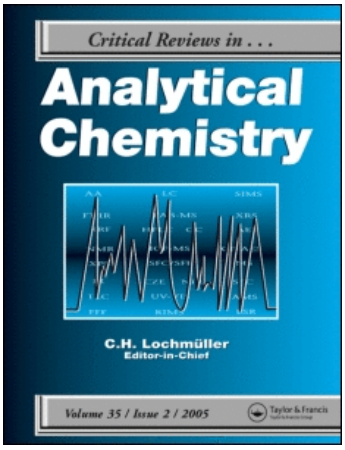

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ENANTIOMERIC SEPARATIONS IN CHROMATOGRAPHY

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I. INTRODUCTION

The resolution of enantiomers (nonsuperimposable, mirror-image isomers) has traditionally been considered one of the more difficult problems in separation science. Enantiomers have identical physical and chemical properties in an isotropic environment except that they rotate the plane of polarized light in opposite directions. A mixture containing equal amounts of enantiomers is referred to as a racemic mixture. Neither racemic mixtures nor solutions of achiral compounds are able to rotate the plane of polarized light.

In the mid-nineteenth century, the French physicist Biot discovered that certain substances had the unusual property of being able to rotate the plane of polarization of a linearly polarized incident light beam.[1,2] These substances, which were said to be optically active or to possess optical rotatory power, were characterized by a lack of symmetry in their molecular or crystalline structure. In 1848, Pasteur reported the first deliberate separation of enantiomers from a racemic mixture.[3] This separation was possible because saturated, racemic solutions of sodium ammonium tartrate form two types of morphologically distinct crystals, each containing a single enantiomer at temperatures below 27°C. In 1874, Van't Hoff and Le Bel independently deduced that the molecular basis of optical activity lay with the "asymmetric carbon atom".[4,5]

Enantiomeric separations are very important in many fields. Some typical fields include chiral synthesis, mechanistic studies, catalysis, kinetics, geochronology, biology, biochemistry, pharmacology, and medicine. There are many methods for the separation of enantiomers. Several nonchromatographic methods classically have been used to isolate optically pure compounds from racemic mixtures. The most generally useful of these methods involves conversion of the racemic mixture to a pair of diastereomers which have different chemical and physical properties and which may be separated by conventional techniques. Separation of the diastereomers can often be achieved by fractional recrystallization. Enantiomeric excesses have also been obtained via microbiological or enzymatic digestion. In this case, the enzyme must preferentially catalyze the reaction of one enantiomer relative to the other. Crystallizations followed by mechanical separation are useful for the few compounds that segregate into morphologically distinct crystals. Another type of crystallization technique involves seeding a supersaturated racemic solution with a small optically pure crystal. The resulting crystals often contain an enantiomeric excess. While all of these techniques have been utilized successfully, none can be considered generally useful, and all are relatively time consuming and tedious. In addition, these methods often fail to afford total separation of enantiomers. Recently there has been a dramatic increase in the number and type of racemic separations achieved by chromatographic methods. The popularity of the chromat-

ographic approach stems from its relative ease and efficiency. In this review, the enantiomeric separations with gas chromatography (GC), liquid chromatography (LC), and thin layer chromatography (TLC) are discussed. It appears that the most interesting research in this area involves the development of new, highly selective stationary phases. A large number of chiral stationary phases (CSPs) were synthesized, and it was found that minor structural modifications in a stationary phase may have a tremendous influence on an enantiomeric separation.

II. GAS CHROMATOGRAPHY

In GC, the separation of enantiomers is generally achieved in two ways. The first involves the use of CSPs.[6-11] The second involves derivatizing a racemate with a chiral group and making a pair of diastereomers, and then separating those on achiral or chiral stationary phases.[6-11] In 1966, Gil-Av and co-workers[12] first made CSPs for GC and demonstrated their usefulness for separating enantiomers. This approach attracted great attention since it enabled one to precisely measure enantiomeric purities in complex mixtures while utilizing very small samples. The use of chiral derivatization methods has a few limitations: (1) active functional groups for forming diastereomeric derivatives are required, (2) it is not easy to get optically pure chiral reagents, (3) individual enantiomers have different reaction rates, and (4) the diastereomeric mixture must be chemically and stereochemically stable.

A. CSPs

1. Amino Acid Derivatives

Gil-Av and co-workers[12] first introduced an enantioselective GC stationary phase which consisted of *N*-trifluoroacetyl (*N*-TFA) L-isoleucine lauryl ester. This liquid phase was used for the separation of *N*-TFA-α-amino acid esters. It showed excellent resolution for volatile *N*-TFA-α-amino acid esters (e.g., *N*-TFA-valine and leucine esters of 2-butanol), but was not suitable for *N*-TFA-alanine esters, 2-heptyl acetate, and α-acetoxypropionate esters of 2-butanol.

Feibush[13] reported a highly efficient diamide-liquid stationary phase, *n*-dodecanoyl-L-valine-*tert*-butylamide. This phase was less polar than dipeptide phases but produced relatively high enantioselectivities. Retention times were generally shorter than on the more polar phases. However, this phase was limited to the separation of amino acid and amino alcohol enantiomers. Also, this phase had relatively high column bleeding at the optimum column operating temperature of 130 to 140°C. To reduce the column bleeding, *N*-docosanoyl-L-valine *tert*-butylamide and *N*-lauroyl-L-valine 2-methyl-2-heptadecylamide were used as the active coating.[14] *N*-TFA isopropyl esters of 14 protein amino acids were studied, and 12 of the 14 compounds were resolved on relatively short columns (2 to 4 m). These packed columns could be used at temperatures as high as 190°C (for *N*-docosanoyl-L-valine *tert*-butylamide) and 180°C (for *N*-lauroyl-L-valine 2-methyl 2-hepadecylamide) without losing their efficiency, even after prolonged use. The influence of structural factors on selectivity of CSPs ($R_1CONHCH[CH(CH_3)_2]CONHR_2$) was studied.[15] Hobo and co-workers studied the stereoselectivity of the chiral diamide stationary phase, *N*-lauroyl-L-valine *tert*-butylamide, diluted with diethylene glycol succinate polyester (DEGS) or squalane for the separation of several *N*-trifluoroacetyl isopropyl esters of amino acids.[16] The addition of DEGS greatly reduced the resolution coefficient. It was thought that this resulted from the blocking of the chiral sites of the stationary phase by the polar solvent. When squalane was added, the resolution coefficient either remained the same or increased. Dilution of the chiral liquid stationary phase with achiral additives may lead to the dissolution of the hydrogen-bonded networks of these dipeptide-analog phases. It was concluded that the unblocked monomeric form of the diamide gave higher retention and stereoselectivity.

Table 1
RESOLUTION FACTORS OF *N*-TFA-*O*-ACYL DERIVATIVES OF AMINOALKANOLS

		Acyl group								
		Propionyl			Isobutyryl			Pivaloyl		
Aminoalkanol	Enantiomer	r[a]	$r_{(D/L)}$[b]	T(°C)[c]	r[a]	$r_{(D/L)}$[b]	T(°C)[c]	r[a]	$r_{(D/L)}$[b]	T(°C)[c]
2-Aminopropan-l-ol	L	14.58	1.040	120	9.40	1.051	140	12.00	1.075	140
	D	15.16			9.88			12.90		
2-Aminobutan-l-ol	L	25.00	1.076	120	14.56	1.089	140	15.86	1.112	140
	D	26.90			15.86			17.64		
2-Aminopentan-l-ol	L	42.28	1.089	120	10.94	1.075	160	11.48	1.097	160
	D	46.00			11.76			12.60		
2-Aminohexan-l-ol	L	69.88	1.087	120	16.86	1.071	160	18.14	1.096	160
	D	76.00			18.06			19.88		
2-Aminoheptan-l-ol	L	51.88	1.071	140	19.86	1.062	170	20.88	1.082	170
	D	55.58			21.10			22.60		
2-Aminooctan-l-ol	L	88.80	1.071	140	31.98	1.067	170	33.14	1.083	170
	D	95.10			34.14			35.90		
2-Amino-3-methyl-butan-l-ol	L	28.44	1.094	120	16.90	1.105	140	18.66	1.125	140
	D	31.12			18.68			21.00		
2-Amino-4-methyl-pentan-l-ol	L	24.10	1.057	140	29.84	1.085	140	30.04	1.130	140
	D	25.48			32.38			33.94		

[a] Corrected retention time (minutes).
[b] L/D, resolution factor, i.e., ratio of the corrected retention time of the L over that of the D enantiomer, calculated with r values expressed to the second decimal place.
[c] Temperature at which good peak resolution and relatively short retention were observed.

From Charles, R. and Gil-Av, E., *J. Chromatogr.*, 195, 317, 1980. With permission.

Koenig and co-workers prepared chiral liquid stationary phases by coupling *S*-α-phenylethylamine with *S*-2-hydroxyisopentanoic acid and *S*-2-hydroxyoctanoic acid.[17] The separations of the enantiomers of racemic amines, amino alcohols, and hydroxy acids were achieved using these stationary phases.

Weinstein and co-workers[18] reported that CSPs containing an amide group and an asymmetric carbon atom, attached to the nitrogen atom [RCONHCH(CH_3)R′], often showed enantiomeric selectivity for amino acids containing *N*-trifluoroacetylamine, *N*-trifluoroacetylamino acid esters, and α-methyl- or α-phenylcarboxylic acid amides. The best efficiency was obtained when R′ was an aromatic group. Particularly good separations were obtained when R′ was an α-naphthyl group, as in *N*-lauroyl-*S*-α-(1-naphthyl)ethylamine. Also, the highest resolution factors were found for aromatic solutes such as *N*-trifluoroacetyl-α-phenylethylamine and α-phenyl butyric acid amides.

Other successful GC separations of enantiomers were reported on modified diamide phases.[19,20] *N*-Docosanoyl-L-valine-2-(2-methyl)-*n*-heptadecyl amide phases[20] were used at column temperatures of up to 200°C. These phases showed excellent stereoselectivity for various compounds (aromatic *N*-TFA amines, *N*-TFA-*O*-acetyl amino alcohol, *N*-TFA-α-methylvaline isopropyl ester, and *N*-TFA-esters of α- and γ-amino acids). Table 1 shows the resolution factors of *N*-TFA-*O*-acetyl derivatives of amino alkanols. Lochmüller and co-workers reported ureide phases (i.e., carbonyl-bis [amino acid esters])[21-24] and examined the separation mechanisms of these phases. The general formula of the ureide phase is shown in Figure 1. Nuclear magnetic resonance (NMR) evidence was interpreted to indicate that only one significant portion of attachment is involved in the formation of diastereoisomeric

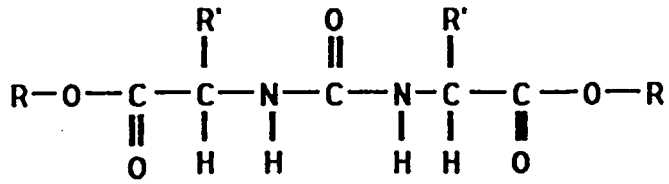


FIGURE 1. General formula of the ureide phase. (From Lochmüller, C. H. and Souter, R. W., *J. Chromatogr.*, 113, 283, 1975. With permission.)

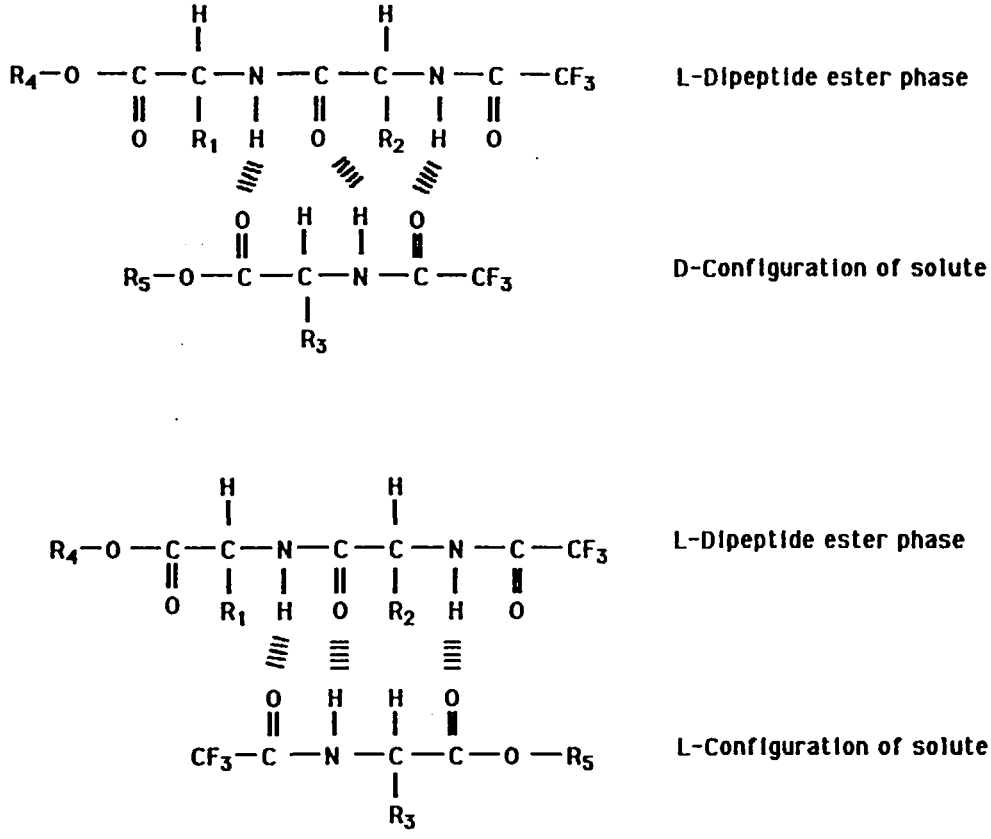


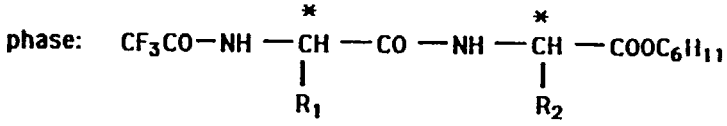
FIGURE 2. Schematic showing the formation of H-bonded diastereomeric association complexes. (From Lochmüller, C. H. and Souter, R. W., *J. Chromatogr.*, 113, 283, 1975. With permission.)

association complexes. This, of course, is highly unlikely given the well-known geometrical requirements (i.e., three-point attachment) for chiral recognition. Also studied were the effects of stationary phase structure on selectivity.[23]

2. Peptide Phases

Gil-Av and co-workers also developed optically active stationary phases composed of *N*-TFA-α-amino acids (e.g., *N*-L-valyl-L-valine isopropylester, *N*-TFA-L-valyl-L-valine cyclohexyl ester,[25] *N*-acetyl-L-valyl-L-valine isopropyl ester,[26] and *N*-TFA-(L-valyl)$_2$-L-valine isopropyl ester[26]). However, these phases had low efficiencies and long analysis times because of limited thermal stability. For example, a *N*-TFA-L-valyl-L-valine cyclohexyl ester stationary phase was used at 110°C. Above 110°C, the column began to bleed, and at temperatures below 110°C, the column efficiency was very low and the peaks were broad.

In peptide phases, the resolution of enantiomers is thought to be through the formation of hydrogen-bonded diastereomeric association complexes between the enantiomeric compounds and asymmetric chiral phases. Figure 2 shows this interaction on a dipeptide ester



	R_1, R_2	abbreviation
N-TFA-L-alanyl-L-alanine cyclohexyl ester	$-CH_3$	ala-ala
N-TFA-L-α-amino-n-butyryl-L-α-amino-n-butyric acid cyclohexyl ester	$-CH_2-CH_3$	aba-aba
N-TFA-L-norvalyl-L-norvaline cyclohexyl ester	$-CH_2-CH_2-CH_3$	nval-nval
N-TFA-L-norleucyl-L-norleucine cyclohexyl ester	$-CH_2-CH_2-CH_2-CH_3$	nleu-nleu

FIGURE 3. Homologous dipeptide stationary phases. (From Parr, W. and Howard P. Y., *Anal. Chem.*, 45, 711, 1973. With permission.)

phase.[24] Parr and co-workers synthesized many dipeptide ester phases.[27-31] They also studied the structural effects on selected dipeptides as stationary phases for the enantiomeric separation of amino acids.[32] Four optically-active dipeptide cyclohexyl esters (*N*-TFA-L-alanyl-L-alanine cyclohexyl ester, *N*-TFA-L-α-amino-*n*-butyryl-L-α-amino-*n*-butyric acid cyclohexyl ester, *N*-TFA-L-norvalyl-L-norvaline cyclohexyl ester, and *N*-TFA-L-norleucyl-L-norleucine cyclohexyl ester) were used (see Figure 3). Separation factors and thermodynamic properties of liquid stationary phase-solute interactions were studied. Table 2 shows relative retention times and separation factors for *N*-TFA-D,L-amino acid isopropyl esters. An increase in the size of the alkyl substituent on the asymmetric centers of the dipeptide solvent produced a greater liquid stationary phase-solute interaction. When the analogous modification was made with the side chain on the α carbon, the interaction decreased.

In order to obtain a dipeptide ester phase of greater thermal stability and higher molecular weight, aromatic amino acids such as phenylalanine were employed (i.e., *N*-TFA-L-phenylalanyl-L-leucine cyclohexyl ester,[29,31,33,34] *N*-TFA-L-phenylalanyl-L-phenylalanine cyclohexyl ester,[35,36] and *N*-TFA-L-phenyl-L-aspartic acid bis [cyclohexyl] ester[35-37]). These phases were able to be used at higher temperatures (130 to 165°C) than the original dipeptide phases and therefore were more effective for separating less volatile enantiomeric amino acid derivatives.

Koenig and co-workers used glass capillaries because they found partial decomposition of some compounds during chromatography with steel capillaries. They reported that steel capillaries promoted the decomposition of solutes (cysteine, serine, and threonine derivatives) to a greater extent than glass capillaries did. The glass capillary columns also possessed higher efficiencies compared to analogous stainless steel capillaries. Some of the stationary phases synthesized and used with glass capillaries include *N*-TFA-L-phenylalanyl-L-phenylalanine cyclohexyl ester, *N*-TFA-L-phenylalanyl-L-leucine cyclohexyl ester, and *N*-TFA-L-phenylalanyl-L-aspartic acid bis (cyclohexyl)ester.[35] Figure 4 shows the separation of racemic TFA-amino acid isopropyl esters on a glass capillary coated with *N*-TFA-L-valine-L-valine cyclohexyl ester.

Table 2
RELATIVE RETENTION TIMES AND SEPARATION FACTORS FOR *N*-TFA-D,L-AMINO ACID ISOPROPYL ESTERS ON FOUR DIFFERENT STATIONARY PHASES

ala-ala			aba-aba			nval-nval			nleu-nleu		
	110°C			100°C			100°C			100°C	
Amino acid	r	α	Amino acid	r	α	Amino acid	r	α	Amino acid	r	α
D-ala	0.289	1.055	D-ala	0.246	1.091	D-ala	0.231	1.100	D-ala	0.228	1.096
L-ala	0.305		L-ala	0.268		L-ala	0.254		L-ala	0.249	
D-aba	0.476	1.064	D-aba	0.341	1.091	D-aba	0.330	1.097	D-aba	0.327	1.091
L-aba	0.507		L-aba	0.372		L-aba	0.362		L-aba	0.356	
D-val	0.382	1.071	D-val	0.366	1.090	D-val	0.364	1.083	D-val	0.366	1.080
L-val	0.409		L-val	0.399		L-val	0.397		L-val	0.396	
D-nval	0.597	1.059	D-nval	0.563	1.086	D-nval	0.553	1.092	D-nval	0.551	1.090
L-nval	0.631		L-nval	0.611		L-nval	0.604		L-nval	0.600	
D-leu	0.844	1.066	D-leu	0.764	1.099	D-leu	0.749	1.110	D-leu	0.748	1.107
L-leu	0.900		L-leu	0.839		L-leu	0.832		L-leu	0.828	
D-ile	0.580	1.078	D-ile	0.535	1.105	D-ile	0.535	1.108	D-ile	0.541	1.101
L-ile	0.625		L-ile	0.591		L-ile	0.592		L-ile	0.595	
D-nleu	0.940	1.064	D-nleu	0.911	1.098	D-nleu	0.903	1.108	D-nleu	0.904	1.107
L-nleu	1.000		L-nleu	1.000		L-nleu	1.000		L-nleu	1.000	
D-*t*-leu	0.387	1.050	D-*t*-leu	0.362	1.052	D-*t*-leu	0.364	1.044	D-*t*-leu	0.368	1.033
L-*t*-leu	0.407		L-*t*-leu	0.381		L-*t*-leu	0.381		L-*t*-leu	0.380	

Note: See Figure 3 for the stationary phase structures and abbreviations. r, Relative retention time; reference compound in all cases is *N*-TFA-L-norleucine isopropyl ester.

From Parr, W. and Howard, P. Y., *Anal. Chem.*, 45, 711, 1973. With permission.

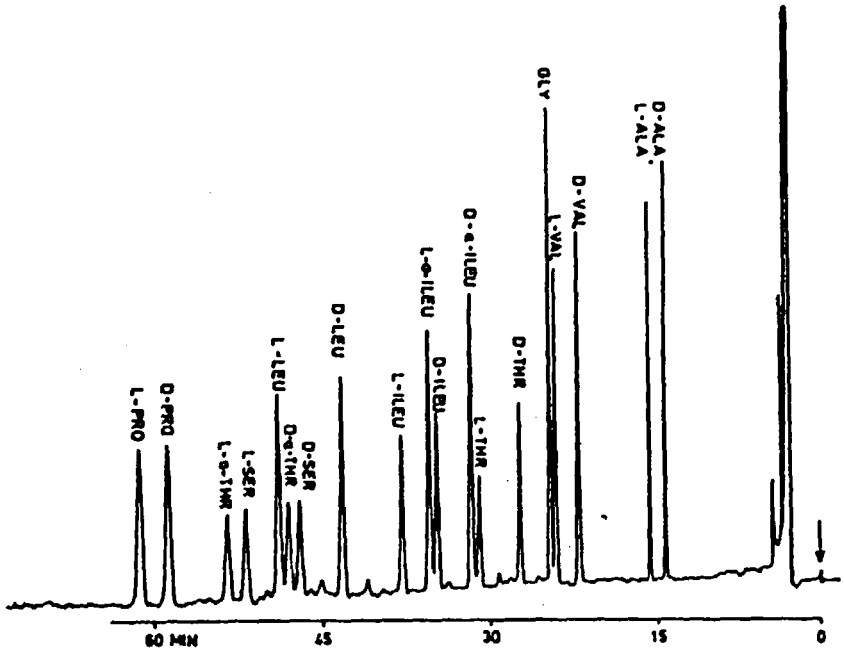


FIGURE 4. Gas chromatogram showing the separation of TFA-amino acid isopropyl esters. (From Koenig, W. A. and Nicholson, G. J., *Anal. Chem.*, 47, 951, 1975. With permission.)

Carbin and co-workers synthesized several systematically substituted, optically active peptide stationary phases (*N*-TFA-L-valyl-L-leucine cyclohexyl ester, *N*-PFP-L-valyl-L-leucine cyclohexyl ester, *N*-TFA-L-leucyl-L-valine cyclohexyl ester, and *N*-TFA-[L-leucyl]$_2$-L-leucine cyclohexyl ester).[38] They studied the effects of systematic changes in the side chains of the peptides and found that the separation factor (α) values were sensitive to changes in the structure of the side group at the ester end.

Some of the dipeptide ester phases were converted to tripeptide phases.[38] *N*-Pentafluoropropionyl (*N*-PFP) groups were substituted for the *N*-TFA group.[29,31,38] Many dipeptide and tripeptide stationary phases have been used in separating amino acids. The tripeptide phases have better thermal stability than dipeptide phases do, but their resolving powers were found to be very similar.[38-40] *N*-TFA-L-methionyl-L-methionine cyclohexyl ester and disulfide and disulfone derivatives of the TFA phases have been synthesized.[41] *N*-TFA-L-methionyl-L-methionine cyclohexyl ester stationary phases gave good separations of *N*-TFA-D,L-amino acid isopropyl esters. Although these phases were stable at temperatures as high as 150°C, increasing the temperature reduced the separation factors. *N*-PFP-derivatized stationary phases have been shown to be stable, giving larger α values and shorter retention times than *N*-TFA phases. In general, the peptide stationary phases that produced the best resolution of separated amino acid derivatives contained *N*-TFA groups or bulky ester groups (e.g., *tert*-butyl, isopropyl, or cyclohexyl ester). Optimal performance of these columns was obtained at lower temperatures.

Oi and co-workers synthesized the *S*-triazine derivative of a tripeptide ester.[42-44] This phase gave good separations of various α-alkyl phenylacetic acid and aryl alkylamine enantiomers. For example, *N,N',N''*-(2,4,6-[1,3,5-triazine]-triyl)-tris-L-valine isopropyl esters (OA-100) gave excellent separations of *N*-TFA-amino acid ester and *N*-TFA-amine enantiomers. However, the enantioselectivity of these phases was insufficient for the resolution of some carboxylic acids and amines; therefore, the determination of the optical purity was impossible.[42-44] OA-200 (*N,N'*-[2,4-(6-Ethoxy-1,3,5-triazine)diyl])-bis-(L-valyl-L-valine isopropyl ester), OA-300 (*N,N'*-[2,4-(6-ethoxy-1,3,5-triazine)diyl])-bis-(L-valyl-L-valyl-L-valine isopropyl ester), and OA-400 (*N,N',N''*-[2,4,6-(1,3,5-triazine)triyl])tris-(Nα-lauroyl-L-lysine-*tert*-butylamide) were used for separating enantiomers of α-hydroxycarboxylic acid esters and some alcohols.[45,46] Racemic 1-phenyl-2,2,2-trifluoroethanol was separated on an OA-400 column. The *S* isomers of the chiral alcohols were found to elute before the *R* isomers. *N*-Lauroyl-(*S*)-proline-(*S*)-1-(α-naphthyl)ethylamide also was used to separate the *R*- and *S*-isomers of 1-phenyl-2-(4-tolyl)ethylamine.[47]

3. Polymer Phases

Frank and co-workers synthesized a CSP named Chirasil-val[48-50] by coupling L-valine-*tert*-butylamide to a copolymer of dimethylsiloxane and carboxy-alkyl-methyl-siloxane of appropriate viscosity and molecular weight. This polymeric GC stationary phase had a higher thermal stability and a lower volatility than the monomeric stationary phases. This polymeric phase was used to separate amino acid and some amino alcohol enantiomers,[51-54] enantiomeric drugs, and metabolites.[55] This phase allowed a greater range of temperatures to be used in the separation of enantiomers by GC. As a result, compounds of lower volatility could be analyzed for the first time. Mass spectrometric detection was also used with this polymeric stationary phase.[56]

A thermally stable chiral phase was made by incorporating L-valine-*tert*-butylamide into the well-known GC stationary phase polycyanopropylmethyl phenylmethyl silicone.[57] The cyano groups of commercially available GC phases (such as OV 225 and Silar 10C) were converted by acid hydrolysis into carboxylic groups, then into acid chlorides, and were finally coupled with L-valine-*tert*-butylamide. This phase was similar to Chirasil-val, but had structural differences (e.g., propyl linkages instead of ethyl linkages to the chiral center

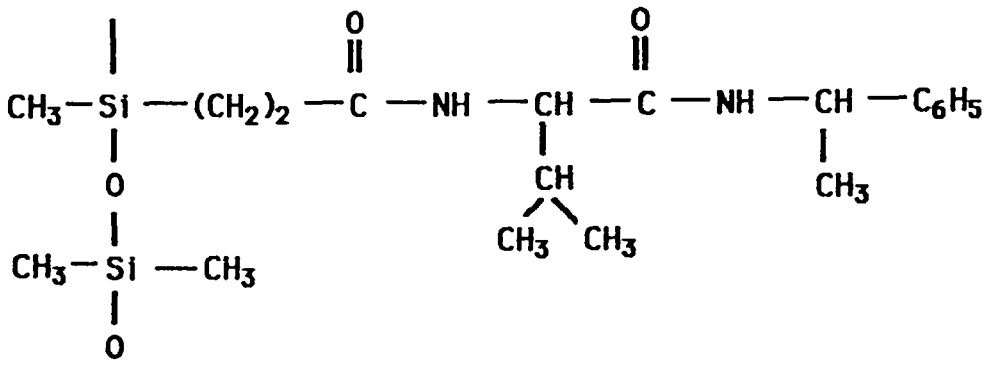


FIGURE 5. The molecular structure of XE-60-*S*-valine-*S*(or *R*)-α-phenylethylamine.

and the presence of phenyl groups on the silicone matrix). This phase was used for the separation of amino acid enantiomers at temperatures ranging from 60 to 230°C. The separation of several protein amino acid enantiomers in one run using temperature programming was demonstrated. This phase also had good enantioselectivity for proline and other secondary amino acid derivatives.[58]

Koenig and co-workers[59] separated chiral aliphatic, aromatic, and monoterpene alcohols as isopropyl urethane derivatives on glass capillary columns coated with XE-60-*S*-valine-*S*-α-phenylethylamide (Figure 5). By forming stable isopropyl urethanes in a facile derivatization step, the enantioselective intermolecular interactions between the racemic alcohols and the CSP were sufficiently enhanced to give enantiomeric resolution. This column was used for the separation of carbohydrate enantiomers as either TFA derivatives or TFA-methyl glycosides.[17,60] A XE-60-*R*-valine-*R*-α-phenylethylamide column was used for the enantiomeric separation of phenolic α- and β-receptor-active drugs after derivatizing with diazomethane and phosgene.[61] Schomburg and co-workers reported modified GC phases for the separation of optically active compounds.[62] Cross-linking experiments for the immobilization of XE-60-L-valine-(*S*)- and XE-60-L-valine-(*R*)-α-phenylethylamine within capillary columns were successfully completed.[62] Another approach to the modification of silicone polymers was reported by Koenig and Benecke.[63] They bonded chiral constituents onto silicone OV-225 after reducing the cyano groups to amino groups with lithium aluminum hydride ($LiAlH_4$), and then coupled benzyloxycarbonyl-L-valine and benzyloxycarbonyl-L-leucine to the amino groups (see Figure 6). These phases separated enantiomers of trifluoroacetylated secondary amines and amino alcohols. It was found that the D amino alcohols had longer retention times than the L enantiomers, while the *S* enantiomers of amines had longer retention times than the *R* enantiomers. These columns were also used for resolving the racemates of arabinitol, fucitol, mannitol, and C5-8,2-aminoalkanes as the trifluoroacetyl derivatives.[64]

4. Miscellaneous Phases

Chiral liquid stationary phases composed of di-*l*-menthyl-(+)-tartrate and di-*dl*-methyl-(−)-malate were used in conjunction with glass capillary columns to separate enantiomers of amino acids, amines, and carboxylic acids.[65] Figure 7A shows the GC separation of *N*-trifluoroacetyl-D,L-leucine isopropyl ester, and Figure 7B shows that of racemic *N*-pentafluoro propyl-α-phenylethylamine. Oi and co-workers also developed some amide-type stationary phases which contained two asymmetric carbon atoms. These were used to separate some carboxylic acid and amine enantiomers.[66] Liquid stationary phases composed of *N*-(1*R*,3*R*)-*trans*-chrysanthemoyl-(*R*)-1-(α-naphthyl)ethylamine phase,[66] which contains two asymmetric carbon atoms attached to both nitrogen and carbon atoms of the amide group,

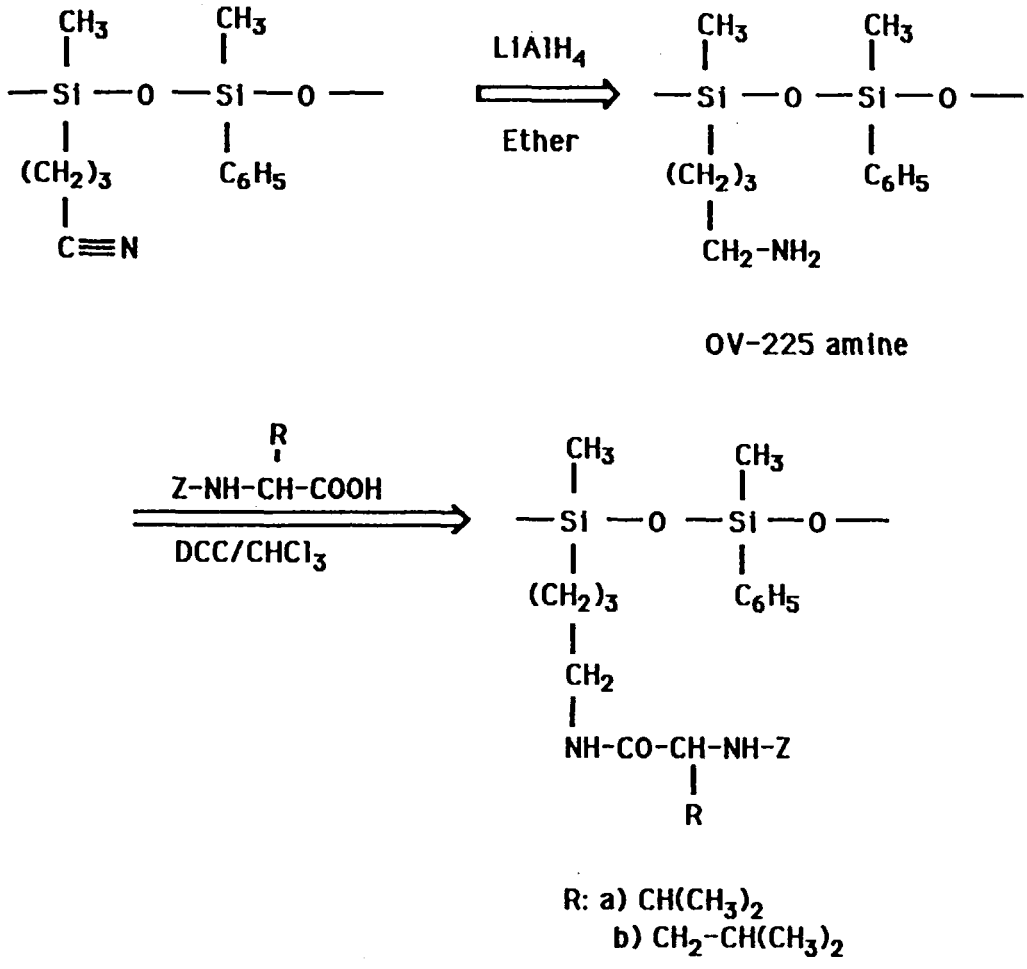


FIGURE 6. Schematic showing the synthetic pathway for the preparation of new chiral stationary phases by reduction of OV-225 polysiloxane. (From Koenig, W. A. and Benecke, I., *J. Chromatogr.*, 209, 91, 1981. With permission.)

showed better enantioselectivity than those composed of *N*-(1*R*,3*R*)-*trans*-chrysanthemoyl-lauryl-amine or *N*-lauroyl-(*R*)-1-(α-naphthyl)ethylamine, both of which contain only one asymmetric center. An *O*-(1*R*,3*R*)-*trans*-chrysanthemoyl-(*S*)-mandelic acid (*R*)-1-(α-naphthyl)-ethylamide stationary phase was synthesized and shown to have good enantioselectivity for chrysanthemic acid ester and 3-(2,2-dichlorovinyl)2,2-dimethylcyclopropanecarboxylic acid esters.[67] Table 3 lists some of the enantiomers separated with this phase, as well as pertinent chromatographic data. α- and β-Cyclodextrin were used by Sybilska and co-workers as stationary phase coatings in the GC separation of enantiomers of α- and β-pinene.[68,69]

B. Derivatization Methods

1. Alcohols

Diastereomers are often separated on conventional, achiral GC stationary phases. Consequently, there are many reports in which racemates have been resolved after derivatization with an optically pure reagent. This method is particularly important for difficult-to-resolve compounds such as branched aliphatic alcohols[70] and 2,3-butanediol esters.[71] The first GC resolution of racemic mixtures of secondary *n*-alkanols as the corresponding diastereoiso-

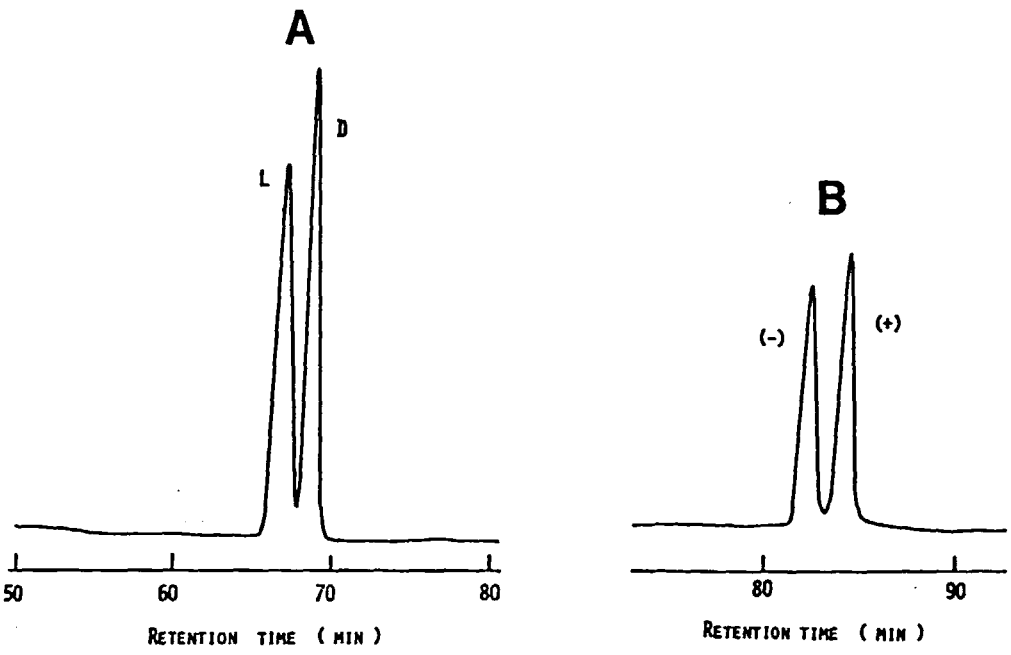
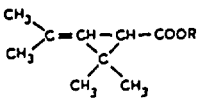
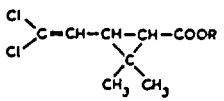


FIGURE 7. Gas chromatogram showing the separation of the enantiomers of (A) *N*-trifluoroacetyl-D,L-leucine isopropyl ester and (B) *N*-pentafluoropropyl-α-phenylethylamine. (From Oi, N., Kitahara, H., and Doi, T., *J. Chromatogr.*, 207, 252, 1981. With permission.)

meric α-hydroxypropionates was reported in 1962.[72,73] Resolution of enantiomorphs by conversion to volatile diastereoisomers followed by rectification and reconversion to the original compounds was done by Bailey and Hass.[74] Partial resolution of racemic 2-butanol and 2-pentanol was done using levorotatory lactic acid. Lactic acid is commonly used as a derivatization reagent because it is inexpensive and easily obtained in pure form.

Bulk dissymmetry at the alcoholic asymmetric carbon atom and the distance between optical centers and their effects on separation have been studied by Rose and co-workers.[75] They investigated the degree of separation of diastereomeric esters of acetylated lactic acid (α-acetoxypropionic acid) as a function of systematic variation in alcohol structure in order to gain insight into the factors responsible for the separation of these esters. Using a 1,2,3-tris(2-cyanoethoxy) propane column, the separation factors of the diastereoisomeric esters of the secondary alcohols (±)-2-*n*-butanol, (±)-2-*n*-pentanol, (±)-2-*n*-hexanol, (±)-2-*n*-heptanol, and (±)-2-*n*-octanol) were found to change from 1.059 to 1.107 in order of increasing chain length. The separation factors of the esters of these alcohols also changed from 1.016 to 1.079 on a D.C. (Dow Corning Corp.) 710 silicone oil column. Gault and Felkin[76] found that the diastereoisomers of unsaturated alcohols or diols are less likely to form an intramolecular hydrogen bond. Among the more effective diastereomeric derivative methods for resolving alcohols include the use of *N*-TFA-(L)-(+)-alanyl ester,[77] *N*-TFA-(*S*)-(−)-prolyl ester,[47] (+)-*trans*-chrysanthemoyl ester,[78] (*S*)-acetoxypropionyl ester,[79] and (*R*)-(+)-1-phenylurethane.[80] A comparison of the easy separation of three different derivatives of racemic alcohols as well as the determination of the configuration of *myrmica* ant 3-octanol was done by Attygalle and co-workers.[81] Three diastereomeric derivatives of 3-(±) octanol were prepared. These were *N*-TFA-(*S*)-(+)-alanyl ester, the *N*-TFA-(*S*)-(−)-proyl ester, and the (+)-*trans*-chrysanthemoyl ester. They used OV-1 and DEGS columns, and the (+)-*trans*-chrysanthemoyl ester was decided to be the most useful derivatizing reagent for the study of the naturally occurring 3-octanol (Table 4).

Table 3
GAS CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS

R	Enantiomer								
		Column temperature (°C)	Retention time* (min)		α**	Column temperature (°C)	Retention time* (min)		α**
			1st peak	2nd peak			1st peak	2nd peak	
CH_3	Cis	100	–	–	–	100	31.83	32.67	1.026
	Trans		9.91	10.12	1.021		41.07	42.10	1.025
C_2H_5	Cis	100	14.31	14.41	1.007	100	44.96	46.04	1.024
	Trans		14.61	14.87	1.018		59.14	60.54	1.024
n-C_3H_7	Cis	100	–	–	–	100	74.56	76.49	1.026
	Trans		25.42	25.97	1.022		98.89	101.4	1.025
iso-C_3H_7	Cis	100	14.68	14.83	1.010	100	44.30	44.94	1.014
	Trans		15.26	15.55	1.019		59.00	60.20	1.020
n-C_4H_9	Cis	100	42.06	42.86	1.019	100	128.8	131.9	1.024
	Trans		43.97	44.92	1.022		173.4	177.5	1.024
$tert$.-C_4H_9	Cis	100	13.53	13.53	1.000	100	44.48	44.48	1.000
	Trans		14.19	14.37	1.013		58.08	58.88	1.014
n-C_6H_{13}	Cis	100	142.4	145.6	1.022	120	118.2	120.6	1.020
	Trans		150.2	153.3	1.021		155.3	158.4	1.020
cyclo-C_6H_{11}	Cis	100	210.7	214.2	1.017	120	173.8	176.5	1.016
	Trans		221.3	226.9	1.025		228.1	233.3	1.023
n-C_8H_{17}	Cis	120	139.5	132.1	1.012	150	79.63	80.69	1.013
	Trans		140.2	142.5	1.016		100.7	102.0	1.013

Note: Chromatography on 40 m × 0.25 mm I.D. glass capillary columns coated with *O*-(1*R*,3*R*)-*trans*-chrysanthemoyl-(*S*)-mandelic acid (*R*)-1-(α-naphthyl)ethylamide. Carrier gas, helium at 0.7 to 0.8 mℓ/min.

* Measured from solvent peak.

** Separation factor calculated from 2nd peak/1st peak retention time ratio.

From Oi, N., Kitahara, H., and Doi, T., *J. Chromatogr.*, 254, 282, 1983. With permission.

Table 4
COMPARISON OF GC PROPERTIES OF 3-(±)-OCTANOL DERIVATIZED WITH DIFFERENT CHIRAL RESOLVING AGENTS ON APOLAR (OV-1) AND POLAR (DEGS), WCOT CAPILLARY COLUMNS

	OV-1						DEGS					
	Retention time (min)				Elution order		Retention time (min)				Elution order	
Derivative	First isomer	Second isomer	α	R	First isomer	Second isomer	First isomer	Second isomer	α	R	First isomer	Second isomer
N-TFA(S)-(+)-alanyl ester	2.7	2.8	1.03	1.2	*R*	*S*	3.1	3.2	1.03	1.2	*R*	*S*
N-TFA-(S)-(−)-prolyl ester	8.6	8.9	1.04	1.8	*R*	*S*	11.4	11.7	1.03	1.2	*R*	*S*
(+)-*trans*-Chrysanthemate ester	8.3	8.6	1.03	1.9	*S*	*R*	2.4	2.5	1.04	1.1	*S*	*R*

Note: Analyses performed isothermally at 150°C (OV-1) and 160°C (DEGS).

From Attygalle, A. B., Morgan, E. D., Evershed, R. P., and Rowland, S. J., *J. Chromatogr.*, 260, 411, 1983. With permission.

Table 5
SEPARATION FACTORS (α) AND OPERATING TEMPERATURES FOR THE SEPARATION OF 3-HYDROXY ACIDS AS *N-tert* BUTYL-CARBAMATE-*tert*-BUTYLAMIDE DERIVATIVES (A) AND *N*-ISOPROPYLCARBAMATE-ISOPROPYLAMIDE DERIVATIVES (B) ON A 25-M FUSED-SILICA CAPILLARY COLUMN OF XE-60-L-VALINE-(*S*)-α-PHENYLETHYLAMIDE

3-Hydroxy acid	α(A)	Column temp (°C)	α(B)	Column temp (°C)
3-Hydroxybutanoic acid	1.030	180	1.022	180
3-Hydroxy-*n*-pentanoic acid	1.029	180	1.016	180
3-Hydroxyisohexanoic acid	1.025	180	1.008	190
3-Hydroxy-*n*-hexanoic acid	1.035	180	1.027	190
3-Hydroxy-*n*-heptanoic acid	1.044	180	1.031	180
3-Hydroxy-*n*-octanoic acid	1.032	190	1.022	190
3-Hydroxy-*n*-nonanoic acid	1.026	210	1.022	210
3-Hydroxy-*n*-decanoic acid	1.026	210	1.021	200
3-Hydroxy-*n*-tetradecanoic acid	—	—	1.018	220

Note: The (*S*)-enantiomers are eluted first.

From Koenig, W. A., Benecke, I., Lucht, N., Schmidt, E., Schulze, J., and Sievers, S., *J. Chromatogr.*, 279, 555, 1983. With permission.

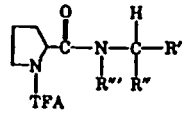
2. Hydroxy Acids and Carboxylic Acids

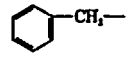
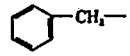
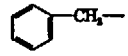
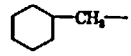
Lactic, α-hydroxyisovaleric, α-hydroxyisocaproic, and 3-phenylacetic acids were separated as (R)-(−)-methyloxycarbonyl methyl esters on trifluoropropylmethylpolysiloxane at 170°C with resolution factors of 1.09, 1.14, 1.15, and 1.14, respectively.[82] The separation of the (+)-3-methyl-2-butyl esters of *O*-trifluoroacetylated (*O*-TFA) or *O*-trimethylsilylated (*O*-TMS) 2-hydroxy acids and of branched carboxylic acids was investigated.[83] Glass capillary columns coated with SE-30 or OV-17 and flame ionization detection were used. The *O*-TFA derivatives were more volatile and showed slightly larger separation factors than the *O*-TMS derivatives. After reacting with isopropyl isocyanate urethane derivatives of racemic α-hydroxy acid esters, the urethane derivatives of chiral α-hydroxy acid esters were separated on XE-60-*S*-valine-*S*-α-phenylethylamide by Koenig and co-workers.[84] They also separated 3-hydroxy acids as *N-tert*-butylcarbamate/*tert*-butylamide derivatives and *N*-isopropylcarbamate/isopropylamide derivatives on fused silica capillary columns of XE-60-L-valine-(*S*)-α-phenylethylamide. The separation factors of *tert*-butyl and isopropyl derivatives are listed in Table 5.[85] Using optical rotation, it was determined that the (*R*) enantiomers of carbamate/amide derivatives were eluted after the (*S*)-enantiomers.

3. Amine

A series of diastereoisomeric *N*-TFA-*S*-prolylamides derived from asymmetric amines and amino acid esters were separated.[86] The data in Table 6 were obtained using two columns in series. One consisted of a relatively nonpolar trifluoropropyl-methyl silicone phase (QF-1), and the other of a relatively polar ethylene-glycol adipate phase (EGA). As can be seen in Table 6, the diastereoisomeric amides were resolved to a greater extent on the polar column. Also, *SS* diastereoisomers elute after the *SR* forms. This indicates that the open-chain structure of the *SS* diastereoisomer has an amide hydrogen more available for interaction

Table 6
SEPARATION DATA OF *N*-TRIFLUOROACETYL-*S*-PROLYLAMIDES



Compound No.	Asymmetric compound	R'	R''	R'''	Column[a]
15	1-Amino-1-phenylethane		CH_3	H	5% QF – 1 A 0.5% EGS B
16	1-Amino-1-cyclohexylethane		CH_3	H	5% QF – 1 A 0.5% EGA B
17	Amphetamine		CH_3	H	5% QF – 1 A 0.5% EGA B
18	Desoxyephedrine		CH_3	CH_3	5% QF – 1 A 0.5% EGA B
19	Phenylglycine ethyl ester		$-COOC_2H_5$	H	5% QF – 1 A 0.5% EGA B
20	Cyclohexylglycine ethyl ester		$-COOC_2H_5$	H	5% QF – 1 A 0.5% EGA B
21	Phenylalanine methyl ester		$-COOCH_3$	H	5% QF – 1 A 0.5% EGA B
22	Cyclohexylalanine methyl ester		$-COOCH_3$	H	5% QF – 1 A 0.5% EGA B

Compound No.	Uncorrected retention of diastereoisomers (min.) SR	SS	Relative retention α	$\Delta(\Delta G^{\ddagger})$ cal/mole	T[b]
15	3.80	4.50	1.20	−175	210
	5.90	8.00	1.37	−284	180
16	3.70	4.15	1.13	−117	210
	2.70	3.35	1.27	−211	180
17	5.15	5.90	1.15	−134	210
	3.10	3.80	1.25	−209	200
18	8.80	9.80	1.12	−108	210
	4.80	5.30	1.11	−98	200
19	10.20	10.20	1.00	...	210
	7.90	8.70	1.10	−89	200
20	10.10	10.90	1.08	−74	210
	4.20	4.80	1.15	−131	200
21	12.00	13.00	1.08	−74	210
	10.40	10.70	1.03	−28	200
22	10.25	10.90	1.07	−65	210
	5.10	5.80	1.14	−122	200

[a] Column A, 5 ft × 1/8 in. of 5% w/w QF-1 on Aeropak 30. Column B, 5 ft × 1/8 in. of 0.5% w/w ethylene glycol adipate on Aeropak 30.

[b] Inert gas peak times T, 180 to 210°C → 0.25 min.

From Westley, J. W., Halpern, B., and Karger, B. L., *Anal. Chem.*, 40, 2046, 1968. With permission.

with the liquid stationary phase than the *SR* form does. The following conclusions were made: (1) the polar phase gave a better separation of diastereoisomeric esters and amides, (2) it was necessary to have the two asymmetric centers close to one another, and (3) the separation was more difficult when polar groups were attached to the asymmetric center.

Koenig and co-workers reported the separation of chiral amines after reacting them with isopropyl isocyanates on a XE-60-*S*-valine-*S*-α-phenylamide stationary phase.[84] In this case the chiral amines were converted into urea derivatives. Also, the separation of chiral amines was reported following derivatization with *N*-fluoroacetyl-L-alanine and its chlorides.[77]

4. Amino Acids

Some amino acids were separated in the form of *N*-trifluoro acetyl (*N*-TFA) esters of 2-*n*-alkahols.[87] Frank and co-workers developed this method as well as another which utilized *N*-pentafluoropropyl isopropyl esters.[48,49,53] The derivatives were prepared by esterification in the presence of HCl and further treatment of the resulting amino ester hydrochlorides in a methylene chloride solution. The resolution of six representative amino acids using 2-butanol and 2-octanol esters of *N*-trifluoroacetylated acids was reported. However, this method had several limitations. It was difficult to separate the enantiomers of proline, pipecolic acid (homoproline), and α-alkylated amino acids, and it was impossible to separate *N*-methylamino acids. Interestingly, isocyanate derivatives of these compounds could be resolved.[85] The formation of *N*-alkylureido derivatives of amino acid esters increased the selectivity of the CSP toward the enantiomers, resulting in complete separations. *N*-Alkylureido/*N*-alkylamide derivatives were formed in one step by reacting isocyanates with *N*-methylamino acids. Using this procedure, *N*-methyl amino acids could be resolved on CSPs (XE-60-L-valine-(*R*)-α-phenylethylamides) for the first time.

Pollock and co-workers reported the resolution of 21 racemic amino acids as TFA-*sec*-butanol ester derivatives on a Carbowax® 1540 column (see Table 7).[88,89] While good resolution was found for most racemates, the *O*, *N* or *S*-di-TFA derivatives of serine, hydroxyproline, and cysteine were not adequate. Some difficult-to-resolve compounds (e.g., tyrosine and tryptophan) required *O*-acetyl-*N*-TFA,*NN*-di-TFA, and *N*-TFA-free OH derivatives for enantiomeric separation.[90] Most amino acids were separated on polar phases such as Carbowax® 20 M, but tyrosine and tryptophan were separated on less polar phases such as DEGS. Some α-amino acids (alanine, valine, leucine, and phenylalanine) were separated as *R*-(−)-methyloxycarbonyl methyl esters on a 5 ft × 1/8 in. column packed with 5% QF-1 on Aeropak 30.[82] The separation factors were 1.06, 1.10, 1.07, and 1.10, respectively.

The *N*-TFA-amino acid methyl ester derivatives were also used for determining the optical purity of these amino acids.[91] Polyfunctionalized amino acids were separated as *N*-TFA-L-prolyl peptide methyl ester derivatives.[92] The use of an optically active acyl group with a known configuration as a derivatizing agent, namely, the L-α-chloropropionyl moiety, for the analysis of amino acid was reported by Lande and Landowne.[93]

C. Conclusion

Several different racemic compounds were separated on a variety of GC liquid stationary phases.The vast majority of these racemates were amino acid derivatives. Most of the CSPs in GC also utilized various amino acids in one form or another. The research on the separation of enantiomers by GC has decreased considerably in the last few years. In this same time period, the research on CSPs in LC accelerated tremendously. There is a need to develop a greater variety of chiral GC phases that will allow the resolution of some underivatized enantiomers. Currently, at least four factors limit the GC separation of enantiomers. One problem (common to all GC) is that the solutes must be volatile. Second, the relatively high column temperatures, for entropic reasons, cause the stability differences between the diastereomeric adsorbates to be quite small and the separation of the enantiomers to be minimal. Moreover, the high column temperature often causes racemization of the chiral stationary phase, resulting in a loss of enantioselectivity with time. There is also some concern over the racemization of the analyte during analysis. Finally, large-scale preparative separations are generally not feasible in GC.

III. LIQUID CHROMATOGRAPHY

Although reports on low-pressure column LC separation of a few racemates appeared in the 1970s, the 1980s is proving to be a major turning point in the field for the following

Table 7
CHARACTERISTIC GC DATA FOR 21 RESOLVED AMINO ACIDS[a]

Amino acid	Retention time[b]	Relative retention time[c]	LD/LL	N	HETP
Valine (LL-DD)	14.7	0.426	1.07	23,104	1.97
(LD-DL)	15.7	.455		21,316	2.14
Alanine	19.1	.553	1.04	12,800	3.57
	19.9	.576		11,302	4.05
α-Aminobutyric	19.1	.553	1.04	12,800	3.57
	19.9	.576		11,302	4.05
Isoleucine and	20.9	.605	1.07	20,357	2.24
(Allo)	22.4	.649		15,267	2.99
Norvaline	31.0	.898	1.05	22,697	2.01
	32.6	.944		30,527	2.00
Leucine	32.4	.939	1.06	36,548	1.25
	34.5	1.00		52,159	0.87
Norleucine	40.1	1.16	1.05	38,677	1.18
	42.0	1.22		43,589	1.05
Serine (di-TFA)[d]	52.3	1.52	1.02	—	—
	53.2	1.54		—	—
Glycine	46.6	1.35	—	18,496	2.47
Proline	52.8	1.53	1.04	127,286	.35
	55.0	1.59		50,755	.30
α-Amino	61.0	1.77	1.02	101,483	.45
Octanoic	62.1	1.80		116,704	.39
Cysteine (di-TFA)[d]	65—75	—	—	—	—
Hydroxyproline[d]	74.9	2.17	1.02	—	—
(di-TFA)	76.2	2.21		—	—
Aspartic acid	82.2	2.38	1.01	—	—
	83.1	2.41		—	—
Threonine	83.6	2.42	1.02	92,153	.49
(mono-TFA)	85.3	2.47		99,396	.46
Methionine	88.8	2.57	1.02	179,538	.25
	90.8	2.63		170,622	.26
Phenylalanine	104.5	3.03	1.03	115,902	0.39
	107.2	3.11		118,807	.38
Glutamic	133.7	3.88	1.03	18,605	2.46
acid	137.8	3.99		23,104	1.98
Ornithine[e]	2.65 hr	—	1.01	—	—
	2.67 hr	—		—	—
Lysine[f]	3.83 hr	—	1.02	—	—
	3.92 hr	—		—	—
β-Aminoisobutyric	23.5	.681	1.04	—	—
	24.5	.710		—	—

[a] Carbowax® 1540: 0.02 in. × 150 ft (Perkin-Elmer K-1540); helium carrier gas, 7.94 mℓ/min; program isothermal at 100°C for 25 min; then program to 140°C at 1°C/min.

[b] Retention times accurate to a SD of ±2.0% or less.

[c] Relative retention times calculated relative to the LD peak of leucine.

[d] Poorly resolved amino acids (only 5 to 30% resolved).

[e] Perkin-Elmer R column (Ucon LB 550-X), 0.02 in. × 150 ft; helium, 10.5 mℓ/min; program 63 to 140°C at 2°C/min.

[f] Same as ornithine except program was isothermal at 140°C; HETP = L/N (mm); $N = 16(X/Y)^2$.

From Pollock, G. E. and Oyama, Y. I., *J. Gas Chromatogr.*, 4, 126, 1966. With permission.

reasons: (1) a tremendous number of new and improved CSPs for high-pressure liquid chromatography [HPLC]) and additives were introduced and and accompanied by a corresponding increase in publications in this area;[94-107] (2) the first widespread commercialization of CSPs occurred during this time; and (3) a number of extensive theoretical and mechanistic studies involving chiral recognition began to appear. The last factor will lead to a greater understanding and optimization of these techniques. There are at least two general approaches for direct LC separation of enantiomers. The first involves the use of CSPs, and the second involves the use of chiral mobile-phase additives in conjunction with achiral or chiral stationary phases. Besides using CSPs or chiral mobile-phase additives, derivatizing a pair of enantiomers with chiral derivatizing agents and using achiral or chiral stationary phases is another option available for the achievement of enantiomeric separations. In this review, CSPs and chiral mobile-phase additives are examined. However, the indirect methods are not included.

A. CSPs

1. Cyclodextrin Phases

Cyclodextrins are cyclic oligosaccharides produced by the action of the enzyme cyclodextrin transglycosylase on starch. Their rough geometry is that of a hollow truncated cone with both ends open.[108-110] These macrocyclic oligomers contain from 6 to 12 D-(+)-glucopyranose units which are bonded through α-(1,4) linkages with all glucose units in the chair conformation (Figure 8). α-Cyclodextrin (α-CD, cyclohexaamylose) has six glucose units, β-cyclodextrin (β-CD, cycloheptaamylose) has seven glucose units, and γ-cyclodextrin (γ-CD, cyclooctamylose) has eight glucose units. These three naturally occurring CDs are commercially available. CDs complex a wide variety of guest organic and inorganic molecules or ions. The internal diameter of α-CD varies from about 4.5 to 6.0 Å, which is a good size for complexing a single five- or six-membered aromatic ring. The internal diameter of β-CD varies from 6 to 8 Å and can easily accommodate molecules the size of biphenyl or naphthalene. The internal diameter of γ-CD varies from approximately 8 to 10 Å. Molecules as large as substituted pyrenes can bind to γ-CD. All cyclodextrins are chiral. The secondary 2- and 3-hydroxyl groups line the mouth of the CD cavity, while the primary 6-hydroxyl groups are at the opposite end of the molecule (Figure 8). Since all of the primary and secondary hydroxyl groups are on the outside of the molecule, the cavity is relatively hydrophobic. Consequently, CDs are able to form inclusion complexes with a variety of water-insoluble and sparingly soluble molecules. Among the more important parameters that determine whether an inclusion complex can be formed are the relative size and geometry of the guest molecule in relation to the dimensions of the host CD cavity. There are several different sizes of CDs, allowing one to separate a variety of enantiomers.

Early papers reported the use of α-, β-, and γ-CD polymer gels for the resolution of mandelic acid derivatives,[111] amino acids,[112,113] and alkaloids[114,115] by column chromatography. However, these polymer gels are not suitable for HPLC due to poor mechanical strength and low efficiency.

It is interesting to consider whether or not CD complexes with chiral molecules satisfy the three-point chiral recognition model.[116] The simplified model in Figure 9 shows a three-point attachment with the hydrophobic group in the cavity and two of the groups projecting radially from the mouth of the CD cavity. There is the possibility of three points of interaction for many inclusion complexes.

A tight CD inclusion complex seems to be a necessary requirement for chiral recognition in many cases. It may be possible that a one- or two-point attachment accompanied by additional specific steric restraints[117,118] could be responsible in some cases for the observed enantioselectivity. Due to the strict stereochemical requirements and the intimacy of the inclusion complex, relative retention on CD-bonded phases may be a useful means of assigning absolute configuration to a series of structurally related compounds.

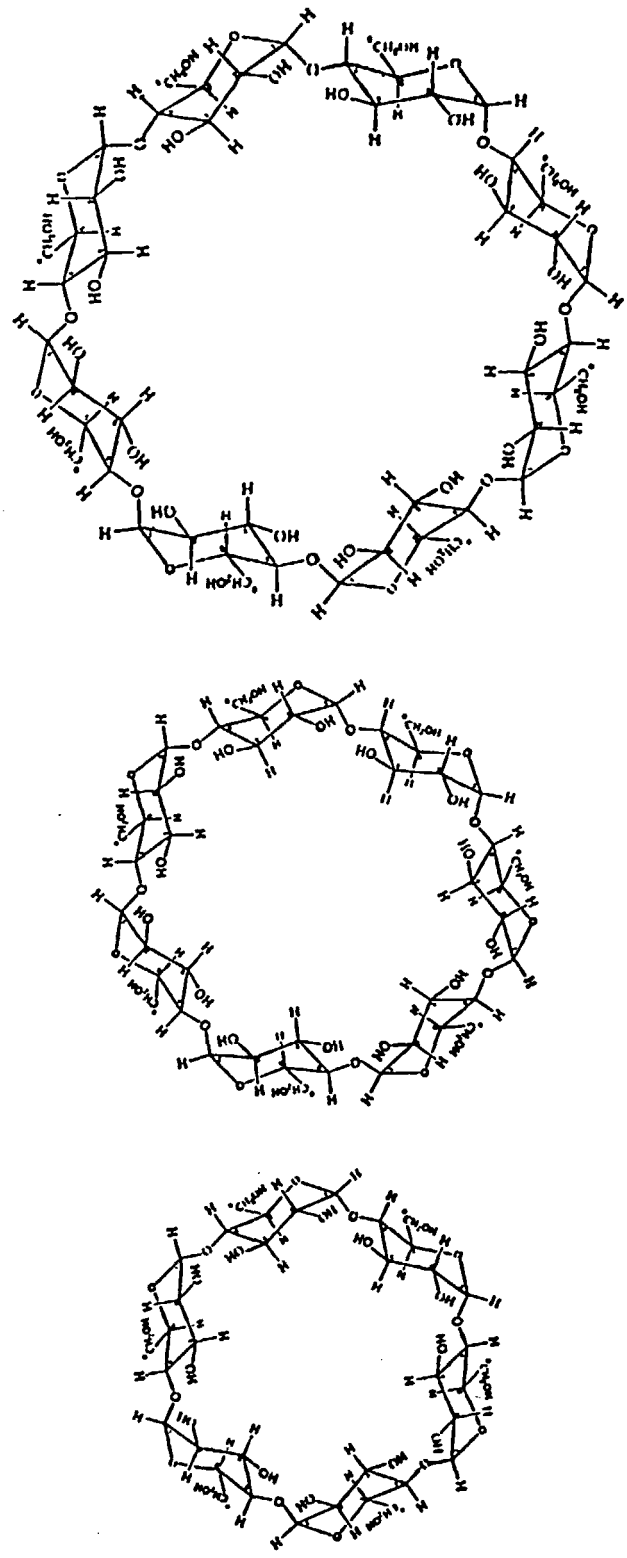


FIGURE 8. Schematic showing the molecular structures of cyclodextrins. (From Armstrong, D.W. and Demond, W., *J. Chromatogr. Sci.*, 22, 411, 1984. With permission.)

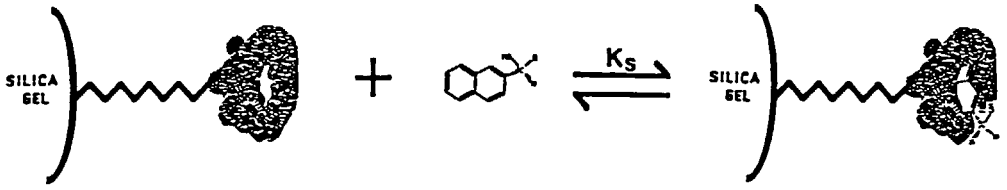


FIGURE 9. A schematic of cyclodextrin bonded to a silica gel support and forming of an inclusion complex with a chiral molecule. (From Armstrong, D. W. and Li, W., *Chromatography*, 2, 43, 1987. With permission.)

Some research groups tried to attach different CDs to silica gel via ethylene diamino linkages.[119-121] However, these packings had several problems: (1) they were hydrolytically unstable, (2) the CD loading was often low, (3) the amines presented affected selectivity, (4) nitroxide formation occurred during synthesis, and (5) the synthesis was often tedious. A recent paper[122] described the preparation and retention characteristics of a CD carbamate-bonded stationary phase which separated enantiomers of mandelic acids and other related compounds. Feitsma and co-workers have reported the separation of the enantiomers of some aromatic carboxylic acids.[123,124]

Other packings which contained no nitrogen-based linkages were developed by Armstrong.[125] These packings were hydrolytically stable under standard LC conditions and were used to resolve a variety of difficult-to-separate isomers and enantiomers.

CD-bonded phases have several advantages over other chiral phases. They were the first CSPs deliberately designed to be used in the reversed phase mode. There are several different cavity sizes, enabling one to separate a variety of different size of enantiomers. Currently, α-CD, β-CD,and γ-CD columns are commercially available (see Table 8) as are acetylated versions of these columns. CD-bonded phases are most often used with water-methanol or water-acetonitrile mobile phases.[125-127] The addition of buffers and/or salts to the mobile phase tends to improve the efficiency significantly in many separations. Changing the pH of the aqueous portion of the mobile phase greatly affects the retention and selectivity of ionizable isomers.[128] Retention and resolution are controlled by altering the amount of organic modifiers (e.g., methanol, acetonitrile, ethanol, etc.) in mobile phases. Generally, retention and resolution decrease with increasing modifier concentration. Recently, separations of enantiomers have been obtained using pure organic modifiers or with very high organic modifier concentrations.[129,130] This is an indication that CD-bonded phases might be useful in normal or mixed-phase modes. Despite the experimental similarities to traditional reversed phase LC, there are a number of significant differences. For example, inclusion complexation plays a significant role in retention. Temperature, ionic strength, and pH effects are generally more pronounced on CD-bonded phases than on reversed phase columns. CD-bonded phases have been used in gradient elution. Currently, CD-bonded phases are the least expensive chiral phases. Preparative separations are possible, and preparative columns are commercially available.

After extensive use (particularly when using mobile phases with a very high percentage of water), the column's efficiency and retention times decrease. This is because the CD cavity is occupied by strongly retained impurities. This problem can be easily solved by washing it with an organic modifier (e.g., absolute methanol, ethanol, or acetonitrile).

CD-bonded phases were used for the separation of metallocene enantiomers, including 13 ferrocene racemates, ruthenocene, and osmocene analogs.[131] Separation of β-naphthyl ester derivatives of amino acids, barbiturates, substituted phenylacetic acid,[132] and dansyl-amino acids also was reported by Armstrong and Demond.[133] Nicotine and nicotine analogs[286] as well as several chiral crown ethers were separated using β-CD bonded columns.[129] Tryptophan, phenylalanine, tyrosine, and their analogs were separated using α-CD-bonded columns.[134]

Table 8
EXAMPLES OF COMMERCIALLY AVAILABLE CHIRAL STATIONARY PHASES[a]

Chiral phase type	Manufacturer[b]	Typical mobile phase used	Representative compounds separated
		π-Complex/Hydrogen Bond	
(*R*)- or (*S*)-*N*-(3,5-dinitrobenzoyl)-phenylglycine, available ionically or covalently bonded and as the racemate	Regis, Baker, Alltech	Normal phase (hexane/isopropanol)	Aromatic sulfoxides; 3-5-dinitro-benzoyl derivatives of amines, alcohols, thiols, amino acids, amino alcohols, and hydroxy acids; aryl-substituted lactams, succinimides, hydontoins, hydroxylphosphonates; oxazolidones; binaphthols, drug derivatives
(*S*)-*N*-(3,5-dinitrobenzoyl)-leucine, ionically or covalently bonded	Regis, Baker	Normal phase (hexane/isopropanol)	Aromatic sulfoxides; 3-5-dinitro-benzoyl derivatives of amines, alcohols, thiols, amino acids, amino alcohols, and hydroxy acids; aryl-substituted lactams, succinimides, hydontoins, hydroxylphosphonates; oxazolidones; binaphthols, drug derivatives
(*R*)- or (*S*)-*N*-(2-naphthyl)-alanine	Regis	Normal phase (hexane/isopropanol)	*Very* high selectivities (αs) for a variety of dinitrobenzoyl-derivatized compounds
(*S*)-(1-α-naphthyl)ethylamine	Sumitomo	Normal phase (hexane/isopropanol)	3,5-Dinitrobenzoyl derivative of amino acids, 3,5-dinitroanilide derivatives of carboxylic acid
(*R*),(*R*)- or (*S*),(*S*)-α-naphthylethyl-aminocarbonyl-valine; several other analogous CSPs based on different amino acid derivatives offered by this company	Sumitono	Normal phase (hexane/isopropanol)	3,5-Dinitrobenzoyl derivatives of amines, amides, amino acid esters, and fungacides; 3,5-dinitroanilide derivatives of carboxylic acids; other derivatives of the above compounds
		Cylodextrin	
β-Cyclodextrin	Advanced Separation Technologies	Reversed phase[c] (aqueous buffers/acetonitrile or	Dansyl and naphthyl amino acids, several aromatic drugs, steroids, alkaloids, metallocenes, binaphthyl crown ethers, aromatic acids

Table 8 (continued)
EXAMPLES OF COMMERCIALLY AVAILABLE CHIRAL STATIONARY PHASES[a]

Chiral phase type	Manufacturer[b]	Typical mobile phase used	Representative compounds separated
α-Cyclodextrin	Advanced Separation Technologies	Reversed phase (aqueous buffers/acetonitrile or methanol)	Barbiturates
γ-Cyclodextrin	Advanced Separation Technologies	Reversed phase (aqueous buffers/acetonitrile or methanol)	Stereoisomers of polycyclic aromatic hydrocarbons
Acetylated β-cyclodextrin	Advanced Separation Technologies	Reversed phase (aqueous buffers/acetonitrile or methanol)	Steroids, PAHs
Chiral Polymers			
Bovine serum albumin	Macherey Nagel	Aqueous buffers	*N*-Aroyl amino acids, aromatic sulfoxides, some drugs
α-Acid glycoprotein	LKB	Aqueous buffers	Wide variety of cyclic and aromatic drugs
Cellulose esters, acetate, benzoate, cinnamate, phenylcarbamate	Daicel	Normal phase (hexane/isopropanol)	Sulfoxide derivatives, organo-phosphorous compounds, and insecticides; barbiturates, cyclic thioamides, disubstituted cyclic racemates
Cellulose tribenzyl ether	Daicel	Normal phase or alcohols	Analogous to above
(+)-Poly(triphenylmethyl methacrylate)	Daicel	Methanol	Binaphthalene derivatives, cyclic insecticides, transdisubstituted racemates, *bis*-azulenes, derivatized glucitol
(+)-Poly(2-pyridyldiphenyl-	Daicel	Methanol	Analogous to above with somewhat different selectivities
Ligand Exchange			
Hydroxyproline	Macherey Nagel	Aqueous buffer/Cu^{2+}	Underivatized and some derivatized amino acids
Amino acid; two types offered	Daicel	Aqueous buffer/Cu^{2+}	Underivatized and some derivatized amino acids

[a] All CSPs utilize silica gel as the support.

[b] Company addresses in alphabetical order: Advanced Separation Technologies, Inc., 37 Leslie Ct., P.O. Box 297, Whippany, N.J. 07981; Alltech Associates, 2051 Waukegan Rd., Deerfield, Ill. 60015; J.T. Baker, Co., 222 Red School La., Phillipsburg, N.J., 00865; Daicel Chemical Industries, Ltd., 8-1, Kasumigaseki 3-chome, Chiyodo-ku, Tokyo; LKB Products, Box 305, S-16126 Bromma, Sweden; Macherey Nagel & Co., Neumann-Neander-Strasse 68, Postfach 307, D-5160 Duren, W. Germany; Regis Chemical Co., 8210 Austin Av., Morton Grove, Ill. 60053; Sumitomo Chemical, Sumitomo Bldg., 5-15, Kitaham, Higashi, Osaka, 541 Japan.

[c] Different selectivity separations are sometimes achieved in 100% organic modifier.

From Armstrong, D. W., *Anal. Chem.*, 59, 84A, 1987. With permission.

The separation of several drug stereoisomers was accomplished on a β-CD-bonded column by Armstrong and co-workers.[135] Figure10 shows a three-dimensional computer-generated projection of the lowest free energy inclusion complexes of *d*- and *l*-propranolol with β-CD. In this configuration, *d*- and *l*-propranolol are placed identically within the CD cavity, and the structures are overlaid exactly at the point of the chiral carbon. Important differences are observed between the complexes of the *d* and *l* isomers with respect to their secondary amine group. In the *d*-propranolol complex, the nitrogen is ideally placed for hydrogen bonding to both a 2- and 3-hydroxyl group of the CD, with respective bond distances of 3.3 and 2.8 Å. The amine in the *l*-propranolol complex is positioned less favorably for hydrogen bonding; the bond distances to the closest 2- and 3-hydroxyls of the CD are 3.8 and 4.5 Å, respectively. This suggests that the *d* isomer would preferentially interact with the β-CD and thereby be retained longer. The principle of three-point chiral recognition is also apparent from this model. Table 9 gives retention data for several racemic drugs using β-CD-bonded columns. Low resolution enantiomers were separated by micro HPLC.[130] One of the advantages of a microcolumn is the high theoretical plate numbers. This is advantageous for difficult-to-separate compounds.

2. π-Complex Hydrogen-Bonding Phases

The three-point chiral recognition model has been used as a basis for the design of several stationary phases. The first commercially successful CSP was developed by Pirkle and Finn.[136] The CSP consisted of (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine attached ionically or covalently to γ-aminopropylsilanized silica gel (Figure 11). These CSP phases were used to separate a series of primary amines, amino alcohols, and amino acid derivatives.[137,138] Later, ionic *N*-(3,5-dinitrobenzoyl) leucine and covalent *N*-(3,5-dinitrobenzoyl) leucine phases were synthesized. The *N*-(3,5-dinitrobenzoyl) leucine phase supplemented the *N*-(3,5-dinitrobenzoyl) phenylglycine packing, thus expanding the applicability of "π-electron acceptor/hydrogen bond" stationary phases for racemic separations by normal phase LC. In some cases, the leucine-based columns separated aromatic solutes more effectively. The CSP and chiral solute could interact by three-point contact (e.g., there might be a π-π donor-acceptor interaction, a hydrogen bond, and a dipole interaction). For example, a dinitrobenzoyl group on the stationary phase is a π-acceptor, so the solute to be resolved should have π-donor groups (e.g., alkyl, *OR*, NR_2 or *SR* substituents). If two diastereomeric adsorbates or enantiomers have sufficiently different energies, separation can be achieved. The success of this approach is evident given the large number and variety of separations reported.

The covalently bonded version of these CSPs can be used with somewhat higher polarity mobile phases, and the enantioselectivity and stability are greater than those of the ionic columns. However, the column should not be stored in water or hydro-organic mobile phases. The ionically bonded CSPs are limited to mobile phases which require no more than 20% propanol (or lower polarity solvents) in hexane.

A number of additional π-complex hydrogen-bonding stationary phases have been synthesized by Pirkle and co-workers,[139-150] and used for separating a wide range of compounds including some mono-ol, epoxide, and diol enantiomers of polycyclic aromatic hydrocarbons such as phenanthrene, chrysene, benz[a]anthracene, monoethylbenz[a]anthracene, 7,12-dimethyl-benz[a]anthracene, dibenz[a,h]anthracene, cholanthracene, 3-methylcholanthrene, and benzo[a]pyrene.[139-158] They have also been used for determining enantiomeric purities and absolute configuration. Being synthetic, these CSPs are typically available in either absolute configuration. Changing the configuration of the CSP changes the elution order of the enantiomers. Several different π-complex hydrogen-bonding normal-phase packings are available. Enantioselectivities (αs) ranged from 1 to 5 for π-electron acceptor columns and 1 to 16 for π-electron donor columns. Enantioselectivities exceeding 100 have recently been reported on a commercial version of this new generation of CSPs.[159] In a recent paper[145],

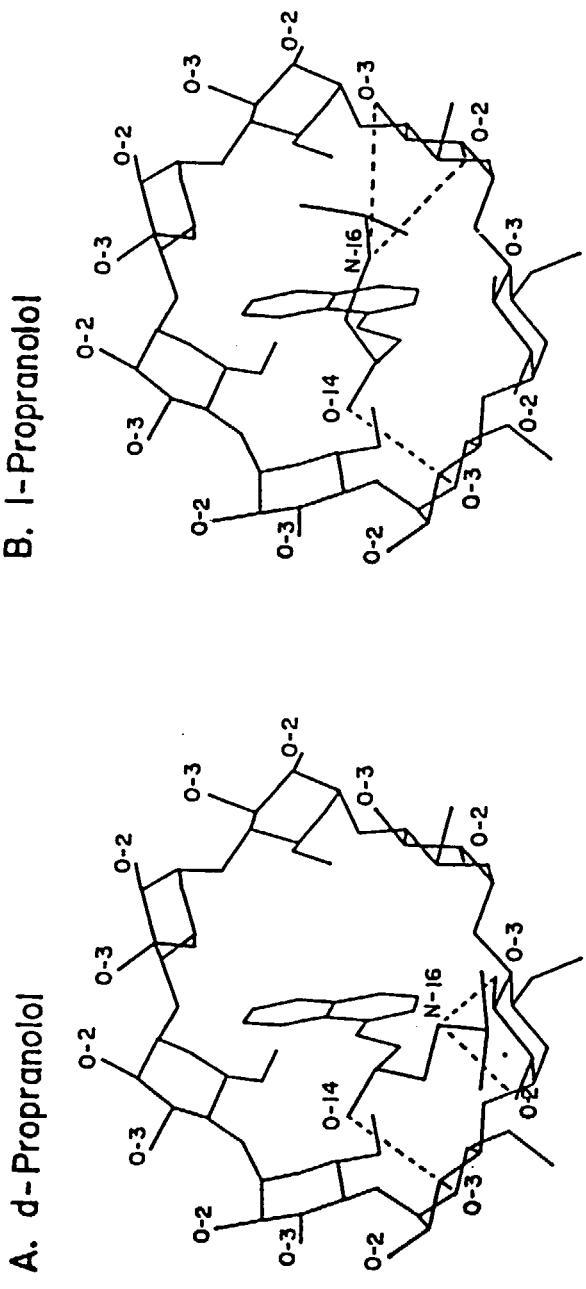


FIGURE 10. Computer projections of inclusion complexes of (A) *d*-propranolol and (B) l-propranolol in β-cyclodextrins taken from X-ray crystallographic data. (From Armstrong, D. W., Ward, T. J., Armstrong, R. D., and Beesley, T. E., *Science*, 232, 1132, 1986. With permission.)

Table 9
STRUCTURE AND SEPARATION DATA FOR ENANTIOMERIC DRUGS

Drug	k'_1[a]	α	R_s	Mobile[b] phase	Column
β-Adrenergic blockers					
Propranolol	2.78	1.04	1.40	25/75	[c]
Metoprolol	3.51	1.03	0.90	32/68	[c]
Antihistamine					
Chlorpheniramine	5.86	1.07	1.51	15/85[d]	[e]
Calcium channel blockers					
Verapamil	2.94	1.03	0.71	[f]	[e]
Nisolidipene	4.13	1.04	0.87	30/70	[c]
Nimodipene	5.09	1.05	1.10	30/70	[c]
Diuretic					
Chlortahlidone	0.50	1.44	1.95	30/70	[e]
Sedative-anticonvulsant					
Hexobarbital	9.39	1.14	1.51	15/85	[g]
Mephobarbital	14.80	1.14	1.60	20/80	[g]
Mephentoin	0.48	1.33	1.83	40/60	[e]
Triazoline	5.00	1.15	1.50	40/60[d]	[e]
Phensuximide	1.97	1.15	1.54	10/90[d]	[e]
Anticorticosteroid					
Aminoglutethimide	7.49	1.03	0.91	[f]	[e]
Nonsteroidal, anti-inflammatory agent					
Ketoprofen	7.67	1.06	1.24	27/73	[c]
Narcotic-analgesic					
Methadone	2.38	1.04	0.81	[f]	[e]
CNS stimulant					
Methylpehnidate	1.17	1.14	1.57	10/90[d]	[e]

[a] Capacity factor of the first elution isomer.
[b] Mobile-phase ratios indicate the relative volume of methanol of 1% aqueous triethylammonium acetate (pH, 4.1) unless otherwise indicated. Flow rates were 1.0 mℓ/min.
[c] Two 25-cm β-CD columns were used in series.
[d] Acetonitrile was used as the organic modifier in place of methanol.
[e] One 25-cm β-CD column was used.
[f] Separation was done with a gradient going from 10/90, acetonitrile/1% TEAA to 20/80, acetonitrile/1% TEAA in 20 min.
[g] One 10-cm β-CDcolumn was used.

From Armstrong, D. W., Ward, T. J., Armstrong, R. D., and Beesley, T. E., *Science*, 232, 1132, 1986. With permission.

a CSP consisting of (*S*)-(−)-omega-siloxyundecanyl *N*-(2-naphthyl) valinate covalently linked to microparticulate silica was shown to be effective for the direct chromatographic separation of the enantiomers of chiral amine and amino acids, 3,5-dinitrobenzamides, and 3,5-dinitroanilinocarbamates derived from chiral alcohols and thiols as their 3,5-dinitrobenzoyl or 3,5-dinitroanilinourea derivatives (see Table 10).

Wainer and co-workers have been involved in separating a variety of chiral compounds of pharmaceutical interest.[160-165] Direct enantiomeric separations of pharmaceuticals were done with commercially available columns (i.e., dinitrobenzoylphenylglycine, etc.). In these studies, pharmaceutical amines were first derivatized with an appropriate reagent. There were several reasons for this preliminary derivatization step. First, the free amines had poor

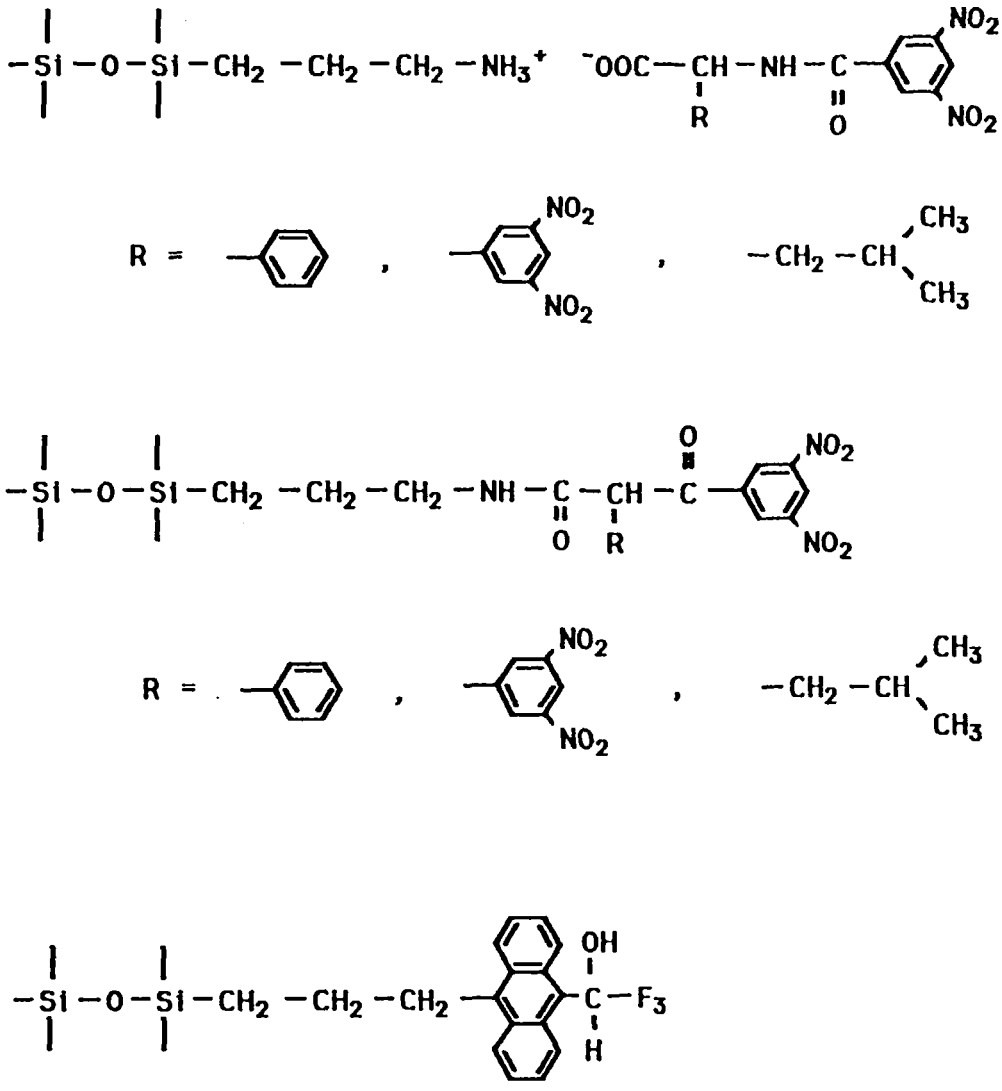
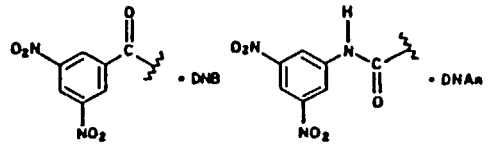


FIGURE 11. Structures of some of the π-complex hydrogen-bond stationary phases developed by Pirkle.

chromatographic properties on CSPs. Second, the derivatization often enhanced enantiomeric selectivity and increased ultraviolet or fluorescence detectability. In a recent paper, the enantiomers of primary, secondary, and some tertiary amines were resolved as carbamate derivatives formed by reaction with β-naphthylchloroformate.[166] A series of 15 derivatized amines were examined, and 12 amines structurally related to amphetamine were separated including pharmacologically important compounds such as methamphetamine and pseudoephedrin.

Oi and co-workers[167-174] focused their attention on the development of new and modified CSPs. The structures of several of these CSPs are shown in Figure 12. CSPs I, II, and III consist of (1*R*, 3*R*)-*trans*-chrysanthemic cid and its amide derivative chemically bonded to γ-aminopropyl silanized silica gel. (*S*)-1-(α-naphthyl)ethylamine (CSP IV and V)2-(4-chlorophenyl)isovaleric acid and its amide derivatives (CSP VI, VII, and VIII) are chemically bonded to aminopropyl silanized silica gel. CSPs II and III, which contain two asymmetric carbon and nitrogen atoms of the amide group, show better enantioselectivity than CSP I.

Table 10
REPRESENTATIVE SEPARATION OF ENANTIOMERS ON A CSP WHICH IS *S*-(−)-OMEGA-SILOXYUNDECANYL *N*-(2-NAPHTHYL)VALINATE COVALENTLY LINKED TO SILICA



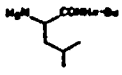
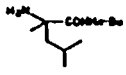
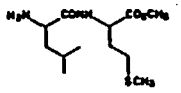
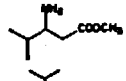
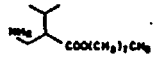
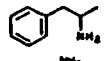
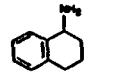
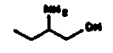
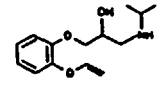
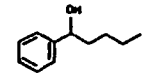
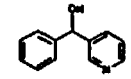
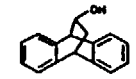
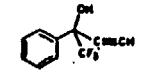
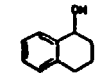
compound	derivative	separability factor (α)	k_1'[9]	R_s[10]	eluent, % v/v 2-propanol in hexane	most retained enantiomer[11]
		α- and *β*-Amino Acid Derivatives				
	DNB	17.66	0.38	10.5	10%	*S*
	DNB	1.45	7.97	4.2	5%	
	DNB	18.66	1.87	18.3	20%	*S,S*
	DNB	1.97	0.93	2.2	5%	*R*
	DNB	1.25	7.33	2.1	5%	
		Derivatives of Amines and Amino Alcohols				
	DNAn	1.19	5.87	1.5	5%	*R*
	DNAn	1.33	3.27	2.8	20%	*S*
	DNAn	1.19	6.37	2.1	5%	
	DNAn	2.42	14.87	10.6	5%	
	DNAn	4.53	1.35	7.7	20%	*S*
	bis-DNAn	1.41	13.2	3.6	20%	
		Derivatives of Alcohols and Thiols				
	DNAn	1.24	3.19	1.8	5%	*S*
	DNAn	2.51	4.87	10.6	5%	
	DNAn	1.20	8.71	2.0	5%	
	DNAn	1.47	4.87	3.2	5%	
	DNAn	1.22	5.35	2.5	5%	*S*
	DNAn	1.10	4.07	0.7	5%	

Table 10 (continued)
REPRESENTATIVE SEPARATION OF ENANTIOMERS ON A CSP WHICH IS *S*-(−)-OMEGA-SILOXYUNDECANYL *N*-(2-NAPHTHYL)VALINATE COVALENTLY LINKED TO SILICA

	DNAn	1.49	3.87	3.7	5%
	DNAn	1.50	2.94	2.7	5%
	DNAn	1.40	9.40	3.5	5%

From Pirkle, W. H. and Pochapsky, T. C., *J. Am. Chem. Soc.*, 108, 352, 1986. With permission.

These phases can separate not only carboxylic acid enantiomers but also amino acid enantiomers. All of these CSPs are normal-phase media. Figure 13 shows the chromatographic separation of racemic *N*-3,5-dinitrobenzoyl-2-octylamine on CSP II and enantiomers of racemic *N*-3,5-dinitrobenzoyl valine methyl ester separated on CSP III. CSP IX is derived from (*R*)- and (*S*)-1-(α-naphthyl) ethylamine with (*S*)-valine chemically bonded to γ-aminopropyl silanized silica. This stationary phase contains two asymmetric carbon atoms attached to two nitrogen atoms of the urea group.[175] The CSPs with the urea group can serve as either a donor or an acceptor in hydrogen bonding. The separations of some ester and alcohol enantiomers do not need any prederivatization steps using CSP IX. Oi's work has shown that there can be significant differences in selectivity for many of the related CSPs. In fact, it has been noted that there are selectivity differences in packings containing the same chiral base molecule but differing in whether they are attached covalently or ionically.

3. Chiral Polymer Phases

A number of enantiomers have been separated on CSPs of natural or synthetic chiral polymers. Typical examples include cellulose, microcrystalline cellulose triacetate, starch, polymerized amino acids, cross-linked polystyrene containing alkaloids, and isotactic (+)-poly(triphenylmethylmethacrylate).[176,177]

Gübitz and co-workers reported the possibility of using small particles of cellulose for the HPLC separation of enantiomers.[178] It was successfully utilized in HPLC by Lindner and Mannschreck.[179] Triacetyl cellulose was used for the HPLC separation of cyclic allene hydrocarbons. Good separations of the enantiomers of (±)*trans*-1,2-diphenyl cyclopropane have been achieved at low pressure (Figure 14). A wide variety of derivatives of cellulose (e.g., cellulose triesters, cellulose trisphenylcarbamate, cellulose nitrate, and cellulose methylcarbamate) have been used to separate racemic compounds.[180-182]

Okamoto and co-workers developed various polysaccharide derivatives of some cellulose stationary phases.[180] Five different polysaccharide derivatives were absorbed on macroporous silica gel (Figure 15). The properties of each of these adsorbents were described. Cellulose triacetate showed chiral recognition for many racemates and is especially effective for substrates with phosphorus atoms as an asymmetric center. However, the degree of chiral recognition generally is not high.

Cellulose tribenzoate showed good chiral recognition for racemates which have carbonyl groups in the vicinity of an asymmetric center. Cellulose trisphenylcarbamate phases have a strong affinity for polar substrates. Cellulose tribenzyl ether-derivatized cellulose is effective as a CSP when protic solvents such as methanol are used as mobile phases. Cellulose tricinnamate phases give long chromatographic retention times and good chiral recognition for many aromatic racemates and for barbiturates. Each adsorbent with polar ester or urethane sorbent sites seems to be able to interact through hydrogen bonding with racemates having –OH or –NH groups (Table 8).

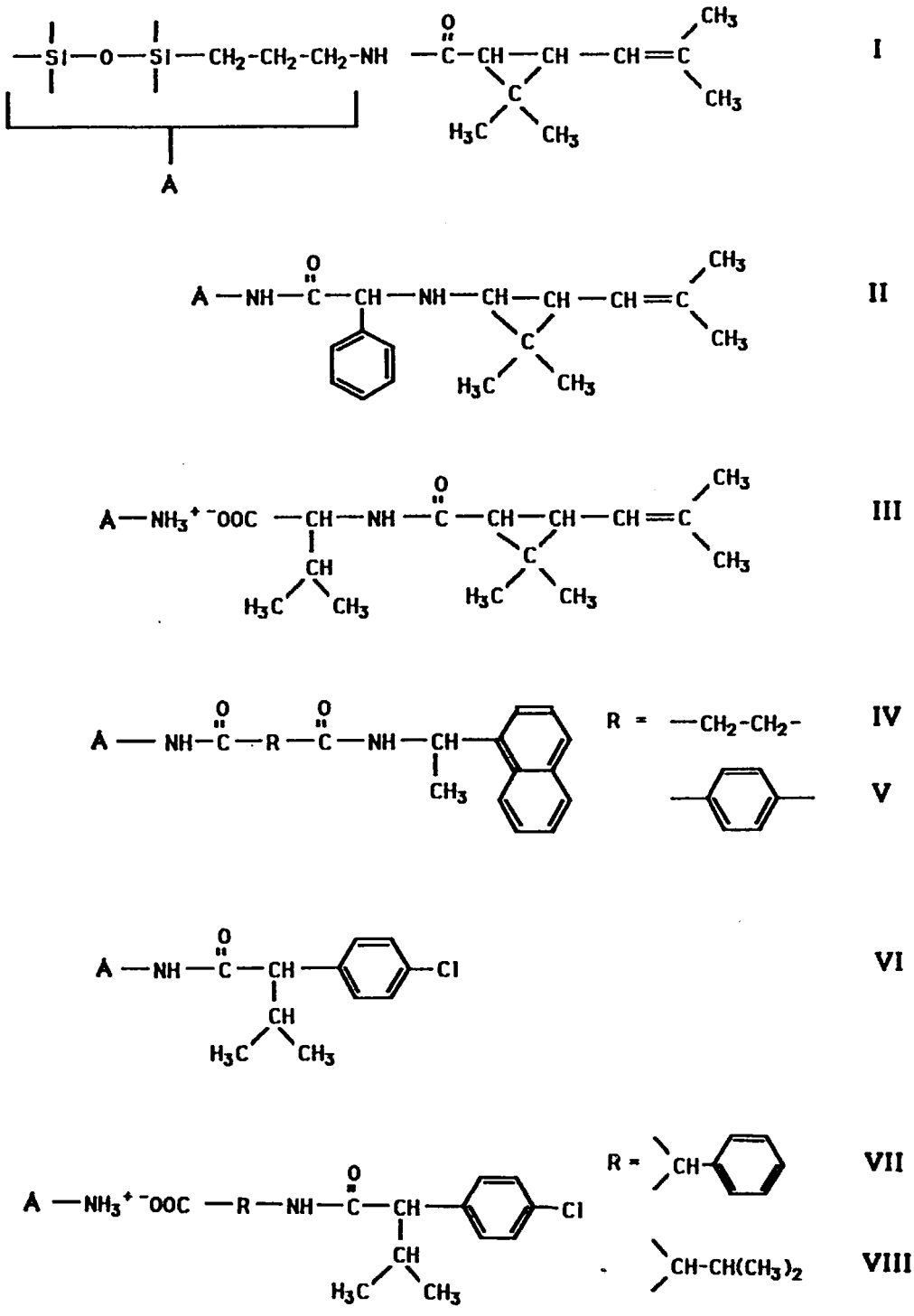


FIGURE 12. Structures of some of the π-complex hydrogen-bond stationary phases developed by Oi.

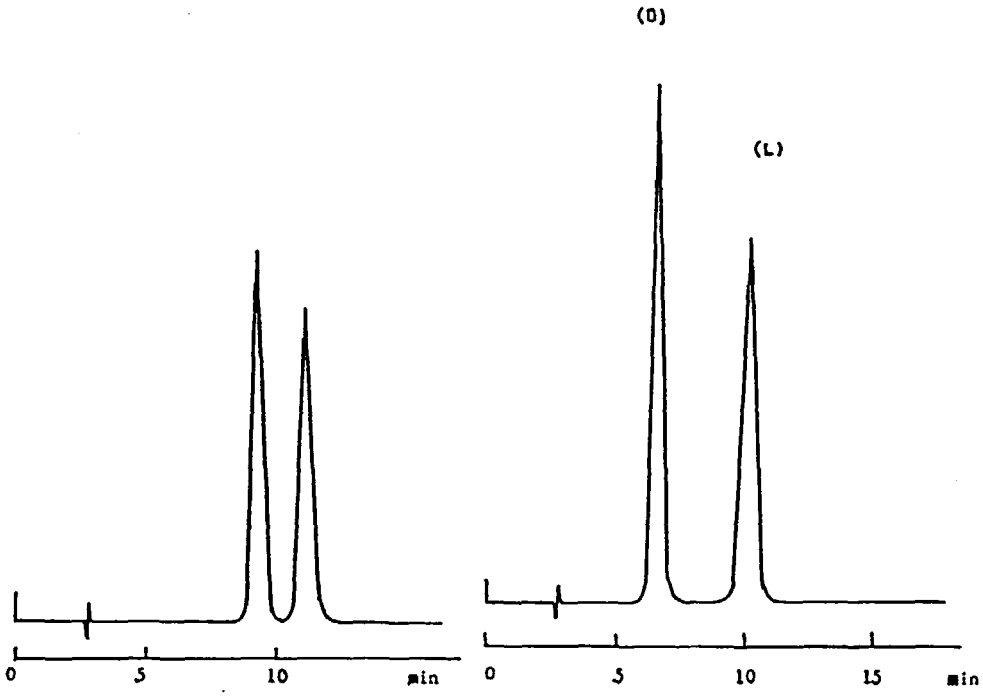


FIGURE 13. Liquid chromatographic separation of racemic *N*-3,5-dinitrobenzoyl-2-octylamine on CSP II and racemic *N*-3,5-dinitrobenzoylvaline methyl ester on CSP III. (From Oi, N., Nagase, M., Inda, Y., and Doi, T., *J. Chromatogr.*, 259, 487, 1983. With permission.)

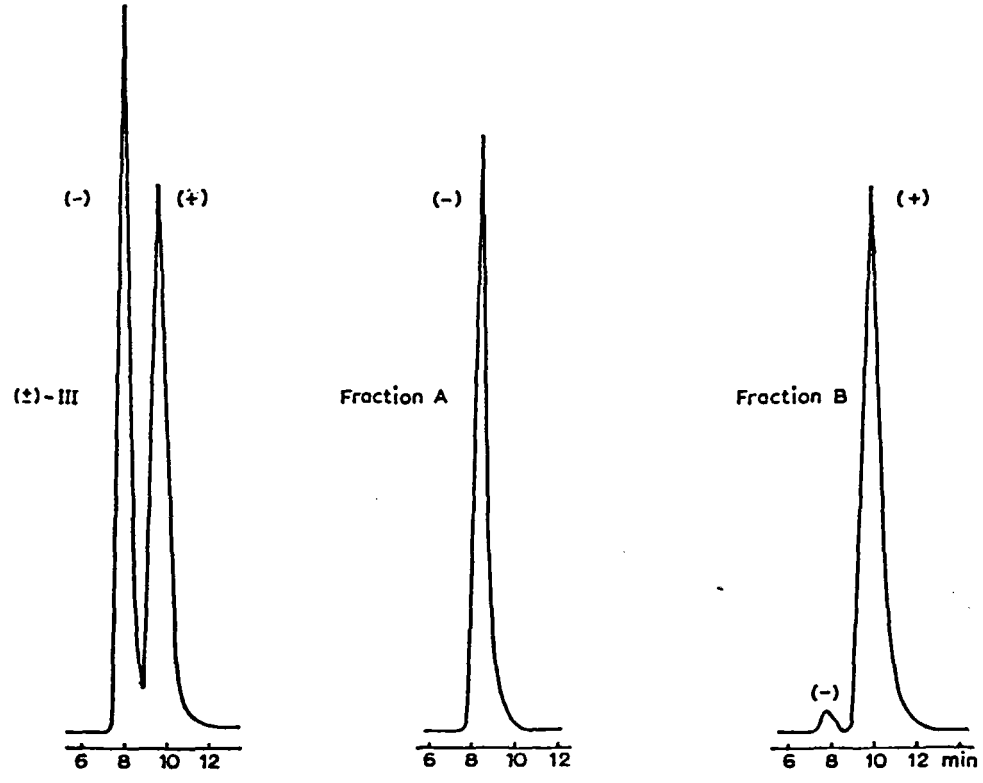


FIGURE 14. Liquid chromatograms of (±)*trans*-1,2-diphenyl cyclopropane in absolute ethanol on triacetylcellulose. (From Lindner, K. R. and Mannschreck, A., *J. Chromatogr.*, 193, 308, 1980. With permission.)

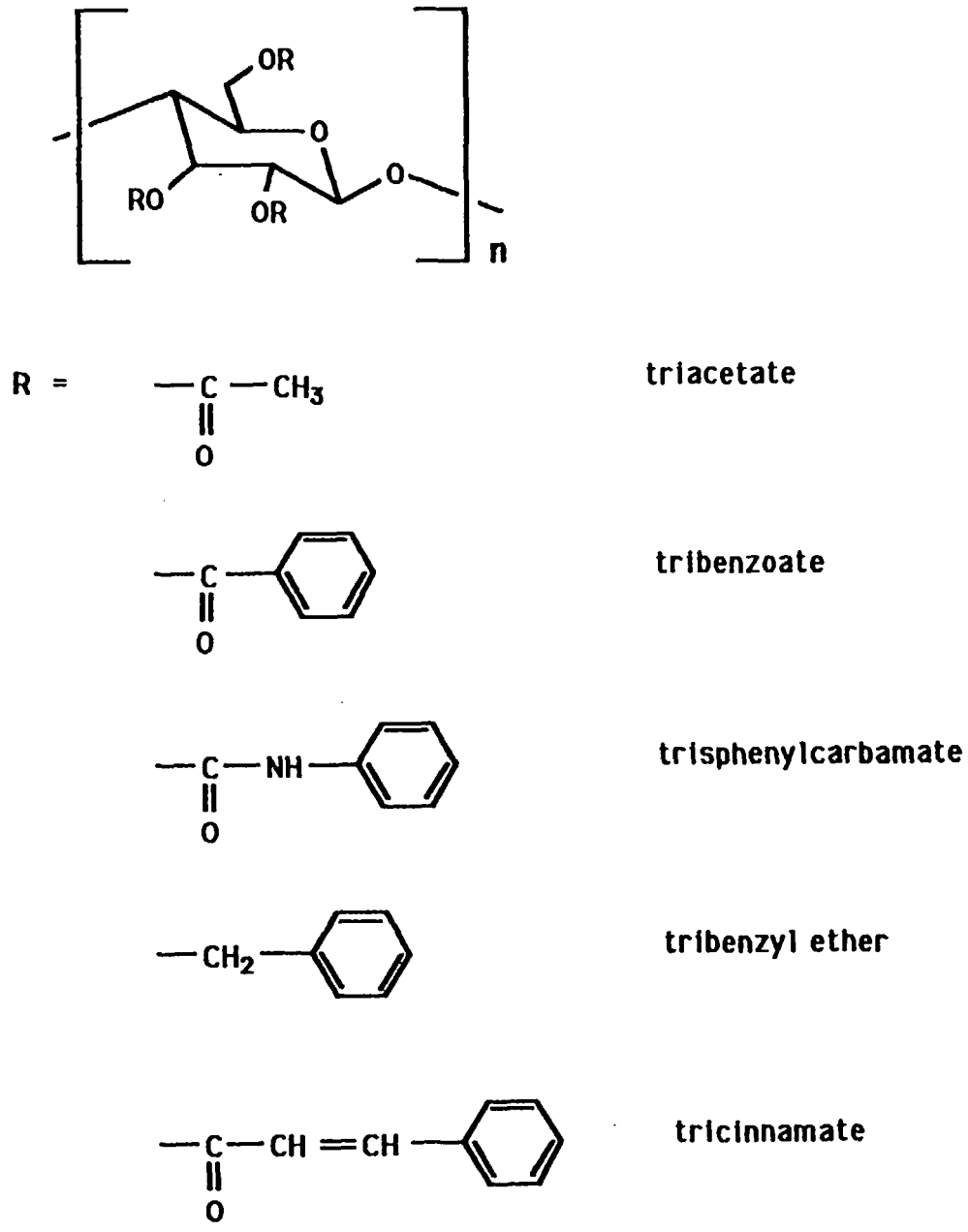


FIGURE 15. Structures of different derivatized cellulose. (From Dappen, R., Arm, H., and Meyer, V. R., *J. Chromatogr.*, 373, 1, 1986. With permission.)

Okamoto and co-workers polymerized triphenylmethyl methacrylate and utilized this polymer as a stationary phase. The polymer's chirality stems from its helicity.[183,184] This polymer is insoluble in common organic solvents when the degree of polymerization exceeds about 60. There are two different approaches for its use as a CSP. First, insoluble (+)-poly(triphenylmethyl methacrylate) (PTrMA) of high molecular weight was ground to a particle size of 20 to 40μm and used directly as a CSP.[185] Second, lower molecular weight-insoluble (+)-poly(triphenylmethyl methacrylate) was adsorbed on macroporous 10-μm diameter silica gel which had been treated with a silanizing agent such as dichlorodiphen-

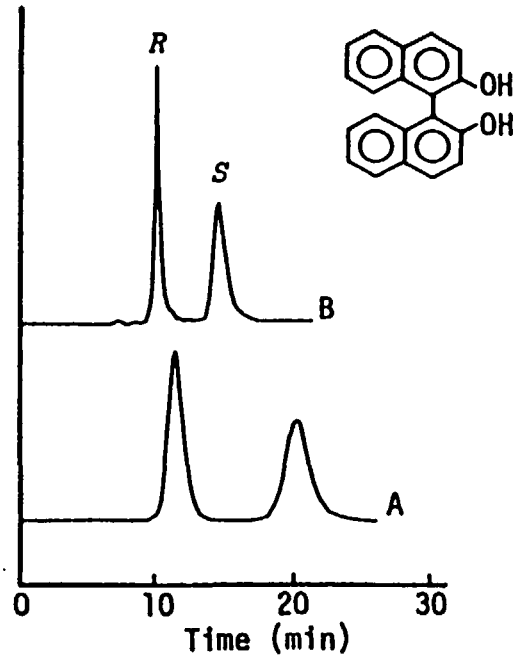


FIGURE 16. Resolution of binaphthol on (A) ground (+)-PTrMA and (B) (+)-PTrMA-coated silica gel columns. (From Okamoto, Y. and Hatada, K., *J. Liq. Chromatogr.*, 9, 369, 1986. With permission.)

ylsilane.[186] The selectivity of the ground PTrMA is different from the coated-type CSP. Although the ground type is particularly useful for separating helicenes,[187,188] the column was not durable enough for practical use.[189] The coated type had greater efficiency and produced relatively rapid separations of some racemates compared to the ground type (Figure 16). For example, the four stereoisomers of the insecticide phenothrin (i.e., (+) and (−), *cis*- and *trans*-3-phenoxybenzyl chrysanthemate) were resolved on the coated stationary phase but not on the ground type. This coating method is a convenient and useful technique for preparing chiral polymer stationary phases for HPLC. It has also been used for a number of the cellulose ester and polysaccharide carbamate CSPs.[190,191] Certainly, one advantage of the coated material is that one can easily and inexpensively make a wide variety of different CSPs. Nonpolar interactions between the packing and a racemic compound appear to be important factors in effective chiral recognition.

Many different kinds of stereochemically and biochemically interesting compounds (such as insecticides and drugs) were separated using coated-type columns,[192-199] but there are some limitations to the mobile phases that can be used. Aromatic hydrocarbons, chloroform, and tetrahydrofuran cannot be used because of the solubility of the polymers. These packings showed higher resolution with methanol eluents than with normal phases, although a hexane-2-propanol mixture is sometimes preferable.

Blaschke and co-workers reported that optically active polyamides and microcrystalline cellulose triacetate CSPs were useful in separating many chiral drugs.[200-206] In this case, copolymerization with cross-linking agents gave three-dimensional polymeric networks. It seemed that chromatographic resolutions were due to an inclusion of the enantiomers in the asymmetric cavities of this network. It was postulated that the fit of the enantiomers in the chiral cavity determined their average residence time in the stationary phases. Hydrogen bonding between the polar groups of the enantiomers and the CO–NH groups of the polymer are assumed to be main adsorbing forces.

New chromatographic investigations on highly selective glycoldimethacrylate-based polymers were described by Wulff and co-workers.[207] With the aid of a chiral template, molecular binding groups could be placed in a highly cross-linked polymer in such a way that they are present as a chiral cavity of specific shape suitable for a given stereochemistry. Polymers of this type possess a high selectivity for racemic resolution of a racemate on the template.

Protein-bonded phases have been used to separate enantiomers of a variety of drugs and derivatized amino acids. Bovine serum albumin linked to agarose was used in classic column chromatography.[208] Currently, the most useful protein HPLC-bonded phases utilize bovine serum albumin or α_1-acid glycoprotein. These were originally developed by Allenmark et al.[209] and Hermansson,[210] respectively. Allenmark and co-workers described the use of a layer of bovine serum albumin on silica for the optical resolution of a series of *N*-aroyl D,L-amino acids[209] and some derivatives of D,L-amino acids.[211] Although several papers have been published on these columns, the separation mechanism of these CSPs is not well understood.[212-217] Capacity factors and separation factors changed with mobile-phase composition. Capacity factors are reduced upon addition of 1-propanol to the mobile phase, indicating the importance of hydrophobic interactions for overall retention.[218] Separation data for several racemic compounds are given in Table 11.

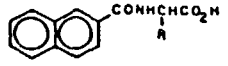
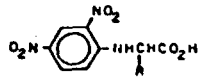
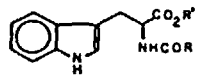
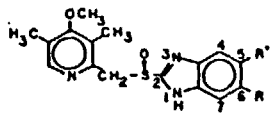
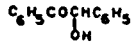
α_1-Acid glycoprotein (orosomucoid) has been immobilized on silica microparticles and was used for separating racemic drug enantiomers.[219-223] The retention of the samples on this stationary phase can be regulated in several ways, either by varying the pH or the ionic strength of the mobile phase or by adding an organic modifier. Separation factors are also affected by the pH and the content of organic modifier in the mobile phase. The ability to regulate retention and enantioselectivity with mobile phase modifiers is one of the advantages of this column. Recent results show that retention and enantioselectivity can be regulated by addition of the tertiary amine *N,N*-dimethyloctylamine (DMOA) to the mobile phase.[224] DMOA decreases the retention and the enantioselectivity of the weaker chiral acids, whereas the retention and the enantioselectivity of the stronger acids increase drastically. Also, the influence of column temperature (between 25 and 80°C) on the separation factor, the separation efficiency, and the retention was evaluated.

Schill and co-workers[225] investigated the applicability of this protein-bonded phase to a broad spectrum of pharmacologically important ammonium compounds. They studied the effect of cationic and neutral mobile-phase modifiers on retention and stereoselectivity. As in the case of the cyclodextrin columns, derivatization is not necessary for retention and resolution of many basic and acidic drugs.

4. Ligand-Exchange Phases

Rogozhin and Davankov first introduced ligand-exchange chromatography for the separation of enantiomers.[226,227] There are two basic approaches to ligand-exchange chromatography. One involves the use of bonded-phase sorbents containing chelating ligands. The other is the use of chiral chelating agents as additives to the mobile phase (see Section III.B.1). The first stationary phases were made from chloromethylated styrene-divinylbenzene copolymers containing chiral chelating ligands such as amino acids.[228,229] Unfortunately, these polymers could not withstand pressure and hence were not applicable to HPLC. After placing the stationary ligand onto the resin, Cu, Ni, or Zn ions were added to the mobile phase and used to separate enantiomers of amino acids. Figure 17 shows a structural model of the mixed ligand copper complex. Due to sterical hinderance of the substituent (*), the D form complexed weakly compared to the L form. In this case, the D form eluted before the L form. The retention, enantioselectivity, and elution order depended upon the complex stability of the constituent ligands and the nature of the applied metal ion. Many chiral polystyrene-type resins which contain different α-amino carboxylic and α-amino phosphonic

Table 11
RACEMIC COMPOUNDS STUDIED WITH RESPECT TO OPTICAL RESOLUTION ON BSA-SILICA (RESOLVOSIL) COLUMNS

No.	*Compound*	*Substituent*	
I		R = CH_2OH	(Ia)
		CH_3	(Ib)
		$CH_2C_6H_5$	(Ic)
II		R = CH_2OH	(IIa)
		CH_3	(IIb)
III		R = CH_3	(IIIb)
		$CH_2C_6H_5$	(IIIc)
IV		R = CH_2CO_2H	(IVa)
		$CH_2CH_2CO_2H$	(IVb)
V		R = R' = H	(Va)
		R = CH_3 R' = H	(Vb)
		R = CH_3 R' = C_3H_5	(Vc)
VI		R = H, R' = Cl	(VIa)
		R = H, R' = Br	(VIb)
		R = H, R' = CH_3	(VIc)
		R = H, R' = $(CH_3)_2CH$	(VId)
		R = H, R' = $(CH_3)_3C$	(VIe)
		R = CH_3OCO, R' = CH_3	(VIf)
VII			

ENANTIOSELECTIVITY OBTAINED ON HPLC OF COMPOUNDS I-VII

Compound no.	*k' (enantiomer)*		*α*	*Mobile phase composition [conc.(M), pH, % 1-propanol]*
Ia	1.3 (L)	2.4 (D)	1.9	Phosphate (0.05, 6.5, 1)
Ib	3.6 (L)	9.9 (D)	2.7	Phosphate (0.05, 6.5, 1)
Ic	15.1 (D)	28.3 (L)	1.9	Phosphate (0.05, 6.5, 1)
IIa	0.45 (L)	2.6 (D)	5.7	Phosphate (0.05, 5.7, 0)
IIb	0.5 (L)	4.9 (D)	9.8	Borate (0.05, 8.0, 0)
IIIb	3.5 (L)	7.1 (D)	2.0	Phosphate (0.05, 8.1, 5)
IIIc	2.95 (L)	9.3 (D)	3.2	Phosphate (0.05, 8.1, 5)
IVa	0.8	2.5	3.1	Phosphate (0.04, 8.1, 2.5)
IVb	0.75	2.2	2.9	Phosphate (0.04, 8.1, 2.5)
Va	1.7 (D)	8.45 (L)	4.9	Phosphate (0.05, 8.15, 3)
Vb	2.95 (D)	6.20 (L)	2.10	Phosphate (0.05, 8.15, 3)
Vc	2.5 (D)	12.7 (L)	5.15	Phosphate (0.05, 6.0, 3)
VIa	12.0	27.0	2.2	Phosphate (0.05, 6.1, 2)
VIb	21.7	58.1	2.7	Phosphate (0.05, 6.1, 2)
VIc	5.3	18.9	3.5	Phosphate (0.08, 5.8, 0)
VId	7.4	48.0	6.5	Phosphate (0.05, 6.1, 2)
VIe	10.6	79.0	7.5	Phosphate (0.05, 6.1, 2)
VIf	19.1	52.6	2.7	Phosphate (0.05, 7.5, 5)
VII	1.7	3.4	2.0	Phosphate (0.05, 8.15, 3)

From Allenmark, S., Bomgren, B., and Boren, H., *J. Chromatogr.*, 316, 617, 1984. With permission.

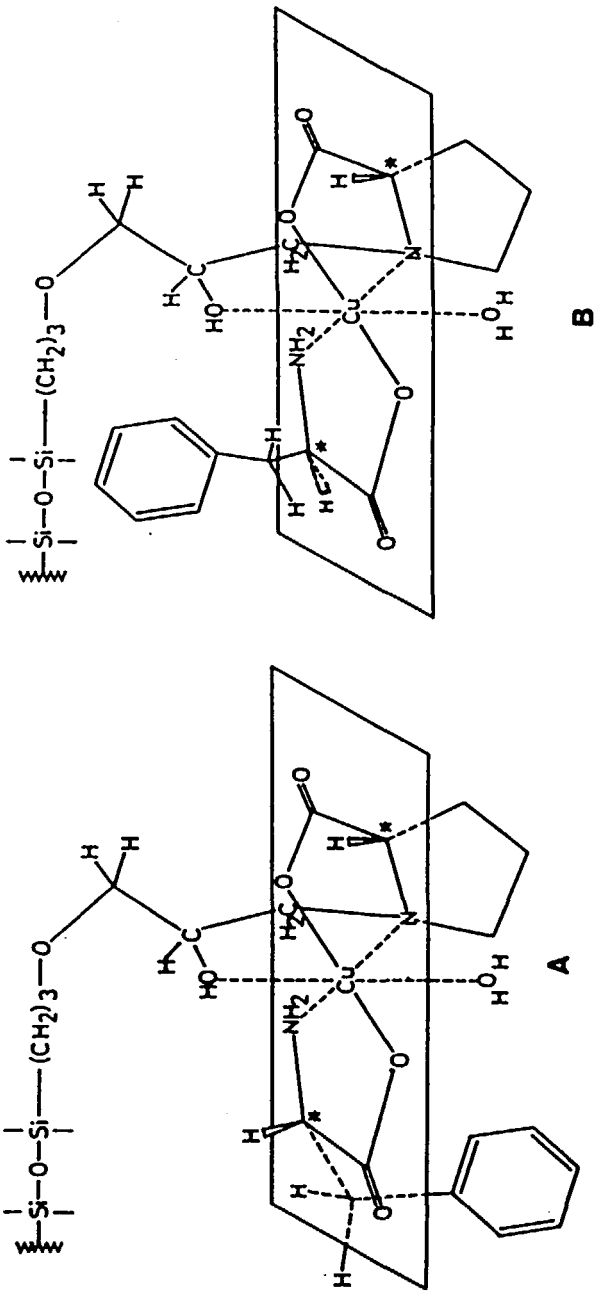


FIGURE 17. Model of the structure of the mixed ligand copper complex. (A) L-Proline-Cu-L-phenylalanine; (B) L-proline-Cu-D-phenylalanine. (From Gubitz, G., *J. Liq. Chromatogr.*, 9, 519, 1986. With permission.)

acid derivatives have been synthesized.[230] A reverse-phase packing coated with *N*-alkyl-L-hydroxyproline was used for separating unmodified α amino acid enantiomers.[231] The extremely high enantioselectivity observed ($\alpha = 16$) was assumed to be a result of a three-site sorbate-sorbent interaction, involving bidentate coordination of a three-site sorbate-sorbent interaction, involving bidentate coordination of two amino acids to a Cu (II) ion in addition to hydrophobic attractions between the hydrocarbon side chains of the amino acids and the *n*-octadecyl groups of the support.

Gübitz and co-workers synthesized similar chemically bonded chiral stationary phases. They were prepared by reacting LiChrosorb Si (dried at 120°C) with 3-glycidoxipropyltrimethoxysilane in benzene. Then the product was reacted with Na L-prolinate in DMF and complexed with Cu (II) by treatment with an aqueous $CuSO_4$ solution.[232] Cu (II), Co (II), Ni (II), and Zn (II) were tested as complexing ions.[233] However, only Cu (II) gave acceptable results. The presence of two polar functional groups ($-NH_2$ and COOH) in the sample molecule was important for enantiomeric separation. The carboxyl group of the bonded amino acid must be free to form a complex with the metal ions. The aqueous mobile phase must also contain the metal ion in order to reduce the loss from the stationary phase. Important factors affecting the selectivity and efficiency of the separations were the pH, ionic strength, and temperature of the mobile phase. High temperatures (40 to 50°C) showed higher separation factors and better column efficiencies. Other amino acids have been bonded to various supports and used in chiral ligand-exchange chromatography. These include L-azetidine carboxylic acid, L-pipecolic acid, and L-phenylalanine.[234,236]

Recently, it was reported that various chelating ligands (bonded to silica gel via 3-glycidoxipropyltrimethoxysilane) were useful for the separation of amino acids, amino acid derivatives, dipeptides, and hydroxy acids.[237] L-Proline, L-hydroxyproline, L-valine, L-histidine, L-phenylalanine, azetidine carboxylic acid, and L-pipecolic acid were evaluated as racemic solutes. Each ligand showed distinct selectivity with L-propylendiamine, L-ephedrine, and L-tartaric acid, which were fixed stationary-phase ligands. Stationary phases containing cyclic amino acids showed a higher enantioselectivity than those containing acyclic aliphatic amino acids.

Hydroxyproline showed a higher enantioselectivity for some amino acids which contained polar groups. This is probably due to the formation of additional hydrogen bonds. The relative enantioselectivities among cyclic acids used as stationary phases were azetidine carboxylic acid < proline < hydroxyproline < pipecolic acid. Table 12 shows capacity factors and separation factors for some amino acids using three different fixed ligands. Preparative separations are possible, and preparative-scale columns are available.

5. Crown Ether Phases

The catalytic and complexing properties of crown ethers are well known.[238] Cram and co-workers research on the complexation of ions by crown ethers led to the development of macrocyclic compounds which showed enantioselectivity.[239-244] Crown ethers have the ability to complex a number of cations (e.g., Na^+, K^+, NH_4^+, etc.) via the nonbonded electrons of the heteroatoms (oxygen, sulfur, or nitrogen) in the crown ether backbone.

The stability of the crown ether-ion complex depends, to a significant extent, on the fit of the ion in the crown ether cavity. For example, the larger crown ether, 18-crown-6, prefers to complex the larger potassium ion over the smaller sodium ion. This selectivity is exactly the opposite for the smaller crown ether, 15-crown-5. Ammonium ions are about the same size of the potassium ions and fit well into the cavity of the 18-crown-6 cyclic crown ether. If the ammonium ion is attached to a chiral atom (as in a protonated amino acid), then the possibility exists for an enantiomeric separation. The optical resolution of the racemates of primary amine salts were achieved using liquid-liquid chromatography.[242] Liquid-liquid chromatography was used with water-$NaPF_6$ or water-$LiPF_6$ solution supported

Table 12
COMPARISON OF THE K′ AND α VALUES (K'_L/K'_D) FOR SOME AMINO ACID ENANTIOMERS ON SORBENTS CONTAINING CYCLIC AMINO ACIDS AS STATIONARY LIGANDS

Amino acid	Si-Pro			Si-Hypro			Si-Pip		
	K'_D	K'_L	α	K'_D	K'_L	α	K'_D	K'_L	α
Ala	1.30	1.30	1.00	1.40	1.40	1.00	1.40	1.25	0.89
Val	2.50	3.80	1.50	2.20	2.60	1.20	1.40	2.68	1.91
Leu	3.10	3.10	1.00	3.40	2.90	0.85	2.08	2.60	1.25
I-Leu	3.00	3.60	1.20	3.40	3.40	1.00	1.70	2.75	1.62
Ser	2.00	3.20	1.60	2.20	3.40	1.55	1.63	2.83	1.74
Met	3.00	3.40	1.10	3.90	3.90	1.00	2.52	3.35	1.33
Lys	2.00	2.20	1.10	1.60	1.60	1.00	1.10	1.55	1.41
Arg	2.40	3.00	1.20	2.40	2.40	1.00	1.55	2.30	1.48
His	6.70	12.10	1.80	7.60	17.60	2.32	5.08	7.93	1.56
Asp	3.20	4.10	1.30	5.90	7.20	1.22	2.52	3.13	1.24
Glu	2.00	2.00	1.00	4.00	4.00	1.00	1.85	2.30	1.24
Pro	2.40	1.40	0.60	3.10	1.30	0.42	1.55	1.18	0.76
Phe	3.20	9.40	2.90	5.20	12.20	2.35	4.03	8.99	2.29
p-Tyr	3.30	10.20	3.10	3.60	15.00	4.17	4.33	14.45	3.34
DOPA	3.40	11.20	3.20	4.40	19.20	4.36	4.48	16.63	3.71
Trp	7.80	27.40	3.50	9.20	39.80	4.33	12.20	31.10	2.55

Note: Pro, proline; hypro, hydroxyproline; pip, pipecolic acid. From Gübitz, G., *J. Chromatogr.*, 9, 519, 1986. With permission.

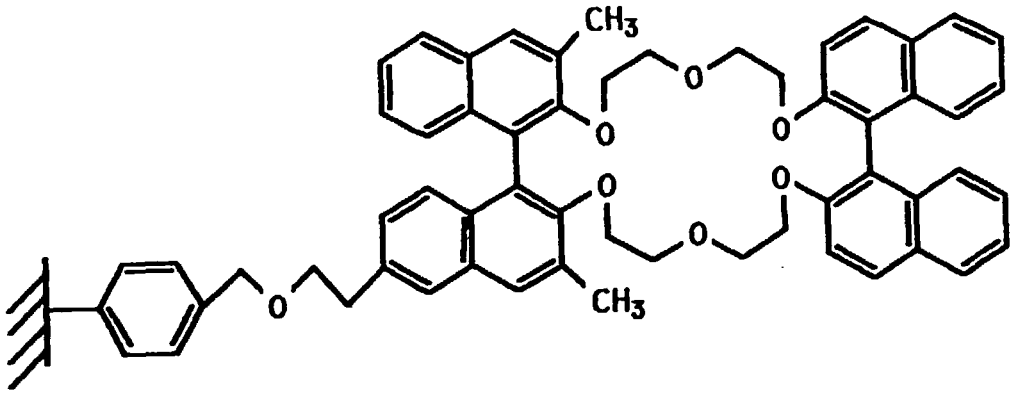


FIGURE 18. Molecular structure of a crown ether which was attached to a styrene-divinylbenzene resin.

on a Celite® or silica gel stationary phase. In this case, the choice of solvent and solute concentration can change the separation factors and resolution.

Optical resolution of α-amino acids and ester salts was accomplished by Sogah and Cram[239] via covalent attachment of the chiral host crown ether (Figure 18) to a macroreticular cross-linked polystyrene *p*-divinylbenzene resin. Separation factors ranged from 26.00 to 1.40 and resolution factors ranged from 4.50 to 0.21. Low temperatures were maintained because chiral recognition decreased with increasing temperature. Curtis and co-workers reported the enantioselective extraction-complexation property of this crown ether toward various racemic ammonium salts.[244]

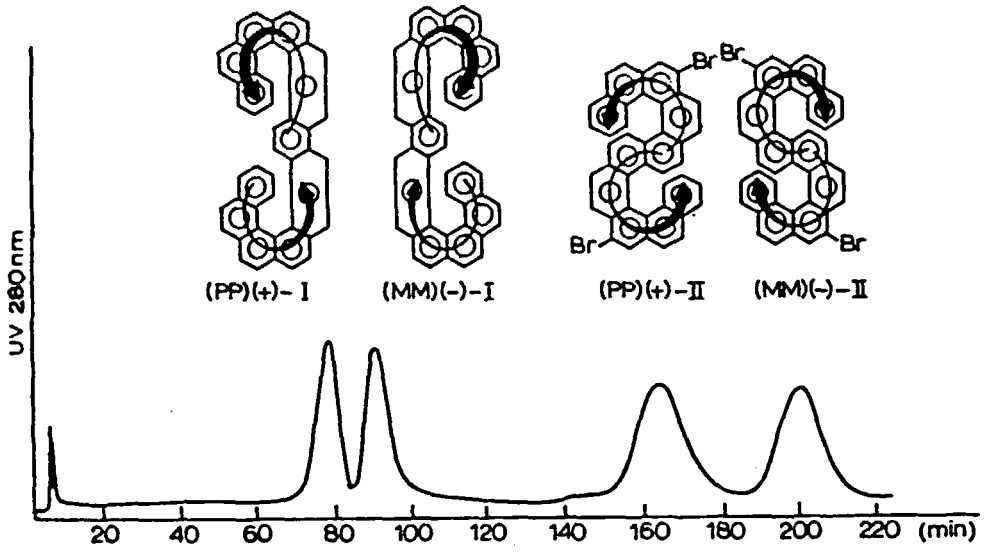


FIGURE 19. Column liquid chromatographic resolution of the racemic double helicenes I and II. (Mikes, F., Boshart, G., and Gil-Av, E., *J. Chromatogr.*, 122, 205, 1976. With permission.)

Although these interesting stationary phases effectively separate some racemic compounds that contain primary ammonium ions, their overall applicability is limited (e.g., the chiral charge transfer stationary phases). Judging from the current cost of many crown ethers, this packing may be prohibitively expensive for large-scale use. It was well known that aqueous solvents and aqueous-organic mixtures tend to decrease the interaction between a crown ether and a guest ion (e.g., as opposed to organic solvents). It is not likely that the enantioselectivity observed in these systems (which utilized organic solvents) will remain in typically reversed-phase mobile phases.

6. Chiral Charge Transfer Phases

In 1955, it was demonstrated that hexahelicene could be resolved from a solution of chiral α-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy) propionic acid (TAPA) by fractional recrystallization.[245] By 1960, resolution was obtained by column chromatography with TAPA-coated silicic acid.[246] Mikes and co-workers extended this technique to HPLC, separating ten racemic helicenes and two double helicenes using a stationary phase of silica coated with TAPA.[247] TAPA and three R(−)-homologues derived from butyric (TABA), isovaleric (TAIVA), and hexanoic (TAHA) acids were used as a stationary-phase coating. It was found that the size of the substituent on the chiral carbon affected the selectivity. Figure 19 shows the separation of the racemic double helicenes. Alumina impregnated with (*S*)-(+)-TAPA has been used as well.[248] TAPA and binaphthyl 2,2′-diylhydrogen phosphate (BPA) has also been bonded to silica gel (see Figure 20). All carbo- and hetero-helicenes could be resolved on TAPA columns, while only helicenes containing heteroatoms or electron-donating substituents were separated on BPA.[249,250] Kim and co-workers reported the resolution of [6]- to [14]-carbohelicenes on silica gel coated with riboflavin[251] (see Figure 20). Lochmüller and Ryall utilized a small 2,4-dinitrophenyl group as the charge transfer acceptor attached to a chiral atom[252] (see Figure 20). Although poor resolution of 1-aza[hexa]helicene and heptahelicene was achieved, Lochmüller and co-workers postulated that the small 2,4-dinitrophenyl group could not have achieved the multiple overlaps with the helicenes as proposed for TAPA. Consequently, it was thought to be possible to achieve chiral discrimination in chromatography with only one or two strong interactions.[253] The fact that this is thought to be geometrically impossible was not considered.[116]

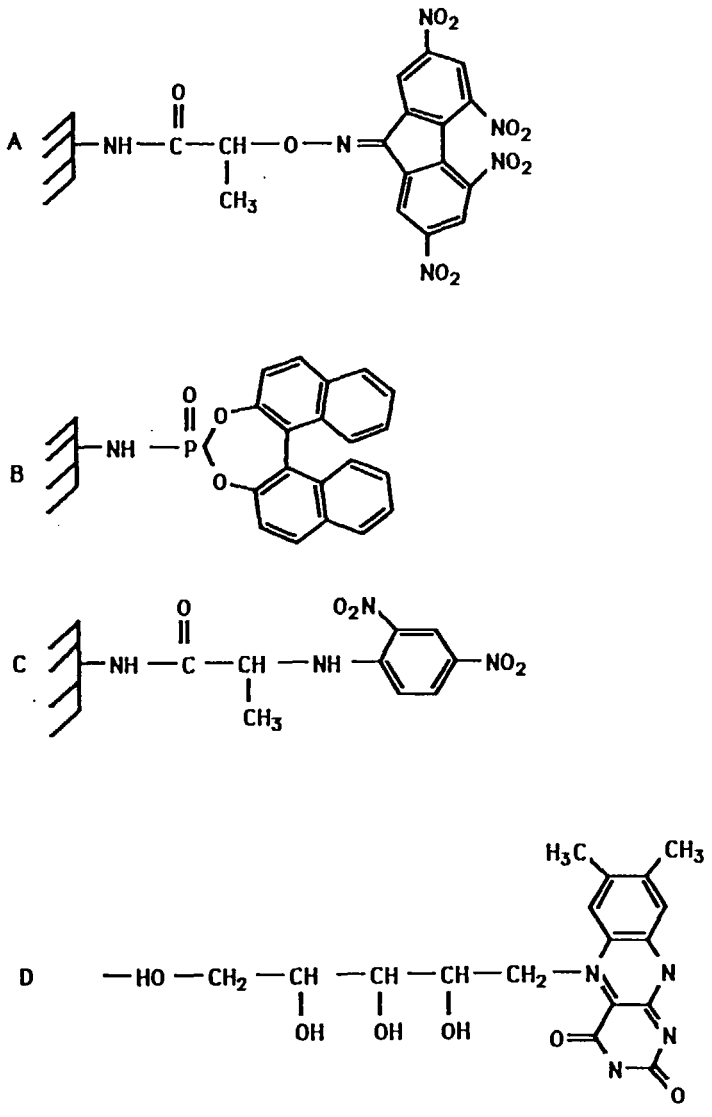


FIGURE 20. Structures of four different charge-transfer adducts that have been bonded to or adsorbed on silica gel to create CSPs capable of separating a variety of helicenes. Again, the chiral molecule in all bonded packing was attached via a γ-aminopropylsilane linkage. (Compound A, TAPA; B, 2,2′-diylhydrogen phosphite XL; C, *N*-2,4-dinitrophenylalanine; D, riboflavin.) (From Armstrong, D. W., *J. Liq. Chromatogr.*, 7, 353, 1984. With permission.)

It is apparent that chiral charge transfer stationary phases are useful for the separation of racemic helicenes. Unfortunately, it has not been demonstrated that they have any wider applicability. As these stationary phases are either coated on silica gel or bonded via an amide linkage through an aminopropylsilane, they should not be used with aqueous or aqueous-organic mobile phases.

B. Chiral Mobile-Phase Additives

Many racemic mixtures can be separated on conventional-achiral LC columns by adding an appropriate chiral additive to the mobile phase. Advantages of this technique are (1) less

expensive conventional packings (such as reversed phases) can be used, (2) chiral additives can be easily changed compared to changing packings, (3) there are a wide variety of possible additives, and (4) the selectivities are sometimes different from those of available chiral stationary phases. Possible disadvantages are (1) some additives must be synthesized, (2) the detection method limits the choice of additives (or vice versa), and (3) the analyte must be subsequently removed from the additive if the former is to be studied in pure form. This is a disadvantage for preparative scale separations.

1. Ternary Complex Additives

Lepage and co-workers[254] and Hare and Gil-Av[255] first reported a useful method for resolving racemic mixtures by adding chiral ligands and metal ions to the mobile phase of conventional LC columns. It was reported that an aqueous eluant of the chiral copper (II)-L-proline complex affected the separation of underivatized amino acid enantiomers on an ion exchange column.[255] The stereoselectivity was ascribed to differences in stability of the diastereomeric amino acid-Cu complexes formed in solution. A simple change in the chirality of the eluent reversed the enantiomeric elution order. The additive consisted of a chiral bidentate ligand which also contained a hydrophobic segment. As a result of the hydrophobic moiety, the ligand associated strongly with reversed phase media. As in ligand-exchange chromatography, a transition metal ion (Cu (II), Zn (II), Cd (II), etc.) must be added to the mobile phase as well. Enantiomers of serine, valine, isoleucine, tyrosine, and phenylalanine were separated.[256] When a L-proline ligand was used, the L-enantiomer was eluted before the D-enantiomer and vice versa. There was no resolution of enantiomers when a racemic proline ligand was used. Racemic solutes (particularly amino acids) form ternary complexes which are retained by the stationary phase. The phase preference of the additive (i.e., stationary or mobile phase) is dependent on the hydrophobicity of the additive and the stationary phase as well as the polarity of the mobile phase. Resolution is based on enantioselectivity effects in the formation of ternary metal complexes, in which one form of enantiomers must be different.

Lindner and co-workers[257] used L-2-isopropyl-4-octyl-diethylene-triamine-Zn (II) to separate all of the optical isomers of dansylamino acids with the exception of dansyl proline. Remarkably high retentions were achieved, and the enantioselectivity values (αs) were found to range from 1.10 (alloisoleucine) to 2.50 (serine). They examined the roles of different metal ions on separations. When Ni (II), Cd (II), and Cu (II) were used, differences in retention and selectivity were found. When Hg (II) was substituted for Zn (II), the order for D,L-dansylamino acid elution was inverted. The resolution of optical isomers of chiral dipeptides using Ni (II) chelates were also shown.

Conditions for the resolution of enantiomers of all the common dansyl derivatized amino acids using the chiral mobile-phase additives L-prolyl-*n*-octylamide-Ni (II) and L-prolyl-*n*-dodecylamide were reported.[258] Variable D,L selectivity was observed, while good column efficiency was maintained. L-Prolyl-*n*-dodecylamide-Ni (II) distributed on the surface of a *n*-alkyl-bonded stationary phase and acted as a chiral immobilized phase. All the common dansyl amino acids were separated within 35 min by using reversed-phase conditions and a linear gradient. Weinstein and Grinberg[259] reported enantiomeric separations of underivatized α methyl and α amino acids on reversed phase columns caused by the addition of Cu complexes of *N,N*-dimethyl-L-valine or *N,N*-dipropyl-L-alanine to the mobile phase.

Many racemic mixtures (e.g., amino acids and dansyl amino acids) were separated using L-phenylalanine-Cu (II),[260-262] *N*-methyl-or *N,N*-dimethyl-L-phenylalanine-Cu (II),[261] L-hydroxyproline-Cu (II) and L-proline,[255,263,264] *n*-alkyl-L-hydroxyproline,[265] and *N*-(*p*-toluenesulfonyl)-L-phenylalanine.[266] L-Aspartame and derivatives of aspartic acid were also separated.[267,268] Lam and co-workers separated the dansyl derivatives of amino acids using Cu(II) and a second amino acid such as L-proline, L-arginine, L-histidine, or L-histidine methyl

Table 13
CAPACITY RATIO (K') AND SELECTIVITY (α) OF D- AND L-Dns-AMINO ACIDS FOR FOUR CHIRAL ELUENTS CONTAINING 2.5-m*M* Cu(II) COMPLEXES OF L AMINO ACIDS

Amino acid	Proline			Histidine			Histidine methyl ester			Arginine		
	k'_L	k'_D	α	k'_L	k'_D	α	k'_D	k'_L	α	k'_D	k'_L	α
Ser	3.7	3.2	0.9	—	—	—	9.3	10.7	1.2	3.0	3.0	1.0
Thr	3.7	4.6	1.2	—	—	—	9.3	9.3	1.0	3.0	3.0	1.0
Ala	5.7	6.6	1.2	1.8	2.4	1.3	11.9	11.9	1.0	4.2	4.4	1.1
αAB	7.3	9.2	1.2	2.2	3.8	1.7	13.6	15.0	1.1	5.7	6.2	1.1
Val	11.4	15.0	1.3	3.2	6.4	2.0	17.3	20.1	1.2	8.3	9.5	1.1
Met	11.4	14.8	1.3	4.4	7.8	1.8	27.2	30.7	1.1	10.3	11.7	1.1
N-Val	14.6	19.0	1.3	3.8	9.0	2.4	23.3	27.6	1.2	11.0	13.0	1.2
I-Leu	—	—	—	7.2	15.4	2.1	40.4	44.7	1.1	17.2	20.8	1.2
Leu	23.9	32.6	1.4	7.2	16.0	2.2	33.0	43.9	1.3	18.5	20.8	1.1
N-Leu	32.6	45.7	1.4	9.4	22.2	2.4	40.4	58.4	1.5	22.3	28.2	1.3
Phe	32.6	52.8	1.6	9.0	9.0	1.0	60.4	77.9	1.3	20.3	23.0	1.1
Trp	41.2	71.2	1.7	7.4	7.4	1.0	93.3	121.8	1.3	24.8	31.2	1.3

Note: Acetonitrile concentration was 15% for the proline and histidine systems and 20% for the histidine methyl ester and arginine systems.

From Lam, S. and Karmen, A., *J. Liq. Chromatogr.*, 9, 291, 1986. With permission.

ester.[269-276] Table 13 shows the separation of D,L-dansyl amino acids using a mobile phase that contained chiral metal complexes of Cu (II) L-proline, L-arginine, L-histidine, and L-histidine methyl ester.

Enantiomers of underivatized α-hydroxy acids, including lactic, mandelic, malic, and tartaric acids have been separated by reversed phase LC using Cu (II)-L-amino acid mobile phases.[277] The hydroxy acids in the column effluent were detected by a post column color reaction with an acidic iron (III) solution. α-Hydroxy acids with additional hydroxy groups and/or carboxyl groups in the β position and α-hydroxy acids with no functional groups in the β position differ in the elution orders, capacity factors, and separation factors. The resolution of amino acids enantiomers was achieved by using (*R,R*)-tartaric acid mono-*n*-octylamide (TAMOA), which complexed copper (II) or nickel (II) ions.[278] Figure 21 shows the separation of some free amino acids using the TAMOA complex as a mobile-phase additive. Cu (II) complexes of *N,N*-di-*n*-propyl-L-alanine were used for the enantiomeric separation of dansyl amino acids by reversed phase HPLC.[279,280]

The micro HPLC separation of dansyl-D,L-amino acids was examined by Takeuchi and co-workers using Cu (II)-L-histidine and Cu (II)-L-histidine methyl ester eluents.[281] It was found that the capacity factor increased with increasing concentration of the chiral additive (Figure 22). The pH also affected the selectivity and the peak shape. The separations were usually better using gradient elution. The capacity factor was found to decrease with increasing pH of the mobile phase. The Cu (II)-L-histidine methyl ester eluent was less selective than Cu (II)-L-histidine.

2. Cyclodextrins

CDs have been used as chiral mobile-phase additives as well. In this case, the CD is adsorbed by the stationary phase and present in the mobile phase as a carrier molecule.

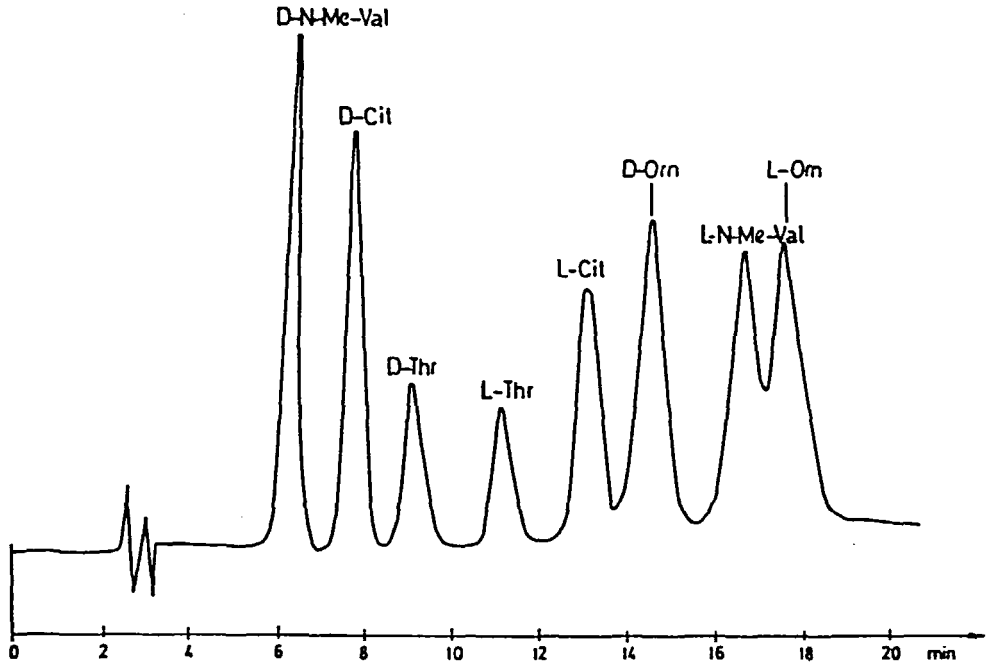


FIGURE 21. Liquid chromatographic separation of D,L-threonine (Thr), D,L-citrulline (Cit), D,L-ornithine (Orn), and D,L-*n*-methyl valine (*N*-Me-val) using the TAMOA-Cu(II) mobile-phase additive. (From Lindner, W. F. and Hirschbock, I., *J. Liq. Chromatogr.*, 9, 551, 1986. With permission.)

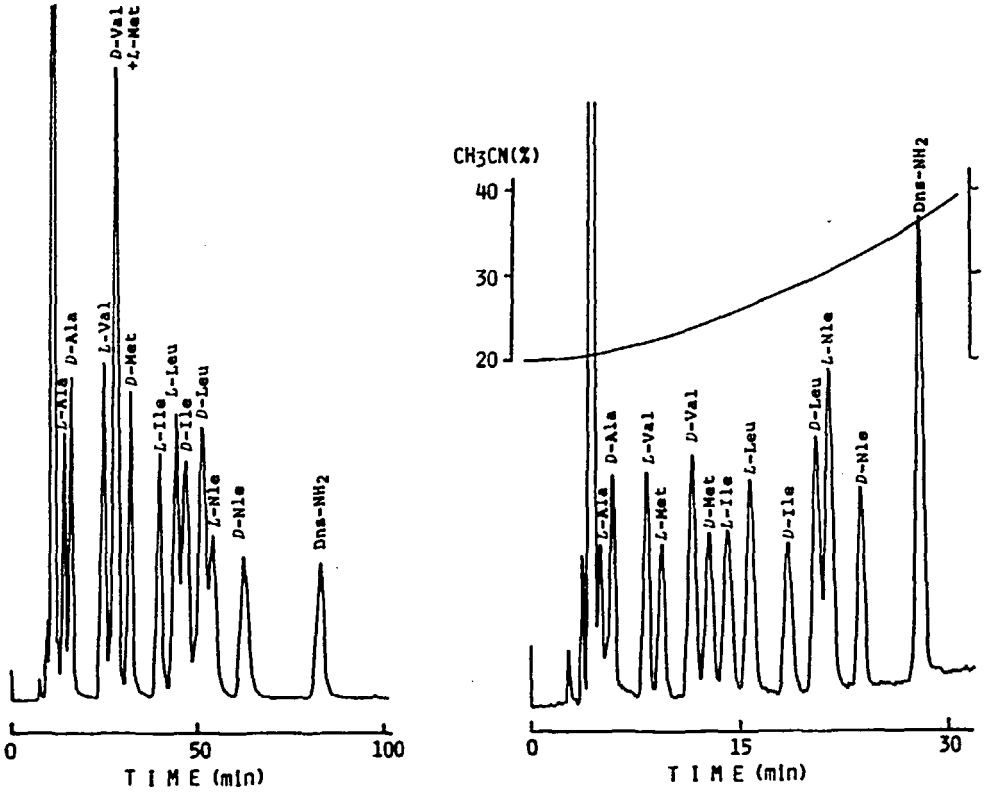


FIGURE 22. Isocratic and gradient separation of dansyl-D,L-amino acids with Cu(II)-L-histidine as the eluent. (From Takeuchi, T., Asai, H., Hashimoto, Y., Watanabe, K., and Ishii, D., *J. Chromatogr.*, 331, 99, 1985. With permission.)

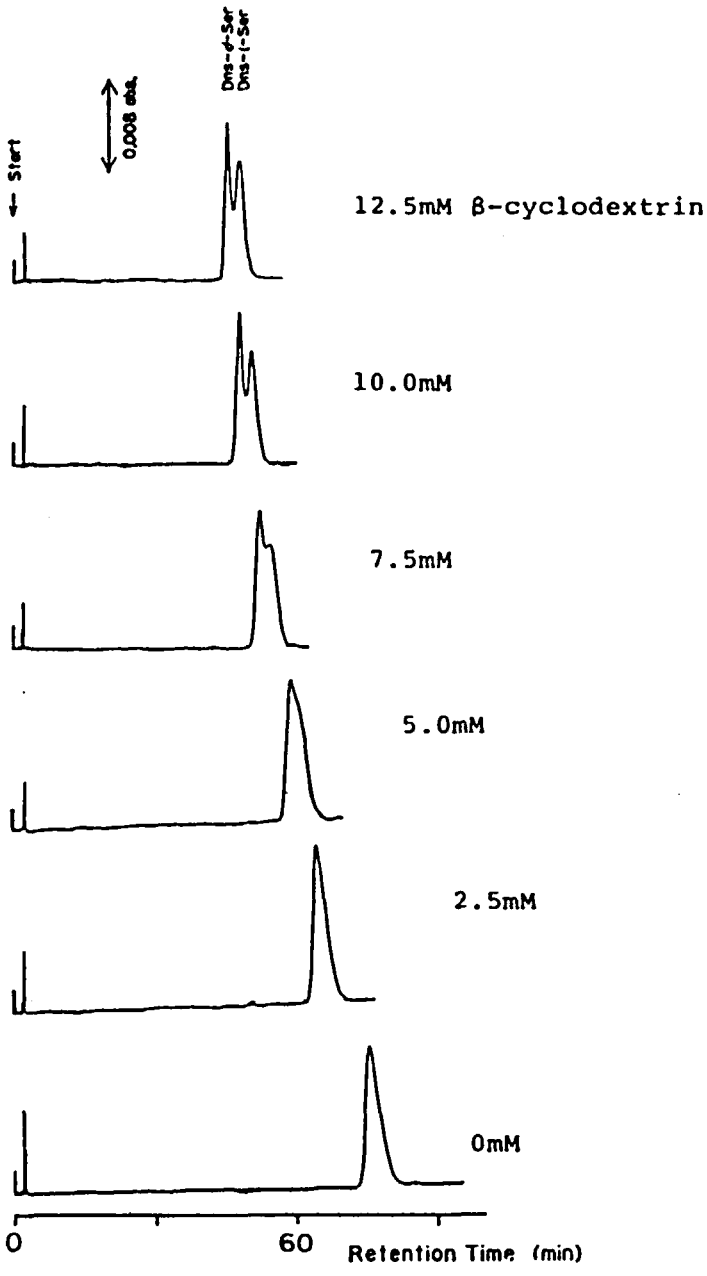


FIGURE 23. Effect of the concentration of β-cyclodextrin on the enantiomeric separation of dansyl serine. (From Takeuchi, T., Asai, H., and Ishii, D., *J. Chromatogr.*, 357, 409, 1986. With permission.)

Debowski and co-workers first used α-CD and β-CD as mobile phase additives for the separation of racemic mandelic acids and mandelic acid derivatives in reverse-phase systems.[282,283] Racemic 1-(2-[3-hydroxyphenyl]-1-phenylethyl)-4-(3-methyl-2-butenyl)piperazine was separated by Nobuhara and co-workers using β-CD as the mobile-phase additives.[284]

Enantiomeric resolution of dansyl amino acids by micro HPLC using β-CD mobile phases has been investigated by Takeuchi and co-workers.[285] They examined the chromatographic parameters which affected enantiomeric resolution. A total of 12 racemic dansyl amino acids were separated in a single chromatogram run with ODS-hypersyl-3 stationary phase. Figure 23 shows the effect of the concentration of β-CD on the enantiomeric separation of dansyl

serine. For separations using β-CD mobile phases, elution orders are reversed compared to separations using β-CD stationary phases. For example, on β-CD bonded phases, the L isomer of dansyl amino acids eluted before its D isomer. However, for β-CD mobile phases, the D isomer of dansyl amino acids elute before the L isomer. β-CD as a mobile-phase additive was used in micro HPLC for the enantiomeric separations of nicotine and nicotine analogs.[286] Mephenytoin and some chiral barbiturates were also separated.[287] Also, CD as a mobile-phase additive was used for the separation of structural isomers in HPLC.[288-290]

3. Other Chiral Additives

Chiral ion interaction agents can sometimes be used to resolve an oppositely charged racemate on conventional reversed-phase columns. In this method, two oppositely charged chiral species are retained on the stationary phase as an overall neutral diastereomeric pair. The binding forces are usually a combination of electrostatic attraction and hydrogen bonding. Solvents of low polarity are normally used to obtain a high degree of ion interaction. The ion pairs can have different distribution properties due to differences in solvation in the mobile phase or due to the different binding strength of the ions to the adsorbent. The three-point attachment is not applicable to diastereomeric ion pairs, and counter ions are usually selected to give a two-point binding with the substrates. This technique was first used by Pettersson and Schill[291-293] to resolve racemic amino alcohols. They used (+)-10-camphor-sulfonic acid as the chiral counter ion. This is a strong acid with a rigid structure due to the bridge in the ring system. Typical chiral ion interaction agents used were quinine, quinidine, (+)-10-camphor-sulfonic acid, albumin, and tartaric acid derivatives. Some alkaloids were also separated by Szepesi and co-workers.[294]

Knox and Jurand reported that chiral recognition can be achieved by adding an optically active Zwitterion pairing agent to the eluent.[295] The separation of racemic mixtures of tryptophan and glycylphenylalanine using L-leucyl-L-leucyl-L-leucine as a Zwitterionic pairing agent was reported. A study of the separation of enantiomers of carboxylic and sulfonic acids as diastereomeric ion pairs with quinine as the chiral counter ion was reported by Pettersson.[296] The enantiomeric selectivity is highly dependent on the structure of the chiral counter ion. Differently modified silica gel adsorbents were used as stationary phases in order to regulate retention and stereoselectivity. The water content of the mobile phase had much influence on retention and stereoselectivity. Very low water contents gave long equilibration times and unstable retention times. The most stable and reproducible chromatograms were obtained when the mobile phase contained 80 to 90 ppm H_2O. Separations also deteriorated when the mobile phase contained higher concentrations of water. Retention was dependent on the properties of the adsorbing stationary phase. The capacity factors decreased with increasing concentrations of the counter ion. This is because of the competitive distribution of the counter ion and the diastereomeric ion pairs to the same stationary-phase adsorption sites. Figure 24 shows the resolution of (±)-*N*-*tert*-butoxycarbonyl-phenylalanine using quinidine as the chiral counter ion.[297] The negative peak is a characteristic system peak for this method. It appeared at the same place on the chromatogram for all runs and was independent of the nature of the injected solute.[296]

In recent papers, a different chiral counter ion, *N*-benzoxylcarbonyl-glycyl-L-proline (ZGP), was added to the organic mobile phase (CH_2Cl_2) and used to separate the enantiomers of amino alcohols.[298,299] Separation factors of 1.2 to 1.4 for enantiomers for β-adrenergic blocking agents (e.g., alprenolol, metoprolol, and propranolol) were obtained. The chiral counter ion was utilized to determine the enantiomeric impurity of <0.1% in *S*-alprenolol and to determine propranol enantiomers in plasma samples.

IV. PLANAR CHROMATOGRAPHY

One advantage of planar chromatography arises from its simplicity. β-CD bonded TLC

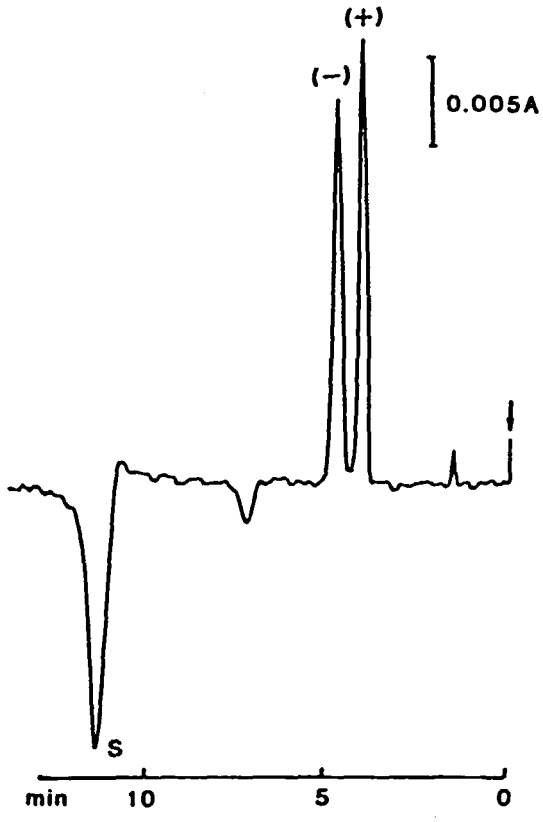


FIGURE 24. Liquid chromatographic resolution of (+/−)-*N*-t-BOC-phenylalanine using an ion interaction agent. (From Pettersson, C. and Shill, G., *J. Liq. Chromatogr.*, 9, 269, 1986. With permission.)

was used to separate optical, geometrical, and structural isomers.[300] A number of different silica gels and binders were evaluated, and planar chromatographic conditions were optimized for the separation of nine racemic mixtures, three diastereomeric mixtures, and six structural isomers. Also, some structural isomers were separated using aqueous solution of CDs as the mobile phases.[301-303]

Cu (II) complexes of *N,N*-di-*n*-propyl-L-alanine were used for the enantiomeric separation of dansyl amino acids on TLC plates[304] and two-dimensional TLC.[305] In the first dimension, dansyl amino acids were separated into components in a nonchiral mode by using a convex gradient elution with aqueous NAOAc buffers and varying concentrations of acetonitrile. In the second dimension, the plates were treated with a chiral Cu complex of *N,N*-di-*n*-propyl-L-alanine and were developed with aqueous acetonitrile-NaOAc buffers. Marchelli and coworkers reported the resolution of D,L-dansyl-amino acids by one- and two-dimensional TLC using a chiral additive in the eluent.[306]

Resolution of optical isomers of D,L-tryptophan, 5-hydroxy-D,L-tryptophan, and 6-hydroxy-D,L-tryptophan were done using paper chromatography and TLC.[307] Racemic amino acids were resolved using cellulose TLC,[308] and racemic 2,2,2-trifluoro-1-(9-anthryl)ethanol was separated by using TLC when (*R*)-*N*-(3,5-dinitrobenzoyl) phenylglycine was bonded to γ-aminopropyl silanized silica gel.[309]

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