphy (HPLC).[26]

In normal-phase chromatography, the stationary phase is more polar than the mobile phase. As illustrated in Figure 1, the retention increases as the polarity of the mobile phase decreases and thus polar analytes are more strongly retained than nonpolar analytes, the opposite of that in reversed-phase liquid chromatography (RPLC). The column packing is either an inorganic adsorbent (silica gel or, less often, aluminum, titanium, or zirconium oxides) or a moderately polar bonded phase (cyanopropyl. (CH3)3-CN; diol,-(CH2)3-O-CH2-CHOH-CH2-OH; or aminopropyl. -(CH2)3-NH2) chemically bonded on a suitable support material, which is usually silica gel. The mobile phase is usually a mixture of two or more organic solvents: nonpolar solvent and strongly or weakly polar solvents, or a mixture of water and an organic solvent (usually acetonitrile) in aqueous normal-phase chromatography (ANP).[26]

III. Principle :

In RPLC, the retained solute molecules are not localized on the surface of the column packing (this behavior resembles a partition process), whereas the adsorbed polar sample and solvent molecules in NPLC are localized on specific polar adsorption sites, to which they are strongly attracted. Neutral compounds can be separated by both RPLC and NPLC, generally with the opposite order of elution, but some differences in the separation selectivity and elution order are usually observed between the two modes. This is illustrated in Figure 2 for the retention factors, k, of some phenylurea herbicides on a

bonded nitrile column in propanol-hexane (normal-phase mode) and propanol-water (reversed-phase mode) mobile phases.[26]

The retention in NPLC increases with increasing polarity (activity) and is proportional to the specific surface area, As, which controls the number of the adsorption sites available for contact with the sample solutes in the column. Water in mobile phases deactivates the adsorbent (decreases x'), as the sites with strongly held adsorbed water are no longer available for adsorption of moderately polar organic compounds. The elution times of analytes generally increase in the following sequence: alkanes < alkenes < aromatic hydrocarbons chloroalkanes <alkylsulphides<ethers <ketones aldehydes esters<alcohols <amides phenols, amines, car-boxylic acids. The retention also depends to some extent on the hydrocarbon part of solutes and generally slightly decreases as the size of alkyl groups increases. However, the adsorption energy of alkyl groups is low and the adsorbed molecules adhere to the adsorbent surface by the polar groups, whereas the hydrocarbon parts of the molecules point from the adsorbent surface toward the mobile phase. The separation in a homologous series is far less effective than in reversed-phase chromatography and usually only a few lowest homologs can be successfully resolved by NPLC. If the localization of the adsorption sites on the surface of a polar adsorbent fits a spatial distribution of polar functional groups in a solute molecule with multiple functional groups, simultaneous interactions of two or more functional groups are possible, which are weaker or absent for molecules with other positions of functional groups. Hence, NPLC (especially on silica gel columns) is usually suitable for separation of positional isomers. Further, differences in the retention of molecules of similar polarities, but different shapes (rigid planar, rod-like, or of flexible chain structure), are often observed and utilized in normal-phase chromatography. Both steric hindrance to the access of a polar functional group to the adsorption site and interactions between vicinal polar groups decrease the adsorption and the retention. Hence, ortho-substituted benzenes are less strongly retained than meta-and para-isomers. For example, o-nitrophenol elutes earlier than m-nitrophenol, whereas p-nitrophenol is retained most strongly in NPLC because of increased acidity caused by mesomeric effect.[26]

Columns and Stationary Phases in NPLC:

Nowadays, most HPLC separations are performed on conventional analytical columns, 10-25 cm long, 3-4.6 mm in diameter, and packed with 3-10 µm uniform particles (recently even with 1-2 µm size). With short 'high-speed columns, 1.5-5 cm long,[26]

simple separations can be accomplished in 1-3 min, with significantly reduced analysis time and solvent consumption. Separa-tions on 'microbore columns, 15-25 cm long and 1-2 mm ID, need even less mobile phase and allow detection of high mass sensitivity. These columns are useful for analyses of small sample amounts and with mass-spectrometric detection. Recently, packed capillary HPLC columns of internal diameter 0.1-0.5 mm have become commercially available. However, the microbore and especially the capillary columns need miniaturized instrumentation to suppress extracolumn contributions of the injector, the detector, and the connecting capillaries to band broadening.[26]

Silica gel:

Macroporous silica gel is the material most often used in NPLC and is also the most important support material for preparing chemically bonded phase packings with a large variety of functionalities. The main advantages of silica particles are their high mechanical strength, compatibility with water and all organic solvents, and dimensional stability of column beds during use with various solvent types and gradient elution, as the wet silica particles do not swell.[26]

Polar-bonded phases:

Cyano, diol, and amino bonded phases used in NPLC are generally prepared by chemical modification of the silica gel surface, like the C18 or C8 phases for RPLC. NPLC separations on these columns are usually more convenient and reproducible than on unmodified silica, as they are less sensitive to the control of mobile-phase water content and require shorter equilibration times after the change of the

mobile phase. However, silica columns usually have a better stability and a longer lifetime, show better isomer selectivity, higher loadability for preparative separations, and are less expensive. Generally, the strength of the interactions with analytes increases in the order: cyanopropyl <diol<aminopropyl silica alumina stationary phases, but selective interac-tions may change this order. Basic analytes are generally very strongly retained by the silanol groups in silica gel and acidic compounds show increased affinities to aminopropyl columns. Aminopropyl and diol columns prefer compounds with proton-acceptor or proton-donor functional groups (alcohols, esters, ethers, ketones, etc.), while dipolar compounds that cannot have proton-donor or proton-acceptor interactions are usually more strongly retained on cyanopropyl than on aminopropyl silica. Chemically bonded phases with other functionalities such as polyethylene glycol, pentafluorobenzene, or alkyls with embedded amide or carbamate groups are generally intended for reversed-phase applications in water-rich mobile phases. However, at high percentage of organic solvent(s) in the mobile phases, the retention of many compounds increases with increasing concentration of acetonitrile or methanol, showing a typical normal-phase behavior.[26]

Alumina:

Several types of porous alumina are available with pore diameters from 6 to 15 nm and surface areas of 70-250m^2 g^1. Its surface is less homogeneous than silica and contains both hydroxyl groups and aluminum ions with a function of Lewis acid sites. By conditioning with acids or bases, the pH of the surface can be adjusted between pH 3 and 9 and can exhibit both anion-exchange (at low pH) and cation-exchange (at high pH) properties. Heating at 200 °C activates the surface of alumina by dehydration; heating at temperatures higher than 900 °C causes irreversible loss of activity. Even though the adsorption properties of alumina are similar to those of silica, there are some differences in selectivity. Alumina favors interactions with a electrons and often yields excellent separation selectivity for compounds with different numbers or spacing of unsaturated (double) bonds, such as polycyclic aromatic hydrocarbons or some steroids. However, it is much less widely used than silica nowadays, because of catalytic properties of its surface, which may cause decomposition or irreversible adsorption of some samples. It is more difficult to bond stationary phases to the surface of alumina than to silica gel, but alumina-based stationary phases are more stable in basic mobile phases.[26]

Zirconium dioxide:

Zirconium dioxide (zirconia) has a polar surface with zirconium atoms as strong Lewis acid sites. Because of empty orbitals, Zr attracts electron-rich Lewis bases such as phosphate anions. Hence, the surface of zirconia has significant cation-exchange properties, giving rise to a mixed-mode retention mechanism. Recently, 3 and 5 µm porous and nonporous spherical zirconia particles have become available for HPLC, with 30 nm pore size and 30 m^2 g specific surface. The primary advantage of zirconia relative to silica is its stability over the entire pH range from 1 to 14 and at temperatures up to 200 °C. Like silica, it is mechanically stable and provides high chromatographic efficiency. Stationary phases based on zirconia surface coated with polybutadiene, polystyrene, carbon, or carbon covalently modified with octadecyl groups are intended mainly for RPLC applications. Even though unmodified zirconia is a much weaker adsorbent than silica in nonaqueous solvents, its NPLC applications in the analysis of steroids and plant genins were reported.[26]

Mobile Phase in Normal-Phase Chromatography:

The polarity and the elution strength, that is, the ability to enhance the elution, generally increases in the following order of most common normal-phase chromatography solvents: hexane heptaneoctane <methylene chloride <methyl-t-butyl ether<ethyl acetate <dioxane <acetonitrile tetrahydrofuran <1- or 2-propanol <methanol. Large changes in selectivity of normal-phase chromatographic separations can be achieved by selecting the solvent with appropriate type of polar interactions. For chromatog raphy on silica gel, normal-phase chromatographic solvents can be classified as nonlocalizing (eg, alkanes, aromatic hydrocar-bons, chloroalkanes), basic-localizing (eg, amines and ethers), and nonbasic-localizing (e.g., esters, nitriles, or nitro compounds). Localizing solvents are strongly attracted to adsorption sites, while nonlocalizing solvents are more or less regularly distributed on the whole

adsorbent surface. The basicity of a solvent is understood as its ability of hydrogen-acceptor interactions with the silanol groups.[26]

Binary mobile phases:

A single solvent only rarely provides suitable separation selectivity and retention in normal-phase systems, which should be adjusted by selecting appropriate composition of a two- or multicomponent mobile phase. Most often, a binary mobile phase is used, containing a weak solvent A, usually an alkane, and a more polar organic solvent, B, whose concentration controls the elution strength of a mixed mobile phase. Change in retention is a regular function of the concentration of B in the mobile phase. As a rule. a twofold increase in percentage of B will cause a two- to threefold decrease in the retention factors, k, depending on the polarity of the strong solvent B. The dependence of the retention in NPLC on the composition of the mobile phase can be characterized more exactly on the basis of the simple adsorption model described by eqn. With some simplification, the dependence of the retention factor, k, on the concentration (volume fraction) of the stronger (more polar) solvent,, in binary mobile phases comprising two solvents of different polarities can be described by eqn [26]:

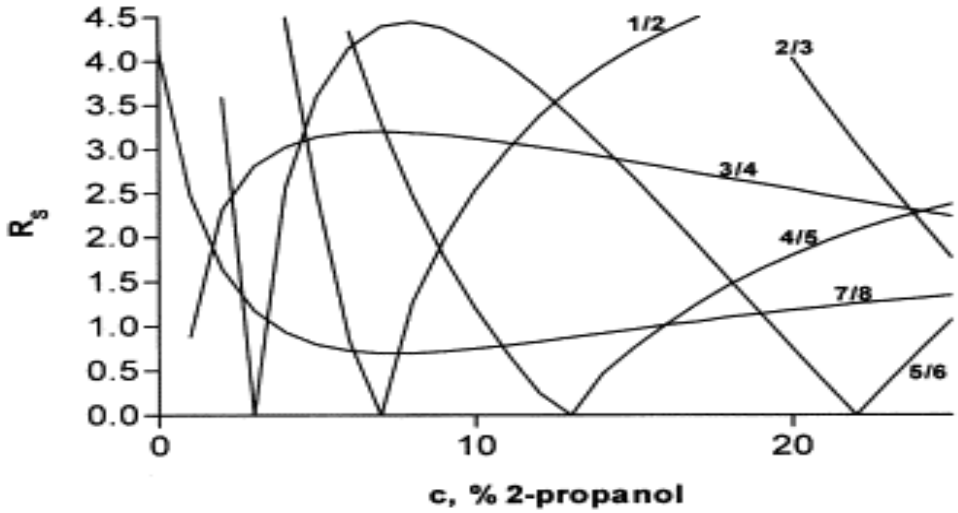
k = k_{0} * varphi ^ (- m)

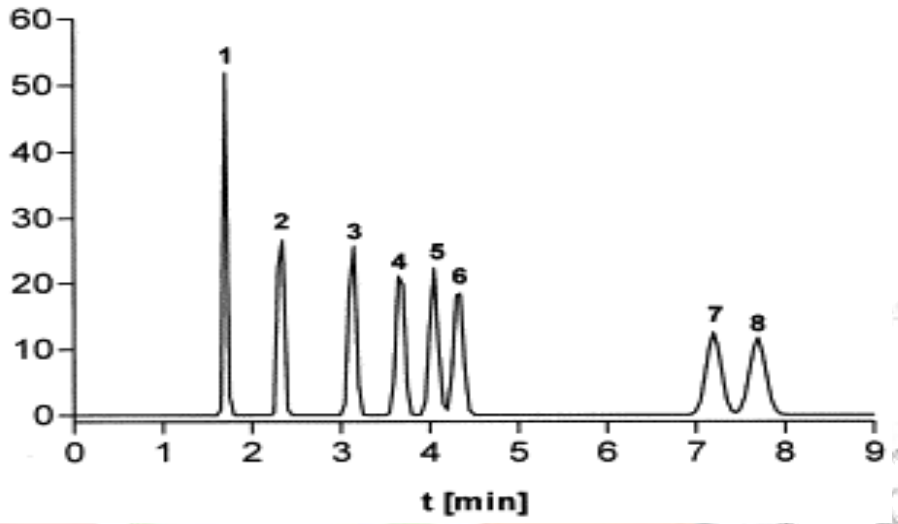
Here, k_{0} and m are experimental constants, which depend on the nature of the solute and on the chromatographic system, but are independent of the concentration of the strong solvent, in the mobile phase. k_{0} is the retention factor in pure strong (polar) solvent, B. The parameter m is a measure of stoichiometric exchange in the displacement model of adsorption, that is, the number of molecules of the strong solvent B necessary to displace one adsorbed sample molecule. Equation applies only for samples strongly retained in the pure nonpolar solvent. A. If the analytes are less strongly retained in pure solvent A, a three-parameter eqn can often describe the effect of changing the mobile phase composition on the retention:[26]

k = (b + a*varphi) ^ (- m)

The constants a, b, and m in eqn [3] depend on the solute and on the chromatographic system. b = (k_{d}) ^ (- n!) where k_{4} is the retention factor in a pure nonpolar solvent. Equation [2] or [3] can be used as the basis of optimization the composition of two-component (binary) mobile phases in NPLC, using a common window diagram or overlapping resolution mapping approach, as illustrated in an example in Figure 3.

Figure 3 Resolution diagram for the isocratic separation of eight herbicides on a Silasorb SPH silica gel column, 7.5 µm, 300 x 4.2 mm ID as a function of the concentration,, of 2-propanol in heptane as the mobile phase (a) and the separation under optimized conditions yielding best resolution of the pairs of compounds 5/6 and 7/8 determined from the window diagram (a) as 19% 2-propanol (b). Solutes: neburon (1); chlorobromuron (2); 3-chloro-4-methylphenylurea (3): desphenuron (4); isoproturon (5); diuron (6); methoxuron (7); deschloromethoxuron (8). Reprinted with permission from Jandera, P. J. Chromatogr. A 1998, 797, 11-22; Elsevier[26].





Complex mobile phases:

Complex mobile phases containing two or more different polar solvents in a nonpolar one can be used to enhance the selectivity in NPLC. Fine tuning of NPLC separations can be based on the 'selectivity triangle' approach, selecting the optimum composition of four-component mobile phases with hexane or heptane as a nonpolar solvent, dichloromethane as a non-localizing solvent, methyl tert-butyl ether as a basic localizing solvent, and acetonitrile or ethyl acetate as a non-basic localizing solvent. The concentration of the nonpolar solvent (a diluter) controls the solvent strength and the concentration ratios of the three polar solvents adjust the selectivity for various sample components. To describe the retention in ternary and more complex mixtures, it is principally possible to use the theoretical model of adsorption chromatography, with elution strength contributed to by all solvents in the mobile phase, but it would be necessary to consider the competition between various solvents in the mobile phase for localized adsorption centers on the adsorbent surface and to correct correspondingly the solvent strength, which is not straightforward in practice.[26]

IV. Applications of Normal-Phase HPLC:

NPLC is most suitable for separation of nonionic and moderately polar compounds, especially for lipophilic samples that are too strongly retained by RPLC. Lipids differing in the number and position of double bonds, tocopherol, carotenoids, fat-soluble vitamins, and steroids in pharmaceuticals can be successfully separated by NPLC on silica gel or alumina columns. Mixed lipid classes in the extracts of animal or plant tissues can be analyzed on silica columns or on columns with bonded polyvinyl alcohol using complex solvent gradients. Gradient-elution NPLC methods were reported for the analysis of natural phospholipids in nervous tissues, meat, etc. Mono-, di-, and triacylglycerol classes in natural oils

can be distinguished on silica gel columns using normal-phase gradient elution, In these applications, either low-wavelength UV detection at 205 nm, or evaporative light-scattering detection is used for quantitative analysis.[26]

Normal-phase HPLC usually offers much improved separation of positional isomers or stereoisomers with respect to RPLC. This is also the reason why normal-phase liquid chromatographic mode with nonaqueous mobile phases is often used for the separation of enantiomers on chiral bonded stationary phases.[26]

NPLC is often the method of choice for separation of water-insoluble synthetic polymers and oligomers with polar repeat monomer units or with polar end groups by the so-called interactive liquid chromatography, which usually offers much better resolution than size-exclusion chromatography for lower polymers with molar masses up to 10000-20000. Because the effect of increasing concentration of the polar solvent B is more significant for polymers than for small molecules.[26]

2. Reverse phase column :

Introduction:

Reversed-phase ultrahigh-performance liquid chromatography (RP-UHPLC) is a fast and highly efficient chromatography method that uses a polar mobile phase and a non-polar stationary phase to separate substances according to their hydrophobicity.[8]

Jorgenson first used the term UHPLC, which stands for ultra-high-pressure liquid chromatography, in 1997. The usage of nano-columns filled with non-porous 1.0-1.5 µm silica-based particles on a prototype system that can withstand extremely high pressures (up to 4100 bar in 1997 and 7200 bar in 2003 was originally described by this group. [8]

In addition to Jorgenson's study, Lee et al. verified that UHPLC could handle pressures of up to 3600 bar. These proofs of concept led to Waters' 2004 commercialization of the first chromatographic system that could withstand 1000 bar of pressure under the brand name Ultra-performance Liquid Chromatography (UPLC), along with narrow-bore columns filled with 1.7-um fully porous particles (FPPs). "Ultra-high-performance liquid chromatography" or "ultra-high-pressure liquid chromatography" are the chosen terms for the technology's name. Alternatively, a few studies have reported on the use of the term very high-pressure liquid chromatography (VHPLC). [8]

More than 100 supports and more than 10 distinct phase chemistries are available from at least 15 sources, and the number of columns filled with totally porous sub-2-um particles has also increased.[8] Regarding particle size (1.5-2 µm), pressure tolerance (600-1400 bar), pH, and temperature ranges, none of these stationary phases are comparable. These significant technological advancements have led to an exponential increase in the number of applications.[8]

UHPLC was primarily employed to accomplish quick separations in the early days of its commercialization. Analysis time can be reduced by a factor of 9 while maintaining the same kinetic performance by using a UHPLC column of 50 x 2.1 mm I.D., 1.7 µm rather than a standard HPLC column of 150 x 4.6 mm I.D., 5 µm. [8]

Because of the enormous number of samples, this high-throughput feature is appealing in chemical, food, and environmental studies where productivity must be increased. The pharmaceutical industry is another industry that is pushing for quick separations.[8] In applications like quality control, pharmacokinetics, and drug metabolism, increased productivity and lower costs are especially necessary during the drug-discovery and development process. The application of UHPLC has various additional advantages besides high-speed separations. Using a variety of examples, this review aims to examine the current and upcoming trends in this technique. [8]

RP-HPLC method growth involves enhancing chromatographic parameters, selecting suitable stationary and mobile phases, and regulating conditions to achieve accurate parting of analytes. Validation of RP-HPLC methods is crucial, ensuring reliability, consistency, and compliance with International Council for Harmonisation (ICH) guidelines. Key validation parameters include accuracy, precision, specificity, detection and quantitation limits, linearity, robustness, and ruggedness.

The importance of RP-HPLC method validation extends to quality control in pharmaceuticals, minimizing product failures and production costs while enhancing productivity and quality. With well-established protocols, validated methods improve reliability across laboratories, ensuring robust and reproducible results. Overall, RP-HPLC and its validation are instrumental in maintaining high standards in pharmaceutical analysis, enhancing product quality, and achieving efficient and accurate separation and measurement of pharmaceutical compounds.[9]

Principal:

Reversed-phase liquid chromatography (RP-LC) is a type of liquid chromatography where organic molecules are separated using polar mobile phases and non-polar stationary phases. In recent years, the reversed phase mode has been used for the great majority of high-performance liquid chromatography (HPLC) separations and studies. [8] The more hydrophobic the sample components, the longer they remain in the system in the reversed phase state.

The following variables impact solute separation and retention in the reversed phase chromatographic system: a. The stationary phase's chemical makeup, or the ligands that are bonded to its surface, and their bonding density, or how much of the surface they cover.[8]

b. The mobile phase's composition, types of bulk solvents whose mixes alter the mobile phase's polarity, hence the term "modifier" for a solvent added to alter the mobile phase's polarity.[8]

c. Additives, like buffers, alter the mobile phase's pH, which in turn alters the solutes' polarity and ionization state. [8]

Methanol and acetonitrile make up the great bulk of the polar organic solvents that are mixed with water to form the mobile phases. These mixtures typically contain a variety of chemicals, including specific additives (EDTA), surfactants (alkyl amines or alkyl sulfonates), and buffers (acetate, phosphate, and citrate). Increasing efficiency, selectivity, and controlling solute retention are the objectives of employing supplements of any form.[8]

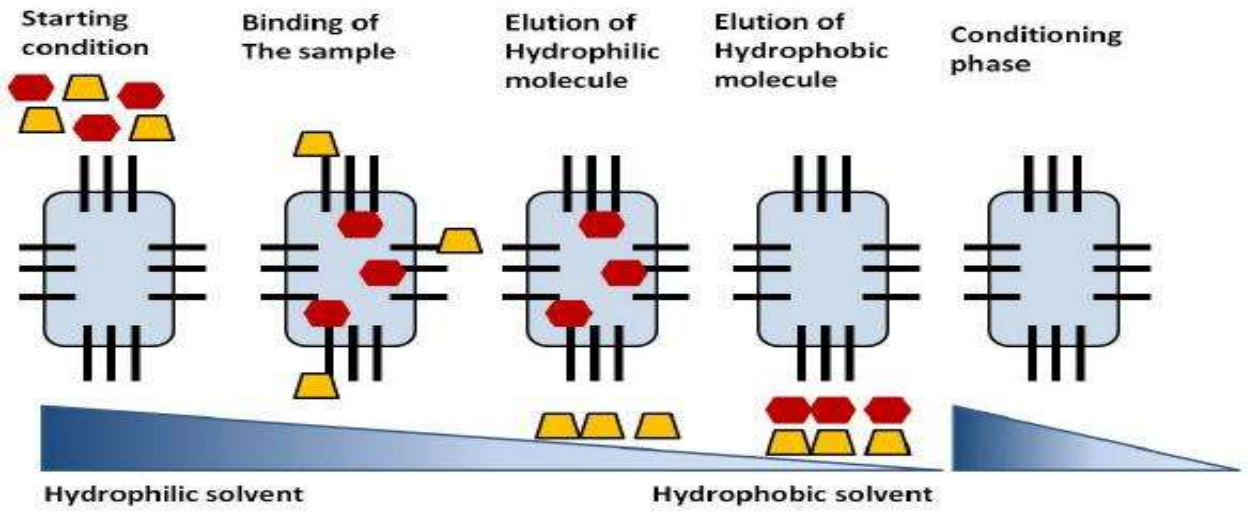


Fig: Steps of reversed phase chromatography.

The RP-HPLC experimental system for the analysis of peptides and proteins usually consists of an n-alkylsilica-based sorbent from which the solutes are eluted with gradients of increasing concentrations of organic solvent such as ace-tonitrile containing an ionic modifier such as trifluoroacetic acid (TFA) (1,2). Complex mixtures of peptides and proteins can be routinely separated and low picomolar-femtomolar amounts of material can be collected for further charac-terization. Separations can be easily manipulated by changing the gradient slope, the operating temperature, the ionic modifier, or the organic solvent composition. The extensive use of RP-HPLC for the purification of peptides, small polypep-tides with molecular weights up to 10,000, and related compounds of pharma-ceutical interest has not been replicated to the same extent for larger polypeptides molecular mass > 10 KDa) and globular proteins. The combination of the traditionally used acidic buffering systems and the hydrophobicity of the n-alkylsilica supports which can result in low mass yields or the loss of biolog-ical activity of larger polypeptides and proteins have often discouraged practi-tioners from using RP-HPLC methods for large-scale protein separations. The loss of enzymatic activity, the formation of multiple peaks for compositionally pure samples, and poor yields of protein can all be attributed to the denaturation of protein solutes during the separation process using RP-HPLC (3-6).[11]

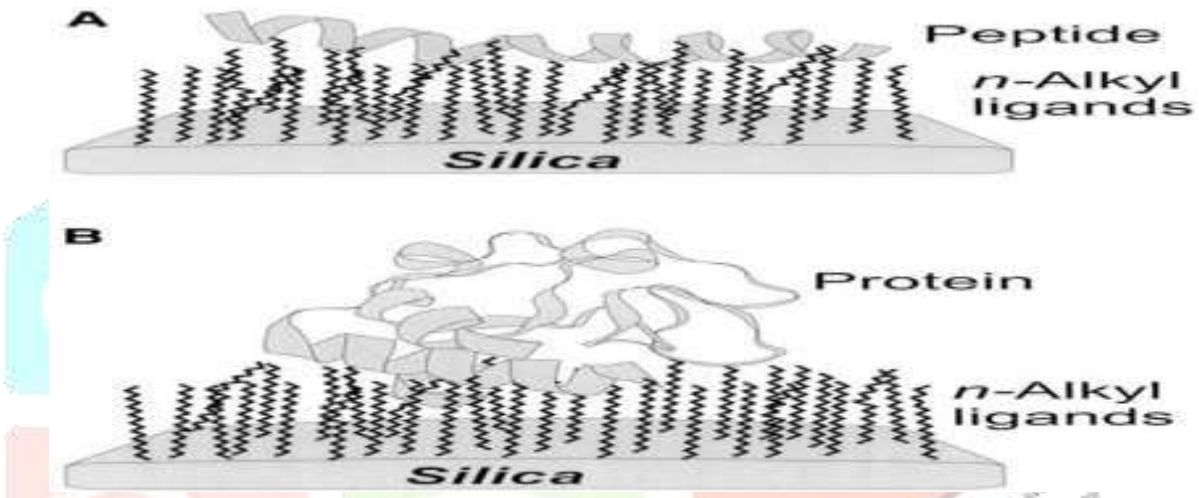


Fig: Schematic representation of the binding of (A) a peptide and (B) a protein, to an RP-HPLC silica-based sorbent. The peptide or protein interacts with the immo-bilized hydrophobic ligands through the hydrophobic chromatographic contact region.

Reversed-Phase Columns:

C8: Octyl Columns.

C18: Octadecyl Columns.

CI: MEBI Columns.

C2: MEB2 Columns.

C3: Propyl Columns.

C4: MEB4 Columns.

C5: Pentyl Columns.

C6: Hexyl Columns. [8]

V. STATIONARY PHASES:

Majors, Dolan, Carr, and Snyder's essay provides a detailed account of the development and history of reversed phase stationary phases. The majority of liquid chromatography runs in the 1970s used solid particles, such as alumina or unaltered silica gel, as the stationary phases. These days, this kind of method is called normal-phase chromatography. [8] Biomolecules with hydrophilic characteristics in the sample firmly adhere to the stationary phase since the stationary phase in this approach is hydrophilic and the mobile phase is non-polar (made up of organic solvents like hexane and heptane). Furthermore, they were difficult to dissolve in the solvents of the mobile phase. [8] Hydrophobic molecules, on the other hand, elute through the polar stationary phase early with insufficient retention because they have less affinity for it. This is the reason why, in order to accept biological chemicals, the silica-based particles were treated with hydrocarbons throughout the 1970s, immobilized or bonded on their surface, and the mobile phases were changed to aqueous and polar in nature. [8]

Silica-based stationary phases:

For a number of reasons, silica gel particles are frequently employed as a stationary phase in high-performance liquid chromatography (HPLC). These include:

1. High surface area: Because silica gel particles have a large surface area, they can interact directly with solutes or, after the bonding of different ligands, they can interact with sample molecules in a number of ways, improving separations.[8]

2. Inertness and chemical and thermal stability: Because silica gel typically does not react with the mobile phase solvents or the substances being separated, it is chemically stable and produces precise, reproducible, and trustworthy analysis.[8]

3. Broad applicability: Silica gel is adaptable and can be altered with different functional groups, which makes it appropriate for a variety of analytes and uses.[8]

4. Effective separation: Silica gel particles' special qualities, such as their large surface area and regulated average particle diameter pore size, enable accurate and effective compound separation in HPLC.[8]

5. Repeatability: High batch-to-batch repeatability is a feature of silica gel particles that is essential for dependable and repeatable HPLC analyses over many years.

6. Control of particle diameter and pore size: It is possible to precisely manage separation depending on molecule size by engineering silica gel to have particular pore diameters.

7. Cost-effectiveness: Because silica is the most plentiful element in the world, its gel is an affordable option for[8]

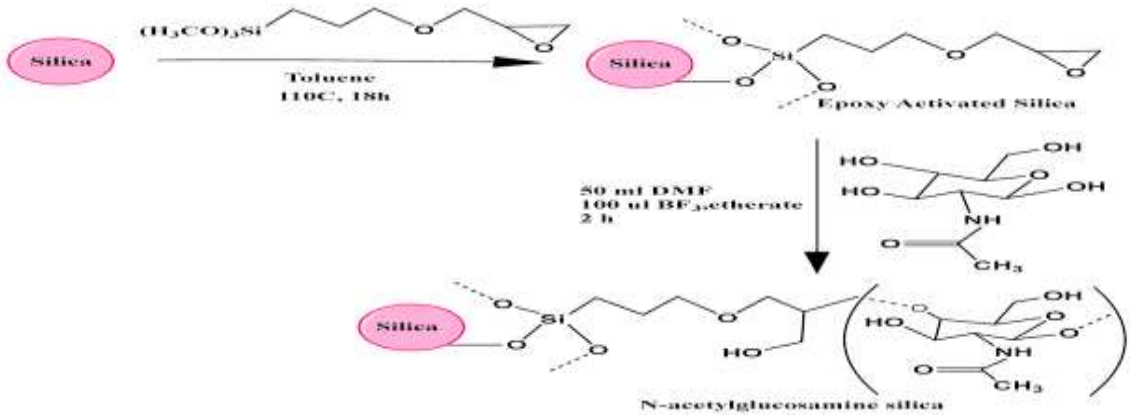


Fig: Silica based stationary phase

MOBILE PHASES:

Boyes and Dong released a thorough paper on the most recent developments and industry best practices for mobile phase selection in reversed-phase chromatography. In reversed-phase chromatography, a mobile phase is made up of aqueous buffers or water mixes to which organic solvents are added in order to selectively elute analytes from a reversed-phase column. Acetonitrile and methanol are the two most often used organic solvents, and they must be miscible with water. Other solvents like ethanol, 2-propanol (isopropyl alcohol), and tetrahydrofuran (THF) can also be utilized. Since the organic solvent is added to the aqueous solution in the mobile phase to change the polarity of the mobile phase, it is also known as a modifier. [8]

Acetonitrile is more transparent than the other solvents in the low UV wavelength range, which makes it the most popular choice for separating molecules with weak or nonexistent chromophores (UV-VIS absorbing groups), like peptides.[8] Acetonitrile offers much lower background absorbance at low wavelengths than the other common solvents, and most peptides only absorb at low wavelengths in the ultra-violet spectrum (usually less than 225 nm). All three solvents are essentially UV transparent, which is key for common reversed phase chromatography because sample components are usually detected by UV detectors.[8]

The pH of the mobile phase can alter the selectivity of some analytes and have a significant impact on analyte retention. Mobile phase buffers can be used to control the ionization of solutes with ionized functional groups, such as amines, carboxyls, phosphates, phosphonates, sulfates, and sulfonates, found in samples. [8]

The retention, selectivity, and resolution of the target analytes can all be impacted by the kind of buffer used, making it a crucial consideration in the creation of the RP-LC technique. There are several things to think about while choosing a buffer for RP-HPLC, such as:[8]

1. The mobile phase's ideal pH is: Selecting a buffer with a pKa that is around the required mobile phase pH is crucial because buffers work best around their pKa value.

2. The buffer's solubility in the organic solvent: The buffer needs to work well with the organic solvent being used in the mobile phase, primarily the typical organic solvents acetonitrile, methanol, and isopropanol that were previously discussed.

3. The buffer's UV cut-off: If UV detection is used, the buffer's UV absorption should be below the wavelength at which the analytes of interest are detected. By doing this, the buffer won't interfere with the analytes' detection. 4. The buffer's compatibility with the detector: The buffer needs to work with the mass spectrometry (MS) apparatus if it is being utilized for detection. Certain buffers, such those that contain phosphate salts, are incompatible with MS detectors because they are not volatile enough and prevent the analytes from being detected by MS by suppressing their ionization. [8]

Applications of RP-UHPLC:

1. Lipid Analysis: RP-UHPLC is used for the separation and identification of lipids in biological samples.

2. Peptide and Protein Analysis: RP-UHPLC is used for the analysis of peptides and proteins, including their identification and quantification. 3. Small Molecule Analysis: RP-UHPLC is used for the analysis of small molecules, such as drugs and metabolites.

4. Enantiomeric Separation: RP-UHPLC can be used for the separation of enantiomers, which are mirror-image molecules [8]

3) Ion - exchange column:

Introduction: The foundation of chromatography is the idea that molecules in a mixture put on a solid surface or into a fluid stationary phase (stable phase) separate from one another as they move with the help of a mobile phase. Molecular characteristics pertaining to adsorption (liquid-solid), partition (liquid-solid), affinity, or variations in their molecular weights are among the elements that have an impact on this separation process.[11] These variations lead certain mixture components to remain longer in the stationary phase and move more slowly throughout the chromatographic system, while other mixture components flow more quickly into the mobile phase and exit the system more quickly, Because pharmaceutical goods must be both safe and effective, the pharmaceutical business is one of the most regulated in the world. Active pharmaceutical ingredients (APIs) impurity and degradation product levels must be closely regulated and adhere to the standards set by global authorities. Chromatography is widely utilized in the pharmaceutical business, from quality control (QC) labs to research and development, and has long been the method of choice for determining the chemical purity of pharmacological ingredients and products [11]. These porous substrates allow for highly customised purification methods that provide product streams that satisfy strict safety, effectiveness, and quality criteria. They are available with a broad variety of ligands, including ion exchange, hydrophobic interaction, affinity, and multimodal. A fluid known as the mobile phase dissolves the mixture and transports it through a structure that contains a different substance known as the stationary phase. The mixture's diverse components separate because they move at different rates. Differential partitioning between the mobile and stationary phases serves as the basis for the separation. The separation is impacted by minute variations in a compound's partition coefficient because they cause variable retention on the stationary phase.[11]

Principal :

For the last 20 years or more, ion chromatography (IC), a well-established analytical technique, has been the method of choice for determining inorganic and tiny organic anions. IC is also used widely for the determination of inorganic cations, but because of the availability of several sensitive, multi-element spectroscopic methods of analysis (especially inductively coupled plasma mass spectrometry ICP/MS), IC assumes less of a dominant role in the field of cation analysis [11], lon exchange extraction (IX) is a particular extraction technique that effectively extracts charged polar solutes (base or acid) from polar media, such as water and less polar solvents. The high-energy electrostatic interaction between the charged functional groups of the analytes and the sorbent is the basis for the separation process in this instance. As a result, the analyte charge determines the choice of sorbent. Primary, secondary, tertiary, and quaternary amines are among the fundamental analytes that are extracted by the cation exchange (CX) column 171. By using the interactions between solute ions and charged sites on the stationary phase, ion exchange chromatography selectively retains or elutes ions according to their electrostatic interactions[11].

Process of separation in chromatography :

The differential movements cause the gradual separation of components over time or space. This process generates a series of distinct zones or peaks as each component exits the system at varying times (in gas or liquid chromatography) or at different locations (as in techniques such as TLC). Factors such as the selection of mobile and stationary phases, temperature, flow velocity, and the physical properties of the components being separated influence the extent of separation [11], AB homeostasis depends on a number of physiological ion exchange activities that are constantly maintained in the kidneys.

Bicarbonate ions (HCO-3) comprise the body's most comprehensive buffer system. Water and carbon dioxide combine to create carbonic acid, which separates into H+ and bicarbonate to refill this system 11, One of the potential options that has been studied is titanium dioxide, or TiO2. According to Marc-hand et al.'s first study, this material may be made from layered potassium titanate K2 Ti4 09 precursor by K+/H+ ion exchange, followed by dehydration [11]. The separation concept is based on differential distribution coefficients between the mobile phase solute and the solid adsorbent stationary phase, resulting in varying retention capacities for both. The adsorption mechanism of the adsorbent material is principally based on chelation and ion exchange interactions with metal ions [11], Proteins, polynucleotides, and other biomacromolecules can interact with ion exchangers because they reveal charged moieties at their surface. A general-purpose and adaptable method for separating proteins and plasmids is ion-exchange chromatography. It's commonly employed for analytical and Preparative purposes 1141, The analytical chemistry community most famously knows Hamish Small as the principal creator of ion chromatography (IC) [11],

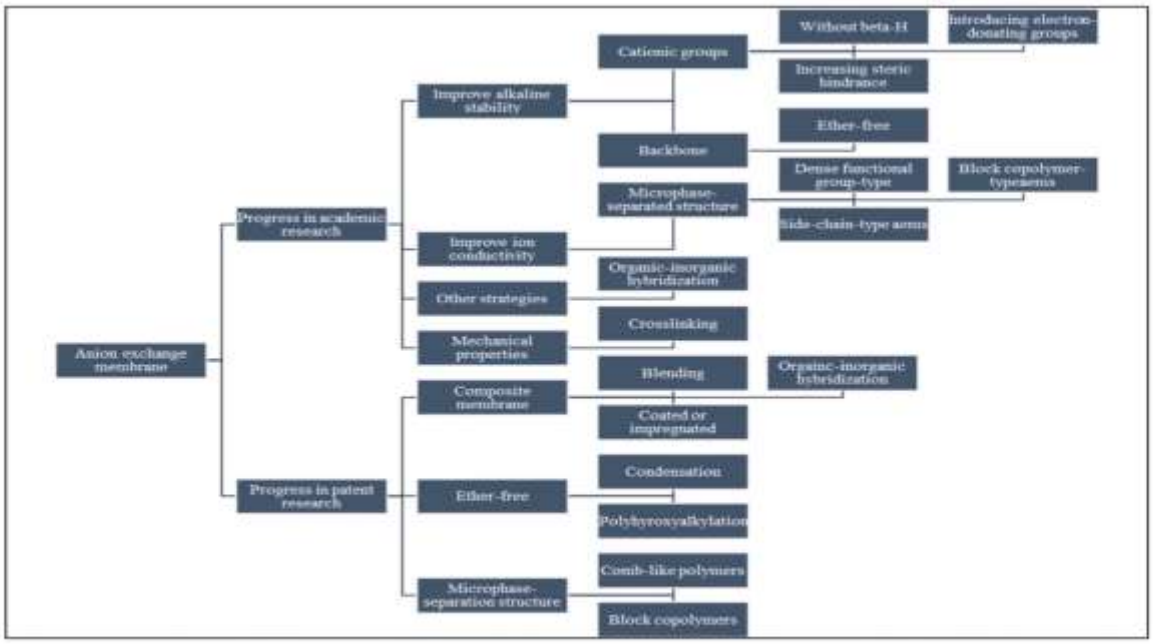


Figure 2 Various strategies and modifications to improve AEM performance, categorized under academic research and patent research. [31]

Ion exchange resins :

To provide a medium for ion exchange, synthetic materials known as ion-exchange polymers (IEPs) include active, electrically charged groups along their backbone. Often referred to as ion-exchange resins, the IEPs are transformed into three-dimensional structures with enhanced structural integrity and resilience when cross-linked by copolymerisation with an appropriate organic linker 1741, An electrically polarised ion exchange resin bed was suggested by Hamish as an ion chromatography suppressor. Because of the "small" size of the ion exchange resin bed, electrochemical regeneration would take place in between each study (or many analyses).[11] The suppressor for anion analysis with sodium hydroxide as the eluent was made comprised of two porous platinum electrodes sandwiched by a little layer of completely sulfonated cation exchange resin. Following investigation of the suppressor,

the sodium form of the cation exchange resin is the predominant form. The compressor is polarised and water is pushed in the opposite direction for regeneration (11)

Ion-exchange membrane :

lon-exchange membranes are frequently used in separation procedures and contemporary electrochemical technologies. An examination of electro mass transfer is necessary for the creation of new materials. The primary focus of this study is on macroscopic transport mechanisms. To understand the membrane selectivity process, however, ion and molecule translation microscopic mobilities must be examined 1251, Target ions are selectively exchanged via ion-exchange membranes (IEMs), which are made up of oppositely charged ions and immobilised ionisable functional groups. IEMs are usually made up of exchangeable counter-ions, immobilised charge groups, and inert substrates. IEM are often divided into two categories based on the kind of immobilised charge groups they contain: i) Cation Exchange Membranes (CEMs) & ii) Anion-Exchange Membranes (AEMS) (26),

Anion exchange membrane :

Depending on the kind of analytes, the mobile phase's composition, and the properties of the analytical support, partitioning, adsorption, and ion exchange may be responsible for the retention mechanism in aqueous organic mobile phases on bare silica columns (30),

Althrough examination of AEM that covers everything from molecular engineering to in-situ performance evaluation, mostly based on the research paths indicated by the structure of already accessible patents, to outline the future development of AEM while considering the current challenges (Figure 2)

Cation ion exchange :

A new pH-based protein separation method using cation exchange chromatography (pH-IEC) that resistant against changes in sample matrix pH and salt content, multi-product, and high-resolution [11],

Matrix effects in ionisation :

The reversible adsorption of charged molecules to immobilised ion groups on a matrix with an opposing charge is known as ion exchange chromatography. Adsorption and sample release from the matrix allow for selective separation. Ion strength and pH are used to equilibrate the exchanger before ion exchange begins.[11] The exchangeable groups are linked to counter-ions during equilibrium. After the sample is introduced and equilibrium is achieved, the molecules experience adsorption and addition with the counter ions are displaced and reversibly bound to the matrix by a suitable charge [11],

Principle of separation :

The fundamental premise of chromatography is that fluid stationary phases (stable phases) and molecular mixtures applied to surfaces or solids separate from one another as they move with the aid of a mobile phase. This separation process is influenced by the molecular characteristics related to affinity, partitioning, adsorption, and changes in their molecular weights. Due to these variances, certain mixture components reach the mobile phase and leave the chromatographic system faster than others. Additionally, some combination components pass through the system more slowly and remain in the stationary phase for longer [11], Thus, three elements form the basis of the chromatography process. A "solid" phase or "a layer of a liquid adsorbed on the surface of a solid support" always makes up the stationary phase[11].

It is the nature of the counterions displaced from the matrix functional groups which determines the IEC format. Thus, with anion-exchange chromatography (LHS), the stationary phase matrix displays a

positively charged functional group with a negative counterion that can be displaced by an anionic sample, thereby enabling matrix adsorption.[11] Cation-exchange chromatography (RHS), with the stationary phase matrix displays a negatively charged functional group with a positive counterion that can be displaced by a cationic sample, again enabling matrix [11]

adsorption with either format, sample desorption from the matrix can concentration within the mobile phase [11]

Ion Exchange Chromatography is based on the ionic interactions between charged particles. The basic elements are:

Stationary phase: A "solid" phase or "a layer of a liquid adsorbed on the surface of a solid support" always makes up the stationary phase. A polymeric resin matrix on the surface of ionic functional groups, such as carboxylic acids and quaternary amines, that have undergone chemical bonding makes up the stationary phase in ion exchange chromatography [11]

Mobile phase: "Liquid" or "gaseous component" are always present in this phase. Ionic solutes are held in place while the mobile phases move over these surfaces by the formation of electrostatic chemical interactions with functional groups. In these kinds, the mobile phase is always liquid (37), The sample is carried across the column by a buffer solution. The ion exchange mechanism and component separation are influenced by the buffer's composition and pH [11]

Ion-Exchange Mechanism: lons use their charge to interact with the stationary phase. lons with a higher affinity for the stationary phase will bind more tightly and elute through the column more slowly, whereas those with a lower affinity will elute more quickly [11]

Elution: Elutes the bound ions out of the column through a change in the composition or concentration of the mobile phase, often as a gradient or stepwise increase in ionic strength[11]

Procedure: When the sample is placed into the column, its ions interact with the oppositely charged groups on the stationary phase. Components are categorised into many groups based on their charge and level of interaction with the ion exchanger. Elution can be achieved by changing the ionic strength or pH of the mobile phase[11]

Application of ion exchange chromatography: [11]

Chromatography is used in many different industries, most notably in the pharmaceutical sector. It is used to classify compounds according to molecular weight and element amount, identify and analyse samples for trace elements, and assess the purity of unknown chemicals. Chromatography is also essential in the production of pharmaceuticals; high-pH anion-exchange chromatography is used for carbohydrates and oligosaccharides, while gas chromatography is used for the separation of volatile mixtures. It may also be used to find contaminants in the pharmaceutical sector [11], The purification of charged proteins and nucleic acids (biomolecules) is a typical use for IEC. Additionally, it is employed in the filtration of water and the process of inorganic ion separation [11],

Many charged or ionisable substances, such as proteins, peptides, enzymes, nucleotides, DNA, antibiotics, vitamins, and more, may be separated and purified using ion exchange chromatography from both natural and artificial sources.[11]

Here is an example of how ion exchange chromatography is used:

Actinide separation: The ion exchange chromatographic technique was initially used to identify the trans plutonium elements of the actinide class.[11] Actinide contraction causes the actinide series to

display elution in the opposite order of the atomic number since some of these elements could only be produced in atom amounts. This turned out to be the only way to find these components. It may be argued that their solitude would not have been possible with any other type of separation.[11]

Organic compound purification: It has been discovered that ions that were initially present in the water have polluted many natural goods that were recovered from it. Such ions can be eliminated via ion-exchange processes.[11]

Pure Reagent Preparation: When using sodium hydroxide solutions for volumetric calculations, carbonate is always present. Errors in acid-base titrations result from this. Running the solutions down a column of a strongly basic anion exchange resin in the hydroxide state is the most straight forward technique for eliminating carbonate. An equivalent amount of hydroxide is produced as the carbonate is absorbed.[11]

4) Size exclusion column:

Introduction:

Size Exclusion Chromatography (SEC) (also known as gel permeation chromatography (GPC)) is a liquid chromatographic technique in which analytes are separated according to their hydrodynamic volume. The technique was invented in the early 1960's [1] and since then it has proven to be a powerful technique for analysis and purification of large molar mass analytes. First reports about SEC used polysaccharide gels [1] (e.g. agarose and dextran gels) and slightly crosslinked polyacrylamide [2] as stationary phases. Whilst these materials were (and still are) applicable for the separation of biopolymers under aqueous conditions[14]

their low chemical and mechanical stability limited their applica-tion at high pressures as well as the use of organic mobile phases. (e.g. THF: DMAC) which are required for the separation of many synthetic polymers. This limitation was addressed by scientists from DOW with the introduction of mechanically stable poly-styrene (poly(Sty)) particles for the separation of synthetic poly-mers [3,4]. It was not long before the technique was extended to methacrylate [5] particles which were used for the separation of water-soluble macromolecules.[14]

Since the size of macromolecules provides information about the chemical, physical, structural and biological characteristics of natural and synthetic macromolecules, SEC is a routine technique in industry, clinical analysis and academic research with applications in the following areas[14]

Synthetic polymers: they represent a very wide group of mate-rials such as plastics, fabrics, coatings, additives and many products used in industry and academic field. Molar mass value is as important as its distribution in a synthetic process as it defines the physico-chemical properties of the product, and SEC allows the determination of both [14]

Given the complexity of protein and peptide-based parenteral therapies, a broad set of complementary techniques are required to moni-tor the critical quality attribute as of intermediate drug substances and drug products. [1.2] As outlined in regulatory agency guidelines, one of these attributes is a quantitative assessment of the aggregation, including dimers and multimers, of the active protein. While numerous techniques have been developed to monitor protein aggregation, size-exclusion chromato-graphy (SEC) has been predominantly favored for routine and validated analyses because of both its speed and reproducibility. [3-6] SEC is also an accurate method if confirmed with an orthogonal method, such as sedimentation velocity analytical ultracentrifugation (SV-AUC). [7-9] The intent of this review is to provide a summary of SEC, including background, theory, and applications with a primary focus on the analysis of peptide and protein aggregates.

Since the early introduction of biologic-based therapeutics, the presence of protein aggregates has been theorized to compromise safety and efficacy. [10] These concerns, which date to the 1980s, have led to routine analysis and quantitation of dimers, trimers, and higher order aggregates for a wide variety of biologic-based therapies, such as insulin, [3-6] recombinant human growth hormone (rGH), [11.12] ar [11.12] and monoclonal antibodies, 18,13,14] Aggregate analyses are typically performed throughout the entire product lifecycle of biotherapies. (8) However, each stage of development may have different assay requirements including robustness, sensitivity, ease of use, and high through-put. These desired attributes have led to a wide variety of techniques for the analytical characterization of biotherapies based on the size of the biomolecules. [8] Commonly used techniques include SV-AUC, [15,16] asymmetric flow field flow fractionation (AF4), [16-18] multi-angle light scattering (MALS), (12.19.20] and SEC. While all of these techniques are frequently used, the dominant method continues to be SEC. [9] [15]

Principal:

Size exclusion chromatography (SEC) is that the separation of mixtures supported the molecular size (more correctly, their hydrodynamic volume) of the components. Separation is achieved by the differential exclusion or inclusion of solutes as they undergo stationary phase consisting of heteroporous (pores of various sizes) cross linked polymeric gels or beads. the method is predicated upon different permeation rates of every solute molecule into the inside of gel particles. Size exclusion chromatography involves smooth interaction with the sample, enabling high retention of biomolecular activity. For the separation of biomolecules in aqueous systems, SEC is mentioned as gel filtration chromatography (GFC), while the separation of organic polymers in non-aqueous systems is named gel permeation chromatography (GPC).(1)

the essential principle of size exclusion chromatography is sort of simple. A column of gel particles or porous matrix is in equilibrium with an appropriate mobile phase for the molecules to be separated. Large molecules are completely excluded from the pores will undergo the space in between the gel particles or matrix and can come first within the effluent. Smaller molecules will get distributed in between the mobile phase of in and out of doors the molecular sieve and can then pass through the column at a slower rate, hence appear later in effluent.[16]

Mechanism of Size Exclusion Chromatography:

Size exclusion also referred to as gel filtration chromatography may be a case of liquid-liquid partition chromatography, during which the solute molecules are get distributed in between two liquid phases, (i) liquid within the gel pores and (ii) liquid outside the gel.

The size exclusion might be explained by Steric Exclusion Mechanism. because the gel particles contain range of pore sizes, small molecules can enter in sizable amount of pores while the massive molecules. will get small number of pores into which they will enter. Thus the various fractions of total pore volume are accessible to molecules of various sizes. Thus, molecules with different sizes will differ in distribution coefficient between these two liquid phase, because the small molecules can enter in additional pores while larger molecules can enter in pores only larger than the molecular size.

the whole volume (Vt) of a column full of a gel that has been swelled by solvent is given by,

$$Vt=Vg+VI+ Vo$$

Where Vg is that the volume occupied by the solid matrix of gel, Vi is that the volume of solvent held within the pores or interstices and Vo is that the free volume outside the gel particles. When mixing or diffusion occurs, the diffusion equilibrium and therefore the retention volume (VR) of the given species is given by

$$VR=V(int.) + Kd\ V(int.)$$

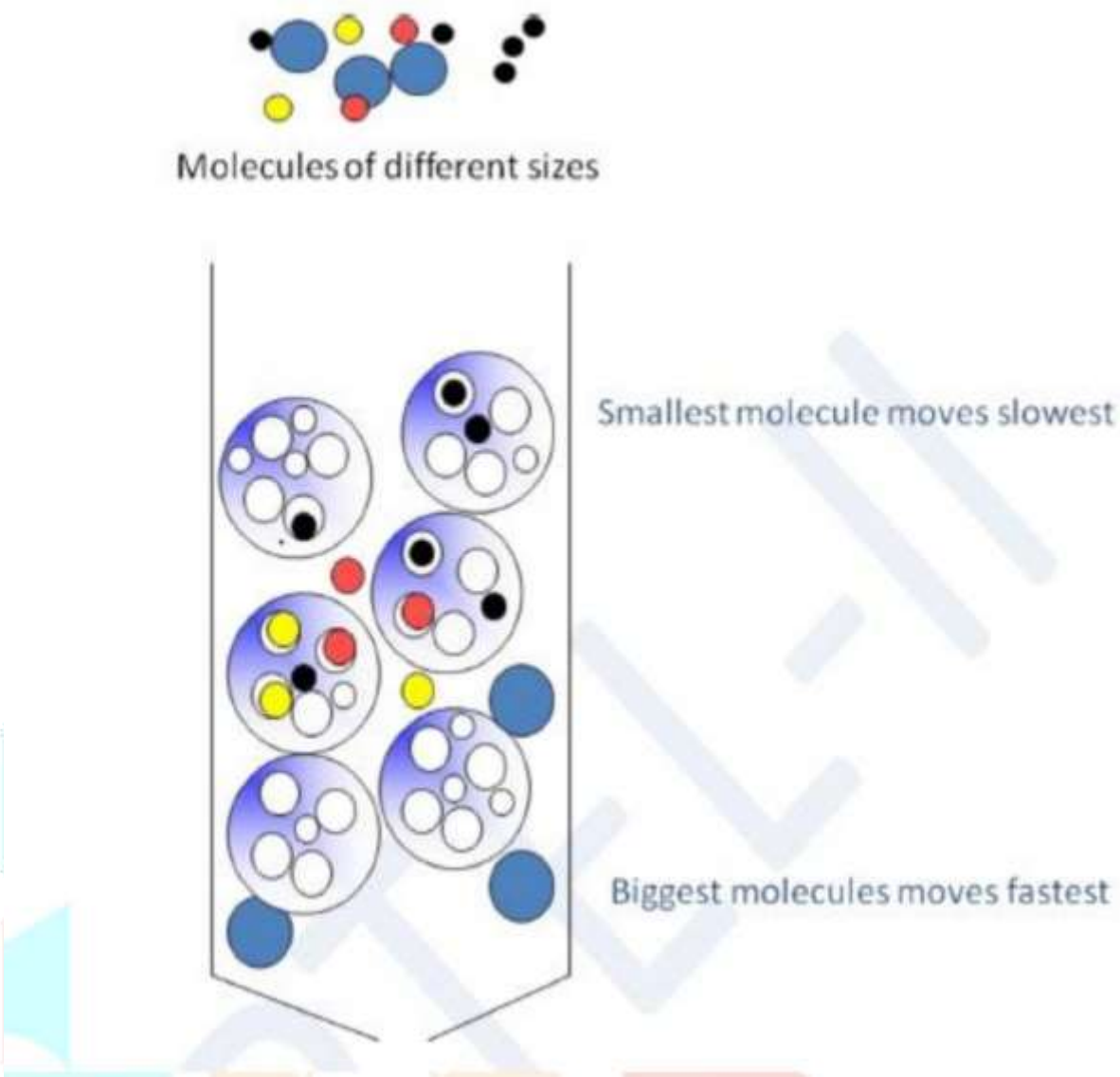


Fig: Theory of Size exclusion chromatography

Stationary phases for SEC:

Polymer particles:

The preferred method for the manufacture of porous polymer particles is suspension polymerization. The approach was invented at the beginning of the 20th century and it is a heterogeneous polymerization process (Fig. 6A) [69]. In suspension polymeriza-tion, droplets of monomers (mono- and di-vinyl monomers having a limited solubility in water) are dispersed in an aqueous solution containing a suspension stabilizer (eg. poly(vinylpyrrolidone), PVP) while mechanically stirring. The suspended monomeric phase contains also a dissolved radical initiator (e.g. azobis-isobutyronitrile, AIBN) and porogenic solvents (also known as porogens or organic diluents) which are essential to obtain a porous material. A dispersion of the droplets inside the continuous phase can form and further copolymerization with a crosslinker results in the for-mation of beads with different appearances such as glassy, opaque, or milky [12]

Different polymerization parameters can affect the porous properties (specific surface area, pore volume and pore size dis-tribution) of the polymer beads. Among them, it can be included polymerization time and temperature, type and concentration of porogen, crosslinker concentration, dispersed/continuous phase ratio, stirring speed, etc. The specific mechanism under which each. reaction variable affects the

porous skeleton of the materials is out of the scope of this review and the reader is suggested to look at the excellent material in the literature dedicated to this topic [12].

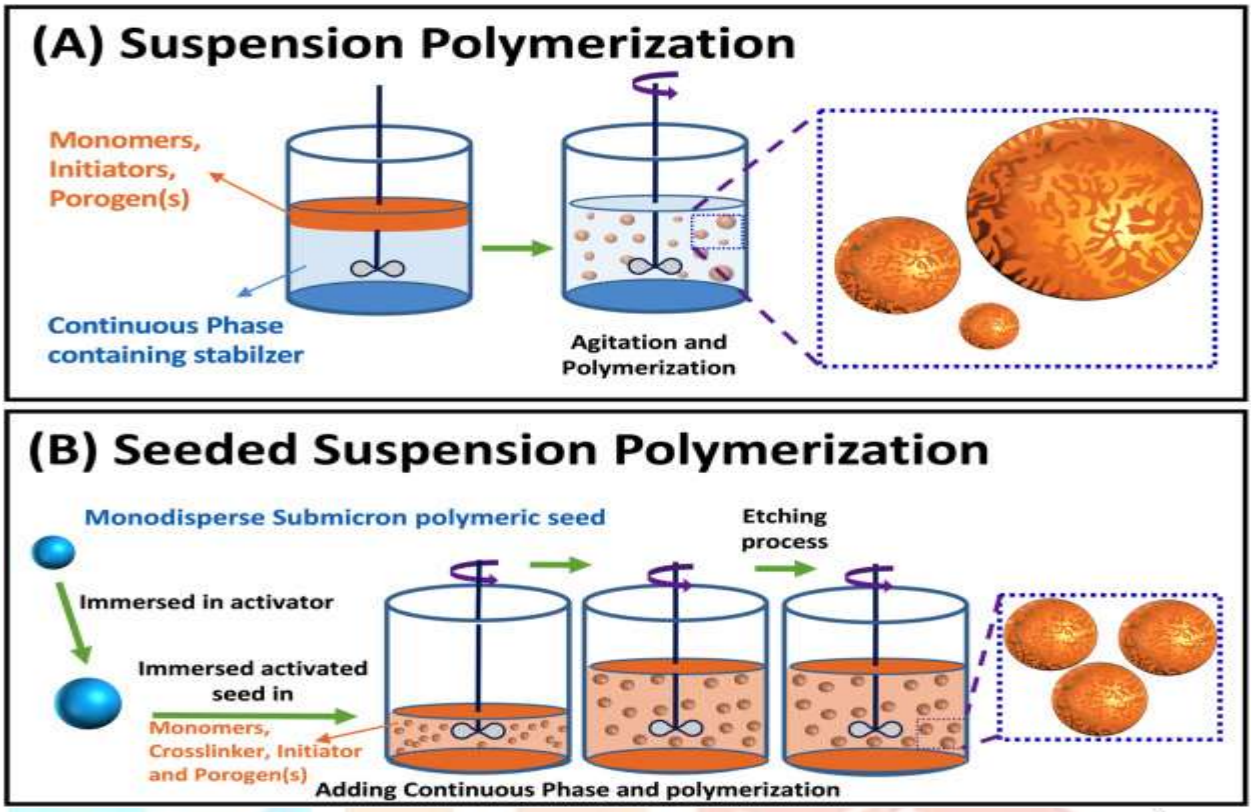


Fig: Schematic of typical synthesis methods for the fabrication of porous polymer particles for SEC.

Use of polymer particles in size exclusion chromatography

Since SEC is a size-based separation, non-specific interactions between the stationary phase and the polymers that are separated should be avoided. In this sense, hydrolyzed poly(GMA-co-EDMA) beads prepared by suspension polymerization were used success-fully for the separations of proteins and poly(Sty) standards (Fig. 8). It is noteworthy that the same column could be used for both hydrophobic and hydrophilic analytes as the poly(GMA-co-EDMA) hydrolysis would increase the polarity of the stationary phase.[12]

Interactions between the analytes and the stationary phase could be due to ion exchange, hydrophobic partitioning, hydrogen bonding or bioaffinity and has been mainly reported for silica sta-tionary phases as the superficial silanols can be responsible for weak cation exchange. These types of interactions can cause shifts in elution times, peak asymmetry (column overloading can also promote peak asymmetry) and changes in the three-dimensional conformation of some analytes such as proteins. leading to an error in the molar mass determination from the Analytica Chimica Acta 1151 (2021) 338244.[12]

calibration curve. When both the analyte and the stationary phase are charged, electrostatic interactions can cause either ion ex-change (when charges are opposite) which delays the elution time of the analyte, or ion exclusion (for same sign charges) which promotes an earlier elution of the analyte. Increasing the ionic strength from the mobile phase can be used to minimize electro-static interactions between the analyte and the stationary phase, but a high salt concentration in the mobile phase can promote hydrophobic interactions leading to analyte retention [12]. For proteins analysis, arginine is commonly used as a mobile phase additive to avoid secondary interactions with the stationary phase. [12]

As discussed previously, the pore size distribution of macro-porous network particles prepared by suspension polymerization can be affected by a careful selection of the reaction parameters. As an

example, the composition of the porogenic mixture was used for the preparation of poly(GMA-co-EDMA) particles with narrower pore size distribution. These porous characteristics. approached those observed for inorganic-based SEC sorbents and this could be beneficial for the separation of biomolecules with narrower molar mass distributions (e.g. globular proteins). How-ever, separation of these biomolecules using the sorbents mentioned above were not demonstrated.[12]

Monoliths: promising materials for SEC columns:

Monoliths are porous materials that can be synthesized as a single piece. As they consist of a continuous porous road, monoliths are alternative materials for SEC columns because they show some advantages over the traditional particulate stationary phases.[12]

In a monolith, the interconnected porous structure promotes high permeabilities, allowing the use of high flow rates with low back pressures on the system. Moreover, the stationary phase structure with flowthrough pores, changes the contribution of mass transfer resistance to the efficiency on the chromatographic system. The characteristics of the mass transfer resistance for silica and organic monoliths are mentioned in sections 3.3.1 and 3.3.2.

According to its chemical composition, monoliths can be organic or inorganic (mainly based on silica), and each has distinctive properties. Table 1 summarizes the main characteristics of organic and inorganic monoliths.[12]

Experimental conditions:

1. Mobile phase and Temperature:

The mobile phase should be completely dissolving the polymer sample during a continuous solution phase, it must be low in viscosity for the SEC system to work in normal pressure range, and it must effectively prevent the polymer molecules from interacting with the stationary phase. Temperature may be a useful parameter to regulate when one or more of those conditions haven't been met but where one is constrained to use an exact mobile phase. Some polymers like polyesters, and polylefins may dissolution only at elevated temperatures. The viscosity of inherently viscous mobile phase might be lowered by raising the temperature.

2. Stationary Phases:

When selecting an optimum stationary phase there are additional criteria to be met; the packing shouldn't interact chemically with the solute i.e. sample it must be completely wetted by the mobile phase but shouldn't suffer adverse swelling effects, it must be stable at the specified operating temperature, and it must have sufficient pore volume and an adequate range of sizes to resolve the sample relative molecular mass distribution. For the high performance SEC, either semi rigid polymeric gels or modified, rigid silica particles are used. The pore size should be in range of 60 to 4000 A°. High performance packing materials have particle size within the range of 5 to 10 µm with efficiencies of several thousand theoretical plates per 15cm column.

3. Sample Size and Mobile Phase flow:

Sample size is defined by both the quantity of the aliquot injected also as by the concentration of the sample solution. the utilization of excessively large sample volumes can cause significant band broadening, leading to loss of resolution and errors in relative molecular mass measurement. The optimum injection volume is going to be function of the dimensions and number of the columns but are going to be range between 25 to 200µL. The flow 1 mL/min are most ordinarily employed for Sets of SEC column and for the only column separation, a flow of 0.5 ml/min is employed.

4. Detectors:

the foremost common sort of universal detectors far and away is that the differential index of refraction (DRI) detector. The differences in index of refraction between a moving sample containing stream and static reference of mobile phase employing a split optical cell. It responds well at a moderate concentration level to most polymeric samples provide that they're different in index of refraction from the mobile introduce which they're dissolved. Despite temperature independence of the SEC separation phenomenon, the DRI is very temperature sensitive as a results of the strong temperature dependence of index of refraction. Thus one normally maintains the DRI during a constant temperature oven together with the columns and injector. The temperature is in any case 5-10°C above ambient.

Other common sorts of concentration detectors are the ultraviolet (UV) and infrared (IR) detectors.IR detector is slightly more sensitive than the DRI detector while the UV detector is several orders of magnitude more sensitive.[18]

Mobile Phase Modifiers:

Numerous studies have evaluated the addition of of organic modifiers or other additives, such as arginine [11.105) to mitigate these secondary inter-actions. These additives are often used to aid in protein recovery. The reduced recovery of aggregates in SEC chromatography is an area of wide concern. [14.105] One strategy often used is the addition of arginine to the mobile phase to reduce secondary interactions. [11.101] Arginine acts as a binder to the analyte in solution, thus preventing it from interacting with the stationary phase. Arakawa analyzed the effect of arginine on protein aggregate quantitation and found an increase in aggregate recovery when arginine was added to the mobile phase. [101] Other studies have also found improvement in peak shape with the use of arginine as a mobile phase additive. [11] Methods using arginine in the mobile phase have been developed for both large biomolecules and small proteins, such as insulin.

Mobile Phase pH:

Mobile phase pH can also be manipulated to reduce secondary interac-tions, (63,65) Varying pH of the mobile phase can perturb the three dimen-sional conformation of the protein, resulting in changes in non-ideal interactions with the stationary phase. These interactions can be predicted based on the relationship between mobile phase pH and the isoelectric point of the protein. Golovchenko et al. [65] demonstrated that at low ionic strengths, ion exchange effects were observed at pH values below the pl of the protein, while ion-exclusion effects were observed at pH values above the isoelectric point of the protein, [65][19]

Applications:

Size exclusion chromatography has been used with great success within the separation of the sugars, polypeptides, protein, liquids, butyl rubbers, polystyrenes, silicon polymers, etc. Size exclusion chromatography (SEC) has been mainly applied to studies of complex, biochemical or highly polymerized molecules.

The main application of SEC is that the separation and characterization of molecules of various relative molecular mass. It become possible to separate molecules of comparable molecular weights by proper selection of the acceptable gel and column length. Another important application is that the separation of huge molecules of the biological origin from inorganic and ionizable species.

The main application of the dimensions exclusion chromatography are as follows:

1. Purification:

The main application of exclusion chromatography is within the purification of biological macromolecules. Viruses, enzymes, hormones, antibodies, nucleic acids and polysaccharides have all been separated and purified by the utilization of appropriate gels or glass granules

2. relative molecular mass determination:

The effluent volumes of globular proteins are largely determined by their relative molecular mass it's been shown that over a substantial relative molecular mass effluent volume is approximately a linear function of the logarithm of the relative molecular mass.

3. Solution concentration:

Solution of high relative molecular mass substances are often concentrated by the addition of dry sephaex G-25 (coarse). Water and low relative molecular mass substance remain in solution. After ten min. the gel is removed by centrifugation, leaving the high molecular material during a solution whose concentration has increased but whose pH and ionic strength are unaltered.

4. Desalting:

By use of a column of sephadex G-25, solution of high relative molecular mass compounds might be desalted. The high relative molecular mass substance moves with the void volume while lower relative molecular mass components are distributed between the mobile phase and crawl.

5. Protein building studies:

Size exclusion chromatography is use to review the reversible binding of aligand to a macromolecules like protein including receptor proteins.[20]

5) Chiral column:

Introduction:

Enantiomers [1] are non-superimposable mirror images of a chiral molecule having similar physical and chemical properties. A large number of drugs, such as B-blockers (metoprolol) [2], antibiotics (D-cycloserin) [3], statins, anticoagulants, anti-inflammatory agents are marketed as racemic mixtures (racemate) having equimolar amounts of Rand S enantiomers of a chiral molecule. These enantiomers present a difference in pharmacological activity or efficacy. Each enantiomer of a chiral molecule can act uniquely, exhibiting different plasma concentration, bioavailability, and toxicology [4]. Therefore, it is of paramount significance to analyze drugs with a chiral center for different bioactivities and to determine the concentration of each enantiomer in biological fluids. However, the similarity in the physical and chemical properties creates a challenge in the separation of enantiomers [5, 6]. Separations can, therefore, be achieved by reacting the sample with a chiral compound or a chiral stationary phase to form diastereomers with different physical and chemical

properties. The United States Food and Drug Administration (USFDA) requires the evaluation of enantiomers as well as the racemic mixtures of a chiral drug before clinical use.

gas Enantiomeric separation can be accomplished by chiral chromatography. Chiral chromatography includes the use of chromatography (GC), supercritical fluid chromatography (SFC), and high-performance liquid chromatography (HPLC). However, chiral HPLC is the most widely used of these methods [4, 7]. Enantioselective determination can also be carried out using hyphenated techniques (LC-MS/MS) [2, 8, 9], micellar electrokinetic chromatography [10, 11] and capillary electrophoresis (CE), the latter being a powerful alternative to chromatographic techniques. Several chiral separation principles successfully applied in HPLC have been transferred to CE due to the advantages of CE employing small amounts of chiral selector and solvents [12]. This article provides an insight into the various chiral stationary phases used in chiral chromatography along with the mechanism of separation and applications.

Need for chiral separation:

The primary goal of chiral separation is to overcome the challenge of similarity in the physical and chemical properties of enantiomers by forming diastereomers which possess different physical and chemical properties. Pharmacokinetic and chiral inversion studies of enantiomers become necessary as pharmacokinetics of enantiomers may be stereoselective and chiral inversions may occur in-vivo or in-vitro [8].

Principle of chiral chromatography:

The ability of the CSP to interact differently with two enantiomers, leading to their HPLC separation is known as chiral recognition. Chiral recognition depends on different interactions such as hydrogen bonding, - interaction, dipole stacking, inclusion complexation, steric bulk between the analyte and the CSP, hydrophobic and electrostatic interaction, charge-transfer interactions, ionic interactions to form transient-diastereomeric complexes.

Chiral separations by HPLC can be achieved by:

i. The direct method, involving the use of a chiral stationary phase (CSP) or a chiral mobile phase additive.

ii The indirect method, involving the use of a chiral derivatizing agent.

1) Direct Separations:

The direct method of separation involves the actual chromatographic separation of molecules that are enantiomerically related to each other [7]. Here, the stationary phase consists of a chiral substance chemically bonded to stationary phase support to form a chiral stationary phase (CSP) [7]. Separation involves an interaction between the CSP and the racemic drugs to form diastereomeric complexes. One of these complexes will have a stronger binding strength than the other, resulting in different retention times for the enantiomeric pair.

Another type of direct method uses a chiral mobile-phase additive (CMPA), which forms a transient diastereomeric complex with the analyte. The resolution of these diastereomeric complexes is then possible by HPLC [7]. This approach is not used due to difficulties such as the requirement for a continuous supply of CMPA, difficulties in detection and poorly shaped peaks. The chiral mobile phase approach represents a simple and flexible alternative, which is, however, not always applicable [13]. The mobile phase which contains the chiral selector cannot be reused.

II) Indirect Separations:

The indirect method is based on chiral derivatization, which involves the reaction of the enantiomers with a chiral derivatizing agent (CAD) to form diastereomeric

derivatives differing in their physical and chemical properties. These diastereomers are then separated on an achiral stationary phase. Chiral derivatization reagents used in indirect methods are chiral Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide), Sanger's reagent (1-Fluoro-2,4-dinitrobenzene), and o-phthaldehyde with N-acetyl-L-Cysteine. This approach circumvents the need for expensive columns with chiral stationary phases and is more flexible; however, the disadvantage of this approach is the additional step which can involve undesirable side reactions, the formation of decomposition products and racemization. Additionally, the chiral derivatization reagent should possess high enantiomeric purity and have derivatizable groups in the analyte [13].[21]

TYPES OF CHIRAL STATIONARY PHASES:

1. Pirkle type (Brush type)

2. Protein-based

3. Cyclodextrin based

4. Polymer-based carbohydrates (polysaccharide-based CSPs)

5. Ligand exchange

6. Macrocyclic antibiotic

7. Chiral crown ethers.

8. Miscellaneous

1.Pirkle type (Donor-Acceptor Columns):

Pirkle type chiral stationary phases are also called as brush type. Separation on these CSPs is based on a three-point attachment between the solute and the CSP. These interactions may be attractive or repulsive in nature. Pirkle columns discriminate enantiomers by binding of one enantiomer with the chiral stationary phase, thereby forming a diastereomeric complex through - bonding, hydrogen bonding, steric interactions, and/or dipole stacking [7]. Pirkle CSP can be categorized into three classes:

(i) π-electron acceptor

(ii) π-electron donor and

(iii) n-electron donor-z-electron acceptor.

Table 1: Some commercially available pirkle-type columns

Trade Name	Bonding	Class
β-Gem 1	N-3,5-dinitrobenzoyl-3-amino-3phenyl-2-(1,1-dimethylethyl)-propanoate	π-electron acceptor
α-Burke 2	Dimethyl N-3,5-dinitro-benzoyl-α-amino-2,2-dimethyl-4-pentyl phosphonate	π-electron acceptor
Leucine	3,5-Dinitrobenzoyl leucine	π-electron acceptor
Whelk-O 1	1-(3,5-dinitrobenzamido)-1,2,3,4- tetrahydrophenanthrene	π-electron acceptor/donor
Phenylglycine	3,5-Dinitrobenzoyl phenylglycine	π-electron acceptor
ULMO	3,5-Dinitrobenzoyl derivative of diphenylethylenediamine	π-electron acceptor/donor
DACH-DNB	3,5-Dinitrobenzoyl derivative of 1,2-diaminocyclohexane	π-electron acceptor
Pirkle 1-J	3-(3,5-Dinitrobenzamido)-4-phenyl-β-lactam	π-electron acceptor

2.Protein-based chiral stationary phases:

While many protein chiral stationary phases have been proposed, six materials with somewhat different characteristics now have been commercialized in columns for chiral separations: bovine and human serum albumin, al- acid glycoprotein (orosomucoid), ovomucoid, cellobiohydrolase (cellulase), and pepsin [7]. The mechanism of chiral interaction between the protein and the analyte involves hydrophobic and electrostatic interactions, hydrogen bonding and charge-transfer interactions may also contribute to chiral recognition. Hydrophobic interactions between the protein and the analyte are affected by percent organic in the mobile phase. As the organic content increases, retention on protein-

based columns decreases. Table2 shows some commercially available protein-based columns.[22]

Table 2: Some commercially available protein-based columns

Trade Name	Chiral Selector
Chiral-AGP	α1- acid glycoprotein (AGP)
Chiral HAS	Human serum albumin (HSA)
Chiral CBH	Cellobiohydrolase (CBH)
Ultron ES-OVM	Ovomucoid
Ultron ES-Pepsin	Pepsin
Resolvosil BSA-7	Bovine serum albumin

3.Polysaccharide chiral stationary phases:

The naturally occurring polysaccharide form the basis for an important group of columns designed for chiral separations [7]. The main polysaccharides are cellulose, amylose, chitosan, dextran, xylan, curdlan, and inulin [16]. However, due to their low-resolution capacities and handling problems, they cannot be used as such and hence, their derivatives have been synthesized [17]..

Polysaccharide-based columns have a high loading capacity and can be used for the separation of a wide range of compounds. Microcrystalline cellulose triacetate (MCT), a product of the heterogenous acetylation of microcrystalline cellulose particles has been one of the first useful column packings developed for chiral HPLC separation [7]. These columns have lower efficiency and therefore are not widely used.

Derivatives of cellulose and amylose exhibit excellent properties for the separation of chiral molecules. The former has a rigid linear structure with a B-(1.4)-D-glucose linkage while the latter has a helical structure with an a-(1,4)-D-glucose linkage. The glucose units have a chair conformation with 2-OH, 3-OH, and 5-CH-OH groups all in the equatorial position [18, 19). The different glucose linkages give rise to the different higher-order structures of these polymers in which the polymers are held by intramolecular and intermolecular hydrogen bonds [20]. Figure 4 shows the chemical structure of cellulose and amylose polymers [18]. The three-dimensional structures of amylose and cellulose polymers are depicted in figure 5 [14].

Polysaccharide-based chiral stationary phases have a wide application in LC-MS due to their high separation efficiency, selectivity, sensitivity and reproducibility under normal and reversed-phase conditions as well as their broad applicability for structurally diversified compounds [21].

The mechanism of chiral interaction on the polysaccharide based chiral stationary phase has not yet been elucidated. However, the following interactions are believed to play a role in the retention:

(i) Hydrogen bonding interactions of the polar chiral analyte with carbamate groups on the CSP;

(ii) π-π interactions between phenyl groups on the CSP and aromatic groups of the solute;

(iii) Dipole-dipole interactions and

(iv) Steric interactions due to the helical structure of the CSP.[23]

Table 3: Polysaccharide-based chiral columns

Type of Adsorbent	Packing Composition Coated on Silica Gel	Column Trade Name
Amylose carbamate	Amylose tris(3,5-dimethylphenylcarbamate)	Chiralpak AD, Chiralpak AD-H
	Amylose tris[(S)-α-methylbenzylcarbamate]	Chiralpak AS, Chiralpak AS-H
Cellulose carbamate	Cellulose tris (phenylcarbamate)	Chiralcel OC
	Cellulose tris(3,5-dimethylphenylcarbamate)	Chiralcel OD, Chiralcel OD-H
	Cellulose tris (3,5-dimethylphenylcarbamate)	Chiralcel OD-R
	Cellulose tris(4-chlorophenylcarbamate)	Chiralcel OF
	Cellulose tris(4- methylphenylcarbamate)	Chiralcel OG
	Cellulose tris(3-chloro-4-methylphenylcarbamate)	Chiralcel OZ-H
Cellulose ester	Microcrystalline cellulose triacetate	Chiralcel CA-1
	Cellulose triacetate	Chialcel OA
	Cellulose tribenzoate	Chiralcel OB, Chiralcel OB-H
	Cellulose tris(4-methylbenzoate)	Chiralcel OJ
		Chiralcel OJ-H
	Cellulose tricinnamte	Chiralcel OK

4.Cyclodextrin chiral stationary phases:

Cyclodextrin (CD) chiral stationary phase is produced by partial degradation of starch by the enzyme cyclodextrin glycosyltransferase followed by enzymatic coupling of the glucose units into a toroidal structure. These glucose units are connected through an a- (1.4) glycosidic linkages. The chair configuration of glucose residues makes the toroidal structure of CD molecule narrower at one end. Figure 7 shows the toroidal structure of the CD molecule [22]. The toroidal structure consists of a hydrophilic outer surface and a hydrophobic inner cavity. The exterior hydrophilic surface results from the secondary 2 and 3-hydroxyl groups lined at the mouth of the CD cavity and primary 6-hydroxyl groups found on the opposite end of the molecule. While the hydrophobic portion results from the glucose oxygens and methylene hydrogens.[24]

Table 4: Commercially available cyclodextrin columns

Trade Name	Support
Cyclobond I 2000	β- Cyclodextrin
Cyclobond II 2000	γ- Cyclodextrin
Cyclobond III 2000	α- Cyclodextrin
Cyclobond I 2000 Ac	β-cyclodextrin, peracetylated
Cyclobond I 2000 SP	β-cyclodextrin, S-hydroxypropyl ether
Cyclobond I 2000 RSP	β-cyclodextrin, R,S-hydroxypropyl ether
Cyclobond I 2000 RN	β-cyclodextrin, R-Naphthylethyl carbamate
Cyclobond I 2000 SN	β-cyclodextrin, S-Naphthylethyl carbamate
Cyclobond I 2000 DMP	β-cyclodextrin, 3,5-Dimethylphenyl carbamate
Chiral CD-PH	phenylcarbamated-ß-cyclodextrin

5.Ligand exchange chiral stationary phase:

Ligand exchange chromatography was introduced by Davankov and Rogozhin. The principle involves the formation of a ternary mixed metal complex between the chiral selector, the analyte and a transition metal such as copper ions that will be at the core of the complex with the enantiomers. The lone electron pairs of the heteroatoms (N, O, S) of the functional groups, belonging to the analyte and selector, occupy definite positions in the coordination sphere of the central metal ion, to result in the formation of the ternary complex [24]. The amino acids act as a chiral selector (ligand). The chiral selector can either be fixed to the stationary phase or added to the mobile phase. In chiral ligand-exchange chromatography, the separation occurs as a result of the exchange of the ligand and the enantiomers on a metal ion. The ligand exchange involves the breaking and formation of coordinate bonds among the metal ions of the complex, ligands, and enantiomers. Therefore, ligand exchange chromatography is useful for the chiral resolution of molecules containing electron-donating atoms such as oxygen, nitrogen, and sulfur [19]. Table 5 lists various ligand exchange-based commercialcolumns.[25]

Table 5: Ligand exchange-based commercial columns

Trade Name	Chiral Selector
Chiralpak WH	L-proline derivatives covalently bonded to silica gel
Chiralpak MA (+)	N,N-dioctyl-L-alanine coated on silica gel
Nucleosil Chiral-1	L-hydroxyproline

6.Macrocyclic chiral stationary phases:

Macrocyclic antibiotics based chiral stationary phases were introduced by Armstrong in 1994. The commonly used macrocyclic antibiotics include rifamycin, glycopeptides (e.g. avoparcin, teicoplanin, ristocetin A, vancomycin, and their analogs), polypeptide antibiotic thiostrepton, and aminoglycosides

(e.g. fradiomycin, kanamycin, and streptomycin). Figure 9 shows the subdivision of macrocyclic antibiotics [25]. Macrocyclic glycopeptides are complex molecules produced by microorganisms in the fermentation broth. The glycopeptides chiral stationary phase is made up of an aglycone portion of fused macrocyclic rings that forms a hydrophobic basket shape, which can include hydrophobic parts of an analyte and a carbohydrate moiety. The macrocyclic antibiotics interact with the analyte through hydrogen bonds, dipole-dipole interactions with the polar groups of the analyte, ionic interactions and π-π interactions. Figure 10 shows the structures of antibiotics used in the preparation of macrocyclic antibiotic CSP [14]. Table 6 lists commercially available macrocyclic antibiotic CSPs.[26]

Table 6: Commercially available macrocyclic chiral stationary phase.

Trade Name	Bonded Macrocyclic Glycopeptide
Chirobiotic V, Chirobiotic V2	Vancomycin
Chirobiotic T, Chirobiotic T2	Teicoplanin
Chirobiotic R	Ristocetin A
Chirobiotic TAG	Teicoplanin aglycone

7.Chiral crown ether:

Crown ether was first developed by Charles Pederson in 1967. These are macrocyclic polyethers that form host-guest complexes with alkali, earth-alkali metal ions and ammonium cations [24]. The main skeleton in the cyclic structure consists of oxygen and methylene groups alternatively placed. The ether oxygens, which are electron donors, remain in the inner wall of the crown cavity and are surrounded by methylene groups in a collar fashion [19]. Two different diastereomeric inclusion complexes are formed. The primary interactions for complexation are hydrogen bonds between the three amine hydrogens and the oxygens of the macrocyclic ether in a tripod arrangement. Ionic, dipole-dipole interactions or hydrogen bonds between the carbocyclic groups and polar groups of the analytes may act as additional supporting interactions [26]. Figure 11 shows the chemical structure of a chiral crown ether [19]. The CROWNPAK CR (-) and CROWNPAK CR (+) columns are composed of chiral crown ether coated on silica-gel.

8.Miscellaneous:

Chiral resolution of some racemic compounds has also been reported on other CSPs such as alkaloid-based CSP, amide and amine-based CSP, acid-based CSP, and synthetic polymer-based CSP.[27]

Table 7: Applications of chiral stationary phases

Sr. No.	Chiral Compound	Separation Technique	Column	Ref. No.
1	S-(-) and R-(+) Metoprolol	Chiral LC-ESI-MS/MS	Chiral Lux amylose-2	[2]
2	Acetyl-glutamine enantiomers	HPLC-MS	Chiralpak AD-H	[8]
3	Pyrrolidine derivatives	Supercritical fluid chromatography	Lux Cellulose-2 and Lux i-Cellulose-5	[27]
4	Letermovir	HPLC	Chiralpak AD	[18]
5	Metolazone	Liquid Chromatography	Chiralpak AD-H	[28]
6	Atenolol, Metoprolol, Propranolol and Bisoprolol	HPLC	Chiralpak AD-H	[29]
7	Ezetimibe and Tramadol	HPLC	Chiralpak AS-H	[30]
8	Ketorolac	RP-HPLC	Chiral AGP	[15]
9	(S)-Tenofovir	RP-HPLC	Chiral AGP	[31]
10	Citalopram and Escitalopram	HPLC	Chiral CD-PH	[32]
11	(-)(R)-and (+)(S)-Chlorpheniramine and its metabolites	HPLC	CYCLOBOND I 2000™	[33]
12	Benidipine Enantiomers	LC-MS	Chirobiotic V	[9]
13	Acetyl-L-carnitine	LC	SUMICHIRAL OA-6100	[34]

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