FEATURE ARTICLE

# Chromatography on chiral stationary phases

**Stig Allenmark[a] and Volker Schurig[b]**

[a]*Department of Organic Chemistry, Göteborg University, S-41296 Göteborg, Sweden*
[b]*Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, D-72076 Tübingen, Germany*

An overview is given of the development of the variety of chiral stationary phase materials that are available today for the direct separation of enantiomers by different chromatographic methods. Applications of these range from trace analytical determinations of enantiomeric composition in biological materials to large scale preparative isolation of enantiopure compounds of industrial importance. These chromatographic techniques are also of interest for studies of mechanism and dynamics, like enantiomerization processes due to chiral inversion taking place in thermally labile chiral molecular structures.

## Introduction

The separation of enantiomers by chromatography can be performed in two modes: indirect method (a), in which off-column conversion of enantiomers into diastereomeric derivatives occurs, by chemical reaction with an enantiomerically pure resolving agent and subsequent chromatographic separation of the diastereomers on a conventional achiral stationary phase; and direct method (b), in which chromatographic separation of the enantiomers takes place on a chiral stationary phase (CSP) containing a resolving agent in high but not necessarily complete enantiomeric purity.

While method (a) involves the formation of diastereomers before separation, method (b) relies on the different diastereomeric molecular association between the chiral, non-racemic stationary phase (selector) and the chiral analyte (selectand). Since diastereomers usually possess different physical properties, an unintended discrimination may arise during detection when using method (a). Also fractionation may occur as the result of incomplete recovery, decomposition and losses during work-up, isolation and sample handling. Furthermore, racemization and kinetic resolution must be absent in the formation of diastereomers by the indirect method (a). Thus, the more refined direct method (b) is preferred in modern enantiomer separation and analysis whenever possible.

Ever since the days of Louis Pasteur, separation of racemates into enantiomers has been a challenge. Still a rather unpredictable matter, the situation has been drastically improved, however, owing to the development of chiral phase systems in chromatography, enabling direct enantiomer separation in a flow system. The crucial issue for the success of these techniques is the chiral material used as the stationary phase in the system. Some early experiments with liquid chromatography on columns packed with lactose[1,2] demonstrated that such separations could be realized; however, no systematic research was made until Gil-Av and co-workers started to use amides and oligopeptides as chiral stationary phases (CSPs) in gas chromatography for the separation of racemic amino acid derivatives.[3]

The chiral discrimination between the enantiomers in this case was caused by a slightly more favourable multiple hydrogen bonding interaction with the stationary phase for the more retained enantiomer. As illustrated by Fig. 1, the CSP and the solute were complementary to each other, forming transient diastereomeric complexes possessing a small difference in stability which caused the resolution.

A similar breakthrough took place in liquid chromatography when in 1966 acetylated microcrystalline cellulose was used with ethanol as the mobile phase to achieve separation of enantiomers.[4] It was shown later[5] that heterogeneous acetylation with almost complete preservation of the microcrystallinity of the cellulose to give a triacetate of each glucose unit could be performed easily, giving a widely useful chiral sorbent. It was also found that the microcrystallinity was essential for the enantioselective properties of the material and that it was lost on dissolution and reprecipitation. It also became clear that this material, at least to some extent, operated by means of inclusion of the solute into molecular cavities, and therefore the term 'inclusion chromatography' was often used to characterize the complex separation process.

## Chiral stationary phases (CSPs) in gas chromatography

The situation prevailing at the outset of enantioselective gas chromatography in the late sixties is vividly described by Gil-Av, the early pioneer in the field:[6]

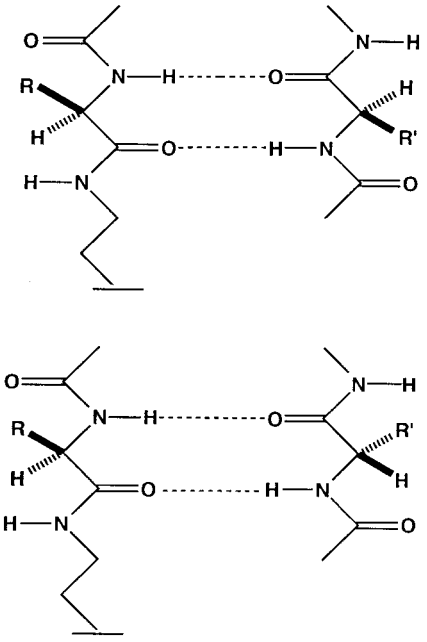


**Fig. 1** Selector–selectand hydrogen-bonding interactions in amide-based CSPs

"When we started this work in 1964, this topic was in a 'state of frustration'. Nobody believed it could be done. In fact, people were convinced that there could not possibly be a large enough difference in the interaction between the D- and L-solute with an asymmetric solvent. This was the feeling people had, even those known as unorthodox thinkers. This view had also some experimental basis, because a number of communications had been published, in which it was claimed that such resolutions could be effected, but nobody was able to reproduce these results, and some of them were shown to be definitely wrong."

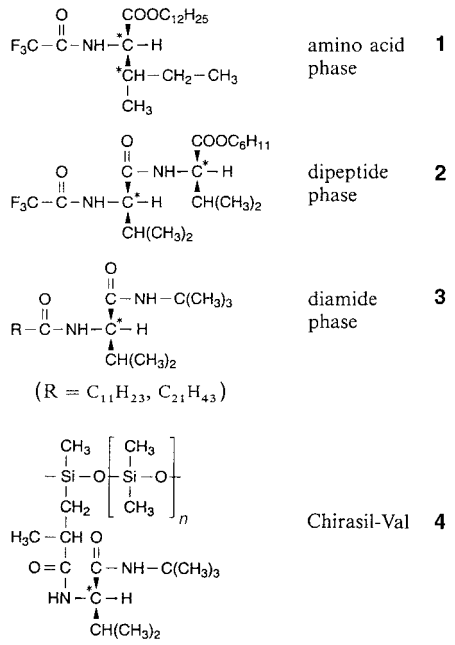
Paradoxically, the situation is almost reversed 30 years later. According to the Chirbase databank most classes of volatile chiral compounds are amenable to enantiomer separation by gas chromatography on various chiral stationary phases (CSPs).[7]

High efficiency, sensitivity and speed of separation are important advantages of enantiomer separation by high resolution capillary gas chromatography. Established ancillary techniques such as multi-dimensional gas chromatography (in series-coupled column operation), use of interfacing and coupling methods (gas chromatography–mass spectrometry, GC–MS) can also be adapted readily for chiral separations. Sensitivity can be extended to the picogram level by GC–MS, electron capture detection (ECD) or element-specific detection. Employing GC–MS selected ion monitoring (SIM) trace amounts of enantiomers present in complex matrices can be detected easily. The direct method (b) is especially useful for chiral analysis when no sample derivatization is required, *e.g.*, by head space analysis whereby volatile enantiomers are directly analysed from the vapour phase of the sample matrix. Owing to the enormous separation power of capillary gas chromatography, contaminants and impurities are usually separated from the analytes and the simultaneous analysis of multi-component mixtures of enantiomers (*e.g.*, the proteinogenic amino acids, Fig. 2) is possible in one analytical run. A prerequisite for the use of method (b) is volatility, thermal stability and resolvability of the chiral analyte. Unless specific derivatization techniques are utilized to increase volatility, capillary supercritical fluid chromatography (SFC) and capillary electrochromatography (CEC) are important complementary methods applicable to nonvolatile analytes. A new development is the use of micro-packed liquid chromatography (μ-LC) or open-tubular liquid chromatography (OTLC).

Enantiomer separation by gas chromatography is routinely performed with three types of chiral stationary phases (CSPs):[8,9] enantiomer separation on chiral amino acid derivatives *via* hydrogen bonding;[6,10–13] enantiomer separation on chiral metal coordination compounds *via* complexation;[14] and enantiomer separation on cyclodextrin derivatives *via* (*inter alia*) inclusion.[15–17]

## Chiral stationary phases based on hydrogen bonding

As mentioned beforehand, the first successful separation of racemic *N*-trifluoroacetyl amino acid esters on a glass capillary column coated with non-volatile *N*-trifluoroacetyl-L-isoleucine lauryl ester **1** (Scheme 1) was achieved in 1966 by Gil-Av *et al.*[3a] Subsequent studies revealed that in the dipeptide phase **2** (Scheme 1) the C-terminal amino acid was not essential for chiral recognition; however, the second amide function was important for additional hydrogen bonding. This observation led to the development of the diamide **3** derived from valine which represents the most efficient selector possessing a single chiral centre.[18] Previously, the chiral selectors were used as non-volatile neat liquids which were coated on the inner capillary surface. Subsequently, Frank *et al.* synthesized Chirasil-type stationary phases by chemically linking the selector to a polysiloxane. Thus, the diamide **3** was coupled *via* the amino function to a statistical copolymer of dimethylsiloxane and (2-carboxypropyl)methylsiloxane of appropriate viscosity.[10] The resulting polymeric CSP Chirasil-Val **4** exhibits excellent gas chromatographic properties for the enantiomer separation of a number of classes of chiral compounds over the temperature range 0–250 °C.[12,16] The simultaneous enantiomer separation of all proteinogenic amino acids in less than 25 min is illustrated in Fig. 2.[11] Enantiomer separation by hydrogen bonding CSPs generally requires derivatization of the analyte in order to increase volatility and/or to introduce suitable functions for additional hydrogen bonding association.




**Scheme 1** Hydrogen-bonding-type CSPs[8]

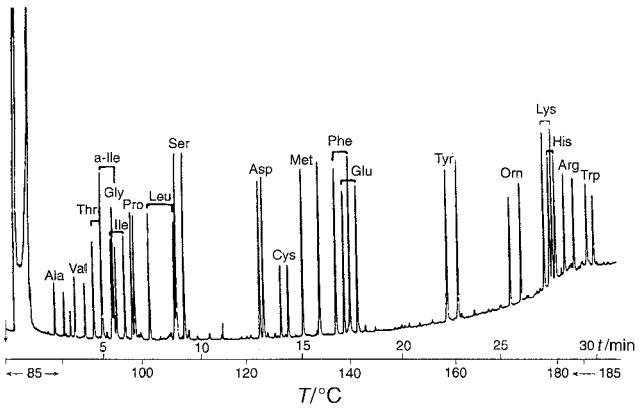
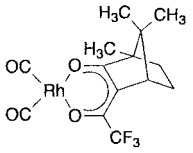



**Fig. 2** Enantiomer separation of proteinogenic α-amino acids as *N*-(*O*,*S*)-pentafluoropropanoyl isopropyl esters (histidine as $N^{im}$-ethoxycarbonyl derivative) on Chirasil-Val **4** (20 m × 0.27 mm id glass capillary, 85–185 °C, 0.35 bar $H_2$). All D enantiomers are eluted before the L enantiomers.[11]

## Chiral stationary phases based on coordination

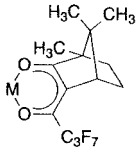
As a first example of enantiomer discrimination by complexation gas chromatography, the chiral metal coordination compound dicarbonylrhodium(I) 3-trifluoroacetyl-(1*R*)-camphorate **5** (Scheme 2) was used for the enantiomer separation of the chiral alkene 3-methylcyclopentene.[14] This method was later extended to oxygen-, nitrogen- and sulfur-containing analytes using manganese(II), cobalt(II) and nickel(II) bis[(3-heptafluorobutanoyl)-(1*R*)-camphorate] as stationary phases dissolved in squalane or dimethylpolysiloxane.[8,14]

In Fig. 3 the enantiomer separation of eight stereoisomeric



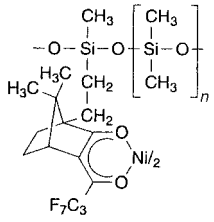
**5**

dicarbonylrhodium(I) 3-trifluoroacetyl-(1*R*)-camphorate



**6**

M = $Mn^{II}_{/2}$, $Ni^{II}_{/2}$, $Co^{II}_{/2}$



**7**

Chirasil-Metal

**Scheme 2** Complexation-type CSPs[8]

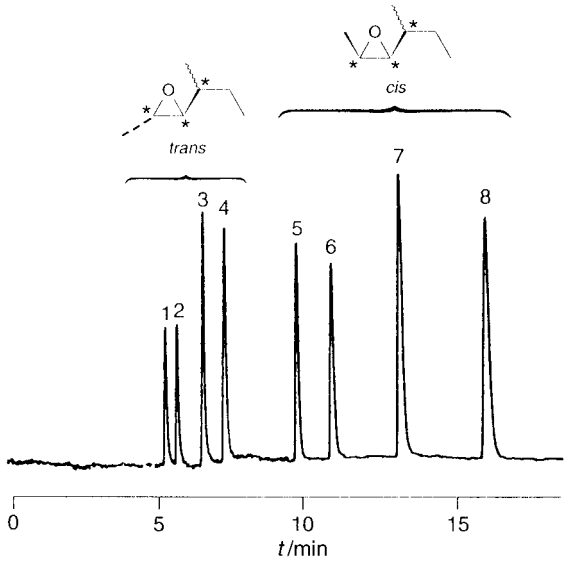



**Fig. 3** Rapid simultaneous separation of eight stereoisomers (enantiomers and diastereoisomers) of 2-methyl-3-(1′-methylpropyl)oxirane on 0.125 M nickel(II) bis[3-heptafluorobutanoyl-(1*R*)-camphorate] **6** in SE 30 (20 m × 0.25 mm id glass capillary, 90 °C, 1 bar $N_2$). Peak assignment:[8] *trans*: 1: 2*S*,3*S*,1′*S*; 2: 2*R*,3*R*,1′*R*; 3: 2*S*,3*S*,1′*R*; 4: 2*R*,3*R*,1′*S*. *cis*: 5: 2*S*,3*R*,1′*R*; 6: 2*R*,3*S*,1′*S*; 7: 2*S*,3*R*,1′*S*; 8: 2*R*,3*S*,1′*R*.

aliphatic oxiranes by complexation gas chromatography is illustrated.

A limitation of the use of coordination-type CSPs **5** and **6** is the low temperature range of operation (25–120 °C). The thermostability has been increased by the preparation of immobilized polymeric CSPs (Chirasil-Metal) **7** (Scheme 2).[19]

Chirasil-Metal stationary phases can also be applied with supercritical carbon dioxide as the mobile phase by capillary supercritical fluid chromatography (SFC).[19] The use of low temperatures in SFC improves the enantioselectivity at the expense of a loss of efficiency.

### Chiral stationary phases based on inclusion

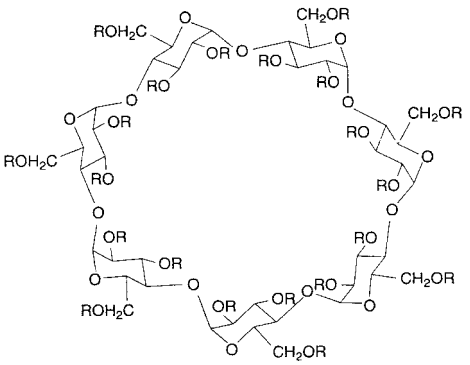
As a first example of enantiomer discrimination by inclusion gas chromatography, native α-cyclodextrin in formamide was employed in a packed column for the enantiomer separation of α- and β-pinene and *cis*- and *trans*-pinane.[20]

Subsequently, it was recognized that alkylated cyclodextrins (CDs) can be used in capillary columns for analytical enantiomer separation. Thus, undiluted permethylated β-cyclodextrin **8** (Scheme 3) was employed above the melting point.[21] Since per-*n*-pentylated and 3-acyl-di-2,6-*n*-pentylated cyclodextrins are liquids even at room temperature, the derivatives **9**–**14** (trade name Lipodex) have been used in the undiluted form for the separation of enantiomers of many classes of compounds on deactivated Pyrex glass and fused silica capillary columns by König.[16] The more polar CD derivatives containing hydroxypropyl, free hydroxy groups or trifluoroacetyl groups, respectively, **15**–**19**, developed by Armstrong *et al.*, were coated on fused silica capillary columns.[22]

In another approach aimed at combining the enantioselectivity of the selectors with the excellent gas chromatographic properties of polysiloxanes, alkylated cyclodextrins like **8** were dissolved in moderately polar silicones such as OV-1701.[15] Thus, the selectors could be employed for gas chromatographic enantiomer separation irrespective of their melting point or possible phase transitions. The separation of enantiomers of saturated cyclic hydrocarbons, devoid of any functionality, is demonstrated in Fig. 4.

The presence of three hydroxy groups which can be regioselectively alkylated and acylated offers an enormous number of possible α-, β- and γ-cyclodextrin derivatives. The readily available permethyl-β-cyclodextrin **8** proved to be the most versatile chiral selector, although for selected applications other derivatives such as octakis(3-*O*-butanoyl-2,6-di-*O*-*n*-pentyl)-γ-cyclodextrin (Lipodex E) **14** are very useful.[16]

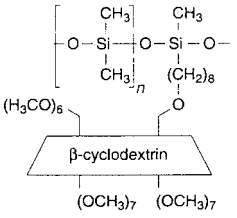
In analogy to Chirasil-Val, permethylated β-cyclodextrin has been chemically linked *via* a mono-6-octamethylene spacer to



R = $CH_3$

heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin **8**

| | | |
|---|---|---|
| hexakis(2,3,6-tri-*O*-pentyl)-α-cyclodextrin | Lipodex A | **9**–**14** |
| hexakis(3-*O*-acetyl-2,6-di-*O*-pentyl)-α-cyclodextrin | Lipodex B | |
| heptakis(2,3,6-tri-*O*-pentyl)-β-cyclodextrin | Lipodex C | |
| heptakis(3-*O*-acetyl-2,6-di-*O*-pentyl)-β-cyclodextrin | Lipodex D | |
| octakis(2,3,6-tri-*O*-pentyl)-γ-cyclodextrin | | |
| octakis(3-*O*-butanoyl-2,6-di-*O*-pentyl)-γ-cyclodextrin | Lipodex E | |
| hexakis[-*O*-{(*S*)-2-hydroxypropyl}-per-*O*-methyl]-α-cyclodextrin | PMHP-α-CD | **15**–**19** |
| heptakis[-*O*-{(*S*)-2-hydroxypropyl}-per-*O*-methyl]-β-cyclodextrin | PMHP-β-CD | |
| hexakis(2,6-di-*O*-pentyl)-α-cyclodextrin | dipentyl-α-CD | |
| heptakis(2,6-di-*O*-pentyl)-β-cyclodextrin | dipentyl-β-CD | |
| heptakis(3-*O*-trifluoroacetyl-2,6-di-*O*-pentyl)-β-cyclodextrin | DPTFA-β-CD | |




Chirasil-Dex **20**

**Scheme 3** Inclusion-type CSPs[8]

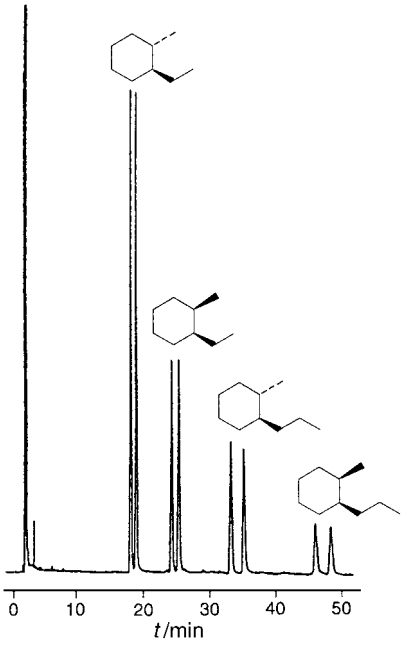
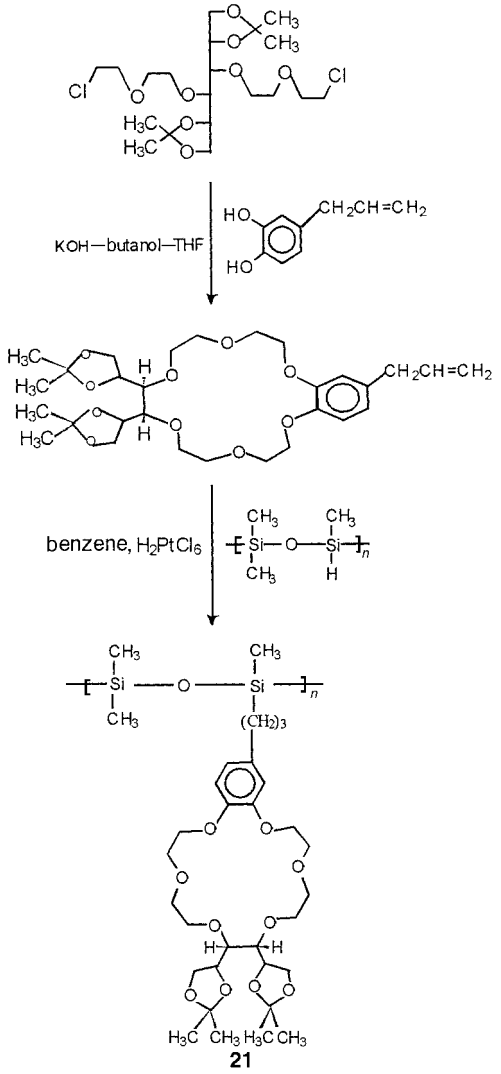



**Fig. 4** Enantiomer separation of *cis*- and *trans*-1-ethyl-2-methylcyclohexane and *cis*- and *trans*-1-methyl-2-*n*-propylcyclohexane on 0.07 M permethyl-β-cyclodextrin **8** in OV-1701 (30 m × 0.25 mm id fused silica capillary, 50 °C, 1.5 bar $H_2$)[8]

a dimethylpolysiloxane backbone to obtain Chirasil-Dex **20** (Scheme 3).[19] The chemically bonded chiral polymers combine the selectivity of the CSP with the efficiency of polysiloxanes, thus affording high resolution capillary columns with an extended range of operating temperatures (0–220 °C). Even separations at temperatures as low as −20 °C are feasible. In addition, fused silica capillary columns coated with Chirasil-Dex **20** possess advantages such as the presence of a non-polar polysiloxane matrix resulting in low elution temperatures for polar analytes, a high degree of inertness allowing analysis of polar compounds without prior derivatization and long-term stability. Chirasil-Dex stationary phases can also be thermally immobilized on the inner surface of fused silica capillary columns. Immobilization of Chirasil-Dex **20** is the prerequisite for use in chiral supercritical fluid chromatography (SFC),[19] open-tubular electro-chromatography (OTCEC) and open-tubular liquid chromatography (OTLC), or a unified approach involving all four methods GC, SFC, LC and CEC and employing a single column.[23]

A new Chirasil-type stationary phase **21** is based on a chiral crown ether derived from mannitol (Scheme 4).[24]




**Scheme 4** Synthesis of Chirasil-man-18C6-C25[24]

## Chiral phase systems in liquid chromatography

Separation of enantiomers by chiral liquid chromatography (CLC) provides a wide variety of possibilities in experimental design and today there is a large number of stationary phases which can be combined with different mobile phase systems to achieve resolution of a racemate.[25] Furthermore, improvements in selective detection techniques, *e.g.*, use of chiroptical[26] and mass spectrometric[27] detection, add to the usefulness of CLC which spans from trace analytical to truly preparative-scale resolutions.

In the following we will review the achievements made in the field of chiral sorbents useful for the direct separation of enantiomers in the liquid chromatographic mode.

Generally, a column for CLC is densely packed with small solid particles constituting the sorbent, *i.e.*, the column bed. A compound migrating through the column will be partitioned between the sorbent and the mobile phase used for elution and its relative affinity to the sorbent will determine its time spent in the column. Consequently, the purpose of a chiral sorbent is to exhibit a different affinity towards the two enantiomers of a racemate applied to the column and thereby cause a difference in their migration rates large enough to cause their complete separation. The sorbent is most often composed of a solid support with a large surface area, usually silica, upon which the chiral stationary phase (CSP) has been immobilized. In this case the CSP constitutes only a surface layer exposed to the mobile phase. Another category of sorbents, lacking the support phase, also exists. Here, the CSP is used in the form of small beads, which are totally porous. This is an alternative for polymeric CSPs, giving a high column capacity at the expense of efficiency. The general design and morphology of chiral sorbents are given in Fig. 5.

In liquid chromatography it is customary to distinguish between normal phase and reversed phase modes of separation, depending upon whether the mobile phase is less or more polar than the stationary phase. Generally speaking, interactions based on hydrogen bonding, charge transfer or Coulombic forces are favoured in normal phase systems, whereas hydrophobic interactions dominate in reversed phase systems. However, intermediate cases, which are difficult to classify, also exist. Table 1 gives a general overview of the various categories and types of chiral sorbents available, ordered roughly in increasing complexity.

It should be emphasized that even if the type of interactions causing retention is often fairly well known, the mechanism behind the chiral discrimination observed is a far more complex

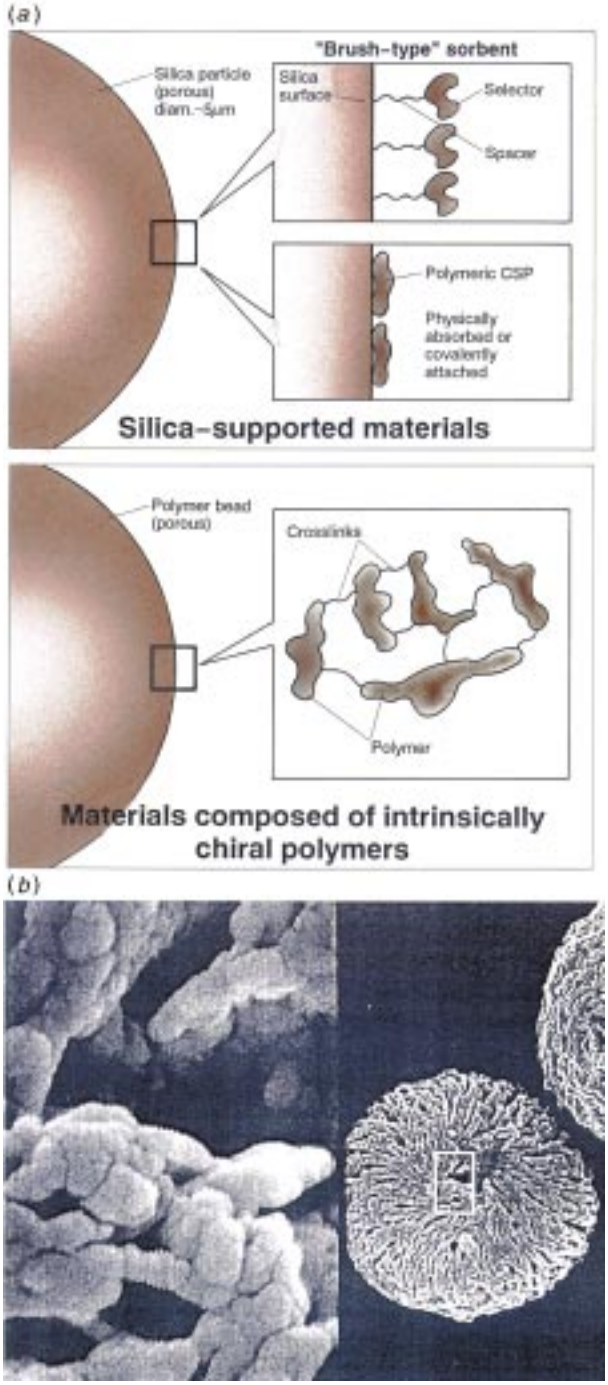



**Fig. 5** (*a*) Morphology of different kinds of chiral sorbents; (*b*) scanning electron microscopic appearance of beads of derivatized cellulose (From ref. 36, reproduced with permission from VCH Verlagsgesellschaft mbH, Weinheim)

problem and in most cases it is incompletely understood. Below some of the more recent advances in the development of chiral sorbents of different categories are summarized.

Naturally occurring macrocyclic peptides (antibiotics produced by fungi) like vancomycin and analogues have been recently exploited as CSPs in reversed as well as normal phase systems.[32] These chiral selectors are of a relatively complex structure [**22**, Fig. 6(*a*)] and the mechanism behind their chiral discrimination ability is virtually unknown, although it has been suggested that they act by a combination of hydrogen bonding, π–π complexation, dipole stacking, inclusion and steric interaction.[32] Vancomycin contains 18 stereogenic centres, whereas the others used, rifamycin B, thiostrepton and teichoplanin, contain 9, 17 and 23, respectively. They represent

**Table 1** Main types of chiral sorbents in liquid chromatography

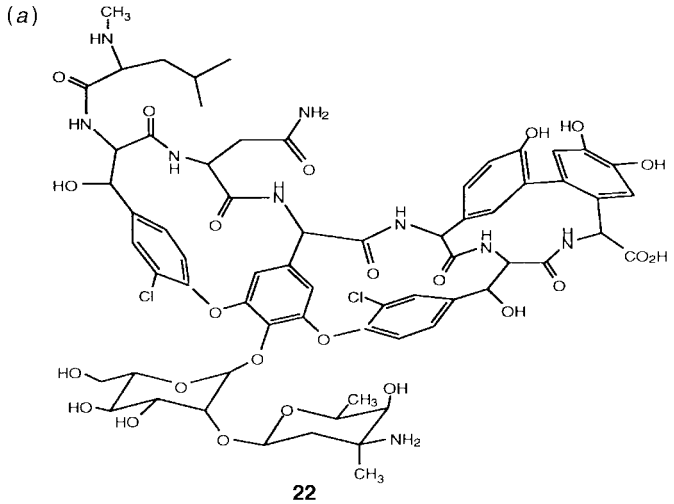
| sorbent | main reference |
|---|---|
| selector-based, brush-type chiral sorbents: | |
| ligand exchange | 28 |
| crown ether inclusion | 29 |
| cyclodextrin interaction | 30 |
| charge transfer complexation | 31 |
| other types: | |
| antibiotics | 32 |
| synthetic receptors | 33 |
| sorbents based on synthetic and natural polymers: | |
| polyacrylamides and polymethacrylates | 34,35 |
| polysaccharides: | 36 |
| esters | |
| carbamates | |
| proteins | 37 |

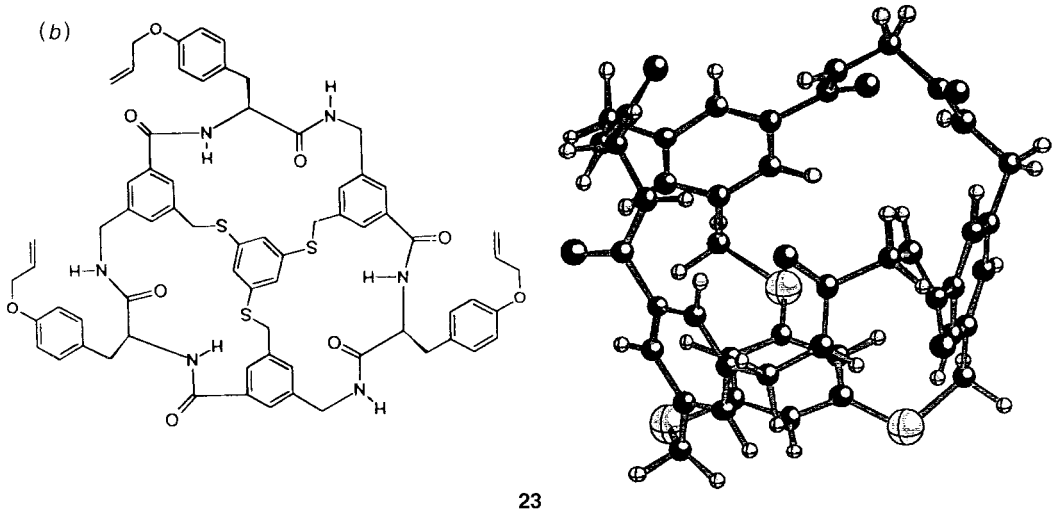
the first type in Fig. 5, *i.e.*, the selector is immobilized *via* a two to three carbon spacer to the silica surface. Columns packed with these sorbents are now commercially available (such as Chirobiotic™, Astec Corp.) and have been used for the resolution of large number of different racemates.

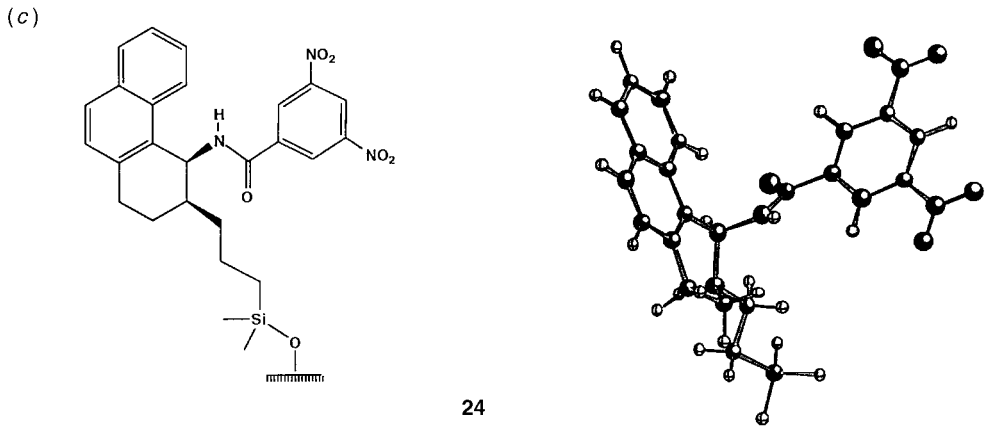
Another complex, basket-shaped selector, although not naturally occurring, but synthesized in the laboratory [**23**, Fig. 6(*b*)], was found to possess almost receptor-like properties towards certain members a series of amides of *N*-acyl amino acids.[33] Note that this selector has $C_3$ symmetry and is based on (*S*)-tyrosine as the chiral building block. Interestingly, when the *N*-Boc methylamide derivatives were resolved on the column, the (*S*)-enantiomers were consistently most retained, whereas for the *N*-3,5-dinitrobenzoyl (DNB) hexylamides the opposite elution order was found. This was interpreted in terms of the operation of two opposing mechanisms, *viz.* inclusion (*i.e.*, binding to the interior of the basket) in the former case and π-stacking in the latter (interactions at the outside of the basket between the π-donating aromatic rings of the selector and the π-accepting DNB groups in the selectand). For some of the racemates the α values were exceptionally high, with $\alpha = 43$ in the case of *N*-Boc-Thr-NHMe, corresponding to a Gibbs free energy difference in binding affinity ($\Delta\Delta G$) of 2.2 kcal mol$^{-1}$. Normal phase conditions, employing dichloromethane with 0.5–1% of methanol as retention modifier, were used for these separations.

Selectors based on aromatic π–π interactions have undergone a dramatic development over the last few years and have recently been designed [**24**, Fig. 6(*c*)] to make use of an additional face-to-edge π–π interaction.[38] As seen in Fig. 6(*c*), a selector of this type is designed to have a cleft, consisting of π-acidic and π-basic aromatic parts directed almost perpendicular to each other, to which one enantiomer is preferentially bound. The enantioselective binding has been shown by NMR studies to be due to a simultaneous face-to-face and face-to-edge π–π interaction.[39] These phases (commercially available as Whelk-O™, Regis Co.) typically operate under normal phase conditions with hexane containing a retention modifier like propan-2-ol.

A series of new CSPs based on *N*,*N*′-diallyl-L-tartardiamide (DATD) as the chiral building block was recently introduced.[40] These sorbents contain *O*,*O*′-diacyl-DATD selector units [**25**, Fig. 6(*d*)] anchored in a network polymeric structure which is covalently bound to the silica matrix [corresponding to the second type in Fig. 5(*a*)]. The columns based on this sorbent operate in a normal phase mode with mobile phases based on hexane with alcohols or ethers as hydrogen bonding modifiers. The nature of the acyl substituents was found to greatly influence the chiral recognition behaviour of these CSPs which are supposed to cause retention and enantiomer discrimination *via* a combination of hydrogen bonding and π–π interactions. Two of the CSPs investigated, **25a** and **25b**, are now commer-



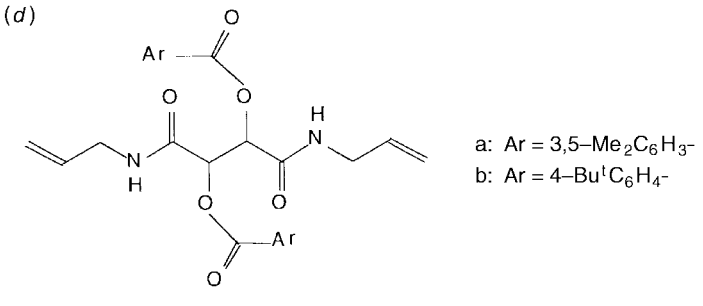




**Fig. 6** Some different types of chiral selectors used in liquid chromatography. (*a*) Vancomycin **22** (used in Chirobiotic-V™), (*b*) $C_3$-symmetric receptor **23** of Gasparrini *et al.*,[33] (*c*) selector **24** based on π–π interaction [used in (*S*,*S*)-Whelk-01™], (*d*) chiral diaroyl-DATD unit **25** used in network polymers (Kromasil-CHI™ DMB and TBB, respectively). In the 3D representation of **23**, the tyrosyl side chains have been omitted for clarity.

cially available as Kromasil® CHI-DMB and CHI-TBB, respectively.

Synthetic polymers as CSPs date back to the early 1970s and the pioneering work of Blaschke and Donow,[34] who created intrinsically chiral polymer particles *via* suspension copolymerization of optically active acrylamides and acrylates with a suitable crosslinker like ethylenediacrylate or methylenebisacrylamide. Some years later, a different type of chiral polymer was prepared for the first time.[35] This was obtained by anionic polymerization of triphenylmethyl methacrylate in the presence of a chiral initiator, leading to polymers which were chiral by virtue of their single-handed helicity. An outline of the reactions used and structures obtained is given in Fig. 7(*a*).

Synthetic polymers based on a different concept, *viz*. polymers containing chiral cavities obtained *via* 'molecular imprinting' techniques[41–43] by the use of chiral templates, constitute another interesting class of materials intended for direct liquid chromatographic resolution purposes. These chiral phases are prepared by first allowing polymerizable molecules, bearing suitable functional groups, to arrange in solution around the template molecules. Then polymerization is initiated to give a solid polymer from which, after grinding and sieving to obtain suitable fine particles, the template is removed by hydrolysis or by a simple washing procedure. When used in an enantioselective chromatographic column the cavities left in the material will cause longer retention of the enantiomer that has been used as the template. The drawbacks resulting from the usually rather low resolution efficiency obtained have been partly overcome by a technique involving deposition of the polymer on silica.

CSPs based on biopolymers of the polysaccharide type have been extensively studied with respect to their chiral recognition mechanism, mainly by NMR spectroscopy.[44] Since the spin–lattice relaxation time, $T_1$, is sensitive to molecular motion, this can be used to probe the selector–selectand dynamics. Further, NOESY spectra give information regarding intermolecular distances. From such data, in combination with results from molecular modelling, a detailed chiral discrimination rationale for the cellulose tris(5-fluoro-2-methylphenylcarbamate)–1,1′-bi-2-naphthol system is now available.[44]

The structurally most complex CSPs, used entirely in the reversed phase mode, are proteins. They are unique in so far as regulation of retention *via* the mobile phase composition can be made in many different ways. The protein immobilized to the silica surface will change its binding characteristics by changing its net charge, conformation and hydrophobicity as a function of the pH, ionic strength, organic solvent modifier or other additive used. Invariably, immobilization of the protein makes use of the terminal primary amino groups in the lysine side chains which are easily reacted with the functionalized silica, either directly or *via* a bifunctional reagent. The most widely used, and commercially available, sorbents thus far are based on serum albumins (BSA, HSA), $\alpha_1$-acid glycoprotein (AGP) and ovomucoid (OVM). Interestingly, a recent study of the last of these has shown that the chiral recognition is not caused by ovomucoid but by another protein from egg white, tentatively named ovoglycoprotein, present as a contaminant.[45] Considerable progress has been made in the understanding of the mechanism of chiral discrimination by proteins *via* a combination of 2D NMR spectroscopy and molecular modelling.[46]

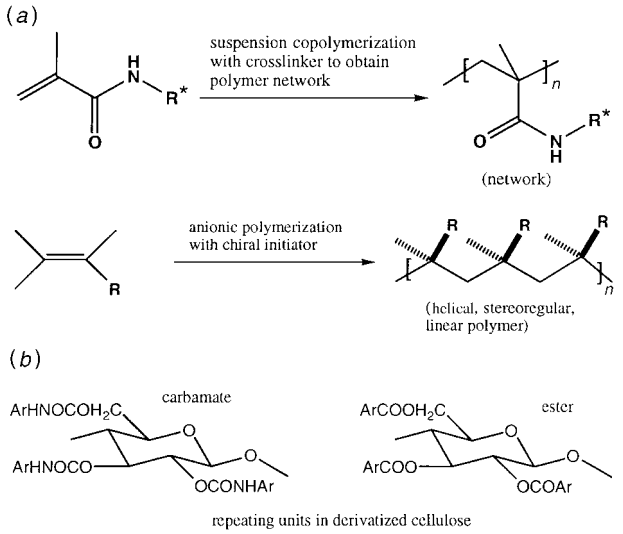



**Fig. 7** (*a*) Synthetic polymers and (*b*) biopolymers used as chiral stationary phase materials

## Current trends in analytical and preparative applications

The real advantage of the direct separation of enantiomers in a flow system appears when the racemate has a structure which precludes the application of classical resolution techniques. For example, it has been possible to resolve hydrocarbon racemates like *cis,trans*-cycloocta-1,3-diene (a representative of a compound with planar chirality) on microcrystalline cellulose triacetate (MCTA).[47] In liquid chromatography the possibility of resolution on a truly preparative scale also exists. Here there are progressive developments taking place with respect to both large-scale production of efficient CSPs and continuously operating chromatographic systems like the recently introduced simulated moving bed (SMB) technique.[48] Basically, SMB technology means that the solute is applied continuously to the bed and the two separated compounds are collected from separate outlets of the system. The bed size can be increased to very large dimensions, enabling large throughputs per time unit. Chiral sorbents which can be prepared easily in large quantities, like MCTA, have already been used successfully on a very large scale.[49] As an example, in 1992 a 500 mm × 450 mm id column packed with 32 kg of MCTA was used for the partial separation of the enantiomers of 1.2 kg of a drug substance in a single run. The separation took *ca.* 13 h and required a flow rate of methanol of 300 ml $min^{-1}$. This clearly shows that preparative separations of enantiomers with chiral phase systems using SMB will be of great importance for the further development of industrial chirotechnology for a variety of applications.

## The temperature effect in chiral separations

A prerequisite for chiral separation by chromatography is a fast and reversible diastereomeric association equilibrium between selectand and selector. Thus, enantioselectivity is not governed by kinetics, but is determined exclusively by thermodynamics. Enantioselectivity is defined by the Gibbs free energy difference $-\Delta\Delta G$ which is related to the chiral separation factor $\alpha$ (*i.e.*, the ratio of the net retention times $t' = t_R - t_M$, of the second and first eluted enantiomer, respectively) by eqn. (1) (this relationship is only valid in the absence of non-enantioselective interactions arising, for example, from achiral matrices used as solvents like polysiloxanes)

$$-\Delta\Delta G = RT \ln\alpha = RT \ln(t'_2/t'_1) \quad (1)$$

The temperature dependence of $-\Delta\Delta G$ is determined by the Gibbs–Helmholtz eqn. (2) ($K_R$ and $K_S$ refer to the association constants of the diastereomers formed between the selector and the enantiomers *R* or *S* of the selectand)

$$-\Delta\Delta G = -\Delta\Delta H + T\Delta\Delta S = RT \ln(K_R/K_S) \quad (2)$$

which may be rewritten as the van't Hoff plot

$$-\Delta\Delta G/T = -\Delta\Delta H/T + \Delta\Delta S = R\ln(K_R/K_S) \quad (3)$$

As expected for a 1:1 association process, $-\Delta\Delta H$ and $\Delta\Delta S$ compensate each other in determining $-\Delta\Delta G$. Therefore, at the isoenantioselective temperature $T_{iso}$ enantiomers cannot be separated.

$$T_{iso} = \Delta\Delta H/\Delta\Delta S \text{ for } -\Delta\Delta G = 0 \quad (4)$$

Below $T_{iso}$, enantiomer separation is enthalpy controlled and the (*R*)-enantiomer is eluted after the (*S*)-enantiomer, while above $T_{iso}$, enantiomer separation is entropy controlled and the (*S*)-enantiomer is eluted after the (*R*)-enantiomer (reversal of the elution order, peak inversion). An example is depicted in Fig. 8.

In most cases, enantioselectivity is enthalpy controlled. Therefore, temperatures as low as possible should be employed to increase the chiral separation factor $\alpha$. Low temperatures should also be used to avoid enantiomerization when the chiral selectand is prone to configurational change.

## Enantiomerization

Owing to peak coalescence, only an unresolved peak will be observed in enantioselective chromatography when a racemic selectand undergoes rapid enantiomerization in the presence of a CSP. Thus, in contrast to *cis*-1-ethyl-2-methylcyclohexane (Fig. 4), only one peak is expected for *cis*-1,2-dimethylcyclohexane which rapidly interconverts from one chiral chair conformation to another representing the non-superimposible mirror image.

If the rate of enantiomerization is comparable to that of the chromatographic timescale of enantiomer separation, a characteristic interconversion profile is observed.[14] It resembles a plateau formation between the unchanged peaks for the enantiomers. By 'dynamic' chromatography, activation parameters of enantiomerization ($\Delta G^{\ddagger}$) can be obtained by comparing experimental and calculated elution profiles. Two examples featuring enantiomerization are shown in Fig. 9.

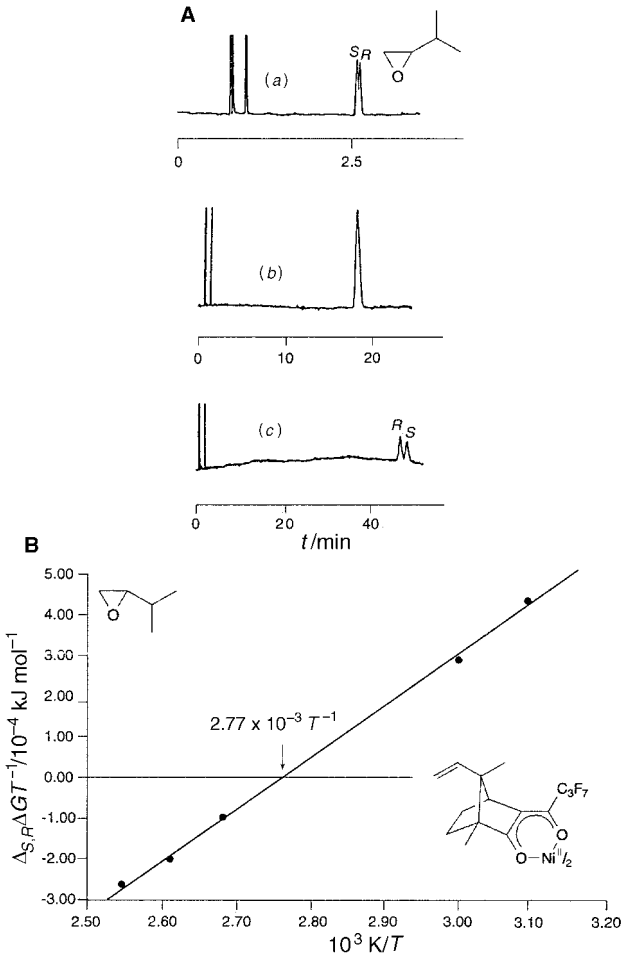



**Fig. 8** **A**, Temperature-dependent reversal of enantioselectivity for the enantiomers of isopropyloxirane by complexation gas chromatography on nickel(II) bis[3-heptafluorobutanoyl-8-(ethylidene)-(1*R*)-camphorate], derivative of **6**. (*a*) 110 °C, (*b*) 70 °C, (*c*) 55 °C. **B**, Linear Van't Hoff plot and determination of the isoenantioselective temperature $T_{iso}$ (89 °C).[8]

## Summary and outlook

The development of chiral stationary phase materials for enantioselective chromatography has been very rapid and today virtually any racemate can be at least partially resolved. The methodologies are rapidly implemented in various processes for different applications, not least within the fine chemicals and pharmaceutical industries where there is often a need for pure enantiomers. This has created a new field of research, *viz.* chirotechnology.

Along with this development there is a need for a better understanding of the phenomenon of chiral recognition at the molecular level. Although this is a complex and difficult problem, progress is being made owing to the continuous improvement of NMR techniques for studies of solution structures of model systems[44,50] as well as of methods for molecular modelling of selector–selectand interactions in docking situations.[51]

The refinement of the chromatographic resolution techniques is perhaps best illustrated by the separations of race-

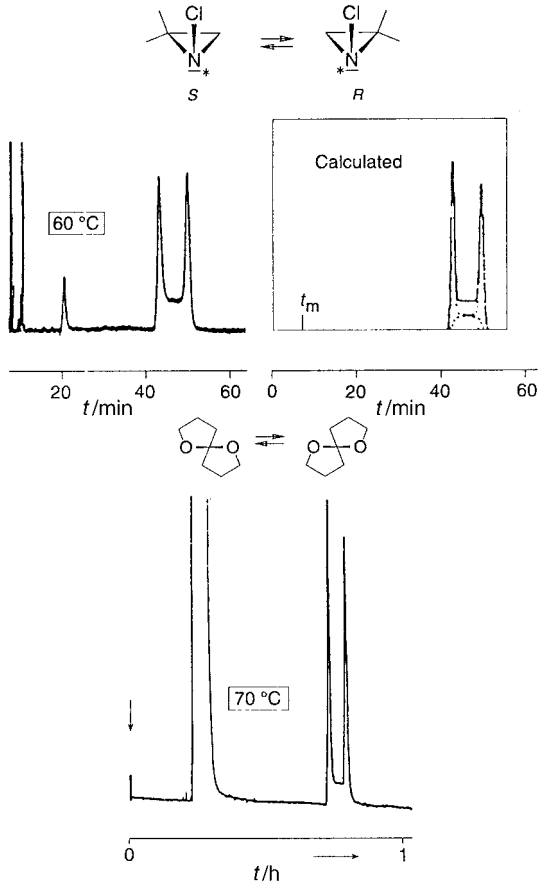



**Fig. 9** Interconversion profiles due to inversion of configuration (enantiomerization) of 1-chloro-2,2-dimethylaziridine and 1,6-dioxaspiro[4.4]nonane by complexation gas chromatography on nickel(II) bis(3-heptafluorobutanoyl-(1*R*)-camphorate) **6**.[8,14]

mates based on chirality solely due to isotopic substitution, as demonstrated recently.[52,53] Furthermore, the strive for miniaturization of analytical separations, *e.g.*, by the use of electro-driven methods, like in capillary column techniques, or of 'chip technology', will lead to new possibilities for the study of extremely small amounts of chiral materials.

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