CHAPTER-1

GENERAL INTRODUCTION TO CHROMATOGRAPHY, VARIOUS CHROMATOGRAPHIC TECHNIQUES AND ITS APPLICATION IN PHARMACEUTICAL AND CHEMICAL INDUSTRUES

1.0 DISCOVERY OF CHROMATOGRAPHY

Chromatography is a physicochemical method for separation of complex mixtures was discovered at the very beginning of the twentieth century by Russian–Italian botanist M. S. Tswett. [1]. In his paper "On the new form of adsorption phenomena and its application in biochemical analysis" presented on March 21, 1903 at the regular meeting of the biology section of the Warsaw Society of Natural Sciences, Tswett gave a very detailed description of the newly discovered phenomena of adsorption-based separation of complex mixtures, which he later called "chromatography" as a transliteration from Greek "color writing" [2]. Serendipitously, the meaning of the Russian word "tswett" actually means color. Although in all his publications Tswett mentioned that the origin of the name for his new method was based on the colorful picture of his first separation of plant pigments (Figure 1), he involuntarily incorporated his own name in the name of the method he invented. The chromatographic method was not appreciated among the scientists at the time of the discovery, as well as after almost 10 years when L. S. Palmer [3] in the United States and C. Dhere in Europe independently published the description of a similar separation processes.

Twenty-five years later in 1931, Lederer read the book of L. S. Palmer and later found an original publications of M. S. Tswett, and in 1931 he (together with Kuhn and Winterstein) published a paper [4] on purification of xantophylls on CaCO3 adsorption column following the procedure described by M. S. Tswett. In 1941 A. J. P. Martin and R. L. M. Synge at Cambridge University, in UK discovered partition chromatography [5] for which they were awarded the Noble Prize in 1952. In the same year, Martin and Synge published a seminal paper [6] which, together with the paper of A.T. James and A. J. P. Martin [7], laid a solid foundation for the fast growth of chromatographic techniques that soon followed.

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between

similar compounds. During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times

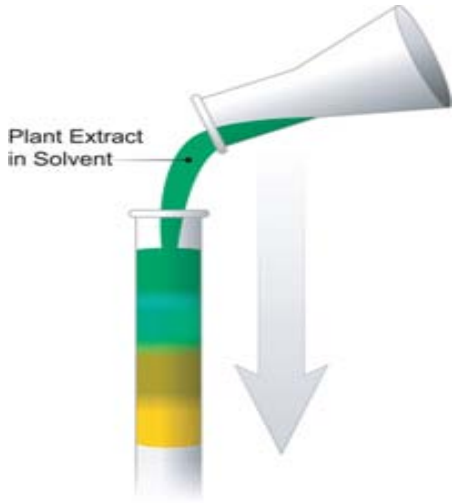

Figure 1: Tswett's Experiment

of compounds being isolated by column chromatography. However, flow rates were inconsistent, and the question of whether it was better to have constant flow rate or constant pressure was debated [8]. High pressure liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of online detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds. By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above the previous techniques. Computers and automation added to the convenience of HPLC. Improvements in type of columns and thus reproducibility were made as such terms as micro-column, affinity columns, and Fast HPLC began to immerge.

By the 2000 very fast development was undertaken in the area of column material with small particle size technology and other specialized columns. The dimensions of the General Introduction typical HPLC column are 100-300 mm in length with an internal diameter between 3-5 mm. The usual diameter of micro-columns, or capillary columns,

ranges from 3 μm to 200 μm [9]. In this decade, sub 2 micron particle size technology (column material packed with silica particles of < 2μm size) with modified or improved HPLC instrumentation becomes popular with different instrument brand name like UPLC (Ultra Performance Liquid Chromatography) of Waters and RRLC (Rapid Resolution Liquid Chromatography) of Agilent.

Today, chromatography is an extremely versatile technique; it can separate gases, and volatile substances by GC, in-volatile chemicals and materials of extremely high molecular weight (including biopolymers) by LC and if necessary very inexpensively by TLC. All three techniques, (GC), (LC) and TLC have common features that classify them as chromatography systems. Chromatography has been defined as follows,

"Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase; ipso facto a separation is achieved" [10].

2. VARIOUS TYPES OF CHROMATOGRAPHY

Chromatography can be classified by various ways (I) On the basis of interaction of solute to the stationary phase [11], (II) On the basis of chromatographic bed shape [12, 13], **(**III) Techniques by physical state of mobile phase

2.1 ON THE BASIS OF INTERACTION OF SOLUTE TO STATIONARY PHASE

2.1.1 ADSORPTION CHROMATOGRAPHY

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.

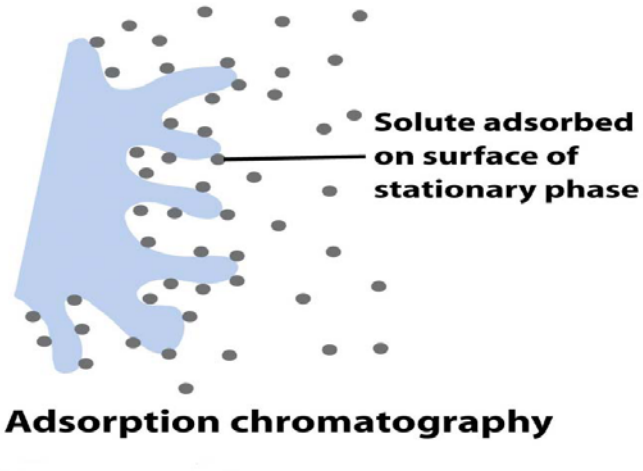

Figure 2: Schematic figure for adsorption phenomenon in chromatography

2.1.2 PARTITION CHROMATOGRAPHY

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid

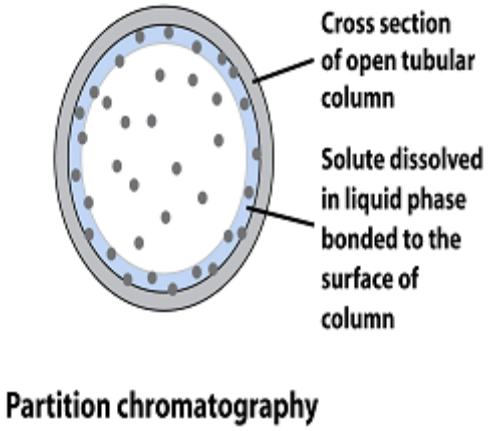

Figure 3: Schematic figure for Partition phenomenon in chromatography

2.1.3 ION EXCHANGE CHROMATOGRAPHY

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.

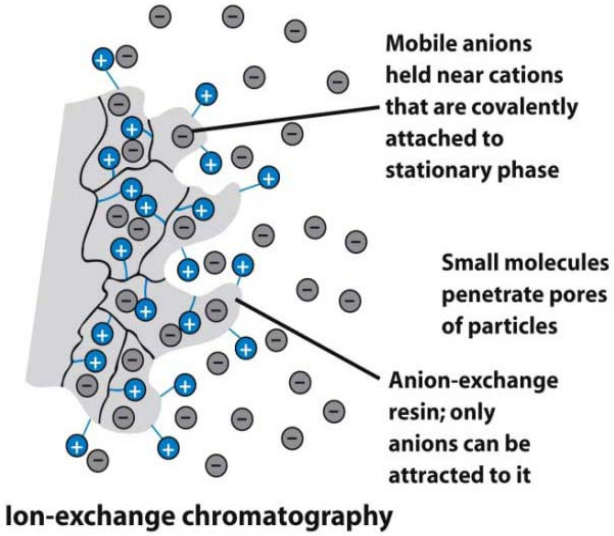

Figure 4: Schematic figure for Ion exchange phenomenon in chromatography

2.1.4 MOLECULAR EXCLUSION CHROMATOGRAPHY

Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size. The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

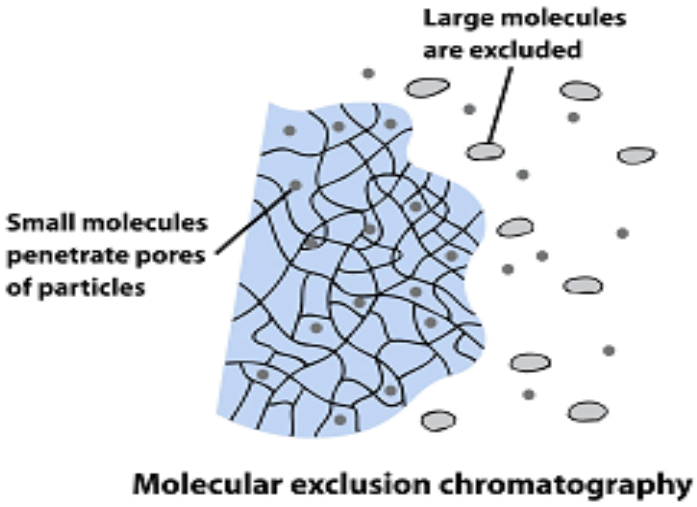

Figure 4: Schematic figure for Molecular Exchange phenomenon in chromatography

2.2 ON THE BASIS OF CHROMATOGRAPHIC BED SHAPE

2.2.1 COLUMN CHROMATOGRAPHY

Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). Differences in rates of movement through the medium are calculated to different retention times of the sample [14].

In 1978, W.C.Still introduced a modified version of column chromatography called flash column chromatography [15] [16]. The technique is very similar to the traditional column chromatography, except for that the solvent is driven through the column by applying positive pressure. This allowed most separations to be performed in less than 20 minutes, with improved separations compared to the old method. Modern flash chromatography systems are sold as pre-packed plastic cartridges, and the solvent is pumped through the cartridge. Systems may also be linked with detectors and fraction collectors providing

automation. The introduction of gradient pumps resulted in quicker separations and less solvent usage.

In expanded bed adsorption, a fluidized bed is used, rather than a solid phase made by a packed bed. This allows omission of initial clearing steps such as centrifugation and filtration, for culture broths or slurries of broken cells.

2.2.2 PLANAR CHROMATOGRAPHY

Planar chromatography is a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (paper chromatography) or a layer of solid particles spread on a support such as a glass plate (thin layer chromatography). Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase. The specific Retention factor (Rf) of each chemical can be used to aid in the identification of an unknown substance.

2.2.3 PAPER CHROMATOGRAPHY

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.

2.2.4 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or

cellulose on a flat, inert substrate. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and to allow for quantification, high-performance TLC can be used.

2.2.5 DISPLACEMENT CHROMATOGRAPHY

The basic principle of displacement chromatography is, "A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities".[17]

There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings.

Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than "peaks".

Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations.

2.3 TECHNIQUES BY PHYSICAL STATE OF MOBILE PHASE

2.3.1 GAS CHROMATOGRAPHY

Gas chromatography (GC), also sometimes known as Gas-Liquid chromatography, (GLC), is a separation technique in which the mobile phase is a gas. Gas chromatography is always carried out in a column, which is typically "packed" or "capillary".

Gas chromatography (GC) is based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often Helium).

The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column). It is widely used in analytical chemistry; though the high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat will denature them), frequently encountered in biochemistry, it is well suited for use in the petrochemical, environmental monitoring, and industrial chemical fields. It is also used extensively in chemistry research.

2.3.2 LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred as high performance liquid chromatography (HPLC).

In the HPLC technique, the sample is forced through a column that is packed with irregularly or spherically shaped particles or a porous monolithic layer (stationary phase) by a liquid (mobile phase) at high pressure. HPLC is historically divided into two different sub-classes based on the polarity of the mobile and stationary phases. Technique in which the stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase, silica as the stationary phase) is called normal phase liquid chromatography (NPLC) and the opposite (e.g. water-methanol mixture as the mobile phase and C18 = octadecylsilyl as the stationary phase) is called reversed phase liquid chromatography (RPLC). Ironically the "normal phase" has fewer applications and RPLC is therefore used considerably more.

Specific techniques which come under this broad heading are listed below. It should also be noted that the following techniques can also be considered fast protein liquid chromatography if no pressure is used to drive the mobile phase through the stationary phase.

2.3.3 AFFINITY CHROMATOGRAPHY

Affinity chromatography [18] is based on selective non-covalent interaction between an analyte and specific molecules. It is very specific, but not very robust. It is often used in biochemistry in the purification of proteins bound to tags. These fusion proteins are labeled with compounds such as His-tags, biotin or antigens, which bind to the stationary phase specifically. After purification, some of these tags are usually removed and the pure protein is obtained. Affinity chromatography often utilizes a biomolecule's affinity for a metal (Zn, Cu, Fe, etc.). Columns are often manually prepared. Traditional affinity columns are used as a preparative step to flush out unwanted biomolecules. However, HPLC techniques exist that do utilize affinity chromatography properties. Immobilized Metal Affinity Chromatography (IMAC) is useful to separate aforementioned molecules based on the relative affinity for the metal (I.e. Dionex IMAC). Often these columns can be loaded with different metals to create a column with a targeted affinity.

2.3.4 SUPERCRITICAL FLUID CHROMATOGRAPHY

Supercritical fluid chromatography is a separation technique in which the mobile phase is a fluid above and relatively close to its critical temperature and pressure.

3. INTRODUCTION TO HIGH PERFORMANCE LC [19]

The acronym HPLC, coined by the late Prof. Csaba Horvath for his 1970 Pittcon paper, originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500 psi. This was called high pressure liquid chromatography, or HPLC.

The early 1970s saw a tremendous leap in technology. These new HPLC instruments could develop up to 6,000 psi of pressure, and incorporated improved injectors, detectors, and columns. HPLC really began to take hold in the mid-to late-1970s. With continued advances in performance during this time (smaller particles, even higher pressure), the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography.

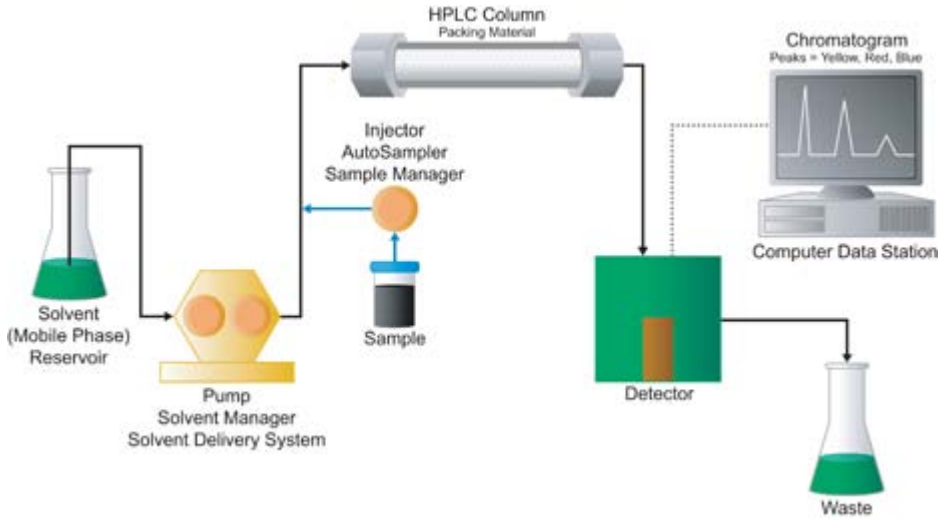

Figure 2: High-Performance Liquid Chromatography [HPLC] System

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals. The components of a basic high-performance liquid chromatography (HPLC) system are shown in the simple diagram in Figure 5 (Schematic Diagram).

A reservoir (Solvent Delivery) holds the solvent (called the mobile phase, because it moves). A high-pressure pump solvent manager is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute.

An injector (sample manager or auto sampler) is able to introduce (inject) the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column. The column contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware. A detector is needed to see the separated compound bands as they elute from the HPLC column (most compounds have no color, so we cannot see them with our eyes).

The mobile phase exits the detector and can be sent to waste, or collected, as desired. When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the eluate containing that purified compound for further study. This is called preparative chromatography. The high-pressure tubing and fittings are used to interconnect the pump, injector, column, and detector components to form the conduit for the mobile phase, sample, and separated compound bands.

The detector is wired to the computer data station, the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to identify and quantitate the concentration of the sample constituents. Since sample compound characteristics can be very different, several types of detectors have been developed. For example, if a compound can absorb ultraviolet light, a UV-absorbance detector is used. If the compound fluoresces, a fluorescence detector is used. If the compound does not have either of these characteristics, a more universal type of detector is used, such as an evaporative-light-scattering detector (ELSD). The most powerful approach is the use multiple detectors in series. For example, a UV and/or ELSD detector may be used in combination with a mass spectrometer (MS) to analyze the results of the chromatographic separation. This provides, from a single injection, more comprehensive information about an analyte. The practice of coupling a mass spectrometer to an HPLC system is called LC/MS.

3.1 ISOCRATIC AND GRADIENT LC SYSTEM OPERATION

Two basic elution modes are used in HPLC. The first is called isocratic elution. In this mode, the mobile phase, either a pure solvent or a mixture, *remains the same throughout the run.* A typical system is outlined in Figure 2.

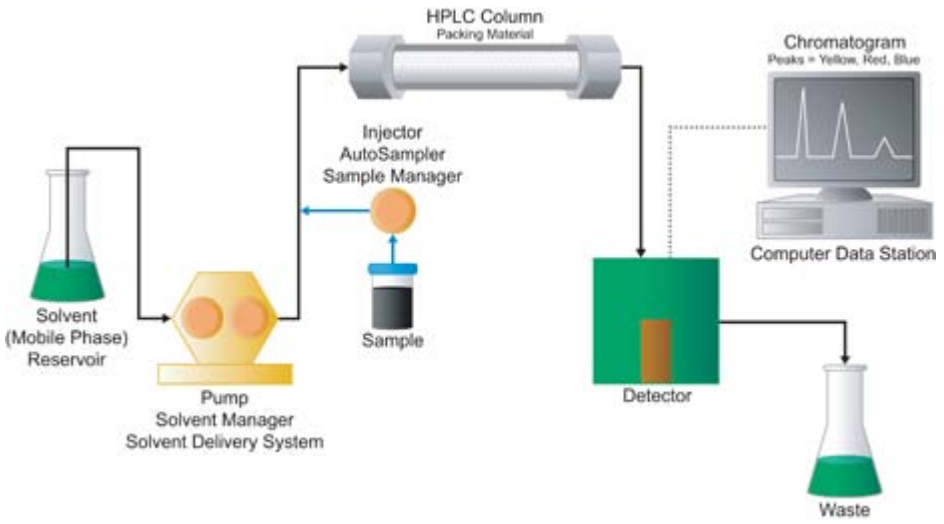

Figure 2: Isocratic LC System

The second type is called gradient elution, wherein, as its name implies, *the mobile phase composition changes during the separation.* This mode is useful for samples that contain compounds that span a wide range of chromatographic polarity. As the separation proceeds, the elution strength of the mobile phase is increased to elute the more strongly retained sample components.

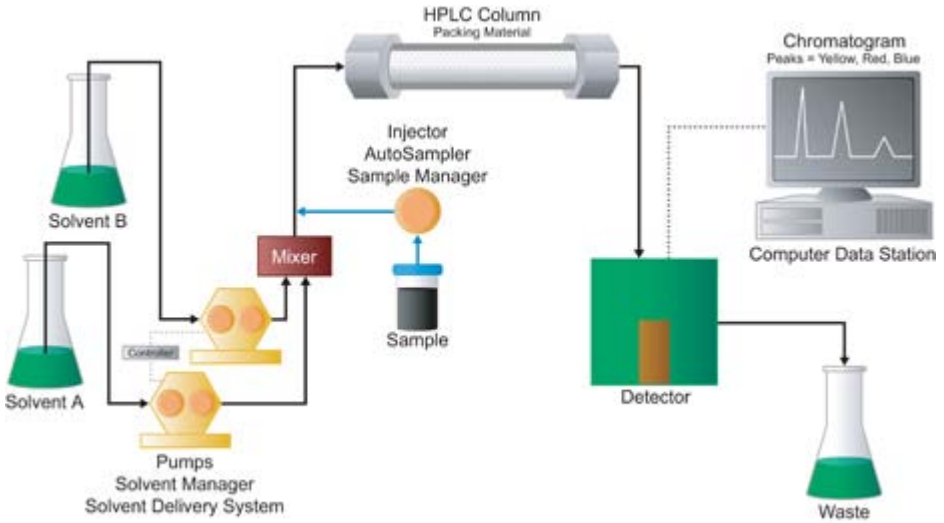

Figure -3: High-Pressure-Gradient System

In the simplest case, shown in Figure 3, there are two bottles of solvents and two pumps. The speed of each pump is managed by the gradient controller to deliver more or less of each solvent over the course of the separation. The two streams are combined in the mixer to create the actual mobile phase composition that is delivered to the column over time. At the beginning, the mobile phase contains a higher proportion of the weaker solvent [Solvent A]. Over time, the proportion of the stronger solvent [Solvent B] is increased, according to a predetermined timetable. Note that in Figure 3, the mixer is downstream of the pumps; thus the gradient is created under *high pressure*. Other HPLC systems are designed to mix multiple streams of solvents under *low pressure*, ahead of a single pump. A gradient proportioning valve selects from the four solvent bottles, changing the strength of the mobile phase over time [see Figure 4].

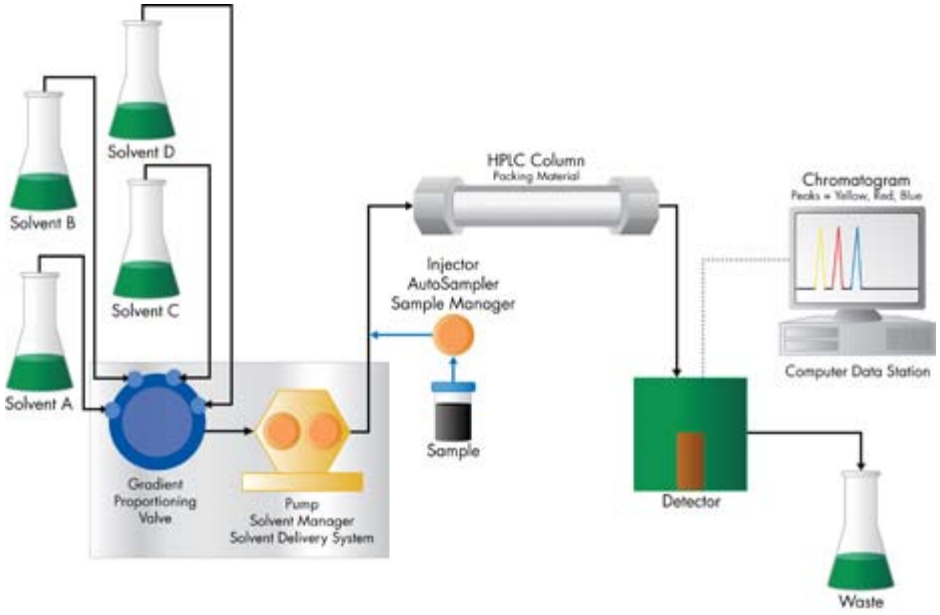

Figure -4: Low-Pressure-Gradient System

Today's HPLC requires very special apparatus which includes the following.

1. Extremely precise gradient mixers.
2. HPLC high pressure pumps with very constant flow.
3. Unique high accuracy, low dispersion, HPLC sample valves.
4. Very high efficiency HPLC columns with inert packing materials.

5. High sensitivity low dispersion HPLC detectors.

6. High speed data acquisition systems.

7. Low dispersion connecting tubes for valve to column and column to detector.

3.1.2 HPLC GRADIENT MIXTURES

HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. This can be complicated in HPLC by the small elution volumes required by many systems. It is much more difficult to produce a constant gradient when mixing small volumes then when mixing large volumes. For low pressure systems this requires great precision in the operation of the miniature mixing General Introduction valves used and low dispersion flows throughout the mixer. For multi-pump high pressure systems it requires a very precise control of the flow rate while making very small changes of the flow rate.

3.1.3 HPLC PUMPS

Because of the small particles used in modern HPLC, modern LC pumps need to operate reliably and precisely at pressures of 10,000 psi or at least 6,000 psi. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical purposes HPLC pumps should have flow rates that range from 0 to 10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. It is extremely difficult to provide a very constant flow rate at very low flow rates. If 1% is considered acceptable then for 1ml/min a flow variation of less than 10µl/min is required. This level of constancy is required because most HPLC detectors are flow sensitive and errors in quantization will result from change in flow rate.

3.1.4 HPLC SAMPLE VALVES

Since sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 psi. For analytical HPLC, the sample volume should be selectable from sub micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml. To maintain system efficiency the sample valve must be designed to have very low dispersion characteristics, this is true not only for flow dispersion but also for the less obvious

problems of dispersion caused by sample adsorption/desorption on valve surfaces and diffusion of sample into and out of the mating surfaces between valve moving parts. It goes without saying that the valves must deliver a very constant sample size but this is usually attained by the use of a constant size sample loop.

3.1.5 HPLC COLUMNS

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. Packing these small particles into the column is a difficult technical problem but even with good packing a great amount of care must be given to the column end fittings and the inlet and outlet connection to keep dispersion to a minimum. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized because of the much lower quantities of both which are required. In particular very expensive optically pure compounds can be used to make Chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents.

3.1.6 HPLC DETECTORS [20-25]

UV/Vis spectrophotometers, including diode array detectors, are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers, electrochemical detectors, mass spectrometers, light scattering detectors, radioactivity detectors or other special detectors may also be used. Detector consists of a flow-through cell mounted at the end of the column. A beam of UV radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes. Fixed (mercury lamp), variable (deuterium or high-pressure xenon lamp), and multi-wavelength detectors are widely available. Modern variable wavelength detectors can be programmed

to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, and then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths, spectra of the eluting peaks and also peak purity.

Differential refractometers detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographic compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds. Fluorimetric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups. Potentiometric, voltametric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols.

In order to give an accurate chromatographic profile the detector sampling (cell) volume must be a small fraction of the solute elution volume. If the detector volume were larger than the elution volume then you would have peaks that appeared with flat tops as the whole peak would be resident in the detector at the same time. This means that as column volumes decrease and system efficiencies increase the volume of the detector cell volume must also decrease. It is odds for the requirement for detector to maintain high sensitivity as this is usually dependant on having a larger cell volume. Again, this requires the very careful design of modern detectors. Many types of detectors can use with HPLC system like UV-Visible or PDA (Photo Diode Array), RI (Refractive Index), Fluorescence, ECD (Electro Chemical Detector), ELSD (Evaporative Light Scattering detector) and many others hyphenated techniques like MS, MS/MS and NMR as well as evaporative IR.

3.1.7 HPLC DATA ACQUISITION

In HPLC data acquisition system the higher sampling rate needed for the rapidly eluting narrow peaks of the HPLC chromatogram. Although the theoretical number of samples needed for good quantization are actually quite small, for real systems a hundred samples or more per peak is recommended; thus, for a 4 sec wide peak, a rate of 25 samples per second may be required. The same data analysis and reporting software can be used as in ordinary LC.

3.1.8 CONCLUSION

HPLC is probably the most universal type of analytical procedure; its application areas include quality control, process control, forensic analysis, environmental monitoring and clinical testing. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with a constant incorporation of new highly selective column packings.

4. INTRODUCTION TO ULTRA PERFORMANCE LC [26]

In 2004, further advances in instrumentation and column technology were made to achieve very significant increases in resolution, speed, and sensitivity in liquid chromatography. Columns with smaller particles (1.7 micron) and instrumentation with specialized capabilities designed to deliver mobile phase at 15,000 psi (1,000 bars) were needed to achieve a new level of performance. A new system had to be holistically created to perform ultra-performance liquid chromatography, now known as UPLC technology.

Basic research is being conducted today by scientists working with columns containing even smaller 1-micron-diameter particles and instrumentation capable of performing at 100,000 psi. This provides a glimpse of what we may expect in the future. UPLC refers

to Ultra Performance Liquid Chromatography. It improves in three areas: chromatographic resolution, speed and sensitivity analysis. It uses fine particles and saves time and reduces solvent consumption [27-31]. UPLC is comes from HPLC. HPLC has been the evolution of the packing materials used to effect the separation. An underlying principle of HPLC dictates that as column packing particle size decreases, efficiency and thus resolution also increases. As particle size decreases to less than 2.5µm, there is a significant gain in efficiency and it's doesn't diminish at increased linear velocities or flow rates according to the common Van Deemter equation [32]. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits which is known as Ultra Performance.

The classic separation method is of HPLC (High Performance Liquid Chromatography) with many advantages like robustness, ease of use, good selectivity and adjustable sensitivity. Its main limitation is the lack of efficiency compared to gas chromatography or the capillary electrophoresis [33, 34] due to low diffusion coefficients in liquid phase, involving slow diffusion of analytes in the stationary phase. The Van Deemter equation shows that efficiency increases with the use of smaller size particles but this leads to a rapid increase in back pressure, while most of the HPLC system can operate only up to 400 bar. That is why short columns filled with particles of about 2µm are used with these systems, to accelerate the analysis without loss of efficiency, while maintaining an acceptable loss of load.

To improve the efficiency of HPLC separations, the following can be done,

(1) Work at higher temperatures

(2) Use of monolithic columns

4.1. USE OF THE UPLC SYSTEM

Elevated-temperature chromatography also allows for high flow rates by lowering the viscosity of the mobile phase, which significantly reduces the column backpressure [35, 36]. Monolithic columns contain a polymerized porous support structure that provides lower flow resistances than conventional particle-packed columns [37-39].

4.1.1 PRINCIPLE

The UPLC is based on the principal of use of stationary phase consisting of particles less than 2 μm (while HPLC columns are typically filled with particles of 3 to 5 μm). The underlying principles of this evolution are governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). The Van Deemter curve, governed by an equation with three components shows that the usable flow range for a good efficiency with small diameter particles is much greater than for larger diameters [40-43]

$$H=A+B/v+Cv$$

Where A, B and C are constants and v is the linear velocity, the carrier gas flow rate. The A term is independent of velocity and represents "eddy" mixing. It is smallest when the packed column particles are small and uniform. The B term represents axial diffusion or the natural diffusion tendency of molecules. This effect is diminished at high flow rates and so this term is divided by v. The C term is due to kinetic resistance to equilibrium in the separation process.

The kinetic resistance is the time lag involved in moving from the gas phase to the packing stationary phase and back again. The greater the flow of gas, the more a molecule on the packing tends to lag behind molecules in the mobile phase. Thus this term is proportional to v.

Therefore it is possible to increase throughput, and thus the speed of analysis without affecting the chromatographic performance. The advent of UPLC has demanded the development of a new instrumental system for liquid chromatography, which can take advantage of the separation performance (by reducing dead volumes) and consistent with the pressures (about 8000 to 15,000 PSI, compared with 2500 to 5000 PSI in HPLC). Efficiency is proportional to column length and inversely proportional to the particle size [44]. Therefore, the column can be shortened by the same factor as the particle size without loss of resolution. The application of UPLC resulted in the detection of additional drug metabolites, superior separation and improved spectral quality [45, 46].

4.1.2 SAMPLE INJECTION

In UPLC, sample introduction is critical. Conventional injection valves, either automated or manual, are not designed and hardened to work at extreme pressure. To protect the column from extreme pressure fluctuations, the injection process must be relatively pulse-free and the swept volume of the device also needs to be minimal to reduce potential band spreading. A fast injection cycle time is needed to fully capitalize on the speed afforded by UPLC, which in turn requires a high sample capacity. Low volume injections with minimal carryover are also required to increase sensitivity [47]. There are also direct injection approaches for biological samples [48, 49].

4.1.3 UPLC COLUMNS

Resolution is increased in a 1.7 μm particle packed column because efficiency is better. Separation of the components of a sample requires a bonded phase that provides both retention and selectivity. Four bonded phases are available for UPLC separations: ACQUITY UPLCTM BEH C18 and C8 (straight chain alkyl columns), ACQUITY UPLC BEH Shield RP18 (embedded polar group column) and ACQUITY UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C6 alkyl) [50]. Column chemistry provides a different combination of hydrophobicity, silanol activity, hydrolytic stability and chemical interaction with analytes.

ACQUITY UPLC BEH C18 and C8 columns are considered the universal columns of choice for most UPLC separations by providing the widest pH range. They incorporate trifunctional ligand bonding chemistries which produce superior low pH stability. This low pH stability is combined with the high pH stability of the 1.7 μm BEH particle to deliver the widest usable pH operating range. ACQUITY UPLC BEH Shield RP18 columns are designed to provide selectivities that complement the ACQUITY UPLC BEH C18 and C8 phases.

ACQUITY UPLC BEH Phenyl columns utilize a trifunctional C6 alkyl tether between the phenyl ring and the silyl functionality. This ligand, combined with the same proprietary endcapping processes as the ACQUITY UPLC BEH C18 and C8 columns, provides long column lifetimes and excellent peak shape. This unique combination of ligand and endcapping on the 1.7 μm BEH particle creates a new dimension in selectivity allowing a quick match to the existing HPLC column. An internal dimension (ID) of 2.1

mm column is used. For maximum resolution, choose a 100 mm length and for faster analysis, and higher sample throughput, choose 50 mm column.

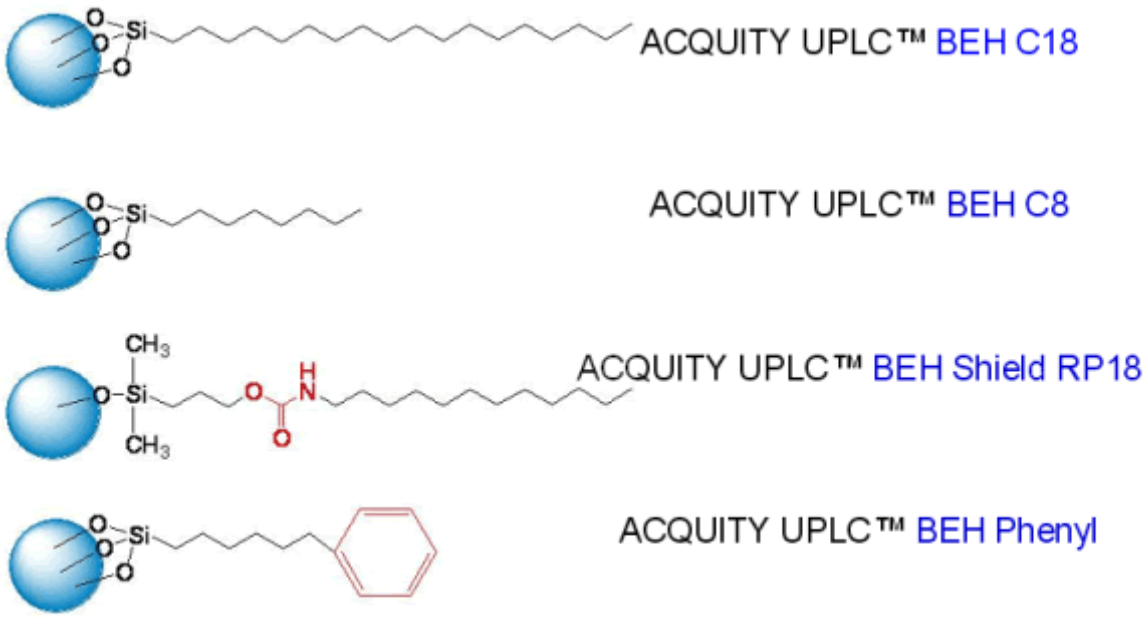

Figure 5: Different column chemistry available for UPLC

Half-height peak widths of less than one second are obtained with 1.7µm particles, which gives significant challenges for the detector. In order to integrate an analyte peak accurately and reproducibly, the detector sampling rate must be high enough to capture enough data points across the peak. The detector cell must have minimal dispersion (volume) to preserve separation efficiency. Conceptually, the sensitivity increase for UPLC detection should be 2-3 times higher than HPLC separations, depending on the detection technique. MS detection is significantly enhanced by UPLC; increased peak concentrations with reduced chromatographic dispersion at lower flow rates promote increased source ionization efficiencies.

The ACQUITY UPLC System consists of a binary solvent manager, sample manager including the column heater, detector, and optional sample organizer. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient. There are built-in solvent select valves to choose from up to four solvents. There is a 15,000-psi pressure limit (about 1000 bar) to take full advantage of the sub-2µm particles.

The sample manager also incorporates several technology advancements. Using pressure assisted sample introduction, low dispersion is maintained through the injection process, and a series of pressures transducers facilitate self-monitoring and diagnostics. It uses needle-in-needle sampling for improved ruggedness and a needle calibration sensor increases accuracy. Injection cycle time is 25 seconds without a wash and 60 sec with a dual wash used to further decrease carry over. A variety of microtiter plate formats (deep well, mid height, or vials) can also be accommodated in a thermostatically controlled environment. Using the optional sample organizer, the sample manager can inject from up to 22 micro titer plates. The sample manager also controls the column heater. Column temperatures up to 65°C can be attained. To minimize sample dispersion, a "pivot out" design allows the column outlet to be placed in closer proximity to the source inlet of an MS detector [51].

For UPLC detection, the tunable UV/Visible detector is used which includes new electronics and firmware to support Ethernet communications at the high data rates. Conventional absorbance-based optical detectors are concentration sensitive detectors, and for UPLC use, the flow cell volume would have to be reduced in standard UV/Visible detectors to maintain concentration and signal. According to Beer's Law, smaller volume conventional flow cells would also reduce the path length upon which the signal strength depends. A reduction in cross-section means the light path is reduced, and transmission drops with increasing noise. Therefore, if a conventional HPLC flow cell were used, UPLC sensitivity would be compromised. The ACQUITY Tunable UV/Visible detector cell consists of a light guided flow cell equivalent to an optical fiber. Light is efficiently transferred down the flow cell in an internal reflectance mode that still maintains a 10mm flow cell path length with a volume of only 500mL. Tubing and connections in the system are efficiently routed to maintain low dispersion and to take advantage of leak detectors that interact with the software to alert the user to potential problems [52].

4.1.4 ADVANTAGES

1. Decreases run time and increases sensitivity.

2. Provides the selectivity, sensitivity, and dynamic range of LC analysis.

3. Maintaining resolution performance.

4. Expands scope of Multiresidue Methods.

5. UPLC's fast resolving power quickly quantifies related and unrelated compounds
6. Faster analysis through the use of a novel separation material of small particle size.
7. Operation cost is reduced.
8. Less solvent consumption.
9. Reduces process cycle times, so that more product can be produced with existing resources.
10. Increases sample throughput and enables manufacturers to produce more material that consistently meet or exceeds the product specifications, potentially eliminating variability, failed batches, or the need to re-work material [53, 54].
11. Delivers real-time analysis in step with manufacturing processes.
12. Assures end-product quality, including final release testing.

4.1.5 DISADVANTAGES

Due to increased pressure requires more maintenance and reduces the life of the columns of this type. So far performance similar or even higher has been demonstrated by using stationary phases of size around 2 μm without the adverse effects of high pressure. In addition, the phases of less than 2 μm are generally non-regenerable and thus have limited use [55, 56].

5. APPLICATIONS OF CHROMATOGRAPHY

In recent times chromatography is most widely used analytical tool for various applications. Hence it could not be possible for include all of it but some of very important and recently developed applications are discussed here,

5.1. AFFINITY CHROMATOGRAPHY: CLINICAL APPLICATIONS [57]

Affinity chromatography is a type of liquid chromatography that makes use of biological-like interactions for the separation and specific analysis of sample components. This work describes the basic principles of affinity chromatography and examines its use in the testing of clinical samples, with an emphasis on HPLC-based methods. Some traditional applications of this approach include the use of boronate, lectin, protein A or protein G, and immunoaffinity supports for the direct quantification of solutes. Newer techniques that use antibody-based columns for on- or off-line sample extraction are examined in detail, as are methods that use affinity chromatography in combination with other analytical methods, such as reversed-phase liquid chromatography, gas chromatography, and capillary electrophoresis. Indirect analyte detection methods are also described in which immunoaffinity chromatography is used to perform flow-based immunoassays. Other applications that are include affinity-based chiral separations and the use of affinity chromatography for the study of drug or hormone interactions with binding proteins. Some areas of possible future developments are then considered, such as tandem affinity methods and the use of synthetic dyes, immobilized metal ions, molecular imprints, or aptamers as affinity ligands for clinical analyte.

5.2. PROTEIN PURIFICATION : MEMBRANE CHROMATOGRAPHY [58]

Adsorption chromatography is increasingly used for protein purification and medical applications. Synthetic membranes have advantages as support matrices in comparison to

conventional bead supports because they are not compressible and they eliminate diffusion limitations. As a result, higher throughput and faster processing times are possible in membrane systems. In this paper, there are various applications of membrane chromatography by focusing on affinity, ion exchange, hydrophobic interaction, reversed-phase and multistage chromatography. The prospects of further development of membrane chromatography are massive.

5.3. LC-MASS SPECTROMETRY IN ANALYTICAL TOXICOLOGY [59]

Liquid chromatography-mass spectrometry (LC-MS), after long-term development that has introduced seven major interfacing techniques, is finally suitable for application in the field of analytical toxicology. Various compound classes can be analyzed, and sensitivities for more or less polar analytes that are as good as or better than those of gas chromatography-mass spectrometry can be obtained with modern interfaces. In addition, because ionization is often softer than classical electron impact, some LC-MS interfaces are able to handle fragile species that are otherwise not amenable to MS. **[59]**

5.4. ENANTIOSELECTIVE CHROMATOGRAPHY AS A ALTERNATIVE FOR THE PREPARATION OF DRUG ENANTIOMERS [60]

The preparative separation of enantiomers by chromatography on Chiral stationary phases (CSPs) has been recognized as being a useful alternative to the more conventional approaches such as enantioselective synthesis and enzymatically catalyzed transformations. The possible contribution of enantioselective chromatography with respect to the preparation of enantiomerically pure compounds is reviewed in the context of the competitive approaches and depending on the application scale, with a special emphasis on the recent progresses achieved in this particular field of separation.

5.5. MICROEMULSION ELECTROKINETIC CHROMATOGRAPHY [61]

Compared to MEKC, the presence of a water-immiscible oil phase in the microemulsion droplets of microemulsion EKC (MEEKC) gives rise to some special properties, such as enhanced solubilization capacity and enlarged migration window, which could allow for

the improved separation of various hydrophobic and hydrophilic compounds, with reduced sample pretreatment steps, unique selectivities and/or higher efficiencies. Typically, stable and optically clear oil-in-water microemulsions containing a surfactant (SDS), oil (octane or heptane), and cosurfactant (1-butanol) in phosphate buffer are employed as separation media in conventional MEEKC. However, in recent years, the applicability of reverse MEEKC (water-in-oil microemulsions) has also been demonstrated, such as for the enhanced separation of highly hydrophobic substances. Also, during the past few years, the development and application of MEEKC for the separation of chiral molecules has been expanded, based on the use of enantioselective microemulsions that contained a chiral surfactant or chiral alcohol.

On the other hand, the application of MEEKC for the characterization of the lipophilicity of chemical substances remains an active and important area of research, such as the use of multiplex MEEKC for the high-throughput determination of partition coefficients (log P values) of pharmaceutical compounds. Emphases are placed on the discussion of MEEKC in the separation of chiral molecules and highly hydrophobic substances, as well as in the determination of partition coefficients, followed by a survey of recent applications of MEEKC in the analysis of pharmaceuticals, cosmetics and health-care products, biological and environmental compounds, plant materials, and foods.

5.6. LC–MS IN FORENSIC & CLINICAL TOXICOLOGY [62]

Liquid chromatographic–mass spectrometric (LC–MS) procedures are available for the identification and/or quantification of drugs of abuse, therapeutic drugs, poisons and/or their metabolites in biosamples (whole blood, plasma, serum, urine, cerebrospinal fluid, vitreous humor, liver or hair) of humans or animals (cattle, dog, horse, mouse, pig or rat). This is relevant to clinical toxicology, forensic toxicology, doping control or drug metabolism and pharmacokinetics.

5.7. LC–MS TO THE CHARACTERIZATION OF FOOD PROTEINS AND DERIVED PEPTIDES [63]

This review describes the development of mass spectrometry off-line and on-line coupled with liquid chromatography to the analysis of food proteins. It includes the significant

results recently obtained in the field of milk, egg and cereal proteins. This paper also outlines the research carried out in the area of food protein hydrolysates, which are important components in foodstuffs due to their functional properties. Liquid chromatography and mass spectrometry have been particularly used for the characterization of food peptides and especially in dairy products.

5.8. AFFINITY CHROMATOGRAPHY IN PROTEOMICS [64]

Affinity chromatography is a powerful protein separation method that is based on the specific interaction between immobilized ligands and target proteins. Peptides can also be separated effectively by affinity chromatography through the use of peptide-specific ligands. Both two-dimensional electrophoresis (2-DE) - and non-2-DE-based proteomic approaches benefit from the application of affinity chromatography. Before protein separation by 2-DE, affinity separation is used primarily for preconcentration and pretreatment of samples. Those applications entail the removal of one protein or a class of proteins that might interfere with 2-DE resolution, the concentration of low-abundance proteins to enable them to be visualized in the gel, and the classification of total protein into two or more groups for further separation by gel electrophoresis. Non-2-DE-based approaches have extensively employed affinity chromatography to reduce the complexity of protein and peptide mixtures. Prior to mass spectrometry (MS), preconcentration and capture of specific proteins or peptides to enhance sensitivity can be accomplished by using affinity adsorption. Affinity purification of protein complexes followed by identification of proteins by MS serves as a powerful tool for generating a map of protein–protein interactions and cellular locations of complexes. Affinity chromatography of peptide mixtures, coupled with mass spectrometry, provides a tool for the study of protein posttranslational modification (PTM) sites and quantitative proteomics.

Quantitation of proteomes is possible via the use of isotope-coded affinity tags and isolation of proteolytic peptides by affinity chromatography. An emerging area of proteomics technology development is miniaturization. Affinity chromatography is

becoming more widely used for exploring PTM and protein–protein interactions, especially with a view toward developing new general tag systems and strategies of chemical derivatization on peptides for affinity selection. More applications of affinity-based purification can be expected, including increasing the resolution in 2-DE, improving the sensitivity of MS quantification, and incorporating purification as part of multidimensional liquid chromatography.

5.9. CLINICAL APPLICATIONS OF GAS CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF STEROIDS [65]

This review article underlines the importance of gas chromatography–mass spectrometry (GC–MS) for determination of steroids in man. The use of steroids labelled with stable isotopes as internal standard and subsequent analysis by GC–MS yields up to now the only reliable measurement of steroids in serum. Isotope dilution GC–MS is the reference method for evaluation of routine analysis of serum steroid hormones. GC–MS is an important tool for detection of steroid hormone doping and combined with a combustion furnace and an isotope ratio mass spectrometer the misuse of testosterone by athletes can be discovered. Finally the so called urinary steroid profile by GC and GC–MS is the method of choice for detection of steroid metabolites in health and disease.

5.10. LC-MS/MS FOR THE ANALYSIS OF PHARMACEUTICAL RESIDUES IN ENVIRONMENTAL SAMPLES [66]

Pharmaceutical residues are environmental contaminants of recent concern and the requirements for analytical methods are mainly dictated by low concentrations found in aqueous and solid environmental samples. This can be determined by LC-MS/MS. Pharmaceuticals included are antibiotics, non-steroidal anti-inflammatory drugs, β-blockers, lipid regulating agents and psychiatric drugs. This can deal by advanced aspects of current LC–MS/MS methodology.

6.0 INTRODUCTION TO ANALYTICAL METHOD VALIDATION

6.1. ANALYTICAL METHOD VALIDATION

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use [67]. All analytical methods that are intended to be used for analyzing any samples will need to be validated. Validation of analytical methods is an essential but time - consuming activity for most analytical development laboratories. It is therefore important to understand the requirements of method validation in more detail and the options that are available to allow for optimal utilization of analytical resources in a development laboratory [68].

6.2. ANALYTICAL METHOD VALIDATION CHARACTERISTICS [69-74]

Typical analytical performance characteristics that should be considered in the validation of the types of procedures described in this chapter are listed below. Each validation characteristic is defined to ensure consistency in usage of terminology and interpretation:

- Accuracy
- Precision
    - Repeatability
    - Intermediate precision
- Specificity
- Detection limit
- Quantitation limit
- Linearity
- Range
- Robustness

6.2.1. ACCURACY

The International Convention on Harmonization (ICH) defines the accuracy of an analytical procedure as the closeness of agreement between the values that are accepted either as conventional true values or an accepted reference value and the value found. For drug substance, accuracy may be defined by the application of the analytical procedure to an analyte of known purity (e.g., a reference standard). For the drug product, accuracy will be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. The ICH document also recommends assessing a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations/three replicates).

Accuracy is usually reported as percent recovery by the assay (using the proposed analytical procedure) of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. The range for the accuracy limit should be within the linear range. Typical accuracy of the recovery of the drug substance is expected to be about 99 –101%. Typical accuracy of the recovery of the drug product is expected to be about 98 – 102%. Values of accuracy of recovery data beyond this range need to be investigated as appropriate.

6.2.2. METHOD PRECISION

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samples of the same homogeneous sample under prescribed conditions. Precision is usually investigated at three levels: repeatability, intermediate precision, and reproducibility. For simple formulation it is important that precision be determined using authentic homogeneous samples. A justification will be required if a homogeneous sample is not possible and artificially prepared samples or sample solutions are used.

6.2.2.1. REPEATABILITY

Repeatability is a measure of the precision under the same operating conditions over a short interval of time, that is, under normal operating conditions of the analytical method with the same equipment. It is sometimes referred to as intra - assay precision.

The ICH recommends that repeatability be assessed using a minimum of nine determinations covering the specified range for the procedure (e.g., three concentrations/three replicates as in the accuracy experiment) or using a minimum of six determinations at 100% of the test concentration. Reporting of the standard deviation, relative standard deviation (coefficient of variation), and confidence interval is required. The assay values are independent analyses of samples that have been carried through the complete analytical procedure from sample preparation to final test result.

6.2.2.2. INTERMEDIATE PRECISION

Intermediate precision is defined as the variation within the same laboratory. The extent to which intermediate precision needs to be established depends on the circumstances under which the procedure is intended to be used. Typical parameters that are investigated include day - to - day variation, analyst variation, and equipment variation. Depending on the extent of the study, the use of experimental design is encouraged. Experimental design will minimize the number of experiments that need to be performed. It is important to note that ICH allows exemption from doing intermediate precision when reproducibility is proven. It is expected that the intermediate precision should show variability that is in the same range or less than repeatability variation. ICH recommends the reporting of standard deviation, relative standard deviation (coefficient of variation), and confidence interval of the data.

6.2.2.3. REPRODUCIBILITY

Reproducibility measures the precision between laboratories. This parameter is considered in the standardization of an analytical procedure (e.g., inclusion of procedures in pharmacopeias and method transfer between different laboratories).

To validate this characteristic, similar studies need to be performed at different laboratories using the same homogeneous sample lot and the same experimental design. In the case of method transfer between two laboratories, different approaches may be taken to achieve the successful transfer of the procedure. The most common approach is the direct - method transfer from the originating laboratory to the receiving laboratory. The originating laboratory is defined as the laboratory that has developed and validated the analytical method or a laboratory that has previously been certified to perform the

procedure and will participate in the method transfer studies. The receiving laboratory is defined as the laboratory to which the analytical procedure will be transferred and that will participate in the method transfer studies. In the direct - method transfer, it is recommended that a protocol be initiated with details of the experiments to be performed and acceptance criteria (in terms of the difference between the means of the two laboratories) for passing the method transfer. Table 1 provides examples of a set of method transfer data between two laboratories.

	Runs	Average percent
Originating Laboratory	12	100.7
Receiving Laboratory	4	100.2

TABLE 1 Result from Method Transfer between Two Laboratories

6.2.4 SPECIFICITY

The ICH defines specificity as the ability to assess unequivocally an analyte in the presence of components that may be expected to be present. In many publications selectivity and specificity are often used interchangeably. However, there are debates over the use of specificity over selectivity and some authorities, for example, the International Union of Pure and Applied Chemistry (IUPAC), have preferred the term selectivity reserving specificity for those procedures that are completely selective. For pharmaceutical application, the above definition of ICH will be used.

For identity test, compounds of closely related structures which are likely to be present should be discriminated from each other. This could be confirmed by obtaining positive results (by comparison with a known reference material) from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. Furthermore, the identification test may be applied to material structurally similar or closely related to the analyte to confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sound scientific

judgment with a consideration of the interferences that could occur. The specificity for an assay and impurity tests should be approached from two angles:

6.2.4.1. IMPURITIES ARE AVAILABLE

The specificity of an assay method is determined by comparing test results from an analysis of sample containing the impurities, degradation products, or placebo ingredients with those obtained from an analysis of samples without the impurities, degradation products, or placebo ingredients. For a stability - indicating assay method, degradation peaks need to be resolved from the drug substance. However, these impurities do not need to be resolved from each other.

For the impurity test, the determination should be established by spiking drug substance or drug product with the appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix. Representative chromatograms should be used.

6.2.4.2. IMPURITIES ARE NOT AVAILABLE

Specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well - characterized procedure or other validated analytical procedure (orthogonal method). This should include samples stored under relevant stress conditions (light, heat, humidity, acid/base hydrolysis and oxidation). For the assay method, the two results should be compared; for impurity tests, the impurity profiles should be compared. Peak homogeneity tests should be performed using PDA or mass spectrometry to show that the analyte chromatographic peak is not attributable to more than one component.

6.2.5. DETECTION LIMIT

The detection limit (DL) is a characteristic for the limit test only. It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. The detection is usually expressed as the concentration of the analyte in the sample.

There are several approaches to establish the DL. Visual evaluation may be used for noninstrumental (e.g., solution color) and instrumental methods. In this case, the DL is determined by the analysis of a series of samples with known concentrations and establishing the minimum level at which the analyte can be reliably detected.

Presentation of relevant chromatograms or other relevant data is sufficient for justification of the DL.

For instrumental procedures that exhibit background noise, it is common to compare measured signals from samples with known low concentrations of analyte with those of the blank samples. The minimum concentration at which the analyte can reliably be detected is established using an acceptable signal - to - noise ratio of 2:1 or 3:1. Presentation of relevant chromatograms is sufficient for justification of the DL.

Another approach estimates the DL from the standard deviation of the response and the slope of the calibration curve. The standard deviation can be determined either from the standard deviation of multiple blank samples or from the standard deviation of they intercepts of the regression lines done in the range of the DL. This estimate will need to be subsequently validated by the independent analysis of a suitable number of samples near or at the DL:

$$LOD = 3.3\ \sigma/S$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve.

6.2.6. QUANTITATION LIMIT

The quantitation Limit (QL) is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. QL is defined as the concentration of related substance in the sample that will give a signal - to - noise ratio of 10 : 1. The QL of a method is affected by both the detector sensitivity and the accuracy of sample preparation at the low concentration of the impurities. In practice, QL should be lower than the corresponding ICH report limit.

ICH recommends three approaches to the estimation of QL. The first approach is to evaluate it by visual evaluation and may be used for noninstrumental methods and instrumental methods. QL is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantitated with acceptable accuracy and precision. The second approach determines

the signal - to - noise ratio by comparing measured signals from samples with known low concentrations of analyte with those of blank samples. QL is the minimum concentration at which the analyte can be reliably quantified at the signal to noise ratio of 10 : 1.

The third approach estimates QL by the equation

$$LOQ = 10\ \sigma/S$$

The slope S may be estimated from the calibration curve of the analyte. The value of σ may be estimated by (1) calculating the standard deviation of the responses obtained from the measurement of the analytical background response of an appropriate number of blank samples or (2) calculating the residual standard deviation of the regression line from the calibration curve using samples containing the analyte in the range of the QL.

Whatever approach is applied, the QL should be subsequently validated by the analysis of a suitable number of samples prepared at the QL and determining the precision and accuracy at this level.

6.2.7. LINEARITY

ICH defines linearity of an analytical procedure as the ability (within a given range) to obtain test results of variable data (e.g., absorbance and area under the curve) which are directly proportional to the concentration (amount of analyte) in the sample. The data variables that can be used for quantitation of the analyte are the peak areas, peak heights, or the ratio of peak areas (heights) of analyte to the internal standard peak. Quantitation of the analyte depends on it obeying Beer's law for the spectroscopic method over a concentration range. Therefore, the working sample

concentration and samples tested for accuracy should be in the linear range. There are two general approaches for determining the linearity of the method. The first approach is to weigh different amounts of standard directly to prepare linearity solutions at different concentrations. However, it is not suitable to prepare solution at very low concentration, as the weighing error will be relatively high. Another approach is to prepare a stock solution of high concentration. Linearity is then demonstrated directly by dilution of the standard stock solution. This is more popular and the recommended approach. Linearity is best evaluated by visual inspection of a plot of the signals as a function of analyte

concentration. Subsequently, the variable data are generally used to calculate a regression line by the least - squares method. At leasfi ve concentration levels should be used. Under normal circumstances, linearity is acceptable with a coefficient of determination (r^2) of ≥0.997. The slope, residual sum of squares, and y intercept should also be reported as required by ICH.

The slope of the regression line will provide an idea of the sensitivity of the regression, and hence the method that is being validated. They intercept will provide an estimate of the variability of the method. For example, the ratios percent of they intercept with the variable data at nominal concentration are sometimes used to estimate the method variability.

For the determination of potency assay of a drug substance or a drug product, the usual range of linearity should be ± 20% of the target or nominal concentration. For the determination of content uniformity, it should be ± 30% of the target or nominal concentration.

6.2.8. RANGE

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. The range is normally expressed in the same units as test results (e.g., percent, parts per million) obtained by the analytical procedure.

For the assay of drug substance orfi nished drug product, it is normally recommended to have a range of 80 – 120% of the nominal concentration. For content uniformity, a normal range would cover 70 – 130% of the nominal concentration, unless a wider and more appropriate range (e.g., metered - dose inhalers) is justified. For dissolution testing, a normal range is ± 20% over the spefi ed range. If the acceptance criterion for a controlled - release product covers a region from 20% after 1 h, and up to 90% after 24 h, the validated range would be 0 – 110% of the label claim. In this case, the lowest appropriate quantifiable concentration of analyte will be used as the lowest limit as 0% is not appropriate.

6.2.9. ROBUSTNESS

Robustness of an analytical procedure is a measure of the analytical method to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness is normally considered during the development phase and depends on the type of procedure under study. Experimental design (e.g., fractional factorial design or Plackett – Burman design) is common and useful to investigate multiple parameters simultaneously. The result will help to identify critical parameters that will affect the performance of the method. Common method parameters that can affect the analytical procedure should be considered based on the analytical technique and properties of the samples:

1. Sample preparation
   - **a**. Extraction time
   - **b**. Sample solvent (pH ± 0.05 unit, percent organic ± 2% absolute)
   - **c.** Membrane filters
   - **d.** Sample and standard stability

2. High - performance liquid chromatography (HPLC) conditions
   - **a.** Mobile - phase composition (pH ± 0.05 unit, percent organic ± 2% Absolute)
   - **b.** Column used (equivalent columns, lots and/or suppliers, age)
   - **c.** Temperature
   - **d.** Flow rate

3. Gas chromatography (GC) conditions
   - **a**. Column used (lots and/or suppliers, age)
   - **b.** Temperature
   - **c.** Flow rate

6.3. PROCESS OF ANALYTICAL METHOD VALIDATION

The typical process that is followed in an analytical method validation is chronologically listed below:

1. Planning and deciding on the method validation experiments
2. Writing and approval of method validation protocol
3. Execution of the method validation protocol
4. Analysis of the method validation data
5. Reporting the analytical method validation
6. Finalizing the analytical method procedure

The method validation experiments should be well planned and laid out to ensure efficient use of time and resources during execution of the method validation. The best way to ensure a well - planned validation study is to write a method validation protocol that will be reviewed and signed by the appropriate person (e.g., laboratory management and quality assurance). The validation parameters that will be evaluated will depend on the type of method to be validated. Analytical methods that are commonly validated can be classified into three main categories: identification, testing for impurities, and assay. Table 2 lists the ICH recommendations for each of these methods. Execution of the method validation protocol should be carefully planned to optimize the resources and time required to complete the full validation study. For example, in the validation of an assay method, linearity and accuracy may be validated at the same time as both experiments can use the same standard solutions. A normal validation protocol should contain the following contents at a minimum:

(a) Objective of the protocol

(b) Validation parameters that will be evaluated

(c) Acceptance criteria for all the validation parameters evaluated

(d) Details of the experiments to be performed

(e) Draft analytical procedure

The data from the method validation data should be analyzed as the data are obtained and processed to ensure a smooth flow of information. If an experimental error is detected, it should be resolved as soon as possible to reduce any impact it may have on later

experiments. Analysis of the data includes visual examination of the numerical values of the data and chromatograms followed by statistical treatment of the data if required.

TABLE 2: VALIDATION PARAMETERS

Type of Analytical Procedure Characteristics	Identification	Testing for impurities		Assay-Dissolution (measurement Only)-Content/Potency
		Quantitation	Limit	
Accuracy	-	+	_	+
Precision	-			
Repeatability	-	+	_	+
Intermediate Precision		+[a]	_	+[a]
Specificity[b]	+	+	+	+
Detection Limit	-	-[c]	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	_	+

Note: −, characteristic not normally evaluated; +, characteristic normally evaluated. a In cases where reproducibility has been performed, intermediate precision is not needed. b Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s). c May be needed in some cases.

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