International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 437-444 www.ijprajournal.com ISSN: 2249-7781

Chromatography – Separation Tool in Bioanalysis

Dipti Dudhade, Divya Rasal, Gautami Kambli, Leena Pawar.
MSc. BIOANALYTICAL SCIENCE student of
Guru Nanak Khalsa Collegeof Arts, Science and Commerce, Matunga, Mumbai.

Date Of Submission: 01-04-2021 Date Of Acceptance: 14-04-2021

ABSTRACT: Chromatography is very important and predominantly used analytical technique. It is used for separation, purification, identification of analyte of interest from mixture and their quantitative and qualitative analysis. There are various types of techniques available for different types of molecules based on their characteristics like size, shape, net charge, hydrophobicity and affinity. The two most essential component in chromatography are stationary phase and mobile phase. These techniques are applicable on large scale.

KEYWORDS: Chromatography, Mobile phase, Stationary phase, Separation, Identification.

I. INTRODUCTION:

Chromatography is one of the most widely used separation technique. The word 'Chroma' means color and 'graph' means writing. It was developed by Mikhail Tswett in 1900;Tswett is called as 'Father of Chromatography'. He was working on separation of plant pigments. It basically makes use of stationary and mobile phase for separation of components from the sample. The sample components are dissolved in the mobile phase. Mobile phase carries the sample into the column, where separation takes place on the basis of partition. Each sample component travels at a different speed and separation occurs in the column. Depending upon nature of phases the sample movement takes place.In each phase the concentration of compound is defined as the partition coefficient K.

$$K=\frac{Cs\ (\text{concentration of the component in stationary phase})}{Cm\ (\text{concentration of the component in mobile phase})}$$

There are different types of chromatographic techniques depending upon their separation principle (Figure 1).The components separated from the mixture are analyzed visually, derivatization is required for colorless samples. Detectors like UV, PDA, MS etc. are used for identification and quantification of components. Depending upon the retention time chromatogram is plotted. Chromatography has various applications in the different industries. In Food Industry, it can be applied for analysis of chemical additives in the food, to determine the shelf life of food substances. In Pharmaceutical Industry, it is used to analyze and identify the amount of chemicals present in the sample and to analyze the purity of drugs. It plays an important role in the development of new drugs. In Chemical Industry, it analyze the toxic compounds present in oils and pesticides also the purity of water samples can be tested. It can also be used for protein, enzyme and insulin purification. It also has many applications in the field of Life Sciences. [1]

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 437-444 www.ijprajournal.com ISSN: 2249-7781

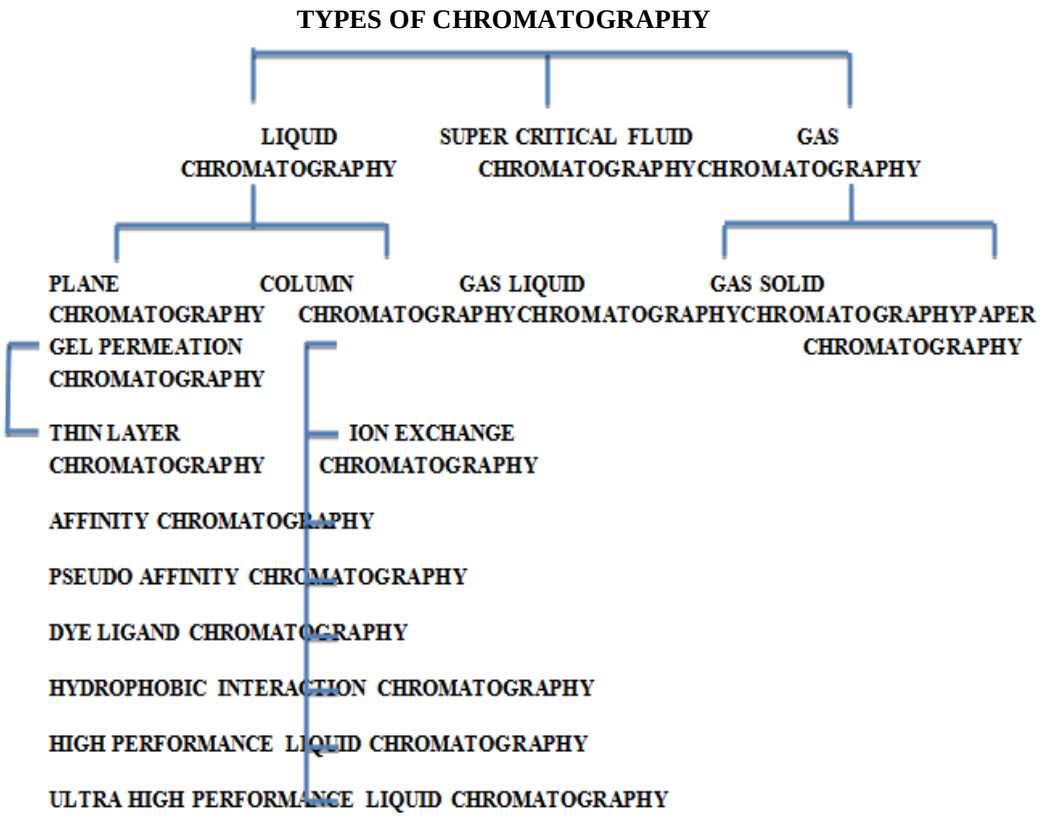

FIGURE 1

PAPER CHROMATOGRAPHY

Paper chromatography is a type of planar chromatography in which stationary phase is coated on a plane surface. Highly purified cellulose paper is used as a supporting matrix for the stationary phase which is mostly water since it is absorbed very well by cellulose. The stationary phase used is mostly polar in nature so less polar mobile phase is used for satisfactory resolution of sample components. Principle of separation is mainly partition (liquid-liquid interaction).The two main techniques employed for development of paper chromatography may be ascending or descending techniques. In ascending technique solvent moves in vertically upward direction by capillary action and the sample is separated. In descending technique solvent moves in downward direction due to the gravitational force. The third one is radial development technique in which circularly cut disc of paper is used and due to capillary action the sample component move in outward direction radially and forms concentring circle of increasing diameter. Fourth technique is known as two-dimensional chromatography, it is a technique in which two different solvent with different eluting properties are used and development is carried out in two directions. It is very simple technique with better efficacy of separation and very less sample is utilized. Paper chromatography technique is used for separation of dyes, pigments, amino acids, hormones, drugs etc. [2]

THIN LAYER CHROMATOGRAPHY (TLC)

TLC was discovered by Izmailov and Shraiber in 1938. The principle of separation is difference in adsorption of molecules. It is a Liquid-Solid adsorption technique. The solutes are separated on the basis of their degree of adsorption on the surface of adsorbent (stationary phase). A plate coated with silica/aluminium oxide is used as stationary phase. The sample is applied onto the plate in form of spots or band. Plate developing chamber is saturated using solvent (mobile phase). Plate is placed into chamber and mobile phase is

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 437-444 www.ijprajournal.com ISSN: 2249-7781

poured (Figure 2). The mobile phase (solvent) by the capillary action rises upward. "Like dissolves like" phenomenon the sample components which are more soluble in solvent move upwards with it. The components having more affinity towards stationary phase will stay near the point of application. Here we evaluate the Retardation factor (Rf value) of components.

$$\text{Rf Value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

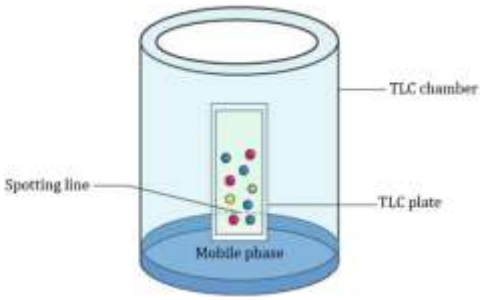

FIGURE 2 -THINLAYER CHROMATOGRAPHY

For visualization of samples UV Cabinets are used. Derivatization using specific reagents is carried out for the colourless samples. TLC is rapid, cost effective and sensitive technique. It is used for identification of compounds, to check purity of compounds. For better separation 2 – Dimensional TLC is carried out. High Performance Thin Layer Chromatography (HPTLC) is modified version of TLC. [3,4,5]

COLUMN CHROMATOGRAPHY

Column chromatography is a type of chromatography in which a single compound is separated from mixture of compounds. This chromatographic technique is used for purification Separated. [7,8]The different types of column chromatography are as follows: [9]

1. Affinity Chromatography
2. Pseudo-Affinity Chromatography
3. Dye-Ligand Chromatography
4. Ion Exchange Chromatography
5. Gel Permeation / Size Exclusion Chromatography
6. Gas Chromatography
7. Hydrophobic Interaction Chromatography
8. High Performance Liquid Chromatography (HPLC)
9. Ultra High Performance Liquid Chromatography (UHPLC)

of biomolecules. In this technique two phases are used i.e. stationary phase and mobile phase. Stationary phase is an adsorbent which has an immobilized surface. During chromatography, the analyte and solvent runs through the stationary phase. Mobile phase in column chromatography is liquid and in the case of gas chromatography mobile phase is gaseous. Mobilephase has a solvent which has no affinity towards the stationary phase. As the name suggest mobile meaning it moves together with the analyte through the column. The stationary phase used is porous matrix. The porous matrix used is agarose, cross linked polyacrylamide and dextran. The separation of mixture is based on the adsorption (Figure 3). [6]

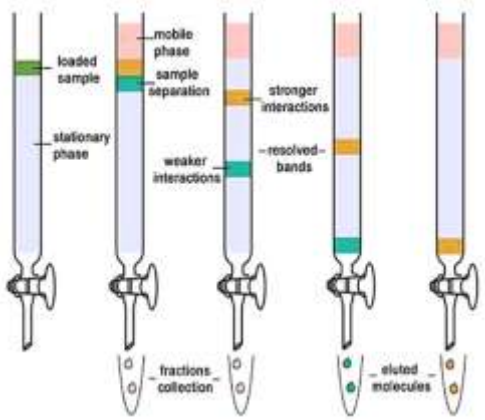

FIGURE 3 - COLUMN CHROMATOGRAPHY

The compounds which are strongly attracted to mobile phase will elute first and in contrast the compounds which are strongly attracted to stationary phase will elute later. In this way, compounds are

AFFINITY CHROMATOGRAPHY

A technique with high specificity was introduced by Pedro Cuatrecasas and his co-workers in 1968. Affinity chromatography is a type of separation chromatography in which separation is based on specific binding interaction between immobilized ligand and its binding partner. In affinity chromatography different types of interaction occurs i.e. antigen-antibody, enzyme-substrate and enzyme-inhibitor interactions. Stationary phase has porous support materials like agarose or any polymer (polyacrylamide, cellulose and silica). Sample can be dissolved in mobile phase i.e. serum, plasma, protein etc.For example when an analyte has to be separated from the mixture, it passes through the column. The analyte

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 437-444 www.ijprajournal.com ISSN: 2249-7781

that selectively binds to the complementary ligand is retained. Other impurities will elute without retention (Figure 4). The advantage of using this technique is that it is very specific, easy to perform, give better yield and throughput. This technique is used for purification of biomolecules, separation of antibodies and purification of antibodies from blood serum.[10,11]

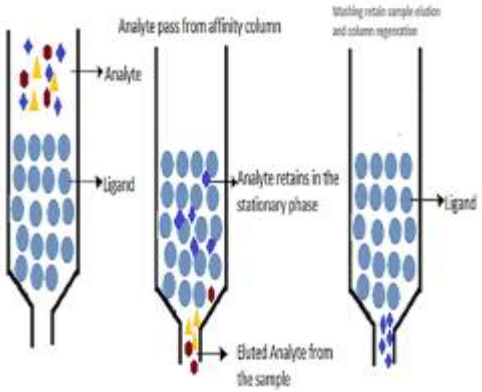

FIGURE4 - AFFINITY CHROMATOGRAPHY

PSEUDO-AFFINITY CHROMATOGRAPHY

Pseudo-affinity chromatography uses dyes, metal chelates as a ligand. [12]The dye used does not have biological relations with targeted protein and they mimic the natural ligands to interact with these proteins. [13] In this type of chromatography pseudo-specific interaction between ligand and molecule to be purified is seen. This interaction gives high efficiency. The adsorption of molecule of interest on ligand shows hydrogen bond and Van der Waals forces of interaction. [12] It is more environment friendly, less costly, widely available and highly stable chromatography technique. This technique is very effective in purifying plasmid DNA from E. coli cell lysate impurities and for intracellular enzyme purification. [13, 14]

DYE LIGAND CHROMATOGRAPHY

Dye ligand chromatography is a type of affinity chromatography. It is also called as dye ligand affinity chromatography. It is a type of purification technique that allows selectivity and high purification. The ligands used in this technique are reactive dyes. To develop a dye ligand chromatography technique, we have to optimize conditions like binding, washing and elution. The most commonly used dye in this chromatography is Cibacron Blue F3GA dye. This dye is widely used for Protein purification on large scale. Before using the dye in column, it has to be purified. This dye can be easily immobilized on matrix and it is resistant to chemical and enzymatic degradation. Here dye act as ligand for protein and binds to the nucleotide binding region of enzyme (enzymatic protein). As this dye has less specificity it can bind on another region of protein. For non-enzymatic proteins, this dye uses mechanism like hydrophobic interaction and ionic forces. [15,16]

ION EXCHANGE CHROMATOGRAPHY (IEC)

Ion Exchange Chromatography (IEC) or Ion Chromatography is a type of chromatography in which ions or polar molecules are separated based on their charge. The separation is due to ionic interactions i.e. reversible exchange of ions between the ions of interest in the sample to the ions present on ion exchanger.Depending upon the separation of ions of interest the ion exchanger is selected. There are two types of ion exchanger:Cationic exchanger will attract positively charged ions as they possess negatively charged groups. Anionic exchanger will attract negatively charged ions as they possess positively charged groups.

These ion exchangers are covalently bonded with the solid matrices. The matrices used can be made up of cellulose, polystyrene, agarose or polyacrylamide. When the mobile phase containing sample is passed through stationary phase that contains ion exchanger of opposite charge, the ions of interest binds to the ion exchanger. These ions can be eluted either by using buffer with varying pH or ionic strength. IEC is widely used, simple and gives higher resolution. It can be used for any charged particles as well as for large molecules separation. It can be used in food and clinical research, fermentation process, water purification, etc. [17]

GEL PERMEATION CHROMATOGRAPHY (GPC)

GPC is also known as Size Exclusion Chromatography, Gel Filtration Chromatography and Molecular Sieve Chromatography. It is a type of partition chromatography where the molecules partition themselves between stationary phase and mobile phase.[18]Basically the molecules are separated on the basis of their size or molecular weight. Stationary phase used here is a column packed with porous glass bead or gel. The sample is dissolved in an appropriate solvent (mobile phase). The sample is allowed to pass through the column. The larger molecules in the sample will

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 437-444 www.ijprajournal.com ISSN: 2249-7781

elute out first as compared to the smaller molecules. Large molecules pass through the interstitial spaces in the column. The smaller molecules enter the porous gel and hence their movement is retarded (Figure 5). [19]The degree of retardation is directly proportional to the time spent by the molecule inside the column. There is a relationship between the particles and molecular sieve gel. The particles distribute themselves

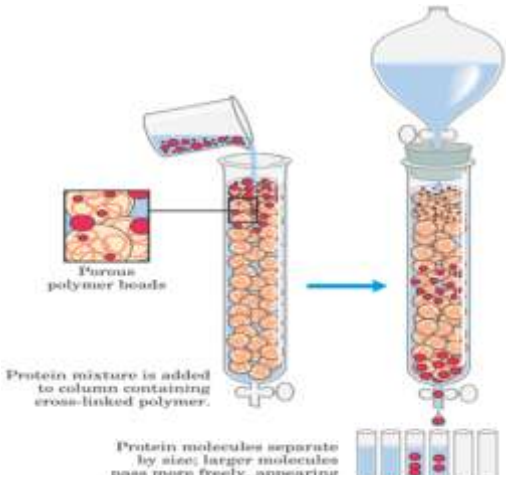

FIGURE 5 - GEL PERMEATION CHROMATOGRAPHY

GAS CHROMATOGRAPHY (GC)

Gas chromatography (GC) is a type of chromatography used to analyse volatile compounds. The separation in GC is based on partitioning of analytes between mobile phase i.e. carrier gas and stationary phase that can be either solid or liquid. The carrier gas used are nitrogen, helium, argon or hydrogen (Figure 6).

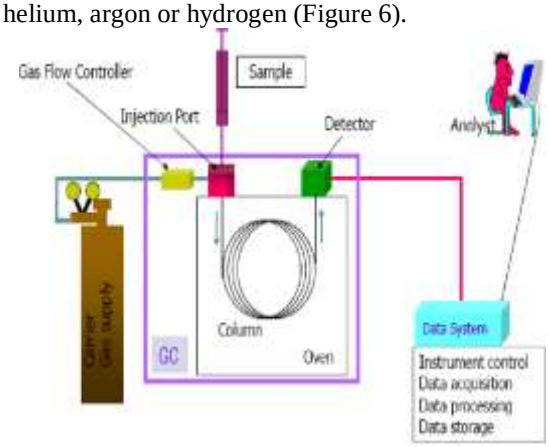

FIGURE 6 - GAS CHROMATOGRAPHY

The sample is injected through the injector port. The sample gets carried away by carrier gas into the column. The sample molecules get separated between the stationary phase and mobile phase based on their affinity. Each components have different retention time based on which they get eluted out and get detected in the detector. The most commonly used detectors are Mass Spectrometer (MS), Thermal Conductivity (TCD), Flame Ionization (FID), Electron Capture (ECD) and Photoionization (PID). GC gives high resolution and sensitivity. It is also easy to perform and requires small amount of sample. GC can be used in petroleum industry, food industry, pharmaceutical industry etc. [20]

between the inner and outer solvent in column. This is defined as distribution co-efficient K_d.For larger molecules K_d= 0 For smaller molecules K_d = 1 [18]$V_e=V_O + K_d (V_t – V_O)$ V_e - Elution Volume, V_O–Void volume, Vt- total volume. Most commonly used gels areSephadex, Agaroseand Polyacryl Amide gel. GPC is used in determination of molecular weight, separation of biological molecules like proteins, nucleic acids.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

Hydrophobic Interaction Chromatography (HIC) also known as salting out. It is widely used for separation and purification of proteins. The separation in HIC is based on differences in the hydrophobicity of protein molecules. The HIC column is packed with porous matrix (agarose or sepharose) to which hydrophobic groups are attached. The sample is introduced into the column containing salt buffer (Ammonium Sulphate) that allows the interaction of hydrophobic or hydrophilic region of proteins with the hydrophobic groups on the matrix (Figure 7). [21]

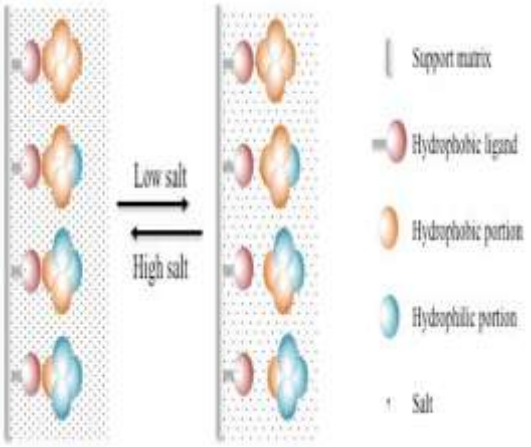

FIGURE 7 -HYDROPHOBIC INTERACTION CHROMATOGRAPHY

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 437-444 www.ijprajournal.com ISSN: 2249-7781

The decreasing gradient of salt buffer is used to elute out the protein molecules based on their increasing order of hydrophobicity. HIC has higher selectivity and less structural damage. Besides protein purification, HIC also be used for monoclonal antibodies purification.[22]

High Performance Liquid Chromatography (HPLC)

HPLC is a type of column chromatography in which stationary phase is coated on the column i.e. column filled with a packing material like silica, polymers, etc. (C18 column is frequently used). The mobile phase used is particularly mixture of aqueous solvent (water) and organic solvent (acetonitrile, methanol and propanol). The separation is carried out by passing pressurized liquid (mobile phase) and sample mixture through a column and it is based upon the affinity of analytes and its relative solubility in stationary phase and mobile phase respectively. The principle of separation is adsorption and the component which has more affinity towards the absorbent travel slower and vice versa. It is widely used analytical technique. Diverse type of component can be separated by using different types and modes of chromatographic separation techniques like normal phase, reverse phase, ion exchange, analytical, preparative etc. Separation of different analytes is carried out by using gradient and isocratic elution techniques of mobile phase.[23]Mobile phase and reservoirs, high pressure pump, injection valve, column, suitable detector like refractive index detector (RI), UV-Visible detector, fluorescence detector, electrochemical detector (ECD)etc. and computer based data acquisition system that gives result of separation in the form of chromatogram which is further interpreted. These are the important components ofHPLC system (Figure 8). [2,23, 24] Van Deemeter equation is important empirical formula for optimization of chromatographic performance. It explains factor responsible for band broadening that results in poor resolution, less efficiency of column.

$$H = A + \frac{B}{\mu} + C\mu$$

Where,

H= Height Equivalent of Theoretical Plate (HETP),A= Eddy Diffusion

B= Longitudinal Diffusion, C= Mass Transfer, μ=Linear velocity. [2]Because of its non-destructive nature, good recovery, better separation and the better speed of analysis, HPLC is widely used for separation of proteins, salts, nucleic acid, polymer, hydrocarbons, enzymes etc. It is widely used technique in drug research and development, pharmaceutical industries and laboratories, quality assurance, forensic analysis etc. [2, 23, 24, 25]

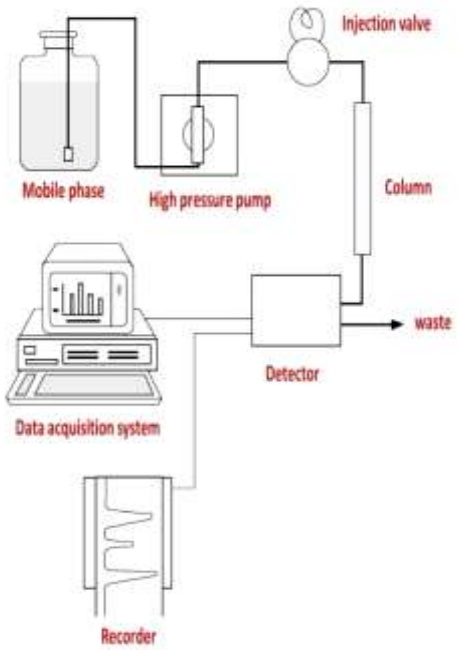

FIGURE 8 - HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (UHPLC)

UHPLC is a modified version of HPLC. It is a liquid chromatography which is based on the principle of partition of analytes between the stationary and mobile phase.It provides improved chromatographic resolution, high speed and good sensitivity. Separation is based on Van Demeter Equation as mentioned above. We make use of special UHPLC column with particle size less than 2 micrometre. Lower the particle size higher is the column efficiency. Commonly used columns are Charged Surface Hybrid [CSH], Ethylene Bridged Hybrid [BEH] etc. The sample gets separated in the column and then gets detected by detectors. Detectors used in UHPLC are UV-Visible, Photodiode Array, Refractive Index and Mass Spectrometer (Figure 9).It is used in determination of pesticides, amino acid determination, impurity profiling, metabolite identification and analysis of food samples. [26,27, 28, 29, 30]

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 437-444 www.ijprajournal.com ISSN: 2249-7781

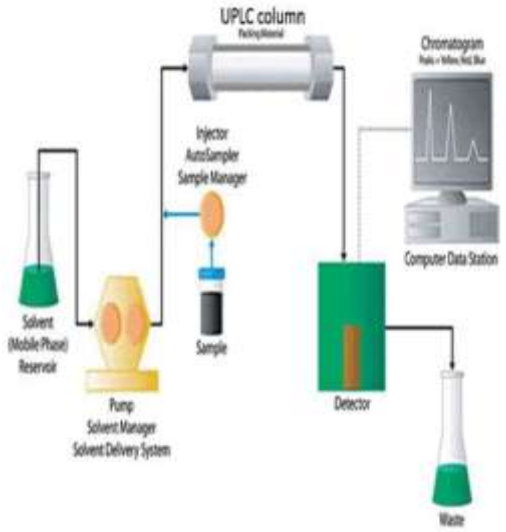

FIGURE 9 – ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

II. CONCLUSION

From past few decades chromatographic techniques has evolved tremendously. There is a shift from paper chromatography to the high throughput column chromatography. Depending upon the type of sample to be analysed we can select an appropriate chromatographic technique. They come in a wide range of cost i.e. from cost effective to high cost. For some methods manual mode is preferred while some are automated. We not only separate components from the mixture but also quantify them by coupling with an appropriate detector. It is used widely in the field of Bioanalytical Science.

REFERENCES

[1]. Ebere, Enyoh&Isiuku, Beniah&Wirnkor, Verla, (2019). Applications of Column, Paper, Thin Layer and Ion Exchange Chromatography in Purifying Samples: Mini Review.

[2]. Priyadarshini, Rekha & Marshall Raj, Gerard &Shewade, Deepak. (2016). Chromatography - the essence of bioanalysis. European Journal of Biomedical and Pharmaceutical Sciences. 3. 366-77.

[3]. Cai, Li. (2014). Thin Layer Chromatography. 10.1002/9780470089941.et0603s08.

[4]. Jyotirmayee, K. & Sarangi, M. (2013). Thin layer chromatography: A tool of biotechnology for isolation of bioactive compounds from medicinal plants. International Journal of Pharmaceutical Sciences Review and Research. 18. 126-132.

[5]. Matysik, Elżbieta& Wozniak, Anna &Paduch, Roman &Rejdak, Robert &Polak, Beata&Donica, Helena. (2016). The New TLC Method for Separation and Determination of Multicomponent Mixtures of Plant Extracts. Journal of Analytical Methods in Chemistry. 2016. 1-6. 10.1155/2016/1813581.

[6]. Akash, M. S., &Rehman, K. (2019). Thin layer chromatography. Essentials of Pharmaceutical Analysis, 157-165. https://doi.org/10.1007/978-981-15-1547-7_12

[7]. How column chromatography works to separate proteins. (n.d.). Retrieved March 27, 2021, from https://www.goldbio.com/articles/article/how-column-chromatography-works-to-separate-proteins#_Toc54883472

[8]. Coşkun, Ozlem. (2016). Separation techniques: Chromatography. Northern Clinics of Istanbul. 3. 10.14744/nci.2016.32757.

[9]. Column chromatography. (2019, October 16). Microbe Notes.

[10]. Magdeldin, Sameh & Moser, Annette. (2012). Affinity Chromatography: Principles and Applications. 10.5772/39087.

[11]. Affinity chromatography. (n.d.). Pharma information Zone. https://pharmainformationzone.blogspot.com/2020/05/affinity-chromatography.html

[12]. Champagne, Jérôme & Delattre, Cédric & Shanthi, C.babu & Satheesh, B. & Duverneuil, L. & Vijayalakshmi, M.. (2007). Pseudoaffinity Chromatography Using a Convective Interaction Media®-Disk Monolithic Column. Chromatographia. 65. 639-648. 10.1365/s10337-007-0225-7.

[13]. Jahanshahi, M., & Ghoreyshi, A., & Khavarpour, M., & Ling, T. (2006). Analysis of Performance ofthe Anion Exchange and Pseudo-Affinity Chromatography for Intracellular Enzyme Purification. Iranian Journal of Chemical Engineering, 3(1), 92-107.

[14]. Caramelo-Nunes, C & Gabriel, Marta & Almeida, Paulo & Marcos, João &Tomaz, Cândida. (2012). Purification of plasmid DNA from clarified and non-clarified Escherichia coli lysates by berenil pseudo-affinity chromatography. Journal of

chromatography. B, Analytical technologies in the biomedical and life sciences. 904. 81-7. 10.1016/j.jchromb.2012.07.023.
[15]. Yokoyama, M. (2018, October 25). Dye-ligand Affinity Chromatography for Protein Purification. News-Medical.Net.
[16]. Boyer P.M and Hsu J. T. (1993). Protein purification by dye-ligand chromatography. Advances in Biochemical Engineering/Biotechnology, 1-44. Doi: 10.1007/bfb0046571.
[17]. Cummins, Phil & Dowling, Oonagh & O'Connor, Brendan. (2011). Ion-Exchange Chromatography: Basic Principles and Application to the Partial Purification of Soluble Mammalian Prolyl Oligopeptidase. Methods in molecular biology (Clifton, N.J.). 681. 215-28. 10.1007/978-1-60761-913-0_12.
[18]. Ó'Fágáin, Ciarán & Cummins, Phil & O'Connor, Brendan. (2017). Gel-Filtration Chromatography. 10.1007/978-1-4939-6412-3_2.
[19]. Wolf, Marcell. (2015). Effective interactions in liquid-liquid phase separated protein solutions induced by multivalent ions. 10.15496/publikation-4822.
[20]. Al-Farga, Ammar & Q.AL-Bukhaiti, Wedad. (2017). Gas Chromatography: Principles, Advantages and Applications in Food Analysis. International Journal of Science Innovations and Discoveries.
[21]. Chen, T. & Zhang, Kelly & Gruenhagen, J. & Medley, C.D. (2015). Hydrophobic Interaction Chromatography for Antibody Drug Conjugate Drug Distribution Analysis. American Pharmaceutical Review. 18.
[22]. O'Connor, Brendan & Cummins, Phil. (2017). Hydrophobic Interaction Chromatography. 10.1007/978-1-4939-6412-3_18.
[23]. Kupiec, Tom. (2004). Quality-Control Analytical Methods: High-Performance Liquid Chromatography. International journal of pharmaceutical compounding. 8. 223-227.
[24]. Malviya, Rishabha& Bansal, Vvipin& Pal, Om & Sharma, Pramod. (2010). High performance liquid chromatography: A short review. Journal of Global Pharma Technology. 2. 22-26.
[25]. Sankar, Ravi &Snehalatha, K &Firdose, Shaik&Babu, Puttagunta. (2019). applications in HPLC in pharmaceutical analysis. International Journal of Pharmaceutical Sciences Review and Research. 59. 117-124.
[26]. Shaikh, Tabrez & Hussain, Sayyed. (2016). ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC): A NEW TREND IN ANALYSIS. World Journal of Pharmaceutical Research.
[27]. Chawla, Gita & Ranjan, Chanda. (2016). Principle, Instrumentation, and Applications of UPLC: A Novel Technique of Liquid Chromatography. Open Chemistry Journal. 3. 1-16. 10.2174/1874842201603010001.
[28]. Dadakova, Eva & Křížek, Martin & Pelikánová, Tamara. (2009). Determination of biogenic amines in foods using ultra-performance liquid chromatography (UPLC). Food Chemistry. 116. 365-370. 10.1016/j.foodchem.2009.02.018.
[29]. Wirtz, Astrid. (2014). Ultra-High Performance Liquid Chromatography and Its Applications. Journal of Separation Science. 37. 10.1002/jssc.201470014.
[30]. Chandraman K. An Updated Review on Ultra Performance Liquid Chromatography. Anal Chem Ind J. 2016;16(15):114.