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Ion-Exchange Chromatography and Its Applications

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1. Introduction

Ion-exchange chromatography (IEC) is part of ion chromatography which is an important analytical technique for the separation and determination of ionic compounds, together with ion-partition/interaction and ion-exclusion chromatography [1]. Ion chromatography separation is based on ionic (or electrostatic) interactions between ionic and polar analytes, ions present in the eluent and ionic functional groups fixed to the chromatographic support. Two distinct mechanisms as follows; ion exchange due to competitive ionic binding (attraction) and ion exclusion due to repulsion between similarly charged analyte ions and the ions fixed on the chromatographic support, play a role in the separation in ion chromatography. Ion exchange has been the predominant form of ion chromatography to date [2]. This chromatography is one of the most important adsorption techniques used in the separation of peptides, proteins, nucleic acids and related biopolymers which are charged molecules in different molecular sizes and molecular nature [3-6]. The separation is based on the formation of ionic bonds between the charged groups of biomolecules and an ion-exchange gel/support carrying the opposite charge [7]. Biomolecules display different degrees of interaction with charged chromatography media due to their varying charge properties [8].

The earliest report of ion-exchange chromatography date back to 1850, Thompson studied the adsorption of ammonium ions to soils [9-11]. Spedding and Powell published a series of papers describing practical methods for preparative separation of the rare earths by displacement ion-exchange chromatography in 1947. Beginning in the 1950s, Kraus and Nelson reported numerous analytical methods which are used for metal ions based on separation of their chloride, fluoride, nitrate or sulfate complexes by anion chromatography [12]. In order to separate proteins an ion exchange chromatographic method was reported by Peterson and Sober in 1956. In modern form ion exchange chromatography was introduced by Small, Stevens and Bauman in 1975 [3]. Gjerde et al. published a method for anion chromatography in 1979 and this was followed by a similar method for cation chromatography in 1980 [12]. Ion-

exchange chromatography has been used for many years to separate various ionic compounds; cations and anions and still continues to be used. The popularity of ion exchange chromatography has been increased in recent years because this technique allows analysis of wide range of molecules in pharmaceutical, biotechnology, environmental, agricultural and other industries [2].

1.1. Ion exchange mechanism

Ion-exchange chromatography which is designed specifically for the separation of differently charged or ionizable compounds comprises from mobile and stationary phases similar to other forms of column based liquid chromatography techniques [9-11]. Mobil phases consist an aqueous buffer system into which the mixture to be resolved. The stationary phase usually made from inert organic matrix chemically derivative with ionizable functional groups (fixed ions) which carry displaceable oppositely charged ion [11]. Ions which exist in a state of equilibrium between the mobile phase and stationary phases giving rise to two possible formats, anion and cation exchange are referred to as counter ion (Figure 1) [1,13]. Exchangeable matrix counter ions may include protons (H^+), hydroxide groups (OH^-), single charged mono atomic ions (Na^+, K^+, Cl^-), double charged mono atomic ions (Ca^{2+}, Mg^{2+}), and polyatomic inorganic ions (SO_4^{2-}, PO_4^{3-}) as well as organic bases (NR_2H^+) and acids (COO^-) [11]. Cations are separated on cation-exchange resin column and anions on an anion exchange resin column [10]. Separation based on the binding of analytes to positively or negatively charged groups which are fixed on a stationary phase and which are in equilibrium with free counter ions in the mobile phase according to differences in their net surface charge (Figure 1) [13-14].
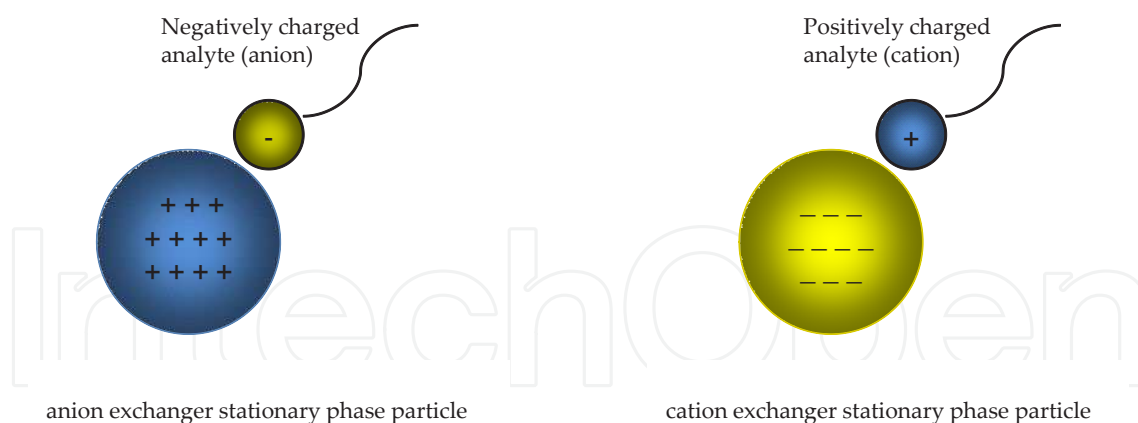

Figure 1. Types of ion exchangers

Ion exchange chromatography involves separation of ionic and polar analytes using chromatographic supports derivatized with ionic functional groups that have charges opposite that of the analyte ions. The analyte ions and similarly charged ions of the eluent compete to bind to the oppositely charged ionic functional group on the surface of the stationary phase. Assuming that the exchanging ions (analytes and ions in the mobile phase) are cations, the competition can be explained using the following equation;

$$S\text{-}X^-C^+ + M^+ \leftrightarrow S\text{-}X^-M^+ + C^+$$
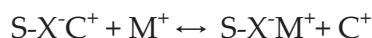
In this process the cation M^+ of the eluent replaced with the analyte cation C^+ bound to the anion X^- which is fixed on the surface of the chromatographic support (S).

In anion exchange chromatography, the exchanging ions are anions and the equation is represented as follow;

$$S\text{-}X^+A^- + B^- \leftrightarrow S\text{-}X^+B^- + A^-$$

The anion B^- of the eluent replaced with the analyte cation A^- bound to the positively charged ion X^+ on the surface of the stationary phase. The adsorption of the analyte to the stationary phase and desorption by the eluent ions is repeated during their journey in the column, resulting in the separation due to ion-exchange [2].

Molecules vary considerably in their charge properties and will exhibit different degrees of interaction with charged chromatography support according to differences in their overall charge, charge density and surface charge distribution. Net surface charge of all molecules with ionizable groups is highly pH dependent [13]. Therefore pH of the mobile phase should be selected according to the net charge on a protein of interest within a mixture is opposite to that of matrix functional group, that it will displace the functional group counter ion and bind the matrix. On the other hand oppositely charged proteins will not be retained. Adsorbed protein analytes can be eluted by changing the mobile phase pH which effect the net charge of adsorbed protein, so its matrix binding capacity. Moreover increasing the concentration of a similarly charged species within the mobile phase can be resulted in elution of bound proteins. During ion exchange chromatography for example in anion exchange as illustrated in Figure 2, negatively charged protein analytes can be competitively displaced by the addition of negatively charged ions. The affinity of interaction between the salt ions and the functional groups will eventually exceed that the interaction exists between the protein charges and the functional groups, resulting in protein displacement and elution by increasing gradually the salt concentration in the mobile phase [11].

Complex mixtures of anions or cations can usually be separated and quantitative amounts of each ion measured in a relatively short time by ion exchange chromatography [10]. In classical ion-exchange chromatography separations have been performed in the open-column mode. Column which is loosely packed with stationary phase as small particles made of 1-2 cm diameter glass. The mobile phase or eluent contains the competing ion and is passed continuously into the column and percolates through it under gravity. Sample mixture is applied to the top of the column and allowed to pass into the bed of ion- exchange material. Eluent flow is then resumed and fractions of eluent are collected at regular intervals from the column outlet. Open column ion-exchange chromatography is very slow due to low eluent flow-rates. Increasing flow rate may result in deteriorated separation efficiency (Figure 3). In modern ion-exchange chromatography the usage of high efficiency ion exchange materials combined with flow-through detection have overcome of these challenges. Separations are performed on the column which is filled with ion-exchanger as particles in uniform size. The particles of ion-exchange material are generally very much smaller than those used for classical open column
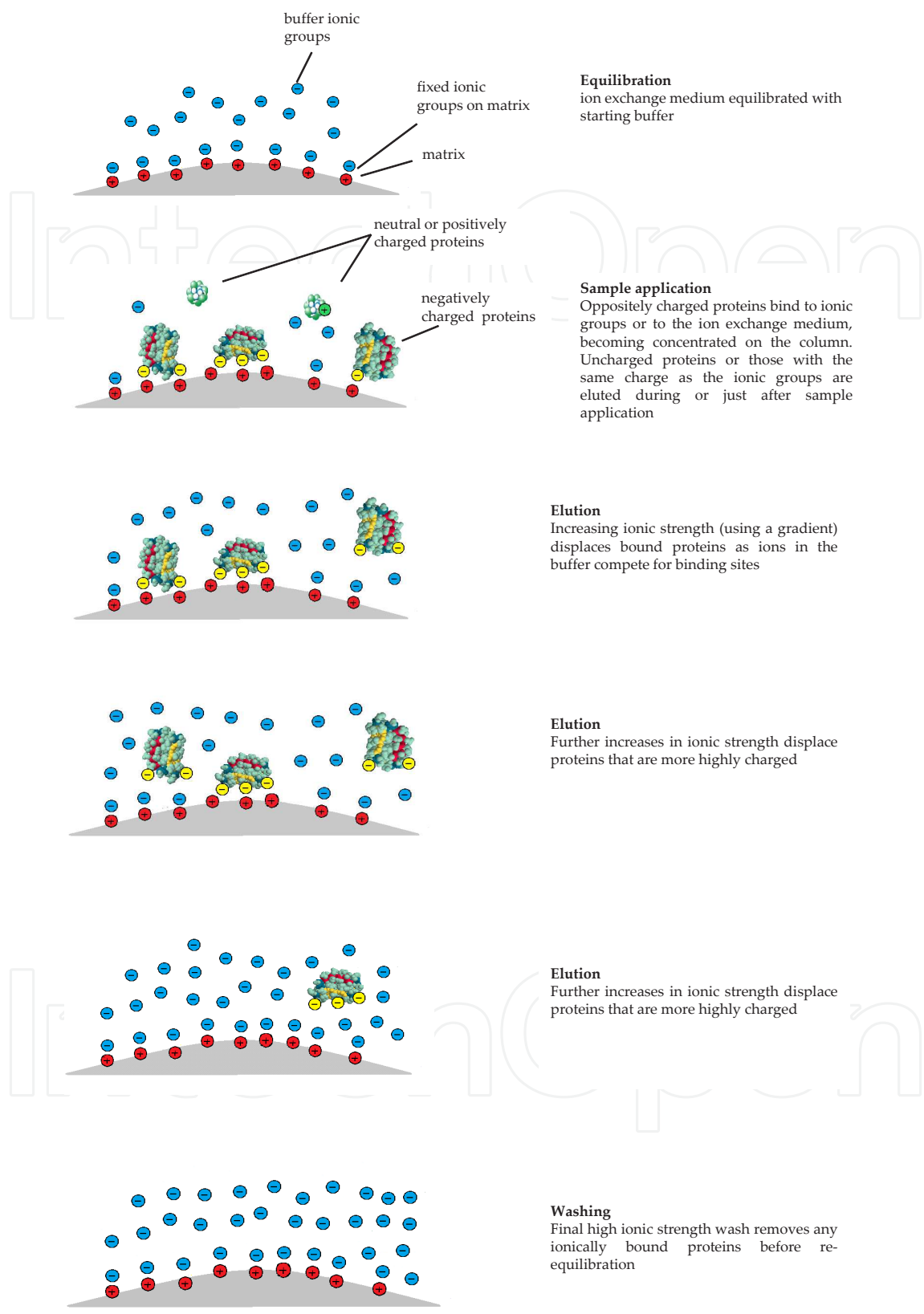
Figure 2. Separation steps in anion exchange chromatography (GE Healthcare)

ion-exchange chromatography [1]. However ion-exchange resins used in modern chromatography have lower capacity than older resins [10]. The eluent must be pumped through the column due to the small particle size of stationary phase. The sample mixture is applied into eluent by the injection port. Finally the separated ions are detected with a flow-through detection instrument [1].
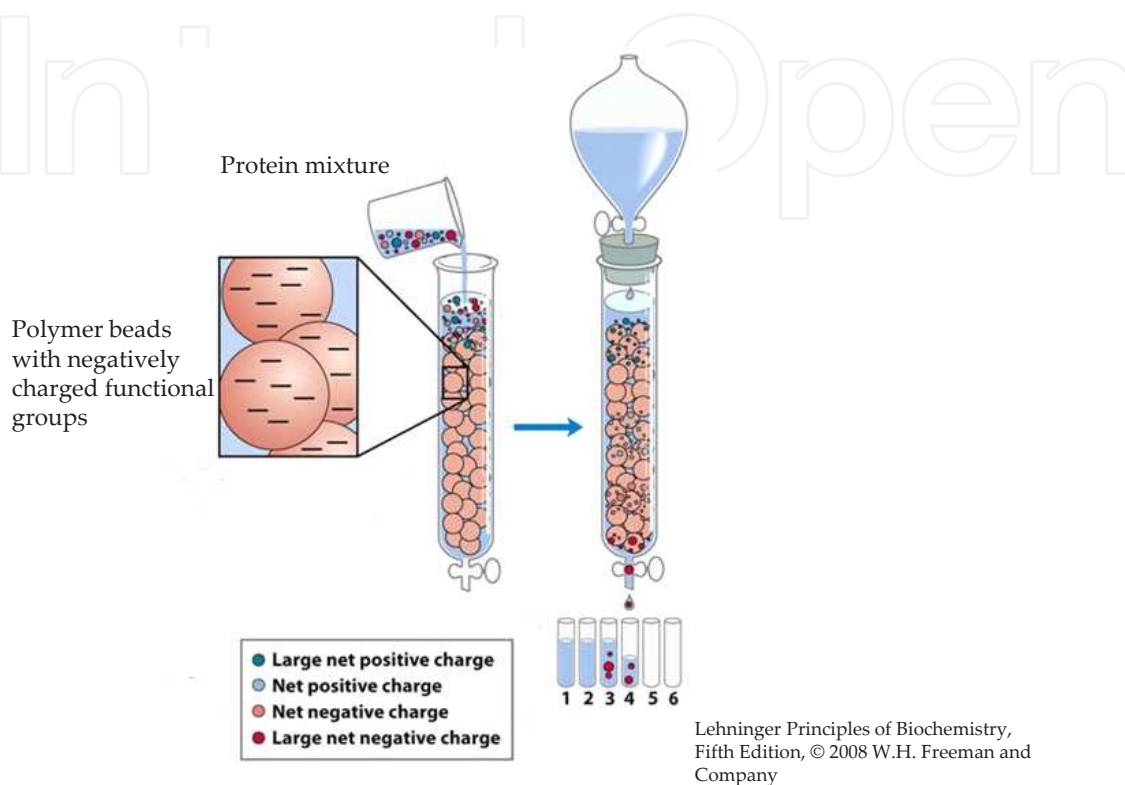

Protein mixture

Polymer beads with negatively charged functional groups

● Large net positive charge
○ Net positive charge
● Net negative charge
● Large net negative charge

1 2 3 4 5 6

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Figure 3. Ion exchange chromatography technique

This technique has been used for the analyses of anions and cations, including metal ions, mono- and oligosaccharides, alditols and other polyhydroxy compounds, aminoglycosides (antibiotics), amino acids and peptides, organic acids, amines, alcohols, phenols, thiols, nucleotides and nucleosides and other polar molecules. It has been successfully applied to the analysis of raw materials, bulk active ingredients, counter ions, impurities, and degradation products, excipients, diluents and at different stages of the production process as well as for the analysis of production equipment cleaning solutions, waste streams, container compatibility and other applications [2]. Wide applicability including high performance and high-throughput application formats, average cost, powerful resolving ability, large sample handling capacity and ease of scale-up as well as automation allow the ion exchange chromatography has become one of the most important and extensively used of all liquid chromatographic technique [11].

Although the extensive use of ion exchange chromatography the mechanism of the separation has not completely been elucidated. A considerable effort has been made to describe the ion exchange process theoretically [3,9]. One of the important disadvantages of this technique is

that this method provides no direct information on events occurring at the surface of the stationary phase, because the ion-exchange equilibrium is always determined by the balance between the solute interaction and the eluent interaction with the active sites of resin [3]. Ion exchange is similar to sorption, since in both cases a solid takes up dissolved sample. The most important difference between them is in stoichiometric nature of ion exchange. Each ion removed from the solution is replaced by an equivalent amount of another ion of the same charge, while a solute is usually taken-up non-stoichiometrically without being replaced in sorption [15]. Stoichiometric displacement based on the mass action law and describes the retention of a solute ion as an exchange process with the counter ion bound to the surface [9]. According to this model, the retention of a protein under isocratic, linear conditions is related to counter ion concentration and can be represented by equilibrium as follow;

$$\log k = -(Z_p/Z_s) \log C_m + \log(\varphi Q)$$

k is the retention factor and C_m is the concentration of the counter ion in the mobile phase. Z_p/Z_s ($= Z$) is the ratio of the characteristic charge of the protein to the value of the counter ion and presents a statistical average of the electrostatic interactions of the protein with the stationary phase as it migrates through the column. The behavior of ion exchange chromatographic system can be explained by stoichiometric models. However, the mechanism of the ion exchange separation is more complex and stiochiometric consideration is inapplicable to long-range mechanisms, such as electrostatic interactions due to the distribution of ions in solution is also influenced by the electrostatic potential [3,6]. Other interactions between solute-solute, solute-solvent and solvent-solvent also contribute to retention and selectivity in ion exchange. For example ion-dipole and dispersion interaction, should be included as important mechanisms. Additionally entropic contribution originating from solvent, such as water, structures around ion exchange sites should also be regarded as important [3]. In addition to these the primary separation mechanism is the electrostatic interaction between ion-exchange sites and counter ions in ion exchange chromatography [6].

An important feature differentiating the ion exchange resins from other types of gels is the presence of functional groups. The groups are attached to the matrix. The ion exchange process between the ions in the solution takes place on these functional groups. The exchange of ions between the ion exchange resin and the solution is governed by two principles:

1. The process is reversible, only rare exceptions are known

2. The exchange reactions take place on the basis of equivalency in accordance with the principle of electro neutrality. The number of milimoles of an ion sorbed by an exchange should correspond to the number of milimoles of an equally charged ion that has been released from the ion exchange [16].

Equilibrium is established for each sample component between the eluent and stationary phases when a sample is introduced into the ion-exchange chromatography. The distribution of component (A) between the two phases is expressed by the distribution coefficient, "D_A".

$$D_A = [A]_r / [A]_m$$

The value of D_A is dependent on the size of the population of molecules of component A in the stationary and eluent phases [1]. As the equilibrium is dynamic, there is a continual, rapid interchange of molecules of component A between the two phases. The fraction of time, fm, that an average molecule of A spends in the mobile phase is given by:

f_m = Amount of A in the mobile phase / Total amount of A

$f_m = [A]_m V_m / [A]_m V_m + [A]_r w$

$= 1/ 1 + D_A (w / V_m)$

$k' = D_A (W / V_m)$

$f_m = 1 / 1 + k'$

w: Weight of the stationary phase

V_m: Volume of the mobile phase [1]

The mechanism of the anion and cation exchange are very similar. When analytes enter to the ion exchange column, firstly they bind to the oppositely charged ionic sites on the stationary phase through the Coulombic attraction [2]. In accordance with Coulomb's law, the interactions between ions in the solute and oppositely charged ligands on the matrix in ion-exchange chromatography are due to the electrostatic forces. Coulomb's law is given by the equation as follow;

$f = q_1q_2/\varepsilon r^2$

f: Interaction electrostatic force

q_1q_2: The charge on ions

: Dielectric constant of the medium

r: The distance between charges.

If the charges on both ions are same (both are positive or negative) the force is repulsive, if they are different (one positive and the other negative) the force is attractive. When the ion charge of the species increase (Divalent ion should interact more strongly than a monovalent ion) and when the dielectric constant decrease (Two oppositely charged molecules increased more strongly in an organic solvent than in water), the interactions increase. On the other hand the distance between the charges increases the interactions decrease. Additionally, other interactions, especially, van der Waals forces participate to the Coulombic forces [2,17].

Ion exchange chromatography, which is also known as adsorption chromatography, is a useful and popular method due to its;

• high capacity,

• high resolving power,

• mild separation conditions,

- versatility and widespeared applicability,

- tendency to concentrate the sample

- relatively low cost [17].

General components of an ion-exchange chromatography are presented as below (Figure 4).

- A high pressure pump with pressure and flow indicator, to deliver the eluent

- An injector for introducing the sample into the eluent stream and onto the column

- A column, to separate the sample mixture into the individual components

- An oven, optional

- A detector, to measure the analyte peaks as eluent from the column

- A data system for collecting and organizing the chromatograms and data

In ion-exchange chromatography, adsorption and desorption processes are determined by the properties of the three interacting entities;

- The stationary phase,

- The constituents of the mobile phase

- The solute [18].

1.2. Stationary phase

Selection of a suitable ion-exchange matrix probably is the most important in ion exchange protocol and is based on various factors such as; ion exchanger charge/strength, linear flow rate/sample volume and sample properties [11]. In ion-exchange chromatography, numerous stationary phases are available from different manufacturers, which vary significantly in a number of chemical and physical properties [6,18]. Stationary phases comprised of two structural elements; the charged groups which are involved in the exchange process and the matrix on which the charged groups are fixed [18]. Ion exchangers are characterized both by the nature of the ionic species comprising the fixed ion and by the nature of the insoluble ion-exchange matrix itself [1].

Ion exchangers are called cation exchangers if they have negatively charged functional groups and possess exchangeable cations. Anion exchangers carry anions because of the positive charge of their fixed groups [15]. The charged groups determine the specifity and strength of protein binding by their polarity and density while the matrix determines the physical and chemical stability and the flow characteristics of the stationary phase and may be responsible for unspecific binding effects [18].

General structure (fibrous or beaded form), particle size and variation, pore structures and dimensions, surface chemistry (hydrophilic or hydrophobic), swelling characteristics of matrix are important factors which effect chromatographic resolution [11,18]. Porosity of ion exchange
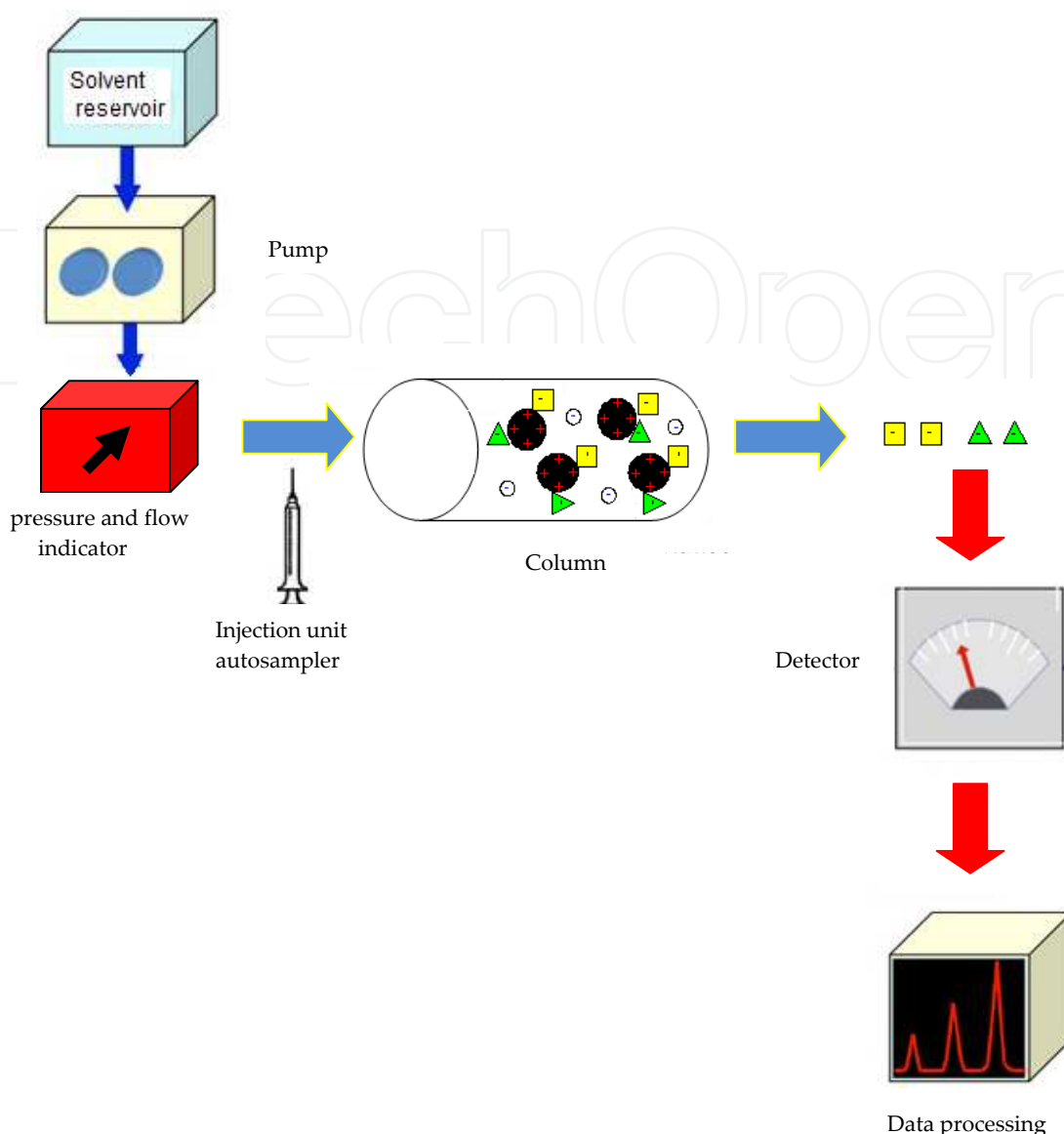
Figure 4. Ion-exchange Chromatography System

beads can be categorized as non-porous, microporous and macroporous. (Figure 5 and Figure 6) [14]. High porosity offers a large surface area covered by charged groups and so provides a high binding capacity [13]. However when compared with beaded matrix fibrous ion exchangers based on cellulose exhibit lower chromatographic resolution [14]. On the other hand high porosity is an advantage when separating large molecules [13] and prefractionation [14]. Non-porous matrices are preferable for high resolution separations when diffusion effects must be avoided [13]. Micropores increase the binding capacity but cause to a band broadening. Another disadvantage of microporous beads is that protein can bind to the surface of the beads near to the pores, so penetration of proteins into the pores can prevent or slow down. These problems are overcome by using macroporous particles with pore diameters of about 600-800 nm which are introduced recently. These kinds of particles behave differently

compared to microporous materials with respect to microflow characteristics the new term perfusion chromatography has been created [14].
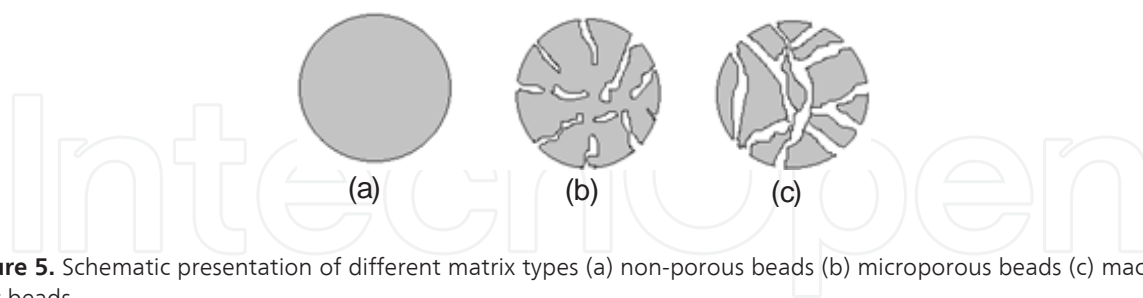

Figure 5. Schematic presentation of different matrix types (a) non-porous beads (b) microporous beads (c) macroporous beads
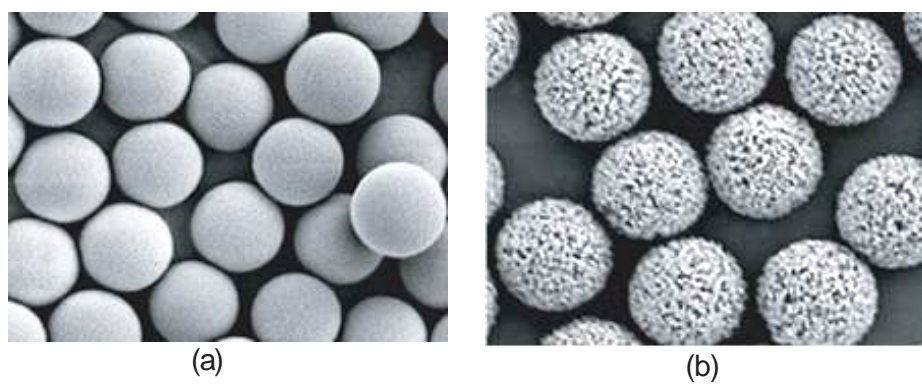

Figure 6. (a) Non-porous beads **(b)** Porous beads

Furthermore a new matrix type which has been recently introduced is based on a completely new principle and exhibits improved chromatographic features when compared with conventional ion exchangers. This matrix which is known as continuous bed does not consist of ion exchange beads or fibers. The matrix is synthesized in the column by polymerization and established from continuous porous support consisting of a nodule chains (Figure 7). The advantages of that matrix are mainly due to the more homogeneous mobile phase flow and short diffusion distances for the proteins. This is explained by the non-beaded form and the unique pore structure of the support [14].

Size, size distribution and porosity of the matrix particles are the main factors which affect the flow characteristics and chromatographic resolution. Small particles improved chromatographic resolution. Stationary phases with particle of uniform size are superior to heterogenous materials with respect to resolution and attainable flow rates. The pore size of ion exchange bead directly effect the binding capacity for a particular protein dependent on the molecular weight of the protein because it determines the access of proteins to the interior of the beads. Binding of large proteins can be restricted to the bead surface only so that the total binding capacity of the ion exchanger is not exploited Pore diameter of 30 nm is optimal for proteins up to a molecular weight of about 200.000 Da [14].

Figure 7. Continuous bed matrix

In order to minimize non-specific interactions with sample components inert matrix should be used. High physical stability provides that the volume of the packed medium remains constant despite extreme changes in salt concentration or pH for improving reproducibility and avoiding the need to repack columns. High physical stability and uniformity of particle size facilitate high flow rates, particularly during cleaning or re-equilibration steps, to improve throughput and productivity [13]. There are pH and pressure limits for each stationary phases. For example pH values higher than 8 should not used in silica based materials which are not coated with organic materials. Matrix stability also should be considered when the chemicals such as organic solvents or oxidizing agents should be required to use or when they are chosen for column cleaning [14].

Matrices which are obtained by polymerization of polystyrene with varying amounts of divinylbenzene are known as the original matrices for ion exchange chromatography. However these matrices have very hydrophobic surface and proteins are irreversibly damaged due to strong binding. Ion exchangers which are based on cellulose with hydrophilic backbones are more suitable matrices for protein separations. Other ion exchange matrices with hydrophilic properties are based on agarose or dextran [14].

Several matrix types and their important properties can be listed as follow;

Matrix materials;

• Cellulose; Hydrophilic surface, enhanced stability by cross-linking, inexpensive

- Dextran; Considerable swelling as a function of ionic milieu, improved materials by cross-linking)

- Agarose; Swelling is almost independent of ionic strength and pH, high binding capacity obtained by production of highly porous particles

- Polyacrylamide; Swelling behavior similar to dextran

- Acrylate-copolymer; High pH stability

- Polystyrene-divinilybenzene; Hydrophobic surface, low binding capacity for proteins

- Coated polystyrene-divinilybenzene; Hydrophilic surface

- Silica; Unstable at pH > 8, rigid particles

- Coated Silica; Hydrophilic surface [14]

In addition to electrostatic interactions between stationary phase and proteins, some further mechanisms such as hydrophobic interactions, hydrogen bonding may contribute to protein binding. Hydrophobic interactions especially occur with synthetic resin ion exchangers such as which are produced by copolymerization of styrene and divinylbenzene. These materials are not usually used for separation of proteins. However new ion exchange materials that consist of styrene-divinylbenzene copolymer beads coated with hydrophilic ion exchanger film were introduced. According to the retention behavior of some proteins, it is considered that coating of the beads so efficient that unspecific binding due to hydrophobic interactions cannot be observed. Silica particles have also been coated with hydrophilic matrix. Acrylic acid polymers are also used for the protein separation in ion exchange chromatography. These polymers are especially suitable for purification of basic proteins [14].

The functional groups substituted onto a chromatographic matrix determine the charge of an ion exchange medium; positively-charged anion exchanger or a negatively-charged cation exchanger [13]. Both exchangers can be further classified as strong and weak type as shown in Table 1. The terms weak and strong are not related to the binding strength of a protein to the ion exchanger but describe the degree of its ionization as a function of pH [14]. Strong ion exchangers are completely ionized over a wide pH range, while weak ion exchangers are only partially ionized a narrow pH range [1,11]. Therefore with strong ion exchangers proteins can adsorb to several exchanger sites. For this reason strong ion exchangers are generally used for initial development and optimization of purification protocols. On the other hand weak ion exchangers are more flexible in terms of selectivity and are a more general option for the separation of proteins that retain their functionality over the pH 6-9 range as well as for unstable proteins that may require mild elution conditions [11]. Alkylated amino groups for anion exchangers and carboxy, sulfo as well as phosphato groups for cation exchangers are the most common functional groups used on ion exchange chromatography supports [14]. Sulfonic acid exchangers are known as strong acid type cation exchangers. Quaternary amine functional groups are the strong base exchangers whereas less substituted amines known as weak base exchangers [1]. Number and kind of the substituents are determined the basicity of amino-groups. Immobilized

tertiary and quaternary amines proved to be useful for ion exchange chromatography. Immobilized diethylaminoethyl and carboxymethyl groups are the most widely used ion exchangers [11].

The ion exchange capacity of an ion-exchanger is determined by the number of functional groups per unit weight of the resin [13]. The total ionic capacity is the number of charged functional groups per ml medium, a fixed parameter of each medium and can be given as mval/ml for small ions. Density and accessibility of these charged groups both on the surface and within the pores define the total binding capacity. Ionic medium and the presence of other proteins if a particular protein is considered also affect the binding capacity. However, under defined conditions, the amount of the certain protein which is bound to ion exchanger is more suitable parameter for determining and comparing the capacity of ion exchange chromatography. Albumin for anion exchangers and hemoglobin for cation exchangers is usually used for this purpose. Determination of the binding capacity before the experiment is generally recommended because the capacity for a particular protein depends on its size and also on the sample composition. The binding capacity of a column can be increased for proteins which are retained on the column at high salt concentrations. The salt concentration is adjusted to a suitable concentration in which the protein of interest tightly bound to the ion exchanger while others which have lower affinity pass through the column without occupying binding sites [14].

Exchange Type	Ion exchange group	Buffer counter ions	pH range	Commercial samples
Strong cation	Sulfonic acid (SP)	Na^+, H^+, Li^+	4-13	Capto®S
				SP Sepharose®
				SP Sephadex®
				TSKgel SP_5PW
Weak cation	Carboxylic acid	Na^+, H^+, Li^+	6-10	CM Cellulose
				CM Sepharose®
				CM Sephadex®
				CM Sepharose® CL6B
				TSKgel CM-5PW
Strong anion	Quaternary amine (Q)	Cl^-, $HCOO_3^-$, CH_3COO^-, SO_4^{2-}	2-12	Q Sepharose®
				Capto®Q
				Dowex®1X2
				Amberlite® / Amberjet®
				QAE Sephadex®
Weak anion	Primary amine Secondary amine Tertiary amine (DEAE)	Cl^-, $HCOO_3^-$, CH_3COO^-, SO_4^{2-}	2-9	DEAE-Sepharose®
				Capto® DEAE
				DEAE Cellulose

Table 1. Weak and Strong type anion and cation exchangers

1.3. Mobile phase (Eluent)

In ion exchange chromatography generally eluents which consist of an aqueous solution of a suitable salt or mixtures of salts with a small percentage of an organic solvent are used in which most of the ionic compounds are dissolved better than in others in. Therefore the application of various samples is much easier [1,3]. Sodium chloride is probably the most widely used and mild eluent for protein separation due to has no important effect on protein structure. However NaCl is not always the best eluent for protein separation. Retention times, peak widths of eluted protein, so chromatographic resolution are affected by the nature of anions and cations used. These effects can be observed more clearly with anion exchangers as compared to cation exchangers [14]. The salt mixture can itself be a buffer or a separate buffer can be added to the eluent if required. The competing ion which has the function of eluting sample components through the column within reasonable time is the essential component of eluting sample. Nature and concentration of the competing ions and pH of the eluent are the most important properties affecting the elution characteristics of solute ions [1].

The eluent pH has considerable effects on the functional group which exist on the ion exchange matrix and also on the forms of both eluent and solute ions. The selectivity coefficient existing between the competing ion and a particular solute ion will determine the degree of that which competing ion can displace the solute ion from the stationary phase. As different competing ions will have different selectivity coefficients, it follows that the nature of competing ion will be an important factor in determining whether solute ions will be eluted readily. The concentration of competing ion exerts a significant effect by influencing the position of the equilibrium point for ion-exchange equilibrium. The higher concentration of the competing ion in the eluent is more effectively displace solute ions from the stationary phase, therefore solute is eluted more rapidly from the column. Additionally elution of the solute is influenced by the eluent flow-rate and the temperature. Faster flow rates cause to lower elution volumes because the solute ions have less opportunity to interact with the fixed ions. Temperature has relatively less impact, which can be change according to ion exchange material type. Enhancement of the temperature increases the rate of diffusion within the ion-exchange matrix, generally leading to increased interaction with the fixed ions and therefore larger elution volumes. At higher temperatures chromatographic efficiency is usually improved [1].

Eluent degassing is important due to trap in the check valve causing the prime loose of pump. Loss of prime results in erratic eluent flow or no flow at all. Sometimes only one pump head will lose its prime and the pressure will fluctuate in rhythm with the pump stroke. Another reason for removing dissolved air from the eluent is because air can get result in changes in the effective concentration of the eluent. Carbon dioxide from air dissolved in water forms of carbonic acid. Carbonic acid can change the effective concentration of a basic eluent including solutions of sodium hydroxide, bicarbonate and carbonate. Usually degassed water is used to prepare eluents and efforts should be made

to keep exposure of eluent to air to a minimum after preparation. Modern inline degassers are becoming quite popular [10].

For separation the eluent is pumped through the system until equilibrium is reached, as evidenced by a stable baseline. The time required for equilibrium may vary from a couple of minutes to an hour or longer, depending on the type of resin and eluent used [10]. Before the sample injection to the column should be equilibrated with eluent to cover all the exchange sites on the stationary phase with the same counter ion. When the column is equilibrated with a solution of competing ion, counter ions associated with the fixed ions being completely replaced with competing ions. In this condition the competing ions become the new counter ions at the ion exchange sites and the column is in the form of that particular ion [1].

Isocratic elution or gradient elution can be applied for elution procedure. A single buffer is used throughout the entire separation in isocratic elution. Sample components are loosely adsorbed to the column matrix. As each protein will have different distribution coefficient separation will achieved by its relative speeds of migration over the column. Therefore in order to obtain optimum resolution of sample components, a small sample volume and long exchanger column are necessary. This technique is time consuming and the desired protein invariably elutes in a large volume. However no gradient-forming apparatus is required and the column regeneration is needless. Alteration in the eluent composition is needed to achieve desorption of desired protein completely. To promote desired protein desorption continuous or stepwise variations in the ionic strength and/or pH of the eluent are provided with gradient elution. Continuous gradients generally give better resolution than stepwise gradients [11].

Additives which are protective agents found in the mobile phase are generally used for maintain structure and function of the proteins to be purified. This is achieved by stimulating an adequate microenvironment protection against oxidation or against enzymatic attacks [14]. Any additives used in ion exchange chromatography, should be checked for their charge properties at the working pH in order to avoid undesired effects due to adsorption and desorption processes during chromatography [13-14]. It is recommended to include in the elution buffer those additives in a suitable concentration which have been used for stabilization and solubilization of the sample. Otherwise precipitation may occur on the column during elution [14]. For example; zwitterionic additives such as betaine can prevent precipitation and can be used at high concentrations without interfering with the gradient elution. Detergents are generally useful for solubilization of proteins with low aqueous solubility. Anionic, cationic, zwitterionic and non-ionic (neutral) detergents can be used during ion exchange chromatography. Guanidine hydrochloride or urea, known as denaturing agents can be used for initial solubilization of a sample and during separation. However, they should use if there is a requirement. Guanidine is a charged molecule and therefore can participate to the ion exchange process in the same way as NaCl during separation process [13].

Commonly used eluent additives which have been successfully used in ion exchange chromatography can be given as follow;

- EDTA; Ethylenediamine tetraacetic acid

- Polyols; Glycerol, glucose, and saccharose

- Detergents;

- Urea and guanidinium chloride

- Lipids

- Organic solvents

- Zwitterions

- Sulfhydryl reagents

- Ligands

- Protease inhibitors [14]

1.4. Buffer

In ion exchange chromatography, pH value is an important parameter for separation and can be controlled and adjusted carefully by means of buffer substances [18]. In order to prevent variation in matrix and protein net charge, maintenance of a constant mobile phase pH during separation is essential to avoid pH changing which can occur when both protein and exchanger ions are released into the mobile phase [11]. By means of buffer substances pH value can be controlled and adjusted. Concentration of H^+ and the buffering component influence the protein binding to the stationary phase, chromatographic resolution and structural as well as functional integrity of the protein to be separated. Thus a suitable pH range, in which the stability of sample is guaranteed, has to be identified. Keeping of the sample function is related with the preservation of its three dimensional structure as well as with its biological activity [18]. A number of buffers are suitable for ion-exchange chromatography. A number of important factors influences the selection of mobile phase including buffer charge, buffer strength and buffer pH [11]. Properties of good buffers are high buffering capacity at the working pH, high solubility, high purity and low cost. The buffer salt should also provide a high buffering capacity without contributing much to the conductivity and should not interact with the ion exchanger functional groups as well as with media [11,17]. The buffering component should not interact with the ion exchanger because otherwise local pH shifts can occur during the exchange process which may interfere the elution. Interactions with stationary phase as well as with additives of the mobile phase and with subsequent procedures may be occur with buffer component and selected pH range. Precipitation of the mobile phase components can be observed for example when phosphate buffer and several di- and trivalent metal ions such as Mg^{+2} and Ca^{+2} are mixed or when anionic detergents (i.e. cholate) are used under acidic conditions or in the presence of multivalent metal ions. Precipitation of metal oxides and hydroxides can occur under alkaline conditions. Buffer components may also affect

enzymatic assays used for screening and analysis of chromatography fractions [14]. The concentration of buffer salts usually ranges from 10 to 50 mM. Commonly used buffers are presented in Table 2 and Table 3 for cation and anion exchange chromatography [17].

Generally, applications of ion exchange chromatography are performed under slightly acidic or alkali conditions, pH range 6.0-8.5 but there are also more acidic and more alkali buffers. Additionally the buffering component should not act as an eluting ion by binding to the ion exchanger. Anionic buffer component such as phosphate or MOPS in cation exchange chromatography and cationic buffers such as ethanolamine, Tris and Tricine in anion exchange chromatography are recommended. Besides interactions of buffer component with stationary phase, there are also possible interactions with additives of the mobile phase. To achieve sufficient buffer capacity the pKa of the buffer component should be as close to the desired pH value as possible difference no more than ± 0.5 pH units. However there are examples of successful separations at which the buffering capacity is very low [17-18]. It has to be considered that the pKa is a temperature dependent value. Performing on ion exchange separation with the same elution buffer at room temperature or in the cold room can have a remarkable effect on the buffer capacity. For optimal binding of a sample ion to an ion-exchanger the ionic strength and thus also the buffer concentrations has to be low in sample and equilibration buffers [18].

Substance	pK_a	Working pH
Citric acid	3.1	2.6-3.6
Lactic acid	3.8	3.4-4.3
Acetic acid	4.74	4.3-5.2
2-(*N*-morpholino)ethanesulfonic acid	6.1	5.6-6.6
N-(2-acetamido)-2-iminodiacetic acid	6.6	6.1-7.1
3-(*N*-morpholino)propanesulfonic acid	7.2	6.7-7.7
Phosphate	7.2	6.8-7.6
N-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid)	7.5	7.0-8.0
N,N-bis(2-hydroxyethyl)glycine	8.3	7.6-9.0

Table 2. Commonly used buffers for cation-exchange chromatography

Substance	pK_a	Working pH
N-Methyl-piperazine	4.75	4.25-5.25
Piperazine	5.68	5.2-6.2
Bis-Tris	6.5	6.0-7.0
Bis-Tris propane	6.8	6.3-7.3
Triethanolamine	7.8	7.25-8.25
Tris	8.1	7.6-8.6
N-Methyl-diethanolamine	8.5	8.0-9.0
Diethanolamine	8.9	8.4-9.4
Ethanolamine	9.5	9.0-10.0
1,3-Diaminopropane	10.5	10.0-11.0

Table 3. Commonly used buffers for anion-exchange chromatography

1.5. Detection

Conductivity detector is the most common and useful detector in ion exchange chromatography. However UV and other detectors can also be useful [10]. Conductivity detection gives excellent sensitivity when the conductance of the eluted solute ion is measured in an eluent of low background conductance. Therefore when conductivity detection is used dilute eluents should be preferred and in order for such eluents, to act as effective competing ions, the ion exchange capacity of the column should be low [1].

Although recorders and integrators are used in some older systems, generally in modern ion exchange chromatography results are stored in computer. Retention time and peak areas are the most useful information. Retention times are used to confirm the identity of the unknown peak by comparison with a standard. In order to calculate analyte concentration peak areas are compared with the standards which is in known concentration [10].

Direct detection of anions is possible, providing a detector is available that responds to some property of the sample ions. For example anions that absorb in the UV spectral region can be detected spectrophotometrically. In this case, an eluent anion is selected that does not absorb UV. The eluent used in anion chromatography contains an eluent anion, E^-. Anions with little or no absorbance in the UV spectral region can be detected spectrophotometrically by choosing a strongly absorbing eluent anion. An anion with benzene ring would be suitable [10]. Usually Na^+ or H^+ will be the cation associated with E^-. The eluent anion must be compatible with the detection method used. For conductivity the detection E should have either a significantly lower conductivity than the sample ions or be capable of being converted to a non-ionic form by a chemical suppression system. When a spectrophotometric detection is employed, E will often be chosen for its ability to absorb strongly in the UV or visible spectral region. The concentration of E^- in the eluent will depend on the properties of the ion exchanger used and on the types of anions to be separated [10].

2. Ion exchange chromatograpy applications

Ion exchange chromatography can be applied for the separation and purification of many charged or ionizable molecules such as proteins, peptides, enzymes, nucleotides, DNA, antibiotics, vitamins and etc. from natural sources or synthetic origin. Examples in which ion exchange chromatography was used as a liquid chromatograpic technique for separation or purification of bioactive molecules from natural sources can be given as below.

Sample 1:

Source:	*Nigella sativa* Linn.
Extraction procedure:	Water extract of *N. sativa* was prepared, dried and powdered. Powder was dissolved in phosphate buffer saline (pH 6.4) and centrifuged at 10.000 rpm for 30 min at 4 ºC. The supernatant was collected as the soluble extract by removing the oily layer and unsoluble pellet. Protein concentration of the soluble extract was determined with Bradford method. Then proteins dialyzed against 0.05 M phosphate buffer (pH 6.4) using 3500 MW cut off dialyzing bags and centrifuged.
Stationary Phase:	XK50/30 column (5 x 15 cm) of DEAE sephadex A50.
Eluent:	0.05 M phosphate buffer (pH 6.4) containing 0.01 M NaCl. Fractions of each were collected with an increasing concentration of NaCl
Detection:	UV detector at 280 nm
Analyte(s):	Number of protein bands ranging from 94-10 kDa molecular mass [19].

Sample 2:

Source:	*Olea europea* L.
Extraction procedure:	Extract was prepared from the leaves and roots of two years old olive plants with water at room temperature. Internal standard as D-3-*O*-methylglucopyranose (MeGlu) was used and added in appropriate volume. Extraction was accomplished by shaking for 15 min and finally the suspension was centrifuged at 3000 rpm for 10 min. Before the injection the aqueous phase was filtered and passed on a cartridge OnGuard A (Dionex) to remove anion contaminants.
Stationary Phase:	Two anion exchange columns Dionex CarboPac PA1 plus a guard column and CarboPac MA1 column with a guard column were used for separation procedure (High Performance Anion Exchange Chromatography).
Eluent:	Eluent was comprised 12 mM NaOH with 1 mM barium acetate. Flow rate was 1 mL/min.
Detection:	Pulsed amperometric detection
Analyte(s):	*myo*-inositol, galactinol, mannitol, galactose, glucose, fructose, sucrose, raffinose and stachyose [20].

Sample 3:

Source:	Soybean
Extraction procedure:	Soybeans were defatted with petroleum ether for 30 min and centrifuged repeating the procedure twice. Then proteins were extracted with 0.03 M Tris-HCL buffer containing 0.01 M 2-mercaptoethanol (pH 8) for 1 hour following by centrifugation (16.250 x g for 20 min at 20 ºC). The supernatant was adjusted to pH 6.4 with 2 M HCl and centrifuged (16.250 x g for 20 min at 2-5 ºC). The precipitate was dissolved in Tris-HCl buffer and the process was repeated in order to obtain purified precipitated fraction containing the 11S globulin. The supernatant obtained after the first

precipitation of the 11S fraction was adjusted to pH 4.8 with 2M HCl and centrifuged (16.250 x g for 20 min at 2-5 ºC). The supernatant was stored at low temperature and the precipitate was dissolved in Tris-HCl buffer (pH 8). The process was repeated to obtain a purified precipitated fraction containing the 7S globulin.

Stationary Phase: Anion exchange perfusion column POROS HQ/10 packed with cross-linked polystyrene-divinylbenzene beads.

Eluent: The starting point for the separation of soybean proteins by HPIEC was the use of a binary gradient where mobile phase was a buffer solution at a certain pH (always pHs higher than the isoelectric pH of soybean proteins, pI = 4.8–6.4) and mobile phase B was the same buffer solution containing as well Msodium chloride. The buffer solutions tried were: phosphate ffer (pH 7 and 12), Tris–HCl buffer (pH 8), borate buffer (pH 9), and carbonate buffer (pH 10). In all cases, the buffer concentration was 20 mM. For every buffer, different gradients were tried. The best separation for ybean proteins was obtained with the borate buffer (pH 9) and gradient starting with an isocratic step at 0% B for 2.5 min and from 0 to 70% B in 14 min (gradient slope, 5%B/min). A fine optimization of the selected gradient enabled a reduction of the analysis time keeping the separation. The final gradient was: 0% for 2 min and from 0 to 50% B in 10 min.

Detection: UV detector at 254 nm

Analyte(s): 11S globulin or glycinin and 7S globulin [21].

Sample 4:

Source: *Cochlospermum tinctorium* A. Rich.

Extraction procedure: The powdered roots of *C. tinctorium* were extracted with ethanol (% 96, v/v) using a soxhlet apparatus to remove low molecular weight compounds. Extraction procedures continue until no color could be observed in the ethanol. The residue was extracted with water at 50 ºC, 2 hour for two times. Obtained extract was filtered through gauze and Whatman GF/A glass fiber filter and then concentrated at 40 ºC in vacuum and dialysed at cut-off 3500 Da to give a 50 ºC crude extract. The extracts was kept at -18 ºC or lyophilized.

Stationary Phase: Anion exchange-DEAE-Sepharose column

Eluent: For obtaining neutral fraction the column was eluted with water firstly. The acidic fractions were obtained by elution of linear NaCl gradient (0-1.4 M) in water. The carbohydrate elution profile was determined using the phenol-sulphiric acid method. Finally two column volumes of a 2 M sodium chloride solution in water were eluted to obtain the most acidic polysaccharide fraction. The relevant fractions based on the carbohydrate profile were collected, dialysed and lyophilized.

Detection: UV detector, 490 nm

Analyte(s): Glucose, galactose, arabinose (in neutral fraction) Uronic acids (Both galacturonic and glucuronic acid), rhamnose, galactose, arabinose and glucose (in acidic fraction) [22].

Sample 5:

Source: Hen egg

Extraction procedure: Fresh eggs were collected and the same day extract was obtained. Ovomucin was obtained using isoelectric precipitation of egg white in the presence of 100 mM NaCl solution. The dispersion was kept overnight at 4 ºC and separated by centrifugation at 15.300 x g for 10 min at 4 ºC. The precipitate was further suspended in 500 mM NaCl solution while stirring for 4 h followed by overnight settling at 4 ºC. After centrifugation at 15.300 x g for 10 min at 4 ºC, the precipitate was freeze dried and stored at -20 ºC. The supernatants obtained during the first step (with 100 mM NaCl

solution) and the second step (with 500 mM NaCl solution) was further used for ion exchange chromatography to separate other egg white proteins. Separation proteins from 100 mM supernatant were allowed to pass through an anion exchange chromatographic column to separate different fractions. The unbound fractions were then passed through a cation exchange chromatographic column to separate further.

Stationary Phase:	High-Prep 16/10 column (Q Sepharose FF)-Anion Exchange Chromatography High-Prep 16/10 column (SP Sepharose FF)-Cation Exchange Chromatography
Eluent:	The column was equilibrated with water and the pH was adjusted to 8.0 before injectionAfter sample injection flow-through fraction was collected using water as the eluent, followed by isocratic elution of the sample using 0.14 M NaCl. Finally the bound fraction was eluted using gradient elution (0.14-0.5 M) of NaCl- Anion Exchange Chromatography.
	The unbound fraction was collected and used as starting material for cation exchange chromatography. The column was equilibrated with 10 mM citrate buffer, which was used as the starting buffer. After sample injection the column was eluted by isocratic elution using 0.14 M NaCl solution followed by gradient elution from 0.14 M to 0.50 M NaCl solution. The fractions were collected and freeze dried-Cation Exchange Chromatography.
Detection:	MS Detector
Analyte(s):	Ovalbumin, ovotransferrin, lysozyme, ovomucin [23].
Sample 6:	
Source	: *Phaseolus vulgaris*
Extraction procedure:	Seeds were grounded and soaked in 20 mM Tris-HCl buffer (pH 7.6) at 4 °C for 24 h. The seeds were blended in a blender to extract the proteins followed by centrifugation (30,000g) at 4°C. Then 450 g/l of ammonium sulphate were added to the supernatant to 70% saturation. The precipitate was removed by centrifugation and the supernatant was extensively dialysed against distiled water. The dialysed protein extract was freeze dried and used for chromatographic separation.
Stationary Phase:	Q-Sepharose Column (3 cm x 7 cm), anion-exchange
Eluent:	The column was equilibrated and initially eluted with 20 mM Tris–HCl (pH 7.6). Elution of the bound fraction was carried out by using 1 M NaCl in the equilibration buffer. All chromatographic steps were performed at the flow rate of 100 ml/h. Further separation selected fraction Q1, which was lyophilised and dissolved in 100 mM Tris–HCl (pH 7.6) buffer was performed onto a FPLC Superdex 75 column at a flow rate 0.5 ml min^{-1}.
Detection:	UV detector, 280 nm
Analyte(s):	A 5447 Da antifungal peptide [24].
Sample 7:	
Source:	Sweet dairy whey
Extraction procedure:	After the cheese making process the sweet whey is produced, it is further processed by reverse osmosis to increase the solids content from approximately 5.5% (w/w) to 14.6% (w/w).
Stationary phase:	Pharmacia's Q- and S-Sepharose anion- and cation-exchange resins
Eluent 1:	For the anion-exchange process; it was found that two step changes, simultaneous in pH and salt concentration were necessary to carry out the anion-exchange separation. A 0.01 *M* sodium acetate buffer, pH 5.8, was used for the starting state or feed loading buffer. After the whey feed was loaded onto the column, one column volume of this

buffer was passed through to wash out any material that did not bind to the resin, including the IgG. Next, two column volumes of 0.05 M sodium acetate, pH 5.0, were passed through the column to desorb those proteins whose pI values were above 5.0. This includes the β-lactoglobulin and bovine serum albumin. This was then followed by two column volumes of 0.1 M sodium acetate, pH 4.0, to finally desorb the α-lactalbumin whose pI range is 4.2–4.5, and thus above that of the passing pH wave of 4.0. After this second step change, the cleaning cycle was then implemented to prepare the column for the next run.

Eluent 2: For the cation-exchange process, it was found that one step change in pH was appropriate to carry out the cation-exchange separation. The buffer used was 0.05 M sodium acetate, pH 5.5, as the starting state or feed loading buffer. One column volume loading of the anion-exchange breakthrough curve fraction was optimum for loading onto the cation-exchange column. After the anion-exchange breakthrough curve fraction was loaded onto the column, one column volume of the initial buffer was passed through to wash out any material that did not bind to the resin. Next a step change in pH was implemented to elute the bound IgG. This was accomplished by passing two column volumes of the buffer, 0.05 M sodium acetate, pH 8.5. As the pH wave of this buffer passed through the cation bed it initiated the elution of the IgG because the upper value of its pI range is 8.3. After this pH step change the cleaning cycle was then implemented.

Detection: UV Detector

Analyte(s): α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulin G and lactose [25].

Sample 8:

Source: *Morus alba* (mulberry) leaves

Extraction procedure: Fresh leaves were homogenized in ice-cold 50 mM Tris–HCl, pH 7.5, containing 0.3 M NaCl, 20 mM diethyldithiocarbamic acid, 5% glycerol, and 2% polyvinylpyrrolidone. The buffer used was 3 ml g^{-1} of the fresh leaves. The homogenate was filtered through a layer of cheesecloth and stored at 20°C for 24 h. After thawing, it was centrifuged at 8000xg, for 40 min at 4°C. The supernatant was collected and ammonium sulfate was added to 70% saturation. The resulting precipitate was recovered by centrifugation at 8000xg for 40 min, redissolved in tris-buffered saline, TBS (50 mM Tris–HCl, pH 7.5 containing 0.3 M NaCl) and dialysed against the buffer overnight at 4°C. The solution was then centrifuged at 13,000xg for 15 min and the supernatant was collected and stored at -20°C. An aliquot of the dialysed ammonium sulfate fraction containing protein was applied to the affinity chromatography on the *N*-acetylgalactosamine-agarose column. And then further separation was performed on Sephacryl S-200 column followed by anion exchange chromatography. Further purification was also performed by anion exchange and gel filtration chromatography

Stationary Phase: Anion-exchange chromatography, a DEAE-Sephacel column (2x9 cm)

Eluent: Equilibrated with 20 mM Tris–HCl, pH 7.5 at flow rate 20 ml min^{-1} and then eluted stepwise with the buffer containing NaCl.

Detection: UV Detector, 280 nm

Analyte(s): Lectins, MLL 1 and MLL 2 [26]

Sample 9:

Source: *Lycium ruthenicum* Murr.

Extraction: Fruits of the plant extracted with hot water yielded a crude polysaccharide sample, CLRP. The carbohydrate of CLRP was 66.2% and protein content was 7.3%. CLRP was a black Polysaccharide sample in which the pigment could not be removed by colum chromatography. To avoid the influence of pigment on the structure analysis, decoloration was performed with 30% H_2O. After decoloration, the carbohydrate content of decolored CLRP was 93.2% and protein content was 4.4%. Decolored CLRP was purified by anion exchange chromatography, yielding five polysaccharide subfractions LRP1, LRP2, LRP3, LRP4, and LRP5.

Stationary Phase: DEAE-cellulose column

Eluent: Distilled water, 0.05–0.50 mol/L $NaHCO_3$ $^-$ solution

Detection: UV Detector, 280 nm

Analyte(s):   Glycoconjugate polysaccharide (LRGP1) [27]

Sample 10:

Source: *Coprinus comatus*

Extraction: Stipe powder of *C. comatus* (100 g) was extracted three times with 1 L 95% ethanol under reflux for 2 h to remove lipid, and the residue was extracted three times with 2 L distilled water for 2 h at 80 °C with intermediate centrifugation (2000 × *g*, 15 min). After concentrating the collected aqueous supernatants to 400 mL (reduced pressure at 40 °C), a precipitation was performed with 3 volumes of 95% ethanol. The precipitate was washed with ethanol and acetone, and then dried at 40 °C, yielding crude polysaccharide material. Crude polysaccharide material was dissolved in 100 mL 0.2 M sodium phosphate buffer (pH 6.0), and after centrifugation the solution was applied to a DEAE-Sepharose CL-6B column.

Stationary Phase: DEAE-Sepharose CL-6B column (3.5 cm × 30 cm).

Eluent: 0.2 M sodium phosphate buffer (pH 6.0), and linear gradient of 0.3–1.5 M NaCl in 0.2 M sodium phosphate buffer (pH 6.0).

Detection: UV Detector, 490 nm (phenol–H_2SO_4) and 500 nm (Folin–phenol)

Analyte(s):   Polysaccharides; disaccharide α,α-trehalose, α-D-glucan, β-D-glucan, α-L-fuco-α-D-galactan [28].

Sample 11:

Source: *Physalisalkekengi* var. *francheti*

Extraction: The dried and defatted fruit calyx extracted with different enzyme Neutral proteinase, Papain and alkaline protease, respectively, in their suitable pH and temperature and then each extract was centrifuged at 5000 rpm for 10 min. The supernatant was concentrated and then precipitated by the addition of ethanol in 1:4 (v/v) at room temperature. The precipitate was dissolved in distilled water and the solution was then washed with sevag reagent (isoamyl alcohol and chloroform in 1:4 ratio), which were centrifuged at 5000 rpm for 15 min and the protein was removed. The supernatant was dialyzed against deionized water for 24 h before concentration under vacuum evaporator at 55 °C. The mixture was precipitated by the addition of ethanol in 1:4 (v/v) at room temperature and the precipitate was freeze dried. Total sugars were determined by the phenol–sulfuric acid assay using glucose as standard.

Stationary Phase: DEAE anion-exchange column

Eluent: The column was eluted first with distilled water, and then with gradient solutions (0.1 M, 0.25 M, 0.5 M NaCl and 0.5 M NaOH), at a flow rate of 0.6 mL/min. The major polysaccharidefractions were collected with a fraction collector and concentrated using a rotary evaporator at 55 °C and residues were loaded onto a Sephadex G-200

gel column (2.5 × 65 cm). The column was eluted with 0.1 M NaCl at a flow rate of 0.3 mL/min. The major fraction was collected and then freeze dried. All of these fractions were assayed for sugar content by the phenol–sulfuric acid method using glucose as standard

Detection: UV Detector, 490 nm

Analyte(s):   Polysaccharides [29].

Sample 12:

Source: *Ornithogalum caudatum* Ait.

Extraction: The whole dried plant was soaked with 95% ethanol to remove the pigments, defats and inactivates enzymes, and refluxed by hot distilled water for 4 h at 90 °C. The aqueous extract was concentrated to 30% of the original volume under reduced pressure in a rotary evaporator, and proteins were removed with Sevag method. The obtained solution was precipitated with 40% ethanol. The supernatant was added by ethanol up to 60%, and kept at 4 °C overnight. The polysaccharide pellets were obtained by centrifugation at 4000 rpm for 15 min, and completely dissolved in appropriate volume of distilled water followed by intensive dialysis for 2 days against distilled water (cut-off M_w 3500 Da). The retentate portion was then concentrated, and centrifuged to remove insoluble material. Finally the supernatant was lyophilized to give crude extract. The crude extract was dissolved in 0.2 mol/L tris (hydroxymethyl) aminomethane hydrochloride buffer solution, and filtered through a filter paper. The solution was passed through an anion-exchange chromatography column. After ion exchange chromatography other chromatographic methods was used for further separations.

Stationary Phase: DEAE-Sepharose fast flow anion-exchange chromatography column (10 × 300 mm)

Eluent: The polysaccharides were eluted with Tris–HCl buffer solution, followed with gradient elution of 0.1–0.8 mol/L NaCl at a flow rate of 0.8 ml/min.

Detection: UV Detector, 486 nm (phenol–sulfuric acid method)

Analyte(s):   Water soluble polysaccharides [30].

Sample 13

Source: *Paecilomyces variotii*

Extraction: After fermentation process, ammonium sulphate was added to the supernatant to give a final concentration of 80% saturation. The ammonium sulphate was added with constant stirring at 4ºC and the mixture stood overnight at 4ºC. The precipitated proteins were separated by centrifugation at 10000 rpm at 5ºC for 30 min. The separated proteins were then re-suspended in a minimum amount of distilled water and the solution dialyzed (using cellulose dialysis tubing) for 24 hrs against distilled water and concentrated by freeze-drying. The partially purified enzyme was dissolved in acetate buffer (20 mM - pH 6.0) and passed through a column

Stationary Phase: Diethylaminoethyl (DEAE) Sepharose column (0.7 x 2.5 cm)

Eluent: Acetate buffer (20 mM - pH 6.0)equilibrated with the same buffer. The solution was passed through the column at a flow rate of 1 mL.mim^{-1} with acetate buffer (20 mM - pH 6.0), followed by a linear gradient from 0-1M NaCl in the acetate buffer. The eluted fractions were collected in an automated fraction collector (Pharmacia Biotech) and the absorbance of the fractions was measured at 280 nm. The major peak fractions were then assayed for tannase activity, and only the fractions possessing tannase activity were pooled.

Detection: UV Detector, 280 nm

Analyte:	Tannase [31]
Sample 14	
Source:	*Castanospermum australe*
Extraction:	50% MeOH extract of seeds
Stationary Phase:	(1) Amberlite IR-120B (500 mL H$^+$ form), (2) Dowex 1-X2 column (3.8×90 cm, OH$^-$form), (3) Amberlite CG-50 column (3.8×90 cm, NH4 + form), (4) Dowex 1-X2 column (3.8×90 cm, OH$^-$ form) (Repeated separation on different ion exchange columns).
Eluent:	0.5 M NH$_4$OH, H$_2$O
Detection:	UV Detection by HPTLC
Analyte(s):	Pyrolizidine alkaloids; fagomine; 6-epi-castanospermine; castanospermine; australine; 3-epi-fagomine; 2,3-diepi-australine; 2,3,7-triepi-australine; 3-epi-australine; 2R-hydroxymethyl-3S-hydroxypyrrolidine; castanospermine-8-O--D-glucopyranoside; 1-epi-australine-2-O--D-glucopyranoside and 1-epi-australine [32].

3. Conclusion

Since the isolation of pharmacologically active substances which are responsible for the activity became possible at the beginning of the 19th century drug discovery researches have increased dramatically [33]. Therefore within the last decade there has also been increasing interest in the liquid chromatographic processes because of the growing pharmaceutical industry and needs from the pharmaceutical and specialty chemical industries for highly specific and efficient separation methods. Several different types of liquid chromatography techniques are utilized for isolation of bioactive molecules from different sources [25]. Ion exchange chromatography is probably the most powerful and classic type of liquid chromatography. It is still widely used today for the analysis and separation of molecules which are differently charged or ionizable such as proteins, enzymes, peptides, amino acids, nucleic acids, carbohydrates, polysaccharides, lectins by itself or in combination with other chromatographic techniques [34]. Additionally ion exchange chromatography can be applied for separation and purification of organic molecules from natural sources which are protonated bases such as alkaloids, or deprotonated acids such as fatty acids or amino acid derivatives [35]. Ion exchange chromatography has many advantages. This method is widely applicable to the analysis of a large number of molecules with high capacity. The technique is easily transferred to the manufacturing scales with low cost. High levels of purification of the desired molecule can be achieved by ion exchange step. Follow-up of the nonsolvent extractable natural products can be realized by this technique [17,35]. Consequently ion exchange chromatography, which has been used in the separation of ionic molecules for more than half a century is still used as an useful and popular method for isolation of natural products in modern drug discovery and it continue to expand with development of new technologies.

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