Separation techniques in biotechnology

Vyas Rama Shivkumar*, Patel Deepa Rasikbhai[1], Prajapati Shailesh Thanaji[2]

*,1,2-Kalol Institute of Pharmacy, Kalol, Kalol(N.G) Gujrat ,382721 ,India

An open access journal

Supporting Information:

Received: 18 January 2019
Accepted: 31 January 2019
Published: 07 February 2019

Competing Interests:
The authors have declared that no competing interests exist.

Corresponding author address

Miss Vyas Rama Shivkumar*
Kalol Institute of Pharmacy,
Kalol,B/H Old Janpath Hotel,
Ahmedabad Mehsana Highway,
Kalol(N.G)-382721,Gujrat
India

ABSTRACT

The development of the prescription drugs brought a revolution in human health. These pharmaceuticals would serve their intent as long as they're free from impurities and are administered in an acceptable quantity. to form medicine serve their purpose numerous chemical and instrumental strategies were developed at regular intervals that are concerned within the estimation of medication.This article is related to separation technique in biotechnology. It provides an excellent deal of help to separate macromolecule and many organic drug .There is a long-run movement towards miniaturisation and integration of separation with preparative or analytical steps. several separation technique during this field like adsorption, partition coefficient, liquid chromatography, HPLC, activity different are studied. As every analytical technique has its own demands, the advances of the on top of technologies are mentioned for various applications in pharmaceutical analysis wherever high-throughput analysis are often meaningful, i.e. during a drug discovery and development setting, and in quality operations.

Keyword: Separation technique, HPLC, chromatography, electrophoresis.

Introduction:

Biotechnological separation techniques are established in more or less every product separation or purification system treat fermentation. These quality control techniques take in adsorption, ion exchange and liquid chromatography on solid supports. The main objective of this article is to place this varied method in perspective, relative to each other, so that collection of the appropriate technique or mixture of techniques may be readily made. Emphasis has been placed on laboratory evaluation techniques, the increase of these techniques and their industrial applications. Special techniques in analytical chemistry additionally play a crucial role within the analysis of the many chemical compounds. Physical separation techniques ar the foremost common, least high-priced and safest. part separation is additionally comparatively simple however tends to be dearer. The specialty chemical and pharmaceutical industries ar the first users of part separation techniques. part separation is merely now being applied to hazardous wastes and plenty of processes are still within the demonstration stage.

Definition [1]**:**Separation method defines as covert ion the mixture of substitute in to one or more distinct product mixture. in which method at least one result of the separation enriched in one or more source of the constitute.

List of separation techniques:

Adsorption, is define as adhesion of ion, atoms or molecules of gas, liquid, dissolved to a surface or solids dissolved to a surface.

- Capillary electrophoresis
- Centrifugation and cyclonic separation
- High-performance liquid chromatography (HPLC)
- Thin-layer chromatography (TLC)
- Countercurrent chromatography (CCC)
- Droplet countercurrent chromatography (DCC)
- Paper chromatography
- Ion chromatography
- Size-exclusion chromatography
- Affinity chromatography
- Centrifugal partition chromatography
- Crystallization
- Decantation

- Electrophoresis, It is separation of organic molecules according to their different interaction with a gel under an electric potential.
- Electrostatic separation, is based on the principle of corona discharge, in which two plates are placed close together and high voltage is applied to separate the ionized particle.
- Elutriation
- Evaporation
- Extraction
- Supercritical fluid extraction
- Liquid-liquid extraction
- Solid phase extraction
- Field flow fractionation
- Dissolved air flotation, it is removal of the suspended solids non-selectively from slurry by bubbles that are generated by air coming out of solution.
- Flotation, It is the recovery of valuable, hydrophobic solids by attachment to air bubbles, and generated by mechanical agitation of an air-slurry mixture, which float, and are recovered.
- Flocculation, is define as separation of solid from a liquid in a colloid, by using the flocculent, which promotes the solid clumping into flock.
- Filtration – Mesh: in which bag and paper filters are used to remove large particulates that are suspended in fluids ,while membrane processes including microfiltration, ultra filtration, nano filtration, reverse osmosis, dialysis utilizing synthetic membranes, separates micrometer-sized or smaller species.
- Fractional distillation
- Fractional freezing
- Magnetic separation
- Precipitation
- Recrystallization
- Scrubbing, separation of solids or gases from a gas stream using liquid.
- Sedimentation, separation using vocal density pressure differences.
- Gravity separation
- Sieving
- Stripping
- Winnowing
- Zone refining
- Sublimation
- Vapor-liquid separation, separates by gravity, based on the Sounders-Brown equation

Adsorption Chromatography [2,12]:

In separation of solutes take place, the partition of the solutes between two liquid phases, in which the original solvent and the film of solvent take place on the adsorption column. Thin-layer chromatography. in which the stationary phase is known as thin layer of an adsorbent such as silica gel coated on a flat plate.Adsorption is the phenomenon in which molecules in a fluid phase take place on a solid surface without any chemical change.

Principle:

Adsorption Chromatography involves the analytical separation of a chemical mixture by passing it over an adsorbent bed that adsorbs different compounds at different rates.

Type of force:

Dipole–Dipole Attraction: This force takes place between polar adsorbent and polar solutes.

Hydrogen bonding: This bond weaker than covalent bonds. Hydrogen bonds are formed between the OH group hydrogen and electronegative atoms such as Oxygen, nitrogen in solutes.

Polarizability.: These forces take place between polar adsorbents and solutes that can polarize such as aromatic compounds.

Weak covalent bonds: this force during complex formation.

Van Der Waals forces: Non polar attraction forces take place between the atoms of nuclei and electrons of other atoms.

ADSORPTION THEORY [11]:

Adsorption theory classify in two parts.

1. Adsorption Isotherm

2. Adsorption Kinetics.

1**. Adsorption isotherm**:

The Freundlich equation or Freundlich adsorption isotherm, it is an empirical relation between the concentrations of a solute on the surface of an adsorbent to the concentration of the solute in the liquid with contact value. In 1909 ,Herbert Freundlich representing the isothermal variation of adsorption of a quantity of gas adsorbed by unit mass of solid adsorbent.

This equation is known as Freundlich adsorption isotherm/ Freundlich adsorption equation. in this relationship where adsorption behavior can be properly depend on the isotherms with a theoretical basis, it is usually appropriate to use such isotherms instead (example :the Langmuir and BET adsorption theories).

Freundlich adsorption isotherm:

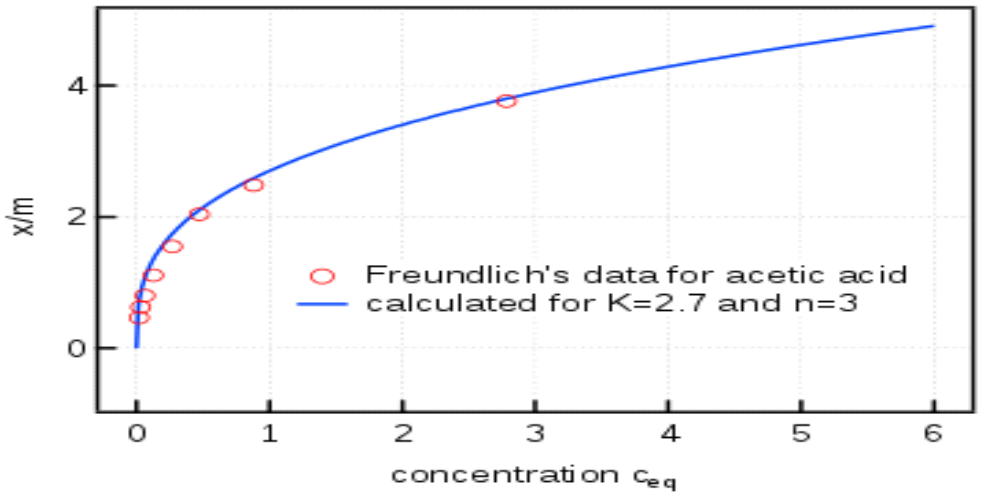

Fig 1 Freudlich Adsorption Isotherm

Freundlich original data for adsorption of acetic acid and a fit according to Freundlich exponential law

The Freundlich adsorption isotherm is mathematically It is written as

log X/M =log K +1/n log P

Where;

x = mass of adsorbate

m = mass of adsorbent

p = Equilibrium pressure of adsorbate

c = Equilibrium concentration of adsorbate in solution.

Here K and *n* are constants for adsorbate and adsorbent at a particular temperature.

At high pressure 1/n = 0.

Limitation of freundlich adsorption isotherm:

This theory was determined that the extent of gas adsorption varies directly with pressure and then it directly varies with pressure raised to the power 1/n until saturation pressure P_s is reached. at that point, the rate of adsorption saturates even applying higher pressure. The Freundlich adsorption isotherm fails at higher pressure. It is used where the solute is unknown, like adsorption of colored material from sugar, vegetable oil etc.

2. Adsorption Kinetics:

The rate of adsorption, R_{ads}, of a molecule onto a surface can be expressed in the similar manner as any kinetic process. Like example, when it is present in terms of the partial pressure of the molecule in the gas phase above the surface:

$$\mathbf{R_{ads} = k' P^{x}}$$

where, k-rate constant

P - Partial pressure

If the rate constant is then it is represent in an Arrhenius form, then we can writing kinetic equation of the form: $\mathbf{R_{ads} = A \exp(-E_a / RT). P^{x}}$

Where E_a = the activation energy for adsorption,

A = pre-exponential (frequency) factor.

The rate of adsorption is governed by

The Rate of molecules at the surface.

The proportion of incident molecules which undergo adsorption.

Ex. we can express the rate of adsorption (per unit area of surface) as a product of the incident molecular flux, F, and the sticking probability, S . $\mathbf{R}_{ads} = \mathbf{S.\ F}$ m^{-2} s^{-}

The flux of incident molecules is present by the Hertz-Knudsen equation

Flux, $F = P / (2\pi mkT)^{1/2,}$ molecules m^{-2} s^{-1}

Where, p- gas pressure [Nm^{-2}]; M-mass (kg) ; T- Temperature.

the adsorbate / substrate system must in the range $0 < S < 1$.

$\boldsymbol{S = f(\theta).\ Exp\ (-E_a / RT)}$

Where, E_a is the activation energy for adsorption and f (θ) is undetermined,

Combining the equations for *S* and *F* yields the following equations for the rate of adsorption:

$$R = \frac{f(\theta).P}{\sqrt{2\pi mkT}} \exp(-E_a / RT)$$

Estimating surface coverage's arising as a result of gas exposure:

If a surface is first clean and after that exposed to a gas pressure under conditions where the rate of desorption is very slow, then the coverage of adsorbed molecules may at first be estimated simply by consideration of the kinetics of adsorption.

As describe above, the rate of adsorption is given by: $\boldsymbol{R_{ads} = S.F}$

i.e.

$$\frac{dN_{ads}}{dt} = SF$$

Where: N_{ads} is the number of adsorbed species per unit area of surface.

In general, this equation must be integrated to obtain an expression for N_{ads}, since the sticking probability is coverage dependent.

However, if it is assumed that the sticking probability is necessary to constant (which may be a reasonable for relatively low coverage), then this integration simply yields:

$$N_{ads} = SFt$$

TYPES OF ADSORBENTS:

1. Carbon and Activated Charcoal

2. Silica Gels, AIuminas and Zeolites.

3. Organic Polymer Adsorbents

2.Carbon and Activated Charcoal: Carbon and Activated Charcoal are characterized by having high surface areas which are made with combined hydrogen and oxygen. These surfaces are consisting of reactive polar groups. It was suggested (29) in 1959 that the surface of these carbons may have carbonyl, hydroxyl, carboxyl and lactones groups. The raw material selected for the preparation of activated charcoal has a definite effect on the adsorbent's properties. Wood, coconut, lignite, bituminous coal, sub bituminous coal, petroleum acid and sludge activated coal have been used as raw materials.

3.Silica gels, alumina's and zeolites: Silica gels can be known as an amorphous, inorganic condensation polymer of silica acid. Silica gel, a typical polar adsorbent, contains four main types of functionality at its surface. The alumina adsorbents surface contains hydroxyl groups of similar structure to the silica gels. However, the potential for ion exchange reactions is greater with alumina compare to silica gel. Calcined alumina is strongly basic and must be washed with acid to produce a neutral or slightly acidic surface. on the alumina strength of the adsorption for aliphatic compounds containing various functional groups has been shown to increase in the following order: -S-, -0-, -C=N, -CO 2-, -CO-, -OH, -N=, -NH,, -SO, -CONH.

3. Organic Polymer Adsorbents:

Ion exchange resin manufacturers have also produced macro porous resins. that is may be used as adsorbents in purification applications. Although these resins may contain very weakly acidic or basic groups, not as ion exchange resins. They are normally used in neutral or slightly acidic solutions anywhere little or no ion exchange activity is displayed.

B. Capillary electrophoresis [13-14]

Capillary electrophoresis is an analytical technique in which separates ions based on their electrophoresis mobility with the use of an applied voltage. The electrophoresis mobility is dependent upon the charge of the molecule, the viscosity of the solution, and the atom's radius. In applying a capillary in electrophoresis had solved some common problems in traditional electrophoresis. For example, the surface to volume ratio is increased by the thin dimensions of the capillaries, which eliminated overheating by high voltages. Increased efficiency and the remarkable separating capabilities of capillary electrophoresis.

Principle:

Capillary electrophoresis separates molecules due to their electrophoresis motilities. A molecule's electrophoresis mobility depends on its charge and how much it is attracted or repelled by the voltage as well as the frictional drag force that resists movement. Here Friction is comparative to the radius of the molecule. Thus, electrophoresis mobility is based on size and charge. The velocity a charged molecule travels down a capillary is the product of its electrophoresis mobility and the applied electric field. Higher voltages so direct to faster velocities and faster separations.

The majority capillary electrophoresis instruments are put up with the negative voltage at the detector end and the positive voltage at the inlet. This way that positively-charged molecules migrate towards the cathode at the end, while negatively-charged molecules migrate the other way. All molecules are seen at the detector however, because there is a bulk fluid flow called electro osmotic flow. The migration arrange is so positively-charged, neutral, and then negatively-charged molecules.

UV-Vis is general and does not require classification as long as the molecule has a double bond. Still, the absorbance depends on the path length, which is little for a 50-µm capillary. A bubble cell/z-cell will increase the path length..

Instrument setup:

A high-voltage power supply, a sample introduction system, a capillary tube, a detector and an output device all are consists of typical capillary electrophoresis system. Some instruments contain a temperature control device to ensure reproducible results. This is since the separation of the sample depends on the electrophoresis mobility and the viscosity of the solutions decreases as the column temperature rises. Each side of the high voltage power supply is related to an electrode.

These electrodes help to stimulate an electric field to initiate the migration of the sample from the anode to the cathode through the capillary tube. The capillary is ready of fused silica and is sometimes coated with polyimide. every side of the capillary tube is dipped in a vial containing the electrode and an electrolytic solution, or aqueous buffer. Before the sample is introduce to the column, the capillary have to be flushed with the desired buffer solution. There is regularly a small window close to the cathodic end of the capillary which allows UV-VIS light to pass through the analyte and measure the absorbance. A photomultiplier tube is as well connected at the cathodic end of the capillary, which enables the construction of a mass spectrum, provided that information about the mass to charge ratio of the ionic species

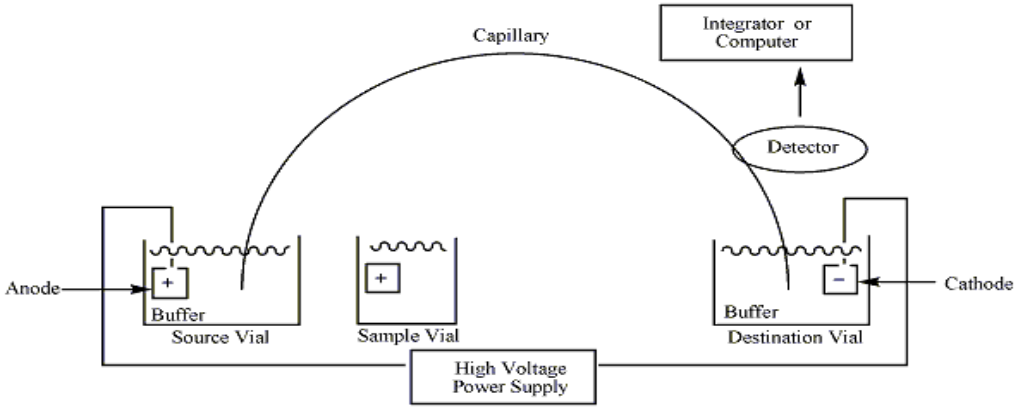

Figure 2: Instrumental Setup

Electrophoresis mobility:

Electrophoresis is the procedure in which sample ions travel under the influence of an applied voltage. The ion undergoes a force that is equivalent to the product of the net charge and the electric field strength. It is also precious by a drag force that is equal to the product of, the translational friction coefficient, and the velocity. This leads to the expression for electrophoresis mobility:

μ EP =q f=q6πηr (1.1) (1.1) μ EP=q f=q6πηr

Where; f for a spherical particle is certain by the Stokes' law; η is the viscosity of the solvent, and rr is the radius of the atom. The rate at which these ions migrate is dictated via the charge to mass ratio. The actual velocity of the ions is indirectly proportional to E, the magnitude of the electrical field and can be determined by the following equation:

v=μ EPE (1.2) (1.2) v=μ EPE

This relationship shows that a better voltage will quicken the movement of the ionic species.

electro osmotic flow:

The electro osmotic flow (EOF) is produce by applying high-voltage to an electrolyte-filled capillary. This flow occurs as the buffer running during the silica capillary has a pH greater than 3 and the SiOH groups lose a proton to become SiO^- ions. The capillary wall after that has a negative charge, which develops a double layer of cations attracted to it. The inner cat ion layer is stationary, while the outer layer is free to move along the capillary. The applied electric field causes the free of charge cations to move toward the cathode creating a powerful bulk flow. The rate of the electro osmotic flow is governed by the following equation:

M EOF=ϵ4πηEζ (1.3) (1.3) μ EOF=ϵ4πηEζ

Where ε is the dielectric stable of the solution, η is the viscosity of the solution, E is the field strength, and ζ is the zeta potential. Because the electrophoresis mobility is greater than the electro osmotic flow, negatively charged particles, which are naturally attracted to the positively charged anode, will separate out as well. The EOF works best with a large zeta potential between the cat ion layers, a large diffuse layer of cations to drag more molecules

towards the cathode, low resistance from the nearby solution, and buffer with pH of 9 so that all the SiOH groups are ionized.

capillary electro separation method:

There are six types of capillary electro separation available: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), Micellar electro kinetic capillary chromatography (MEKC), capillary electro chromatography (CEC), capillary iso electric focusing (CIEF), and capillary isotachophoresis (CITP). They can be classified into constant and alternating systems as shown in Figure 3. A constant system has a background electrolyte acting throughout the capillary as a buffer. This can be broken down into kinetic and steady-state (varying electrolyte composition) processes.

A alternating system keeps the sample in distinct zones separated by two different electrolytes.

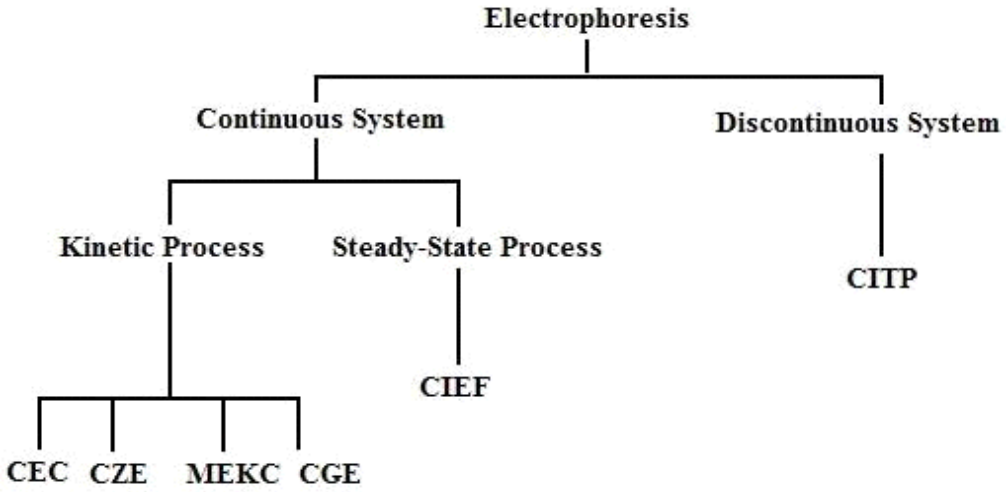

Figure:3 Categorization of Electrophoresis Techniques

Capillary zone electrophoresis:

Capillary Zone Electrophoresis (CZE), also known as free solution capillary electrophoresis, it is the the majority normally used technique of the six methods. A mixture in a solution can be separated into its character components quickly and easily. The separation is based on the differences in electrophoresis mobility, which is directed proportional to the charge on the molecule, and inversely proportional to the viscosity of the solvent and radius of the atom. The

velocity at which the ion moves is directly proportional to the electrophoresis mobility and the magnitude of the electric field.

The fused silica capillaries have silanol groups so as to become ionized in the buffer. The negatively charged SiO^- ions pull towards you positively charged cations, which form two layers—a stationary and diffuse cat ion layer. In the presence of an applied electric field, the diffuse layer migrates towards the negatively charged cathode creating an electrophoresis flow (μ e p μ e p) that drags bulk solvent along with it. Anions in resolution are involved to the positively charged anode, but get swept to the cathode as well. Cations with the major charge-to-mass ratios separate out first, followed by cations with reduced ratios, neutral species, anions with smaller charge-to-mass ratios, and finally anions with better ratios. The electro osmotic velocity can be adjusted by altering pH, the viscosity of the solvent, ionic strength, voltage, and the dielectric constant of the buffer.

Capillary gel electrophoresis:

CGE uses separation based on the disparity in solute size as the particles migrate throughout the gel. Gels are helpful because they minimize solute diffusion that causes zone broadening, stop the capillary walls from absorbing the solute, and limit the heat transfer by slowing down the molecules. A normally used gel apparatus for the separation of proteins is capillary SDS-PAGE. It is a very susceptible system and simply requires a little amount of sample.

Micellar Electro Kinetic Capillary Chromatography (Mekc):

MEKC is a separation technique so as based on solutes partitioning between micelles and the solvent. Micelles are aggregates of surfactant molecules that form when a surfactant is additional to a solution above the critical micelle concentration. The aggregates have polar negatively charged surfaces and are logically attracted to the positively charged anode. Because of the electro osmotic flow toward the cathode, the micelles are pull to the cathode as well, but at a slower rate. Hydrophobic molecules will waste the majority of their time in the micelle, while hydrophilic molecules will travel quicker through the solvent. When micelles are not present,

neutral molecules will migrate with the electro osmotic flow and no separation will occur. The attendance of micelles results in a retention time to where the solute has small micelle interaction and retention time tmc where the solute strongly interacts. Neutral molecules will be divided at a time between to and tmc. Factors that involve the electro osmotic flow in MEKC are: pH, surfactant concentration, additives, and polymer coatings of the capillary wall.

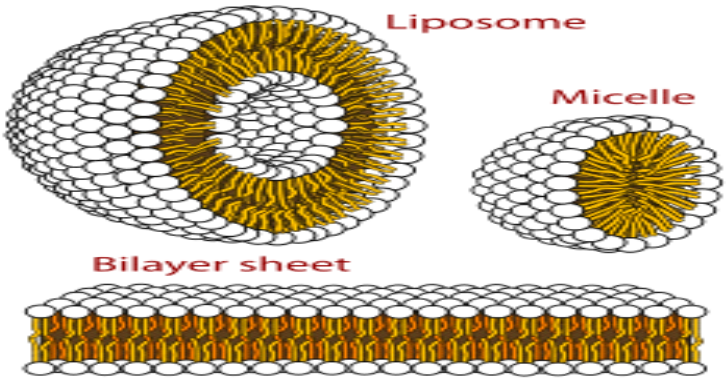

Figure .4: Liposome Bilayer.

Capillary Electro Chromatography (CEC):

The separation mechanism is a packed column parallel to chromatography. The mobile liquid passes larger than the silica wall and the particles. An electro osmosis flow occurs because of the charges on the stationary surface. CEC is parallel to CZE in that they both have a plug-type flow compared to the pumped parabolic flow that increases band broadening.

Capillary Isoelectric Focusing (CIEF): CIEF is a technique normally used to separate peptides and proteins. These molecules are call zwitterionic compounds for the reason that they contain both positive and negative charges. The charge depends on the functional groups close to the main chain and the near pH of the environment. In addition, each molecule has a specific Iso electric point (p I). When the neighboring pH is equal to this pI, the molecule carries no net charge. To be clear, it is not the pH value where a protein has all bases deprotonated and all acids prorogated, but rather the value where positive and negative charges cancel out to zero. At a pH below the p I, the molecule is positive, and then negative when the pH is above the p I.

as the charge changes with pH, a pH gradient be able to used to separate molecules in a mixture. Through a CIEF separation, the capillary is filled among the sample in solution and typically no EOF is used (EOF is removed by using a coated capillary). while the voltage is applied, the ions will migrate to a region where they turn into neutral (pH=pI). The anodic last part of the capillary sits in acidic solution (low pH), while the cathodic end sits in basic solution (high pH). Compounds of equal Iso electric points are "focused" into sharp segments and remain in their specific zone, which allows for their distinct detection.

Calculating PI:

An amino acid with n Ionizable groups with their respective pKa values pK_1, pK_2, ... pk_n will have the pI equal to the average of the group pkas: $p\ I = (pK_1+pK_2+...+pk_n)/n$. Most proteins have many Ionizable side chains in addition to their amino- and carboxyl- terminal groups. The pI is dissimilar for every protein and it can be theoretically calculated according to the Henderson-Hasselbalch approximation, if we know amino acids composition of protein. In order to experimentally establish a protein's pI 2-Dimensional Electrophoresis (2-DE) be able to used. The proteins of a cell lysine are useful to a pH immobilized gradient strip, upon electrophoresis the proteins migrate to their pI within the strip. The next dimension of 2-DE is the separation of proteins by MW using a SDS-gel.

Capillary Isotachorphoresis (CITP):

CITP is the single method to be used in a discontinuous system. The analyte migrates in successive zones and every zone length can be measured to find the quantity of sample present.

Capillary Electrophoresis Versus High Performance Liquid Chromatography (HPLC):

CE has a level flow, compared to the pumped parabolic run of the HPLC. The level flow results in narrower peaks and better resolution (Figure 1.41.4).

CE has a better peak capacity when compared to HPLC—CE uses millions of theoretical plates.

HPLC is extra carefully developed and has many mobile and stationary phases that can be implemented.

HPLC has extra complex instrumentation, while CE is simpler for the operator.

HPLC has such a broad variety of column lengths and packing, whereas CE is partial to thin capillaries.

Both techniques use similar modes of detection. So can be used complementary to one another.

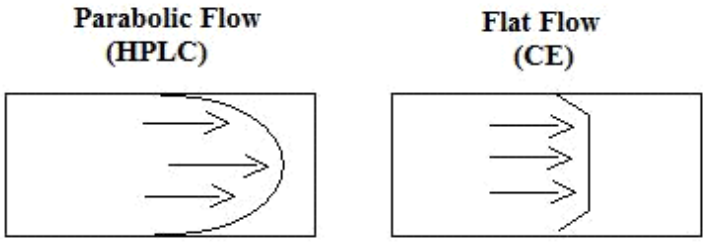

Figure 5. HPLC versus CE flow profiles

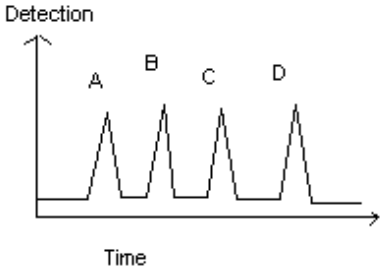

Figure 6: Detection Times

Application:

Capillary electrophoresis can be used for the simultaneous resolve of the ions NH_4^{+}, Na^{+}, Mg^{2+} and Ca^{2+} in saliva.The main use of CE by a forensic biologist is typing of STR from biological samples to generate a profile from highly polymorphic genetic markers which differ between individuals. new emerging uses for CE include the detection of specific mRNA fragments to help out identify the biological fluid or tissue source of a forensic sample. One of the major application of CE in forensic science is the development of methods for amplification and detection of DNA fragments by polymerase chain reaction (PCR) which has to lead to quick and dramatic advances in DNA typing in forensic. DNA separations are carry out by thin CE, 50-mm, fused silica capillaries packed with a sieving buffer.

High-Performance Liquid Chromatography (HPLC):

Principle:

When a mixture of compounds is passed throughout the HPLC column, it separate into its components before it exits from the column. The basic principal of HPLC is the partitioning the analyte between the solid phase and the mobile phase. The principle of Chromatography based on. Chromatography is mostly a technique used to separate the components contained in a sample mixture based on the principle of differential adsorption of substance by the adsorbent.

Instrumentation:

Eluants, Injectors, Columns:

Phase of HPLC – Partition chromatography, Adsorption chromatography , Ion chromatography ,Size exclusion chromatography.

mobile phase – are stored in individual reservoirs connected to the pumping system – must be free of particles that can clog up components & free of bubble forming gases that find trapped in column or detector Three essential ways to degas solvents

1) Suction filter (0.4 or 0.2 µm)

2) Ultrasonic ate (with vacuum)

3) He purge

HPLC sample injectors are totally 6 port valves that are overfilled by syringe give extreme accuracy & precision – typical volumes are 10 to 50 µL but can be larger

Columns -

Guard columns are generally used previous to the analytical column to protect & raise lifetime of column – operator frequently slurry or dry packs short guard column regularly with same or parallel packing used in analytical column (old column material

Detectors for HPLC:

Three types

1) Filter instrument – optical filters, Hg lamp

2) Variable wavelength – monochromatic

3) Diode array detector- provide spectra

Filter based UV-vis detector – Typically set at 254 nm using the most prominent band in Hg spectrum – can also use 313, 365, 334 nm and other lines as well

Variable wavelength detectors – use continuum source like (D 2 or H 2) & a monochromatic, select any λ, less sensitive

PDA - D 2 or H2 source, disperse & focus on diode array, get complete spectrum every 1 sec, powerful, expensive, less sensitive, lots of data generated

Fluorescence Detector

In general fixed wavelength filter fluorometer excitation filter & emission filter be able to changed for particular λ of interest gives selectivity based on the ability to exhibit fluorescence - excitation wavelength - emission wavelength changeable λ monochromator based fluorescence detectors also presented Filter based detectors usually more sensitive

Refractive index detector (RI) :

Responds to almost all solutes but has poor sensitivity – detects changes in refractive index as sample passes throughout as long as solute has different RI than solvent – analogous to TCD in GC.

Electrochemical Detection:

Amperometric – fix potential & measure current

Conduct metric – measure conductivity

Coulometric – fix potential & integrate

Volta metric – vary potential & measure

Potentiometric measure potential be able to use 2 or 3 electrode design with Pt or carbon electrodes.

THIN LAYER CHROMATOGRAPHY [15]:

TLC is a technique for analyzing mixtures through separating the compounds in the mixture. TLC be able to used to help find out the number of components in a mixture, the distinctiveness of compounds, and the purity of a compound. By observing the appearance of a product or the disappearance of a reactant, it be able to used monitor the progress of a reaction. TLC is a susceptible technique - microgram (0.000001 g) quantities can be analyzed by TLC - and it takes little time for an analysis (about 5-10 minutes).It it consists of three steps, spotting, development, and visualization.

Step

First the sample to be analyzed is dissolved in a volatile solvent to produce a very dilute (about 1%) solution. Spotting consists of by a micro pipette to transfer a small quantity of this dilute solution to one end of a TLC plate, in this casing a thin layer of crushed silica gel that has been coated onto a plastic sheet. The spotting solvent rapidly evaporates and leaves at the back a small spot of the material. Development consists of placing the bottom of the TLC plate into a deep pool of a development.

Producing the Chromatogram:

Start with a very easy case - just try to show that a particular dye is infact a mixture of simpler dyes.

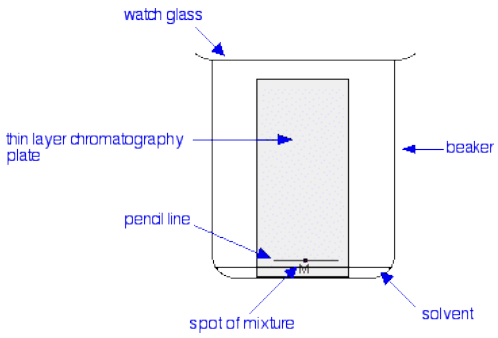

Fig 7. Chromatograph production

Visualization:

Colored compounds are simple – the spots can be directly observed after development. for the reason that most compounds are colorless but, a visualization method is needed. The silica gel on the TLC plate is impregnate through a fluorescent material that glows under ultraviolet (UV) light. A spot will hold up with the fluorescence and show as a dark spot on a shining background.

The R_f value is used to quantify the movement of the materials along the plate. R_f is equal to the distance traveled by the substance divided by the distance traveled by the solvent. Its value is always between zero and one.

Countercurrent Chromatography : Countercurrent chromatography is a liquid chromatography (LC) method that uses two immiscible liquid phases without any solid support. Countercurrent chromatography is unsuitable while the two liquid phases do not flow countercurrent to each other.

Droplet counters current chromatography:

A new structure of countercurrent chromatography, named droplet counter-current chromatography, has been developed. This all-liquid separation method is base on the partitioning of solute among a stable stream of droplets of moving phase and a column of near stationary liquid phase. Milligram quantities of dinitrophenyl (DNP) amino acids were separated with efficiency comparable to that of gas chromatography.

Principle: The principle of separation base on the partition of a solute between two immiscible solvents .The relative proportion of solute passing into each of the two solvents resolute by partition coefficient.

Instrument:

Two types of instruments are available like

- CPC manufacture
- Multi coil countercurrent system

Operation:

At first pump the stationary phase solvent into the channel as the rotor is spinning at low speed.

Followed by mobile phase solvent at the rotation speed essential for separation.

As the mobile phase flows steadily example was applied.

Multi Coil Counters Current Chromatography: Instrument consists of two or three identical multilayer coils set symmetrically around the rotor frame of the centrifuge every coil column beneath goes planetary motion.

Advantage:

No require of solid support as the stationary phase,

100% recovery of the compounds,

Many partition systems can be prepared

Low solvent consumption.

Paper Chromatography [11]:

The analytical method used to separate colored chemicals or substances It is mainly used as a teaching tool, having been replaced by other chromatography methods, such as thin-layer chromatography.A paper chromatography variant, two-dimensional chromatography involves Using two solvents and turning the paper 90° in between.This is helpful for separating complex mixtures of compounds having parallel polarity, for example, amino acids. The retention factor (R *f*) defined as the ratio of the distance traveled by the solute to the distance traveled by the solvent. It is used in chromatography to measure the amount of retardation of a sample in a stationary phase relative to a mobile phase. R *f* values are frequently expressed as a fraction of two decimal places.If R_f value of a solution is zero, the solute remains in the stationary phase and thus it is immobile. If R_f value = 1 then the solute has no affinity for the stationary phase and travels with the solvent front.

TYPES:

Descending:

Increase of the chromatogram is complete by allowing the solvent to travel down the paper. Here, mobile phase is located in solvent holder at the top. The spot is reserved at the top and solvent flows down the paper from above.

Ascending:

In which the solvent travels up the chromatographic paper. Both descending and ascending paper chromatography are second-hand for the separation of organic and inorganic substances. The sample and solvent move upward.

Ascending-descending:

This is the hybrid of both of the on top of techniques. The upper division of ascending chromatography can be folded over a rod in order to allow the paper to become descending after crossing the rod.

Radial:

This is also called circular chromatography. A circular filter paper is in use and the sample is deposited at the center of the paper. After drying the spot, the filter paper is joined horizontally on a Petri dish containing solvent, so with the aim of the wick of the paper is dipped in the solvent. The solvent rises from first to last the wick and the components are separated into concentric circles.

Two-dimensional:

In this method a square or rectangular paper is used. at this time the sample is useful to one of the corners and development is performed at a right angle to the direction of the first run.

Crystallization [8]:

Crystallization is a separation procedure, widely applied in the chemical and pharmaceutical industry. The principle of crystallization is related to the limited solubility of a compound in a solvent at a certain temperature, pressure, etc. The crystals formed have very standard internal structure, the basis of which is called the crystal lattice. The formation of such a extremely ordered structure prohibits unfamiliar molecules from being incorporated into the lattice, a solid product of high purity is obtained. The simultaneous arrangement and purification of a solid product make crystallization a main operation in the process industry.The main phenomena that occur during crystallization can be described by the following quantities:

Method:Crystal formation can be divided into two types, where the first type of crystals is composed of a cat ion and anion, also known as a salt, such as sodium acetate. The second type of crystals is composed of uncharged species, for example menthol.Crystal formation can be achieved by various methods, such as: cooling, evaporation, addition of a second solvent to reduce the solubility of the solute (technique known as anti solvent or drown-out), solvent layering, and sublimation, changing the cat ion or anion, as well as other methods.The creation of a supersaturated solution does not guarantee crystal formation, and frequently a seed crystal or scratching the glass is essential to structure nucleation sites.

Precipitation: It is the formation of a solid in a solution or inside another solid during a chemical reaction or by diffusion in a solid. When the reaction take place in a liquid, the solid created is called the precipitate, or when compacted by a centrifuge, a pellet. The liquid remaining above the solid is in either case called the supernatant or supernatant.

Equipment

1. **Cooling crystallizers**

2. Evaporative crystallization

3. Evaporative crystallizers

4. DTB crystallizer.

Ion Exchange Chromatography [18-19]:

Ion chromatography is a chromatography process that separates ions and polar molecules based on their affinity to the ion exchange. It works on about any kind of charged molecule including large proteins, small nucleotides, and amino acids. However, ion chromatography must be complete in conditions that are one unit away from the iso electric point of a protein.Separation and purification operations with ion exchange resins involve the reversible interchange of ions

between a functionalized insoluble resin (the ion exchange material) and an Ionizable substance in solution.

Principle: Ion Exchange Chromatography related to charge-charge interactions between the proteins

Theory:

1. Selectivity**.**

2. Kinetics**.**

1. **selectivity**: The selectivity which a resin has for a variety of ions is affected by many factors. These factors include the valence and size of the exchange ion, the ionic form of the resin, the total ionic strength of the solution, crosslink age of the resin, the type of functional group and the nature of the non-exchanging ions.

2. **kinetics**: Kinetics of ion exchange is usually considered to be controlled by mass transfer in ion exchange resin particles or in the immediately surrounding liquid phase. The theory used to describe mass transfer in the particle is based on the Nernst-Planck equations developed by Helfferich, which accounted for the effect of the electric field generated by ionic diffusion, but excluded convection.

Column Chromatography:

Chromatography is define as the substances to be separated are introduce on the top of a column packed with an adsorbent , pass all the way through the column at different rates that depend on the affinity of each substance for the adsorbent and for the solvent or solvent mixture, and are usually collected in solution they pass from the column at different time.

CLASSIFICATIONS OF CHROMATOGRAPHY

Types of column chromatography are generally used:

1. Adsorption chromatography: Process which separates lipid mixtures chiefly according to their relative polarities.

2. Ion-exchange chromatography:Process which is based on the value of ionic groups present in some lipid molecules.

1. ADSORPTION CHROMATOGRAPHY [12]: The separation is based on differences in the degree of adsorption of lipid mechanism on top of a solid and immobilized phase.The retention results in a range of mechanisms together with hydrogen bonding, Van der Waals' forces and also ionic bonding. The solid phase is relatively polar and the more polar the lipid, the more strongly is it adsorbed. Thus, the lipids are eluted by increasingly polar solvents. This method has a low resolution when used at low pressure but has a high performance when run at high pressure by a stationary phase made of fine particles (HPLC). The previous is restricted to the fractionation of complex mixtures into two or three less complex ones, the later on being adopted to analyze and measure purified fractions.

2.AFFINITY CHROMATOGRAPHY [10,12]: The affinity purification process can be idealized into the selective adsorption of enzyme A from a mixture of proteins. After the unabsorbed contaminating proteins are washed from the column, enzyme A can be eluted from the ligand and recovered. This technique has been used successfully to purify enzymes, proteins, antibodies, oligonucleotides and nucleic acids.

Principle: Affinity chromatography is difference in interactions' strength between the different bimolecular inside a mobile phase, and the stationary phase. The stationary phase is first loaded into a column with mobile phase containing a variety of bimolecular from DNA to proteins (depending on the purification experiment). Then, the two phases are acceptable time to bind. A wash buffer is after that poured during a column containing both bound phases. The wash buffer removes non-target bimolecular by disrupting their weaker interactions with the stationary phase. Target bimolecular have a much higher affinity for the stationary phase, and remain bound to the stationary phase, not being washed away by wash buffer. An elution buffer is then poured through the column containing the remaining target bimolecular. The elution buffer disrupts

interactions between the bound targets bimolecular with the stationary to a much greater extent than the wash buffer, effectively removing the target bimolecular. This purified solution contains elution buffer and target bimolecular, and is called elution.

:

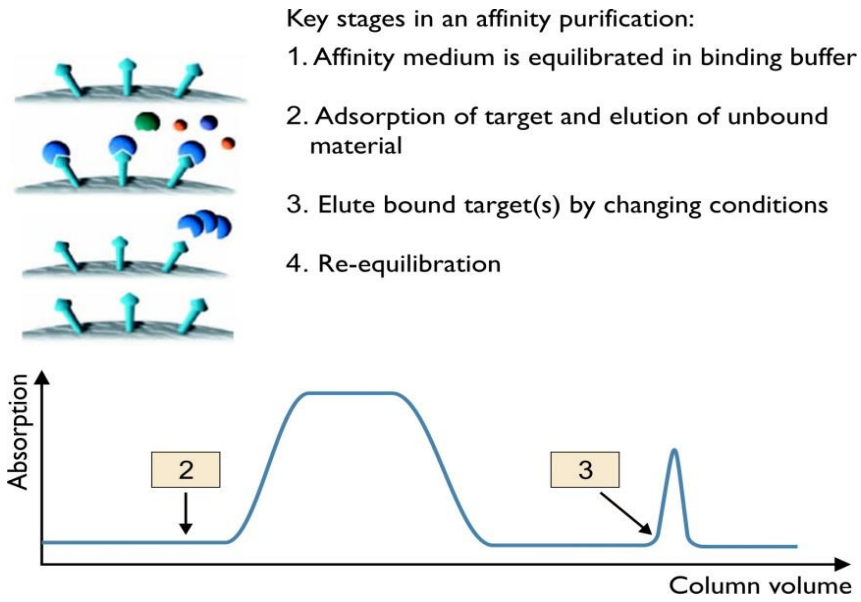

Fig 8 Stage in affinity chromatography

3.Vacuum Columns [Vacuum Liquid Chromatography (VLC)]:

The adsorbent is applied dry keen on a sintered glass funnel. The sample is useful by dry method or as solution. Then the mobile phase is additional portion by portion and vacuum is useful after each portion to collect each fraction.

OTHER SEPARATION METHOD [15]:

1. Liquid liquid extraction
2. Solid liquid extraction
3. Solid solid extraction

1. **Liquid- Liquid extraction**: Liquid–liquid extraction. Liquid–liquid extraction (LLE), also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubility's in two different immiscible liquids, usually water and an organic solvent. Liquid-liquid extraction can also be achieved by using inert solid materials such as diatomaceous earth, a sand-like porous material commercially available in several forms such as Kieselguhr, Diatomaceous earth or Hydrometrics. These materials can be new to the liquid in the batch mode or, more often, as a support in a chromatographic column.

Application of liquid-liquid extraction:

1.Extraction of nitrobenzene after reaction of HNO3 with toluene in H2SO4

2.Extraction of methylacrylate from organic solution with perchlorethylene

3.Extraction of benzylalcohol from a salt solution with toluene

4.Extraction of caprolactam from ammonium sulfate solution with benzene

5.Extraction of acrylic acid from wastewater with butanol

.

2. Solid- Solid extraction: Solid-phase extraction is a trial preparation process by which compounds that are dissolved in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties.

Conclusion:

In the area of biotechnology, separation techniques are urgently needed to meet demands for ultra-high purity and yield. Thus, a variety of technique are being residential to address these wants. Modification of existing techniques have become available for separating compounds from complex sample matrices. Normally, biological compounds for the pharmaceutical and biotechnology industry must be obtained at greater than 99.9% purity (sometimes greater than 99.99%) while maintaining high yield. In a few area of chemistry this quantity of purity would cause problems; in biotechnology it is still trickier to achieve because of the complex sample matrices. In adding, the compounds of attention may be very parallel to impurities in the sample matrix, and the compounds could be denatured by sure solvents and high temperature. In particular, three areas of biotechnology have presented scientists with troubles in separations, cell separations, DNA-RNA separations, and protein-peptide separations.

References:

1. Dechow F J, Reed & Carnrick Pharmaceuticals, New Jersey U .S. A;Noyes publications, 1989,1-449.
2. H ERICH, AG Hoffman, Interrelations of Chemistry and Biotechnology – IIIt, Switzerland, 1992; 64(1):168-184.
3. J William.,A.Palladino, Introduction to Biotechnology, approach 2nd edition,1-32. Zurich, Process Enginring Laboratory II, 2018.
4. R Pierluigi, Z Andrea, R Barbara, R Aldo., M Elisa, Field-flow fractionation and biotechnology, TRENDS in Biotechnology ; 2005,23(9) .
5. Karlström Amelia, Hober Sophia. Chromatography method for protein purification,Royal Institute of Technology, Albanova University Center..
6. Freundlich theory https://en.wikipedia.org/wiki/Freundlich_equation.
7. Fraction flow theory http://www.cyberlipid.org/fraction/frac0004.htm
8. Capillary electrophoresis theory https://www.google.co.in/search?q=capillary+electrophoresis&oq=CAPILAY+ELECTRO&aqs=chrome.1.69i57j0l5.29886j0j7&sourceid=chrome&ie=UTF-8
9. Paper chromatography theory https://en.wikipedia.org/wiki/Paper_chromatography
10. Adsorptions chromatography theory https://www.scribd.com/doc/21403933/Adsorption-ChromatographyCapillary electrophoresis theory https://en.wikipedia.org/wiki/Capillary_electrophoresis
11. . Capillary electrophoresis theory https://www.jove.com/science-education/10226/capillary-electrophoresis-ce
12. Thin layer chromatography https://www.chemguide.co.uk/analysis/chromatography/thinlayer.html
13. Extraction technique

https://memberfiles.freewebs.com/49/71/47557149/.../cpc%20by%20vinay.ppt Crystallization theory https://en.wikipedia.org/wiki/Crystallization.

14.Affinity chromatography https://en.wikipedia.org/wiki/Affinity_chromatography.

15..Affinity chromatography

http://elte.prompt.hu/sites/default/files/tananyagok/IntroductionToPracticalBiochemistry/ch06s04.html.