with 2-propanol have been found in some instances to improve the separation.

Functionalized Cellulose Phases

In the overwhelming majority of cases enantiomers can be separated on these columns without derivatization. In a few instances esterification of the carboxyl group and the introduction of a benzoyl group into the molecule have been found advantageous.

Conclusions and Future Trends

It is indisputable that the direct chromatographic enantioseparations based on chiral stationary phases has greatly surpassed the importance of indirect separations based on covalent chiral derivatization. The fact that the overwhelming majority of papers reporting on the development and application of chiral separations are from these fields does not necessarily reflect the real of the contribution of covalent chiral derivatization in the solution of practical problems in pharmaceutical and biomedical analysis. In some areas (e.g. the determination of small concentrations of D-amino acids in various biological samples, etc.) the well-established classical methods are still in use. It is also remarkable that the combination of chiral derivatization with the use of chiral stationary phases has proved to be suitable for the solution of very delicate problems (separation of several enantiomers and diastereomers of drugs with more than one chiral centre). It should also be noted that the combination of chiral chromatography with fluorescence derivatization enables the limit of detection of enantiomeric impurities to be decreased to the 10 ppm. level. These tendencies are expected to continue in the near future.

See also: **II/Chromatography: Liquid:** Derivatization. **III/Chiral Separations:** Amino Acids and Derivatives; Capillary Electrophoresis; Cellulose and Cellulose Derived Phases; Chiral Derivatization; Countercurrent Chromatography; Cyclodextrins and Other Inclusion Complexation Approaches; Gas Chromatography; Ion-Pair Chromatography; Ligand Exchange Chromatography; Liquid Chromatography; Molecular Imprints as Stationary Phases; Protein Stationary Phases; Supercritical Fluid Chromatography; Synthetic Multiple Interaction ('Pirkle') Stationary Phases; Thin-Layer (Planar) Chromatography.

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Countercurrent Chromatography

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Introduction

Countercurrent chromatography (CCC) is a generic term for support-free liquid/liquid partition chromatography, which can be used for the separation of a variety of enantiomers by dissolving a chiral selector (CS) in the liquid stationary phase. The method eliminates the time-consuming procedure which involves chemically bonding the chiral selector to a solid support for chiral chromatography. It also provides an important advantage over the conventional methods in that the same column can be used for the separation of various enantiomers simply by

dissolving the suitable chiral selector in the liquid stationary phase.

In the past various hydrostatic CCC systems, such as droplet CCC, rotation locular CCC (RLCCC) and centrifugal partition chromatography (CPC), have been used for the separation of chiral compounds. None of those techniques, however, is considered satisfactory for preparative purposes in terms of sample size, resolution and/or separation time. Recently, the hydrodynamic CCC system termed high speed CCC has remarkably improved the technique so that highly efficient separations can be achieved in a short elution time. This technique has been successfully applied to the separation of racemates using a Pirkle-type chiral selector. In this method analytical (milligram) and preparative (gram) separations can be performed simply by adjusting the amount of chiral selector in the liquid stationary phase in the standard separation column. Gram quantities of enantiomers can be obtained by pH-zone-refining CCC, a new preparative separation technique recently developed for the separation of ionizable compounds (see pH-zone-refining CCC). In addition, the present method allows computation of the formation constant of the chiral-selector complex, one of the most important parameters for studies on the mechanism of enantioselectivity.

Standard High speed CCC Technique for Chiral Separation

The separations are performed using a commercial high speed CCC centrifuge equipped with a set of multilayer coil separation columns. The two-phase solvent system is selected in such a way that the target analyte has a partition coefficient ranging from 0.5 to 1 in the CS-free solvent system. When the organic phase is used as the stationary phase, the chiral selector may be made hydrophobic by attaching a long hydrocarbon chain to enhance both solubility and retention in the stationary phase.

In each separation, the column is first filled about half way with the CS-free stationary phase. This is followed by introduction of the CS-containing stationary phase (about 60% of the total column capacity) by discharging the excess amount of CS-free stationary phase from the other end of the column. In this way some amount of CS-free stationary phase (about 10% of the stationary phase retained in the column) remains at the end of the column during separation to absorb carried-over CS from the mobile phase which would contaminate the eluted fractions. After the sample solution is injected through the sample port, the mobile phase is pumped into the column while the column is rotated at the required speed, usually 800–1000 rpm. If desired separation can be carried out by successive injection of samples without replenishing the CS-containing stationary phase.

Figure 1 shows the separation of four pairs of dinitrobenzoyl (DNB)–amino acid enantiomers by the standard CCC technique using a two-phase solvent system composed of hexane/ethyl acetate/methanol/10 mM HCl with *N*-dodecanoyl-L-3,5-dimethylanilide as a CS in the organic stationary phase. Because of its high hydrophobicity ($K > 100$), this CS has high solubility in the organic stationary phase and is almost entirely partitioned into the stationary phase. All analytes were well resolved in 1–3 h. This chiral selector is similar to the 'Pirkle'

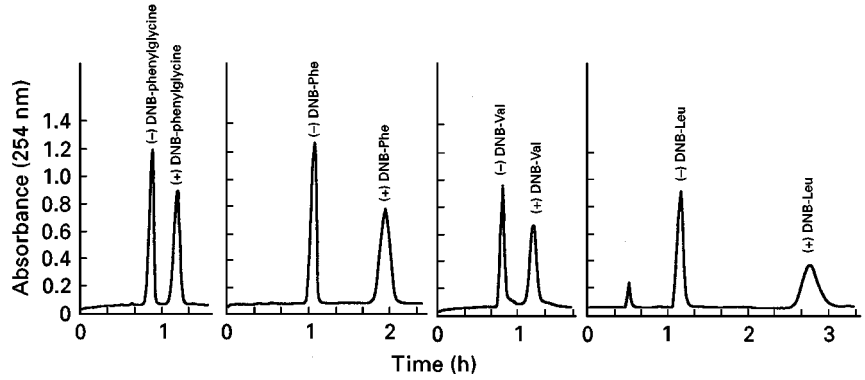

Figure 1 Separation of four racemic pairs of DNB-amino acids by the standard high speed CCC technique. Experimental conditions: apparatus: multilayer coil high speed CCC centrifuge with a semipreparative column of 1.6 mm i.d. and 330 mL capacity; solvent system: hexane/ethyl acetate/methanol 10 mM HCl (8 : 2 : 5 : 5, v/v/v/v), *N*-dodecanoyl-L-proline-3,5-dimethylanilide (CS) (2 g) was added to the organic stationary phase (200 mL) as a chiral selector; samples: from left to right, (±)-DNB-phenylglycine, (±)-DNB-phenylalanine, (±)-DNB-valine, and (±)-DNB-leucine, 5–10 mg of each dissolved in 5 mL of solvent consisting of equal volumes of each phase; flow rate: 3.3 mL min^{-1}; revolution: 800 rpm; analysis of fractions: optical rotation and circular dichroism; stationary phase retention: 65% of the total column capacity.

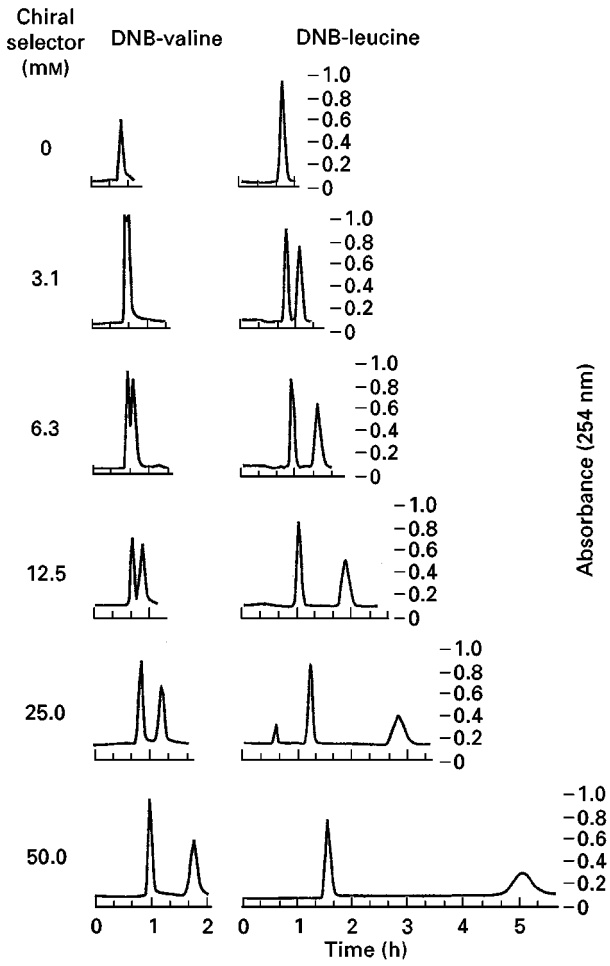

Figure 2 Effects of the amount or concentration of CS on the separation of DNB–amino acid racemates. In all resolved chromatograms, the first peak represents (−)-enantiomer and the second peak (+)-enantiomer. Experimental conditions: apparatus and column: see the Figure 1 caption; sample: racemic DNB–amino acid mixture consisting of DNB-valine and DNB-leucine, each 5–10 mg dissolved in 2 mL of solvent (1 mL of each phase): solvent system: hexane/ethyl acetate/methanol/10 mM HCl (8 : 2 : 5 : 5, v/v/v/v); stationary phase: upper organic phase with CS ranging from 0 to 4 g in 200 mL as indicated; mobile phase, lower aqueous phase; flow rate: 3 mL min^{-1}; revolution: 800 rpm.

chiral stationary phase which has been introduced for the HPLC separation of racemic DNB–amino acid t-butylamides. For CCC separation an *N*-dodecanoyl group was covalently attached to the CS molecule to increase its hydrophobicity so that it is almost entirely partitioned into the organic stationary phase.

The effect of the CS concentration in the stationary phase on the peak resolution was investigated by a series of experiments as shown in **Figure 2**. As the CS concentration was increased, the separation factor and peak resolution were increased as indicated by eqn [6]. The results clearly imply an important technical strategy for the present method: the best peak resolution is attained by using a saturated solution of the CS in the stationary phase in a given column, and the resolution is further improved by using a longer and/or wider-bore coiled column which can hold a greater amount of CS in the stationary phase.

The preparative capability of the system was investigated on the separation of DNB-leucine enantiomers by varying the CS concentration in the stationary phase. The results shown in **Figure 3** indicate that the sample loading capacity is mainly determined by the CS concentration in the stationary phase, i.e. the higher the CS concentration, the greater the peak resolution and sample loading capacity. As mentioned earlier, the standard HSCCC column (typically 300 mL in capacity) can be used for both analytical and preparative separation simply by adjusting the CS concentration in the stationary phase.

pH-Zone-refining CCC for Chiral Separation

pH-Zone-refining CCC is a powerful preparative technique that yields a succession of highly concentrated rectangular solute peaks with minimum overlap where impurities are concentrated at the peak boundaries. This technique has been applied to the resolution of DNB-amino acid racemates using a binary two-phase solvent system where the retainer acid and chiral selector were added to the organic stationary phase and the eluent base to the aqueous mobile phase. **Figure 4** shows a typical chromatogram of a DNB-leucine racemate obtained by pH-zone-refining CCC. The pH of the fraction (dotted line) reveals that the peak is evenly divided into two pH zones with a sharp transition. Compared with the standard CCC technique, the pH-zone-refining CCC technique allows separation of large amounts of analyte in a shorter elution time.

Determination of Formation Constant of CS–Enantiomer Complex

The present technique has an advantage over conventional HPLC by allowing the determination of the formation constant (K_f) for the CS–enantiomer complex which is useful for developing the CS for chiral chromatography.

In a schematic view of the portion of the separation column in **Figure 5**, enantiomers (A_+ and A_-) are partitioned between the organic stationary phase (upper half) and the aqueous mobile phase (lower half). In the organic stationary phase enantiomers form a CS complex [$CSA_\pm$] according to their formation constants ($K_{f\pm}$). In this situation:

$$D_\pm = ([A_\pm]_{org} + [CSA_\pm]_{org})/[A_\pm]_{aq} \qquad [1]$$

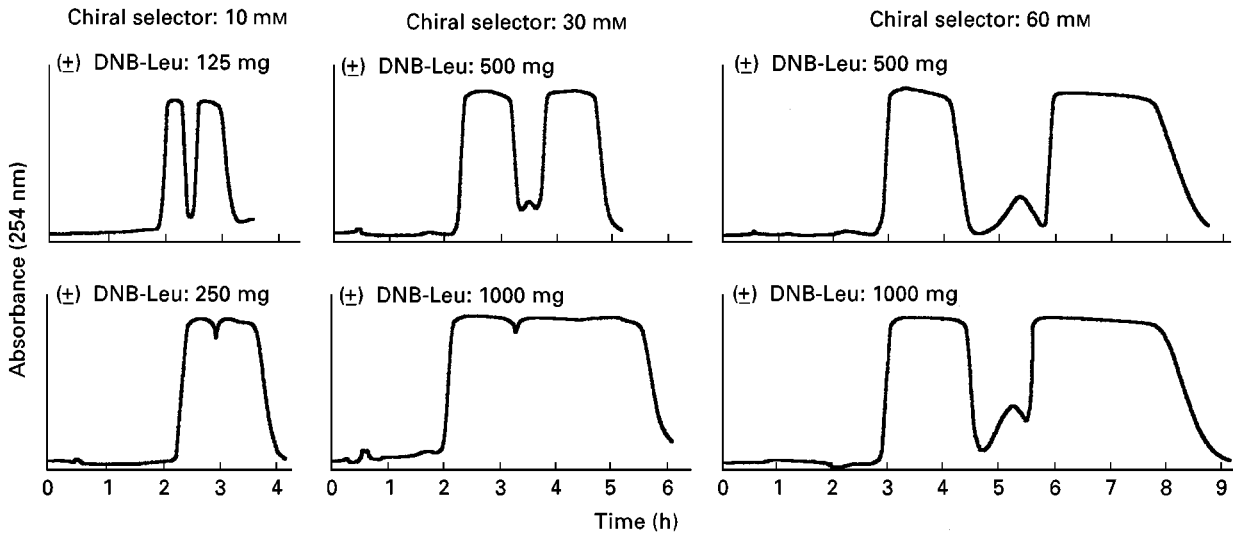

Figure 3 Preparative separation of DNB-leucine racemate by high speed CCC. Experimental conditions: solvent system: hexane/ethyl acetate/methanol/10 mM HCl (6 : 4 :5 : 5) where the organic stationary phase containing CS at 10 to 60 mM as indicated; samples: (±)-DNB-leucine 125–1000 mg dissolved in 10–45 mL of solvent. (For other conditions, see the Figure 1 caption.)

$$D_0 = [A_\pm]_{org}/[A_\pm]_{aq} \quad [2]$$

$$K_{f\pm} = [CSA_\pm]_{org}/[CS]_{org}[A_\pm]_{org} \quad [3]$$

where D_0 is the partition coefficient of the analyte in a CS-free solvent system (this is also called the partition ratio) and $D_\pm$ is the partition coefficient in a CS-containing solvent system. From these equations, the partition coefficient of the analyte is given by the following equation:

$$D_\pm = D_0(1 + K_{f\pm}[CS]_{org}) \quad [4]$$

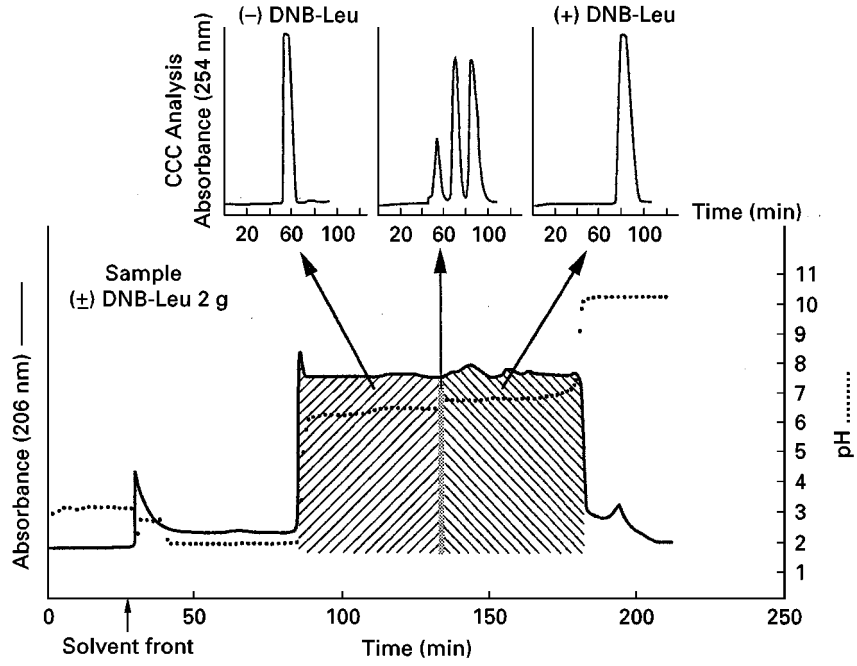

Figure 4 Separation of DNB-leucine racemate by pH-zone-refining CCC. Experimental conditions: apparatus: see the Figure 1 caption; solvent system: methyl t-butyl ether/water; stationary phase: upper organic phase to which trifluoroacetic acid (40 mM) and CS (40 mM) were added; mobile phase: lower aqueous phase to which aqueous ammonia was added to 20 mM; sample: (±)-DNB-leucine 2 g; flow rate: 3.3 mL min^{-1}; revolution: 800 rpm; analysis: chirality by analytical HSCCC (see Figure 1) and pH by a portable pH meter. Note that the analysis of the fraction from the mixing zone (middle chromatogram) shows three peaks corresponding to (−)-DNB–leucine, impurity and (+)-DNB–leucine from left to right.

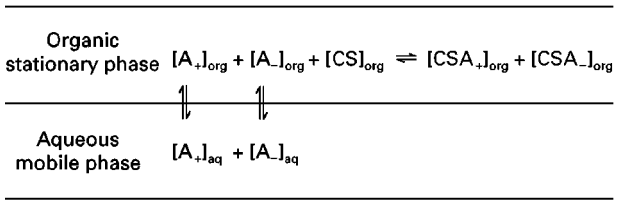

Figure 5 Schematic diagram of simple chemohydrodynamic equilibrium between the racemates ($A_\pm$) and chiral selector (CS) in the separation column.

Here, $[CS]_{org}$ is the difference between the initial concentration of the CS and the concentration of the $CSA_\pm$ complex, i.e.:

$$[CS]_{org} = [CS]_{initial} - [CSA_\pm]_{org} \quad [5]$$

When $[A_\pm]_{org} \ll [CS]_{initial}$, $[CS]_{org}$ approaches $[CS]_{initial}$ hence, eqn [4] may be rewritten:

$$D_\pm \approx D_0(1 + K_{f\pm}[CS]_{initial}) \quad [6]$$

The validity of eqn [6] has been examined by a series of experiments, the results of which indicated that the separation factor (D_+/D_-) is increased as expected by increasing the concentration of chiral selector in the stationary phase (Figure 2).

For computation of K_f, eqn [6] can be modified into a more convenient form:

$$(D_\pm - D_0)/D_0 \approx K_{f\pm}[CS]_{initial} \quad [7]$$

where $D_\pm$ and D_0 can be computed from the chromatograms obtained with and without the chiral selector in the stationary phase.

Using eqn [7] the formation constant ($K_{f\pm}$) has been determined by a series of experiments where small amounts (0.1–0.2 mg) of enantiomers were separated at various CS concentrations in the stationary phase. **Figure 6** is drawn by plotting the $(D_\pm - D_0)/D_0$ values from each enantiomer against the initial CS concentration in the stationary phase where the formation constant is computed from the slope of the straight line. These results indicate that the method is useful for computing the formation constants of various analyte–CS pairs.

General Chemodynamic Model in Chiral CCC

This second model deals with more generalized condition where the ionic analytes are dissociated in the aqueous mobile phase. This approach can be useful for predicting the feasibility of chiral resolution by pH-zone-refining CCC.

In Figure 5, which shows the chemodynamic equilibrium in a portion of the separation column, if the analytes are ionizable (e.g. acids) they will be partially dissociated to form anions $[A^-_\pm]_{aq}$ which are almost insoluble in the organic phase.

In this equilibrium state, the following set of equations is given for each racemate:

$$D_\pm = ([A_\pm H]_{org} + [CSA_\pm H]_{org})/([A_\pm H]_{aq} + [A^-_\pm]_{aq}) \quad [8]$$

$$D_0 = [A_\pm H]_{org}/[A_\pm H]_{aq} \quad [9]$$

$$K_a = [A_\pm H]_{aq}/[A^-_\pm]_{aq}[H^+]_{aq} \quad [10]$$

$$K_{f\pm} = [CSA_\pm H]_{org}/[CS]_{org}[A_\pm H]_{org} \quad [11]$$

where $D_\pm$, D_0, K_a, and $K_{f\pm}$ represent the partition coefficient, the partition ratio, the dissociation constant, and the CS–complex formation constant for each racemate, respectively. From these equations, we obtain:

$$pH_{Z\pm} = pK_a + \log\{D_0/D_\pm)(1 + [CS]_{org}K_{f\pm}) - 1\} \quad [12]$$

In pH-zone-refining CCC, the peak resolution is mainly determined by the difference in pH between

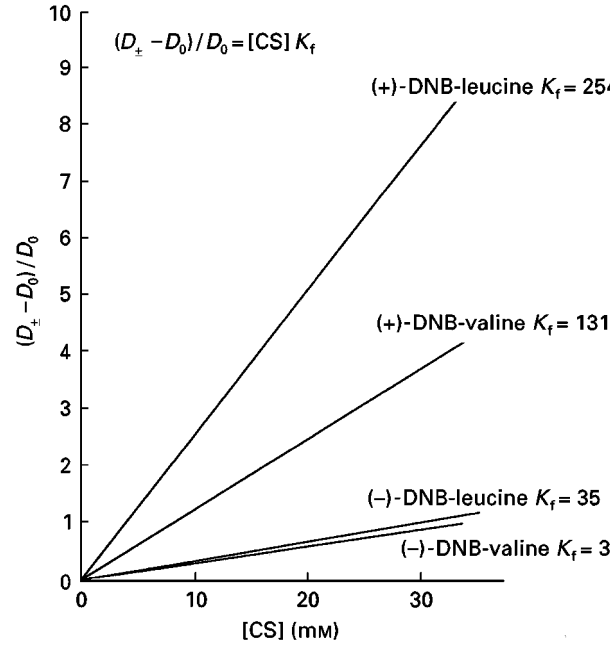

Figure 6 Determination of formation constant of CS-DNB-amino acids by the standard HSCCC technique. The diagram was produced by plotting $(D_\pm - D_0)/D_0$ (eqn [7]) against the initial CS concentration in the organic stationary phase. The slope of each line indicates the formation constant (K_f) of the corresponding enantiomer.

the two zones, i.e.:

$$\Delta pH_{Z\pm} = \log\{([CS]_{org/Z+}K_{f+} + 1 - D_0/K_r)/ ([CS]_{org/Z-}K_{f-} + 1 - D_0/K_r)\} \quad [13]$$

where $[CS]_{org/Z+}$ and $[CS]_{org/Z-}$ are the free CS concentrations in the A_+ and A_- zones, respectively. When $K_{f+} > K_{f-}$, $[CS]_{org/Z+} < [CS]_{org/Z-} < [CS]_{initial}$. Eqn [13] indicates that chiral resolution can be improved by increasing D_0/K_r and/or choosing the CS with a large K_{f+}/K_{f-} value. It also implies that increasing the CS concentration will yield higher peak resolution.

Advantages of Chiral CCC

Countercurrent chromatography can be applied to the separation of enantiomers by dissolving a suitable chiral selector in the liquid stationary phase in analogy to binding the CS to the solid support. The HSCCC technique has the following advantages over the conventional chromatographic technique using a solid stationary phase:

1. The method permits repetitive use of the same column for a variety of chiral separations by choosing appropriate chiral selectors.
2. Both analytical and preparative separations can be performed in a standard CCC column by adjusting the amount of CS in the liquid stationary phase. The method is cost effective especially for large scale preparative separations.
3. The separation factor and peak resolution can be improved by increasing the concentration of CS in the stationary phase.
4. The method is very useful for investigation of the enantioselectivity of the chiral selector including determination of formation constant and separation factor.
5. pH-Zone-refining CCC can be applied to chiral separation allowing a large scale separation in a shorter separation time.

See also: **II/Chromatography:** Chromatography: Instrumentation. **Chromatography: Liquid:** Column Techonology. **III/Chiral Separations:** Amino Acids and Derivatives; Capillary Electrophoresis; Cellulose and Cellulose Derived Phases; Countercurrent Chromatography; Crystallization; Cyclodextrins ad Other Inclusion Complexation Approaches; Gas Chromatography; Ion-Pair Chromatography; Ligand Exchange Chromatography; Liquid Chromatography; Molecular Imprints as Stationary Phases; Protein Stationary Phases; Supercritical Fluid Chromatography; Synthetic Multiple Interaction ('Pirkle') Stationary Phases; Thin-Layer (Planar) Chromatography. **Zone Refining Countercurrent Chromatography**.

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Crystallization

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Methods for obtaining optically active compounds in enantiopure form are commonly classified into three categories: utilization of chiral pool starting materials (stereoselective multistep synthesis), creation of chirality from achiral precursors (asymmetric synthesis) and separation of racemates into their enantiomer constituents (resolution). This last method can be carried out in a variety of ways: crystallization processes, chromatography of racemates on chiral stationary phases and kinetic resolution mediated by chiral reagents or enzymes.

The crystallization methods comprise several variants: (1) direct crystallization of enantiomer mixtures, (2) separation of diastereoisomer mixtures – the so-called classical resolution – and (3) crystallization-induced asymmetric transformation. The first two were discovered by Louis Pasteur in 1848 and 1853, respectively. The third was first reported in 1913. At the turn of the 21st century, these methods are in wide use in laboratory-scale separations as well as in industry for the preparation of pharmaceutical and agrochemical active principles. To cite but a few examples, hundreds to thousands of tonnes of commercially important materials such as (−)-menthol, (*S*)-(+)-naproxen, L-α-methyldopa, D-phenylglycine and the pyrethroid insecticide deltamethrin are produced by such methods.