PART I

PRINCIPLES, MECHANISM, AND INSTRUMENTATION
1.1 INTRODUCTION

Ionic methods of separation have been used since the industrial revolution in Europe to reduce hardness of water. In the mid-nineteenth century, British researchers treated various clays with ammonium sulfate or carbonate in solution to release calcium. In the early twentieth century, zeolite columns were used to remove interfering calcium and magnesium ions from solutions to permit determination of sulfate. Ionic separation procedures were used in the Manhattan project to purify and concentrate radioactive materials needed to make atom bombs. Peterson and Sober [1] reported in 1956 a chromatographic method based on ion exchange to separate proteins. However, ion chromatography (IC), in its modern form, was introduced in 1975 by Small et al. [2]. The technique has since gained significant attention for the analysis of a wide variety of analytes in pharmaceutical, biotechnology, environmental, agricultural, and other industries. Several books and chapters on IC have provided a detailed review of its principles and instrumentation [3–5]. In 2000, United States
Pharmacopeia-National Formulary (USP-NF) had only a few monographs that described test methods involving IC [6] and no general chapter on this technique. However, the number of monographs that include one or more IC-based test procedures has increased dramatically in the last 10 years. In addition, the current USP-NF [7] contains two general chapters on IC (<345> and <1065>) and at least four general chapters that include IC-based test methods (<1045>, <1052>, <1055>, <1086>), indicating its importance as a chromatographic technique for the analysis of pharmaceutical drug substances, products and excipients. In General Chapter <1065>, entitled “Ion Chromatography”, USP-NF describes ion chromatography as “a high-performance liquid chromatography (HPLC) instrumental technique used in USP test procedures such as identification tests and assays to measure inorganic anions and cations, organic acids, carbohydrates, sugar alcohols, aminoglycosides, amino acids, proteins, glycoproteins, and potentially other analytes” [7].

This chapter will present an introduction to IC providing an outline of its principles and applications in the analysis of active and inactive ingredients, counter-ions, excipients, degradation products, and impurities relevant to the analysis of pharmaceutical, biologic and biotechnology-derived therapeutic and prophylactic products.

1.2 WHAT IS ION CHROMATOGRAPHY?

Modern IC is a form of HPLC, just as normal phase, reversed-phase and size exclusion chromatographies are different forms of HPLC. The separation in IC is based on ionic (or electrostatic) interactions between ionic and polar analytes, ions present in the eluent, and ionic functional groups derivatized to the chromatographic support. This can lead to two distinct mechanisms of separation—(a) ion exchange due to competitive ionic binding (attraction), and (b) ion exclusion due to repulsion between similarly charged analyte ions and the ions derivatized on the chromatographic support. Separation based on ion exchange has been the predominant form of IC to-date. In addition, chromatographic methods in which the separation due to ion exchange or ion exclusion is modified by the hydrophobic characters of the analyte or the chromatographic support material, by the presence of the organic modifiers in the eluent or due to ion-pair agents, resulting in better resolution that were not achieved otherwise, have gained popularity recently (mixed mode separation).

Numerous studies have been conducted in the last 30 years to understand the details of the mechanisms of ion-exchange and ion-exclusion chromatographies and the effect of different elution parameters, including flow rate, salt concentration, pH, presence of organic solvents, and temperature, on them. The current chapter is not meant to provide a comprehensive review of the studies. Rather, it is meant to provide a general introduction to both types of IC explaining in a qualitative non-mathematical approach how they work, what types of analytes are suitable for separation by ion-exchange and ion-exclusion chromatographies, and the effect of different factors on their performance.
1.3 ION-EXCHANGE CHROMATOGRAPHY

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. That is, a column used to separate cations, called a cation-exchange column, contains negatively charged functional groups. Similarly, an anion-exchange column, which separates anions, is derivatized with positively charged functional groups. Ion-exchange chromatography has been widely used in the analysis 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.

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 represented by the following scheme:

\[ S^- X^+ + M^+ \leftrightarrow S^- X^+ M^+ + C^+ \] (1)

In this process, the cation \( M^+ \) of the eluent exchanges for the analyte cation \( C^+ \) bound to the anion \( X^- \) derivatized on the surface of the chromatographic support (S). If, on the other hand, the exchanging ions are anions, it is called anion-exchange chromatography and is represented as:

\[ S^- X^+ A^- + B^- \leftrightarrow S^- X^+ B^- + A^- \] (2)

in which, the anion \( B^- \) of the eluent exchanges for 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 as they travel along the length of the column, resulting in the separation due to ion-exchange [8].

1.3.1 Mechanism

The mechanism of the two processes, cation exchange and anion exchange, are indeed, very similar. In the first step of the process, analyte ions diffuse close to the stationary phase and bind to the oppositely charged ionic sites derivatized on the stationary phase through the Coulombic attraction. The Coulombic force of interaction \( f \) between the two ions in solution, in its simplified form, is given by the equation,

\[ f = \frac{q_1 q_2}{\varepsilon r^2} \] (3)

in which \( q_1 \) and \( q_2 \) are charges on two ions, \( \varepsilon \) is the dielectric constant of the medium, and \( r \) is the distance between them. In most of the ion chromatographic separations,
except when organic solvents are included as modifiers, the medium is water (solutions of acids, alkalis or salts). Therefore, we can consider $\varepsilon$ to be a constant. If the charges on both ions are similar (either both positive or both negative), the force is repulsive. Where they are dissimilar (one positive and the other negative), the force is attractive. We need to remember two basic principles of thermodynamics to understand the mechanism. (1) Attractive force between two oppositely charged ions results in decrease in enthalpy ($H$) and free energy ($G$). (2) The thermodynamic principles favor the process in which the free energy change is negative.

In a column, the bound analyte ions face competition from similarly charged ions present in the eluent as they compete for binding to the same oppositely charged ionic sites of the stationary phase. For example, the negatively charged analyte ions and the negative ions present in the eluent both compete for the positively charged sites on the stationary phase. Overcoming binding due to the ionic attraction between negatively charged analyte ions and the positively charged ionic site of the stationary phase requires 'work' and leads to an increase in free energy (and enthalpy) of the system and, as such, is not thermodynamically favorable. However, the increase is overwhelmingly compensated by the decrease in free energy (and enthalpy) due to the binding of the negative ions of the eluent because the concentration of the negative ions of the eluent is overwhelmingly greater than that of the analyte ion concentration. To illustrate this with a simple example, the typical concentration of an eluent in IC ranges between 10–100 mM (in some cases, as low as 1 mM or as high as 500 mM). However, the typical concentration of each analyte is in the micromolar to sub-micromolar range. Thus, the concentration of the eluent ion is $10^4$–$10^5$ fold higher than that of the concentration of the analyte ion. The energy input needed to displace an analyte ion from the stationary phase is significantly less than the energy released due to attractive interactions between the stationary phase ion and the overwhelmingly larger number of ions in the eluent resulting in a decrease of free energy and the overall process is thermodynamically favored.

When ionic or polar analytes enter an ion-exchange column, they first bind to the charged sites of the stationary phase in a layer. As different amounts of energy are needed to unbind different analytes from the stationary phase, due to differences in charge density and other factors (see later), the desorption takes place at a different rate and/or requires different concentrations of eluent ions. This leads to separation of the analytes—the analyte requiring lesser energy is desorbed (eluted) earlier from the stationary phase. This adsorption-desorption phenomenon continues from layer to layer as the analytes travel along the length of the chromatographic column, increasing separation between the analytes (Figure 1.1). In an optimized separation procedure, the analytes are resolved when they exit the column.

Equation (3) predicts that the force of attraction between a monovalent analyte ion with one unit of charge (e.g., chloride) and an ionic site on the stationary phase will be lesser than that between a divalent analyte ion (e.g., sulfate), which has two units of charge, and the same stationary phase ionic site. Thus, a higher concentration of eluent ion will be necessary to displace a divalent ion from the stationary phase than that required to displace a monovalent ion, resulting in a separation of the two by IC, and the monovalent ion will be eluted from the column earlier than a divalent ion.
Similarly, a trivalent ion will bind the stationary phase more strongly than a divalent ion and will be eluted from the column after the divalent ion.

The above discussion, however, does not explain separation of monovalent ions from an ion exchange column. It is conceivable that we should consider the charge density on the surface of an ion rather than its actual charge, since the ions, particularly those of interest in the analysis of pharmaceutical drugs, are not point masses and the underlying assumption of equation (3) is that the charges are points. A larger monovalent ion (e.g., chloride) will have less charge density than a smaller monovalent ion (e.g., fluoride), since both have a total of one unit of charge. Thus, fluoride ion is expected to bind more strongly on a stationary phase than chloride, require a higher eluent concentration to displace, and elute later from the column. So, when a mixture of fluoride, chloride and bromide is chromatographed on an IC column, bromide is expected to be eluted first (being the largest and therefore having the lowest charge density among the three ions), then chloride and then fluoride. In reality, however, the elution order is found to be reversed. For example, when a mixture of different anions are eluted from an IonPac AS11 column with sodium hydroxide [9], fluoride ion is eluted first, then chloride and then bromide. In fact, the results from the same example show that when a mixture of fluoride, chloride, bromide, nitrate, acetate, and benzoate, all of which are monovalent ions, are eluted from an IonPac AS11 with sodium hydroxide [9], the elution sequence of the ions is,

\[
\text{Fluoride} > \text{acetate} > \text{chloride} > \text{bromide} > \text{nitrate} > \text{benzoate}
\]  \quad (4)

With the exception of acetate, it appears that a smaller ion is eluted earlier than a larger ion. Similarly, when a mixture of trivalent ions, phosphate and citrate, are eluted from an IonPac AS11 column with sodium hydroxide, the less bulkier phosphate ion is eluted before the bulkier citrate ion [10]. That is, the elution sequence is the reverse of what is expected based on their charge densities.

It is of interest to note that the sequence in which these ions are eluted from the column closely resembles the Hofmeister series (or the lyotropic series) [11]. It is
conceivable that the mechanism of separation is somehow related to the mechanism that led to the Hofmeister series [12]. The binding of the analyte ions to the ions on the stationary phase followed by competitive desorption by similar ions present in the eluent, as discussed above, indeed, represent only part of the overall process. Water molecules play a very critical role in the overall process.

An ion in aqueous solution (or for that matter in solution of a polar solvent) does not exist as a free ion. It is hydrated (or generally speaking solvated) with several molecules of water (or solvent). The hydration extends over several layers of water molecules, primarily through coordinate bond formation, formation of hydrogen bonds, and Van der Waals type ion-dipole and dipole-dipole interactions, depending on the nature and charge of the ions, forming a hydration sheath around each ion. The thickness of this sheath is roughly proportional to the charge density of the ion. The water molecules of the sheath interact with the molecules of the bulk water through ion-dipole and dipole-dipole interactions and thereby become part of an overall water structure. Thus, when an eluent ion binds to the stationary phase, it has to free itself from this structure. While free energy (G) is reduced due to the attractive binding between the oppositely charged ions, a considerable amount of free energy is required to break the water structure. However, the ion that was exchanged out of the stationary phase due to the above binding has the same charge as the ion that exchanges in. The former ion immediately forms its own water structure in the solution. While energy needs to be put in to unbind the ion, a significant amount of free energy is released due to the formation of the water structure. Schematically, the overall process can be described as:

\[
\begin{align*}
\text{Destruction of water structure of the eluent ion} & \quad \rightarrow \quad \text{Increase in } G \\
\text{Binding of the eluent ion to the stationary phase} & \quad \rightarrow \quad \text{Decrease in } G \\
\text{Unbinding of an analyte ion from the stationary phase} & \quad \rightarrow \quad \text{Increase in } G \\
\text{Formation of the water structure around the analyte ion} & \quad \rightarrow \quad \text{Decrease in } G 
\end{align*}
\]

The overall change in free energy is a combination of the free energy changes of the individual steps. A smaller ion will have a high charge density. So, it will be able to form a significantly extended water structure around it resulting in a large decrease in free energy. Thus, a smaller monovalent ion (e.g., fluoride) is eluted from the column earlier than a larger monovalent ion (e.g., chloride) because of a larger reduction of free energy as a result of extended hydration around it. Oxygenated ions such as acetate can form a significantly thicker hydration sheath around it than is expected from its charge density. The oxygen atoms present in these ions can form strong hydrogen bonds with hydrogen atoms of water in the initial layer. Subsequent layers of hydration are formed through hydrogen bonding among the water molecules as well as due to strong ion-dipole and dipole-dipole interactions. Such ions in solution can form a very stable structure permitting a large decrease in the free energy. Thus, even though acetate ion is bulky it is eluted earlier from the column than the chloride and bromide ions, which are smaller than acetate.
1.3.2 Eluent

Typically the eluents used in ion exchange chromatography are acids, alkalis or salt solutions, and do not contain an organic solvent (however, see later). The extremes of pH conditions offered by acids or alkalis help ionize polar molecules into ions. An excellent example is the ionization of neutral sugars and alditols under the high pH conditions, typically 10–500 mM sodium hydroxide, used in High Performance Anion Exchange Chromatography (HPAEC). However, such applications will require analyte molecules to be stable in the acid or alkali used as the eluent. This sometimes limits the application of IC in the analysis of pharmaceutical drugs because the analyte may not be stable under the extreme pH conditions of acids or alkalis. If the analyte molecules are ionic or strongly polarized, elution by salt solutions or buffers of controlled pH conditions, often provide an excellent opportunity for separation by IC. [Using acids or alkalis as eluents has an additional advantage, when suppressed conductivity detection is used. This will be discussed later.]

The elution can be isocratic or with increasing salt concentrations, either by batch or gradient elution, or by altering pH of the eluent. Less tightly bound ions are eluted initially; more tightly bound analytes are eluted either under altered elution conditions (e.g., higher salt concentration or different pH) or simply later, resulting in separation. When gradient elution is used, the peak is expected to be slightly asymmetric and the tailing factor [7] is expected to be greater than 1. As an analyte band travels through the column (Figure 1.1), the eluent behind it has a concentration higher than the concentration at which it is eluted. So, the back of the band cannot bind to the column but can diffuse through the eluent. However, the eluent concentration at the front of a band is lower than the concentration at which it is eluted. It, therefore, binds to the column and its diffusion is restricted.

Changing eluent pH can change the ionic characters of the analytes and/or the functional groups on the chromatographic support. Thus, an anion may become less ionic at a lower pH. However, the actual ionic character depends on the pKₐ of the acid containing the anion (A⁻), which is the negative logarithm of the equilibrium constant of the following equilibrium:

\[
A^- + H^+ \leftrightarrow HA
\]  

(5)

The further the elution pH is from the pKₐ, the more ionic it will be. Thus, the anion with a lower pKₐ value (more acidic) will be eluted after an anion with a higher pKₐ value (less acidic). Similarly, a cation having a lower pKₘ value (more basic) will be eluted after a cation with a higher pKₘ value (less basic).

1.3.3 Organic Solvents

Sometimes small quantities of organic solvents (organic modifier) are added to IC eluent to achieve better separation, to reduce hydrophobic interaction with the column packings, and for improving chromatographic/peak parameters (e.g., theoretical plate, resolution, peak shape). We now need to consider the $\varepsilon$ term used in Equation 3
above to understand the effect of organic modifiers. The dielectric constant of water is around 80 at 20°C. The value of this parameter is below 50 for most of the organic solvents. Thus, when organic solvents are added to an aqueous eluent, the dielectric constant of the medium is decreased. This results in a tighter binding of the analyte and eluent ions to the stationary phase because this term appears in the denominator in Equation 3, which alters the elution pattern.

Inclusion of organic solvents also affects the formation of water structure around an ion by (a) altering the forces of ion-dipole and dipole-dipole interactions and hydrogen bonding due to altered dielectric constant, and (b) interferes with the formation of water structure by inserting itself into the structure. The forces of ion-dipole and dipole-dipole interactions, which, in turn, also affect hydrogen bond formation, are governed by the Coulomb’s Law of interaction (Equation 3). The force of such interaction is, thereby, altered by the inclusion of organic solvents. However, the impact will not be significant when a small quantity of organic solvent is used.

The polar organic solvent molecules, particularly those containing oxygen atoms, also enter into the hydration sheath by forming hydrogen bonds. However, they cannot form as extensive a hydrogen bond network as water due to the hydrophobic nature of such molecules and their larger size, thereby weakening the water structure. Thus, less free energy is needed to break such structures as an eluent ion binds to the stationary phase. Similarly, there is a lower reduction of free energy when the analyte ion is released into the eluent.

Inclusion of an organic solvent also reduces the effect of hydrophobic association between the analyte molecules and the stationary phase. In particular, when the analyte has a significant hydrophobic surface, as is the case for many pharmaceutical drugs, it often shows a broad peak in IC due to its interaction with the hydrophobic surface of the chromatographic support. Inclusion of a small quantity of organic solvent often results in sharper peaks thereby improving peak characteristics and other chromatographic parameters (e.g., resolution) by reducing the effect of hydrophobicity.

### 1.3.4 Other Factors

The dissociation constants of analytes vary with temperature, although the extent of variation is usually small. This does not have any effect on the chromatographic profile, where the analytes are fully ionized under the conditions of chromatography. However, the retention times of analytes that are not fully ionized will vary slightly with temperature. This variation does not pose a significant problem because samples relevant to pharmaceutical applications are usually run with a reference standard. Thus, ion-exchange chromatography is typically run under ambient or near ambient temperatures. Similarly, pressure does not affect elution profiles, as the effects of pressure on dissociation constants are negligible. However, the columns should be operated at their optimum operating pressures (or pressure range) to maintain high performance.

Since ion-exchange chromatography involves binding and unbinding of analyte ions to charges on the surface of the chromatographic support, it is critical that analyte ions are able to diffuse to the chromatographic support to bind to it and diffuse away from the support when desorbed. Therefore, the flow rate must be such as to
1.4 ION-EXCLUSION CHROMATOGRAPHY

Introduced by Wheaton and Bauman in 1953 [13], Ion-exclusion Chromatography uses strong cation- or anion-exchange chromatographic supports to separate ionic, polar, weakly polar, and apolar analytes, and has been used in the analysis of organic acids, alcohols, glycols and sugars. In contrast to ion-exchange chromatography, the charge on the functional groups on the chromatographic support is the same as the charge on the analyte ion. That is, to separate negatively charged or negatively polarized analytes, the chromatographic supports are derivatized with negatively charged functional groups (typically, sulfonate). Similarly, analytes with positive charge or polarity are separated using a chromatographic support that carries positive charges (most frequently, quaternary ammonium ions).

1.4.1 Mechanism

Although the actual mechanism of separation is not fully understood, it is widely held that the separation is effected by partition of analytes between the stationary phase and the mobile phase across a hypothetical semipermeable Donnan membrane. This theory will be discussed briefly in this chapter. An alternate explanation is presented in Chapter 2 of this book.

Water molecules bind to the ionic functional groups of the chromatographic support through coordination, hydrogen bond, and Van der Waals type ion-dipole interaction forming hydration spheres around the functional groups. Water molecules in this hydration sphere and also those trapped in the interstitial spaces (and pores) of the resins are immobilized around the chromatographic support forming the stationary phase system. As a fully ionized analyte in the mobile phase approaches a stationary phase containing like charges (e.g., chloride ion approaching a stationary phase around a sulfonate-derivatized resin), it is strongly repelled by the similar charge. The repulsion is Coulombic and the repulsive force is given by the Equation 3 above. The repulsive force increases rapidly as the ionic analyte approaches the stationary phase because the Equation 3 contains the $r^2$ term in the denominator. The repulsion does not permit the ionic analytes to come more than a certain distance from the stationary phase system forming the outer surface of the hypothetical Donnan membrane (Figure 1.2) and such analytes elute from the column without being retained.

When an apolar molecule approaches the same stationary phase, it experiences no repulsion as the $q$ term corresponding to an apolar molecule in Equation 3 is zero. So, it can freely penetrate deep into the immobilized water layer, which permits it to stay longer in the column. Such molecules partition back and forth at different layers as it they travels along the length of the column. Thus, an apolar analyte is
eluted from the column well after ionic and polar analytes. A polar analyte, which has partial separation of charges within the molecule (forming a dipole), experiences less repulsion than an ion but more than an apolar molecule. Thus, the degree of penetration of such an analyte is in between an ion and an apolar molecule and it is eluted from the column in between ionic and apolar analytes.

It is also clear from Equation 3 above that the force of repulsion experienced by a polar analyte depends on its dipolar character. An analyte that is more polar has more ionic character, thus, experiences greater repulsion and, therefore, will penetrate less into the stationary phase and will be eluted earlier from the column, compared to a less polar analyte. Thus, less and less polar molecules elute later and later from the column and an apolar molecule elutes at the end resulting in separation.

However, it appears that the partition mechanism does not fully explain many of the separations achieved by ion-exclusion chromatography. Additional mechanisms seem to play some role in the process (see Chapter 2 of this book).

**Hydrophobic Properties** of analyte molecules play an important role in the separation. Molecules with extended hydrophobic surface are retained longer in the column due to stronger hydrophobic association with the stationary phase system. For example, the elution times of aliphatic carboxylic acids become longer as the length of the alkyl groups increases [14]. The elution order of a mixture of the first three aliphatic carboxylic acids is:

formic acid > acetic acid > propionic acid.

Calculations based on their pKₐ values indicate that these three aliphatic carboxylic acids are strongly ionized in solution (60–97%). Thus, they should come out close
to the void volume of the column based on the partition mechanism discussed above. Although formic acid is eluted close to the void volume, the other two are eluted later. Similarly, higher aliphatic amines (e.g., butylamine, pentylamine, diethylamine) show longer elution time due to the hydrophobic character of their long aliphatic chains. The elution times are reduced and the peak shapes are considerably improved when an organic solvent is included in the mobile phase [15].

π–π interaction also plays a role in the separation by ion exclusion chromatography when the support contains a double bond or an aromatic ring (e.g., polystyrene). For example, acrylic acid, which contains a double bond, elutes after propionic acid. Aromatic acids, which contain a benzene ring show long retention time on the column [14].

Hydrogen bonding is an important factor, particularly in the separation of molecules that contains several hydroxyl groups, e.g., carbohydrates. These molecules are retained longer by the stationary phase, presumably due to hydrogen bonding with the hydration sphere of the stationary phase system.

Steric factors also play a role in ion-exclusion chromatography. Molecules with bulkier groups are excluded earlier. For example, a dicarboxylate (e.g., oxalate) is eluted earlier than a monocarboxylate (e.g., acetate) when eluted with 7.5 mM sulfuric acid. An iso-carboxylic acid (e.g., iso-butyric acid) is eluted earlier than the corresponding normal carboxylic acid [14].

Complexation with the positive counter-ion of the chromatographic support also plays a role in the separation of analytes containing hydroxyl groups (e.g., sugars). Calcium and lead forms of a cation-exchange resin are often used to separate neutral monosaccharides.

1.4.2 Eluent

Based on the partition mechanism discussed above, it is conceivable that deionized water can be used as the eluent during ion-exclusion chromatography. However, several problems have been encountered [14–16]. Although water is found suitable for the resolution of very weak acids, such as carbonic and boric acids, or very weak bases, strong or even moderately strong acids and bases are too ionized in water to be separated. They are not retained sufficiently due to their high degree of ionization and are eluted within the void volume or close to the void volume without adequate resolution. Secondly, the peaks are often fronted, broad, and/or significantly tailed, due to factors other than pure partition mechanism described above. Typically, dilute solutions of strong acids and alkalis are used in the separation of anionic (e.g., carboxylic acids) and cationic (e.g., amines) solutes, respectively, to overcome the problem. Sulfuric, hydrochloric and aliphatic sulfonic acids are widely used. The strong acids suppress ionization of carboxylic acids permitting them to be resolved. Phosphoric acid and perfluorobutyric acid have been used successfully for the separation of weaker acids. Amines are separated using dilute alkalis, such as sodium hydroxide. It is interesting to note that eluents of the same pH, when used with the same stationary phase, produce very similar chromatographic profiles, irrespective of the nature of the acid.
used as the eluent. The choice of actual acid to be used as eluent, therefore, is often determined by the detection system to be used.

Sometimes, addition of organic solvents to aqueous eluents leads to reduction of run time, sharper peaks and higher resolution because organic solvents minimize the hydrophobic effects. The organic solvent to be used and its concentration are determined by its compatibility with the detection system.

1.4.3 Other Factors

Ion-exclusion chromatography is usually run at ambient temperature, however, higher resolution is obtained at an elevated temperature because the partition rate is increased and the hydrophobic effect is reduced. In some cases, pure water is used as eluent at 60–80°C. [However, note that many analytes, including almost all proteins and some of the pharmaceutical drug molecules, are not stable at such a high temperature.] The efficiency of separation increases with decreased flow rate because it is necessary to permit sufficient time to the analyte molecules to diffuse into the hydration sphere of the stationary phase system to achieve optimal separation. A flow rate in the range of 0.3–0.5 mL/min is recommended for most separations. Ion-exclusion chromatography requires columns that are usually large in size, typically 30 cm, because a considerable volume of chromatographic support material is necessary to provide sufficient occluded liquid to obtain a stationary phase that permits separation of solutes of similar characteristics.

1.5 INSTRUMENTATION

Figure 1.3 shows a schematic of the set up of an IC system. An examination of the figure shows that the set up closely resembles that of a typical HPLC system. The components include an autosampler, a high-pressure pump, an injection valve with sample loop of suitable size (typically, 10–250 μL), a guard column, an analytical column, an optional suppressor or a post-column reagent mixing system, a flow-through detector, and a processing system ranging from a data-processing integrator to a computerized system management unit, which contains software to run the system using pre-programmed method and schedule (sequence) files, perform data acquisition and processing to crunch out the final results.

Since the mobile phase generally contains dilute acids, alkalis or salt solutions, the components in contact with mobile phase are typically made of a completely metal free inert material, such as polyetheretherketone (PEEK). A conventional HPLC system also may be used provided that its components are made of materials that are compatible with the mobile phase. Following suitable preparation, the sample is introduced through the injection valve. After optional chemical suppression or other post column treatment of the effluent, the analyte is detected using a suitable detection system (see later). Because IC typically uses an ionic mobile phase, a suppression of background conductivity of the eluent is often necessary prior to conductometric detection, when such a detector is used, although nonsuppressed conductometric detection has been
used in pharmaceutical analysis, particularly when water, weak acids or weak bases are used as eluents, as is common in ion-exclusion chromatography.

A detailed description of each of the individual components of an IC system is beyond the scope of this chapter. Furthermore, with the exception of the detector system, including the suppressor, and the need to have metal-free components for most IC applications, the components are no different from those used in a traditional HPLC system. A brief discussion on the suppressor and the detectors used in IC is provided below.

1.6 DETECTION

Any suitable detector can be used for the detection and quantitation of analytes by IC. The choice of detector depends upon the nature of the analyte molecules. This may include the universal refractive index (RI) detector, UV detector for analytes that absorb UV, fluorescence detector for analytes that contains fluorophores, or radiochemical detectors, where appropriate [cf. 7]. However, traditionally, IC is associated with electrochemical detectors. So, only a discussion of the electrochemical detector systems is included in this chapter. It is not the intention of this chapter to suggest that other types of detectors should not be used with IC. Indeed, they should be, if the application dictates. However, the ability of electrochemical detectors is less appreciated in the pharmaceutical industry, presumably because mechanisms of action of these detectors are less understood compared to those of the traditional photometric detectors mentioned above.

Two types of modern electrochemical detectors are widely used in IC—conductivity (suppressed and nonsuppressed) and pulsed amperometry.

1.6.1 Conductivity Detection

When a constant voltage is applied across two electrodes between which the effluent from a column flows, a current is generated because the effluent contains ions or
polar molecules. The strength of the current is proportional to the conductivity of the solution, which, in turn, is proportional to the concentration of ionic species in solution and their ion conductances. The concentration is the number of ions carrying electricity. The ion conductance of an ion determines its ability to carry electricity. The ions present in effluent provide the background (baseline) conductivity of a chromatographic profile. The additional conductivity due to an analyte ion or a polar molecule, when they are present in the effluent, provides the peak, which is proportional to its concentration. Different analytes at the same concentration show different peak areas (or peak heights) due to the difference in their ion conductances.

The problem, however, is that the conductivities of effluent solutions are often significantly higher than the conductivities of the analytes, simply because, as mentioned above, the concentrations of ions in effluent are $10^4 - 10^5$ higher than that of the analytes, particularly in ion-exchange chromatography. Thus, early attempts to apply conductivity measurement to IC had significant limitations.

1.6.1.1 Suppressed and Nonsuppressed Conductivity Detections. This limitation was overcome when Small et al. [2] introduced the concept of suppressed IC. Small et al. used a packed-bed suppressor in the hydroxide form to achieve sensitive detection of the ions by chemically modifying the effluent before it enters the conductivity detector. The suppression was achieved by converting the mineral acid eluent to water and thereby obtaining a very low background signal and low noise, while converting the analyte to its base form, which is fully dissociated and actually carries more current than the analyte itself, thereby increasing the sensitivity of the detection (see later). In this system, the effluent containing $HA$ ($A$ being the anion) passes through the suppressor that exchanges $A^-$ for $OH^-$ to produce water, which does not conduct electricity. Noise is proportional to the background signal and elimination of the background electrolyte lowers the noise, provides more stable baseline and improves analyte sensitivity. However, in 1979, Gjerde et al. [17] reported an IC method in which the analytical column is directly linked to a conductivity detector without any suppressor. The methods employed a low capacity analytical column and dilute solutions of weak acids or bases as eluents to achieve low background signals.

The question then is, to suppress or not to suppress. The conductivity of an electrolyte, $MX$, is given by the following equation:

$$C = c_{MX} \Lambda_{MX} = c_{MX}(\lambda_M + \lambda_X)$$

(6)

where $C$ is the conductivity of the electrolyte, $c_{MX}$ is the concentration of $MX$ in Normality (N), $\Lambda_{MX}$ is the equivalent conductance of the electrolyte $MX$, and $\lambda_M$ and $\lambda_X$ are equivalent ion conductances of $M^+$ and $X^-$ ions, respectively (including their respective waters of hydrations). The ion conductances of a few common ions are shown in Table 1.1.

To understand suppressed and nonsuppressed detection, let us consider two identical cation-exchange chromatographic runs of the analyte $MX$ using a strong acid, $HA$, as the eluent (where $A$ is an anion), with the difference that in the first system the effluent first passes through a suppressor before entering the conductivity cell, whereas in the second system the effluent flows directly through the conductivity cell.
### TABLE 1.1. Equivalent Ion Conductances of Common Ions

<table>
<thead>
<tr>
<th>Cation</th>
<th>Eq. Ion Conductance (mho)</th>
<th>Anion</th>
<th>Eq. Ion Conductance (mho)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(^+)</td>
<td>349.8</td>
<td>OH(^-)</td>
<td>198.0</td>
</tr>
<tr>
<td>K(^+)</td>
<td>73.5</td>
<td>Br(^-)</td>
<td>78.4</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>50.1</td>
<td>Cl(^-)</td>
<td>76.3</td>
</tr>
<tr>
<td>Li(^+)</td>
<td>38.7</td>
<td>HCO(_3)(^-)</td>
<td>44.5</td>
</tr>
<tr>
<td>NH(_4)(^+)</td>
<td>73.4</td>
<td>SO(_4)(_2)(^-)</td>
<td>79.8</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>53.1</td>
<td>Acetate</td>
<td>40.9</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>59.5</td>
<td>Propionate</td>
<td>35.8</td>
</tr>
</tbody>
</table>

In the second (nonsuppressed) system, the analyte signal is measured by the conductivity difference (ΔC) between MX and HA, given by the following equation:

\[
\Delta C = c_M[(\lambda_M - \lambda_H) + (\lambda_X - \lambda_A)] \tag{7}
\]

where \(c_M\) is the concentration of M\(^+\) (same as the concentration of MX) in the effluent. The change in conductivity in Equation (7) is around \(-300 \pm 30\) times \(c_{MX}\) because the equivalent ion conductance values of anions are within approximately \(\pm 30\) of each other, except when the anion is the hydroxyl ion (Table 1.1). However, hydroxyl ion cannot be used in a cation-exchange chromatography in which an acid is used as the eluent. The result is, therefore, a negative peak, which can be viewed as a positive peak by reversing the signal polarity of the detector. In the suppressed system, the MX first passes through the suppressor, in which both X\(^-\) and A\(^-\) are converted to OH\(^-\). Most of the OH\(^-\) combines with H\(^+\) of the acidic eluent to form water. Therefore, the analyte is now detected essentially in a background of pure water, resulting in a positive analyte response, given by the equation:

\[
\Delta C = c_M(\lambda_M + \lambda_{OH}) \tag{8}
\]

The change in conductivity in Equation (7) is around \(250 \pm 20\) times \(c_{MX}\) (\(C_M = c_{MX}\)) resulting in a positive peak (the equivalent ion conductances of cations are within range of approximately \(\pm 20\), except when the cation is H\(^+\)).

The above calculation appears to indicate that the nonsuppressed conductivity is about as much or more sensitive than the suppressed conductivity detection. However, we have not taken into consideration the difference in baseline conductivities. In suppressed conductivity, the eluent that passes through the detector is essentially pure water with the baseline conductivity approaching zero, compared to baseline conductivities of 1000–1500 µS when a strong acid is used as the eluent in a nonsuppressed detection system and lower when a weak acid is used because a weak acid is not fully dissociated. Thus, a peak equivalent to the conductivity of 250 times \(c_{MX}\) is observed against a background of essentially zero conductivity with suppressed conductivity detection. The same chromatography produces a peak equivalent to around 300 times \(c_{MX}\) in a background of 1000–1500 µS when nonsuppressed conductivity is used.
As the baseline conductivity is proportional to the concentration of ions in the eluent, it is critical to use dilute solutions of weak acids and bases when nonsuppressed conductivity detection is employed because they are slightly dissociated, even in dilute solutions. Consequently, it is necessary to use low-capacity ion-exchange columns. However, the capacity is much less of a factor while choosing a column when suppressed conductivity detection is used, because high eluent concentrations may be used without any significant change in the background conductance, as long as the suppressor capacity is not exceeded.

The baseline conductivities (noise) in a typical suppressed conductivity detection is found to be <0.5 nS (using strong acids or alkalis) while the same is ~10 nS (using weak acids or alkalis) with nonsuppressed detection. Thus, considering the above example,

\[
\text{Signal-to-noise ratio for the suppressed system} = \frac{250 \cdot c_{MX}}{0.5} = 500 \cdot c_{MX}
\]

\[
\text{Signal-to-noise ratio for the nonsuppressed system} = \frac{300 \cdot c_{MX}}{10} = 30 \cdot c_{MX}
\]

Thus, suppressed conductivity detection provides about an order of magnitude better signal-to-noise ratio than the nonsuppressed system.

Furthermore, Detection Limit and Quantitation Limit are related to the signal-to-noise ratio [cf. 18]. Thus, both validation parameters are expected to be an order of magnitude lower when suppressed conductivity detection is used compared to nonsuppressed detection, attributing greater detection and quantitation sensitivity to the former technique.

When gradient elution is used, the baseline changes continuously with nonsuppressed conductivity detection. This makes peak area (or height) measurement less accurate. The baseline does not change when gradient elution is used in conjunction with suppressed conductivity detection.

However, for analytes that form weak bases from the suppressor reaction, such as NH$_4^+$, a nonlinear calibration curve has been observed. Thus, a quadratic curve fit is typically required for acceptable correlation of the calibration curve (see Chapter 4 of this book for more details). A linear calibration curve is observed using nonsuppressed conductivity detection.

1.6.1.2 Mechanism of Suppression. Although originally introduced by Small et al. [2], chemical suppressors are seldom used today. The suppressors that are widely used today operate electrolytically. The design and the mode of operation of electrolytic suppressors from different manufacturers vary to some degree in details but the basic mechanism of their operation is essentially the same, which will be discussed here. More recently suppressors have been developed which recycle the eluent back to the eluent delivery chambers, thereby resulting in reduction of the operating cost. These suppressors work only in conjunction with electrolytic eluent generation systems where the feed from the eluent chamber is water. The mechanism of operation of such suppressors is discussed elsewhere in this book (see Chapter 4).

To explain the mechanism of operation of electrolytic autosuppressors, let us consider anion suppression in the effluent from an anion-exchange column (Figure 1.4).
The analyte, $X^-$, having $Na^+$ as the counter-ion, is eluted from the column by the eluent, sodium hydroxide. As shown in Figure 1.4, the effluent enters into the suppressor through the central chamber enclosed by a “semi-permeable membrane”, which permits transfer of positive charges under the influence of an electric field but not transfer of negative charges (for an anion suppressor) nor transfer of material by diffusion. The central chamber has an anode chamber on one side and a cathode chamber on the other. Water is pumped into both the cathode and the anode chambers. When an electric field is applied, water in both chambers undergoes electrolysis. In the anode chamber, the electrolysis generates hydrogen ion and oxygen molecules. Similarly, hydroxyl ion and hydrogen is generated in the cathode chamber. Hydrogen ion travels across the membrane from the anode chamber into the central chamber and sodium ion moves out of the central chamber into the cathode chamber. Hydroxyl ion of the eluent binds to the hydrogen ion in the central chamber to form water. Sodium ion that has moved out of the central chamber is replaced by hydrogen ion that has moved in. The central chamber now contains $H^+X^-$ (instead of $Na^+X^-$) in pure water, which moves to the detector. Thus, the acid form of the analyte $X^-$ in pure water enters the detector and the eluent is converted to pure water, which provides essentially zero background.

Similarly, when a cation suppressor is used, the “semi-permeable membrane” permits transfer of negative charges only under an electric field and the analyte cation with hydroxyl counter-ion in pure water goes to the detector.

The eluent is converted to pure water only when the eluent is either an acid or an alkali. If a salt is used as an eluent, the anion will combine with the hydrogen ion
produced by the electrolysis of water to form the corresponding acid when an anion suppressor is used (e.g., HCl if NaCl is used in the eluent). Similarly, the suppression will produce the hydroxyl form of the cation, if a cation suppressor is used. The suppression will not lead to near zero background under such conditions, however, the background could be still acceptably low if the acid form of the anion is a very weak acid or the hydroxyl form of the cation is a very weak base.

1.6.2 Pulsed Amperometric Detection

Used typically in combination with high-performance anion-exchange chromatography (HPAEC), pulsed amperometric detection (PAD) has proved to be a powerful tool in the detection of mono- and oligosaccharides, alditols, amino acids and peptides without requiring any sample derivatization.

At high pH, the analytes are oxidized at the surface of the gold electrode by the application of pulses of positive potentials. The current (hence amperometry) generated is proportional to the analyte concentration, which therefore can be detected and quantitated. When a single potential is applied to the electrode, oxidation products that deposit on the electrode surface gradually “poison” the electrode surface, resulting in loss of analyte signal. To prevent signal loss, the electrode surface is cleaned by a series of potential pulses that are applied for fixed time periods after the detection potential. Repeated application of a series of potentials designated \( E_1, E_2, E_3, \ldots \), over defined time periods \( t_1, t_2, t_3, \ldots \) constitutes the basis of pulsed amperometry. The series of potentials applied for defined time periods is referred to as a waveform.

The potential \( E_1 \) applied over the time period, \( t_1 \), is subdivided into two time periods related to two functions. In the initial part, called the delay period \( (t_d) \), time is allowed to permit the current to stabilize due to changing potentials so that only stable current from analyte oxidation is measured during the second part of \( E_1 \), the detection period \( (t_{det}) \), also known as integration time \( (t_i) \), as data acquisition takes place to integrate peak areas during this time. The time periods during which each potential is applied, is of the order of a millisecond or less so that data acquisition is continuous for all practical purposes.

Several waveforms are used in the analysis of different molecules. They were developed to increase detection sensitivity, and minimize the sensitivity to dissolved oxygen, the baseline drift, and the loss of electrode surface, when used continuously.

Typically, an Ag/AgCl reference electrode is used as half electrode (in combination with gold electrode) in PAD. The principles and applications of PAD are described in further details in Chapter 3.

REFERENCES
