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Novel chiral stationary phases with multiple-interaction centers

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NOVEL CHIRAL STATIONARY PHASES WITH MULTIPLE-INTERACTION CENTERS

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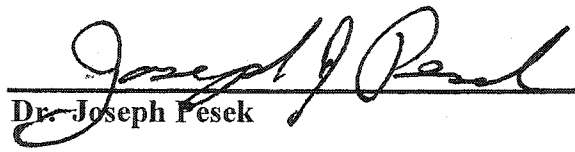

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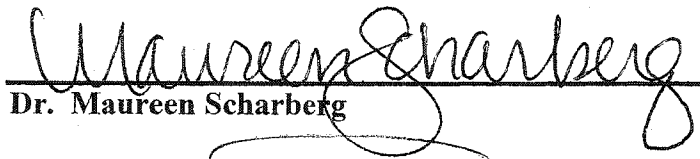

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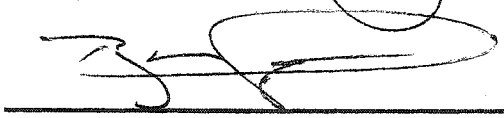

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ABSTRACT

NOVEL CHIRAL STATIONARY PHASES WITH MULTIPLE- INTERACTION CENTERS

By Vandana. Ramakrishnan

This thesis addresses the topic of chiral stationary phases with multiple interaction centers. Chirality deals with the asymmetry of an entity. In chemistry, this concept explains why an object and its mirror image are non-superimposable. This very property has been used in the synthesis of chiral stationary phases for chromatographic separations. Both gas and liquid chromatography columns have been researched in this review.

These types of phases were designed specifically to separate enantiomeric compounds. William Pirkle was the pioneer in the synthesis of Chiral Stationary Phases (CSPs) and Dalgliesh proposed the theoretical model by which the separations occurred. The primary focus of this review is the effect of the presence of an extra chiral center on a chiral stationary phase. It has been concluded that, the presence of an extra chiral center does improve separation of certain enantiomers when compared to conventional stationary phases.

ACKNOWLEDGMENTS

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I would also like to thank my family, especially my parents, my brother, my grandmother, my husband, and my son for being understanding and supportive of the long road it took me to get this work completed successfully.

TABLE OF CONTENTS

PAGE NUMBER

LIST OF TABLES

Table **Page Number**

LIST OF FIGURES

Figure **Page Number**

CHROMATOGRAPHY- GENERAL PRINCIPLES

Introduction:

In 1903, the Russian botanist, Michael Tswett, separated plant pigments on a chalk column. Since the pigments separated as two bands of differing colors (yellow and green), he called the process "**chromatography**." This term is derived from the Latin word and literally means "**writing with color**." Following this achievement, there was a lull in the research done in this area. It picked up again in the 1940's, starting with paper chromatography, followed by gas chromatography (Martin and James, 1952). The search for a robust, high-performing analytical tool reached an apex with the introduction of high-performance liquid chromatography in the 1970's.[1-3]

HPLC was initially referred to as **h**igh-pressure **l**iquid **c**hromatography because high pressure was used to force the solute mixture in the mobile phase through the stationary phase. The name, however, seemed to emphasize that pressure was the one and only critical factor involved in separation. Once it was determined that column length, flow rate, stationary phase packing modes, and particle size were all factors in optimum separation, the name was changed to **h**igh **p**erformance **l**iquid **c**hromatography.[4]

The other techniques mentioned above, like gas chromatography, thin layer chromatography, paper chromatography are still in use today. However, once the performance parameters were optimized, HPLC became the analytical tool of choice for a

majority of industrial applications. Examples of a few fields of application include the food industry, biochemical, pharmaceutical and agricultural.

Chromatography- Basic Principles:

Chromatography can be briefly summarized as the separation of a mixture of solutes in a mobile phase through a (packed) stationary phase. The kind of mobile and stationary phases depend on the kinds of materials undergoing separation. Other factors affect separation (temperature, pressure, pH, polarity) and these are changed to suit the need of the experiment. In HPLC, using high pressure to improve separation is a feature. Described below in Figure 1 is a general setup of a HPLC system.

General setup of a high performance liquid chromatography system:

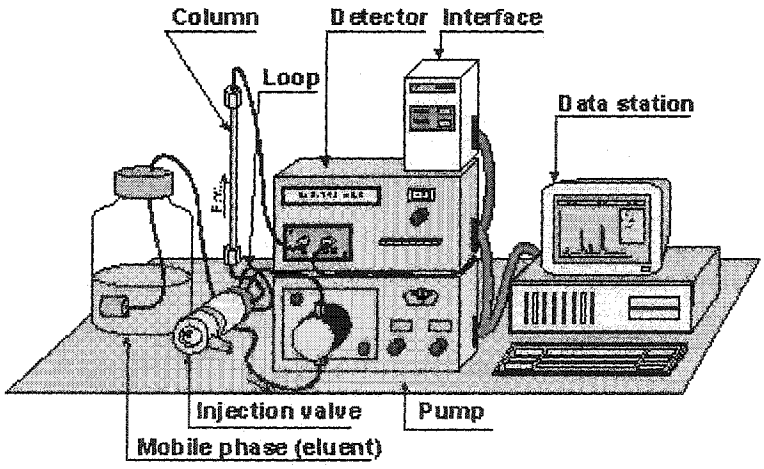


Figure 1. HPLC system (Reprinted with permission). [4]

The major components involved in a HPLC system are:

Pump: The pump performs the function of keeping a constant flow of mobile phase (containing solutes) through the column containing the stationary phase.

Mobile Phase: The mobile phase is the medium in which the components to be resolved are carried through the column. In the case of Liquid Chromatography (LC), the mobile phase is a liquid and as a result the different properties can influence the separation. Some of the common features of the mobile phase that make a difference in the separation process are pH, polarity, flow rate and viscosity.

Stationary Phase: Although each part of a system is equally important, the stationary phase in a HPLC system is very critical. It is the column where the actual resolution of components occurs as the mobile phase carrying the solutes passes through it. In an LC system, the stationary phase is solid, a coated solid or a chemically modified solid. As with the mobile phase, the stationary phase too has some key features that affect separation such as chemical selectivity, affinity, particle size, packing density and pore size.

Injector: The injector introduces the sample solution into the mobile phase just ahead of the entry into the column (containing the stationary phase) from the top.

Detector: As each of the components elute from the column, the detector senses a signal from them. There are various kinds of detectors based on the properties of the eluting substances that produce corresponding signals. Some commonly detectable eluent properties are UV-visible absorbance, fluorescence, radioactivity, and electrochemical changes.

Interface: The signals emitted from the detector are converted into computer-readable (and more graphic) data when passed through an interface. Typically such interfaces (an AD converter) contain software to process these data.

Data Station: The digitized data is retrieved as a graph printout, which represents the data collected from the chromatographic experiment. This graph, generally called the **chromatogram**, shows the elution order of the separated components as a series of peaks (Figure 2). It is a general principle of chromatography that sharper peaks in the chromatogram imply better resolution. With on-line data becoming standard, it has become common practice for chromatographers to attach computers to HPLC systems digitize and store the data for future use.[5]

Parameters of Characterization:

Let us consider a hypothetical separation process, where a mixture containing components A and B was injected into the HPLC system and in the process B elutes out first. Such a chromatogram would appear as shown in Figure 2 below.

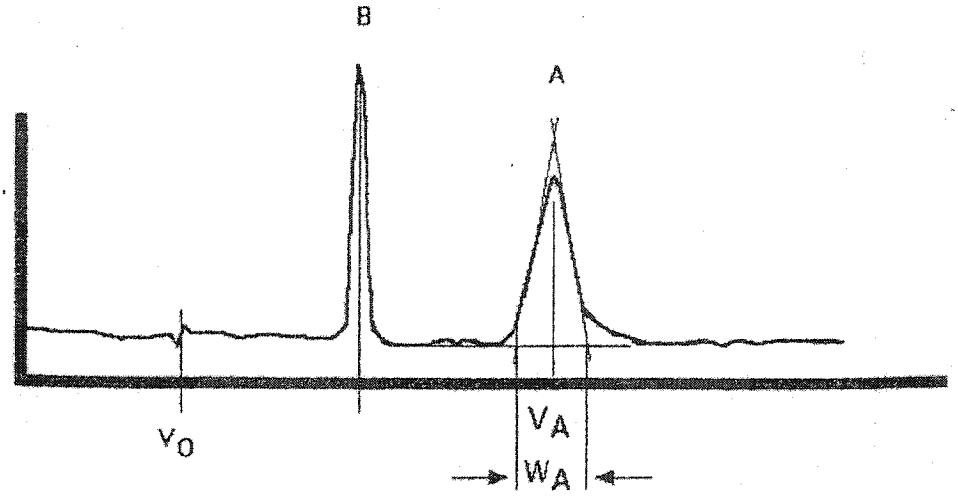


Figure 2. Separation model chromatogram.[6]

The next step in the chromatographic process is interpreting the data. The chromatograph as shown in Figure 2 is a function is a plot of elution time vs. absorbance. Before the separation parameters can be characterized, several terms must be defined.

V_0= Void volume: This parameter represents the volume of mobile phase present in the column exclusive of the volume occupied by the stationary phase. It is common practice to determine the void volume by running a non-retained solute through the column.

Retention Volume: This term represents the volume of the mobile phase that is needed to elute a component from the column. In the experiment, illustrated in Figure 2,

V_A= the retention volume of component A.

V_B= the retention volume of component B.

Once these factors have been tabulated, the next step involves using them to calculate other parameters that characterize the solute or separation process. Three important terms used in this regard are **k′** (capacity factor or retention factor), α (selectivity factor or separation factor) and **N** (efficiency). Another factor needed for the measurement of N is called the peak width, represented by W_X, where X refers to either A or B depending on the component efficiency being calculated. The measurement for peak width starts from the first inflection at the base of the peak across to where the peak ends (see Figure 2).

The capacity factor (**k′**) is a measure of how long a solute is retained on the column. Retention is a function of affinity of the solute to the stationary phase. The stronger the attraction between the solute and the column material, the longer is the retention. The

capacity factor is expressed as a ratio of the adjusted elution volume of the solute to the void volume as shown below.

The separation factor (α) is a measure of selectivity of the column for any pair of solutes. The term α is expressed as a ratio of the retention volumes of the eluted components, second eluted solute: first eluted solute.

The plate count or the efficiency factor (N) is a measure of peak sharpness. By visual inspection, it can be determined that, a sharper peak leads to better resolution. Upon mathematical calculation (see equation below), if N is less than 1000, resolution is said to be poor and if N ≥ 5000-10, 000, resolution is good.[3,6]

Using the chromatogram from the described experiment (B elutes first, followed by A as shown in Figure 2), the terms can be calculated as follows:

$$k' = (V_X - V_0)/ V_0$$

$$\alpha = k' A/ k' B$$

$$= (V_A - V_0)/ (V_B - V_0).$$

$$N = 16(V_X/ W_X)^2$$

It is also common to tabulate the efficiency as a function of half peak-width. But since this method does not correct satisfactorily for peak tailing, the above convention is preferred.

Chromatography Classification:

There are several factors that control how separation is achieved in chromatography. There are two basic types of liquid phase partition chromatography based on the stationary phase-mobile phase properties.

Normal Phase: In this chromatographic process, the stationary phase is relatively polar (hydrophilic) compared to the mobile phase (which is apolar or hydrophobic).

Reverse-Phase: The stationary phase is hydrophobic (apolar) when compared to the mobile phase (polar, hydrophilic).

Besides the two basic classifications, there is yet another characterization for a whole different class of stationary phases- **Chiral Stationary Phases (CSPs)**. Such phases use the property of chiral recognition to distinguish between two structurally similar components (enantiomers) in the mobile phase. Examples include enantiomers of drugs like thalidomide, naproxen.

Chirality:

The word originates from the Greek word "cheiro", which refers to hand or handedness. The property where an object and its mirror image are non-superimposable, is defined as chirality.[7]

Importance of Chirality:

All life processes are inherently chiral and therefore specific in structure and function. A case in point is the synthesis of only L- or laevorotatory amino acids in the human body, which makes proteins and enzymes chiral because their synthesis involves only one isomer. This feature makes it impossible for the other amino acid isomer (D-form or

dextrorotatory) to be acceptable in the recognition mechanism during precisely controlled, structurally specific enzyme-receptor interactions. This very feature of chirality's has simplified the endless metabolic choices made in any living system. Other chiral molecules include carbohydrates and nucleosides. Chiral compounds are in many products in the pharmaceutical, food, petrochemical and organic chemicals industries.

Although Louis Pasteur in 1848 manually separated the optical isomers of sodium ammonium tartrate crystals (thus, first describing molecular asymmetry), it was Vant Hoff and Le Bel who first proposed the *idea of chirality.*[8] It was their theory that described the chiral carbon atom as having a tetrahedral arrangement with bonds directed towards the four corners occupied by different moieties.[7,8] Enantiomers can be differentiated by the spatial arrangement of moieties around a chiral center. They are stereoisomers that differ in their property to rotate plane-polarized light in the right, dextro- D (+) direction or the left, laevo- L (-) direction. A general method for calculating the number of possible enantiomers of a chiral molecule is done using the formula $\mathbf{2^n}$, where n is the number of chiral centers. The use of D and L terminology is common in carbohydrate chemistry. However, the Cahn-Ingold-Prelog designated nomenclature is prevalent for all other purposes. They have chosen to name the enantiomers as the R (rectus) or the S (sinister), depending on the arrangement of moieties around the stereogenic (chiral) center. Rules have been laid out for what constitutes an R form or an S form.[9]

An equimolar mixture of two enantiomers is referred to as a racemic mixture. Such compounds are structurally similar and resolving them needed a different approach than

conventional HPLC, which was used for predominantly achiral resolution on achiral phases. The concept of chiral recognition was then introduced into the resolution process.

Stationary phases which have the element of chirality incorporated into them to aid in the resolution process were called **Chiral Stationary Phases (CSP)**. Such phases have a chiral selector, which preferentially interacts with a particular enantiomer in a racemic mixture, thus aiding in the process of **selective enantiodiscrimination** and therefore resolution. Given that CSPs could resolve enantiomers, Dalgleish (1952) proposed a theory for chiral recognition and resolution. It was called the **three-point interaction theory** and is still used in explaining the dynamics in the mode of interaction of enantiomers with the chiral selector on a CSP.[10]

He proposed that, in a chromatographic process involving a CSP and a mobile phase containing enantiomers; effective resolution depended on the kind of interaction between the CSP and the analyte. Chiral recognition and enantiomeric resolution takes place when three "specific, simultaneous and discrete interactions" take place between one of the enantiomers and the chiral selector on the CSP.[10] However for *resolution* to occur, one of these interactions had to be stereochemically dependent. In a chromatographic situation, the mobile phase containing the enantiomers would be passed over the stationary phase. The success of these interactions, especially the key "steric" interaction, would depend upon the orientation between the enantiomers and chiral selector in the stationary phase. This process would lead to the formation of a transient, but relatively stable diastereomeric complex with one enantiomer. Since the other

enantiomer could not experience this identical interaction with the CSP, it would be eluted out first. These interactions could include attractive, repulsive, hydrogen bonding, and pi-pi interactions. The various types of interaction affect the complex formation and further, resolution. This principle is demonstrated in the figure below, where A, B, and C are the CSP sites and A′, B′, and C′ are the enantiomer complementary sites. Enantiomer I undergoes the 3-point interaction, while II does not. So II will be eluted first.[11]

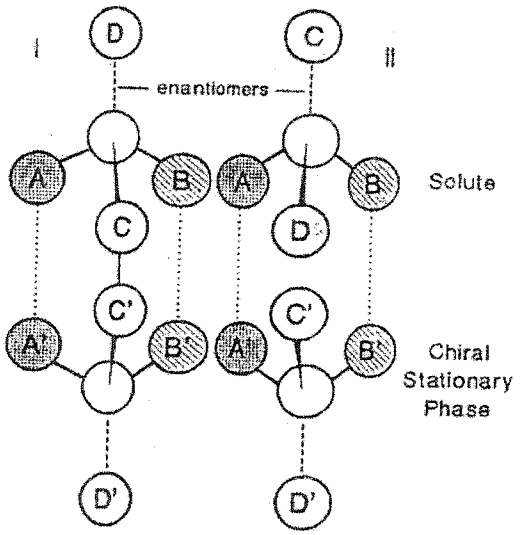


Figure 3. Illustration of the "3-point interaction" model. (Copyright 1984 Aster Publishing Corporation). [11]

The need for better chiral recognition mechanisms was demonstrated in the 1960's after the thalidomide tragedy. This drug was administered to pregnant women as a racemic mixture, to counter the effects of morning sickness and to induce sleep. What

was not known then was that drug molecules were capable of passing through the placenta into the fetus. The result was horrific birth defects, which were later traced back to thalidomide. It was eventually discovered that the S- form is teratogenic and the R-form is safe for use. However, since the drug inter-converts *in vivo*, its use has been stopped. This incident highlighted the need and requirement that drugs be tested for pharmacological effects in both their R+ (left) and S- (right) forms.[12]

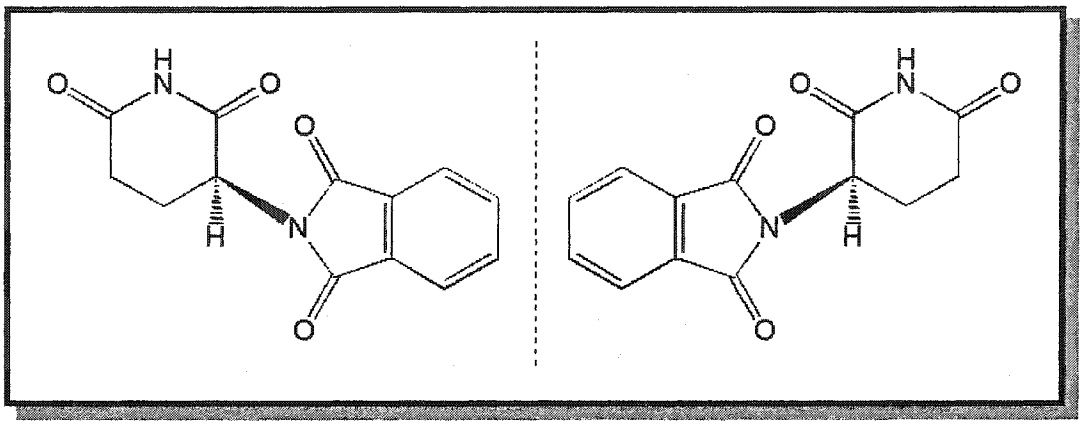


Figure 4. Optical isomers of thalidomide (Reprinted with permission).[12]

Not only did the thalidomide tragedy reinforce a need for improving enantioseparation, it has since become common practice for large pharmaceutical companies to maintain large libraries of all compounds synthesized and test each enantiomers independently for pharmacological effects. This data is then reviewed frequently depending on the drug's use.

CHIRAL STATIONARY PHASES – A REVIEW

CSPs can be classified based on the types of interactions between the solute and the chiral selector on the phase (Refer Table 1).

Classification	Interaction
Type I	Attractive interactions, hydrogen bonding, π- π interactions and dipole interactions.
Type II	Attractive interactions and inclusion complex formation.
Type III	Retention by formation of inclusion complexes within chiral cavities.
Type IV	Ligand exchange mechanisms with metal (chiral additives) complexes.
Type V	Hydrophobic and polar interaction with bound protein phases.

Table 1. Classification of chiral stationary phases based on interactions.[7]

Once the three-point interaction theory by Dalgleish became accepted as the model for explaining chiral separations, the next step was to synthesize chiral stationary phases. The knowledge of the basic principles of chirality was a starting point in the synthesis of CSPs that would be used to separate enantiomers. William Pirkle designed the very first kind of CSP in 1979. This phase was synthesized by bonding the chiral anthryl alcohol

to silica and it was used to resolve the racemates of a wide variety of aliphatic and aromatic compounds like sulfoxides, lactones and alcohols, to name a few.[13]

Type I Chiral Stationary Phases:

The first phases designed by Pirkle and associates were termed "brush-phases," since it appears like the selector is protruding out of the stationary support.[7,11] These phases were prepared by attaching using a spacer link to attach a chiral moiety to an achiral support like silica. Shown below in Figure 5 is a schematic diagram of such a CSP.

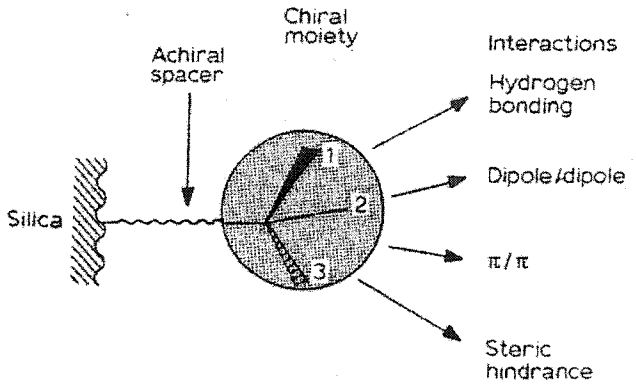


Figure 5. Type I Chiral Stationary Phases and types of interactions which can be involved in interactions.[7]

As can be inferred from Figure 5 and Table 1, these phases can undergo a variety of interactions involving hydrogen bonding, dipole interactions and pi-pi interactions. Amino acids and their derivatives made good chiral selectors because they are inherently chiral in nature. Instances of amino acids and their derivatives attached to a silica support were the 3, 5 dinitrobenzoyl derivatives of phenylalanine and leucine. In terms of low cost

analysis, these phases proved viable because the chiral selectors, were, for the most part, easily procured. Hence large-scale preparation of these phases was not a problem. Drugs were the analytes of choice for separation on these phases. These phases showed excellent resolving capacity within their limits. One major drawback was the frequent need for derivatization of the column. This compromised on efficiency and eventually the life of the column itself.

An example of a Type I column is (R)-N- (3, 5-dinitrobenzylbenzoyl) phenylglycine (covalent), which is a π-acceptor phase.

Type II Chiral Stationary Phases:

While experimenting with naturally available materials that exhibited intrinsic chirality, cellulose was considered as a good starting point. Most biopolymers such as proteins, enzymes and polysaccharides are natural choices because their monomers are themselves chiral. Cellulose and its derivatives were chosen as a starting point for racemic (enantiomeric) resolution. Economically, it was a viable choice because of its abundance in nature. Stereochemically, cellulose proved to be a highly organized polymer with a high degree of stereoselectivity.

Cellulose is an unbranched polysaccharide made of repeating units (>1000) of D-glucose linked end to end via α-1, 4 linkages (Figure 6). Each long cellulose chain is held along with its neighbors by hydrogen bonding facilitated by the hydroxyl groups protruding off the glucose molecule.[13] This bonding helps adjacent chains twist together to form a rope-like configuration (helix) that is capable of forming cavities within. In an

analysis of racemic mixtures, analytes are trapped within these cavities and resolved based on the attractive forces that are encountered inside the cavity.

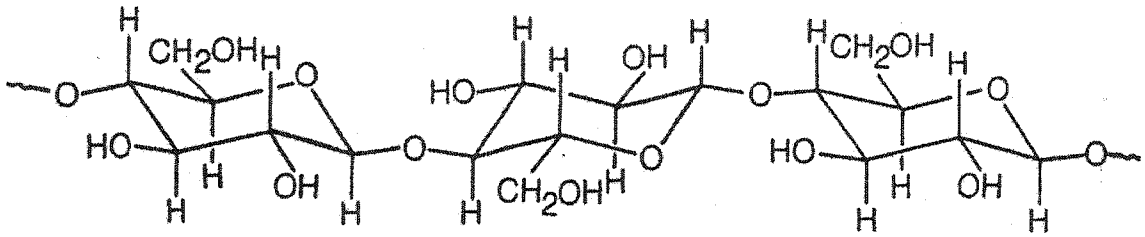


Figure 6. Structure of cellulose. [13]

For chiral HPLC to be viable, a CSP made of cellulose needs to fulfill certain criteria. They constitute mechanical strength, rigidity, an ability to withstand a variety of solvents and concentrations, exhibit specific binding properties (for specific compounds) unique to their helical cavities for higher resolution and be amenable to preparative scale HPLC. As seen with Type I phases, derivatization of the hydroxyl groups of cellulose did not seem to compromise its stereoselectivity or its integrity as a CSP. This property was widely used to synthesize different kinds of cellulose-based phases. Example of one such phase is microcrystalline cellulose triacetate (MCT). The MCT phase showed a refined stereoselection for a variety of racemic compounds particularly those that contained aromatic rings. This would stand to reason because rings would provide a good stereochemical orientation when "trapped" inside a helical cavity. The major disadvantage of cellulose-based phases was their tendency to swell in organic solvents and collapse under high pressures.

Type III Chiral Stationary Phases:

These phases have a mechanism of action wherein the stationary phase has a cavity in which an enantiomer (from a racemic mix) is trapped, while the other isomer elutes out due to its inability to sustain any kind of interaction within the cavity. Classic examples of these phases are cyclodextrin-based phases.

Cyclodextrins (CD) are torus shaped oligosaccharides made up of 6-12 repeating units of D-glucose linked by a α-1, 4 linkages. CDs are named α–, β– or χ- cyclodextrin depending on the number of glucose molecules involved in the linkage. β–CD is the most commercially popular CD. Figure 7 below illustrates the cross-sectional view of a β- cyclodextrin molecule.[14]

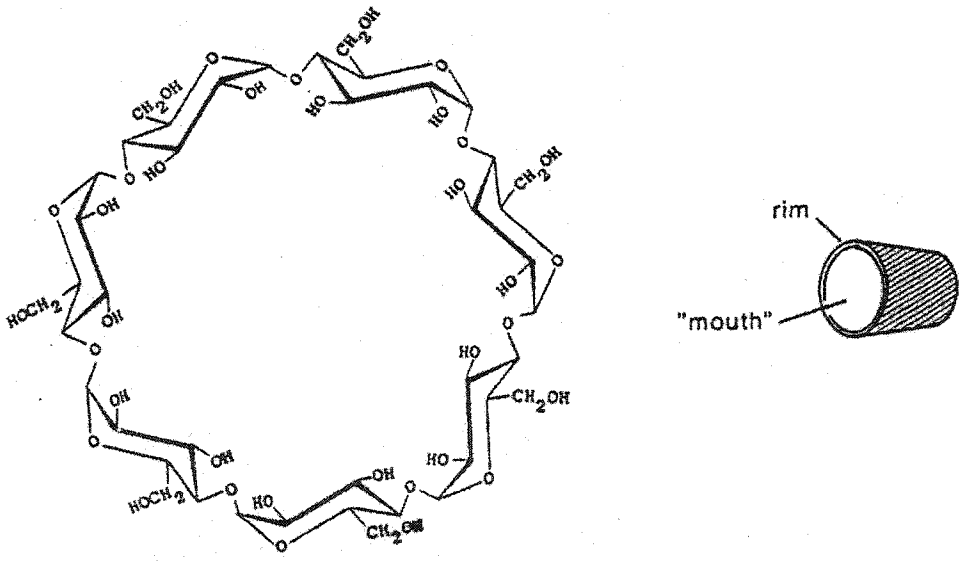


Figure 7. The chemical structure of β-cyclodextrin molecule and its assumed conformation in an aqueous solution. [14]

A side view of a cyclodextrin molecule gives it an appearance of a truncated cone. The interior of the cone is highly hydrophobic and the exterior is hydrophilic owing to

the hydroxyl groups "sticking out." The phases that we have reviewed (I and II) so far operate in the normal phase mode (polar stationary phase, apolar mobile phase). Cyclodextrin is an example of a stationary phase that operates in the reverse-phase mode (apolar stationary phase, polar mobile phase). The chirality of this molecule arises from the chiral centers contributed by the arrangement of individual glucose molecules within the "cone." This configuration is ideal for the resolution of bulky drug enantiomers. Figure 8 demonstrates the geometry of a β–CD molecule.[13]

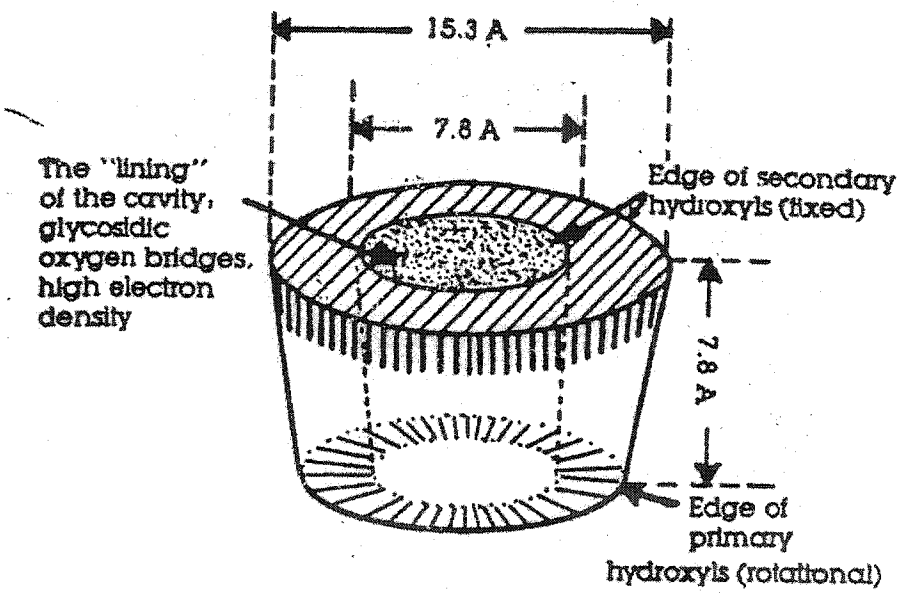


Figure 8. Geometry of beta-cyclodextrin. From *Cyclobond Handbook.* [13]

The CD molecule is covalently linked to a silica support. The mechanism of action of a CD molecule can be explained by a "guest-host" analogy. The interior of the cone acts as the "host" area. When a mixture of racemes to be analyzed is passed through a chiral column containing CD as the immobilized phase, the "guest" enantiomer gets "trapped" inside the cavity because it undergoes attractive or repulsive interactions depending on the environment inside the cavity. The enantiomer unable to undergo these interactions elutes out first. Examples of inclusion complex interactions include hydrogen bonding and electrostatic interactions (Figure 9).[13]

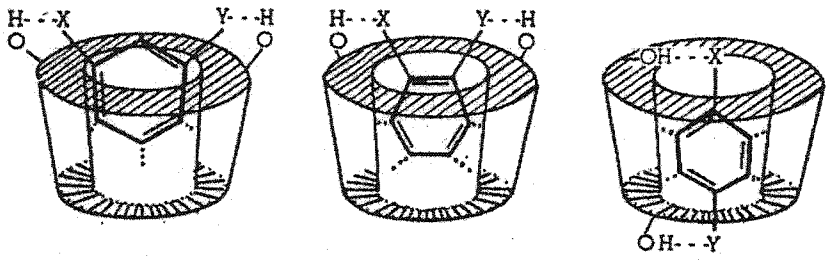


Figure 9. Inclusion complex schematic. From *Cyclobond Handbook.*[13]
-----Hydrogen bonding
.....Electrostatic association
X **Hydrophobic groups that can enter cavity**
Y **Polar groups that can hydrogen bond**

Drugs of different kinds (antihistamines, anti-inflammatory, steroids) can be resolved on these columns. These columns can be operated only at pHs above 3.0, below which they degrade. On the other hand, they are easy to maintain and regenerate, which makes them a preferred stationary phase. The resolving capacity of the stationary phase over time is fairly reliable.

Type IV Chiral Stationary Phases:

Karger and his coworkers first used the technique of CLEC (Chiral Ligand Exchange Chromatography) while Davankov and his team perfected the technique later on.[14] An amino acid-metal ion complex is immobilized on a solid support to form these columns. Ions commonly used are copper, cobalt and zinc. The mixture to be resolved is passed through this column.

The enantiomer forms a diastereomeric chelation complex with the metal ion (Figure 10). Water is added to add an element of steric hindrance. The enantiomer that does not undergo chelation is eluted out first. The mobile phase also contains the metal ions complexed to the amino acid to replenish any metal ion loss during resolution. These columns find most application for resolution of enantiomers of amino acid derivatives.[9]

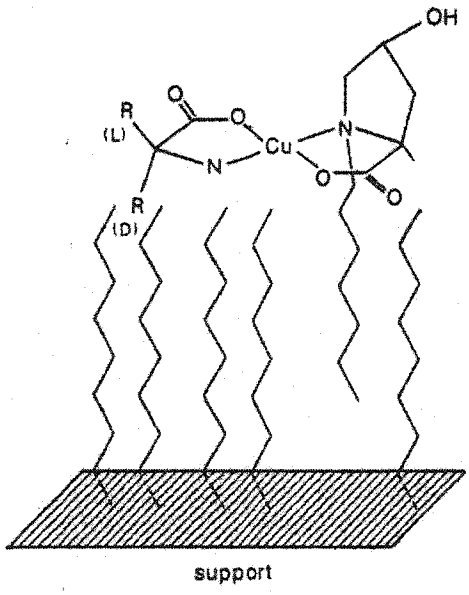


Figure 10. Structures proposed for the mixed-ligand sorption complexes. [14]

Type V Chiral Stationary Phases:

The human body (or any living organism) is a typical example of a chiral system. All metabolic reactions that take place inside the organism are driven and controlled by the chirality of the factors involved- sugars, amino acids, proteins and enzymes and their substrates. Most of the pharmacological interactions of drugs with their receptors are chiral. With this in mind, it was natural that proteins were thought of as being a good choice for enantioseparations. Proteins immobilized to a solid support acts as a chiral selector in these phases. These are one of the most popular kinds of CSPs in use today. Proteins are instinctive choices for some of the following reasons:

- Amino acids are chiral, so a protein (amino acid polymer) is also inherently chiral.
- Conformational change occurs when pH is varied, which is a useful factor in stereoselectivity.
- Addition of mobile phase modifiers into a stationary phase containing a protein as a chiral selector also induces conformational changes, which alter the enantioselectivity of the stationary phase.

Some of the common proteins that are commonly used as chiral selectors are BSA (bovine serum albumin), human serum albumin, α_1-AGP (human alpha 1 acid glycoprotein) or orosomucoid. BSA was one of the first to be used; however, α_1-AGP is the most widely used in industry today. For proteins to become chiral selectors, they

have to be first immobilized on a solid support like silica. Cross-linking is one such method of immobilization.

Proteins are able to undergo a variety of interactions with enantiomeric isomers like electrostatic, hydrophobic ones. Separation on protein phases is carried out in the reverse phase mode. Conformational changes can be brought about in a protein by changing one or more of available mobile phase parameters like pH, concentration of the modifier or the ionic strength. These changes can be tailored to direct the enantioseparation.[15] A disadvantage with certain types of protein phases is inability to withstand extreme stress (temperature, solvent concentration). Figure 11 below represents a general mechanism of interactions between an immobilized protein and an enantiomer in the mobile phase.

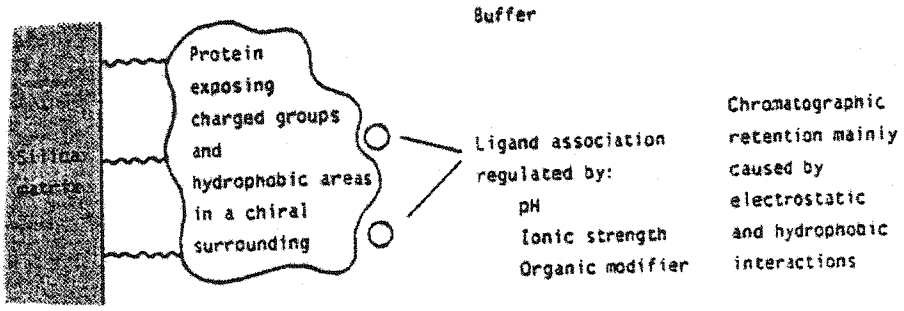


Figure 11. Basic types of interaction between a ligand analyte and an immobilized protein in aqueous media.[15]

Human α_1- acid glycoprotein (Human AGP):

One protein that is most preferred over others of the same genre is the human α_1- acid glycoprotein (AGP). This serum glycoprotein is found in abundance in human plasma

and is involved in non-specific binding of drugs in the system, which makes it a popular choice as a chiral selector. AGP is a 181 amino acid polypeptide, acidic in nature and has an isoelectric point at 2.7. It is known to have one major drug-binding site. Immobilizing it on a silica support can make a chiral stationary phase using this protein. It has the advantage of tolerating organic solvents, high temperatures and wide pH ranges. This phase acts in the reverse-phase mode. Changing the pH or the concentration of mobile phase modifiers changes the drug-binding properties of the protein. This property has been used to study the effectiveness of AGP as a chiral selector, first done in 1983. The first generation column was called Enantiopac and the second generation is called Chiral-AGP.[16] Examples of drug enantiomers resolved on AGP-phases are ibuprofen, fenobarbitol and naproxen. Figure 12 is a schematic representation of human α_1- acid glycoprotein (AGP).[9]

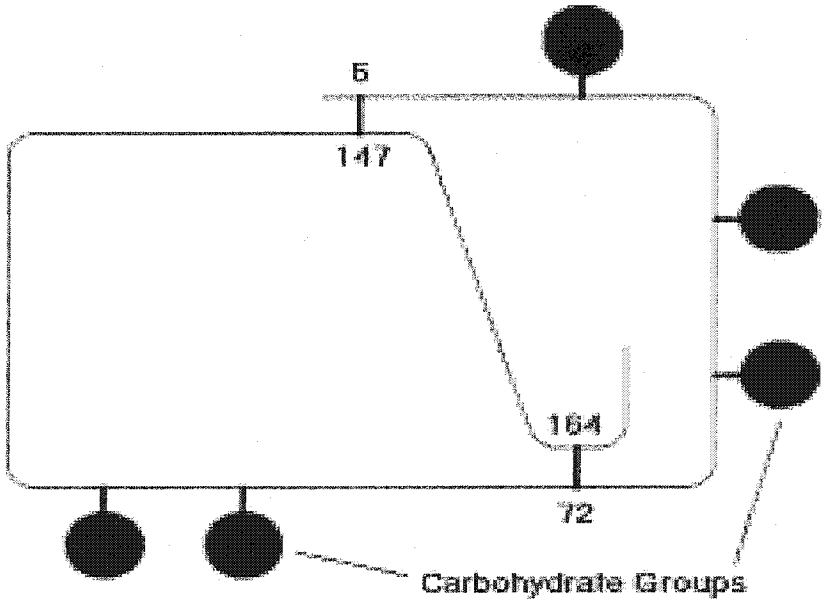


Figure 12. Schematic representation of AGP (Reprinted with permission).[9]

Multiple-Interaction Center Chiral Stationary Phases:

All phases mentioned above have predominantly single-interaction centers. These worked for a variety of enantioseparations. Once this technique was perfected, the search was on for more refined methodologies of chiral selection. This quest led to the proposal of the theory of a stationary phase with multiple-interaction centers. The rationale used was that the more the number of chiral selectors (centers), the more opportunity there would be for specific, simultaneous yet discrete interactions. If the above three parameters could be assumed to happen, then it was only natural to conclude that the degree of enantiodiscrimination would consequently be better defined. To this end, the idea of chiral phases with multiple-interaction centers evolved rapidly.[13]

Many laboratories favor *in-situ* preparation of multiple-interaction CSPs because of the ease and economy involved. Although the concept of a multiple-interaction center stationary phase was full of possibilities, a balance between theory and feasibility needed to be maintained. While deciding on starting material for the synthesis of such phases, it was intuitive that chiral moieties that demonstrated a high degree of resolution would be a logical place to start. Two choices that presented themselves and were taken into account before synthesizing such phases were:

1. Amino acids, natural or synthetic (with a high degree of enantiomeric purity) seem to be the primary choice as starting materials. The fact that they can usually be used "as is" without any prior racemization (object changing to its mirror image formation) lends added credence.

2. Racemic materials (or material that require prior racemization) also were considered as building blocks for CSPs and were not excluded merely because of their "modified" status.

The chemistry for the preparation of a multiple-interaction center CSP most often involved attachment of the chiral component to silica. The abundant availability of n-aminopropylsilanized silica made this commercially viable. Six significant research attempts have been reviewed in an attempt to understand the role played by multiple chiral centers in enhancing enantioseparation.

The work of Naobumi Oi and Kitahara in this area has been of significant interest because they focused primarily on CSPs with multiple interaction centers. Their pioneering work was on the separation of optical isomers by gas chromatography. Work was started on the premise that an extra chiral center would improve resolution. The success they had with this experiment led them to try the same principle with a variety of phases in liquid chromatography. Of all the works discussed here, this particular group has been prolific in their work with multiple-interaction center CSPs.[17-20]

M.J.B Lloyd based his work on previous experiments done by Pirkle and associates who found that chiral amide bonded phases had a wide range of applications. Pirkle's work involved synthesizing and comparing two diastereomeric phases and two enantiomeric phases based on (α)-1-naphthylethylamine. Amino acid enantiomers were separated on these phases.[21]

Curt Pettersson and Carlo Gioeli used acetyl quinine bonded to silica (by silanization) as their CSP, where acetyl quinine acts as the chiral selector. This phase has been used in

the reverse-phase mode for the separation of amino acids and carboxylic acid derivatives. According to them, the reversed-phase system had advantages in bioanalysis over the normal phase because it involved direct injection of aqueous samples.[22]

Anne-Françoise Aubry et al used α_1-acid glycoprotein (AGP) immobilized on silica as a chiral selector. This phase was used to separate three anti-malarial drugs-chloroquine, enpiroline and mefloquine. They have investigated the properties (retention and selectivity) of the phase upon changing the pH and the addition of a modifier dimethyloctylamine (DMOA). The effect on two of the three drugs, chloroquine and enpiroline led them to conclude the involvement of a multiple-site mechanism in which both competitive and allosteric interactions were involved. [23]

SYNTHESIS AND APPLICATIONS OF MULTIPLE-INTERACTION CENTER CHIRAL STATIONARY PHASES (CSPs)

N- (1*R*, 3*R*) - *trans*- CHRYSANTHEMOYL (R)-1-(α-NAPHTHYL) ETHYLAMINE AS A STATIONARY PHASE FOR THE SEPARATION OF OPTICAL ISOMERS BY GAS CHROMATOGRAPHY.[17]

This paper represents one of the first works of the more prolific team of researchers in this area-Naobumi Oi and Hajimu Kitahara. It had been already established that gas chromatography carried out with a chiral liquid phase improved the selectivity for combinations of optical isomers of certain classes of compounds. It was decided to test this theory by incorporating a second chiral selector to understand the degree to which this would influence selectivity of an enantiomeric mixture.

The enantioselectivity of three kinds of phases for a variety of mixtures of compounds were studied. Examples of tested compounds included racemic mixtures of nitriles, amines, amino acid propyl esters, amides and other kinds of compounds (Refer to Table 2). The formulae of the three phases are as outlined below:

Phase I: N- (1*R*, 3*R*)-*trans*-chrysanthemoyl (*R*)-1-(α-naphthyl) ethylamine (contains two chiral centers, with asymmetric carbon atoms attached to both the nitrogen and carbon atoms of the amide group).

Phase II: N- (1*R*, 3*R*)-*trans*-chrysanthemoyl laurylamine.

Phase III: N- lauroyl (*R*)-1-(α-naphthyl) ethylamine.

This work is considered one of the first in the attempt to separate nitriles. The figures below (13, 14, and 15) demonstrate the separation capabilities of phase I for various enantiomeric mixtures.

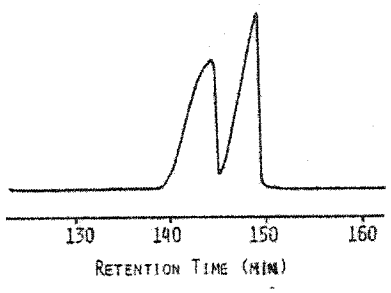


Figure 13. Gas chromatogram of racemic 2- (4-chlorophenyl) isovaleronitrile. Column: glass capillary collumn (40m x 0.25mm I.D.) coated with N-(1R, 3R)- trans-chrysanthemoyl (R)-1-(α-naphthyl) ethylamine. Temperature: 80°C. Carrier gas (helium) flow rate: 1.0 mL/min.[17]

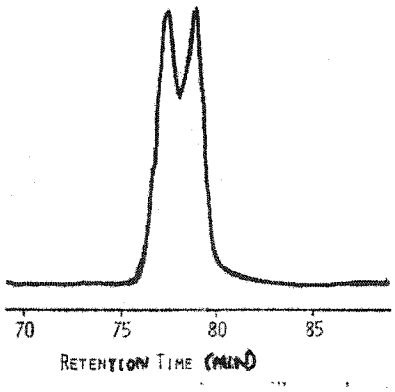


Figure 14. Gas chromatogram of racemic ethyl cis-chrysanthemate. Chromatographic conditions as in Figure 13.[17]

	Phase I	Phase II	Phase III
	α**	α**	α**
Amines			
2-Octyl amine***	1.048	1.000	1.060
1-Phenyl ethylamine§	1.167	1.009	1.146
Amino acid propyl esters***			
Alanine	1.063	1.011	1.031
Valine	1.059	1.019	1.044
Leucine	1.044	1.022	1.000
Carboxylic tert.-butyl amides.			
3,3-Dimethyl-2-ethyl butyric acid	1.070	1.025	1.000
2-Bromo-3,3-dimethyl butyric acid	1.152	1.000	1.129
cis-Chrysanthemic acid	1.032	1.000	1.040
trans-Chrysanthemic acid	1.092	1.074	1.000
Carboxylic acid ethyl esters			
cis-Chrysanthemic acid	1.015	1.000	1.000
cis-3-(2,2-Dichlorovinyl)-cyclopropanecarboxylic acid	1.017	1.000	1.000
Nitriles			
2-Phenyl propiononitrile	1.016	1.000	1.000
2-(2-Fluorophenyl)-isovaleronitrile	1.028	1.000	1.000
2-(4-Chlorophenyl)-isovaleronitrile§§	1.033	1.000	1.000
Alcohols			
1-Phenyl ethanol	1.014	1.000	1.000
Pantoyl lactone	1.030	1.000	1.000
Menthol	1.018	1.000	1.000

**Separation factor calculated by second peak/ first peak

§Resolved as N-pentafluoropropionyl derivative
§§Chromatographed at a room temperature of 120°C

Table 2. Gas chromatographic separation of enantiomers: Chromatographed on 40m x 0.25mm I.D. glass capillary columns. Column temperature: 100°C. Carrier gas; helium at 0.6-1.2mL/min. [17]

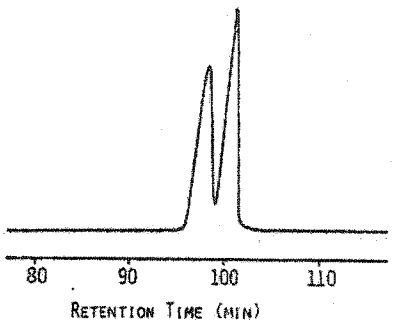


Figure 15. Gas chromatogram of racemic menthol. Chromatographic conditions as in Figure 13. [17]

Results and Discussion:

As defined in Chapter 1, the separation efficiency of a column for a mixture of compounds is characterized by the k′ (retention factor) and α (separation factor). The higher the value of the latter, a better separation is said to have occurred. By examining the chromatograms and their corresponding values in Table 2, the following conclusions can be drawn about the separation capability of Phase I.

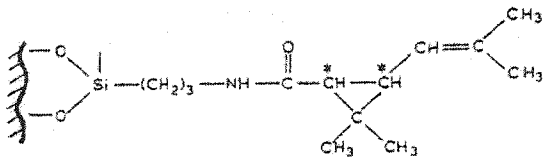
In Figure 13 it can be seen that, the two enantiomers of 2-(4-chlorophenyl)-isovaleronitrile have been well resolved by Phase I, while phases II and III could not do so (α values in both cases, 1.000, Table 2). Figure 14 shows that although there seems to be poorer resolution in the peaks of ethyl *cis*-chrysanthemate by Phase I, the separation is still better defined than Phases II and III (α values in both cases, 1.000, Table 2). Figure 15 shows the chromatogram for the resolution of menthol. Phase I gave a good resolution of both enantiomers, whereas Phases II and III were unable to resolve satisfactorily (racemic menthol, α values in both cases, 1.000, Table 2).

A closer study of all the separation factors of Phases II and III with respect to separation of the various racemic mixtures of the enantiomers shows that whereas these two gave α values of 1.000 (indicating poor resolution or no resolution), Phase I gave a better degree of enantioseparation. One factor that may have contributed to this result could be steric hindrance due to the extra chiral center. The presence of this center would enhance the stereomeric dynamics between stationary phase and the functional groups in the analyte, thus contributing to a better resolution. Phase I has not resolved certain racemic mixes (eg. ethyl *cis*-chrysanthemate), when compared to the other two phases, but it certainly seems that the presence of the extra chiral center was a major contributing factor in the enhanced resolving capacity.

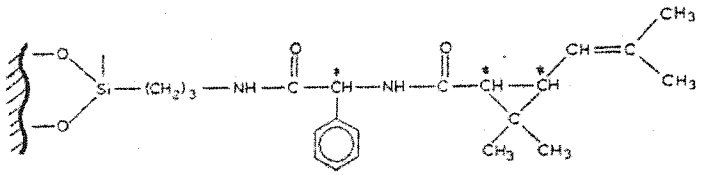
HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS ON (1R, 3R)-*trans*-CHRYSANTHEMIC ACID AND ITS DERIVATIVES BONDED TO SILICA GEL.[18]

The previous review (based on gas chromatography) was based on the presence of an extra chiral center in a stationary phase and its effect on the separation properties of the phase on enantiomeric mixtures. The same group decided to apply the principle (extra chiral center) to liquid chromatography with N- (1R, 3R) - *trans*-chrysanthemoyl-R-1-(α-naphthyl) ethylamine, which contains two asymmetric carbon atoms attached to both nitrogen and carbon atoms of the amide group. Three novel phases (named I, II, and III) were synthesized using (1R, 3R) - *trans*-chrysanthemic acid. The amide derivatives of

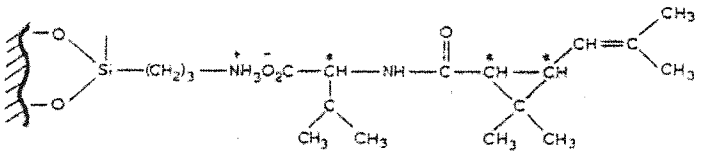
these phases were chemically attached to silica gel and their degrees of separation were tested for various enantiomeric mixtures. The mobile phases used were n-hexane-isopropanol and n-hexane-1, 2-dichloroethane-ethanol mixtures. The structures of the three phases are shown in Figure 16.



(I)



(II)



(III)

Figure 16. Structures for phases I, II, and III. [18]

The solutes that had to be separated were derivatized prior to use and were used as N-acetyl esters and N-3, 5, dinitrobenzoyl (DNB) esters. In Phase II, D-phenyl glycine was used to enhance chirality whereas in Phase III, L-valine was used. Table 3 shows the α and k' values for all three phases for the racemic mixtures.

Compound	Phase I			Phase II			Phase III		
Amines:	α*	*k'***	MP***	α*	*k'***	MP***	α*	*k'***	MP***
N-Acetyl-1-phenylethylamine	1.05	4.22(R)	4	1.08	6.02(R)	4	1.00	4.32	4
N-Acetyl-1-(□-naphthyl)ethylamine	1.00	3.69	4	1.10	4.63(R)	4	1.00	3.72	4
N-3,5-Dinitrobenzoyl-1-(□naphthylethylamine)ethylamine	1.07	1.65(S)	4	1.07	8.21(R)	2	1.19	5.05(R)	4
N-3,5-Dinitrobenzoyl-1-phenyl2-(4-tolyl)ethylamine	1.00	1.19	4	1.15	5.75(R)	2	1.14	3.92(R)	4
N-3,5-Dinitrobenzoyl-*sec.*-butylamine	1.00	2.02	4	1.12	4.24(R)	4	1.00	4.85	4
N-3,5-Dinitrobenzoyl-2-octylamine	1.10	0.92	4	1.30	2.00	4	1.10	2.92	4
Amino acids:									
N-Acetylalanine methyl ester	1.00	5.50	4	1.00	5.82	4	1.06	5.55(D)	4
N-3,5-Dinitrobenzoylalanine methyl ester	1.00	2.38	4	1.91	6.81(D)	4	2.26	4.28(D)	4
N-3,5-Dinitrobenzoylalanine *n*-butylamide	1.04	2.11(L)	4	3.21	1.78(D)	5	2.02	2.04(D)	5
N-Acetylvaline methyl ester	1.00	2.14	4	1.07	2.53(L)	4	1.05	2.68(D)	4
N-3,5-Dinitrobenzoylvaline methyl ester	1.00	0.69	4	2.59	1.91(D)	4	1.90	1.54(D)	4
N-3,5-Dinitrobenzoylvaline *n*-butylamide	1.00	0.44	4	4.00	0.62(D)	5	1.75	0.56(D)	5
N-Acetylphenylalanine methyl ester	1.00	1.50	4	1.15	2.44(L)	4	1.00	3.00	4
N-3,5-Dinitrobenzoylphenylalanine methyl ester	1.00	1.04	4	1.96	3.42(D)	4	1.78	2.62(D)	4
N-3,5-Dinitrobenzoylphenylalanine *n*-butylamide	1.00	0.49	4	3.35	0.99	5	2.11	0.76	5
Carboxylic acids									
1-Methylphenylacetic acid isopropylamide	1.03	5.00(S)	1	1.00	6.00	1	1.00	4.65	1
1-Isopropyl-(4-chlorophenyl)acetc acid *tert.*butylamide	1.19	.84(S)	1	1.00	1.09	1	1.25	1.08(S)	1
1-Methylphenylacetic acid 3,5-dinitroanilide	1.09	4.08(R)	4	1.07	8.33(R)	4	1.16	10.75(S)	4
1-Isopropyl-(4-chlorophenyl)acetc acid 3,5-dinitroanilide	1.07	4.26(S)	4	1.00	7.41	4	1.16	18.48(S)	4
1-Bromo-2,2-dimethyl butyric acid 3,5-dinitroanilide	1.06	4.43(S)	4	1.25	6.25(R)	4	1.07	13.92(S)	4
trans-Chrysanthemic acid 3,5-dinitroanilide	1.07	2.23	4	1.07	3.71(S)	4	1.14	5.99(R)	4
Alcohols									
1-(2,4-Dichlorophenyl)-4,4-dimethyl-2(1,2,4-triazol-1yl)-1-penten-3-ol (S-3308)	1.13	1.04(R)	3	1.50	1.69(R)	3	1.00	3.57	3
1-(4-4-Chlorophenyl)4,4-dimethyl-2(1,2,4-triazol-1-yl)1-penten-3-ol (S-07)	1.11	1.36(R)	3	1.31	2.33(R)	3	1.00	4.26	3

Table 3. High-performance liquid chromatography separation of the enantiomers on chiral stationary phases.[18]

(Chromatographic parameters of Table 3).
***The separation factor of the enantiomers (α) is the ratio of their capacity ratios**

****k' is the capacity factor for the initially eluted enantiomer.**

*****Mobile phases: 1=isopropanol-*n*-hexane (0.5:99.5); 2=isopropanol-*n*-hexane (5:95); 3=*n*-hexane-1, 2-dichloroethane-ethanol (700:90:9); 4=*n*-hexane-1, 2-dichloroethane-ethanol (100:20:1); 5=*n*-hexane-1, 2-dichloroethane-ethanol (48:15:1).**

Results and Discussion:

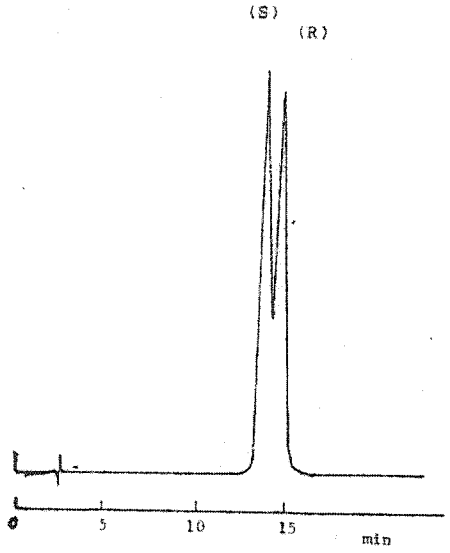


Figure 17. Chromatographic separation of the enantiomers of racemic-1- isopropyl- (4-chlorophenylacetic acid) acetic acid 3, 5-dinitroanilide on chiral stationary phase I. Chromatographic conditions as seen in Table 3. [18]

Figure 17 displays a chromatogram for resolution by Phase I (which has only one chiral center). As can be observed, although two peaks are seen for both R- and S-isomers, they have not been baseline resolved (α= 1.07, and k′(S enantiomer) = 4.26). Comparison of these values for Phases II and III, show that while Phase II did not exhibit any selectivity for this compound, Phase III showed better resolution. This can be

attributed to the role played by the position of the functional group in the solute and the steric effects it might have experienced interacting with the multiple chiral centers present in the stationary phase.

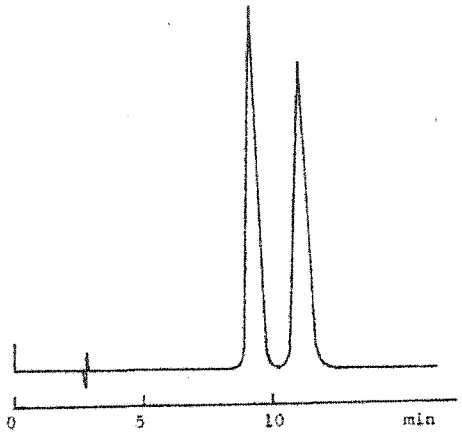


Figure 18. Chromatographic separation of the enantiomers of racemic N-3, 5-dinitrobenzoyl- 2-octylamine on chiral stationary phase III. Chromatographic conditions as in Table 3. [18]

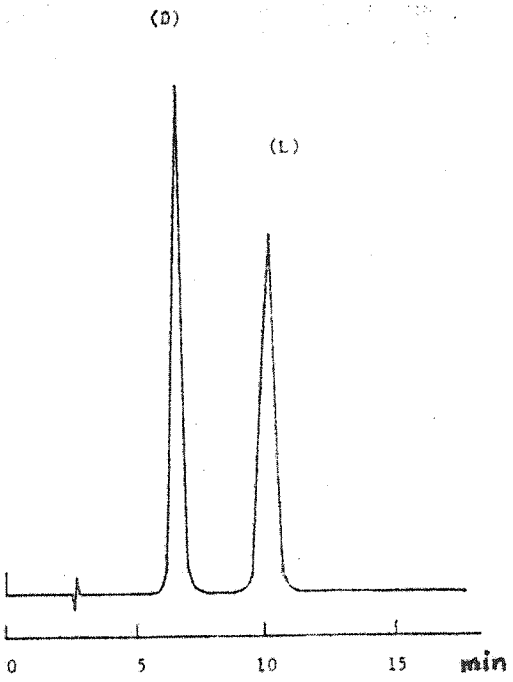
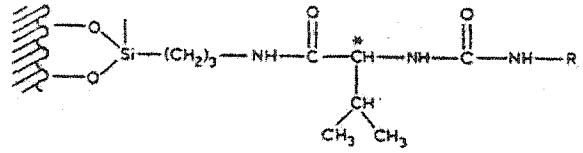


Figure 19. Chromatographic separation of the enantiomers of racemic N-3, 5-dinitrobenzoylvaline methyl ester on chiral stationary phase III. Chromatographic conditions as in Table 3. [18]

Figures 18 and 19, on the other hand, show a distinct separation of enantiomers. It is interesting to note that both of the test compounds in this case were dinitrobenzoyl (DNB) derivatives. The presence of the DNB moiety can play a key role in the steric effects facilitating the interaction dynamics. This leads to the conclusion that chiral separations do indeed, follow Dalgleish's 3-point theory where the degree of enantioseparation is controlled by steric hindrance. The presence of the extra chiral center on both Phases II and III influence the chemistry to an extent that there is a major role played in the formation of diastereomeric complexes with the solutes. Hydrogen bonding and hydrophobic interactions could have contributed in addition to the steric effects. The energy differential affects the order of appearance of the eluted enantiomers, thus enabling a clear resolution and sharper peaks. An examination of the α and k' (Table 3) values shows consistently better values for Phases II and III. These phases showed good separation values for most of the tested compounds although alcohol esters were resolved to a lesser degree by Phase III. In comparison, Phase II showed superior values for both parameters. Notable α values were seen for the separation of fungicide S-3308 isomers. Phases II and III showed overall better performances when compared to Phase I.

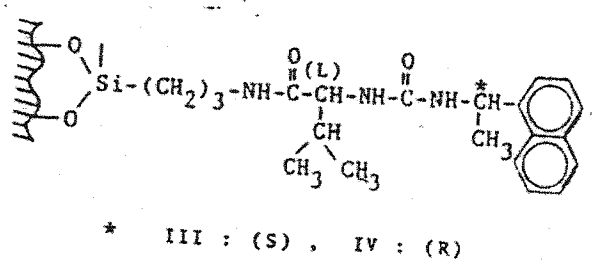
ENANTIOMER SEPARATION BY HPLC WITH SOME UREA DERIVATIVES OF L-VALINE AS NOVEL STATIONARY PHASES.[19-20]

There can be other types of starting material being used for synthesizing CSPs. While considering materials that would make potentially good precursors, it was proposed that amino acids, because of their inherent chirality and abundant availability were intuitive choices. This review deals with separations by novel CSPs prepared by chemically bonding urea derivatives of L-valine to γ-aminopropyl silanized silica (Phases I and II which are, respectively, N- (tertbutylaminocarbonyl)-L-valyl aminopropyl silica gel and (R)-1- (α-naphthyl) ethylaminocarbonyl-aminopropyl silica gel). Here, the asymmetric carbon is attached to the nitrogen of the amide groups. Phases I and II contained a single asymmetric center. This was then improved upon by the addition of a second chiral center derivatizing the R- and S- versions with L-valine and bonding it to silica to obtain Phases III and IV. Different mobile phase combinations were used as illustrated in the table (Table 4). The performance of both phases III and IV in separating various enantiomeric mixtures of amino acid esters, amines, alcohols and miscellaneous compounds was tested and the results discussed after studying the chromatograms. As seen in previous papers, the amino acids were derivatized to their dinitrobenzoyl (DNB) derivatives. One reason this process is carried out is that the DNB group promotes steric effects in the separation process and also act as a participant in potential π-π interactions between the solute and an appropriate chiral center on the stationary phase.



R = (I) isopropyl, (II) *tert.*-butyl, (III) phenyl

Figure 20. Structures of Phases I and II.[19]



* III : (S) , IV : (R)

Figure 21. Structures of Phases III and IV.[20]

Results and Discussion:

The separation capabilities of phases III and IV have been compiled in Table 4. Figures 22 and 23 show sample separation abilities if phases III and IV.

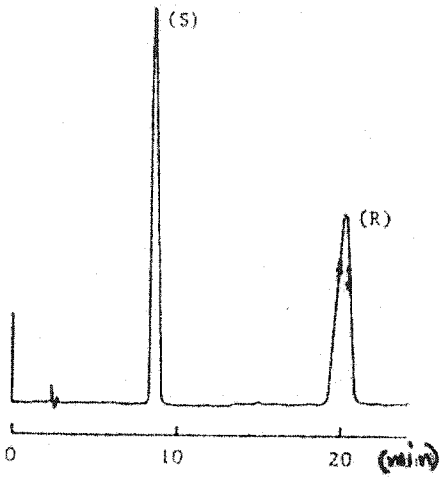


Figure 22. Chromatographic separation of the enantiomers of racemic 2- phenylpropionic acid as 3, 5- dinitroanilide derivatives upon chiral stationary phase III. Chromatographic conditions as in Table 4.[20]

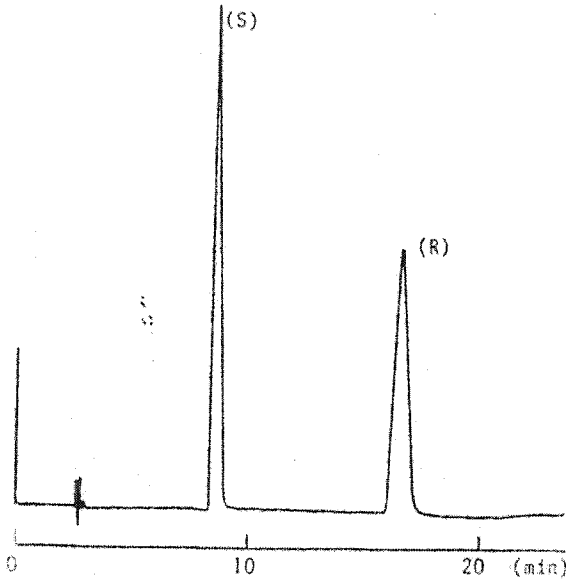


Figure 23. Chromatographic separation of the enantiomers of racemic 1- phenylethylamine as N-3, 5-dinitrobenzoyl derivative upon chiral stationary phase IV. Chromatographic conditions as in Table 4.[20]

Compound	Phase III			Phase IV		
	α	k'1	MP	α	k'1	MP
Amino acids	1.15	3.87	A	1.15	4.87	A
Alanine [a]	1.28	1.8	A	1.32	2.18	A
Valine [a]	1.30	1.65	A	1.52	1.95	A
Leucine [a]	1.86	1.40	C	1.73	2.13	C
Alanine [b]	1.77	0.77	C	1.91	1.20	C
Valine [b]	1.44	0.79	C	1.29	1.66	C
Leucine [b]						
Amines [c]						
1-Phenylethylamine	1.98	1.54	C	2.31	2.44	C
1-(α-Naphthyl)ethylamine	3.94	1.37	C	3.83	2.53	C
2-Octylamine	1.20	2.74	D	1.25	5.00	D
Carboxylic acids [d]						
2-Phenylpropionic acid	2.89	2.56	C	2.32	3.05	C
2-(4-Chlorophenyl)isovaleric acid	2.02	2.10	C	2.50	2.48	C
2-Bromo-3,3-dimethylbutyric acid	2.16	1.78	C	2.00	2.41	C
Alcohols [e]						
1-Phenylethanol	1.54	1.43	C	1.29	1.61	C
1-(α-Naphthyl)ethanol	1.44	1.70	C	1.34	1.88	C
2-Octylalcohol	1.14	2.54	D	1.05	3.36	D
Others [f]						
Fenpropathrin	1.22	6.00	E	1.12	5.35	E
S-3308*	1.16	3.22	E	1.20	2.90	E
S-3307**	1.11	4.23	E	1.15	3.92	E
Allethrolone	1.04	9.59	B	1.09	10.6	B
Propargyllone	1.02	15.7	B	1.07	18.6	B

a) Resolved as N-acetyl O-methylester derivatives

b) Resolved as N-3,5-dinitrobenzoyl O-methylester derivatives

c) Resolved as N-3,5-dinitrobenzoyl derivatives

d) Resolved as N-3,5-dinitroanilide derivatives

e) Resolved as 3,5-dinitrophenyl urethane derivatives

f) Resolved directly

* 1-(2,4-Dichlorophenyl)-4,4-dimethyl 1-2-(1,2,4-triazol-1-yl)-1-penten-3-ol

** 1-(4-Chlorophenyl)-4,4-dimethyl 1-2-(1,2,4-triazol-1-yl)-1-penten-3-ol

Table 4. HPLC Separation of enantiomers upon chiral stationary phases. [20]

The separation factor of the enantiomers, α, is the ratio of their capacity factors, k'. k'_1 is the capacity factor for the initially eluted enantiomer.
Mobile phases (MP):
A= n-hexane-isopropanol (93:7);
B= n-hexane-isopropanol (39:1);
C= n-hexane-dichloromethane-ethanol (15:4:1);
D=n-hexane-dichloromethane-ethanol (50:10:1);
E=-hexane-dichloromethane-ethanol (100:20:1);
F= n-hexane-dichloromethane (500:1).
Flow rates of 1.0mL/ min were typically used.

From Table 4, it can be observed that both phases show consistently superior values of α for all classes of separated compounds, especially for carboxylic acid mixtures. Figure 22 shows the efficiency of Phase III in resolving the 3, 5-dinitroanilide derivative of a carboxylic acid. The peaks are sharp, well separated and baseline resolved. Similarly, Figure 23 shows the separation characteristics of Phase IV for racemic 1-phenylethylamine. Both these solutes have a bulky aromatic group on them and both chiral phases have a second chiral center being contributed by the urea moiety. The former would undergo pi-pi interactions with the latter, thus contributing to the enhanced enantioseparation. Another factor that could play a role in resolution would be the potential for hydrogen bonding between the amide group (on the CSP) and the solute.

The α and k′ values for separation of the same class of compounds by Phases III and IV were compared to those of Phases II and I and were found to be better. This can be attributed to the increased separation capability rendered by the second chiral constituent.

PREPARATION AND EVALUATION OF NEW CHIRAL STATIONARY PHASES FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION OF ENANTIOMERS.

COMPARISON OF ENANTIOMERIC AND DIASTEREOMERIC PHASES BASED ON (α)-1-NAPHTHYLETHYLAMINE FOR THE RESOLUTION OF DERIVATIVES OF AMINO ACID ENANTIOMERS.[21]

This review examines three different kinds of phases (two diastereomeric) and one enantiomeric phase based on R-phenylglycyl-R/ S- (α)-1-naphthylethylamide and glycyl-S-(α)-1-naphthylethylamide respectively. They were evaluated for their separation capacity of amino acid esters under different mobile phase combinations. These phases will be referred to as I, II, and III.

Phase I: based on R-phenylglycyl S-(α) naphthylethylamide

Phase II: based on R-phenylglycyl R-(α) naphthylethylamide

Phase III: based on glycyl-S-(α) naphthylethylamide.

Phase III is a single enantiomer phase and I and II are diastereomeric (Figure 24).

The mobile phase combinations used were dichloromethane-hexane and isopropanol-hexane. The compounds resolved were 3, 5-DNB derivatives of amino acids. Apart from being a potential pi-pi interaction facilitator, the DNB derivative has a high molar extinction coefficient (in the UV spectrum), which makes it a viable choice for spectroscopy studies and enabling trace quantity detection of samples. The performance of Phases I and II (two chiral centers) have been compared with respect to separation capacity of Phase III (single center phase).

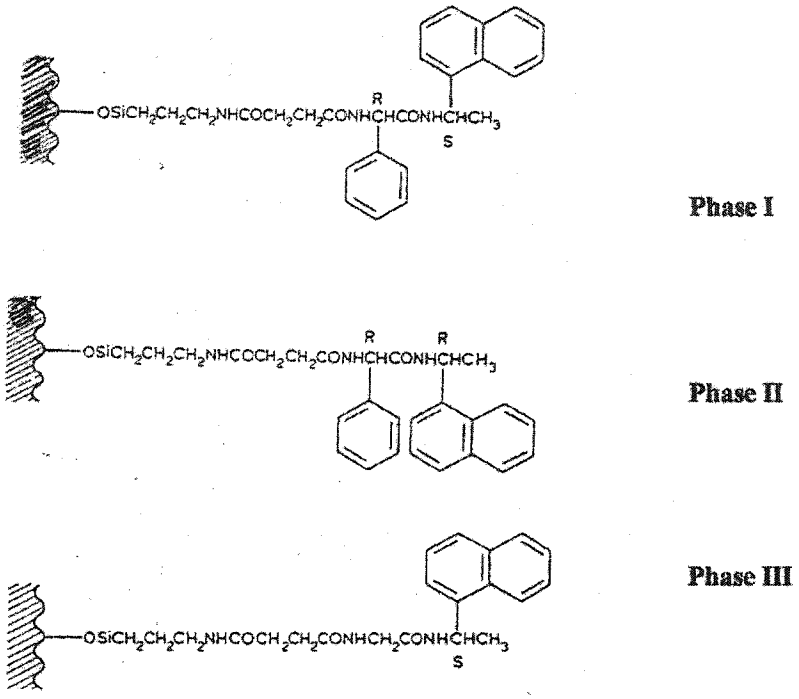


Figure 24. Three new chiral phases based on (α)-1-naphthylethylamine.[21]

Amino acid der.	Phase I				Phase II				Phase III			
	k'	*α*	*MP***	*R/S****	*k'*	*α*	*MP***	*R/S****	*k'*	*α*	*MP***	*R/S****
Val	0.67	1.85	A	*R*	0.71	1	A	-	0.59	1.35	A	*R*
Asp	1.04	1.27	A	*R*	1.12	1	A	-	1.15	1.14	A	*R*
Ala	1.57	1.32	A	*R*	1.69	1	A	-	1.57	1.2	A	*R*
Phe-Ala	1.11	1.38	A	*R*	1.05	1	A	-	0.95	1.2	A	*R*
Met	1.22	1.6	A	*R*	1.39	1	A	-	1.23	1.43	A	*R*
Pro	1.6	1	B	-	18.34	1	C	-				

Table 5. Comparison of chiral Phases I, II and III using dichloromethane-hexane mobile phase.[21]

Amino acid der.	Phase I				Phase II				Phase III			
	k'	*α*	*MP***	*R/S****	*k'*	*α*	*MP***	*R/S****	*k'*	*α*	*MP***	*R/S****
Val	5.49	2.03	A	*R*	4.26	1.79	A	*S*	4.05	1.89	A	*R*
Asp	14.77	1.2	A	-	13.61	1.19	A	-	13.09	1.22	A	-
Ala	8.35	1.54	A	*R*	7.23	1.54	A	*S*	6.73	1.52	A	*R*
Phe-Ala	13..4	1.26	A	*R*	9.5	1.54	A	*S*	9.24	1.4	A	*R*
Met	4.55	1.58	B	*R*	3.75	1.59	B	*S*	3.55	1.7	B	*R*
Ser	11.41	1.31	B	*R*	11.46	1.28	B	*S*	9.89	1.24	B	*R*
Threo	6.07	1.4	B	*R*	6.15	1.24	B	*S*	5.58	1.33	B	*R*
Tyr	12.11	1.47	C	*R*	11.77	1.25	C	*S*	9.39	1.39	C	*R*
Trp	10.46	1.19	C	*R*	8.6	1.17	C	*S*	7.26	1.33	C	*R*
Iso-his	9.21	1.33	A	*R*	9.72	1.42	A	*S*	8.31	1.32	A	*R*
Pro	4.01	1	A	-	4.08	1	A	-	3.6	1	A	-

Table 6. Comparison of chiral Phases I, II and III using isopropanol-hexane phase.[21]

Mobile Phase legends:

Table 5:

A= dichloromethane-hexane (80:20) @ 1mL/ minute.

B= dichloromethane-hexane (20:80) @ 1mL/ minute.

C= dichloromethane- hexane (40:60) @ 2mL/ minute.

Table 6:

A= isopropanol-hexane (10:90) @ 2mL/ minute.

B= isopropanol -hexane (20:80) @ 2mL/ minute.

C= isopropanol - hexane (30:70) @ 2mL/ minute.

The mechanism involved in separation may include hydrogen bonding between the amide group and the carboxyl group. Hydrophobic and pi-pi interactions between the

phenyl group and the dinitrobenzoyl groups may also affect the degree of interaction between the solute and stationary phase.

The differences in mobile phase combinations and proportions made a noticeable difference in the separation features and have been outlined in the tables above (Tables 5 and 6). From Table 5 it can be deduced that mobile phase composition had no effect on the resolution capacity of Phase II. All the α values were 1 (no peak resolution for the constituents in the solute). However, Phase I showed consistently better performance over phase III. A significant feature of the separation properties of this phase was the eluting out of non-polar and totally protected amino acids (derivatized with 3, 5-dinitrobenzoyl group). The derivatization adds bulkier side groups that affect the kind of interaction with the chiral centers present on the phase. The presence of the second chiral center in Phase I (R-phenylglycine) in combination with the mobile phase combination aided in the resolving capacities of the CSP.

From Table 6 it can be concluded that, Phase I still showed superior performance for all combinations of mobile phase and for all amino acid derivatives (polar or non-polar) with greater discrimination for the polar amino acid derivatives like serine, threonine, and tyrosine. Phase II showed better resolution here when compared to the dichloromethane-hexane combination. One common feature in the separation properties of all three phases with any combination of mobile phases was that they were uniform in their inability to resolve 3, 5-DNB proline isomers. The cyclic and rigid molecular structure of proline in tandem with the derivatization (DNB group) could be instrumental in the inability to interact with any component of the stationary phase satisfactorily. While steric factors

seem to facilitate enantioseparation (Dalgleish's theory), such separation would also have to be thermodynamically viable to occur. It is possible that the proline isomers were not able to interact as satisfactorily as the other amino acid isomer derivatives. Overall, the separations of the three phases can be summarized as Phase I > Phase III > Phase II. It certainly seems that in this case all other separation parameters being equal (solutes tested, mobile phase combinations), the extra chiral center in the diastereomeric Phase I played a significant role in enhancing the selectivity of the CSP over the single center enantiomeric Phase III.

SEPARATION OF ENANTIOMERIC ACIDS USING IMMOBILIZED ACETYLQUININE AS A CHIRAL STATIONARY PHASE.[22]

Quinine has been used for a while as a viable chiral selector in previous trials for separation of enantiomers. The paper reviewed here describes the work done using acetylquinine as a chiral selector in the stationary phase in combination with an organic mobile phase. The chromatography was done in the reverse-phase mode and the effects of pH, mobile phase (acetate buffer and methanol in specific ratios) concentration and modifier were studied.

Acetylquinine was attached to a silica support by a silanization reaction. One version is non-end capped (will be referred to as acetylquinine silica, Phase I) and the other is end capped (acetylquinine silica, Phase II). Phase II was prepared by the addition of hexamethyldisilazane to Phase I. All experiments were carried out at 23°C. Methanol

was used as an organic modifier to influence stereoselectivity without compromising retention.

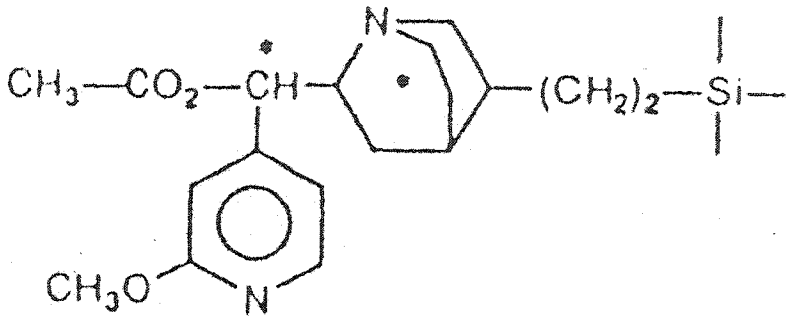


Figure 25. Structure of end capped Phase II (*denotes chiral centers).[22]

Results and Discussion:

The results of the separation and the separation features of both phases (retention times and capacity factors) for a variety of amino acid derivatives and carboxylic acid esters are shown in Tables 7 and 8.

Solid phase: Acetylquinine silica I.Mobile phase:acetate buffer (pH4.92, *I*=0.02*M*)-methanol (85:15).
Dansyl-5-dimethylamino-naphthalene-1-sulfonyl,
CBZ-benzoxy carbonyl,
Boc- tertiary butoxy carbonyl.

Solute	k´1	α
CBZ-leucine	15.3	1.04
CBZ-Valine	12.5	1.09
CBZ-Phenyl-Alanine	26.3	1.1
Boc-Phenyl-Alanine	12.4	1.06
Dansyl-Phenyl Alanine	67.2	1.1

Table 7. Separation of amino acid derivatives on Phase I.[22]

Retention times for these enantiomer derivatives seemed to be dependant on the bulkiness of the substituent group and the steric effects rendered. The presence of an iso-propyl group in the valine makes it more bulky and therefore the chiral selector would not be able to retain it as long as, perhaps, leucine. In comparing benzoxy carbonyl (CBZ)-phenylalanine and tertiary butoxy carbonyl (Boc-) phenylalanine, it can be seen that the k′ values are markedly different. The former is retained longer than the latter and the bulkiness of the substituent (CBZ- vs. Boc-) and charge-based interactions between the acetylquinine and phenyl groups can also play a role in the stereochemical dynamics.

Solid Phase:Acetylquinine silica II.
Mobile Phase: acetate buffer (pH 5.34, *I*-0.1*M*)-methanol (6:4)
k´1= Capacity factor for first eluted enantiomer and
k'2= Capacity factor for the second eluted enantiomer
α =. ratio of k'2/ k'1

Acid	k´1	α
2-(2-chlorophenoxy) propionic acid	5.21	1.09
2-(3-chlorophenoxy) propionic acid	5.75	1.12
2-(4-chlorophenoxy) propionic acid	5.36	1.15
2-(4-bromophenoxy) propionic acid	6.39	1.17
2-(4-iodophenoxy) propionic acid	8.54	1.21
Haloxyfoc	35.7	1.12

Table 8. Separation of halogenated phenoxypropionic acid enantiomers on Phase II. [22]

An examination of the retention values and separation factors of all the separated solutes shows that Phase II has demonstrated good resolution and stereoselection for the different derivatives. A key observation is the change in retention as the chlorine atom moves from the ortho- to the para- position. The stereogenic effects of the presence of

one halogen atom with respect to the other on the aromatic ring can explain this phenomenon. This can cause an increase in the interactions between the acetylquinine on the stationary phase and the solute. Also, increased retention of one of the enantiomers would result in the other eluting out first, thus marking a time lag between the two. This in turn would increase selectivity of the column for the retained enantiomer. Another point to note is the difference in stereoselectivity when the halogen atom itself is different. For example, the k′ for the iodo- derivative compared to the chloro- derivative. Once again, the size is the key here. Iodine being a bulkier substituent would enable a different kind of interaction when compared to its chlorine or bromine counterpart. The size and therefore orientation of the iodo- moiety would help increase steric effects. This combination of effects (size and orientation) would prevent easy movement along the column and hence the increase the retention. Whereas for the chloro- and bromo- moieties, passage through the column would be relatively faster, resulting in decreased retention.

Phase II gives higher stereoselectivity for the enantiomers tested than Phase I (comparison of values of Tables 7 and 8), while giving time- optimized results. The retention times of the enantiomers were lower without compromising selectivity. It was seen that regulating the methanol concentration in the mobile phase, further reduced retention times with no compromise in selection properties of Phase II. These results led the team to conclude that, all other experimental conditions being equal, the extra chiral center in acetylquinine Phase II facilitated enhanced enantioseparation.

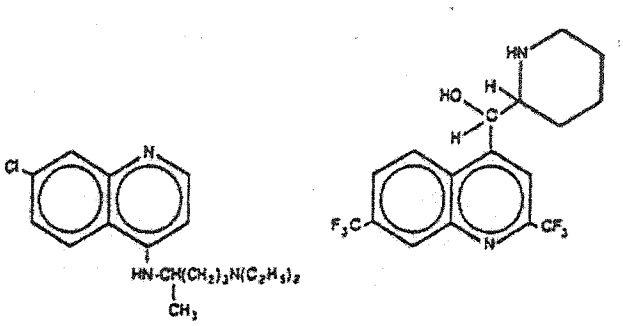
ENANTIOSELECTIVE CHROMATOGRAPHY OF THE ANTIMALARIAL AGENTS CHLOROQUINE, MEFLOQUINE AND ENPIROLINE ON A α_1 – ACID GLYCOPROTEIN (AGP) CHIRAL STATIONARY PHASE- Evidence for a Multiple-Site Chiral Recognition Mechanism.[23]

The chiral selector in this experiment is human plasma protein α_1-AGP (acid glycoprotein). It has been used in chiral separations since 1983. Some of its useful features include stability, tolerance for high temperatures and organic solvents, variable pH. It exhibits reverse-phase character and has been used in bioanalysis for detection of components in plasma and urine.

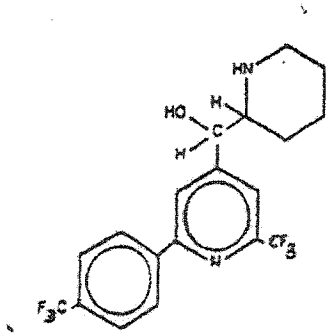
The trade name for the first generation phase synthesized using AGP is called EnantioPac and the second-generation phase (by Chromtech) is called Chiral- AGP. Currently it is one of the more popular chiral phases because of its ability to separate a variety of compounds.

In the investigative review, AGP was used as a chiral selector because of the variation in properties that it exhibits when subjected to changes in pH or the concentration of an organic modifier (e.g., dimethyl octylamine, (DMOA)). These changes in properties can be used to study the affinity of the binding site in the protein for different kinds of compounds. To do this the chromatographic experiment was designed as a study on the effect of mobile phase pH and mobile phase concentration of modifier, dimethyloctylamine (DMOA) on retention and selectivity. The samples studied were the anti-malarial drugs such as chloroquine, mefloquine and enpiroline (Figure 26).

Chloroquine is a drug that has been used for antimalarial treatment for a long time. Mefloquine has recently gained acceptance for use, while the medical efficacy of enpiroline is still under analysis. The temperature was ambient, flow rate @ 0.9 mL/min and the elution orders for the solutes were: (-)/ (+) for mefloquine, (+)/ (-) for enpiroline and (-)/ (+) for chloroquine. The results of the study are summarized in Tables 9 and 10.



Chloroquine Mefloquine



Enpiroline

Figure 26. Structures of Solutes used in the study.[23]

Solute	pH 5	pH 6	pH 7
Mefloquine			
k´1	4.53	18.31	32.56
k´2	7.73	30.86	45.49
α	1.71	1.68	1.39
Enpiroline	**pH 5**	**pH 6**	**pH 7**
k´1	6.37	15.93	38.5
k´2	6.37	15.93	38.5
α	1.00	1.00	1.00

Table 9. Effect of mobile phase pH (absence of DMOA).[23]

In Table 9, the effects of increasing the pH on the retentive powers of the protein (but without addition of the organic modifier, DMOA, to the mobile phase) have been examined. From Table 9, it can be inferred that with an increase in pH, there is a dramatic increase in retention times and a slight decrease in enantioselectivity for mefloquine. On the other hand, enpiroline shows an equally noticeable rise in retention times for both enantiomers, with insignificant changes in enantioselectivity. The higher retention at pH 7 (conformational change of the protein) can be attributed to Coulombic attractions between the drug enantiomer and the negatively charged protein.

The effects of increasing both pH and concentration of the cationic modifier (DMOA) are shown in Table 10. The changes undergone by the stationary phase and therefore the effect on the stereoselective properties can be examined in detail by studying the values obtained.

[DMOA]	pH	0	1mM	2mM	3mM
Enpiroline	pH 5	1.00	1.00	1.00	1.00
	pH 7	1.09	1.13	1.17	1.18
Mefloquine	pH 5	1.49	1.29	1.5	1.38
	pH 7	1.17	1.00	1.00	1.00
Chloroquine	pH 5	1.00	1.00	1.00	1.00
	pH 7	1.01	1.08	1.07	1.08

Table 10. Effect of DMOA concentration on stereoselectivity (α) at pH 5 and 7.[23]

For chloroquine, at pH 5, there is no effect of DMOA concentration on selectivity.At pH 7, upon addition of the first 1 mM of DMOA, there is an increase followed by loss of selectivity upon subsequent additions. For enpiroline at pH 5, there is no effect of DMOA addition. At pH 7, there is a noticeable increase in selectivity upon subsequent additions of DMOA. As for mefloquine, enantioselectivity was lost upon subsequent additions of DMOA at both pHs.

It was observed that at pH 5, the drugs eluted in the following order, chloroquine, enpiroline, mefloquine. At pH 7, the elution order was, enpiroline, chloroquine, mefloquine.

Effect of pH:

In a chiral stationary phase, especially one where an immobilized protein is involved, one must consider that conformational changes will occur when the mobile phase composition or pH is changed (pI of AGP is at 2.7). Small changes in pH can add or

remove hydrogen ions from side chain groups on the surface of a protein, without causing any major alteration to the conformation. The results obtained in this experiment are consistent with the above theory. It can be observed that AGP does indeed seem to undergo some degree of conformational change upon pH change. This conformational change affects the binding properties of the proteins with the drugs, as can be seen by the change in selection properties (for the malaria drugs) when the pH is altered.

Effect of altering DMOA concentration:

A modifier works by affecting the binding of the solute to the protein through competitive or allosteric interactions. Allosteric interactions are those where binding of one ligand influences the binding of another (by facilitating or decreasing the affinity of subsequent ligands). Competitive interactions are those where one ligand binds to a site in competition with another. When the authors examined the effect DMOA had on the CSP, they concluded that the solutes bind to a site that was subject to competitive displacement by DMOA. However, they also explained the increase in α by suggesting that due to the increase in pH and concomitant change in the DMOA concentration, there may be additional sites made (temporarily) available on the AGP, which were sensitive to allosteric interactions. Since there was an increase in retention at the initial addition followed by subsequent loss of retention, it could mean that increasing [DMOA] increased competitive binding of DMOA to the site. This explains why the drug enantiomers eluted faster with lower retention times upon later DMOA additions and pH increments. Such a change could explain the differences in enantioselectivity seen with

and without the addition of the DMOA. These were the results that led the authors to propose that there could exist a multiple-site-multiple-recognition mechanism at work. However, at the conclusion of the study, it was still uncertain as to the number of such involved sites or their definitive characteristics.

CONCLUSION:

Chiral stationary phases were designed to improve the chromatographic resolution of enantiomers. Pirkle and his associates were among the pioneers in the field of designing the first chiral chromatographic columns. Dalgleish postulated the three-point interaction theory, which explains how chiral recognition is achieved. The very phenomenon of chirality ensures that there are virtually an endless number of chiral compounds and their resolution is a major research challenge.

Enantiomeric selectivity is usually achieved through a combination of chiral stationary phase and mobile-phase conditions. Different versions of chirality were used. These included a chiral stationary phase, chiral selectors immobilized onto an achiral support and addition of chiral additives into the mobile phase. Different chiral selectors are being incorporated into stationary phases in an attempt to improve their selectivity for racemic mixtures of enantiomers. Experiments were designed to find the optimum separation conditions.

Once these techniques proved successful and reproducible results were obtained, the next step was to take the principle a step further. The idea proposed was that extra chiral

centers in the column could only improve enantioselectivity. Such multiple centers would increase the number of specific, discrete interactions between the chiral analytes and the chiral center on the column. Amino acids were the preferred starting materials for many CSPs because of their inherent chiral nature. Other racemic mixtures of compounds also were found feasible for chiral columns synthesis.

The CSPs were synthesized *in situ* and the solutes were derivatized according to the need of the experiment. HPLC was the preferred method of choice in all the papers reviewed. This is due to the fact that quick and reproducible results were obtained using this technique. With a single exception, all research attempts focused on the separation of amino acid, alcohol, acid or amide derivatives. All these reviews have been able to support the theory that the presence of an extra chiral center improves chiral resolution. The promising results obtained in these experiments could be used to increase the applicability of multiple chiral center CSPs in industry.

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