CHAPTER II
HISTORICAL

Chirality of a molecule was first reported in 1815 by the French physicist Jean-Baptiste Biot. He found that α-quartz rotated the plane of polarized light[91]. However, the first chiral separation, which laid the foundation for stereochemistry, was reported in 1848 by Louis Pasteur[92-93]. Because of the hemihedral facets on the crystals of racemic sodium ammonium tartrate, he was able to separate the mirror image crystals of the isomers by the use of a magnifying glass and tweezers. Characterization of the physical properties of individual enantiomers, (in which the only difference lies in the opposite rotation of a plane polarized light) led Pasteur to postulate that the enantiomers have different three-dimensional arrangements and on the macroscopic and microscopic levels they are mirror-images of each other[94]. Furthermore, he advanced the field by studying the influence of one chiral compound upon another and introduced the technique of resolution via diastereoisomer formation[95]. This separation of enantiomers by diastereomer formation is the basis of many modern chromatographic separation.

In 1874, the Dutch physical chemist Jacobus Hendricus van't Hoff[96] and the French chemist Achille Le Bel[97] independently theorized that the molecular basis of chirality that was first observed by Pasteur was an asymmetric carbon. The asymmetric carbon proposed by Van't Hoff had the correct tetrahedral shape, whereas Le Bel, proposed a square pyramid.[98]

It is interesting to note that Pasteur's discovery of spontaneous enantiomeric resolution applies only to rare cases in which each isomer crystallizes separately and in a recognizable morphologic form. For more than a century, spontaneous resolution, as well as diastereomeric separation and differential enzymatic reactivity were the only methods employed for enantiomeric separations. In all of these techniques the separation and characterization of enantiomers require a chiral environment.

2.1 Chromatographic Methods

There are various methods for enantiomeric analysis which do not require the separation of enantiomers. These include polarimetry, nuclear magnetic resonance, isotopic dilution, calorimetry, and enzyme techniques. The disadvantages in all these techniques, however, are the need for pure chiral samples and their relative slowness. a typical analytical problem requires separation and quantitation of enantiomers and sometimes identification of the levorotatory or dextrorotatory enantiomer. Both gas chromatography (GC) and high performance liquid chromatography (HPLC) provide fast and accurate methods for enantiomeric separation and allow quantitation of both mass (and optical rotation for HPLC) if appropriate detection devices are used.

Chromatographic methods are considered the most useful for chiral separation[99]. There are two approaches: indirect, which utilizes derivatizing agents, and direct, which uses chiral stationary phases or chiral mobile phase additives. Chromatographic chiral separation dates back in 1939 when Henderson and Rule[100] demonstrated the separation of *d,l-p*-phenylenediiminocamphor on *d*-lactose. Both Kotake[101] et al. in 1951, and Dalgliesh[102] in 1952 utilized paper chromatography, a cellulose support, to separate chiral amino acids. Although Dalgliesh was not the first to observe such separations, he correctly attributed the enantioselectivity to adsorption by cellulose and proposed the Three Point Rule for asymmetric recognition. This rule was later restated by Pirkle[103] as "Chiral recognition requires a minimum of three simultaneous interactions, with at least one of these interactions to be stereochemically dependent."

In 1960, Klem and Reed[104] first reported the use of a silica gel support for chiral HPLC chromatographic separation. Racemic naphthyl ether and phenanthrene were partially separated on silica gel that was impregnated with (+)-α-(2,4,5,7-tetranitro-9-fluorenylideneamino-oxy)propionic acid.

The first successful GC direct enantiomeric separation was reported by Gil-Av, Feibush, and Charles in 1966[105]. The first stationary phase, N-TFA-L-isoleucine lauryl ester, resolved derivatized amino acids as N-TFA isopropy, 2-butyl, and t-butyl esters,

with the n-alkyl esters only partially resolved. From then on, other chiral stationary phases for GC[106] and LC[107] enantiomeric separations have been synthesized and developed.

2.1.2 Indirect Chromatographic method

In the indirect method, a racemic mixture is made to react with a chiral reagent to form a pair of diastereomers and then chromatographed using an achiral column[108]. Because diastereomers possess different physiochemical properties, they can be separated in an achiral environment. The advantages of the indirect approach are the following: (1) less expensive, i.e., conventional chromatographic columns can be used; (2) flexible because various achiral columns and mobile phase conditions, as in HPLC, can be used; (3) numerous types of derivatization chemistry are available and the cost of each reagent may be less expensive than for a chiral column; and (4) different selectivities can be achieved. On the other hand, the disadvantages of this method are: (1) long analysis time that include sample preparation and verification of the derivatization chemistry; (2) inconvenience, specifically in preparative chromatography, when reversal of derivatization is needed to recover the pure enantiomers[109]; (3) the need to synthesize non-commercially available pure derivatizing reagent; and (4) biased results for enantiomeric composition[110] due to partial racemization of derivatizing agent or unequal reaction rates.

The indirect method of separating enantiomers in GC or HPLC has been used for many years and is still commonly performed for various purposes[111]. For example, in the bioanalytical field, the indirect method is frequently used because separation of diastereomers from a complex matrix on nonchiral HPLC is simplified by the greater flexibility in selecting appropriate stationary and mobile phase conditions.

Detailed and comprehensive treatments of the chromatographic separation of diastereomers are given by Lindner[112] and Souter[113].

2.1.3 Direct Chromatographic Methods (Chiral Mobile Phase Additives)

Direct separation of enantiomers on an achiral column using a chiral mobile phase additive is applied only in HPLC. In GC the mobile phase is an inert carrier gas, where the possibility of selective interactions with the analyte or the stationary phase is minimal. However, in HPLC, the mobile phase is a dynamic part of the system that influences both analyte and stationary phase interactions.

In this method, enantiomeric separation is accomplished by the formation of a pair of transient diastereomeric complexes between racemic analyte and the chiral mobile phase additive. Chiral discrimination is due to differences in the stabilities of the diastereomeric complexes, solvation in the mobile phase, and/or binding of the complexes to the solid support. The three major approaches in the formation of diastereomeric complexes[114] are: (1) transition metal ion complexes (ligand exchange); (2) ion pairs; and (3) inclusion complexes. A detailed discussion of each mode is given by Allenmark[115] and Ahuja[116].

Many racemic mixtures can be separated on conventional achiral LC columns by using an appropriate chiral mobile phase additive. Additives such as α, β, and γ-cyclodextrins have been successful. Advantages of this technique[117] are as follows: (1) less expensive conventional LC columns can be used; (2) a wide variety of possible additives are available; and (3) different selectivities from the chiral phases can be obtained. However, the problems with this technique[118] include: (1) many chiral additives are costly, and sometimes, have to be synthesized; (2) the mode of operation is complex, and (3) incovenient for preparative applications because the chiral additive must be removed from the enantiomeric solutes.

2.1.4 Direct Chromatographic methods (Chiral Stationary Phases)

Enantiomeric separation by using chiral HPLC stationary phases (CSPs) is based on the formation of transient diastereomeric analyte-CSP complexes between the enantiomers and the chiral molecule that is an integral part of the stationary phase.

At present, there are over a hundred CSPs for HPLC that are commercially available. According to Wainer[119], there are five major classes of HPLC-CSPs based on the type of analyte-CSP complexes formed. The Type 1 or "Pirkle" phase forms analyte-CSP complexes by attractive-repulsive interactions, mainly by π electron donor-acceptor mechanisms. The Type 2, exemplified by derivatized cellulose, involves attractive interactions followed by inclusion into chiral cavities. The Type 3 CSPs, such as cyclodextrin and crown ethers, form inclusion complexes. In the Type 4 CSP, the analyte is a part of a diastereomeric metal complex (chiral ligand-exchange chromatography). The Type 5 CSP is a protein, e.g., bovine serum albumin, and the analyte-CSP complexes are based on the combination of hydrophobic and polar interactions. Table I shows some examples of commercially available CSPs and the mode of chromatographic analysis that can be carried out.

Table I. Classification of Chiral Stationary Phases (CSPs)

Type	Description	Examples	Mode (modifiers)
1	Pirkle-type (π-donor or π-acceptors)	DNB-phenylglycine, DNB-leucine, naphthylalanine	Normal phase (polar)
2	Attractive interactions followed by inclusion (derivatized cellulose)	Chiralcel OA, OB, OD, OF, OJ	Normal phase (polar)
3	Inclusion (cyclodextrins, polyacrylates, polyacrylamides, crown ethers)	Cyclobond I, II, III; Chiralpak OP, OT; Chiralcel CR	Reversed phase (aqueous acetonitrile or methanol)
4	Ligand exchange	Proline, hydroxyproline	Reversed phase (aqueous buffers)
5	Proteins	Albumin, glycoprotein	Reversed phase (aqueous buffers)

Type 1 CSP: Pirkle Type and Related CSPs

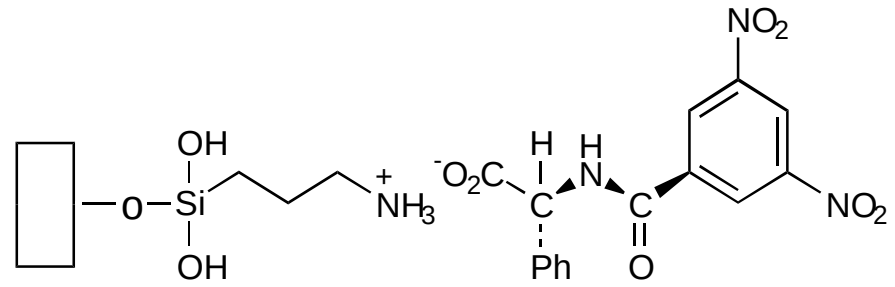
Pirkle's group[120] at the University of Illinois is generally acknowledged as the inventor of Type 1 CSP, although various CSPs of similar type have been developed by Japanese[121-122] and European[123-126] laboratories. They have advanced this area by developing amino acid derivatives containing π-acidic or π-basic aromatic group that are ionically or covalently linked to aminopropyl silica. Furthermore, Pirkle has done extensive mechanistic studies utilizing computer modelling of diastereomeric complex interaction and various homologous series studies to elucidate the chiral recognition mechanism of the CSP.

Enantiomeric separation is achieved by the following interactions: (1) π-π interactions between the π-donor and π-acceptor aromatic rings of racemic analyte and CSP, and vice versa; (2) hydrogen bonding involving secondary amines and carbonyl groups on the CSP with acidic proton, hydroxyl, and amino groups on the analyte; (3) dipolar interactions as in dipole stacking; and (4) steric interactions arising from the bulky nonpolar groups attached near the chiral center of the CSP that provide conformational control. Figure 3 shows a Pirkle CSP of N-(3,5-dinitrobenzoyl)phenylglycine ionically or covalently bonded to a silica support.

Type 1 CSPs can separate a wide spectrum of racemic compounds, such as alkyl and aryl carbinols, aryl substituted hydantoins, lactams, succinamides, phthalides, sulfoxides, sulfides, amides and imides. For racemic analytes having polar functional groups, e.g., amino, carboxyl, and hydroxyl, they have to be made less polar by derivatization because underivatized they interact too strongly with the CSP. Derivatization involves conversion of the amino group to amide, urea, or carbamate functionality; carboxyl conversion to ester, anilide, or amide; and hydroxyl conversion to ester and carbamate[126]. Recently, Pirkle[127] has developed a hybrid column with both π-acid (3,5-dinitrobenzoyl) and π-base (naphthyl) groups. It can resolve a variety of compounds, including underivatized carboxylic acids.

The mobile phases used for Type 1 CSPs usually contains a nonpolar organic solvent with various amounts of polar modifier, e.g., hexane and 2-propanol.

(a)



(b)

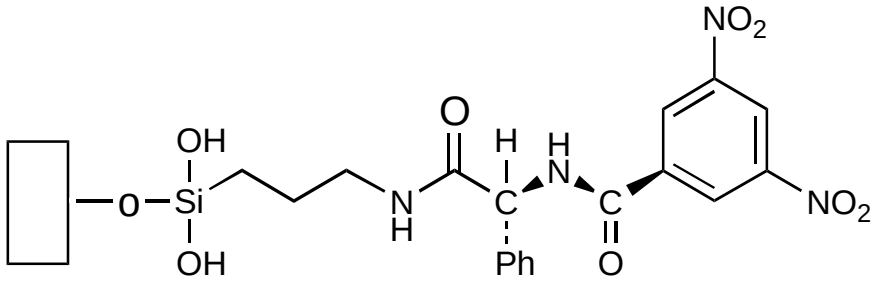


Figure 3. Type 1 CSP: Pirkle N-(3,5-dinitrobenzoyl)phenylglycine (a) ionically or (b) covalently bonded to a aminopropylsilica.

Type 2 CSPs: Derivatized Cellulose and Related CSPs

Cellulose is a crystalline polymer composed of a linear poly-β-D-1,4-glucoside. Energy calculations support a helical structure in which each glucose residue lies near the helical axis but is twisted relative to the previous one[128]. At the supramolecular level, cellulose contains ribbonlike polymer chains that are held together by intramolecular and intermolecular hydrogen bonds that results in the formation of sheets; the sheets then stack

vertically and are staggered 1/2 a glucose unit (Fig. 4). Thus, chiral cavities are created between sheets, further imparting chiral character[129]. Unmodified cellulose was reported to resolve amino acids and derivatives[130-131], diaminocarboxylic acids[132], synthetic alkaloids[133], and cathecins[134].

Although crystalline cellulose shows chiral recognition, it does not make practical CSPs[135]. Poor resolution and broad peaks are obtained due to slow mass transfer and slow diffusion through the polymer network. The highly polar hydroxyl groups

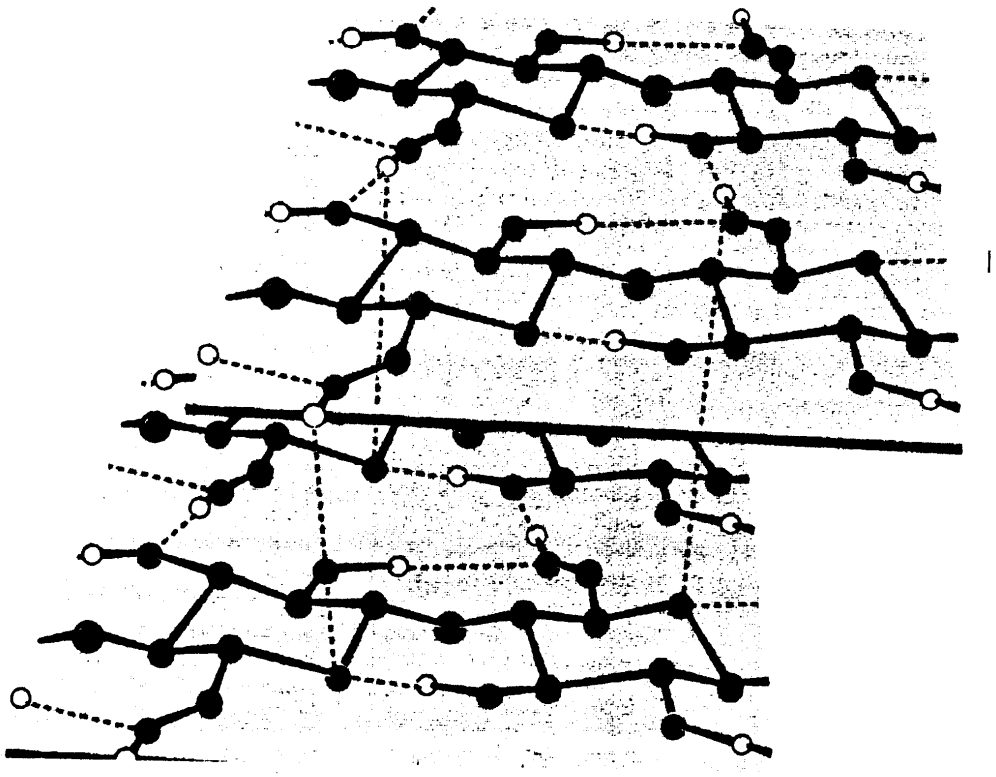


Figure 4. Structural diagram of unmodified cellulose showing the β-(1,4) linkage of D-glucose residues and intramolecular and intermolecular hydrogen bonding resulting in fomation of sheets. (From ref. 129)

characteristic of cellulose often lead to nonstereoselective binding between the analyte and cellulose. In addition, cellulose is unable to withstand normal HPLC pressures. Derivatization of cellulose brings about practically useful CSPs with high chiral recognition mechanisms that can separate a wide range of racemic compounds. Okamoto *et al*. developed the first commercially successful series of cellulose triacetates initially coated[136] and later bonded[137] to a silica gel support. The other Type 2 CSPs that are commercially available from the Daicel Chemical Industries have been developed by Okamoto[138] and co-workers. A list of commercially available Type 2 CSPs is given in Table II.

The derivatized cellulose CSPs have been studied extensively[140-143]. However, it was Wainer *et al*.[144-145] in 1986 who first concluded that the chiral recognition mechanism, which is generally accepted for most of the Type 2 CSPs, involves attractive interactions followed by the steric fit of the molecules into the chiral surface. That is, the analyte-CSP complexes are formed first by attractive interactions and chiral discrimination then takes place due to the steric fit of racemic analyte into the chiral cavity. The attractive interactions include hydrogen bonding, dipole-dipole, and π-π interactions.

Some derivatized amyloses[146] (Table II) are also classified as Type 2 CSPs. They exhibit similar behavior in discriminating racemic analytes. Amylose is a natural polymer possessing the same constituents as cellulose but differing in the glycosidic linkage, in this case a helical α-D-1,4-glucoside. The chiral recognition mechanism for the amylose CSPs, as concluded by Wainer *et al*.[147], involves attractive interactions and steric fit of analytes into chiral cavities, but the latter is no longer critical for chiral discrimination. The chiral recognition mechanism is said to be conformationally driven. If the derivative group of an amylose CSP is the same as that of cellulose, the expected enantioseparating ability of two CSPs should be complementary[148]. Although amylose and cellulose have the same D-glucose constituents but they have different higher order structures due to difference in the glucose linkage. Fig. 2 shows the structure of the Type 2 CSPs used in this research, Chiralpak AD Chiralcel OD. Both CSPs have the same derivatized glucose constituents.

Table II. Commercially Available Type 2 Chiral Columns (After Okamoto, ref 139.)

Polysaccharide Derivative	Trade Name	Distributor
Cellulose triacetate (coated on silica gel)	Chiralcel OA	Daicel
Cellulose tribenzoate (coated on silica gel)	Chiralcel OB	Daicel
Cellulose trisphenylcarbamate (coated on silica gel)	Chiralcel OC	Daicel
Cellulose tris(3,5-dimethylphenyl-carbamate (coated on silica gel)	Chiralcel OD Chiralcel OD-R	Daicel
Cellulose tris(4-chlorophenylcarbamate) (coated on silica gel)	Chiralcel OF	Daicel
Cellulose tris(4-methylphenylcarbamate) (coated on silica gel)	Chiralcel OG	Daicel
Cellulose tris(4-metylbenzoate) (coated on silica gel)	Chiralcel OJ	Daicel
Cellulose tricinnamite (coated on silica gel)	Chiralcel OK	Daicel
Amylose tris(3,5-dimethylphenylcarba-mate (coated on silica gel)	Chiralpak AD	Daicel
Amylose tris [(S)- phenylethylcarbamate) (coated on silica gel)	Chiralpak AS	Daicel

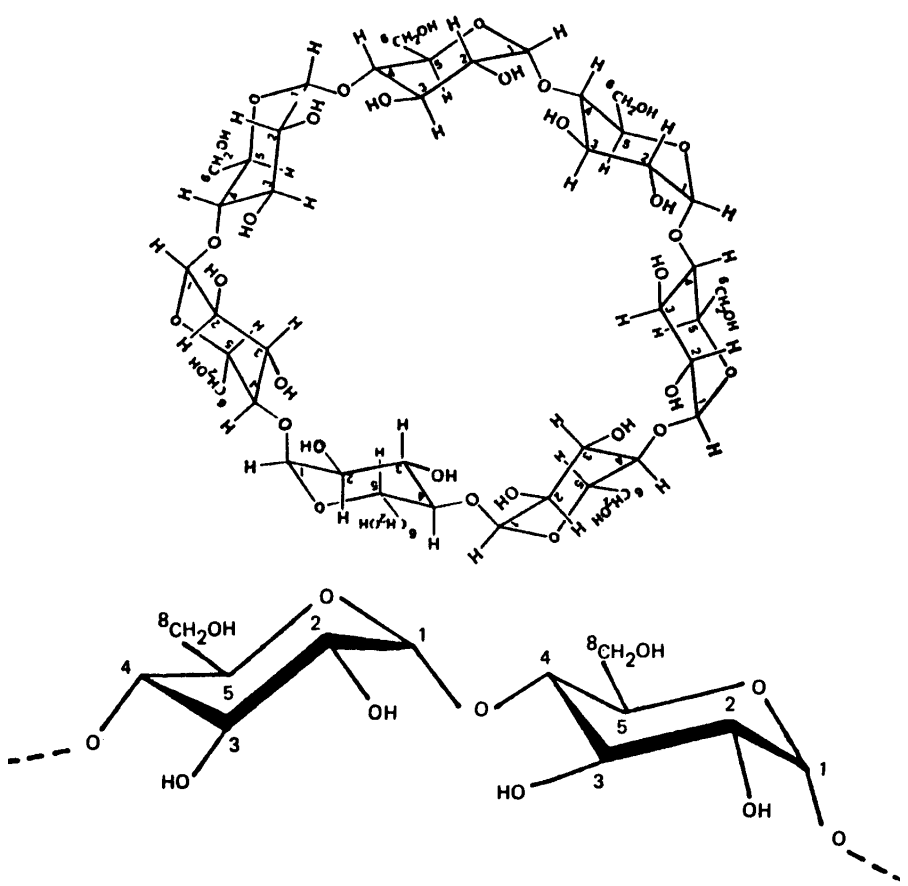
Type 2 CSPs of derivatized cellulose and amylose developed by Daicel Chemical Industries, Japan, are reported to separate a wide range of racemic compounds. These include axially and planar asymmetric compounds, metal containing compounds, compounds possessing chiral sulfur or phosphorus atoms, hydrocarbons, halides, cyano compounds, ketones, amines and their derivatives, acids and their derivatives, alcohols and their derivatives, ethers and oligomers[149]. Most of the commercially available derivatized cellulose and amylose CSPs are adsorbed onto a macroporous silica gel support[150]. It is believed that the higher order structure of a Type 2 CSP greatly influences its enantioseparating ability.

Type 3 CSPs: Inclusion

Chiral separations based on inclusion are achieved through a mechanism by which the guest molecule is included into the cavity of a host molecule. The exterior of the host molecule generally possesses functional groups that act as steric barriers or interact with with the guest molecule in a fashion that induces enantioselectivity.

The most often used CSP of this type is cyclodextrin bound to a silica support. Cyclodextrins[151] are cyclic oligomers with 6 (α), 7 (β), or 8 (γ) D-glucose units through α-1,4-linkage that adopt a tapered cylindrical or toroidal shape (Fig. 5). The toroid has a maximum diameter ranging from 5.7 Å (α-cyclodextrin) to 9.5 Å (γ-cyclodextrin) with a depth of about 7 Å. The interior of the toroid is hydroxyl free and hydrophobic which favors enantioseparation of partially nonpolar compounds via selective inclusion. Since inclusion is critical for chiral discrimination, α-cyclodextrin is suitable for small racemic molecules and γ-cyclodextrin CSP is better for big molecules. Since cyclodextrin as a whole is hydrophilic, aqeuous mobile phases are used. Derivatized cyclodextrins[152] have also been developed to vary the enantioseparating ability.

(a)



(b)

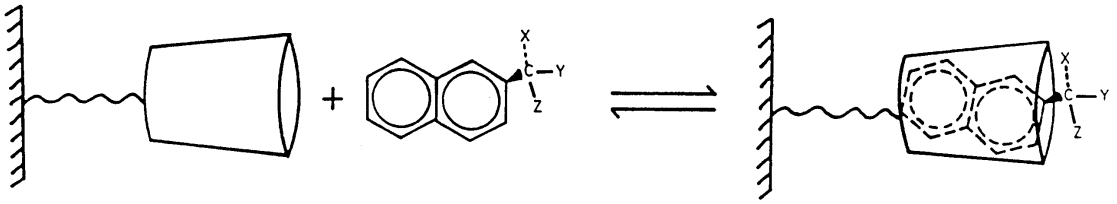


Figure 5. Type 2 CSP: (a) Structural diagram of β-cycldextrin and two of the glucose residues that illustrate the details of the α-(1,4) glycosidic linkage and (b) the schematic diagram of cyclodextrin bonded to a silica gel support forming an inclusion complex with a chiral molecule. (From ref. 151)

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Armstrong[153-154] has studied extensively the cyclodextrins and popularized them as both CSPs and mobile phase additives. Cyclodextrins possess excellent selectivities for a wide range of racemic compounds: amines, alcohols, carboxylic acids,epoxides, and others. At present, cyclodextrin is most often used for chiral separation as a chiral stationary phase in both GC and LC, and as mobile phase additives in LC, GC, and CE.

Crown ethers are classified as Type 3 CSPs. Unlike cyclodextrin, the host-guest interaction within the chiral cavity is hydrophilic in nature. Crown ethers are heteroatomic macrocycles with repeating units of (-X-C_2H_4-) where X, the heteroatom,is commonly oxygen but may also be a sulfur or nitrogen atom. Crown ethers[155], especially 18-crown-6 ethers, can complex inorganic cations and alkylammonium compounds. This inclusion interaction is based mainly on hydrogen bonding between the hydrogens of the ammonium group and the heteroatom of the crown ether. Additional electrostatic interaction occurs between the nitrogen and the crown ether's oxygen lone pair electrons. Crown ether CSPs are made up of crown ethers covalently bonded to a silica gel or polystyrene matrix. It was Cram and co-workers[156] who first demonstrated the enantioseparating ability of crown ether for a number of amino esters and amino acids. Shinbo *et al.*[157] found that chiral separations can also be performed on a reversed phase column that was dynamically coated with a crown ether. This crown ether has been utilized in a commercially available column, Crownpak CR (Daicel Chemical Industries, Japan) and has been used to separate a large number of amines, amino alcohols, amino acids, and amino esters.

Other Type 3 CSPs are made up polyacrylates and polyacrylamides as the chiral selectors.

Type 4 CSPs: Ligand Exchange

As early as 1968, the ligand exchange technique first offered a complete and reliable separation of enantiomers of amino acids and some chiral compounds that form complexes with metal cations.

Ligand exchange chromatography (LEC)[158] involves the formation of a reversible coordination complexes between a bidentate analyte, a divalent metal ion, and a chiral

ligand immobilized in the stationary phase. The metal ion is a transition metal, usually Cu^{+2}, and the chiral ligand is an amino acid, e.g., proline. The stability of such complexes is highly dependent on the transition metal used: Cu^{+2} complexes are generally the most stable and are preferred in LEC applications. Factors affecting selectivity and efficiency of the enantioseparations include the pH, ionic strength, and column temperature. Ligand-exchange chromatography was first reported by Davankov's group[159-160] in Moscow in 1971. They used immobilized L-proline residues on styrene-divinyl benzene resins in the presence of Cu^{+2} to separate racemic amino acids.

Gubitz *et al.*[161] in 1983 improved efficiencies of the original Type 4 CSP by using a silica gel, instead of the styrene-divinyl benzene packing material. Karger *et al.*[162-163] in 1983 further improved the efficiency and enantioselectivity of such packing by incorporating an inert n-butyl or n-decyl spacer chains between the ligand groups interacting with the metal atom and the silica support.

Ligand exchange chromatography, however, is limited only to racemic analytes that act as a bidentate ligand for Cu^{+2} or other comparable ions, e.g. amino acids. Alternatively derivatization is needed for the enantiomers to be separated.

Type 5 CSPs: Proteins

Proteins are polymers composed of chiral units (L-amino acids) and are known to bind small molecules. A protein can be immobilized on solid support by a variety of bonding chemistries; the choice of bonding chemistry affects the selectivity of the CSP obtained. In 1973, Stewart and Doherty[164] first reported the use of agarose-bonded bovine serum albumin (BSA) for chiral resolution of racemate amino acids. This was followed by the devolopment of other protein CSPs. Allenmark *et al.*[165] in 1982 reported agarose-bonded BSA, and Hermansson[166] in 1983, a silica bonded α1-acid glycoprotein. Other protein CSPs are ovomucoid[167], human serum albumin[168], avidin[169], and cellobiohydrolase[170].

The retention mechanism for Type 5 CSPs is most likely based on hydrogen bonding, ionic interactions, or other interactions[171]. The mobile phases are generally aqueous

buffers that are used in limited pH ranges with organic modifiers. Retention and stereoselectivity can be manipulated by varying the mobile phase composition, pH, and column temperature. Although the capacity of protein column is limited, they offer a broad range of applicability. The Type 5 CSPs are used to separate neutral, cationic, and anionic racemic species.[172]

2.2 Temperature Dependence of Chiral Separation

Variation of column temperature has an effect on retention and enantioselectivity of chiral analytes on a CSP. For example, an increase in temperature generally decreases the retention of analytes due, on a molecular level, to their lower affinity with the stationary phase and therefore their faster migration through the chromatographic column. However, the enantioselectivity may either increase or decrease depending on the type of interaction mechanism. Thus, a change in temperature can be used to optimize enantioseparation. From the retention behavior and measurements of thermodynamic parameters - enthalpy, entropy, and Gibbs free energy of association between the enantiomers and CSP, and enthalpy-entropy compensation analysis[173] some information on the mechanism for enantioseparation can be obtained.

The temperature studies in Chiral HPLC utilized the methods developed for reversed phase HPLC and are sufficiently noteworthy to be reviewed here.

In 1978, Melander, Campbell and Horvath[174] used the enthalpy-entropy compensation method to conclude that the same retention mechanism was operating for the separation of a homologous series of analytes on the different hydrocarbonaceous (reversed phase) stationary phases at varying mobile phase compositions and temperature conditions. These authors used the retention data collected at the different chromatographic conditions and plotted the natural logarithm of capacity factors (another expression for retention) against the reciprocal of absolute temperature (van't Hoff plots). From the slopes of van't Hoff plots, the enthalpy changes for the partitioning of analytes between stationary and mobile phases were measured. A compensation plot (natural logarithm of capacity factors against the measured enthalpies) was then constructed and it gave a

linear plot suggesting that a single, partitioning mechanism existed for the interaction between analyte and the stationary phase.

Mathematically, the relationship for enthalpy-entropy compensation is given by

$$\Delta H^\circ = \beta \Delta S^\circ + \Delta G^\circ_\beta \qquad (2.2a)$$

where ΔG°_β is the Gibbs free energy change at the compensation temperature β and ΔH° and ΔS° are the standard enthalpy and entropy changes, respectively. From the above equation, the Gibbs free energy change is independent of temperature, since changes in enthalpy are compensated by entropy. A detailed discussion of the theory of enthalpy-entropy compensation method is given in Chapter III.

In 1986, Hancock *et al*.[175] utilized column temperature data for the studies of protein and peptide separations by reversed phase HPLC. The authors noted that, in contrast to many biochemical systems in which an increase in temperature results in increased interaction, in the separation of peptides an increase in temperature decreased the retention. Furthermore, the separation of insulin proved to be entropy controlled, and the separation of a lipid-binding peptide was enthalpy driven.

These conclusions were inferred by means of van't Hoff plots[176] obtained from

$$\ln k = -\Delta H^\circ / RT + \Delta S^\circ / R + \ln \varphi \qquad (2.2b)$$

where k, is the retention factor (another expression for retention); φ is the volume ratio of the stationary and mobile phases; **R** is the gas constant; **T** is the absolute temperature, and ΔH° and ΔS° are the enthalpy and entropy changes, respectively, when the analyte interacts with the stationary phase. If the measured value of ΔH° is greater than ΔS°, the separation is said to be enthalpy controlled. For enthalpy driven separations, lowering the temperature enhances separation. The reverse is true for an entropy driven separation that is characterized by a larger ΔS° value than ΔH°. A detailed discussion of this theory is given in Chapter III.

Cole and Dorsey[177] in 1992 utilized van't Hoff plots and the measured enthalpy and entropy parameters to study the retention mechanism in reversed phase HPLC. In their study, the entropy contribution increased proportionally to the enthalpy contributions when the bonding density of the sorbent surface was increased. The authors concluded

that partitioning, rather than adsorption, must be the "relevant model of retention" for the analytes on a reversed phase stationary phase.

Although the above studies are for the separation of achiral compounds on achiral stationary phases, they are discussed because of the use of temperature and vant Hoff plots to help understand the retention mechanism involved. Similar experiments have been used in this research to investigate the chiral recognition mechanisms for the CSPs used in this research.

One of the first studies to utilize temperature for the optimization of chiral separation was by Feitsma *et al.*[178] in 1985. The enantioseparation of aromatic carboxylic acids on β-cyclodextrin was improved by raising the temperature to 55°C. At 25°C enantiomeric resolution of the acids was poor because the second eluting peak was broad and tailing. There was an overall increase in resolution from 1.6 to 1.65 when the temperature was 55°C. Although there was no explanation for the increase in resolution, it appears that the major effect may simply due to less adsorption at the higher temperature and reduced tailing.

In 1986, Mazzo *et al.*[179] optimized the chiral separation of an aromatic carboxylic acid, MK-286, an anti-hypertensive drug at 0°C. The CSP was a Pirkle-type covalent dinitrobenzoyl-L-phenylglycine using a mobile phase of methanol/aqueous buffer. The resolution was improved from 0.75 to 1.25 when the temperature was lowered form 25°C to 0°C. This is probably an enthalpy driven improvement, resulting from increased selective interactions, like hydrogen bonding.

Pescher et al.[180] in 1986 studied the temperature dependence, coupled with mobile phase effects, of the enantioseparation of tertiary phosphine oxides on a Pirkle-type dinitrobenzoylalanine CSP. The mobile phase was hexane/chloroform/ethanol (25/66.9/8.1). In this study the enantioselectivity was enhanced at lower temperature which compensated for the loss in efficiency due to the increase in the mobile phase viscosity (slower mass transfer). The enantioselectivity changed from 1.52 to 2.33 when the temperature was decreased from 50°C to -15°C. The authors further noted that low temperatures are useful for separating extremely thermolabile compounds.

An interesting temperature dependence study was reported by Weaner *et al.*[181] in 1988. The enantioseparation was for fatty acid esters and amide epoxides on a Pirkle-type CSPs using a hexane/2-propanol eluent system. Maximum resolution occured at different temperatures for the ester and amide enantiomers, with the overall profile of resolution versus temperature being an inverted parabola. For the ester, the maximum resolution was 1.6 between 0 and 5°C and for the amide, 1.7 at 38°C. Enantioselectivity of the ester decreased rapidly above 0°C, but for the amide it reached a peak at 10°C and stayed constant up to 40°C. These results were attributed to the weak analyte-CSP interaction for the ester, in contrast to the strong (probably hydrogen bonding) amide-CSP interaction.

From this study of Weaner *et al.*[182] , it is important to point out that: (a) enantioseparation on a CSP is dependent on the solute structure which defines the type and extent of interaction with the CSP; and (b) the nonlinearity dependence of enantioselectivity with temperature, i.e. showing a maxima, can be attributed to the degree of solute-CSP interaction.

Gilpin *et al.*[183] in 1991, reported a maxima in plots of ln *k* versus the absolute temperature (van't Hoff plots). In the study, D- and L-tryptophan were enantioseparated on a bovine serum albumin CSP, at varying temperatures and pH. The authors speculated that the initial increase in ln *k* with temperature, which is "thermodynamically inconsistent with a simple retention mechanism", may be due to either gross changes in protein surface orientation or induced conformational changes of the protein, which then provide an increased number of binding sites. It was concluded that the unusual curve and nonlinearity of the van't Hoff plots was due to a change of the retention mechanism.

Armstrong *et al.*[184] in 1992 utilized only temperature dependence and calculated thermodynamic parameters from van't Hoff plots and enthalpy-entropy compensation method to study the enantioselective retention mechanism of derivatized cyclodextrin GC CSPs. A homologous series of TFA derivatized amines, TFA derivatized diols, and alkyl esters of 2-bromobutyric acid were the probe molecules. Based on the results, the probe molecules were divided into two groups: (a) Group I compounds that gave a nonlinear compensation plot and a large enthalpy and entropy values, and (b) Group II compounds

that gave a linear compensation plot and lower enthalpy and entropy values. It was concluded that the Group I compounds were enantioseparated by an inclusion mechanism complex. On the other hand, the Group II compounds were resolved by external, multiple association with the cycodextrin (top, side, and / or bottom surfaces). The authors further noted that the enantioselective retention mechanism of some compounds may be temperature dependent, e.g., more inclusion at lower temperatures.

The importance of Armstrong's work for the research on chiral recognition mechanism lies in the possibile similarity of multiple retention mechanism between cyclodextrin and the Type 2 CSPs, Chiralcel OD and Chiralpak AD. It was noted previously that the general recognition mechanism for the derivatized cellulose and amylose CSPs involves both attractive interactions and inclusion or steric fit into chiral cavities. If the attractive-repulsive interactions and inclusion mechanisms are competing, temperature studies may determine which is dominant, e.g., van't Hoff plots and measurements of thermodynamic parameters.

There are additional reports on the use of column temperature to optimize HPLC chiral separations. For the Type 2 CSPs, Witte *et al.*[185] in 1992 reported the effect of temperature on the enantioseparation of different aminotetralins on cellulose tris(3,5-dimethylphenyl-carbamate), Chiralcel OD. The resolution of aminotetralins with π-electrons not directly bonded to the hydrogen bonding of the molecule was enhanced by lowering the temperature. This result was attributed to π-π interactions that are more pronounced at lower temperatures. According to the authors, "the π-π interaction is such a weak force that at higher temperatures the energy of the analyte and the CSP is so high that the π-π interaction is easily disturbed". It was also noted that the aminotetralins which have either the primary or secondary amine group and possess a sufficiently short N-alkyl substituent showed a decrease on resolution at lower temperatures, but all other racemic amino aminotetralins showed an increase of resolution. This result was explained in terms of the hydrogen bonding interaction , which is a stronger force and less influenced by temperature.

Another temperature study that is worth mentioning is that of Cabrera *et al.*[186] in 1994. Oxazepam and prominal were enantioseparated on a chemically bonded β-

cyclodextrin , LiChroCART Chiradex, using a reversed phase eluent. A decrease in temperature caused an increase in the retention of both analytes, but the enantioselectivity for the former was increased and of the latter was decreased. These results were explained in terms of thermodynamic parameters that control the enantioseparation. Oxazepam which favored the low temperature was said to be an enthalpy controlled enantioseparation. Prominal, on the other hand, that favored a high temperature for the enantioseparation was said to be entropy driven.

In addition, Smith, Taylor, and Wilkins[187] in 1995 reported the chiral separations of a series of related compounds within a series of potassium channel activator analogues at different temperatures. The CSP was cellulose tris(3,5-dimethylphenylcarbamate), Chiralcel OD, and the mobile phase was a mixture of hexane and 2-propanol. By thermodynamic measurements, the analog that showed better enantioseparation at low temperature was enthalpy controlled. This result was attributed to a hydrogen bonding phenomenon that is important in the chiral discrimination. For the other analog that favored the high temperature, its enantioseparation was said to be entropy controlled. The authors concluded that the π-π interactions were important in the chiral recognition process.

Lastly, Booth and Wainer[188] in 1996 investigated the retention mechanism of the amylose tris(3,5-dimethylphenylcarbamate) CSP, Chiralpak AD, for mexilitine and for a series of structurally related compounds using the enthalpy-entropy compensation method coupled with the quantitative structure enantioselective retention relationship (QSERR) method. The enthalpy-entropy compensation analysis, which was used to determine a multiple retention mechanism, suggested that there were two retention mechanisms for the CSP. The authors further noted that these mechanisms were based on either the presence or absence of secondary hydrogen bonding groups.

From the cited works, temperature dependence studies that include retention behavior, measurements of thermodynamic parameters, enthalpy, entropy, and Gibbs free energy of association between the enantiomers and CSP, and enthalpy-entropy compensation analysis, provide important information for the enantioselective retention

mechanism. However, these studies do not give a complete description of the mechanism for the separation of a chiral compound on a CSP.

2.3 Mobile Phase Studies (Polar Modifiers)

In liquid chromatography, the separation of a chiral compound on a CSP is thought of an adsorption-desorption process[189]. The mobile phase molecules compete with the racemic analyte for the active binding sites of the CSP. After column equilibration, the polar modifier molecules occupy the adsorption active sites of the CSP. As the analyte "plug" enters the column, analyte molecules begin displacing mobile phase molecules from interaction sites of the CSP. The relative affinities of the racemic analyte versus the mobile phase species for the CSP active sites determine the retention, *k*, for a given analyte, and the difference in affinities between the analyte enantiomers and the mobile phase determine the enantioselectivity, α. A decrease in retention when different mobile phase is used means that the analyte has lesser affinity for the CSP compared to the different mobile phase, and the analyte molecules have a difficulty in displacing the different mobile phase molecules.[190]

Mobile phase studies help elucidate the possible chiral recognition mechanism for a CSP. Variation of the mobile phase composition enables one to help predict the dominant interactions that influence retention, and the interactions that are critical to enantioselectivity.

2.3.1 Alcoholic Mobile Phase Modifier

As shown in Table I, Type 1 and Type 2 CSPs generally utilize normal phase eluent with a polar modifier, such as an alcohol. As noted in the previous discussion, the chiral selectors of the Type 1 and Type 2 CSPs are polar and are capable of forming hydrogen bonds. Alcohol as the polar modifier, by virtue of its functionality, is also capable of forming hydrogen bonds.[191] The alcohol molecules, therefore, compete with the analytes for the active adsorption sites of the CSP.

In 1984, Zief *et al.*[192] reported the enantioseparation of 2,2,2-trifluoro-1-(9-anthryl)ethanol on covalent R-N-(3,5-dinitrobenzoyl)phenylglycine (a Pirkle-type CSP) using hexane with either ethanol, 2-propanol, or *tert*-butanol as the polar modifier. The polarity of the alcoholic mobile phase modifier was kept constant, thus, the only difference lay in the steric structure of the alcohols. Of the three alcohols tested *tert*-butanol gave the best enantioselectivity, $\alpha = 1.62$ vs. $\alpha = 1.56$ for 2-propanol and $\alpha = 1.33$ for ethanol. This increase in α was attributed mainly to an increase in the *k* of the primarily bound enantiomer while the *k* of the first eluted enantiomer was essentially unchanged. The authors concluded that an increase in the bulk of the alcohol decreased its absorption on the CSP.

Pescher *et al.*[193] in 1986 reported similar results in the resolution of enantiomeric phosphine oxides on Pirkle-type dinitobenzoylalanine CSP. The mobile phases used were a mixture of hexane and alcohol, and the polarity of the alcoholic mobile phase modifier was kept constant. The enantioselectivity of the system increased with increasing bulk of the alcohol.

Macaudiere *et al.*[194] in 1986 reported the resolution of a series of enantiomeric amides using a Pirkle-type CSP under SFC conditions. The amides were chromatographed using carbon dioxide modified with an alcohol. The results of the study indicated that at constant k, the stereoselectivities are greater for alcohols with bulkier side groups close to the hydroxyl moiety.

So far, all studies cited are about the influence of alcoholic mobile phase modifiers on *k* and α on a Pirkle-type CSP. In all these studies, the enantioselectivity increases with an increasing bulk of the modifier. In practical terms, this means that the bulkiest modifier should be used to optimize chiral separations.

Studies of the effect of the alcoholic mobile phase modifiers for the Type 2 CSPs have also been made. Mobile phase effects on *k* and α on the cellulose triacetate CSP were investigated by Koller *et al.*[195] in 1983. In their study, the eluent was changed from methanol to ethanol-water (96:4) to 2-propanol. Three enantiomeric solutes were chromatographed. The *k* of each solute increased throughout the study while the effect on α varied according to the structure of the solute. The authors concluded that the polarity

of the eluents may not be the key to their elution power. It was further noted that if the analytes were included into chiral cavities of the cellulose triacetate CSP, the elution power may be determined primarily by the size of the eluent. This suggests that the bulk of the alcoholic mobile phase modifiers affects the steric environment of the chiral cavities. It must be remembered that the higher order structures of the derivatized cellulose influences the chiral separation process.

Wainer *et al.*[196] in 1987 investigated the effect of the bulk of an alcoholic mobile phase modifier on k and α of a series of amides and alcohols on the cellulose tribenzoate CSP. The mobile phases used consisted of hexane and alcohols. At a constant concentration of ethanol in hexane, *k* of each solute increased with decreasing polarity of the alcoholic modifier while α varied according to the structure of solute. The results of their studies indicated that as in the Pirkle-type CSP both the analyte and alcoholic mobile phase modifier compete for chiral and achiral binding sites on the CSP. The bulk around the hydroxyl moiety of the alcoholic mobile phase modifiers played a role in this competition. In addition, the results also suggested that like the cellulose tribenzoate CSP, the alcoholic mobile phase modifiers may bind to sites near or at the chiral cavities of the CSP changing the steric environment at these cavities and, thus, the stereoselectivity of the CSPs.

2.3.2 Mobile Phase Effects (Acidic Modifier)

The majority of the enantioseparations of chiral acidic and basic compounds on Type 2 CSPs require the addition of acidic mobile phase modifiers[197-198]. However, there have not been any in depth studies on the influence of the acidic mobile phase modifiers on retention and enantioselectivity in Type 2 CSPs. Okamoto *et al.*[199] in 1989 reported the successful enantioseparation of 2-arylpropionic acids on tris(3,5-dimethylphenylcarbamate)s of amylose and cellulose using 1% trifluoroacetic acid (TFA) as the acidic mobile phase modifier. These authors mentioned that TFA helped in the adsorption-desorption process. In 1996, Booth *et al.*[200] studied the chiral discrimination mechanism in amylose tris(3,5-dimethylphenylcarbamate), Chiralpak AD, for 2-

alkylarylpropionic acids and 1% TFA as the acidic modifier. Tang[201] in 1996 attempted to explain the role of TFA on the enantioseparation of ibuprofen and ketoprofen on tris(4-methylbenzoate), Chiralcel OJ. According to her results, small amounts of TFA only improved the enantiomeric resolution of both analytes because they were already partially resolved without TFA. Tailing peaks without TFA and longer retention times were due to the strong interactions of ibuprofen and ketoprofen with the accessible silanol groups of the packing material. That is, TFA essentially deactivated the CSP resulting in less peak tailing and therefore, better resolution. Furthermore, Tang explained that TFA displaced the analytes acidic group on silanol sites of CSP and suppressed the ionization of the weakly acidic silanol groups.

2.4 Chiral Recognition Mechanism Studies

Predicting the chiral recognition of a CSP for a particular racemic compound is a difficult task because of the complexity of the process. The preferential interaction of a CSP with one of the enantiomers, known as chiral recognition, reflects only those sets of interactions that are important for enantioselectivity causing differential retention of enantiomeric analytes.

2.4.1 Studies Involving Variation of Structural Features of a Common Skeleton

In this method a homologous or analogous series of chiral compounds are used as test probes carefully designed to interact with the chiral stationary phase. Retention factors and enantioselectivities are evaluated and then correlated with the structural features of the probe molecules. As long as the differences in retention are substantial, this method can be used with confidence and has the advantage of requiring only very small amounts of compounds.

In 1980, Pirkle *et al.*[202] used the chiral derivatives of a 3,5 dinitrobenzoylamines, alcohols and thiols as test probes for a fluroanthrylalcohol CSP covalently bonded to a silica support. The results obtained confirmed the theory of π-π interactions between the

nitrobenzoyl and anthracene moieties (π-electron acceptor and donor, respectively), and hydrogen bonding between the benzoyl carbonyl group and the hydroxyl proton on the CSP.

Hesse and Hagel[203], Blaschke[204], and Francotte *et al*.[205], to cite works specifically on derivatized cellulose CSPs, used various racemic phenyl-substituted analytes to study the chiral recognition mechanism of microcrystalline cellulose triacetyl (CTA-I). The authors proposed that the mechanism involved the penetration of the aromatic portion of the solute into the cavities that are formed between the D-glucose units of the CSP. The observed stereoselectivity was due to differences in fit or inclusion of the enantiomers of the analytes in the cavities of the CSP. The mechanism is similar to the inclusion (host-guest) mechanism proposed by Armstrong *et al*.[206] for the cyclodextrin-based CSPs.

Wainer *et al*. in 1986[207], studied the chiral recognition mechanism for another type of derivatized cellulose CSP. Chiral amides were used and the CSP was cellulose tribenzoate. The results indicated that the formation of the diastereomeric analyte-CSP complex is based on hydrogen bonding, π-π and amide dipole interactions. These interactions not only formed the complexes but also positioned the analyte on the CSP. In addition, the magnitude of the enantioselectivity of the enantiomeric amides was dependent upon the differences in the steric fit of the asymmetric portion of the analyte in a chiral cavity of the CSP. It should be noted that the proposed mechanism here is different from that of CTA-I, which is the formation of inclusion complex.

Since the higher structural order of the derivatized cellulose CSPs influences its enantioseparating ability, Wainer and co-workers[208] followed up their study by exploring the influence of mobile phase on retention and enantioselectivity. This was to verify if steric fit into chiral cavity was critical to chiral discrimination. Inclusion in this context means that the formation of diastereomeric complexes involves inclusion of analytes in chiral cavities followed by attractive interactions. It must be remembered that the higher order structure of derivatized cellulosed CSPs affects its enantioseparating ability.

In 1987, Wainer *et al*.[209] investigated again the chiral recognition of the same CSP but for chiral aromatic alcohols. In this study, both the influence of temperature and mobile phase were explored. They concluded that a similar mechanism existed, where the

first attractive interaction was hydrogen bonding between the alcohol moiety of the analyte and the carbonyl oxygen of the ester moiety.

In 1992, Witte *et al.*[210] studied the chiral recognition mechanism for the cellulose tris(3,5-dimethyphenylcarbamate), Chiralcel OD using twenty one different aminotetralins. In the study, the effects of temperature and mobile phase were also investigated. As mentioned before, hydrogen bonding was the dominant interaction between CSP and solute. Furthermore, according to the authors, the analyte-CSP "free" π-π interaction greatly enhanced the separation.

Overall, the use of structurally related compounds can allow inferential conclusions to be drawn about mechanisms of interactions. The method is reliable as long as the chromatographic data is reproducible and of high precision. In addition, if other studies are performed such as temperature dependence and mobile phase effects, a better understanding of the chiral recognition process by correlation of retention and enantioselectivity using homologous or analogous series can be achieved. The information obtained from the study of specific molecular interactions operating during a chromatographic process may be exploited to gain the ability to predict enantioseparations, as well as to further the development of highly efficient and even customized CSPs.

2.4.2 Thermodynamic Studies

The basic concept was described earlier in Section 2.2. Thermodynamic approaches for the study of chiral recognition mechanism also utilize homologous or analogous series of compounds, and the retention behavior and the corresponding enantioselectivities at different temperatures are evaluated. The thermodynamic parameters, free energy, enthalpy, and entropy of association between the enantiomers and CSP are then calculated. Entropy and enthalpy data from the graphs of the logarithm of capacity factor versus the reciprocal of absolute temperature (van't Hoff plots) are utilized to indicate the possible chiral recognition mechanism. However, the results of this study do not give a complete description of the chiral recognition process. The study could best evaluate the

number of mechanisms for a CSP, e.g. single or multiple mechansms[211], and whether the enantioseparation is enthalpy or entropy controlled.

2.4.3 Quantitative Structure Enantioselective Retention Relationships (QSERR)

The QSERR approach to study the chiral recognition process also utilizes a homologous or analogous series of chiral compounds carefully designed to interact with the chiral stationary phase. The retention data are subjected to multiparameter regression analysis or factor analysis against various non-empirical molecular descriptors, which reflect the structural features of the analytes. Directed by the initial QSERR equations, molecular modelling is then used to either expand or reject the hypotheses proposed via the equations in order to predict retention and enantioselective separations based on the structures of the solute and the CSP. Only a few studies have been made using this approach because of its complexity and its requirement of a sophisticated computer program. This approach is generally termed as Quantitative Structure Retention Relationship[212] (QSRR) which was first developed in reversed phase HPLC. Three chromatographers, Kaliszan[213], Jandera[214], and Jinno[215] have been resposible for the development of this approach.

Kaliszan *et al.*[216] used this approach to study the retention and chiral recognition mechanism for a series of benzodiazipines chromatographed on a human serum albumin CSP. The QSERR analysis also provided information on the structure of the HSA-benzodiazepine binding site, which was consistent with the structure derived from X-ray crystallographic studies.

In 1996, Booth and Wainer[217] investigated the chiral recognition mechanism of the amylose tris(3,5-dimethylphenylcarbamate) CSP, Chiralpak AD, for α-alkyl arylcarboxylic acids using the QSERR method. Their study indicated that the chiral recognition process involves initial hydrogen bonding interactions between the solute and CSP, insertion of a solute into a cavity on the surface of the CSP, and the stabilization of the analyte-CSP complex by formation of an additional hydrogen bond within the cavity. The chiral recognition process was said to be “conformationally driven”.

Booth and Wainer[218] also studied the chiral recognition mechanism of mexiletine an a series of structurally related compounds on Chiralpak AD. By application of QSERR coupled with thermodynamic approaches, two separate mechanisms were identified. As mentioned before, the mechanisms are based on either the presence or absence of secondary hydrogen-bonding groups.

The QSERR approach is valuable to chiral recognition mechanism studies because it may describe exactly which part of the analyte and CSP is involved and to what extent this is responsible for the separation. However, the disadvantages of this model include its complexity, the need for specialized computer hardware and software, and the expertise to use it. Lack of descriptors for the mobile phase molecules may also be a problem.[219]

2.4.4 Enthalpy-Entropy Compensation

A complementary approach with QSERR to investigate chromatographic retention mechanism involves enthalpy-entropy mechanism[220]. Analogously to QSEERs, enthalpy-entropy compensation manifests itself as a linear dependence of the overall free energy change on the corresponding enthalpy change for intrinsically similar physico-chemical phenomena. Linearity in plots of logarithm of capacity factors, measured at an appropriate reference temperature, and the corresponding enthalpies for the particular chromatographic process are indicative that the analytes display similar retention mechanism in a given system. Like the QSERR, this method requires test probes of similar structures.

The enthalpy-entropy compensation method, like the thermodynamic approach, is used mainly to predict a single or multiple mechanism for a CSP. This does not give a specific description of the chiral recognition process. The detailed methodology of this approach is discussed in Chapter III.

2.4.5 Molecular Modelling

Molecular modelling studies are directed towards the understanding where and how chiral discrimination takes place in a CSP. Most of the microscopic modelling for the chiral recognition mechanism of a CSP utilizes theory which implements the following: quantum mechanics, molecular mechanics, molecular dynamics, Monte Carlo simulations, and molecular graphics[221]. Several molecular modelling studies have been made for all the CSPs. An example is the molecular modelling studies done by Camilleri *et al.*[222] for the enantioseparation of oxiracetam and related molecules on cellulose triphenyl carbamate CSP. In the study, calculations of minimum energy configurations were performed for the first individual molecules, then various conformations for the docked pair. With the aid of molecular modelling, the authors were able to identify the possible interactions leading to stereoselection.

For the other molecular modelling studies, the reader is directed to the Lipkowitz[224] review in 1995.

A shortcoming of the computer modelling approach is the lack of considerations for the mobile phase effects. It must be noted that the mobile phase greatly influences in the enantiomeric separation on a CSP. In addition, this is a complex method and requires a sophisticated computer hardware and software, as well as the expertise of the analyst.