CHAPTER 1
INTRODUCTION

1.1 High Performance Liquid Chromatography [1-2]

Liquid chromatography (LC) is the general term given to chromatographic methods in which the analyte is distributed between a liquid mobile phase and either a liquid or a solid stationary phase. If the stationary phase is a liquid, this type of chromatography is called liquid-liquid chromatography. If the stationary phase is a solid, however, the technique is known as liquid-solid chromatography.

After extensive research and development in the 1970s, LC became a fast, efficient, selective, and widely applicable separation tool. An important and useful quality of LC is that it can be used for the separation of thermally labile and non-volatile compounds. This technique can also be used to separate samples that are hundreds of milligrams in the relatively short period of several hours.

Although LC is similar to GC in basic ways, a number of differences exist between the two. For example, both the stationary and mobile phases influence the separation in LC, unlike in GC where only the stationary phase affects the separation. Another significant difference between the two methods is that a typical LC system experiences lower diffusion rates because the mobile phase has a higher viscosity and density. Although LC columns do have higher resistance to flow and back pressures, they generally have much larger sample capacities. Finally, LC tends to be more experimentally complex than GC.

A wide variety of stationary phases, including those with size exclusion and ion-exchange properties, can be used with LC. Common

stationary phases include immiscible liquids, liquid films coated on solid supports, organic compounds bonded to solid supports, inorganic solids like silica or alumina, and solid porous organic polymers.

In general, LC systems contain long, narrow columns packed with a small particle stationary phase under high pressure. The operating conditions can vary widely though depending upon the sample being analyzed.

The most basic type of liquid chromatography is column chromatography. In this technique, a mobile phase moves through a column packed with large diameter stationary phase particles. Typically, only gravity or low air pressure is applied to the system. This type of liquid chromatography does not offer good efficiency, resolution, or selectivity. In order to improve the utility of the technique, high performance liquid chromatography (HPLC) was developed.

High performance liquid chromatography is unquestionably the most widely used of all of the analytical separation techniques. The reasons for the popularity of the method are its sensitivity, its ready adaptability to accurate quantitative determination, its suitability for separating nonvolatile species or thermally fragile ones and its widespread applicability to substances that are of prime interest to industry.

1.1.1 Retention Mechanisms

During a separation, the solute distributes itself between the mobile phase and the stationary phase. The amount of time it is retained in each phase depends on the analyte's affinity for the phase. Liquid chromatographers can take advantage of four different retention mechanisms to achieve separation: partition, adsorption, size-exclusion, ion exchange and affinity.

Partition

In partition chromatography, which is also called liquid-liquid chromatography, the solute distributes itself between two immiscible phases.

The stationary phase in this technique is often a liquid coated on a solid support. The liquid mobile phase caries the solute through the liquid stationary phase.

The retention time of the solute depends upon the length of time it resides in the stationary phase. The extent of the partitioning of the solute is governed by its solubility in each of the phases, which are of differing polarities.

In the first partition chromatography experiments, the stationary phase was polar (e.g. ethylene glycol or silica) while the mobile phase was a non-polar organic liquid like hexane. Chromatographers found that increasing the polarity of the mobile phase (i.e., making it more like the stationary phase) decreased the elution times of the analytes. This technique became known as normal-phase chromatography (NPC) simply because it was the first system in use.

In normal-phase chromatography, the separation depends on the interactions of the analytes with the polar stationary phase.

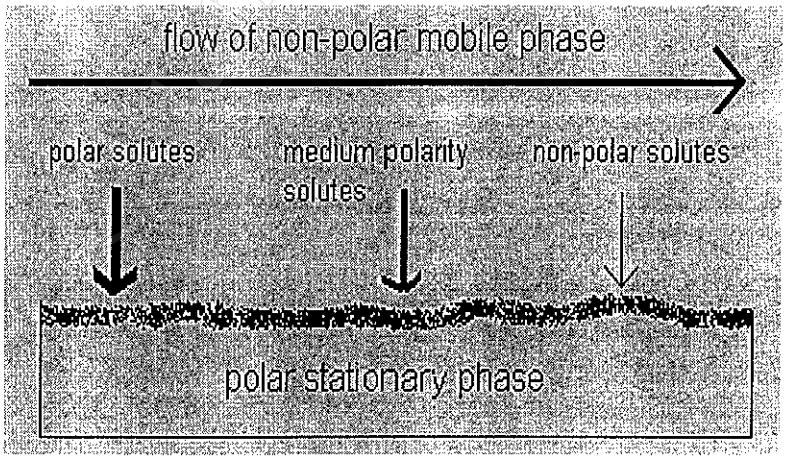

Figure 1.1 Schematic of the interaction in NPC.

The least polar component of the sample is eluted first because it is not very miscible with the polar stationary phase. The medium polarity components elute later, followed by the polar components that interacted strongly with the stationary phase. Although normal-phase chromatography is best suited for the separation of polar samples, strongly polar molecules may interact so strongly with the stationary phase that they cannot be eluted from the column in a reasonable amount of time. Therefore, the polarities of the two phases are deemed to be switched and the technique is called reversed-phase chromatography (RPC). Reversed-phase chromatography systems utilize a non-polar stationary phase like a hydrocarbon or an alkyl-bonded silica and a polar mobile phase such as water or acetonitrile. In these systems, increasing the polarity of the mobile phase (i.e., making it less like the stationary phase) increases the elution times of the analytes.

Unlike in normal-phase chromatography, both the polarity and the size of the analyte influence retention. In RPC, the hydrophobicity (which is dependent upon polarity and size) of the analyte is what governs retention. The stable hydrogen bonding of the aqueous mobile phase is interrupted by the presence of the analyte. The larger the analyte, the larger its hydrophobic interactions with the mobile phase and the longer it remains in the stationary phase.

In RPC, the more polar components of the sample are weakly retained and are eluted first. The large, non-polar components of the sample, however, are the most strongly retained.

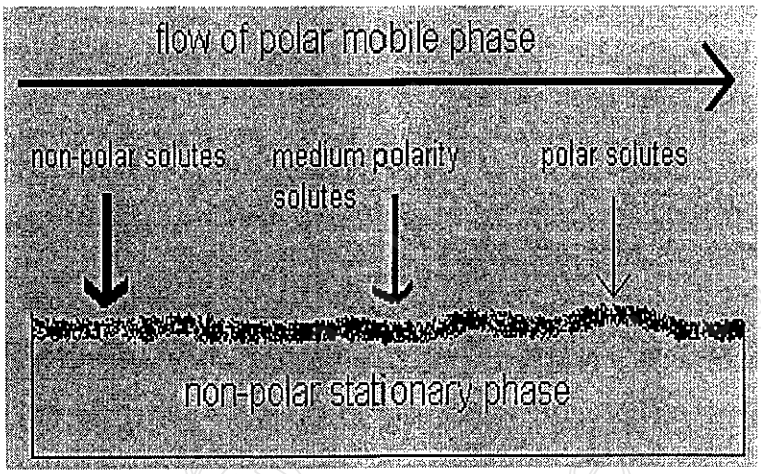

Figure 1.2 Schematic of the interaction in RPC.

Reversed-phase chromatography is a versatile technique and, depending upon the choice of phases, can be used for the separation of non-polar, polar, and ionizable samples.

In reverse phase chromatography, the packing is non-polar and the solvent is polar with respect to the sample. Retention is the result of the interaction of the nonpolar components of the solutes and the nonpolar stationary phase. Typical stationary phases are nonpolar hydrocarbons, waxy liquids or bonded hydrocarbons (such as C_{18}, C_8, C_4, etc.) and the solvents are polar aqueous-organic mixtures such as methanol-water or acetonitrile-water.

The C_{18}, C_8, and phenyl bonded phases are most often used in the reverse phase mode. It has been estimated that 60-90 % of all analytical LC separations are done on bonded phases in the reversed phase mode. Bonded phases made by covalently bonding a molecule onto a solid stationary phase are intended to prepare "liquid coatings" which will be permanent. Silica is a reactive substrate to which various functionalities can be attached or bonded. The functionalities most widely bonded to silica are the alkyl (C_{18} and C_8), aromatic phenyl, and cyano and amino group.

General characteristics of reversed phase chromatography

- Broad scope which allows sample types with a wide range of polarities and molecular weights to be separated.
- General rapidity of mobile phase column equilibration during methods development and gradient regeneration.
- General ease of use.
- Applicability to separation of ionic or ionizable compounds by manipulating secondary chemical equilibrium such as ionization control and ion pairing in the aqueous mobile phase.
- Buffering the mobile phase in the pH range from 2 to 5 with one of the common buffers, the ionization of the weak acids can be suppressed or controlled allowing them to be retained in their neutral form. Similarly weak bases can be retained in their neutral form at pH 7-7.5.
- For strong acids and bases ionization control cannot be employed because the stability of alkyl bonded phases is diminished below pH 2 and above pH 7.5. Highly hydrophilic weak acids and bases often remain difficult to retain with ionization control. In such cases ion pair reversed phase chromatography can be used.
- The possibility of special selectivity such as structural or steric are achievable by specific mobile phase additives:
- Metal ions are capable of binding to organic compounds in a very selective method which is used for ligand exchange chromatography. The selectivity generated in these metal ion phase systems is based in part on differences of the solute (ligand) binding strength to the metal ion. An alternate approach is the addition of various chelating agents (4-

dodecyldiethylene-triamine - C_{12} dien) in combination with a metal ion. The type and strength of the metal chelate complex-solute binding can be greatly varied depending upon the chemical environment surrounding the metal ion as determined by the chelating agent added.

In general, partition chromatography is useful for the separation of low- to medium-molecular weight samples of intermediate polarity. It is particularly useful because it can separate members of a homologous series (molecules which have the same functional groups) as well as low-polarity compounds that are difficult to separate with other methods. Also, the presence of water in the mobile phase is not a complication as it is with adsorption methods.

Adsorption

Adsorption chromatography uses the retention of the solute by the intermolecular forces of the solid stationary phase to achieve separation. In general, non-polar solutes are eluted by non-polar solvents and polar solutes are eluted by polar solvents.

The most commonly used adsorbents are silica and alumina, but other materials such as charcoal, cellulose, and calcium carbonate are also used. The adsorbent is active; its level of activity is determined by its polarity and number of adsorption sites. The presence of water in the system will decrease the activity of the stationary phase by adsorbing to its surface sites.

Both the solvent and the solute compete for surface sites on the stationary phase. The proper selection of the solvent is therefore very important for the elution of the analyte.

In general, adsorption chromatography is useful for the separation of low- to medium-polarity samples with molecular weights below 1000. Peak fronting and tailing are common disadvantages to this technique.

Size-exclusion

Size-exclusion chromatography (SEC) is based on the sieving principle. In SEC, the stationary phase particles are manufactured with a wide range of pore sizes, causing the stationary phase to behave like a molecular sieve. As a result of the sieving action, the solutes are separated on the basis of size, with the larger ones eluting first.

Ion-exchange

Ion-exchange chromatography (IEC) is based on the principle that opposites attract. Ion-exchange chromatography is used to separate charge analytes and therefore occurs as a result of interaction between a charged solute and an oppositely charged, solid stationary phase. Ion-exchange chromatography can be applied to any solute that can acquire a charge in solution. Thus, even carbohydrates, which are largely uncharged below pH 12, can be separated by ion-exchange chromatography at sufficiently high pH.

Affinity

Affinity chromatography is based on the lock-and-key mechanism prevalent in biological systems. The retention mechanism is very specific, but the technique is more time-consuming and more expensive than those employing other retention mechanism.

1.1.2 Instrumentation

A HPLC system is basically composed of 1) a pump, 2) an injector, 3) a column, 4) a detector, as shown in **Figure 1.3**. The pump keeps liquid flow at a constant rate. Sample is injected by the injector, it is carried into the column and separated into its components. Each component elutes from the column one by one and is detected.

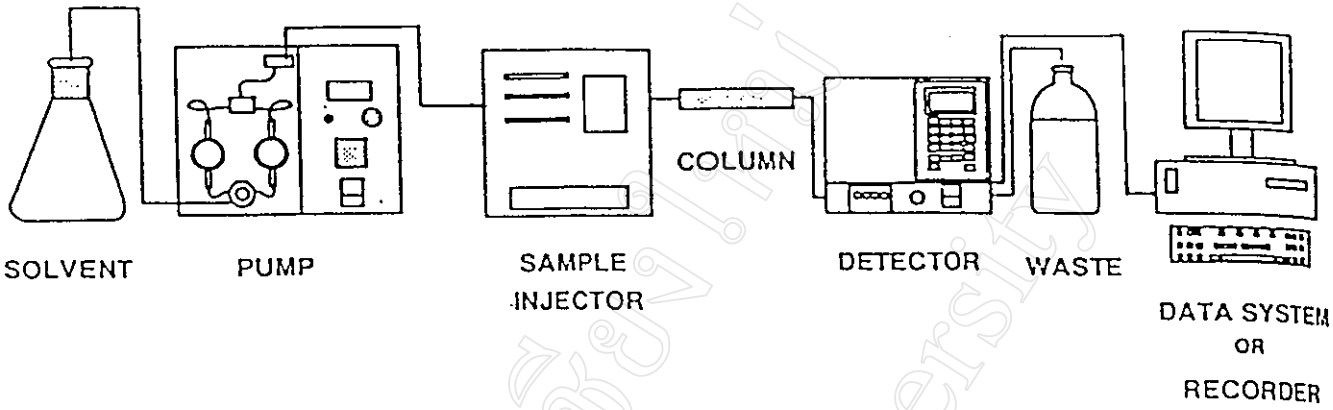

Figure 1.3 Block diagram of high performance liquid chromatograph.

1.1.3 Stationary phase

Reversed-phase stationary phases appear to be the most generally useful of the stationary liquid phases. The most common stationary phase in RP-chromatography are those in which a functional group is chemically attached to a silica support (bonded phases). The most popular bonded phases are the alkyl groups, such as $-CH_3$, $-C_4H_9$, $-C_8H_{17}$ and $-C_{18}H_{37}$, phenyl (C_6H_5) groups, cyano[$(-CH_2)_3CN$] groups and amino [$(-CH_2)_3NH_2$] groups, with retention increasing exponentially with chain length. These packing materials, with some restrictions on the pH of the eluent used, provide good hydrolytic stability and are resistant to solvent stripping within normal column operating pressures. Newer reversed phase packings, based on graphitised carbon and on rigid, porous microparticulate beads comprising a polystyrene/divinylbenzene matrix, are available, which through more polar than ODS can tolerate a much wider range of eluent pH (1-13) [3]. A list of several reversed phase stationary phases which are used for HPLC is given in **Table 1.1**.

Table 1.1 Commercially available reversed phase packing materials [3]

Packing materials	Phase	Particle size (μm) and shape	Surface area (m^2g^{-1})	Carbon load (%)	End capping
Lichosorb	C_8	5,7,10 irregular	250	7 monomeric	-
Hypersil	C_8	5,10 spherical	50	3.0 monomeric	yes
Phenomenex Ibsil	C_8	3,5,10 spherical	165	7.5 monomeric	yes
Nucheosil 100	C_8	3,5,10 spherical	350	9 monomeric	no
Partisil	C_8	5,10 irregular	350	8.5 monomeric	yes
Selectosil	C_8	3,5,10 spherical	330	8 monomeric	no
Spherisorb	C_8	3,5,10 spherical	220	6 monomeric	yes
Ultremex	C_8	3,5,10 spherical	200	8 monomeric	yes
Hypersil	C_{18}	3,5,10 spherical	170	10.0 monomeric	yes
Lichosorb	C_{18}	5,7,10 irregular	500	17 monomeric	no
Nucheosil 100	C_{18}	3,5,10 spherical	350	14 monomeric	yes
Nucleosil Ibsil	C_{18}	3,5,10 spherical	165	11.0 monomeric	yes
Selectosil	C_{18}	3,5,10 spherical	330	13.0 monomeric	yes
Spherisorb (1)	C_{18}	3,5,10 spherical	220	7 monomeric	partial
Ultremex	C_{18}	3,5,10 spherical	200	13.0 monomeric	yes
Zorbax	C_{18}	3,5,7 spherical	330	20 monomeric	yes

1.1.4 Mobile phase

Retention in reversed-phase chromatography is a function of sample hydrophobicity whereas the selectivity of the separation results almost entirely from specific interactions of the analyte with the mobile phase [4]. The intelligent selection of the type of stationary phase for the separation is made and selectivity is adjusted by modifying the mobile phase. The selection of the mobile phase for a particular separation is thus a very important consideration in HPLC. A suitable solvent will preferably have low viscosity, be compatible with the detection system, so as not to interfere with the measurement by the detector and it must be able to completely dissolve the sample without reaction with analytes; for example, if an UV- absorption detector is used, the solvent

cannot absorb ultraviolet radiation, be readily available in a pure form, and, if possible, have low flammability and toxicity.

It is not unusual, and indeed in many instances it is preferable, to use mixtures of solvent as the mobile phase rather than a single pure solvent [5]. The mobile phase used in reversed-phase chromatography are based on a polar solvent, typically water, to which a less polar solvent such as acetonitrile or methanol is added. **Table 1.2** lists polarity indices and other properties for a number of solvents that are employed in HPLC.

Table 1.2 Properties of common chromatographic mobile phases [6]

Solvent	Viscosity,cP[a]	Polarity Index,P′	Eluent Strength[b]
Fluoralkanes[c]	0.4-2.6	<-2	-0.25
Cyclohexane	0.90	0.04	-0.2
n-Hexane	0.30	0.1	0.01
1-Chlobutane	0.42	1.0	0.26
Carbon tetrachloride	0.90	1.6	0.18
i-Propyl ether	0.38	2.4	0.28
Toluene	0.55	2.4	0.29
Diethyl ether	0.24	2.8	0.38
Tetrahydrofuran	0.46	4.0	0.57
Chloroform	0.53	4.1	0.40
Ethanol	1.08	4.3	0.88
Ethyl acetate	0.43	4.4	0.58
Dioxane	1.2	4.8	0.56
Methanol	0.54	5.1	0.95
Acetonitrile	0.34	5.8	0.65
Nitromethane	0.61	6.0	0.64
Ethylene glycol	16.5	6.9	1.11
Water	0.89	10.2	Large

[a]The centipoise is a common unit of viscosity; in SI units, 1 cP = 1 mN . s . m^{-2}

[b]On Al_2O_3, multiplication by 0.8 gives ε^0 on SiO_2.

[c]Properties depend upon molecular weight. Range of data are given.

1.1.5 Detectors for HPLC [6]

The function of the detector in HPLC is to monitor the column effluent and afford a means of detecting solutes their in. Detectors function according to many different principles but all output is an electrical signal which is proportional to some property of the analyte. The choice of detectors is often dictated by the chemical characteristics of the analyte species and this choice may subsequently determine which eluent is used and also possibly which stationary phase and mode of chromatography. The detector response will be related to the amount of the analyte in the column effluent though different analytes will respond to differing extents and hence the detector must be calibrated with respect to each of the analytical species of interest.

The ideal HPLC detector should have the following characteristics.

- high sensitivity
- good stability and reproducibility
- linear response over several orders of magnitude
- small internal volume minimizing zone broadening
- a short response time independent of flow rate
- insensitive to changes in temperature and pressure
- high reliability and ease of use
- similar response to analytes or selective response to analyte classes
- non-destructive

UV-Vis detectors [5,7]

UV-Visible absorption detectors are the most widely used detectors in liquid chromatography. As most organic compounds have some useful absorption in the UV region, these detectors are fairly universal in application, although sensitivity depends on how strongly the sample absorbs

and the availability of a transparent mobile phase at the wavelength of maximum absorption.

The operation of spectrophotometric detectors is based on the measurement of the absorbance of monochromatic light by the sample in accordance with the well-known Beer-Lambert law. Most detectors provide an output in absorbance units which is linearly related to sample concentration over range of 10^4 to 10^5. Detection limits are in the low to subnanogram range in favorable circumstances. Some properties of UV-Vis detectors are given in **Table 1.3**.

Table 1.3 Some properties of UV-Vis detectors

Response	Selective
Typical sensitivity	Nanogram
Linear range	10^5
Flow rate sensitive	No
Temperature sensitive	No
Type	Nondestructive

1.1.6 Chromatographic theory

Chromatography involves the separation of the components of a mixture by virtue of differences in the equilibrium distribution or partition coefficient, K, of the components between two phases: the mobile phase and the stationary phase [8-9]. If C_s and C_m are the concentrations of a component in the stationary phase and mobile phase, respectively, then;

$$K = C_s / C_m \qquad (1.1)$$

The easily measured parameter, which can also be used for qualitative analysis, is the retention time, t_R, of a chemical species. The retention is the time required to elute a sample component from the stationary phase, i.e., it is the time required for the mobile phase to sweep the component from the stationary phase. A similar and also useful parameter is the retention volume, V_R. The retention volume is the volume of the mobile phase required to elute the sample component from the stationary phase. The retention volume and the retention time are related to each other by

$$V_R = F. t_R \qquad (1.2)$$

The plate theory [10]

In the chromatographic model proposed in the plate theory, the chromatographic column is considered to consist of a number of thin sections or "plates," each of which allows solute to equilibrate between the stationary and mobile phases. The greater the number of the theoretical plates (N), the more efficient the column is considered to be. The movement of a solute along to the column is viewed as a stepwise transfer from one theoretical plate to the next. The thinner the theoretical plates, the greater the number that can be envisaged within a given length of column. These terms are related as follows :

$$H = L/N \qquad (1.3)$$

Where L is the length of the column (millimeters). Thus, the smaller the height equivalent to a theoretical plate (HETP, or H), the greater is the efficiency of the column. In general, the H value is smaller for small stationary phase particle sizes, low mobile phase flow rates, less viscous mobile phases, higher separation temperatures, and smaller solute molecule sizes.

Column efficiency can be expressed by the approaches of chromatographic theory. A dimensionless measure of column efficiency, N, is called the plate number or number of theoretical plates. A large plate number system is highly efficient. These values allow us to express the number of theoretical plates as given in **Equation 1.4.**

$$N = 16(t_R / w_b)^2 = 5.54(t_R / w_{1/2})^2 \qquad (1.4)$$

Where t_R is the retention time of solute, w_b is the base peak width $w_{1/2}$ and is the width at half-height.

The rate theory [11]

The rate theory is based on the rate of mass transfer between two phases, diffusion rate of solute along the column, eluent flow rate and hydrodynamics of the mobile. Therefore, factors may be concluded and shown as the relationship between column efficiency and variables in column composition and analytical conditions as expressed by the van Deemter equation :

$$H = A + B/u + Cu \qquad (1.5)$$

Where A = eddy diffusion term
B/u = longitudinal diffusion term
Cu = mass transfer term

Retention time and capacity factor

Retention time, t_R, is the time a sample component takes after injection to reach the detector. A component that is not retained by the column

will travel at the same rate as the eluent, reaching the detector in time t_0, known as the dead time or solvent front (**Figure 1.4**).

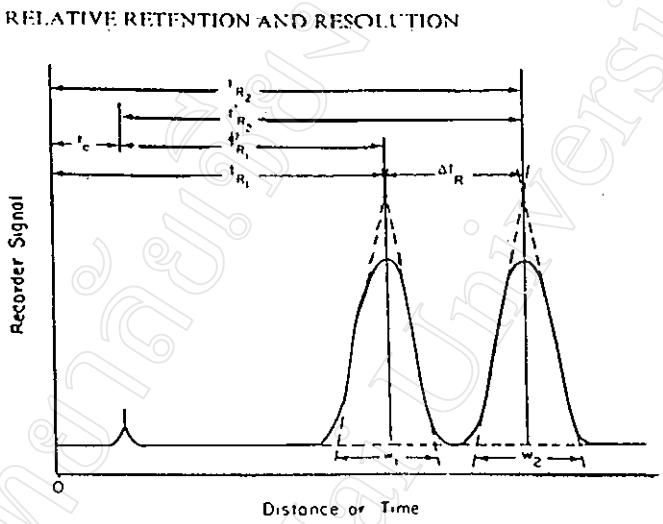

Figure 1.4 Chromatogram of two solutes illustrating the evaluation of retention times [10].

When developing a method, it is useful to calculate the capacity factor, k′, which is independent of column dimension, flow rate and practical packing size.

$$k' = (t_R - t_0)/ t_0 \qquad (1.6)$$

k′ provides important information about the quality of a separation. When k′ is small, resolution is poor. If k′ is made large, the analysis time may be too long, or any gain in resolution may be cancelled out by peak-

broadening. Ideally, k′ should be between 2 and 10. In practice, 1<k′>20, is satisfactory.

In HPLC, the capacity factor is varied by changing the solvent strength of the eluent. It is usually quickest to start with a strong eluent and make it weaker as needed. A rule of thumb is that k′ changes by three time for each 10% change in the organic component of the eluent [12].

Selectivity factor

The selectivity factor (α) describes the separation of two sample peaks relative to each other. By definition, α is always greater than 1.

$$\alpha = k'_2 / k'_1 \qquad (1.7)$$

$$\alpha = \frac{(t_{R2}-t_0)}{(t_{R1}-t_0)} \qquad (1.8)$$

α is a measure of the selectivity of the column, i.e., its ability to discriminate between two sample components. The larger the value of α, the easier it is to separate two components. In practice, α should lie between 1.1 and 1.4 [12].

Resolution

The resolution, R_S, is the degree of separation between two components of a sample.

$$R_S = \frac{(t_{R2} - t_{R1})}{0.5(w_1 + w_2)} \qquad (1.9)$$

R_S can be calculated from a chromatogram by measuring the peak width in units of time. To measure for partially resolved peaks, the unresolved part of the peak must be extended down to the baseline to obtain the peak width. For good results, should be greater than 1 (**Figure 1.5**) [6,12].

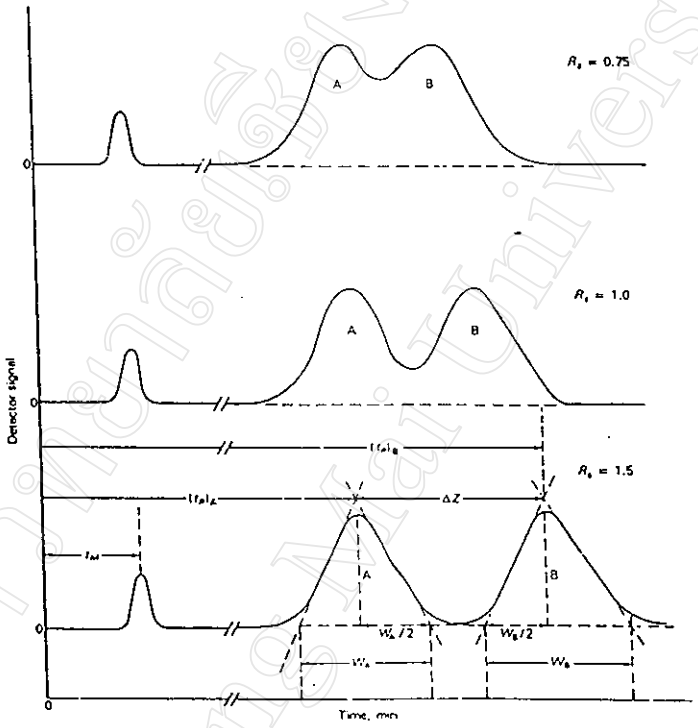

Figure 1.5 Separations at three resolutions. Here, $R_S=2\Delta Z/ (w_A+ w_B)$.

1.2 Ion interaction chromatography

Ion interaction chromatography (IIC), also known as reversed-phased ion-pair chromatography (RP-IPC), is a popular separation mode of HPLC. It is primarily used for the separation of mixtures of ionic and/or ionizable compounds, often in the presence of neutral solutes. The technique is based on the addition of amphiphilic (surface-active) ions to the mobile phase

in order to enhance the retention of ionic sample components. Other important application areas of IIC include the separation of inorganic ions, detection enhancement with UV-active ionic additives and the separation of enantiomers.

In IIC systems, numerous mobile phase variables (ion interaction type and concentration, ionic strength, eluent pH, organic solvents) can be used to control solute retention and separation selectivity. The broad choice and combination of these variables allow for the separation of complex sample mixtures containing ionic/ionizable and neutral solutes [13].

The analysis of strong acids or strong bases is one example of the accomplishment of IIC. In this analysis, the pH of the eluent is adjusted in order to encourage ionization of the sample; for acids pH 7.5 is used, and for bases pH 3.5 is common. Retention is then altered by including in the mobile phase a bulky organic molecule having a charge opposite from that of the ion to be analyzed. The counter ion is the ion interaction or ion-pairing reagent. Three basic models have been proposed to describe the ion interaction mechanism: the ion-pair model, the dynamic ion-exchange model, and the ion-interaction model.

The ion-pair model postulates that, the ion-pairing reagent contains bulky organic substituents the ion pair, which is formed in the mobile phase between the solute and the ion-pairing reagent, is hydrophobic in character and will therefore adsorb onto the hydrocarbon stationary phase. The longer the alkyl chain on the ion pairing agent, the less polar is the ion pair, the greater is the affinity of the ion pair for the stationary phase, and the longer is its retention.

The dynamic ion-exchange model proposes that it is the unpaired organic counter ion that adsorb to the surface of the non-polar stationary phase, forming a dynamic equilibrium between ion-pairing reagent in the mobile and ion interaction reagent adsorbed to the surface of the stationary phase. This

interaction causes the column to behave as an ion exchanger, and sample ions are therefore separated on the basis of conventional ion-exchange mechanism.

The ion-interaction model can be viewed as an intermediate between the two previous models and processes of the formation of an electrical double layer at the stationary phase surface. As in the previous model, it is suggested that a dynamic equilibrium is established between the ion-pairing reagent adsorbed onto the stationary phase and that free in solution. However, this model proposes that to this primary layer of charge is attracted a second layer of loosely held ions of opposite charge. Transfer of solutes through the double layer to the stationary phase is then a function of both electrostatic effects and the solvophobic effects responsible for retention in reversed-phase chromatography.

A list of ion interaction reagents is given in **Table 1.4**. An alkylsulfonate is a good first choice for basic solutes, whereas quaternary amines are useful for acidic solutes. Just as in reversed-phase chromatography, the most popular solvent combinations in ion interaction reversed-phase chromatography are water/methanol and water/acetonitrile. The major limitation, however, is the solubility of the ion interaction reagent. If the ion interaction reagent is not soluble in the organic modifier, precipitation may occur within the chromatographic system. Thus, it is wise to check the solubility of the proposed ion interaction reagent in the least polar solvent to be used.

Retention in ion interaction chromatography is affected by the type, size, and concentration of the ion interaction reagent. The larger and more concentrated the reagent, the better able it is to form ion pairs and the longer the retention time of the solutes. Retention is also affected by the nature and concentration of the organic modifier. The more lipophilic the modifier and the more concentrated it becomes, the shorter is the retention time of the sample solutes [14-15].

Table 1.4 Ion interaction reagents [14]

Type	Main applications
Quaternary anines, e.g., tetramethylammonium, tetrabutylammonium, and palmityl-trimethylammonium ions	For strong and weak acids, sulfonated dyes, carboxylic acids
Tertiary amines, e.g., trioctylamine	Sulfonates
Alkyl and aryl sulfonates, e.g., methane or heptane sulfonic acid, camphorsulfonic acid	For strong and weak bases, benzalkonium salts, catecholamines
Perchloric acid	Forms very strong ion pairs with wide range of basic solutes
Alkyl sulfonates, e.g., lauryl sulfate	Similar to sulfonic acids; yields different selectivities

Ion Interaction Chromatography is a method for improving the separation of charged analytes. In the resolution of organic ions with conventional HPLC methods, use of ion pair reagents can enhance peak shape and retention time when common remedies such as modifying eluent ratios or changing stationary phase fail.

In ion interaction chromatography, a hydrophobic cation or anion is added to the mobile phase. This enhances the retention time of oppositely charged analytes. It is very powerful tool for manipulating the selectivity of a separation. The retention of neutral analytes is nearly unaffected. Analytes with opposite charge to the ion interaction reagent are retained longer, and the retention of analytes with the same charge as the ion interaction reagent is reduced [16].

Typical anionic ion interaction reagents are long-chain sulfonic acids such as hexyl-, heptyl-, or octylsulfonic acid. Cationic ion interaction reagents are for example, the tetrabutylammonium ion or the cetyltrimetylammonium ion. These reagents are typically added to the mobile phase at a concentration around 5-10 mmol/L. At low concentration of the reagent, the retention of the oppositely charged analytes increases directly proportionally to the concentration of the reagent. At higher concentrations, the increase in retention levels off. At equal concentration, more hydrophobic ion interaction reagents, specifically, those with a longer chain length, increase the retention more than less hydrophobic reagents. At what concentration the increase in retention levels off depends on the mobile-phase composition and the chain length of the ion interaction reagent.

The reagent is adsorbed onto the surface of the packing. Its surface concentration depends on the mobile-phase composition and the concentration of the reagent. It can be shown that the retention charge of an analyte depends primarily on the molar surface concentration of the pairing reagent. One can superficially interpret the increased retention of the analytes as an ion-exchange mechanism that is superimposed on the normal reversed-phase retention mechanism. However, UV-absorbing ion interaction reagents such as a cetylpyridinium ion has been used to detect non-UV-absorbing analytes. A positive signal is obtained at the point where the analytes elute, accompanied by negative signal in a fixed position of the chromatogram. When the ion interaction reagent is injected itself, a positive signal is obtained at the same retention time as the negative peak obtained when the analytes are injected. This phenomenon is interpreted as stemming from the need to maintain charge balance as the analyte is adsorbed on the stationary phase.

Retention can be influenced by the type and the concentration of the interaction reagent, the ionic strength, and the pH of the mobile phase, as well as the concentration and type of the organic modifier to the mobile phase.

The technique therefore opens the window for additional parameters that can be used to influence the selectivity of a separation.

Many investigators recommend that columns used for ion interaction chromatography should be dedicated for this application and should not be used for regular reversed-phase chromatography. The reason for this is not clear. Apparently, ion interaction reagents are difficult to remove from silica-based reversed-phase packings [17-18].

1.3 Anions analysis by ion interaction chromatography

Literature methods for the determination of inorganic anions based on both ion chromatography and ion interaction chromatography published before 1984 have been exhaustively reviewed by Haddad and Heckenberg [19]; and a review by Marina et al. Published in 1989 was devoted to HPLC applications in the analysis of inorganic species [20].

Ion interaction methods can be also advantageously used in the determination of metals through formation of anionic complex species. Concerning the application of ion interaction methods in the determination of inorganic anions in real samples, a sensitive application concerns the determination of impurities in analytical grade reagents [21]. Many examples can be found in the fields of environmental, clinical and food chemistry. Typical inorganic anions have been determined in tap [22-28] and surface waters [24-25,28-29], sea or lagoon waters [28-33] atmospheric precipitation as rain, snow, aerosols in correlation with temperature, urban and rural sites [34].

Examples of applications in clinical chemistry are the determination of nitrite and nitrate in human saliva [35], of bromide in blood [36] and of arseniate in urine. Food chemistry applications are the determination of inorganic and organic anions in wines [37] and in fruit juices [38] and of iodide in commercial salt [35].

Ion interaction methods offer, with respect to ion chromatography, advantages of lower cost in relation to both instrumentation and columns, and can be advantageously employed in laboratories where only conventional HPLC systems are available. On the other hand, resolution and sensitivity are comparable to those obtained in ion chromatography, assuming that a suitable ion interaction reagent is chosen [18].

Nitrate ion (NO_3^-) exists in a number of salts, the most common of which are potassium nitrate and sodium nitrate. Nitrate is a wide spread contaminant of ground and surface waters worldwide [39-40]. Nitrate is a potential human health threat especially to infants, causing the condition known as methemoglobinemia, also called "blue baby syndrome". Nitrate is converted in the gut to nitrite, which then combines with hemoglobin to from methemoglobin, thus decreasing the ability of the blood to carry oxygen. Infants are more susceptible to nitrate toxicity than older children or adults. Fatalities are rare, but subacute methemoglobinemia can be asymptotic while affecting development, making the condition particularly insidious [41-42]. In addition, nitrate-containing wastes are produced by many industrial processes including paper and munitions manufacturing. Because agriculture is implicated in the nitrate pollution problem, farmers and rural communities are the most threatened populations [39].

Bromate ion (BrO_3^-) is not a natural component of water but may be formed during the disinfection of drinking water using ozone or a combination of ozone and hydrogen peroxide. The concentration of bromide in raw water is a major factor in the formation of bromate. The bromine in well water is primarily inorganic. The major natural sources of bromide in groundwater are saltwater intrusion and bromide dissolution from sedimentary rocks. Sewage and industrial effluent as well as road and agricultural runoff may also contribute to elevated bromide levels in surface waters. Limited data indicate that concentrations in drinking-water are generally less than 90 μg/L.

Bromate has been found to induce a very high incidence of kidney tumors in male and female rats and peritoneal mesotheliomas in male rats [43].

Thiocyanate ion (SCN^-) commonly exists in the forms of potassium thiocyanate and sodium thiocyanate. Thiocyanate has levels in effluent streams downstream from a plant outlet can not be underestimated. Though not as toxic as cyanide, thiocyanate is harmful to aquatic life. Thiocyanate is a common constituent of hydrometallurgical solutions . It is formed when pyretic materials are leached with solutions containing cyanide. For efficient plant control it is important continuously to monitor and determine the level of thiocyanate and the subsequent consumption of free cyanide in these process solutions [44].

Iodate ion (IO_3^-) and iodide (I^-) ions, like other anions in the above mentioned paragraphs, are always present in the forms of potassium iodate and potassium iodide. Iodate and iodide may be harmful if swallowed, may cause irritation to skin, respiratory tract, affect blood, kidneys, central nervous system. In addition, chronic ingestion of iodides may produce "iodism", which may be manifested by skin rash, running nose, headache and irritation of mucous membranes, weakness, anemia, loss of weight and general depression may also occur. [45-46].

1.4 The objectives of the study

This research work is aimed to investigate the effect of chromatographic variables on the separation of anions; namely, iodate (IO_3^-), bromate (BrO_3^-), nitrate (NO_3^-), iodide (I^-) and thiocyanate (SCN^-) ions, by ion interaction chromatography. These anions are selected on the basis that they are singly negatively charged ions with the differences on their geometrical structures. Therefore the charge distribution of these anions will certainly exert some influences on the separation behaviors by this chromatographic method. Among the variables to be studied, these include the type and concentration of

ion interaction reagent, the flow rate and pH of the mobile phase, the concentration of organic modifier and the wavelength of detection. Once the chromatographic conditions are optimized, they will be employed for the analysis of the real water samples.