# Liquid Chromatography

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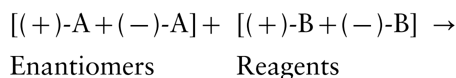
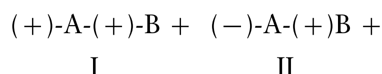
A pair of enantiomers only differ in their optical rotation, but their physical properties such as melting point, boiling point, refractive index and solubility are identical. On the other hand, the physical and chemical properties of a pair of diastereomers are different. For the separation of enantiomers by liquid chromatography, it is essential to form a diastereomeric complex in the mobile phase or in the stationary phase or to convert to diastereomers. The former is called the direct method, and is based on the addition of chiral additives to the eluent or the use of chiral stationary phases, resulting in the formation of a diastereomeric complex in the mobile or stationary phase. The second indirect method is based on the reaction of the enantiomers with a homochiral reagent, resulting in the formation of a pair of diastereomers. This article deals with both the indirect and direct methods using liquid chromatography.

## Purity of Chiral Derivatization Reagent or Chiral Selector

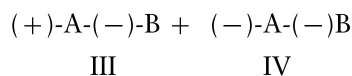
For the chiral derivatization method, there is one point which necessitates careful interpretations of the results. When the enantiomeric mixture of (+)-A and (−)-A are derivatized with the chiral derivatizing reagent of (+)-B, it often includes (−)-B as a chiral impurity. If the reagent B is 100% optically pure, two diastereomers (+)-A-(+)-B (I) and (−)-A-(+)B (II) are formed. If reagent B is not optically pure, additional diastereomers (+)-A-(−)-B (III) and (−)-A-(−)B (IV) are formed.



[(+)-A+(−)-A]+ [(+)-B+(−)-B] →
Enantiomers Reagents



(+)-A-(+)-B + (−)-A-(+)B +
I II



(+)-A-(−)-B + (−)-A-(−)B
III IV

Reaction products

The enantiomers of A to be separated and determined as their diastereomeric derivatives (I and II) can be resolved as the respective peaks on an achiral stationary phase. However, the peaks of the products III and IV produced with (−)-B overlap with the peaks of II and I, respectively, on achiral stationary phases, because II and III, and I and IV are enantiomeric pairs. Therefore, if the optical purity of the derivatization reagent is not known or not taken into consideration, the optical purity of the target compound will not be determined accurately. Further, this is the case when the separation of enantiomers is carried out by the use of chiral additives to the eluent on achiral stationary phases.

In contrast, direct resolution of enantiomers using chiral stationary phases does not have the drawbacks described above. We can easily determine 0.1% or 0.05% of the antipode using chiral stationary phases.

## Indirect Methods

The indirect method, involving reaction with a homochiral reagent, is an efficient technique for the separation of many enantiomers. It is essential that the chiral derivatization proceeds completely in both enantiomers, and that racemization does not occur. The indirect methods are unsuitable for analysis of enantiomers in a standard sample and pharmaceutical preparations, because the low amount of the antipode level should be determined, and are unsuitable for preparative purposes. However, they are suitable for trace analysis of enantiomers in complex matrices such as biological samples and environmental samples because of the introduction of highly sensitive tags. These include UV-visible, fluorescent and electrochemical tags. Fluorescence derivatization is the most effective to determine the target compound in complex matrices in terms of sensitivity and/or selectivity. **Table 1** shows the fluorescence derivatization reagents (**Figures 1** and **2**) used for the separation of enantiomers bearing amino, keto, hydroxyl and carboxyl groups.

## Direct Methods

Direct methods using chiral mobile-phase additives can separate many enantiomers by addition of chiral selectors to an eluent on achiral stationary phases. However, for preparative applications, the additives must be removed. Separations on chiral stationary phases can be used for both analytical and preparative purposes. At the present time, the trend is to use chiral stationary phases for the separation of enantiomers by liquid chromatography whenever possible.

**Table 1** Fluorescence derivatizating reagents used for the separation of enantiomers

| *Reagent* | *Enantiomer* | *Separation mode* |
|---|---|---|
| OPA + N-protected L-cystein | Amino acids, amino alcohols, baclofen, tranylcypromine | Reversed-phase |
| FLEC | Amino acids, methamphetamine, ephedrine, atenolol | Reversed-phase |
| DANE | Amino acids, ibuprofen, indoprofen, naproxen, loxoprofen | Normal-phase |
| NEIC | Propranolol, nadolol, prenylamine, betaxolol, acebutolol | Reversed-phase and normal-phase |
| Methyl-BNCC | Hydroxyls, $\beta$-hydroxy acids, propranolol, penbutolol | Normal-phase |
| NBD-Apy | *N*-acetyl amino acids, antiinflammatory drugs | Reversed-phase |
| NBD-Pro-COCl | Alcohols, amine | Reversed-phase |
| NBD-ProCZ | Ketones | Reversed-phase |
| NBD-PyNCS | Amines, $\beta$-blockers, amino acids, peptides | Reversed-phase |
| FLOPA | Ibuprofen, $\alpha$-phenylcyclopentyl acid | Reversed-phase and normal-phase |
| FLOP-Cl | Amino acids, peptides | Reversed-phase and normal-phase |
| MNE-OTf | $\alpha$-Methoxyphenylacetic acid, propranolol | Reversed-phase |

See Figures 1 and 2 for definitions of abbreviations.

## Chiral Stationary Phases

Many chiral selectors are adsorbed or immobilized covalently on to liquid chromatography supports. The chiral stationary phases obtained are classified into two types with respect to their general structure. One type is based on synthetic or natural polymers, which are totally or intrinsically chiral; the other type is based on a chiral selector of low molecular weight. Chiral stationary phases can be further classified into five types based on the solute and chiral stationary-phase interactions:

- Type I: the diastereomeric complexes of the solute and chiral stationary phase are formed by attractive interactions such as hydrogen-bonding, $\pi$–$\pi$, dipole stacking, etc., between the solute and chiral stationary phase.
- Type II: the primary mechanism for the formation of the solute and chiral stationary phase complex is through attractive interactions, but inclusion complexes also play an important role.
- Type III: the solute enters into chiral cavities within the chiral stationary phase to form inclusion complexes.
- Type IV: the solute is a part of a diastereomeric metal complex; this is called chiral ligand exchange chromatography.
- Type V: the chiral stationary phase is a protein and the solute and chiral stationary phase complexes are based on combinations of hydrophobic, electrostatic and hydrogen-bonding interactions.

**Table 2** shows commercially available chiral stationary phases which are classified into these five types.

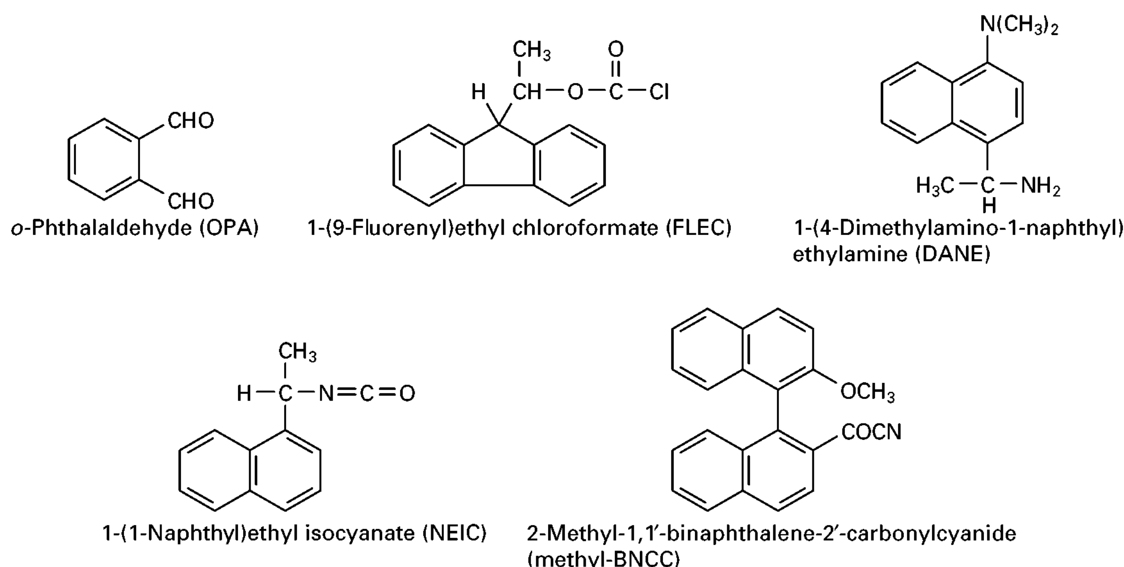



**Figure 1** Structure of reagents (see Table 1).

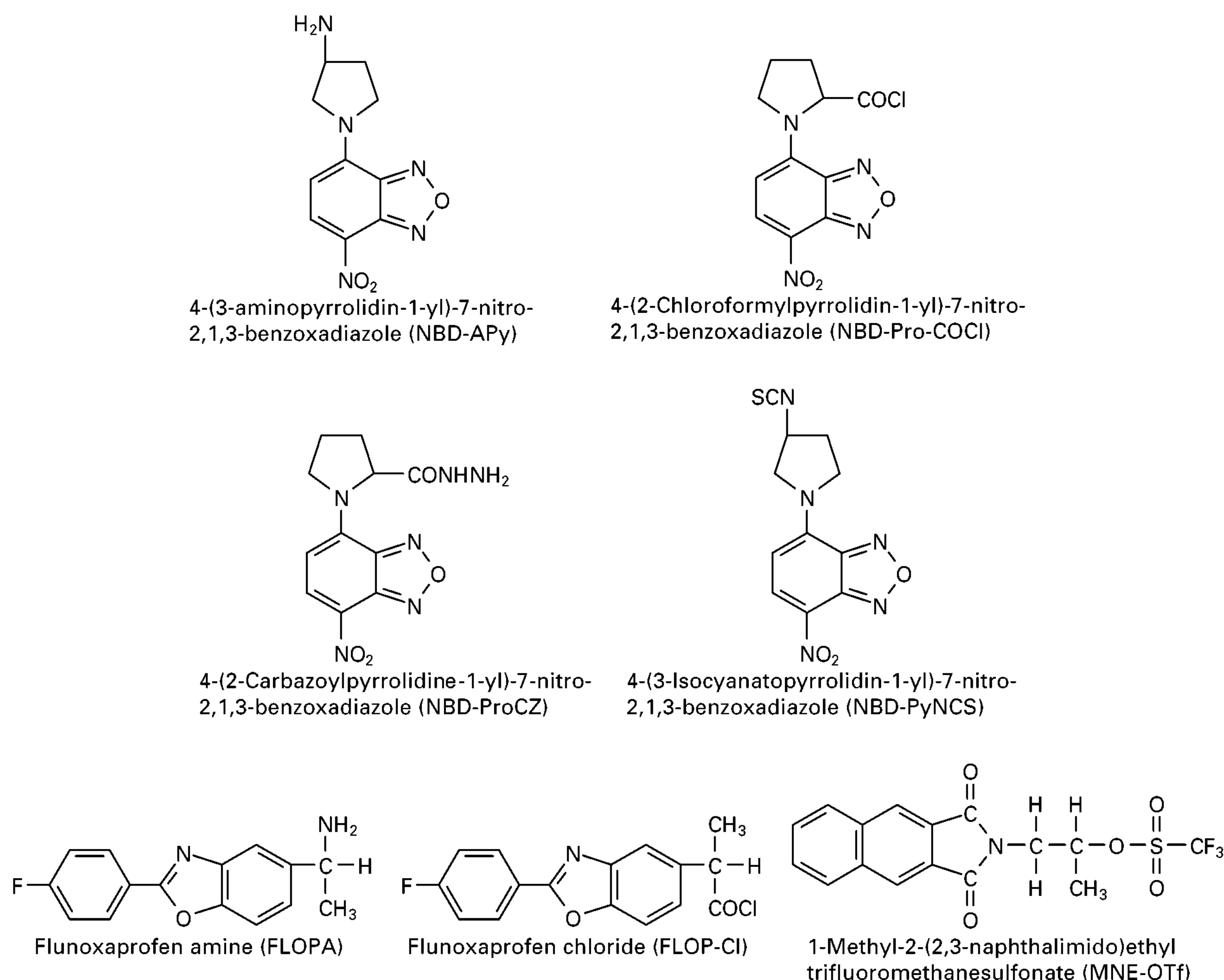



**Figure 2** Structure of reagents (see Table 1).

## Type I Chiral Stationary Phases

These stationary phases were based on aromatic $\pi$-acid (3,5-dinitrobenzene) and $\pi$-base (a naphthalene moiety) derivatives. In addition to $\pi$–$\pi$ interaction sites, they have hydrogen-bonding and dipole–dipole interaction sites provided by an amide, urea or ester moiety. By nuclear magnetic resonance measurements in solution, three interactions are proposed to occur in the more favoured diastereomeric complex between an *N*-(3,5-dinitrobenzoyl)-$\alpha$-amino amide and an *N*-(2-naphthyl)-$\alpha$-amino ester: a $\pi$–donor–acceptor interaction and two hydrogen-bonding interactions, as shown in **Figure 3**. In the development of these stationary phases, the reciprocality concept was introduced as follows: if optically active A resolves the enantiomers B, then optically active B resolves the enantiomers of A. As a result, $\pi$–acid and $\pi$–base compounds have been separated using chiral stationary phases based on $\pi$–base and $\pi$–acid derivatives. Since a combination of simultaneous $\pi$–$\pi$ and hydrogen-bonding interactions in the nonpolar solvent used as the mobile phase play an important role in chiral recognition, as described above, these stationary phases are mainly used in the normal-phase mode, although it is possible to separate some enantiomers in the reversed-phase mode.

Recently, chiral stationary phases based on the macrocyclic antibiotics, vancomycin and teicoplanin, have been developed. These stationary phases can separate many enantiomers in both normal- and reversed-phase modes.

## Type II Chiral Stationary Phases

Underivatized saccharides such as cellulose and starch have been used as chiral stationary phases. Cellulose, which contains *c.* 200 glucose units (microcrystalline cellulose), has been extensively used for the chiral resolution of highly polar compounds such as amino acids, amino acid derivatives and diaminodicarboxylic acids. The chiral recognition ability

**Table 2** Chiral stationary phases for liquid chromatography

| *Type* | *Ligand* | *Trade name* |
|---|---|---|
| Type I | *N*-(3,5-Dinitrobenzoyl)-D-, *N*-(3,5-Dinitrobenzoyl)-L-phenylglycine | D-,L-Phenylglycine |
| | *N*-(3,5-Dinitrobenzoyl)-D-, *N*-(3,5-Dinitrobenzoyl)-L-leucine | D-,L-Leucine |
| | Naphthyl-D-, naphthyl-L-alanine | D-, L-Naphthylalanine |
| | Naphthyl-D-, naphthyl-L-leucine | D-,L-Naphthylleucine |
| | *N*-(3,5-Dinitrobenzoyl)-(*R*)-1-naphthylglycine | Sumichiral OA-2500 |
| | *N*-(3,5-Dinitrobenzoyl)-aminocarbonyl-L-valine | Sumichiral OA-3100 |
| | *N*-[(*S*)-(1-Naphthyl) ethylaminocarbonyl]-L-valine | Sumichiral OA-4000 |
| | (*R*)-, (*S*)-1-Naphthylethylamine | LC-(*R*)-, LC-(*S*)-Naphthyl urea |
| | Teicoplanin | Chirobiotic T |
| | Vancomycin | Chirobiotic V |
| Type II | Microcrystalline cellulose triacetate | Chiralcel CA-1<br>Cellulose triacetate |
| | Cellulose *tris*(4-methylbenzoate) | Chiralcel OJ, OJ-R |
| | Cellulose tribenzoate | Chiralcel OB, OB-H |
| | Cellulose triacetate | Chiralcel OA |
| | Cellulose tricinnamate | Chiralcel OK |
| | Cellulose *tris*(3,5-dimethylphenylcarbamate) | Chiralcel OD, OD-H, OD-R |
| | Cellulose *tris*-phenylcarbamate | Chiralcel OC |
| | Cellulose *tris*(4-methylphenylcarbamate) | Chiralcel OG |
| | Cellulose *tris*(4-chlorophenylcarbamate) | Chiralcel OF |
| | Amylose *tris*(3,5-dimethylphenylcarbamate) | Chiralpak AD |
| | Amylose *tris*((*S*)-1-phenylethylcarbamate) | Chiralpak AS |
| | Poly-*N*-acryloyl-(*S*)-phenylalanine ethylester | ChiraSpher |
| | (+)-Poly(triphenylmethyl methacrylate) | Chiralpak OT(+) |
| | (+)-Poly(diphenyl-2-pyridylmethyl methacrylate) | Chiralpak OP(+) |
| Type III | 2,2′-Diphenyl-1,1′-binaphthol derivatives of 18-crown-6 | Crownpak CR(+)<br>Crownpak CR(−) |
| | $\alpha$-, $\beta$-, $\gamma$-Cyclodextrin | Cyclobond III, I, II |
| | $\beta$-, $\gamma$-Cyclodextrin | ChiraDex, ChiraDex Gamma |
| | $\beta$-Cyclodextrin derivatives | Cyclobond I Ac, SP, RSP, SN, RN, DMP, PT[a];<br>Ultron ES-PhCD[b]; Nucleosil $\beta$-PM[c] |
| | $\alpha$-, $\gamma$-Cyclodextrin derivatives | Cyclobond III Ac, II Ac |
| Type IV | L-Hydroxyproline | Nucleosil Chiral-1 |
| | 2-Amino-1,2-diphenylethanol | Chiralpak WE |
| | *N*,*S*-Dioctyl-D-penicillamine | Sumichiral OA-5000 |
| Type V | Bovine serum albumin | Resolvosil BSA-7, BSA-7PX<br>Ultron ES-BSA<br>Chiral-BSA |
| | Human serum albumin | Chiral-HSA<br>Chiral-HSA |
| | $\alpha_1$-Acid glycoprotein | Chiral-AGP |
| | Ovomucoid | Ultron ES-OVM |
| | Avidin | Bioptic AV-1 |
| | Cellulase | Chiral-CBH |
| | Pepsin | Ultron ES-Pepsin |

[a] Ac, Acetylate; SP, (*S*)-2-hydroxypropyl ether; RSP, racemic 2-hydroxylpropyl ether; SN, (*S*)-naphthylethylcarbamate; RN, (*R*)-naphthylethylcarbamate; DMP, 2,6-dimethylphenylcarbamate; PT, *para*-toluoyl ester.
[b] PhCD, Phenylcarbamate.
[c] $\beta$-PM, Permethylate.

of the cellulose is based on the microcrystallinity because, when treated with dilute alkali, cellulose loses its chiral recognition ability, resulting in a stable amorphous form.

It was found that microcrystalline cellulose triacetate preserved microcrystallinity and had excellent chiral recognition ability. In contrast, microcrystalline cellulose triacetate precipitated from a solution

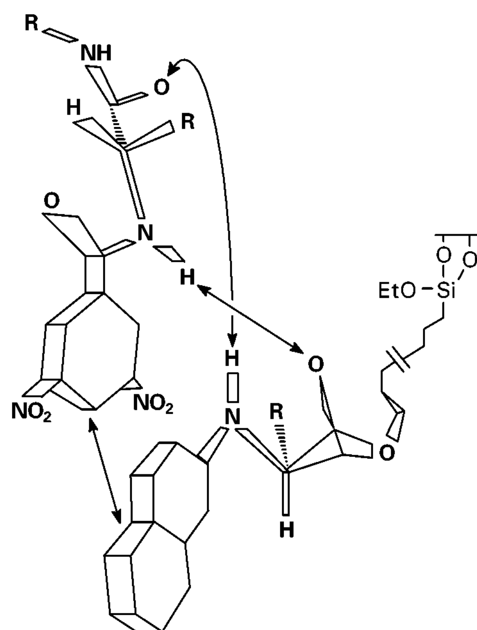



**Figure 3** The more favoured diastereomeric complex between an *N*-(3,5-dinitrobenzoyl)-α-amino amide and an *N*-(2-naphthyl)-α-amino ester. A π–donor–acceptor interaction and two hydrogen-bonding interactions are indicated by arrows. (Reproduced with permission from Pirkle WH and Pochapsky TC (1987) *Advances in Chromatography*, vol. 27, p. 116. New York: Marcel Dekker.)

has another morphology, and different chiral recognition properties, compared with microcrystalline cellulose triacetate. The chiral stationary phases based on the cellulose triacetate precipitated from a solution are prepared by coating the polymer on a silica gel matrix. Many chiral stationary phases prepared by this technique are commercially available as triacetate, tribenzoate, trisphenylcarbamate, tribenzyl ether and tricinnamate derivatives of cellulose.

Chiral stationary phases based on cellulose and amylose derivatives could separate 78% of racemates examined. However, the disadvantage of stationary phases based on the cellulose and amylose derivatives coated on silica gels is the restriction of the eluents used; the coated polymer is soluble in some eluents and removed. Polysaccharide derivatives chemically bound to silica gel overcome this problem. The coated and the chemically bound polymers showed different chiral recognition properties.

With regard to the chiral recognition mechanism, the interaction of the solute and the chiral stationary phase based on cellulose phenylcarbamate derivatives has been investigated by computational chemistry and nuclear magnetic resonance measurements of the complex. It was found that π–π and hydrogen-bonding interactions play an important role in chiral recognition of the solute.

## Type III Chiral Stationary Phases

Type III chiral stationary phases include cyclodextrin (CD), polymethacrylate and crown ether stationary phases. The solute enters into chiral cavities to form inclusion complexes and the relative stability constants of the resulting diastereomeric complexes are different. The cavities of CD and polymethacrylate are hydrophobic, while those of crown ethers are hydrophilic.

α-, β- and γ-CD are cyclic oligosaccharides containing 6, 7 and 8 β-1, 4-D-glucoside units, respectively. Because an aromatic portion of a molecule can enter into the chiral cavity, solutes having an aromatic moiety at, or adjacent to, the chiral centre are well resolved. Acetyl, hydroxypropyl, naphthylethyl carbamate and phenyl carbamate derivatives of CDs have been prepared and used for chiral resolution of many solutes which cannot be separated using native CDs. Chiral stationary phases based on CDs and derivatized CDs have been predominantly used in reversed-phase mode.

Chiral stationary phases based on polymethacrylate have been prepared using a chiral monomer such as (*S*)-acryloylphenylalanine, and achiral monomers such as triphenylmethyl methacrylate and diphenyl-2-pyridylmethyl methacrylate together with chiral cation catalysts. In normal-phase mode, chiral stationary phases based on these polymers can separate many racemic solutes that are difficult to resolve by other methods because of a lack of functionality.

Crown ethers, which are synthetic macrocyclic polyethers, can form selective complexes with various cations. Chiral crown ethers covalently bound or adsorbed on to silica supports can separate enantiomeric ammonium compounds such as amino acid esters, amines and amino alcohols. The multiple hydrogen-bonding interactions between the ammonium group and the ether oxygens play an important role in the chiral recognition.

## Type IV Chiral Stationary Phases

Chiral ligand exchange chromatography is based on the formation of diastereomeric ternary complexes that involve a transition metal ion (M), chiral ligand (L), and the enantiomers of the racemic solute (*R* and *S*). Of all the transition metals examined [Cu(II), Ni(II), Zn(II), Hg(II), Co(III), Fe(III), etc.], Cu(II) forms the most stable complexes, and cyclic amino acids such as L-proline and L-hydroxyproline are the best chiral selectors, when bound to a polymer or silica support. The diastereomeric mixed chelate complexes formed in this system are represented by the formulas L-M-*R* and L-M-*S*. When these

complexes have different stabilities, the less stable complex is eluted first, and the enantiomeric solutes are separated. The enantiomers resolved include amino acids, amino acid derivatives, 2-amino alcohols, barbiturates and hydantoins.

## Type V Chiral Stationary Phases

Chiral stationary phases based on a protein are of special interest because of their unique properties of stereoselectivity and because they are suited for separating a wide range of enantiomeric mixtures. Protein-based stationary phases so far developed have included albumins such as bovine and human serum albumin, enzymes such as trypsin, $\alpha$-chymotrypsin, lysozyme and pepsin, and glycoproteins such as $\alpha_1$-acid glycoprotein from human or bovine serum, cellobiohydrolase I from the fungus *Trichoderma reesei*, ovomucoid (in fact, ovoglycoprotein), avidin and ovotransferrin from egg whites, and flavoprotein (riboflavin-binding protein) from egg whites and yolks. The advantages of protein-based stationary phases generally include the use in reversed-phase mode, enantioselectivity for a wide range of compounds and direct analysis without derivatization. The disadvantages are low capacity, lack of column ruggedness and limited understanding of the chiral recognition mechanism. These stationary phases are mainly used for analytical purposes.

With regard to the chiral recognition mechanism, hydrophobic, electrostatic and hydrogen-bonding interactions take place between the chiral stationary phase and the solute.

## Chiral Mobile Phase Additives

There are no fundamental differences between the techniques using chiral stationary phases and chiral

**Table 3** Chiral selectors used as mobile-phase additives

| *Chiral selector* | *Enantiomers* | *Stationary phase* |
|---|---|---|
| **Metal complex** | | |
| L- or D-Proline + Cu(II), L-Phenylalanine + Cu(II), *N*-Methyl-L-phenylalanine + Cu(II), *N,N*-Dimethyl-L-phenylalanine + Cu(II), (*R*, *R*)-Tartaric acid mono-1-octylamide + Cu(II) | Amino acids | Cation exchanger, ODS, OS |
| L-Propyl-*n*-octylamide + Ni(II), L-Proline + Cu(II), L-Arginine + Cu(II), L-Histidine + Cu(II) | Dansyl amino acids | ODS, OS |
| (*R*, *R*)-Tartaric acid mono-1-octylamide + Cu(II) | $\beta$-Amino alchols | ODS |
| **Uncharged additives** | | |
| 1,1′-Binaphthyl derivatives of 18-crown-6 | Amino acids | ODS |
| *Cyclodextrins* | | |
| $\alpha$-Cyclodextrin | $\alpha$-, $\beta$-Pinene | ES |
| $\beta$-Cyclodextrin | Propranolol, pseudoephedrine, salsolinol, thalidomide, dansyl amino acids | Porous graphite carbon, CN, ODS |
| $\gamma$-Cyclodextrin | Norgesterol | ODS, CN |
| TM-$\beta$-Cyclodextrin | Benzoin, ethyl mandelate | Si |
| | 5-Butyl-1-methyl-5-phenylbarbituric acid, Dansyl phenylalanine | ODS |
| CM-, CE-$\beta$-Cyclodextrin | Aminoethylbenzodioxane derivatives, hexobarbital | BS |
| *Chiral acid* | | |
| 10-Caphorsulfonic acid | Amino alcohols | Diol |
| Z-Glycyl-L-proline | Amino alcohols, *N*-alkylated-2-aminotetralines | Diol |
| *Chiral amine* | | |
| Alprenolol, quinine, quinidine, cinchonidine | 10-Caphorsulfonic acid, *N*-(1-phenylethyl) phthalamic acid, *O*-methylmandelic acid, *O*-methoxy-$\alpha$-trifluoromethyl-phenylacetic acid, 2-phenylacetic acid, naproxen | Diol |

ODS, octadecylsilyl; OS, octylsilyl; ES, ethylsilyl; CN, cyanopropylsilyl; TM, heptakis(2,3,6-tri-*O*-methyl); Si, silica gels; CM, carboxymethyl; CE, carboxyethyl; BS, butylsilyl; Z, *N*-benzoxycarbonyl.

mobile phase additives. This means that all chiral selectors covalently bound to supports can be used for the separation of enantiomers by addition to the mobile phase. With chiral mobile phase additive techniques, there are at least two possible mechanisms; one is that the chiral mobile phase additive and the enantiomers may form diastereomers in the mobile phase. Another is that the stationary phase may be coated with the chiral mobile phase additive, resulting in diastereomeric interactions with the enantiomeric pairs during chromatography. It is thought that both mechanisms occur depending on both the stationary and mobile phases used.

The techniques using chiral mobile phase additives can be divided into three categories: metal complexation used in chiral ligand exchange chromatography, the use of various uncharged additives, and the ion-pairing techniques used for charged analytes. **Table 3** shows chiral selectors used as mobile phase additives.

*See also:* **II/Chromatography: Liquid:** Derivatization; Mechanisms: Chiral; **III/Chiral Separations:** Cellulose and Cellulose Derived Phases; Chiral Derivatization; Cyclodextrins and Other Inclusion Complexation Approaches; Ligand Exchange Chromatography; Ion-Pair Chromatography; Protein Stationary Phases; Synthetic Multiple Interaction ('Pirkle') Stationary Phases. **Inclusion Complexation: Liquid Chromatography.**

## Further Reading

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# Molecular Imprints as Stationary Phases

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In 1949, Frank Dickey published what can be considered the first paper on a molecularly imprinted synthetic material. The work was inspired by the theories of Dickey's mentor Linus Pauling, who had suggested that the primary structures of all polypeptides constituting the antibodies are the same and that the diversity originates from folding directed by physical contact with the antigens. Even if Pauling's hypothesis on antibodies later turned out to be incorrect, his ideas laid the foundation for the concept of molecular imprinting. Consistent with these early studies, molecular imprinting can be defined as a method in which the selectivity of a material for a chosen molecule is induced by the presence of the molecule during the preparation, assembling or rearrangement of the material.

Dickey's studies were followed by several other investigations in the same direction, but it was not until the 1970s that the field of molecular imprinting started to mature to its present form. Wulff introduced a new approach that allowed the introduction of functional groups at defined positions in synthetic network polymers. This approach is often referred to as covalent molecular imprinting, to distinguish it from the noncovalent approach developed by Mosbach and his co-workers in the early 1980s.

The field of molecular imprinting is growing rapidly. Molecularly imprinted polymers (MIPs) have found application as stationary phases for chiral separations and solid-phase extractions, as synthetic antibodies in competitive ligand-binding assays, as recognition elements in sensors and as catalysts of chemical reactions. The concepts of molecular imprinting will be described briefly here. For a more detailed description of the imprinting principle and the preparation of molecularly imprinted polymers, see 'Affinity Separation: Imprint Polymers' in this encylopedia. The remainder of this paper focuses on the use of molecularly imprinted polymers as chiral stationary phases (CSPs).