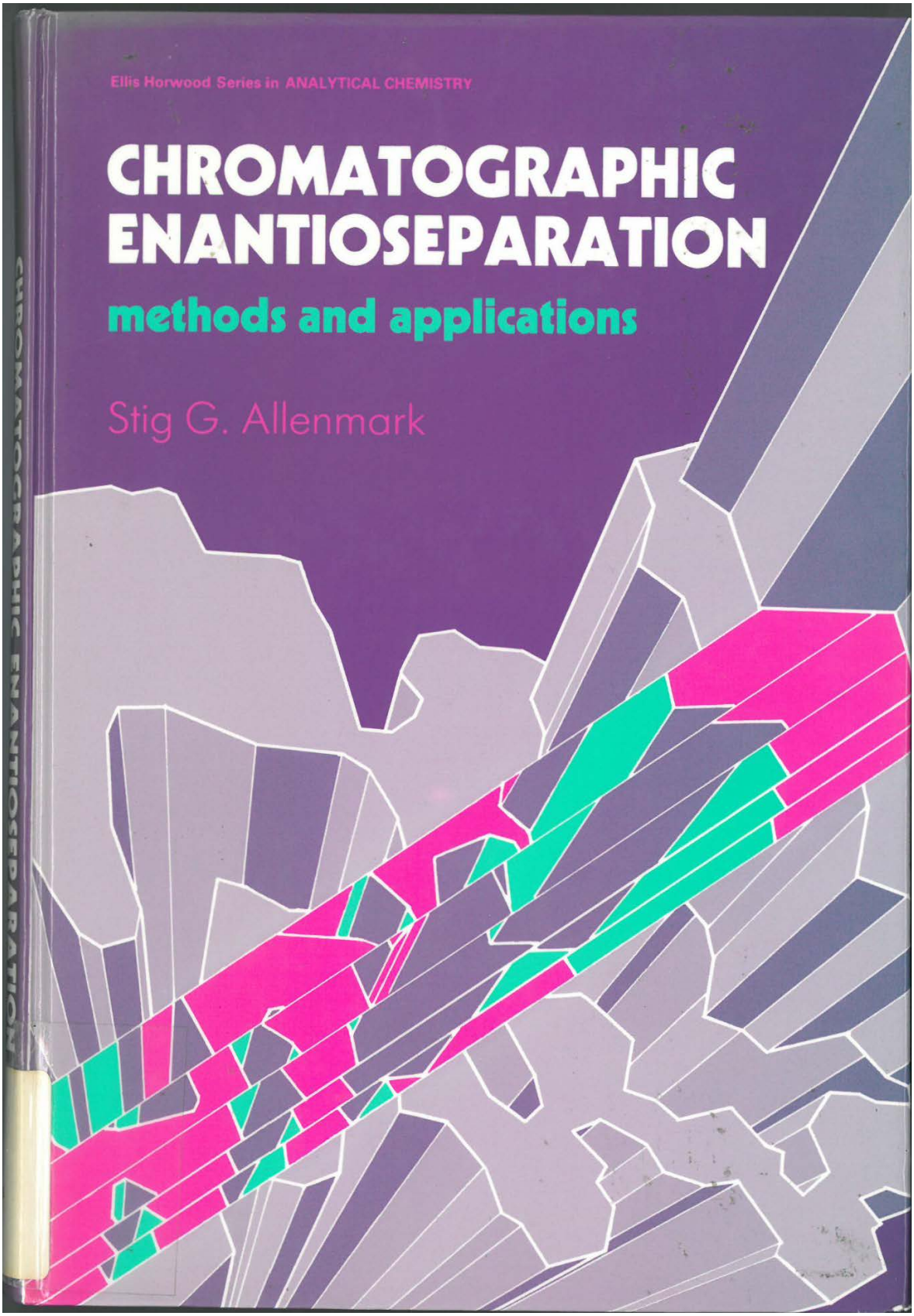


CHROMATOGRAPHIC ENANTIOSEPARATION: Methods and Applications

STIG G. ALLENMARK
Laboratory of Microbiological Chemistry
University of Gothenburg, Sweden

ELLIS HORWOOD LIMITED
Publishers · Chichester

Halsted Press: a division of
JOHN WILEY & SONS
New York · Chichester · Brisbane · Toronto

First published in 1988 by
ELLIS HORWOOD LIMITED
Market Cross House, Cooper Street,
Chichester, West Sussex, PO19 1EB, England
The publisher's colophon is reproduced from James Gillison's drawing of the ancient Market Cross, Chichester.

Distributors:
Australia and New Zealand:
JACARANDA WILEY LIMITED
GPO Box 859, Brisbane, Queensland 4001, Australia
Canada:
JOHN WILEY & SONS CANADA LIMITED
22 Worcester Road, Rexdale, Ontario, Canada
Europe and Africa:
JOHN WILEY & SONS LIMITED
Baffins Lane, Chichester, West Sussex, England
North and South America and the rest of the world:
Halsted Press: a division of
JOHN WILEY & SONS
605 Third Avenue, New York, NY 10158, USA
South-East Asia
JOHN WILEY & SONS (SEA) PTE LIMITED
37 Jalan Pemimpin # 05–04
Block B, Union Industrial Building, Singapore 2057
Indian Subcontinent
WILEY EASTERN LIMITED
4835/24 Ansari Road
Daryaganj, New Delhi 110002, India

British Library Cataloguing in Publication Data
Allenmark, S. G. (Stig G.), *1936–*
Chromatographic enamtioseparation.
1. Chromatography
I. Title
543'.089

Library of Congress Card No. 88–1092

ISBN 0–84312–988–6 (Ellis Horwood Limited)
ISBN 0–470–21080–X (Halsted Press)

Typeset in Times by Ellis Horwood Limited
Printed in Great Britain by Hartnolls, Bodmin

Table of contents

Preface 9

List of Symbols and Abbreviations 11

1 Introduction 13
Bibliography 18
References 18

2 The development of modern stereochemical concepts
2.1 Chirality and molecular structure 19
2.1.1 Molecules with asymmetric atoms 19
2.1.2 Other types of chiral molecular structures 20
2.2 Definitions and nomenclature 23
Bibliography 26
References 26

3 Techniques used for studies of optically active compounds
3.1 Determination of optical or enantiomeric purity 27
3.1.1 Methods not involving separation 27
3.1.1.1 Polarimetry 27
3.1.1.2 Nuclear magnetic resonance 29
3.1.1.3 Isotope dilution 31
3.1.1.4 Calorimetry 33
3.1.1.5 Enzyme techniques. 33
3.1.2 Methods based on separation 34
3.2 Determination of absolute configuration 35
3.2.1 X-Ray crystallography with anomalous scattering 36
3.2.2 Spectroscopic (ORD, CD) and chromatographic methods based on comparison 37
Bibliography 40
References 40

4 Modern chromatographic separation methods
4.1 A review of basic chromatographic theory 42
4.2 Instrumentation 46
4.2.1 Gas chromatographic instrumentation 46
4.2.2 Liquid chromatographic instrumentation 50
4.3 Separation of enantiomers by means of covalent diastereomers — a survey 51
4.3.1 Gas chromatography 54
4.3.2 Liquid chromatography 54
Bibliography 62
References 62

5 Theory of chiral chromatography for direct optical resolution
5.1 The prerequisite for enantioselective interaction with the chiral stationary phase 64
5.2 Some general aspects regarding chiral recognition models and chromatographic enantioselectivity 66
5.2.1 Co-ordination to transition metals 66
5.2.2 Charge-transfer interaction 68
5.2.3 Inclusion phenomena 69
5.3 Some thermodynamic and kinetic considerations 70
5.3.1 Temperature effects 70
5.3.2 Peak coalescence due to enantiomerization phenomena 71
Bibliography 74
References 74

6 Chiral gas chromatography
6.1 The development of chiral stationary phases based on hydrogen bonding 75
6.1.1 Amino-acid and oligopeptide derivatives 75
6.1.2 Polysiloxane-bonded phases 79
6.2 Phases based on chiral metal complexes 82
6.3 Phases based on inclusion effects 83
6.4 Relative merits of the various modes of chiral gas chromatography . . . 84
Bibliography 88
References 88

7 Chiral liquid chromatography 90
7.1 Chiral stationary phases based on naturally occurring and synthetic polymers 91
7.1.1 Polysaccharides and derivatives 91
7.1.1.1 Polysaccharides 91
7.1.1.2 Polysaccharide derivatives 95
7.1.2 Derivatives of polyacrylamide and similar synthetic polymers . . . 103
7.1.2.1 Sorbents based on chirally substituted synthetic polymers . . . 103
7.1.2.2 Sorbents based on isotactic linear polymethacrylates of helical conformation 106

7.1.3 Synthetic polymers containing 'grafted' chiral cavities 108
7.1.4 Proteins 111
7.1.4.1 Immobilized albumin 112
7.1.4.2 Immobilized α_1-acid glycoprotein (orosomucoid) 113
7.2 Bonded synthetic chiral selectors 119
7.2.1 Crown ethers ('host–guest' complexation) 119
7.2.2 Metal complexes (chiral ligand exchange) 120
7.2.3 Selectors based on charge-transfer complexation 124
7.2.4 Selectors based on hydrogen bonding 129
7.2.5 Other types of selectors 130
7.3 Techniques based on addition of chiral constituents to the mobile phase 132
7.3.1 Metal complexation 133
7.3.2 Uncharged chiral mobile phase additives 134
7.3.3 Ion-pairing techniques 137
Bibliography 140
References 141

8 Analytical applications in academic research and industry
8.1 Amino-acids 146
8.1.1 The enantiomer labelling technique 147
8.1.2 Other applications of chromatographic amino-acid resolution . . . 147
8.2 Stereochemical problems in natural product chemistry 149
8.2.1 Pheromone stereochemistry 150
8.2.2 Structure elucidation of polypeptide antibiotics and related natural products 153
8.2.3 Miscellaneous applications 156
8.3 Pharmaceutical applications 158
8.3.1 Neutral or weakly acidic or basic pharmaceuticals 158
8.3.1.1 Barbiturates 158
8.3.1.2 Hydantoins 159
8.3.1.3 Benzothiadiazines and structurally related compounds 160
8.3.1.4 Benzodiazepinones 163
8.3.1.5 Miscellaneous compounds 165
8.3.2 Protolytic (charged) pharmaceuticals 168
8.3.2.1 Amphoteric compounds 168
8.3.2.2 Basic (cationic) compounds 171
8.3.2.3 Acid (anionic) compounds 176
8.4 Studies of microbial and enzymatic reactions 177
8.4.1 Enzymatic and microbial alkene epoxidation 178
8.4.2 Microbial amide and ester hydrolysis 180
8.4.3 Asymmetric ketone reduction by yeast organisms 181
8.5 Miscellaneous applications and techniques 183
8.5.1 Determination of enantiomerization barriers 183
8.5.2 Determination of configuration from chromatographic data 185
8.5.3 Evaluation of enantiomeric purity from chromatographic partial optical resolution (Mannschreck's method) 185

Bibliography 188
References 188

9 Preparative scale enantioseparations — need, progress and problems 192
Bibliography 199
References 199

10 Future trends
10.1 New detector systems. 200
10.2 Column improvements 203
10.3 Supercritical fluid chromatography 206
Bibliography 206
References 206

11 Experimental procedures for the synthesis of chiral sorbents
11.1 Techniques for the preparation of chiral sorbents by derivatization of polysaccharides 208
11.1.1 Preparation of microcrystalline cellulose triacetate (MCTA) 208
11.1.2 Preparation of silica coated with cellulose triacetate 209
11.1.3 Preparation of silica coated with cellulose triphenylcarbamate . . . 209
11.2 Polymerization procedures used to obtain chiral synthetic polymer materials 209
11.2.1 Preparation of poly[(S)-*N*-acryloylphenylalanineethyl ester] 209
11.2.2 Preparation of polycellulose(triphenylmethyl methacrylate) 211
11.3 Techniques used for the binding of chiral selectors to silica. 211
11.3.1 Preparation of 3-glycidoxypropyl-silica 212
11.3.2 Large-scale preparation of (R)-*N*-(3,5-dinitrobenzoyl)-phenylglycine covalently silica-bound sorbent 212
11.3.3 Preparation of (S)-(−)-α-*N*-(2-naphthyl)leucine 213
11.3.4 Hydrosilylation of (R)-*N*-(10-undecenoyl)-α-(6,7-dimethyl-1-naphthyl) isobutylamine 213
11.3.5 Preparation of silica-bonded (S)-1-(α-napththyl)ethylamine 213
11.3.6 Preparation of silica-bound polyacrylamide and polymethacrylamide 214
Bibliography 214
References 214

Appendix: Commercial suppliers of chiral columns for GC and LC. 216

Index 218

7

Chiral liquid chromatography

Though the concept of chiral GC is quite clear, viz. that enantiomers are separable with a chiral stationary phase, the situation is more complex for chiral LC. Here we can distinguish between two fundamentally different cases, depending upon whether enantiodifferentiation takes place through a chiral recognition effect by the stationary phase or by a chiral constituent of the mobile phase forming a diastereomeric complex *in situ* during the chromatographic process. From an *experimental* point of view, a clear distinction can be made between the use of a chiral column (i.e. a column containing a CSP) together with an achiral mobile phase, and the use of an achiral column together with a chiral mobile phase. In the latter case, however, the actual mode of chiral separation will depend on the relative affinity of the chiral constituent for the stationary phase and the analyte, respectively. In one extreme case, the chiral constituent may become strongly adsorbed on the stationary phase, thereby converting it into a CSP, and the separation process would then be regarded as a chiral recognition by the CSP thus generated. In the opposite case, the chiral constituent has a much lower affinity for the stationary phase than for the analyte. This means that diastereomeric complexes are generated in the mobile phase and the separation takes place as a normal LC separation of diastereomers. Between these two extreme cases there is probably a range of mixed retention modes. In other words: are diastereomeric complexes formed at the surface of the chromatographic sorbent, or in the mobile phase, or both?

Chiral sorbents for LC may further be classified with respect to their general structural types. Some are based on synthetic or natural polymers and are totally and intrinsically chiral. Others consist of chiral selectors of low molecular weight which are bound to a hard, incompressible matrix, usually silica. There are also sorbents consisting of polymers anchored to silica in order to give improved column performance.

A rough classification of the various modes of chiral LC is given in Table 7.1. This is mainly based on the general nature of the chromatographic sorbent, without regard to the type of retention process involved, which is often quite complex and difficult to evaluate in any detail.

Table 7.1 — Summary of the main methods used for direct optical resolution by liquid chromatography

Site of chiral selector	Basic principle	Capacity	Column efficiency
Stationary phase (CSP)	Use of an intrinsically chiral, polymeric stationary phase, either of natural origin (polysaccharides and derivatives, proteins) or synthetic (synthetic polymers with chiral substituents or 'grafted' chiral cavities)	Analytical to preparative	Low to moderate, depending on whether a support material is used or not
Stationary phase (CSP)	Use of bonded synthetic chiral selectors	Analytical to preparative	Moderate to fairly high
Mobile phase (CMP)	Addition of chiral constituents to the mobile phase system used. Column achiral, usually an alkyl-silica used in reversed-phase mode	Analytical	Moderate to fairly high

7.1 CHIRAL STATIONARY PHASES BASED ON NATURALLY OCCURRING AND SYNTHETIC POLYMERS

Owing to the early recognition of the chiral nature and ready availability of many natural products, particularly carbohydrates, such compounds were among the first to be tried as sorbents for optical resolution by LC. As early as 1938 a partial resolution of a racemic camphor derivative was obtained on a column packed with lactose [1]. Lactose remained a column material of interest for some years and was used with success in the first nearly complete chromatographic chiral resolution described in the literature, which took place in 1944, when Tröger's base was resolved on a 0.9 m long lactose column [2]. The resolving capacity of a polysaccharide, viz. cellulose, was first realized by the observation that a racemic amino-acid could occasionally give two spots in paper chromatography [3–5]. Dalgliesh advanced his three-point interaction theory in 1952 on the basis of results from paper chromatography of racemic amino-acids [6]. Other early findings on direct optical resolution of amino-acids by means of paper chromatography [7] and cellulose thin-layer chromatography (TLC) [8] were reported. This led to further use of cellulose and cellulose derivatives, as well as investigations of starch and cyclodextrins for the purpose of chiral LC. At present, derivatives of a large number of natural polysaccharides are under investigation as potential chiral sorbents.

The principle of using chiral polymers has also been exploited with many different types of totally synthetic materials, by various approaches, and the results seem to be very promising.

Further, the enantioselectivity of proteins, first observed by studies of binding equilibria in solution (for reviews see [9,10]), has been successfully utilized for analytical chiral LC.

7.1.1 Polysaccharides and derivatives

7.1.1.1 Underivatized polysaccharides

A. Cellulose

The linear polysaccharide cellulose represents the most common organic compound of all. Its chemical constitution is that of a linear poly-β-D-1,4-glucoside (Fig. 7.1a).

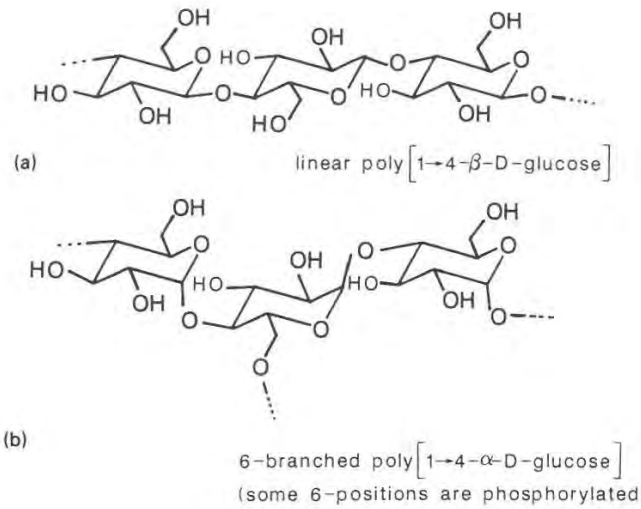


Fig. 7.1 — The chemical structure of (a) cellulose and (b) amylopectin, the main components of starch.

It forms very long chains containing at least 1500 (+)-D-glucose units per molecule. The molecular weight of cellulose ranges from 2.5×10^5 to 1×10^6 or more. In a cellulose fibre these long molecules are arranged in parallel bundles and held together by numerous hydrogen bonds between the hydroxyl groups. In the native state cellulose is therefore built up from partially crystalline regions. These are not regenerated on precipitation of cellulose from solution. As seen from Fig. 7.1a the (+)-D-glucose repetitive unit contains five chiral centres and three hydroxyl groups. All the ring substituents are equatorial.

It has been found that partial hydrolysis of natural cellulose with dilute mineral acid can yield a material with a high degree of crystallinity because hydrolytic cleavage will take place preferentially in the amorphous regions. Such a material contains ca. 200 glucose units per chain and is usually called 'microcrystalline cellulose' [11]. It is marketed as 'Avicel' by several chemical companies.

Although derivatives of cellulose have been used in most recent research efforts and successful resolutions, very good results have also been obtained by the use of unmodified cellulose and are therefore worth mentioning. The compounds resolved are, without exception, highly polar with multiple sites for hydrogen bond formation. Some typical results are summarized in Table 7.2.

In a recent work [22], it was found that on treatment with dilute alkali cellulose will lose its enantioselective properties owing to a transformation of the native, metastable form into a rearranged and stable amorphous form.

Table 7.2 — Examples of optical resolutions performed by liquid chromatography on cellulose

Type of compound	LC mode	References
Amino-acids, amino-acid derivatives	Paper	[3–5,7,12]
	Thin layer	[8,13,14]
	Column	[15–17]
Diaminodicarboxylic acids	Column	[18]
Synthetic alkaloids	Column	[19,20]
Cathecins	Column	[21]

B. Starch

The other widespread polysaccharide, also built from (+)-D-glucose units, is starch. Its structure is more complex than that of cellulose. It is composed of ca. 20% amylose and 80% amylopectin, the latter being an insoluble fraction. Both are entirely composed of (+)-D-glucose units, linked by α-glucoside bonds. Whereas amylose is a linear polymer, amylopectin is branched by C_1–C_6 connections (Fig. 7.1b).

Depending upon the source, there are different particle sizes of starch available. The material obtained from potatoes is relatively coarse (60–100 μm) and has been favoured for column chromatography. Despite its ready availability and non-swellable properties in aqueous media, which give good flow properties, it has so far found very little use.

As in the case of cellulose, starch appears to be most suitable for polar aromatic compounds. Its use for resolution of atropisomers with structures containing polar substituents has been particularly well documented [23–27]. These separations show a very pronounced dependence on the nature of the mobile phase, and are especially influenced by the ionic strength. Figure 7.2 illustrates the chromatographic behaviour of a starch column after application of racemic 2,2′-dinitrodiphenic acid and elution with 1*M* sodium citrate buffer, pH 7.7, at 60°C. The separation factors obtained are quite satisfactory but the column efficiency is modest.

C. Cyclodextrins

As early as 1908 it was discovered by Schardinger [28] that new crystalline carbohydrates, so-called dextrins, were formed if starch was subjected to degradation by a micro-organism, *Bacillus macerans* [29]. These compounds were found to be normal β-1,4-D-glucosides, but cyclized to rings of 6–12 units. Those with the three smallest rings (6–8 units) have been called α-, β-, and γ-cyclodextrins (CD), respectively, and form inclusion complexes with various compounds of the correct size. The diameter of a β-CD ring is 8Å and its volume is ca. 350Å^3. The stability of the inclusion complex is largely dependent on the hydrophobic and steric character of the guest. These phenomena make CDs, particularly β-CD, which is easily available, highly promising for use in chiral LC.

The major development in chiral LC with cyclodextrins started with the technique of using them as mobile phase additives in TLC experiments [30–32]. This technique has also been applied with success to column chromatography and will be treated in Section 7.3. Earlier some efforts to use cross-linked cyclodextrin gels for

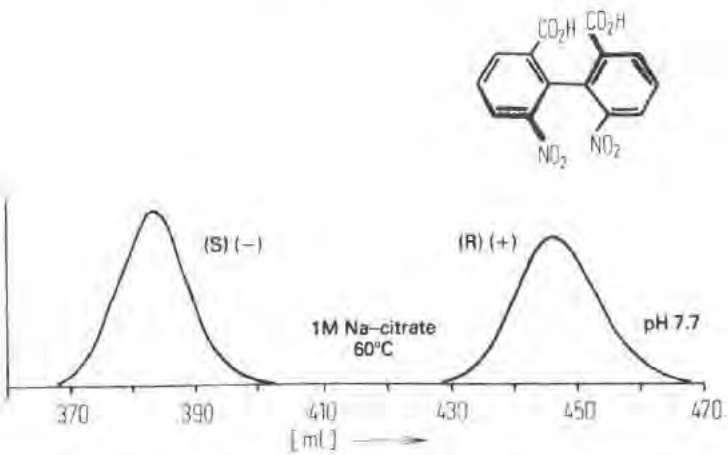


Fig. 7.2 — Optical resolution of atropisomers on a starch column. (Reprinted, with permission, from H. Hess, G. Burger and H. Musso, *Angew. Chem.*, 1978, **90**, 645. Copyright 1978, Verlag Chemie GmbH).

chromatographic purposes had also been made [33–36]. The first attempts to immobilize CDs on solid supports were made quite recently [37,38]. By an improvement in coupling techniques, a highly efficient β-CD silica-bonded phase column is now available [39,40].

Since the formation of inclusion complexes with CDs in aqueous systems is based mainly on hydrophobic interaction, it is logical that a CD column operates entirely in a reversed-phase mode. Consequently, the mobile phase systems normally used are the same as those used in ordinary reversed-phase LC, usually methanol/water or acetonitrile/water. This also means that buffers can be used to control the pH and possibly affect the retention of charged solutes.

The rather special type of solute–CSP interaction present in the case of immobilized CDs deserves particular attention. The inclusion complexes formed are of great interest, not only from a theoretical point of view. This field, which belongs to 'host–guest' chemistry (like the crown-ether complexes which will be treated in Section 7.2.1), is important in achieving better understanding of the role and function of ordered molecular complexes in biological systems.

The conformation of a cyclodextrin in an aqueous system is generally assumed to approximate to a truncated cone, Fig. 7.3, possessing a hydrophobic internal surface. Hydrophobic molecules such as benzene or hexane, which can diffuse in and out of the cavity, are reversibly adsorbed on this surface. Retention of a hydrophobic solute should largely be dependent on the efficiency of the contact with the interior of the cavity. Enantioselectivity is likely to be associated with the chiral structure at the entrance of the cavity, caused by the exposed 2- and 3-hydroxy groups in the glucose units. If the solute is of a suitable size, allowing good contact with the internal surface and hence restricted in movement, then a different interaction between the chiral

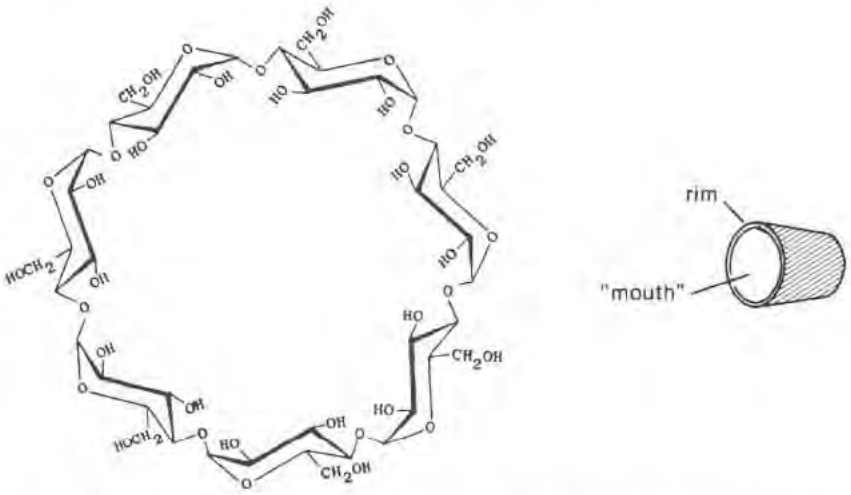


Fig. 7.3 — The chemical structure of β-cyclodextrin and its assumed conformation in an aqueous solution.

cavity entrance and the substituents of the two enantiomers may cause a difference in both the complexation constants and the chromatographic k' values.

The effect of the mobile phase on the enantioselectivity appears to be large. In general, both the k' and α values tend to decrease with increasing content of organic modifier. In most cases, methanol as a retention modifier will decrease α to a lesser degree than acetonitrile. Retention on CD columns is markedly affected by the temperature, decreasing rapidly to zero between 60 and 80°C in most cases. This might be due to an increased conformational mobility of the ring with increased temperature.

7.1.1.2 Polysaccharide derivatives

A. Cellulose triacetate

In 1966 Lüttringhaus and co-workers [41,42] found that a partially acetylated cellulose (described as a 2.5 acetate) could be used with ethanol to achieve optical resolution in column chromatography. Some years later another German research group carefully investigated the heterogeneous acetylation of native (microcrystalline) cellulose and found that a triacetate could be prepared with almost complete preservation of the microcrystallinity and excellent resolving properties [43]. They pointed out that the microcrystallinity was essential for the enantioselective properties of the material, since the optical resolution power was totally lost on dissolution and reprecipitation. The metastable state of the material was evident from these experiments as the change was found to be irreversible. On the basis of the results from these investigations it was concluded that microcrystalline cellulose triacetate

(MCTA) gave retention by means of inclusion of the solute into molecular cavities in the chiral matrix. Therefore the term 'inclusion chromatography' was used [44,45].

Owing to the availability and low cost of 'microcrystalline' cellulose (Avicel), the well described technique of its acetylation and the interesting properties of the product, MCTA has been the subject of extensive research during the last decade. Large columns can be packed with this cheap material and relatively large quantities of sample can be used, permitting preparative work. A typical example of a resolution on MCTA is shown in Fig. 7.4.

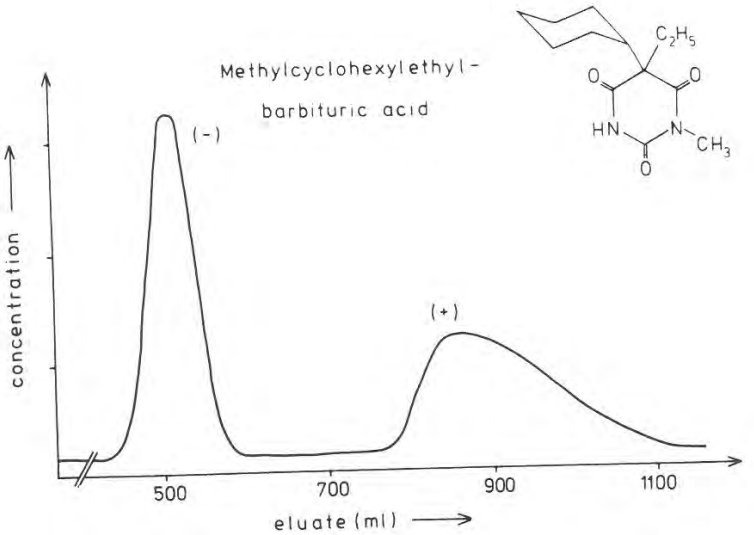


Fig. 7.4 — Separation of the enantiomers of 205 mg of (±)-methylcyclohexylethylbarbituric acid on 210 g of MCTA. (Column 85 × 2.5 cm, ethanol 96%, flow-rate 50 ml/hr). (Reprinted from G. Blaschke, *J. Liquid Chromatog.*, 1986, **9**, 341 by courtesy of Marcel Dekker, Inc.).

It is very important that MCTA is allowed to swell in boiling ethanol before being packed into a column. Ethanol (95%) is a good medium for swelling, and does not dissolve MCTA.

The inclusion mode of retention of solutes on MCTA is consistent with the very different chromatographic behaviour shown by benzene and mesitylene (1,3,5-trimethylbenzene), the first being much more strongly retained owing to better permeation into the cavities. 1,3,5-Tri-*tert*-butylbenzene is totally excluded and therefore of use for void volume determinations [46].

Further evidence for the inclusion model of retention is the fact that very non-polar solutes, lacking functional groups, can be resolved. Thus, racemic *trans*-1,2-diphenylcyclopropane is easily resolved into its antipodes on MCTA [47]. Optical resolutions of a great number of structurally quite different compounds on MCTA

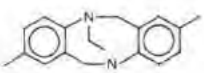
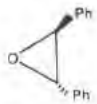
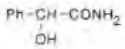
have been described to date, many on a preparative or semipreparative scale. This will be treated further in Chapter 9.

The main drawbacks of MCTA are its compressibility and relatively large, irregular and inhomogeneous particle size. This means that preparative columns can only be run at very low linear flow-rates (cf. the conditions used in Fig. 7.4). The latter problem can be partially solved by grinding and careful fan-sieving of the material. However, since MCTA requires a swollen state to function well, reduction of the compressibility is a more difficult problem.

An exhaustive study of the influence of the supramolecular structure of cellulose triacetate on the chromatographic optical resolution of several racemates was recently made by Francotte *et al.* [48]. Their results confirmed the original ideas by Hesse and Hagel [43–45] that the inclusion of low molecular-weight chiral molecules into a specific spatial arrangement of the glucose units of the polysaccharide chains is of fundamental importance for the chiral discrimination process.

During experiments with deposition of CTA into silica gel particles, it was found by a Japanese research group [49,50] that, although the dissolved and reprecipitated polymer had apparently lost most of its microcrystalline structure, the new material still possessed some resolving capacity. The results presented in Table 7.3 are

Table 7.3 — A comparison between microcrystalline (I) and reprecipitated (II) cellulose triacetate sorbents in optical resolutions under identical conditions

Compound	Sorbent	k_1'	k_2'	α
	I	2.61 (−)	5.36 (+)	2.05
	II	0.59 (+)	0.91 (−)	1.53
	I	7.82 (+)	11.3 (−)	1.45
	II	0.94 (−)	1.23 (+)	1.31
	I	2.08	3.08	1.48
	II		0.80	1.00
	I		10.3	
	II		0.46	

Column: 250 × 4.6 mm. Eluent: ethanol. The signs denote the optical rotation of the enantiomer eluted. (Reprinted from T. Shibata, I. Okamoto and K. Ishii, *J. Liquid Chromatog.*, 1986, **9**, 313 by courtesy of Marcel Dekker, Inc.).

illustrative. As expected, all the k' values found are much lower than in MCTA, particularly for benzene. Further, the α values are diminished and a reversal of the elution order appears. However, a much higher column efficiency is found, which often more than compensates for the lower α values. This new material also permits

use of solvents other than ethanol, and of higher flow-rates for a faster chromatographic run.

The mechanism of retention by these CSPs appears to be highly complex and not yet satisfactorily elucidated. Many factors have been found to play important roles, such as the average molecular weight of the polymer, the molecular weight distribution, the solvent used for deposition onto the support and the nature of the support [51]. Nevertheless, the substantial improvements obtained by the presence of a rigid support and the wider choice of mobile phases are evident. This is illustrated by Fig. 7.5 which shows chromatograms of racemic *trans*-stilbene oxide under four

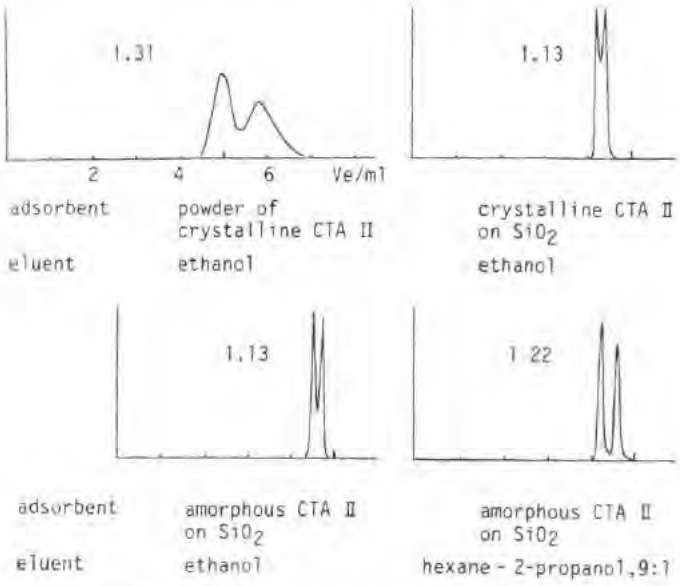


Fig. 7.5 — Optical resolution of racemic *trans*-stilbene oxide by two different types of reprecipitated CTA under various conditions. (Reprinted from T. Shibata, I. Okamoto and K. Ishii, *J. Liquid Chromatog.*, 1986, **9**, 313 by courtesy of Marcel Dekker, Inc.).

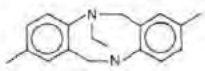
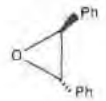
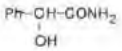
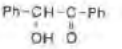
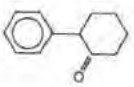
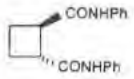
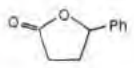
different conditions. The effect of the silica support on column efficiency, as well as that of the mobile phase on α, is quite clear. The degree of crystallinity of the CTA obtained by reprecipitation was established by means of X-ray powder diffraction.

These results, which demonstrated that the microcrystallinity of CTA is not an absolute requirement for efficient chiral recognition, has renewed interest in the use of carbohydrate-based polymers for optical resolution, with vigorous research activity, particularly in Japan. Derivatives of cellulose and other polysaccharides are now under extensive investigation.

B. Other derivatives of cellulose

There are essentially four types of derivatives that are easily prepared from cellulose by modification of the free hydroxyl groups, viz. organic esters, nitrates, carbamates (obtained by reaction with isocyanates) and ethers. Of these the esters and carbamates have been shown to be potentially the most useful. Table 7.4 shows the

Table 7.4 — Examples of separation factors (α) obtained in optical resolutions on various derivatives of cellulose

Compound	Substituent on cellulose					
	$COCH_3$	NO_2	COPh	CH_2Ph	CONHPh	COCH=CHPh
	1.31 (+)	1.33 (+)	1.0 (−)	1.34 (+)	1.32 (+)	2.82 (+)
	1.22 (−)	1.61 (−)	1.47 (+)	1.0	1.32 (+)	1.15 (+)
	1.08	1.10	1.0	1.0	1.0	1.0
	1.05 (−)	1.0 (−)	1.12 (+)	1.0	1.0 (+)	1.08 (−)
	1.07 (+)	1.14 (+)	1.47 (−)	1.0	1.14 (−)	1.26 (−)
	1.13 (−)	1.22 (−)	2.06 (+)	1.0	1.25 (+)	1.52 (−)
	1.39 (+)		1.17 (−)	1.0		1.07 (−)

Column: 250 × 4.6 mm. The cellulose derivatives were coated (20–22%) on LiChrospher Si-1000. The α values were not all obtained under identical conditions. The signs denote the optical rotation of the first-eluted enantiomer. (Reprinted from T. Shibata, I. Okamoto and K. Ishii, *J. Liquid Chromatog.*, 1986, **9**, 313 by courtesy of Marcel Dekker, Inc.).

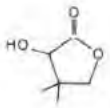
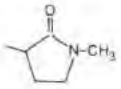
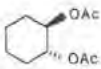
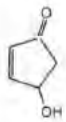
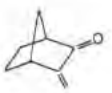
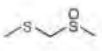
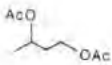
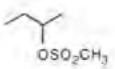
derivatives investigated so far, with the α values for some racemic compounds resolved. Note that the elution order of the enantiomers on CTA is the reverse of that on cellulose tribenzoate (CTB).

It seems to be quite clear that the better properties exerted by the ester and carbamate derivatives, compared to the ethers, are associated with the polar carbonyl groups, which cause increased retention of polar solutes. An investigation

of the k' values obtained for a series of solutes of increasing polarity, by the use of a CTB-column and a tribenzylcellulose column gave very consistent results. If the k' ratio [with k'(CTB) as the denominator] were to be calculated for such a series, it would be found to range from <2 for saturated and chlorinated hydrocarbons, through 2 for aromatic hydrocarbons with non-polar substituents, to >3 for amides, alcohols, lactones, sulphoxides and aliphatic nitro compounds [52].

An important property of the CSPs based on cellulose derivatives is their usefulness for optical resolution of chiral aliphatic compounds. Quite often, an aromatic substituent will promote separation, but it is by no means essential. In Table 7.5 some non-aromatic compounds resolved on cellulose derivative columns are given together with α values obtained.

Table 7.5 — Various non-aromatic compounds resolved on derivatives of cellulose

Compound	α (on triacetate)	Compound	α (on tribenzoate)
	1.22		1.21
	1.31		1.44
	1.23		1.41
	1.61		1.80
	1.21		

Conditions as described under Table 7.4. Elution orders were not established. (Reprinted from T. Shibata, I. Okamoto and K. Ishii, *J. Liquid Chromatog.*, 1986, **9**, 313 by courtesy of Marcel Dekker, Inc.).

In summary, the five most useful cellulose derivatives possess properties characterized [53] below.

Triacetate—for many racemates, especially effective for substrates with a phosphorus atom as an asymmetric centre. In general low separation factors.

Tribenzoate—for racemates with carbonyl group(s) in the neighbourhood of an asymmetric centre.

Trisphenylcarbamate—for polar racemates. Sensitive to the molecular geometry of the substrates.

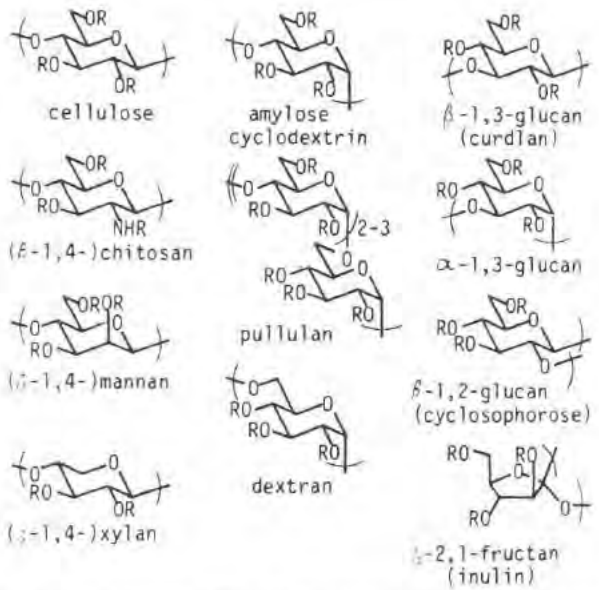
Tribenzyl ether—effective with protic solvents as mobile phases.

Tricinnamate—for many aromatic racemates and barbiturates. High retention times.

These silica-coated CSPs have been commercialized and columns ("Chiralcel") are marketed by Daicel Chem. Co. (see Appendix for details).

C. Derivatives obtained from other polysaccharides

Derivatives of a variety of polysaccharides other than cellulose have been prepared and many of them show interesting properties. Of the polysaccharides shown in Scheme 7.1 pullulan was the least promising, as its benzoate failed to resolve any of



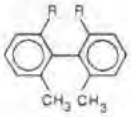
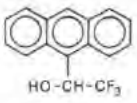
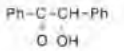
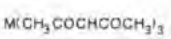
Scheme 7.1 — Polysaccharides used as ester and phenylcarbamate derivatives to produce new CSPs

the compounds tested. This was taken as an indication of the importance of a certain structural regularity. Pullulan has the $1 \rightarrow 6$ linkage randomly distributed over every 3–4 α-$1 \rightarrow 4$ linkages.

It has now been demonstrated that many readily available polysaccharide

derivatives can be used as CSPs for optical resolution by LC. Recently, a series of phenylcarbamate derivatives of various carbohydrate polymers was investigated [54]. Many of these new CSPs were found to give better resolution of certain racemates than the corresponding cellulose derivatives. The data collected in Table 7.6 give some insight into the resolving capacities of the particular chiral sorbents investigated.

Table 7.6 — Examples of compounds which have been optically resolved on phenylcarbamate sorbents derived from various polysaccharides

Compound		Sorbent derived from
		cellulose amylose
	R = OH R = NO_2	cellulose, amylose cellulose
		chitosan xylan
		xylan
	M = Co, Cr	inulin

Data obtained from Y. Okamoto, M. Kawashima and K. Hatada, *Polymer Preprints*, 1984, **33**, 1607. With permission.

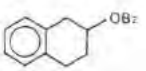
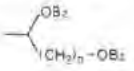
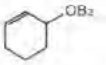
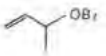
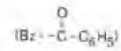
As already pointed out, very little is known about the mechanisms of enantioselection by the polysaccharide derivatives. The recent finding that aliphatic hydrocarbons, such as *cis,trans*-1,3-cyclo-octadiene (**1**), can be optically resolved on

1

MCTA [55] is hard to interpret without the assumption of an inclusion effect, i.e. a steric discrimination caused by a difference in the permeability of the enantiomers into chiral cavities, leading to non-identical retention times on the column. For compounds bearing polar substituents, however, there appear to be contributions from hydrogen bonding and dipole–dipole interactions. Contrary to what might have

been expected, it has turned out that conformationally rigid solutes are not better resolved than more flexible ones [56]. This result, exemplified in Table 7.7, illustrates the complexity of the chiral recognition mechanism in these CSPs. It is reasonable to assume that a certain conformation of a substrate molecule is necessary for a good steric fit causing enantioselection. If such a conformation cannot be attained, there will be no resolution. It is also conceivable that great differences in these steric requirements will be present among the various polysaccharide derivatives [51].

Table 7.7 — Separation factors obtained from optical resolution of a series of benzoates and dibenzoates on silica coated with cellulose tribenzoate

Compound	α	Compound	*n*	α
	1.16		1	1.14
	1.00		2	1.74
	1.21		3	1.44
			4	1.83

Data obtained from T. Shibata, I. Okamoto and K. Ishii, *J. Liquid Chromatog.*, 1986, **9**, 313. (Reprinted by courtesy of Marcel Dekker, Inc.).

7.1.2 Derivatives of polyacrylamide and similar synthetic polymers

As we have seen in Section 7.1.1, carbohydrate biopolymers are very useful and readily available chiral starting materials, which require only simple derivatization procedures to yield selective column materials for enantioseparation. Synthetic chiral polymers, however, cannot be produced without the use of a chiral reagent or a chiral catalyst. In the first case, a chiral derivatization of a suitable monomer is performed and the product is then polymerized to form a polymer network having chiral substituents (Fig. 7.6a). In the second case, the monomer is polymerized under the influence of a chiral catalyst, which will produce an optically active polymer since the stereoregulatory influence of the catalyst will yield an isotactic polymeric structure of a certain preferred helicity (Fig. 7.6b). Here, the chirality of the polymer is inherent, i.e. it is caused only by the helical structure.

7.1.2.1 Sorbents based on chirally substituted synthetic polymers

This type of sorbent has been successfully developed by Blaschke and co-workers [56–62]. So far the work has been concentrated on polyacrylamide and polymethacrylamide derivatives, where the chiral substituents originate from an optically active amine or amino-acid component. By the use of a suspension polymerization

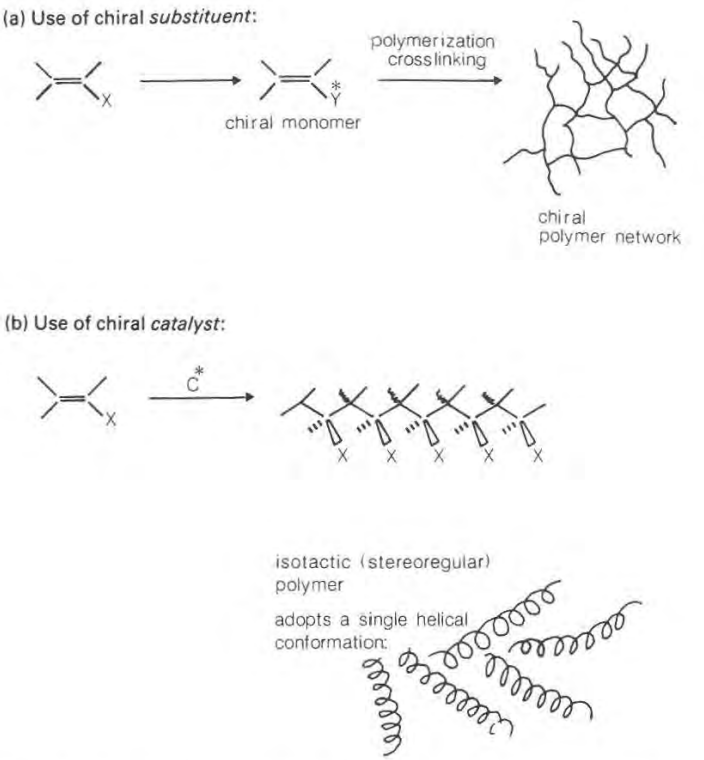


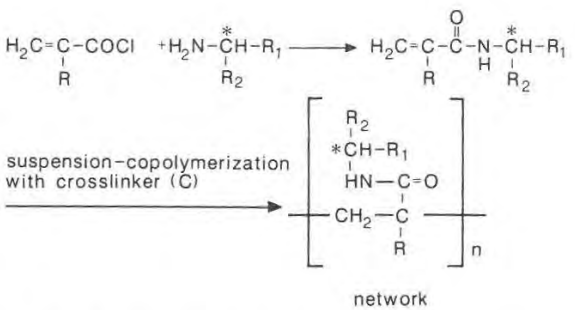
Fig. 7.6 — Two different routes to chiral sorbents based on synthetic polymers. (a) From chiral monomer; (b) from achiral monomer.

technique, polymer particles of desired mean diameter and acceptable size homogeneity can be obtained. Free-radical initiation is used and the porosity of the gel particles is regulated by the relative amount of cross-linking agent added. The particles swell in organic solvents and the material is only used in low-pressure LC systems.

The resolving capacity of these sorbents is highly dependent on a variety of factors. Apart from the substituents on the polyacrylamide backbone, the degree of cross-linking, the nature of the cross-link and the mobile phase compositions are the most essential considerations. Systematic investigations of these factors began in 1973 when partial resolution of doubly radiolabelled (^{3}H and ^{14}C) mandelic acid and mandelamide was studied by analysis of the enantiomer composition of eluted fractions by liquid scintillation counting [56]. Since the $^{3}H/^{14}C$ activity ratio is proportional to the enantiomer composition this detection technique permits a rather precise determination of α even at very low resolution.

The method is, of course, limited to those cases where it is possible to radiolabel the two enantiomers of a compound with the appropriate radioisotope. Accordingly, the elution profiles of both enantiomers are obtained in a single chromatographic experiment.

The preferred route to these polymers was shown to be that given in Scheme 7.2.



R= H, CH_3; R_1= CH_3, COOR'; R_2= alkyl or aryl groups

C= $(H_2C{=}CH{-}CO_2CH_2)_2$ (preferred)

Scheme 7.2 — Synthetic routes to the chiral polyacrylamides and polymethacrylamides.

The great variation of performance with substituents is evident from Table 7.8,

Table 7.8 — Optical resolution ability of a series of variously substituted polyacrylamide sorbents. (Reprinted, with permission, from G. Blaschke, *Angew. Chem.*, 1980, **92**, 14. Copyright 1980, Verlag Chemie GmbH).

R	R_1	R_2	R'	Optical yield (%)	
				Mandelic acid	Mandelamide
H	CH_3	C_6H_5	—	28	35
CH_3	CH_3	C_6H_5	—	8	81
H	CH_3	*cyclo*-C_6H_{11}	—	12	35
CH_3	CH_3	*cyclo*-C_6H_{11}	—	58	92
CH_3	CH_3	1-naphthyl	—	0	97
H	CH_3	*p*-I-C_6H_4	—	0	0
H	COOR'	CH_3	C_2H_5	14	18
H	COOR'	C_6H_5	C_2H_5	46	34
H	COOR'	$C_6H_5CH_2$	C_2H_5	51	96
H	COOR'	*p*-OH-$C_6H_4CH_2$	C_2H_5	0	0
H	COOR'	$C_6H_5CH_2$	*tert*-C_4H_9	89	80

which indicates that two of the most useful polymers are those corresponding to (1) $R = H$, $R_1 = CO_2R'$, $R_2 = C_6H_5CH_2$, $R' = C_2H_5$ and (2) $R = CH_3$, $R_1 = CH_3$, $R_2 = cyclo\text{-}C_6H_{11}$.

These substituted polyacrylamides have been found particularly well suited to polar compounds with functional groups capable of hydrogen bonding. It is therefore logical that relatively non-polar mobile phases have been found most useful. Combinations of hydrocarbons, ethers and possibly small amounts of an alcohol are typical; examples include toluene–dioxan, hexane–dioxan and toluene–dioxan–methanol mixtures. Protic co-solvents such as methanol strongly decrease the retention and therefore normally form less than 10% of the mobile phase composition.

Although hydrogen bonding is apparently the major binding contribution to retention of the solute, the mechanism of enantiomer discrimination appears to be quite complex. As in the case of polysaccharide-derived sorbents, enantioselection is assumed to be caused by inclusion phenomena, i.e. the binding groups in the asymmetric cavities into which the solute enantiomers are thought to diffuse are more favourably located for one of the antipodes, which therefore will be preferentially retained.

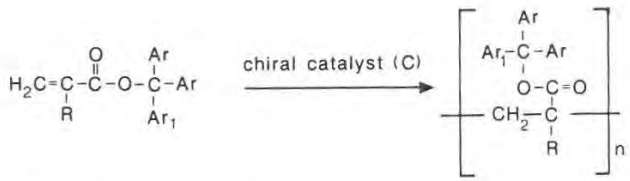
Columns packed with sorbents of this kind have mainly been used for semipreparative work (sample amounts between ca. 1 and 250 mg) on racemic pharmaceuticals. These applications will be treated in Chapter 9. An interesting recent modification of the sorbent consists of a silica-bonded non-cross-linked polyacryloyl (S)-phenylalanine ethyl ester. This has been used for analytical HPLC and gives considerably improved column efficiency [63].

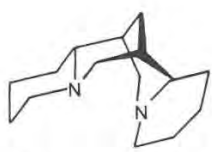
7.1.2.2 Sorbents based on isotactic linear polymethacrylates of helical conformation

A vinyl polymer with chirality caused only by its helicity was first prepared in 1979 by Okamoto and co-workers [64,65]. Optically active poly(triphenylmethyl methacrylate) was then obtained according to Scheme 7.3 by asymmetric anionic polymerization of triphenylmethyl methacrylate under the influence of a chiral initiator in toluene at low temperature. The success of the reaction is strongly dependent on the initiator used, which is based on a complex between an optically active diamine and butyllithium or lithium amide. Both (−)-sparteine–butyllithium, and (+)-(2S,3S)-dimethoxy-1,4-bis(dimethylamino)butane–lithium amide, were found to give the (+)-polymer in good yield [66]. At a degree of polymerization greater than ca. 70 the polymer is insoluble in most common organic solvents.

The material can be used as such after grinding and sieving to a particle size averaging ca. 30 μm [67]. However, a more efficient and durable chromatographic sorbent is obtained by adsorption of the low molecular-weight soluble fraction of the polymer on silanized silica (10 nm, or 1000 or 4000 Å) [68].

It is perhaps not surprising that this CSP turned out to be excellent for optical resolution of racemic aromatic hydrocarbons of linear and planar chirality [69]. The compounds in Fig. 7.7. (p. 108) are examples of such hydrocarbons that are all resolved with high α values. They are characterized by high hydrophobicity and a rigid molecular geometry. A difference in the extent to which the enantiomers would interact with the helical CSP (in which the triphenylmethyl group is assumed to attain





Scheme 7.3 — Asymmetric polymerization reaction used to produce the right-handed helical poly(triarylmethyl methacrylates).

a propeller-like conformation) is therefore very reasonable on intuitive grounds. This is particularly true in the case of hexahelicene, for which the highest α value (>13) was reported. The enantiomer most strongly retained on the (+)-CSP is the (+)-form , which has P- (right-handed) helicity. Since it was found that the (+)-CSP interacts very strongly with itself, but only weakly with the (−)-polymer, it is very likely that the (+)-CSP also has P-helicity [70,71]. The same P-helicity of the most strongly retained enantiomer was also found for all other compounds investigated that possessed this type of chirality. The situation is shown in Fig. 7.7 This correlation means that chromatographic retention data can be used for determination of absolute configuration of these types of compound.

Because the structure of this CSP is not cross-linked and the coated-silica version of the sorbent is based only on physical adsorption, some limitations are put on the choice of mobile phase, for solubility reasons. Thus, aromatic hydrocarbons, chloroform and tetrahydrofuran (which dissolves the polymer) should be avoided. To date, methanol has been the preferred mobile phase [51] and there is generally a tendency towards increase in α value with increasing polarity of the solvent, but retention times may be unacceptably long in many cases.

The use of these sorbents with protic mobile phase systems is disadvantageous owing to the solvolytic instability of the ester bond. Thus in methanol the CSP gradually undergoes solvolysis to yield methyl triphenylmethyl ether as a by-product. (It should be remembered that the trityl group is commonly used for protection of carboxyl groups in peptide synthesis and is easily removed under weakly acidic conditions). It is therefore advisable to work at low temperature with alcohols as eluents.

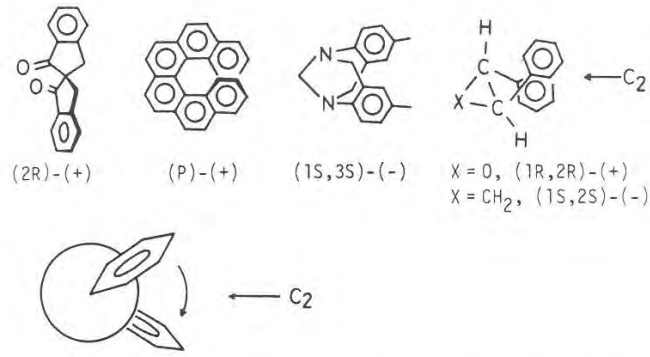
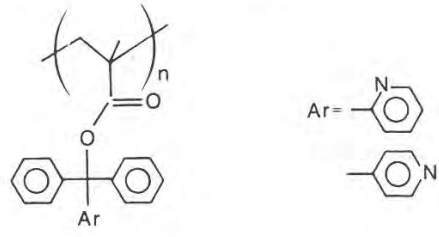


Fig. 7.7 — Schematic representation of the helical CSP-structure and absolute configurations of most strongly retained enantiomers, showing their resemblance to (P)-(+)-hexahelicene. (Reprinted from Y. Okamoto and K. Hatada, *J. Liquid Chromatog.*, 1986, **9**, 369 by courtesy of Marcel Dekker, Inc.).

Partly in order to reduce the ester lability, a series of similar polymers, (**2**), has been prepared. In general all of these gave inferior resolution although the 2- and 4-pyridyl analogues, when used with hexane–2-propanol eluent mixtures, gave interesting results for some polar solutes [72]. It has been suggested that hydrogen bonds involving the pyridyl groups may play a role in these cases.



2

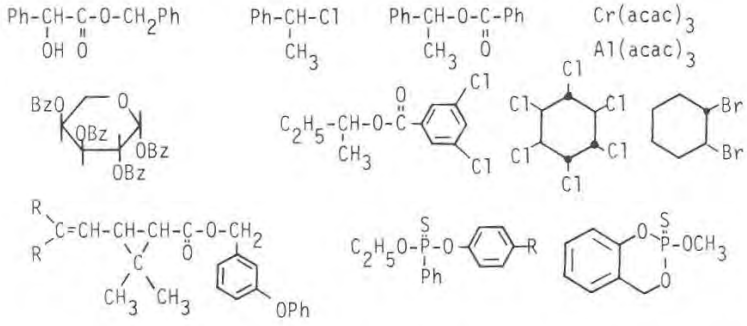
It is further of interest that compounds without aromatic substituents have been resolved on the (+)-poly(triphenylmethyl methacrylate) CSP, viz. the trisacetylacetonates of cobalt, chromium and aluminium [73,74].

The usefulness of this CSP is evident from Scheme 7.4, in which the various compounds resolved are given.

Columns containing this type of optically active synthetic polymers coated onto 10-μm silica are available (as "Chiralpak") from Daicel (see Appendix for details).

7.1.3 Synthetic polymers containing 'grafted' chiral cavities

As early as 1949 Pauling presented the idea of constructing, by synthetic means, polymer network cavities which should fit only one of two enantiomers [75].



Scheme 7.4 — Various compounds optically resolved on (+)-poly(triphenylmethyl methacrylate) sorbents.

Basically, the principle rests on an imitation of an enzyme's binding site, which can usually be regarded as a chiral cavity or cleft in the protein, often highly specific with respect to binding of substrate enantiomers because of the precise steric requirements for multiple bond attachment. Because the experimental technique can be compared with making a plaster cast from an original template it has also been called 'molecular imprinting'. Thus, the molecules of a particular compound act as templates around which a rigid polymeric network is cast. This procedure, which is conceptually straightforward but most delicate to carry out in practice, can be summarized in three distinct steps.

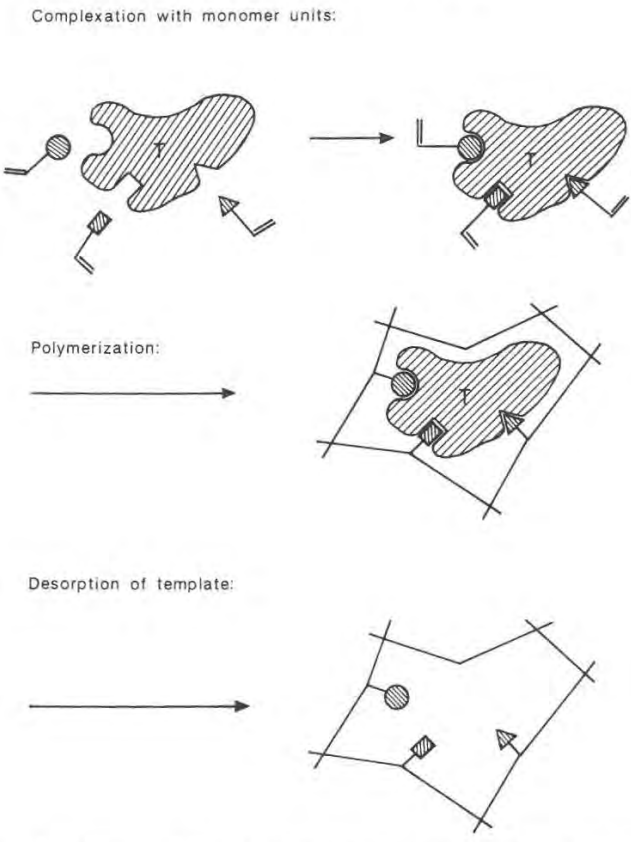
(1) Formation of a complex between the (chiral) compound used as template and a polymerizable monomer.

(2) Polymerization with cross-linking to form a rigid matrix.

(3) Removal of the template, either simply by some washing technique, or by means of a hydrolytic or similar reaction, which has to be used in the case of covalent attachment to the template. These steps are visualized in Scheme 7.5.

Extensive research on this technique has been carried out since 1977 when it was shown that a polystyrene sorbent, prepared with the use of an optically active template, could be used for partial resolution of the corresponding racemate [76–80]. The preferred method has been to use the rapid and reversible formation of boronic acid ester bonds between a carbohydrate structure and a vinyl-substituted phenylboronic acid (Fig. 7.8). In this way the monomer units are covalently attached to the template (cf. Scheme 7.5, step 1). Polymerization and cross-linking with a divinyl compound, followed by hydrolysis and washing out of the template will then yield the chiral sorbent.

The chromatographic performance of this kind of sorbent is shown in Fig. 7.9. The chromatogram illustrates one of the difficulties associated with the technique, viz. the necessity of fast mass-transfer to reduce band broadening. Although boronic ester formation is a very fast process, it is still somewhat too slow for chromatographic purposes. It is important to keep in mind that chromatographic sorption–de-



Scheme 7.5 — The principle of 'molecular imprinting' with formation of a defined arrangement of binding groups within the microcavity.

sorption equilibria are always based on non-covalent interactions, except for some protein separations by affinity chromatography where thiol–disulphide interchange may contribute.

In accordance with these facts, it was found that the column efficiency could be improved by increasing the temperature and also by acid–base catalysis, induced by the addition of ammonia to the mobile phase [81].

The choice of the mobile phase is complicated by the requirement that the polymer should be resistant to swelling in the medium, as deformation of the cavities will otherwise occur, with loss of selectivity. A mixture of acetonitrile with 4–6% of water and 2–8% of concentrated ammonia solution has been found to be very useful. The flow-rate has a pronounced influence on the result and very low flow-rates are usually necessary.

Interestingly, the k' values increase with increasing temperature. The increase is larger for the most strongly retained enantiomer, leading to an improved α-value.

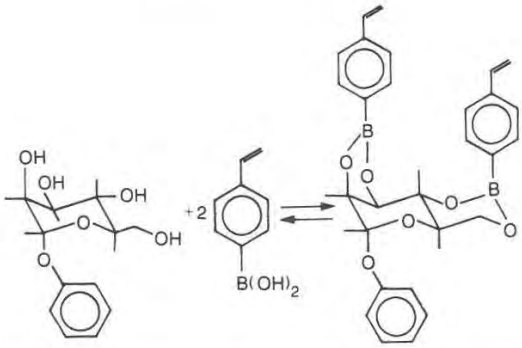


Fig. 7.8 — Introduction of polymerizable vinyl groups into phenyl-β-D-mannopyranoside by esterification with 4-vinylbenzeneboronic acid.

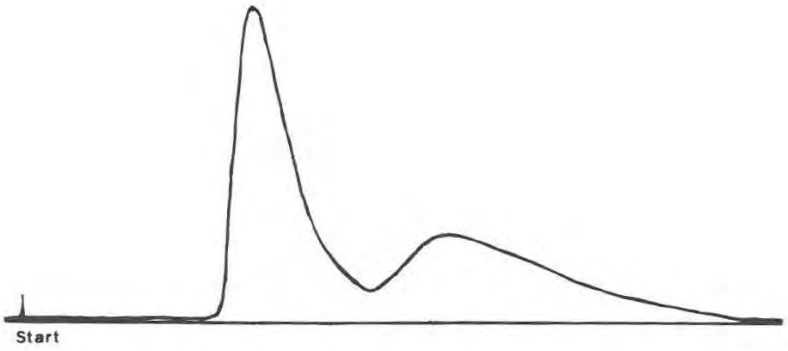


Fig. 7.9 — Elution profile from the chromatography of phenyl-β-D,L-mannopyranoside on macroporous polymer imprinted with the D-enantiomer. (Mobile phase: acetonitrile with 4% concentrated ammonia solution and 5% water, flow-rate 0.1 ml/min. Sample amount: 200 μg). (Reprinted, with permission, from G. Wulff, H.-G. Poll and M. Minarik, *J. Liquid Chromatography*, 1986, **9**, 385 by courtesy of Marcel Dekker, Inc.).

7.1.4 Proteins

The complicated molecular structure of proteins makes them very interesting for binding studies. The technique of affinity chromatography developed from the knowledge of the ability of certain protein–ligand pairs to form very strong complexes. Such pairs could be derived from natural systems, such as enzyme–substrate analogue, enzyme–cofactor, hormone–receptor protein, etc., but it was soon realized that many 'unnatural' synthetic compounds could also show very strong binding power (high affinity) for proteins.

The availability and importance of serum proteins, particularly serum albumins, have made them preferred models for binding studies. It was known from Scatchard analyses that binding to a protein involves multiple equilibria, i.e. a number of binding sites, some of which have different affinity for the ligand. It therefore seemed quite probably that the net result, the overall binding constant, could be different for the two enantiomers in a racemate. Further, from the knowledge concerning the substrate enantioselectivity often shown by enzymes, the presence of high affinity sites with enantiomer-differentiating ability would be expected in other proteins as well.

A number of studies of solution equilibria between serum proteins and various ligands, particularly pharmacologically active compounds, have been made, which demonstrated significantly different binding constants for the respective enantiomers [82,83]. Such effects could, however, be demonstrated more clearly by chromatographic techniques. Thus, in 1973 the previously known higher affinity of L-tryptophan for bovine serum albumin (BSA) was used to separate the enantiomers on a column packed with a BSA–Sepharose gel [84]. Elution of the D-form was performed with a borate buffer (pH 9), then the L-form was desorbed by changing to dilute acetic acid. The technique was later used for determination of the enantioselectivity of certain drugs for serum albumins [85,86]. In the last few years analytical chiral LC-methods based on the use of immobilized proteins as stationary phase materials have been rapidly developed and shown to be useful for a variety of resolution problems.

7.1.4.1 Immobilized albumin

Early work with the use of low-pressure LC-systems and isocratic elution with phosphate and borate buffers from columns packed with BSA coupled to Sepharose, demonstrated that the optical resolution of charged solutes such as the free amino-acids tryptophan, kynurenine [3-(2-aminobenzoyl)alanine] and their 5- and 3-hydroxy derivatives, respectively, is extremely dependent on the pH of the mobile phase [87]. It was further shown that compounds with chirality at a sulphur atom, such as methyl *o*-carboxyphenyl sulphoxide, could be resolved and that, in addition to pH, the ionic strength of the mobile phase had a great influence on retention and resolution [88].

A significant improvement in column performance and ease of operation was obtained with the introduction of a silica support (spherical 7 or 10 μm particles, 300 Å average pore diameter) for the immobilized albumin [89]. Analytical columns packed with such albumin-silica sorbents ("Resolvosil") are useful in combination with aqueous buffered eluents for optical resolution of a variety of racemates.

As with the previously described polymeric CSPs, the mechanism of chiral recognition is complex and not yet known in any detail. However, the major contributions to the overall retention have been elucidated from systematic investigations of the effects of solute structure and mobile phase composition. In many respects the albumin-silica sorbents act like alkyl-silica reversed-phase materials. Alkanols, preferably l-propanol, can be used as retention modifiers, causing faster elution due to a decrease in hydrophobic interaction with the sorbent. There are essentially three variables by means of which the mobile phase system can be optimized for a particular resolution problem, viz. the pH, ionic strength and organic

solvent-modifier [90]. There appear to exist simultaneous contributions mainly from charge and dipole interactions on the one hand and hydrophobic interactions on the other. Further, effects from hydrogen bonding and charge-transfer interaction are likely to be present. The large effects of the mobile phase on the k' values of the solute enantiomers are understandable in view of the dynamic properties of a protein with respect to charge distribution and conformation. BSA consists of no less than 581 amino-acids in a single chain (m.w. 6.6×10^4) and its higher structure is to a large extent determined by the 17 disulphide bridges present in the molecule. At pH 7.0 its net charge is −18 and its isoelectric point is 4.7. Thus, as is well-known from enzyme chemistry, a solvent change may cause changes in a binding site of a protein by charge as well as conformational effects.

Depending on the charge of the solute, a pH change of the mobile phase may cause either increased or decreased k' values. Whereas amines and free amino-acids bearing a positive charge are more strongly retained at higher pH, the reverse is true for negatively charged solutes. Systematic studies of a series of *N*-benzoyl-D,L-amino acids have given some insight into the mechanism of interaction between a solute and the protein. The effect of various mobile phase parameters on the k' and α values is shown in Fig. 7.10. First, it is evident that retention is increased to a large extent by increasing the hydrophobic character of the amino-acid (SER < ALA < PHE). Secondly, the increasing negative net charge of the protein with increasing pH will cause a decreased k' for all six species (due to the effect on ionic interaction). Next, the effect of buffer concentration could be interpreted as an increased ionic adsorption at low ionic strength. The small but significant increase in k' for the most strongly retained species at the highest buffer concentrations is probably an effect originating from increased hydrophobic interaction. Because ionic (coulombic) and hydrophobic interactions are oppositely influenced by the ionic strength, the two effects should overlap, giving a minimum in solute adsorption (k') at a certain point. Finally, the effect of an organic solvent-modifier is obvious; it has always been found to decrease the retention of the solute, the effect being largest for the most hydrophobic compounds.

The effect of pH and ionic strength on the retention of uncharged solutes is small, but significant, and attributable solely to changes in the binding sites of the CSP. Addition of 1-propanol causes a decrease in retention comparable to that found for charged solutes, indicating that it affects mainly the binding contribution caused by hydrophobic interaction. This is also shown by the very large effect of the chain length of 1-alkanols on retention, the higher alkanols being much more effective competitors for the binding sites, and causing faster elution of the solute.

The flexibility conferred by regulating retention through changes in the mobile phase is shown in Scheme 7.6, which gives an idea of the procedure used in the search for optimal conditions for optical resolution of a racemate on a "Resolvosil" column.

Very large α values, actually not desirable in practical applications, can be obtained on these columns [91,92]. However, adjustment to give adequate baseline resolution with short retention times is quite easy (Fig. 7.11).

7.1.4.2 Immobilized α_1-acid glycoprotein (orosomucoid)

Despite the great potential of proteins as CSPs, only one other than albumin has so far been investigated. This is a human plasma protein called α_1-acid glycoprotein

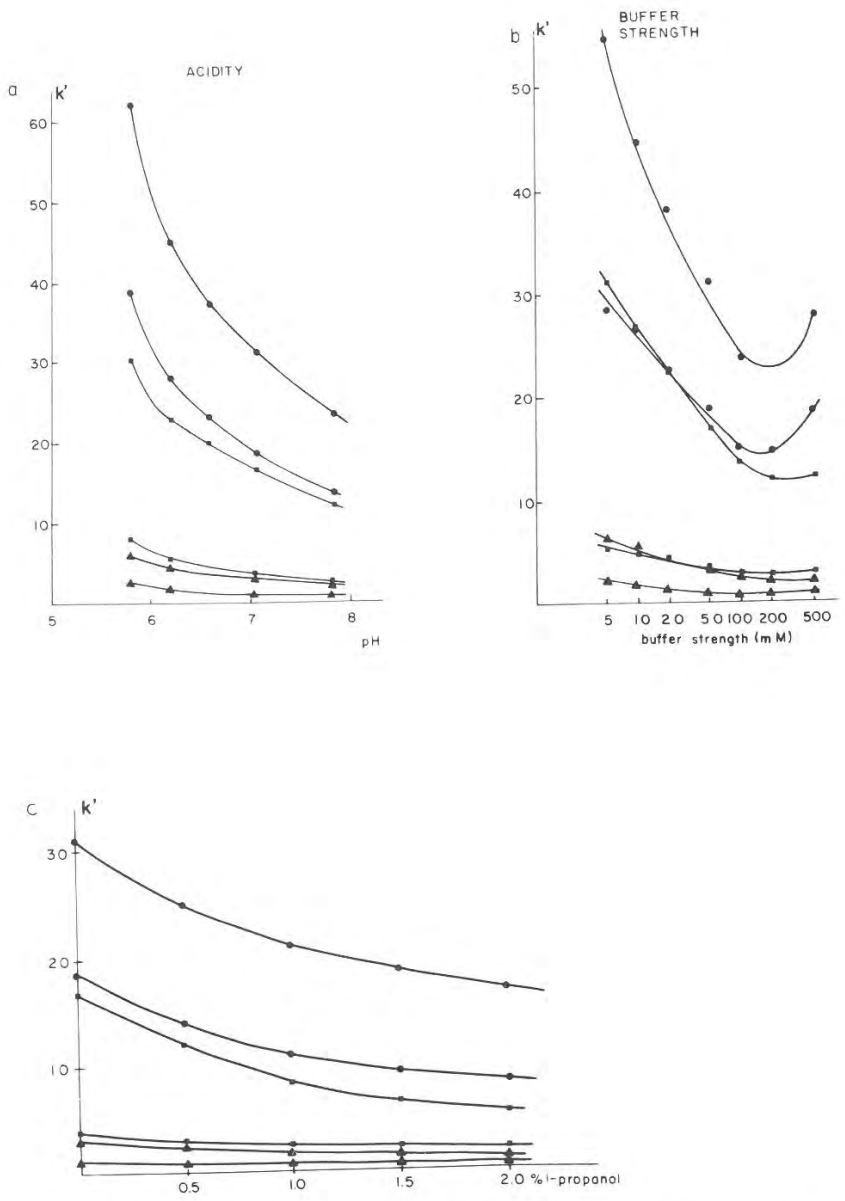
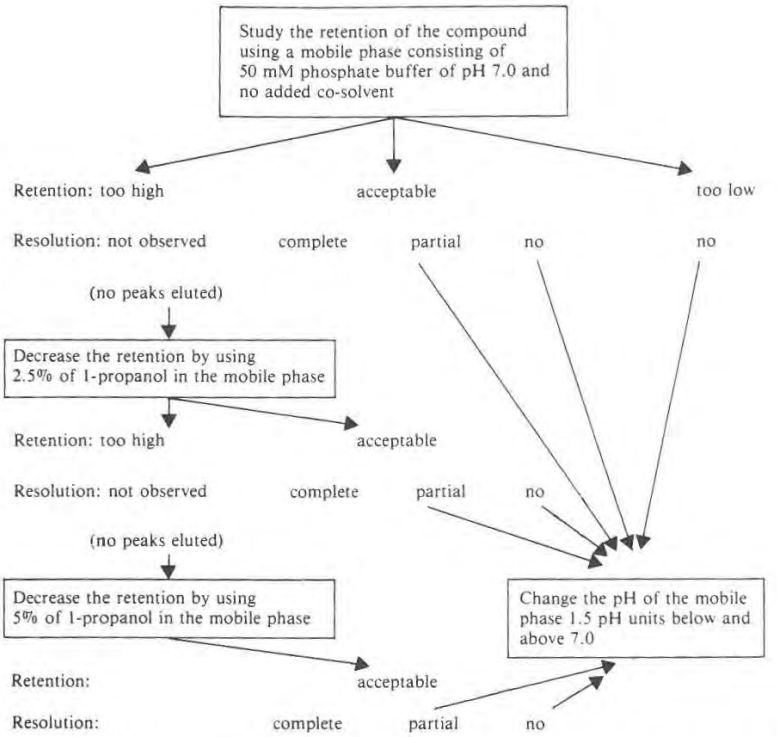


Fig. 7.10 — Retention (k') of the enantiomers of *N*-benzoyl-D,L-serine (▲), -alanine (■) and -phenylalanine (●) as a function of: (a) pH, (b) buffer strength, and (c) organic modifier (1-propanol). (Reprinted, with permission, from S. Allenmark, B. Bomgren and H. Borén, *J. Chromatog.*, 1984, **316**, 617. Copyright 1984, Elsevier Science Publishers B.V.).



Scheme 7.6 — Optimization procedure in optical resolution by systematic changes in mobile phase composition. (Reprinted, with permission, from S. Allenmark and B. Bomgren, in *Affinity Chromatography — A Practical Approach*, P. G. D. Dean, W. S. Johnson and F. A. Middle (eds.), p. 108. Copyright 1985, IRL Press, Oxford.).

(AGP) or orosomucoid, present in a concentration of 55–140 mg per 100 ml of plasma. It has been claimed that AGP is the main cationic binding protein in the human organism [93].

The chromatographic sorbent is composed of a 300-Å silica support in which the protein has been immobilized by a functional group transformation followed by cross-linking to form aggregates which are large enough to be held within the pores. The procedure is based on the fact that the orosomucoid molecule contains five carbohydrate units, which together constitute ca. 45% of the molecular weight. Oxidation with sodium metaperiodate converts the primary alcohol functions of these sugar units into aldehyde groups. Incorporation of this modified protein into the silica, followed by raising of the pH of the buffer solvent, yields cross-linking by Schiff-base formation. Hydrolytically stable bonds are then formed by reduction of the imino functions with sodium cyanoborohydride [94]. The immobilization chemistry is outlined in Scheme 7.7. Columns packed with this sorbent are manufactured under the name "EnantioPac".

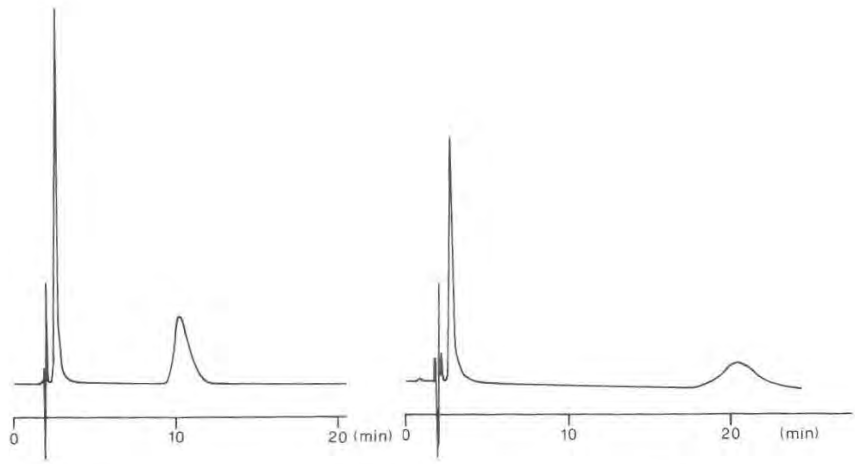


Fig. 7.11 — An example of the effect of the mobile phase on resolutions; chromatogram of D,L-kynurenine under two slightly different conditions: (a) pH 7.1, (b) pH 7.6. (S. Allenmark and S. Andersson, unpublished work).

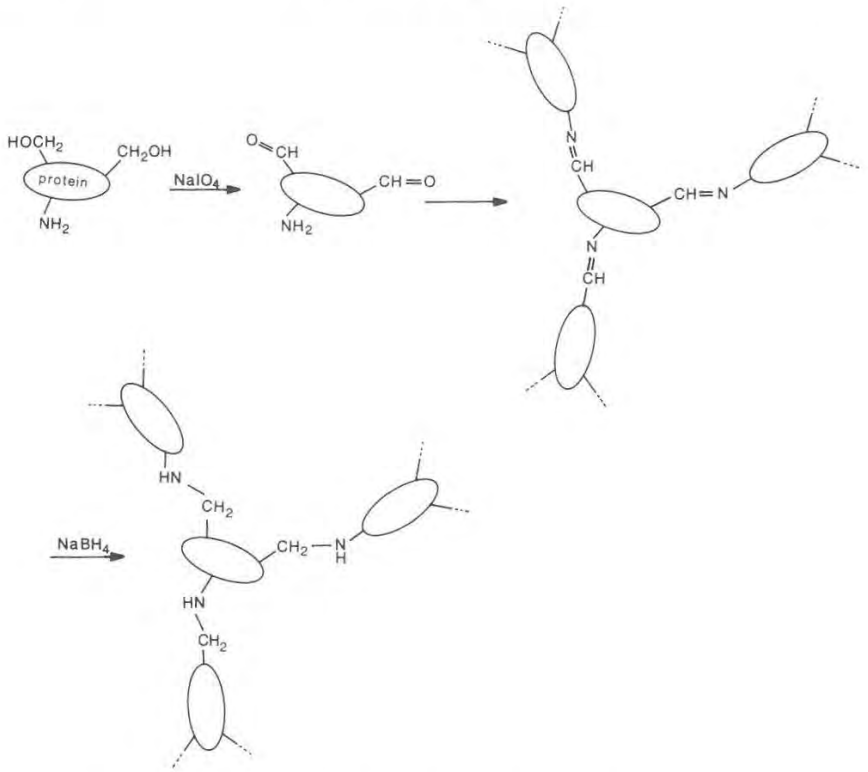
In the unbound native form AGP has an isoelectric point of 2.7, i.e. it is ca. 2 p*I*-units more acidic than albumin. Its molecular weight is 4.1×10^4. Sialic acid (14 residues in all) present in the sugar units is presumed to be involved in the binding of ammonium-type compounds at neutral pH [95], a process thought to be associated with some enantioselectivity.

Basically, the solute retention is governed by the same types of primary interaction processes as in albumin-silica and the methods used for regulation of retention and resolution are essentially the same, although 2-propanol has been the preferred alkanol modifier [96]. Further, cationic as well as anionic modifiers have been used. An analysis of their effects on the retention of charged solutes has shown consistency with an ion-pair equilibrium model for the solute–sorbent interaction [97]. Thus, the same principles which are widely used [98] for the regulation of retention of charged solutes in ordinary reversed-phase chromatography can be applied to optical resolutions on "EnantioPac" columns. This again shows the importance of hydrophobic binding sites in protein CSPs.

Because the use of such ion-pairing retention modifiers is of great importance for practical applications such as drug analysis, to which we will return in Chapter 8, let us briefly outline the theoretical background of ion-pair chromatography [99].

We assume that the solute, S, is a basic compound which exists in the protonated form, HS^+, at the pH used for the separation. A charged ion-pairing compound Q^+X^- is added to the mobile phase. The distribution of the solute between the mobile and the stationary phase will then be influenced by the concentration of the added compound.

We know from Chapter 4 [Eq. (4.3)] that the capacity ratio, k', can be expressed



Scheme 7.7 — Reactions used for immobilization of the protein by cross-linking.

as $k' = Kq$, where K is the distribution constant (C_s/C_m) of the solute and q denotes the phase ratio (V_s/V_m).

It can be shown [99] by consideration of the equilibria involved, that the capacity ratio of the solute of interest, k'_{HS^+}, will take the form:

$$k'_{HS^+} = \frac{qK^\circ K_{HSX}[X^-]}{1 + (K_{QX}[Q^+][X^-])} \tag{7.1}$$

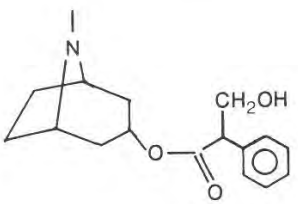
where K° is the monolayer capacity.

The validity of this expression can be experimentally verified. Rearrangement will yield the expression:

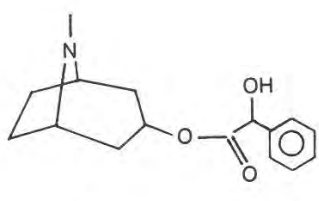
$$\frac{[X^-]}{k'_{HS^-}} = \frac{(1 + K_{QX}[Q^+][X^-])}{qK^\circ K_{HSX}} \tag{7.2}$$

Thus, a plot of $[X^-]/k'_{HS^+}$ against $[Q^+][X^-]$ should be linear if the model is correct.

An example of the effect of sodium octanoate ($X^- = C_8H_{17}CO_2^-$) on the optical resolution of atropine (**3**), and homoatropine (**4**), on an "EnantioPac" column is shown in Fig. 7.12. Note that the linear relationships mean that the α values are unchanged, as predicted from the assumption of a single binding site.



3



4

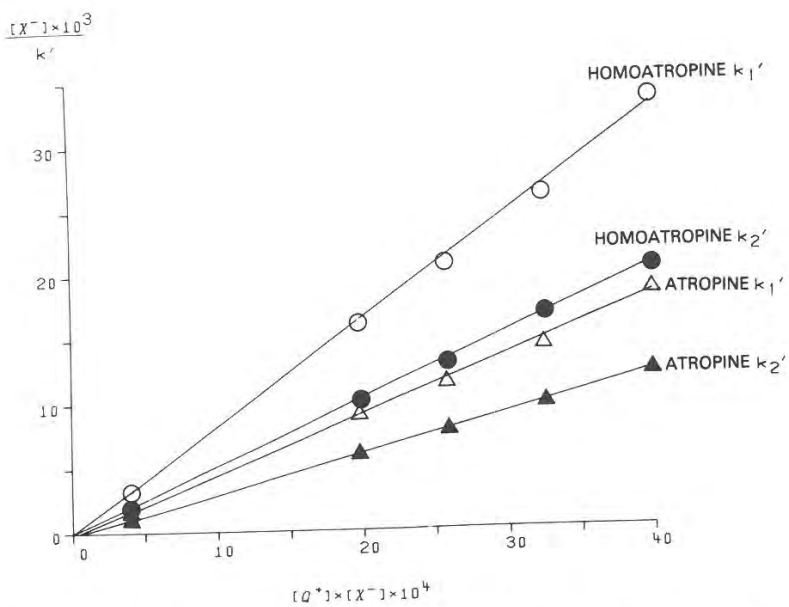


Fig. 7.12 — Application of the ion-pair distribution model to the retention of the enantiomers of atropine and homoatropine. Mobile phase: octanoic acid in 0.02*M* phosphate buffer, pH 7.0. (Reprinted from G. Schill, I. W. Wainer and S. A. Barkan, *J. Liquid Chromatog.*, 1986, **9**, 641 by courtesy of Marcel Dekker, Inc.).

The effect of hydrophobic charged modifiers, as well as the effects of pH and various alkanols, on a variety of ionic solutes have been extensively studied by Schill and co-workers [97,100] and by Hermansson and Eriksson [101].

In summary, although generalizations are difficult to make in the field of chiral separations with proteins, some observations, common to both types of columns described, are noteworthy.

(1) Alkanol modifiers generally decrease retention of both enantiomers. Most probably, this effect is due to their tendency to reduce hydrophobic interaction between the protein surface and the solute. Since hydrophobic interaction is an essential part of the sorption equilibrium, any reduction will cause faster elution from the column. The effect on the enantiomeric separation factor, however, will depend on the relative decrease in retention of the two enantiomers. Often, but not always, α will either be reduced or essentially unchanged.

(2) The effect of pH on retention is markedly larger for charged than for uncharged solutes. Generally, a decrease in pH gives reduced retention for cationic solutes and an increased retention for anionic solutes. The effects on α are often quite large but difficult to predict or systematize.

7.2 BONDED SYNTHETIC CHIRAL SELECTORS

The CSPs described under this heading are all characterized by their well-defined molecular structures bonded to some solid support, usually silica. These low molecular-weight chiral compounds, here called selectors, have often been chosen on a more rational basis because their enantioselective properties can often be evaluated from NMR studies on their solutions. This also means that the elution order from use of sorbents based on such chiral selectors is often predictable from a chiral recognition mechanism.

7.2.1 Crown ethers ('host–guest' complexation)

Macrocylic polyethers are known under the name crown ethers because molecular models of them often resemble a crown in shape. Their ability to form strong complexes with metal cations as well as substituted ammonium ions has led to very extensive research [102]. One interesting field is their use as phase-transfer agents, where the formation of lipophilic alkali-metal ion inclusion complexes is utilized for the transfer of alkali-metal salts into organic solvents [103]. The field is now known as a branch of 'host–guest' complexation chemistry, mimicking Nature's principle for structural recognition, so common in biological regulatory systems.

The first successful synthesis of optically active crown ethers [104,105] led Cram and his collaborators to investigate their use for optical resolution purposes [106]. This resolution principle was then transferred into an LC separation technique by the use of a chiral crown ether in the mobile phase or covalently bound to a silica support [107]. Such a chiral host is able to discriminate between enantiomeric ammonium compounds, such as esters of D,L-amino-acids, because the multiple hydrogen bonds formed between the ammonium group and the ether oxygens will, for steric reasons, lead to a less stable complex with one of the enantiomers.

The situation is shown by Fig. 7.13. The immobilized selector, the optically active crown ether of (R,R)-configuration, acts as the host, incorporating a guest, repre-

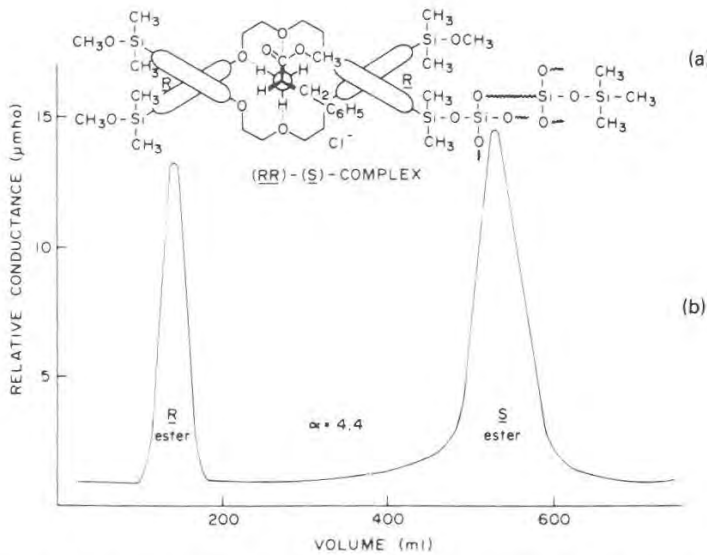


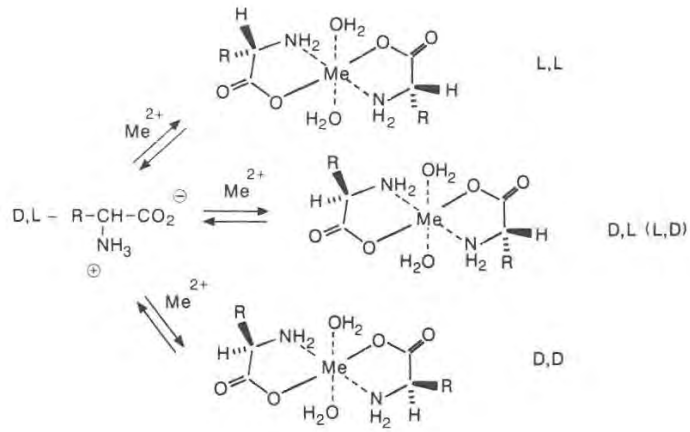
Fig. 7.13 — (a) Structure of the optically active crown ether used for optical resolution. (b) Chromatographic resolution of methyl phenylalaninate hydrochloride. (Reproduced from L. R. Sousa, G. D. Y. Sogah, D. H. Hoffman and D. J. Cram, *J. Am. Chem. Soc.*, 1978, **100**, 4569, with permission. Copyright 1978, American Chemical Society).

sented by the (S)-form of methyl phenylalaninate (as hydrochloride). All three protons of the ammonium group are hydrogen-bonded to crown ether oxygen atoms. The conformational freedom within the complex is rather restricted and the (S)-enantiomer will permit an energetically more favourable conformation in the complexed form. Note that the crown ether, despite its apparent symmetry, can exist in four optically active forms because it is derived from the atropisomeric binaphthol units, which are resolvable owing to hindered rotation. The structure shown is obtained from the (R)-enantiomer.

7.2.2 Metal complexes (chiral ligand exchange)

The ability of transition metals to participate in complex formation was early exploited for the purpose of enantiomer separation. The pioneering work was carried out by Davankov, who published his first papers on the new technique, chiral ligand exchange chromatography (CLEC) as early as 1970 [108–110]. The method used was to immobilize L-proline on a chloromethylated styrene–divinylbenzene copolymer and to utilize the ternary complex formation in the presence of copper(II) ions and amino-acid anions (Scheme 7.8).

Because diastereomeric complexes are formed from amino-acid ligands of opposite configuration, any difference in stability of these complexes will, of course,



Scheme 7.8 — Bidentate transition-metal amino-acid ternary complex formation.

result in different chromatographic mobilities of the amino-acid enantiomers. Since the first successful experiments, numerous papers on CLEC have been published, and it is today by far the most widely studied method for chiral LC. Some of the most important results from these investigations are summarized below.

(1) Of all chelating metal ions studied [Cu(II), Ni(II), Zn(II), Hg(II), Co(III), Fe(III), etc.], copper(II) forms the most stable complexes and seems to have the greatest potential for liquid-chromatography purposes.

(2) Cyclic amino-acids, such as L-proline and L-hydroxyproline, form, together with copper(II), the most enantiodifferentiating chiral selectors.

(3) The method of immobilization and the nature of the matrix are highly important.

(4) In general, an unchanged enantiomeric elution order is obtained for a series of different bidentate amino-acids from a given column.

The requirement of sufficient stability of the ternary complex means that this condition can only be fulfilled by a very limited number of racemic compounds. Since five-membered ring formation is favoured, compounds such as α-amino- and α-hydroxy-acids are the most suitable. Not surprisingly, it has also been found that β-amino-acids (where formation of a six-membered ring is necessary) are difficult to resolve by CLEC [111]. The number of co-ordinating groups influences the relative complex stability. Bidentate ligands, such as the neutral amino-acids lacking other polar substituents, therefore show an elution order (on a polystyrene L-Pro or L-HO-Pro sorbent) which is opposite to that obtained with, for example, the acidic amino-acids (Asp, Glu), which are terdentate.

A way to rationalize these and other results is to consider the various possibilities of stabilizing the sorption complex by electron donation to the metal atom through solvation or other types of ligand participation along the axial directions in the

complex. It is assumed that water molecules normally stabilize the complex by co-ordination in an axial position. Therefore, complex stability will be highly dependent on substituent effects causing changes in this solvent participation. Such effects are very likely to take place, owing to the fixed geometry and reduced conformational mobility in the sorption complex. This also explains the importance of the matrix used. The situation is illustrated in Fig. 7.14.

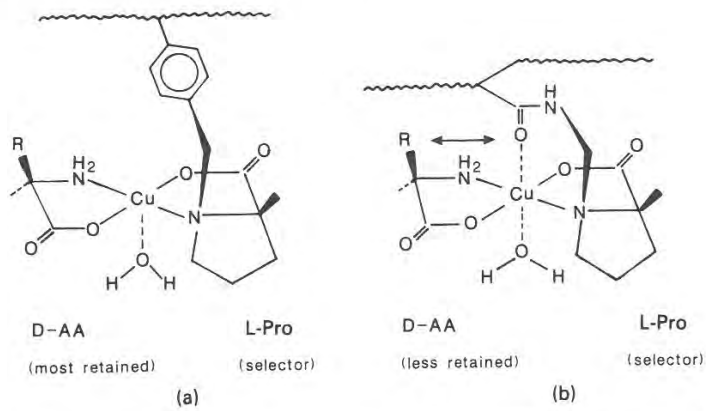


Fig. 7.14 — Steric effect on solvent participation in ternary complexes formed with an immobilized liagand.

If we start by looking at the situation for the polystyrene–L-Pro sorbent (a), we find that in the two possible diastereomeric sorption complexes formed by a bidentate ligand, the L–L complex will be the least stable because of steric hindrance to solvation. Therefore, the elution order in this case will be L prior to D. With a terdentate ligand, on the other hand, a participation by a neighbouring group, in this case a carboxylate group, will produce a more efficient stabilization than the water molecule. As this participation is only possible in the L–L complex, this will be the most stable and the elution order will consequently be D prior to L. Now, considering the polyacrylamide–L-Pro sorbent (b), the effect of the polar functions in this matrix has to be taken into account. Here, the strongly electron-donating carbonyl groups of the polyacrylamide will participate [112]. In this case it is assumed that the effect of the participation of the amide carbonyl group in complex stabilization (the polyacrylamide–L-Pro behaves as a terdentate ligand) will be reduced in the L–D complex owing to steric interference with the amino-acid side-chain R. This view is further supported by experimental stability data for analogous low molecular-weight model systems [113]. The elution order of bidentate α-amino-acids, D prior to L, is fulfilled by the compounds investigated, with proline as an exception. The separation factor is strongly increased with the size and branching of the substituent R [114].

The intensive research in this field since 1970 has resulted in a variety of sorbents for CLEC, based on various matrices (polystyrene, polyacrylamide, polymethacry-

late, silica) and chiral selectors (where *N*-anchored L-Pro, L-allo-HO-Pro and L-HO-Pro have been among the most useful). A summary is given in Table 7.9.

Table 7.9 — Various types of sorbents used for CLEC

Immobilized ligand	Matrix	Metal ion	Racemates resolved
Various L (and D)-AA	polystyrene	Cu^{2+}, Ni^{2+}, Zn^{2+}	Various D,L-AA, 2-amino-alcohols, mandelic acid
L-Pro	polystyrene	Cu^{2+}	D,L-Pro
N-Carboxymethyl-L-Val	polystyrene	Cu^{2+}	D,L-AA
(R)-*N*,*N*′-dibenzyl-1,2-propandiamine	polystyrene	Cu^{2+}	D,L-AA
L-Pro, L-AA	polyacrylamide	Cu^{2+}	D,L-AA
L-Pro	silica	Cu^{2+}	D,L-AA
L (and D)-Pro, L-HO-Pro, L-Val, L-His	silica	Cu^{2+}, Ni^{2+}, Co^{3+}, Zn^{2+}	D,L-AA
L-Pro-NH_2	silica	Cu^{2+}	D,L-Trp, D,L-Tyr, D,L-Phe
L-Pro-NH_2	silica	Cd^{2+}	Dns-D,L-AA, barbiturates, hydantoins
t-BOC-L-Pro-NH_2 or L-Val-NH_2	silica	Cu^{2+}	D,L-AA, Dns-D,L-AA
Linear polyacrylamide–L-Pro-NH_2	silica (adsorption)	Cu^{2+}	D,L-AA
N-Acyl-L-Val-NH_2	silica		*N*-acyl-D,L-AA ester

Use of silica as a support has significantly improved the normally quite low column efficiency and facilitated the application of modern LC-technology. Despite this, the effective plate height is still comparatively large. This is undoubtedly due to a slow exchange process, on the chromatographic time-scale, in the sorption complex. Therefore, a gain in resolution is usually obtained by an increase in column temperature to around 50°C [115].

The mobile phase is always an aqueous, usually buffered, system containing a low concentration (ca. 0.1–1.0 m*M*) of the complexing metal ion. The use of a water-miscible organic solvent such as methanol or acetonitrile as a modifier is sometimes advantageous. Use of sorbents comprised of a linear polyacrylamide/Cu(II) phase adsorbed onto silica was also found to give slow ligand exchange kinetics [114].

Techniques for covalent binding of amino-acids to silica have been developed [116–129]. A series of silica-bound amino-acids as fixed ligands was investigated by Gübitz and co-workers [121–123] who found that on complexation with Cu(II) ions cyclic amino-acids show higher enantioselectivity than aliphatic ones, and phenylalanine as a fixed ligand gives an elution order for all amino-acids (i.e. L- prior to D-forms) that is the reverse of that obtained with the other ligands investigated.

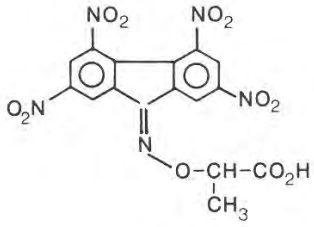
Methods utilizing non-covalent immobilization of the amino-acid/metal complex by hydrophobic interaction with a reversed-phase (alkyl-silica) sorbent, have also been developed and extensively investigated. Even though some of these techniques actually do not require any chiral additive in the mobile phase [130], they represent

borderline cases, and because of their similarity to other methods based on the combination of a reversed-phase non-chiral column and a mobile phase containing a chiral additive, they are presented in Section 7.3.

7.2.3 Selectors based on charge-transfer complexation

The chiral selectors described in this section are characterized by the operation of an aromatic π–π bonding interaction as an essential element of the retention process. Interactions of this type are well known and are found to take place between so-called π-donor and π-acceptor molecules. A π-donor will have a tendency to lose an electron because the resulting positive charge will be accommodated by the π-system. Conversely, a π-acceptor can readily stabilize a negative charge and has therefore a tendency to accept an additional electron in its π-system. In this way a π-donor/acceptor pair will form a complex when a charge can be transferred from the donor to the acceptor molecule. The strength of such complexes may be considerable. Often the donor molecule is called a π-base and the acceptor a π-acid.

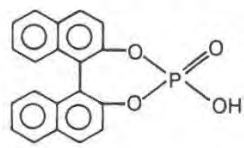
One of the earliest applications of charge-transfer (CT) complexation to the problem of optical resolution by LC was carried out in the 1960s by Klemm and co-workers [131,132]. Polycyclic aromatic compounds, including the helicenes, were known to form CT-complexes with acceptors such as nitroaromatics. An interesting resolving agent based on π-acidity had been designed and synthesized by Newman and co-workers [133–135]. This compound, optically active α-(2,4,5,7-tetranitro-fluorenylideneamino-oxy)propionic acid (TAPA, **5**) was shown to be capable of resolving a variety of aromatic π-bases by CT-complex formation [131–133].



5

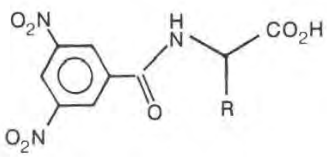
By the use of a TAPA-impregnated chromatographic support, Klemm succeeded in partial resolution of some aromatic ethers and hydrocarbons. The ability of TAPA to resolve chiral aromatic hydrocarbons lacking functional groups attracted much interest, and resolutions of a variety of helicenes by LC on TAPA bound to silica or physically adsorbed on silica or alumina have been published, notably by Gil-Av and co-workers [136,137] and by Wynberg and co-workers [138,139]. Other resolutions based on this principle have also been reported [140–142].

Among other CT-complexing chiral selectors suggested are *N*-dinitrophenyl-L-alanine [143] and binaphthylphosphoric acid (**6**) [144,145], which have also been found useful for resolutions of helicenes.

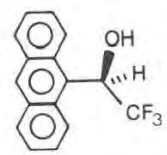


6

A breakthrough in the use of CT-adsorption for optical resolution by LC was made by the introduction of *N*-(3,5-dinitrobenzoyl)amino-acids (**7**) as chiral, immobilized CT-acceptor ligands. This approach, taken by Pirkle and co-workers [146–148], was preceded by the use of an optically active anthryl carbinol, viz. (R)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol ("Pirkle's alcohol") (**8**), for the resolution of racemic 2,4-dinitrophenyl methyl sulphoxide by recycling on a silica column saturated with the anthryl carbinol selector† [149]. The successful results led to a chiral sorbent based on this optically active π-base covalently bound to a silica support. This sorbent was used for optical resolutions of a variety of π-accepting racemic solutes, including sulphoxides, amines, amino-acids, hydroxy-acids, lactones, alcohols, amino-alcohols and thiols [150].



7



8

The great success of the technique was interpreted by Pirkle, in the framework of Dalgliesh's three-point interaction theory, as being a result of a combination of simultaneous π–π interaction and hydrogen bonding in the non-polar solvent used as the mobile phase [151]. The solute–sorbent interaction proposed as a chiral recognition model is shown in Fig. 7.15.

Since this chiral sorbent had shown excellent properties with respect to 3,5-dinitrobenzoylated racemic compounds such as amino-acids, application of the principle of 'reciprocal' behaviour (meaning that if optically active *A* resolves the enantiomers of *B*, then optically active *B* resolves the enantiomers of *A*) led to the synthesis of (R)-*N*-(3,5-dinitrobenzoyl)phenylglycine as a π-acidic chiral selector. This could be very conveniently used when ionically bound to 3-aminopropyl-silica in combination with 0–20% of 2-propanol in hexane as a mobile phase. Studies of various substituted anthryl carbinols on this CSP contributed to a large extent to a better understanding of the mechanism behind the enantioselective adsorption, which often resulted in high α values [146].

† These early experiments were carried out in a glass column, which permitted direct observation of the red charge-transfer complex formed between the alcohol and the sulphoxide. Moreover, the intensity of the colour varied inversely with temperature, demonstrating reversible complexation.

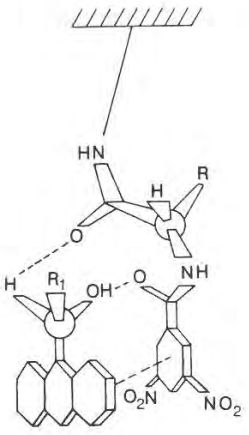


Fig. 7.15 — Pirkle's chiral recognition model. The charge-transfer complex formation causes simultaneous additional enantiomer-dependent contacts between the partners.

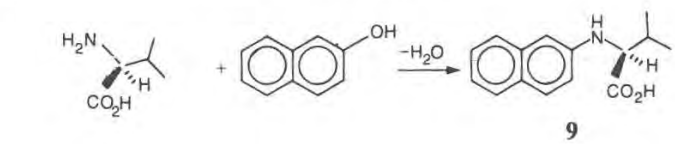
It was found that substituents, R_1, in the anthryl carbinol system, that changed the π-basicity also changed the α values in the same direction. Further, α appeared to increase with the size of R_1. An additional functional group present in R_1 had a minor influence, unless it could competitively interact with the CSP or essentially alter the conformational behaviour of the molecule.

In order to obtain a stable, covalent bond to the aminopropyl-silica support and hence a wider choice of mobile phase, the DNB-amino-acid was reacted with the amino terminal of the silica with EEDQ as condensing agent. Interestingly, the amide-bonded phases differed significantly from their ionically bound analogues when used under identical conditions.

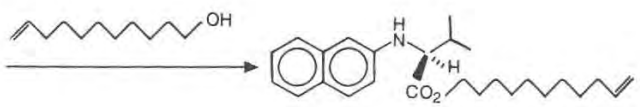
The two most successful CSPs of type (**7**), also commercialized, are those based on phenylglycine and leucine [$R = CH_2C_6H_5$ and $CH_2CH(CH_3)_2$] [152].

The reciprocal aspect of chiral recognition was further utilized as a strategy for the design of another series of CSPs. It had been observed [153] that a long-chain ester of *N*-(2-naphthyl)-D,L-alanine was resolved on an (S)-*N*-(3,5-dinitrobenzoyl)-leucine column with a very large α value (10.5). Therefore, new CSPs, comprised of a naphthyl-amino-acid anchored to the silica by a long-chain ester function, were prepared [154,155]. It may be instructive to take a brief look at the synthetic route leading to these sorbents, as exemplified by the case of valine [$R = CH(CH_3)_2$] in Scheme 7.9.

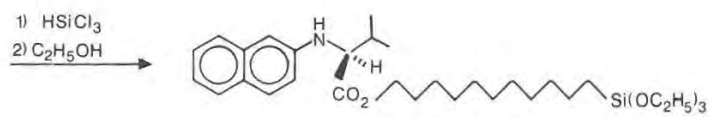
First, L-valine is reacted with 2-naphthol in a modified Bucherer reaction, yielding (S)-(−)-*N*-(2-naphthyl)valine (**9**). This product is then esterified with 10-undecen-1-ol by acid catalysis, yielding (**10**). The next step involves a hydrosilylation reaction, meaning that trichlorosilane is added to the terminal double bond, with chloroplatinic acid as catalyst. The chiral trichlorosilane obtained is then converted (without purification) into the corresponding triethoxysilane (**11**). The latter is then bound to silica.



9



10



11

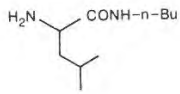
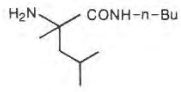
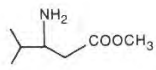
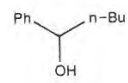
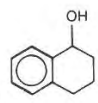
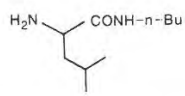
Scheme 7.9 — Synthetic route to immobilized, π-donating chiral selectors.

The enantioselective properties of these sorbents are shown in Table 7.10 for a series of derivatives of amines and alcohols. In order to make full use of the π-donating capacity of the naphthylamino function in the CSP, the 'selectand' (the analyte) must carry a good π-acceptor such as the 3,5-dinitrophenyl group. This can be introduced rather easily by acylation (forming amides and esters) or by carbamoylation with an isocyanate (forming urea derivatives and carbamates). These key reactions are shown in Scheme 7.10.

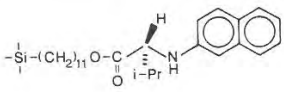
The data presented in Table 7.10 give some indications that derivatives of α-amino-acids are preferred over the β-isomers as selectands and also that α-methyl substitution in the α-amino-acid is highly unfavourable from the point of view of chiral recognition. It is also noteworthy that very simple aliphatic amines and amino-alcohols are well resolvable as DNAn derivatives. Further, a pronounced decrease in separability is found in more polar eluents. Although many factors are involved here, it is reasonable that in a reversed-phase system a significant contribution to overall retention stems from hydrophobic interaction. In many cases this effect should be rather unselective. This is most likely the main cause of the large increase in the k' values of the α-amino-acid derivatives with increased mobile phase polarity, leading to a drastic reduction in α but no change in elution order.

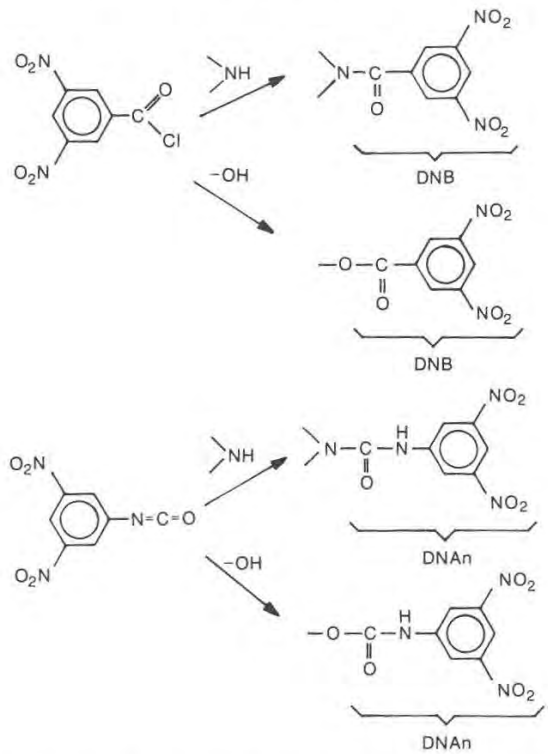
Other applications of the reciprocity principle have led to various new chiral bonded selectors acting as π-donors. these include the hydantoin- [156], arylalkyl-amine- [157–162] and phthalide-based [163] CSPs. The reciprocity principle and the importance of competing (opposite sense) chiral recognition processes has recently been reviewed and discussed [164].

Table 7.10 — Chromatographic data of 3,5-dinitrophenylated derivatives of some amino-acids, amines and alcohols. (Reprinted from W. H. Pirkle and T. C. Pochapsky, *J. Am. Chem. Soc.*, 1986, **108**, 352, with permission. Copyright 1986, American Chemical Society).

Compound	Derivative	k'_1 (enantiomer)	α	2-Propanol in hexane (%)
	DNB	0.38 (R)	17.66	10
	DNB	7.97	1.45	5
	DNB	0.93 (S)	1.97	5
	DNAn	5.87 (S)	1.19	5
	DNAn	3.27 (R)	1.33	20
	DNAn	3.19 (R)	1.24	5
	DNAn	5.35 (R)	1.22	5
	DNB	9.0 (R)	2.61	Reversed-phase conditions: 50% methanol–water

DNB = 3,5-dinitrobenzoyl-, DNAn = 3,5-dinitroanilido-

CSP: 



Scheme 7.10 — Derivatization reactions used for introduction of the π-acceptor function into the analyte.

7.2.4 Selectors based on hydrogen bonding

By an extension of the principle used in gas chromatography on chiral amide stationary phases, viz. multiple hydrogen bonding, Hara and associates constructed a series of selectors for optical resolution by liquid chromatography [165–167]. They assumed that the hydrogen bond formation in the liquid stationary phase of the chiral GC method used by Charles *et al.* [168] (cf. Section 6.1.1) could also be utilized in LC when combined with a non-polar mobile phase. The suggested principle of optical resolution by diastereomeric selector–solute complexes involving two hydrogen bonds is shown in Fig. 7.16.

The selectors were constructed from L-valine and D-tartaric acid as optically active starting materials. Various *N*-acyl derivatives of L-valine were prepared and covalently bound to 3-aminopropyl-silica. From tartaric acid an analogously amide-bound isopropyl amide was synthesized, as well as the free selector, di-isopropyl tartaric diamide [(R,R)-DIPTA], and used as a mobile phase additive. The various selectors are represented in Scheme 7.11.

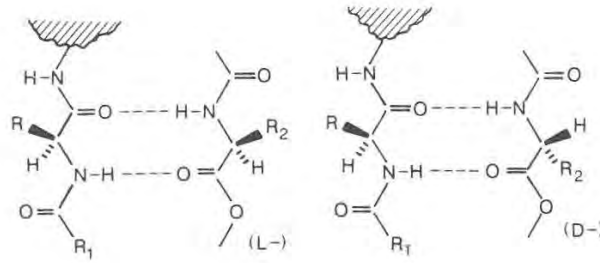


Fig. 7.16 — Diastereomeric sorption complexes formed by a two-point hydrogen-bonding interaction.

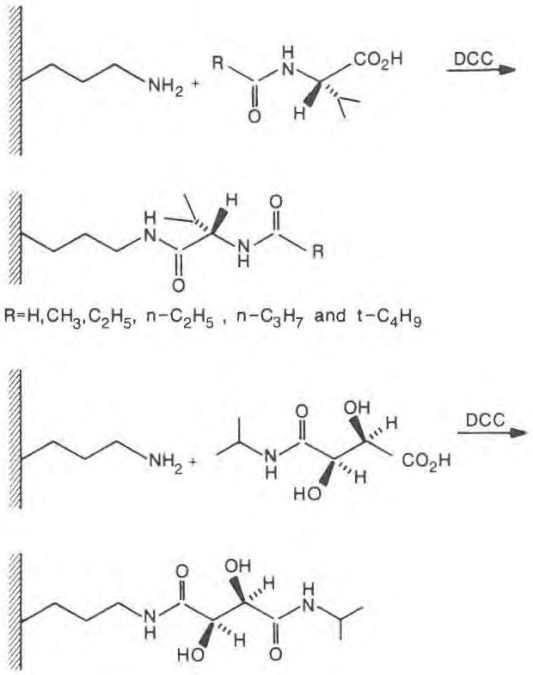
As expected, the *N*-acyl-L-valine CSPs were able to discriminate between the enantiomers of *N*-acylamino-acid esters in non-aqueous mobile phase systems (usually hexane/2-propanol mixtures) [169]. It was found that (*N*-formyl-L-valylamino)propyl-silica gave optimal results in tests on a series of *N*-acetylamino-acid methyl esters. Further, a change to a *tert*-butyl ester function caused increased α values. The D-enantiomers always eluted before the L-forms. This means that a solute–sorbent association, as depicted in Fig. 7.16 for the most strongly retained enantiomer, can be formulated. Interestingly, the same stereochemistry was found when *N*-acetyl-L-valine-*tert*-butylamide was added to the mobile phase as a selector. An implication from this association stereochemistry is that the complex corresponding to the most strongly retained enantiomer will have both amino-acid α-substituents located on the same side and directed away from the surface of the silica matrix.

The mobile phase effects observed were completely consistent with the hydrogen bond association model. Capacity factors increased in a predictable way with decreasing 2-propanol content in hexane, meaning that there is then less competition by the solvent for hydrogen-bonding sites of the CSP. The same effect could be achieved by substituting 2-propanol for less competitive, aprotic, solvents such as chloroform, dichloromethane and diethyl ether.

The number of possibilities of hydrogen-bond formation is increased considerably in the selector based on tartaric acid. There is also a greater conformational freedom which would allow an increased adaptability for association with a wide range of compounds and therefore, in principle, also for broader enantioselectivity. This immobilized selector is quite analogous to (R,R)-DIPTA and was developed on the basis of the successful use of the latter as a mobile phase additive, as will be described in Section 7.3.

7.2.5 Other types of selectors

Though the bonded selectors described in Sections 7.2.1–7.2.4 are reasonably well understood in terms of their chiral recognition behaviour, a number of other selectors have been developed [170–179] which probably act by more complicated

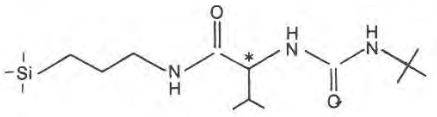


Scheme 7.11 — Routes to immobilized, hydrogen-bonding chiral selectors.

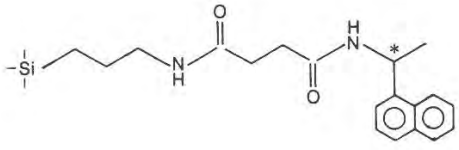
and less well understood mechanisms.

Very promising CSPs based on amide [175–177] or urea [178, 179] derivatives have recently been prepared and studied by Oi and colleagues. The latter phases all contain an asymmetric carbon atom directly attached to a urea nitrogen atom (**12**,**14**), (Scheme 7.12). Thus, **12** contains L-valine as the chiral component [coupled to 3-aminopropyl-silica as the *N*-(*tert*-butylaminocarbonyl) derivative], **13** the chiral 1-(α-naphthyl)ethylamine substituent, and **14** contains both these chiral elements.

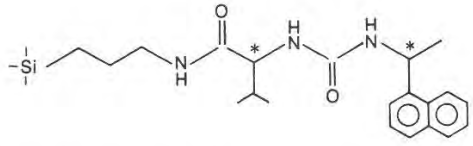
It seems most likely that phase **12** which is structurally similar to the diamide phases used in GC and by Hara in LC, operates by a combination of hydrogen bonding and steric effects. Results from the use of phase **13**, however, indicated that additional charge-transfer interactions could contribute, because resolution of compounds with π-accepting substituents was facilitated. Interestingly, Oi found that phase **14**, which embodies two chiral centres, was superior to the first two, owing to its broader applicability. It gave particularly good resolution of *N*-3,5-dinitrobenzoyl derivatives of amines, 3,5-dinitroanilide derivatives of carboxylic acids and 3,5-dinitrophenylurethane derivatives of alcohols. Some esters and alcohols were also well resolved without prior derivatization.



12



13



14

Scheme 7.12 — Amide and urea-linked chiral selectors developed by Oi.

7.3 TECHNIQUES BASED ON ADDITION OF CHIRAL CONSTITUENTS TO THE MOBILE PHASE

Modern reversed-phase HPLC sorbents (C_2–C_{18} silicas) are excellent materials for achievement of high column efficiency. The principle of using mobile phase additives in reversed-phase LC in order to regulate the retention behaviour of an analyte is widely used today. Basically, the technique makes use of the well-known phenomenon of ion-pair formation in organic media, where the partition of a positively charged analyte such as a protonated amine will be greatly influenced by the nature of the counter-ion (cf. Section 5.2). By the use of an optically active counter-ion, diastereomeric ion-pairs will be formed, which may be well separated on an achiral ordinary reversed-phase column. As discussed previously, the technique of using amphipilic additives can be regarded as a dynamic coating of the achiral sorbent, i.e. a physical immobilization of the amphiphile. Thus, if this additive is optically active, the achiral sorbent will be converted into a chiral one.

Consequently, there is no fundamental difference between the techniques given below and those described above. The ionically bound Pirkle-phases (Section 7.2.3) are excellent CSPs as long as they are used in non-polar solvents where the mobile phase has very little tendency to displace the selector from its adsorption sites. Under these conditions, therefore, the mobile phase can be used without any added selector. In cases where the selector is immobilized by strong hydrophobic interaction to an alkyl-silica or other hydrophobic matrix, the situation may be similar but normally a constant coverage of the matrix with the selector will require its presence in the mobile phase.

Many of the principles already described, which are based on covalently bound chiral phases, can also be applied to the technique of adding the chiral selector to the

mobile phase. They can be divided into three categories, viz. the metal complexation used in CLEC, the use of various uncharged additives, and finally the ion-pairing techniques used for charged analytes.

7.3.1 Metal complexation

Karger was the first to use metal complexation for CLEC applications [180, 181]. By the use of chiral triamines (L-2-ethyl- and L-2-isopropyl-4-octyldiethylenetriamine — each of which has a hydrophobic C_8-substituent) as mobile phase additives in the presence of Zn(II) and other transition metal ions, a series of dansyl amino-acids was well resolved on a C_8–reversed-phase column. A similar system utilized L-prolyl-*N*-octylamide and Ni(II) and was successfully applied to the same type of resolutions with a C_{18}-column [182].

The technique was studied in detail by Davankov *et al.* [130] with the intention of arriving at a useful method for analytical as well as preparative resolution of free amino-acids. *N*-Alkyl-L-hydroxyprolines (C_7, C_{10} and C_{16} chain lengths) were used to coat a C_{18}-silica column and Cu(II) acetate (0.1m*M*) in methanol/water (15/85 v/v) was used as the mobile phase. All additives were adsorbed by means of strong hydrophobic interaction with the C_{18} sorbent, giving essentially no column bleed. Therefore, it was possible in this case to use the mobile phase without the chiral additive, provided the water content was high enough. The postulated structures of the mixed-ligand sorption complexes formed are shown in Fig. 7.17.

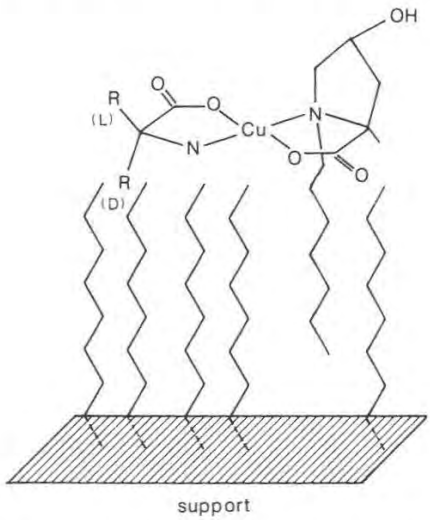


Fig. 7.17 — Structures proposed for the mixed-ligand sorption complexes.

In general, decreasing alkyl chain length was found to give larger separation factors. The reason for this is obscure as the amounts of chiral additive adsorbed could not be determined. The effects caused by a variation of the mobile phase

composition are essentially as follows: (a) an increase in pH (above 5.5) causes increased retention and larger separation factors, (b) a decrease in Cu(II) concentration causes a small increase in retention but no significant effects on separation factors, (c) increasing the ammonium acetate concentration strongly decreases the retention but the effects on the separation factors are quite complex.

As pointed out previously (cf. Section 7.2.2), the relative stabilities of the mixed-ligand sorption complexes formed in CLEC are highly dependent on the method used for immobilization. In the present case, where the selector is physically immobilized by hydrophobic interaction, the elution order of all amino-acids is $k'(\text{L}) < k'(\text{D})$. This experimental result, combined with the mobile phase effects, points to the enantioselective mechanism outlined in Fig. 7.17. The *N*-alkyl chains of the selector are assumed to be oriented parallel to the C_{18}-chains. By co-ordination with a Cu(II) ion, this fixed ligand will adopt a conformation such that the hydroxypyrrolidine ring and its *N*-alkyl group extend in a direction opposite from the main co-ordination plane of the Cu(II) chelate. Therefore, in a mixed-ligand sorption complex formed by the D-enantiomer of the analyte, the α-substituent of this enantiomer will be directed towards the hydrophobic (C_{18}) sorbent surface. This will cause an increased stabilization by means of hydrophobic interaction. An L-enantiomer, on the other hand, will lack this possibility and will therefore be eluted faster than the D-enantiomer.

The same principle was used in a technique based on coating alkyl-silicas with *N*-decyl-L-histidine [183]. The separation factors were generally lower than those found when using *N*-alkyl-L-hydroxyproline selectors, but were still useful for determination of enantiomer composition. As in the previous case, the highest enantioselectivity was found for the amino-acids with the largest α-substituents (alkyl and aryl groups).

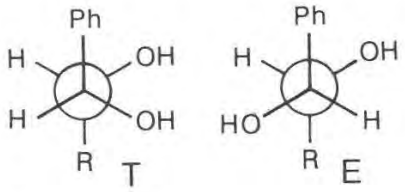
If the alkyl chain of the chiral selector is omitted, conditions are obtained under which there should no longer be any strong hydrophobic interactions with the alkyl-silica and no actual physical immobilization of the selector. Consequently, the chromatographic process might then be best thought of as an *in situ* generation and separation of diastereomeric complexes in a reversed-phase mode. A variety of methods based on this principle of chiral metal complexation in the mobile phase have been developed during recent years and these are summarized in Table 7.11.

7.3.2 Uncharged chiral mobile phase additives

As described in Section 7.2.4, many diamide-type CSPs give rise to enantioselective hydrogen-bond interactions and these bonded selectors should therefore also be useful as additives in a non-polar mobile phase in normal-phase chromatography. Under these conditions they are adsorbed rather strongly on a silica surface, which can then be regarded as coated with a CSP. In particular, *N*-acetyl-L-valine *tert*-butylamide (**15**) and (R,R)-DIPTA (**16**; Section 7.2.4), have been found useful for the optical resolution of a variety of polar solutes [208–211]. From the point of view of chiral recognition, the behaviour shown by the *threo*- and *erythro*- forms of solutes containing a 1,2-diol structure (exemplified by **17**) is quite relevant. Whereas the *threo*-compounds (T) yield larger separation factors with increasing bulkiness of the substituent R, the reverse is found for the *erythro*-forms (E). This is quite understandable in view of the preferred conformations (Scheme 7.13) of the two forms,

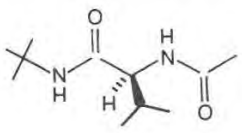
Table 7.11 — Chiral metal complexes used as mobile phase additives for optical resolution by CLEC

Selector	Metal ion	Stationary phase	Analyte	References
L-proline	Cu^{2+}	octyl-silica	amino-acids	[184–186]
L-proline	Cu^{2+}	octyl-silica	dansylamino-acids	[187]
L-proline	Cu^{2+}	silica	thyroid hormones	[188]
L-proline	Cu^{2+}	cation exchanger	amino-acids	[189]
L-histidine	Cu^{2+}	octyl-silica	amino-acids	[186, 190]
L-histidine methyl ester	Cu^{2+}	octadecyl-silica	amino-acids	[191, 192]
L-arginine	Cu^{2+}	octyl-silica	amino-acids	[186]
L-phenylalanine	Cu^{2+}	octadecyl-silica	aromatic amino-acids	[188]
L-phenylalanine	Cu^{2+}	octadecyl-silica	mandelic acids	[193]
L-aspartic acid monoalkylamides	Cu^{2+}	octadecyl-silica	amino-acids	[194–196]
L-aspartyl-L-phenylalanine methyl ester (Aspartame)	Cu^{2+}, Zn^{2+}	octadecyl-silica	amino-acids	[197, 198]
N,N-dipropyl-L-alanine	Cu^{2+}	octadecyl-silica	dansylamino-acids methylamino-acids	[199–202]
N,N-dialkyl-L-amino-acids	Cu^{2+}	octadecyl-silica	amino-acids	[203]
N-(*p*-tosyl)-L- (and D-) phenylalanine	Cu^{2+}	octadecyl-silica	amino-acids	[204, 205]
L-amino-acids	Cu^{2+}	octadecyl-silica	hydroxy-acids	[206]
(R,R)-tartaric acid monooctylamide	Cu^{2+}, Ni^{2+}	octadecyl-silica	amino-acids	[207]

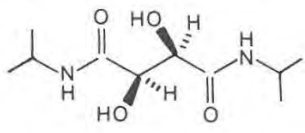


Scheme 7.13 — The role of conformational stability for the substituent effect in *erythro*- and *threo*-isomers on α.

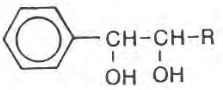
which become more populated as the steric requirements of R increase. Because a two-point hydrogen-bond formation between the CSP and the solute will require a *gauche* conformation of the two hydroxyl groups in **17**, it is clear that the experimental results strongly support such a mechanism of formation.



15



16



17

Another type of uncharged, chiral selector which has been used as a mobile phase additive is cyclodextrin (CD), mainly in the β-form. This is generally used in a reversed-phase system employing C_{18}-silica and an aqueous buffer system [212–214]. The first studies were performed on substituted mandelic acids, and it was shown that substituent effects were very large, and retention decreased with increasing pH or increasing β-CD concentration. Complete optical resolution ($\alpha = 1.8$) was achieved for *o*-chloromandelic acid at pH 2.1 and with 14.4m*M* β-CD in the buffer, otherwise separation factors were low and decreased with increasing pH.

Further insight into the mechanism of enantioseparation by this technique was obtained recently from a study of some barbiturates and related compounds [215]. It was assumed that the analyte (G) was present in an uncharged form ($pH < pK_a$), that it formed a 1:1 inclusion complex with the cyclodextrin molecule, and that the properties of the sorbent were unaffected by the added β-CD. With these assumptions the following equilibria can be written:

$$G_m + \beta\text{-CD} \overset{K_G}{\rightleftharpoons} (G\beta\text{-CD})_m$$

(mobile phase equilibrium)

$$G_s \overset{k'_G}{\rightleftharpoons} G_m$$

$$(G\beta\text{-CD})_s \overset{k'_{(G\beta\text{-CD})}}{\rightleftharpoons} (G\beta\text{-CD})_m$$

(mobile phase/ stationary phase equilibria)

A system of this kind will yield an observed capacity ratio k', which can be expressed by Eq. (7.3). Rearrangement gives Eq. (7.4).

$$k' = \frac{k'_G + k'_{(G\beta\text{-CD})} K_G[\beta\text{-CD}]}{1 + K_G[\beta\text{-CD}]} \tag{7.3}$$

$$k' = \frac{k'_G - k'}{K_G[\beta\text{-CD}]} + k'_{(G\beta\text{-CD})} \tag{7.4}$$

Plots of k' as a function of $(k'_G - k')/[\beta\text{-CD}]$ were linear, as expected from Eq. (7.4), and thus in accordance with 1:1 stoichiometry of the complex. The value of k'_G is, of course, easily obtained experimentally as k' in the absence of β-CD. From the slopes and intercepts of such plots values of K_G and of $k'_{(G\beta\text{-CD})}$ are readily determined for both enantiomers. Interestingly, this led in the case cited [215] to the result that the barbiturates were optically resolved only by virtue of different stability constants (K_G) of the diastereomeric β-CD inclusion complexes, as the calculated $k'_{(G\beta\text{-CD})}$ values were found to be close to zero (in fact they were slightly negative). This means that the enantiomer corresponding to the least stable complex will be preferentially retained.

In contrast to this result it was found that a substituted hydantoin (mephenytoin) was optically resolved predominantly by a difference in the retention of the diastereomeric β-CD inclusion complexes formed, as the calculated $k'_{(G\beta\text{-CD})}$ values differed by a factor of almost 3. However, the slightly different K_G values obtained resulted in a decreased α value. This difference in behaviour was interpreted as due to incomplete immersion of the hydantoin in the β-CD cavity, a condition which should be different for the two enantiomers. Retention here is essentially caused by the part of the molecule which protrudes from the cavity.

Even if further experimental data are needed in order to fully verify the suggested mechanisms, the analysis given emphasizes the importance of the stability of the complex. Accordingly, the enantiomer differentiation observed can arise from either or both of the following effects:

(1) the differences in the complex stability constants, $K_{(-)\text{-G}}$ and $K_{(+)\text{-G}}$;
(2) the differences in retention of the complexes, i.e. their adsorption on the sorbent, $k'_{(-)\text{-complex}}$ and $k'_{(+)\text{-complex}}$.

It is also quite important to realize that these effects may work in opposite directions and in extreme cases yield $\alpha = 1$.

7.3.3 Ion-pairing techniques

As a logical extension and elaboration of the ion-pair chromatographic technique [216, 217], a chiral counter-ion, (+)-10-camphorsulphonic acid, was introduced as a mobile phase additive for the optical resolution of some amino-alcohols [218]. The amino-alcohol in its protonated form combines with the camphorsulphonate by electrostatic interaction to form diastereomeric complexes. It is assumed that a second interaction, a hydrogen bond between the keto group and the hydroxy group, respectively, of the two partners, is present and gives rise to the different chromato-

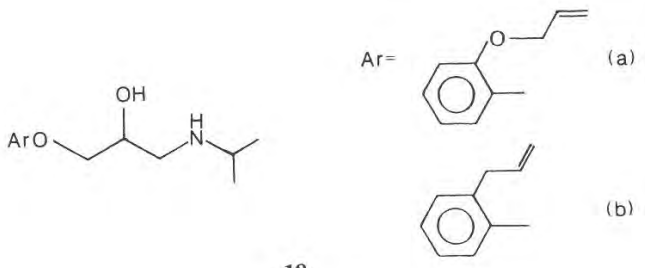
graphic retentions observed (Fig. 7.18). The separations were conducted in a normal-phase mode on silica or diol-silica [219] by using methylene chloride with a small amount of a polar retention modifier.

Methylene chloride is an excellent ion-pair solvent and it is most likely that the enantiomer separation should be regarded as a separation of labile diastereomeric ion-pair complexes, i.e. the chiral discrimination occurs in the mobile phase. As expected, the capacity factors decrease considerably with increasing concentration of the polar component of the mobile phase.

The requirement of a two-point interaction for enantioselection is supported by results illustrating the structural effects of the analyte on the separation factor (Table 7.12). No optical resolution takes place when the hydroxyl and amino groups are separated by more than two carbon atoms or if the hydroxyl group is absent. Further, oxprenolol (**18**a) is not resolved, probably due to an internal hydrogen bond between the hydroxyl group and the oxygen atom of the allyloxy group.

Improved results were obtained with the use of an *N*-protected dipeptide, *N*-carbobenzoxycarbonyl-glycine-L-proline, as the chiral counter-ion. In these cases α values as high as 1.4 were obtained for some of the amino-alcohols [220].

These results encouraged a reciprocal use of the ion-pair system for the enantioseparation of racemic sulphonic and carboxylic acids with optically active amino-alcohols as counter-ions. It was then found that whereas alprenolol (**18**b), with its binding groups located in a flexible alkyl chain, give a low degree of stereoselectivity [221], the situation was considerably improved by the use of compounds with rigid ring systems such as quinine, quinidine and cinchonidine [222].



18

These and related techniques have recently been reviewed by Pettersson and Schill [223].

Although the use of chiral counter-ions for optical resolution by the technique described has proved to be successful for certain applications, the chromatographic system is rather complex and the effects of the many factors involved, on retention and resolution, are not always easy to interpret [222]. Some of these important factors are given below.

(1) The chromatographic sorbent. The surface properties of the strongly polar silica-sorbent are critical and it has been found [222] that diol-silica (which is a

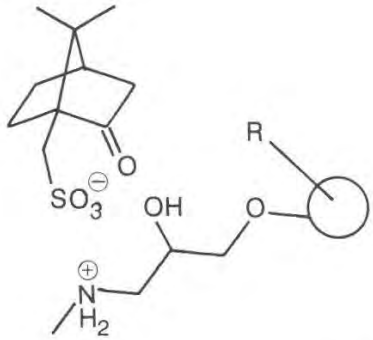
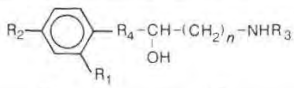


Fig. 7.18 — Proposed interaction between the camphorsulphonate ion and the amino-alcohol to account for the chromatographic enantioselectivity observed.

Table 7.12 — Separation factors obtained in chiral ion-pair chromatography of some amino-alcohols with camphorsulphonate as a counter-ion. (Reprinted, with permission, from C. Pettersson and G. Schill, *Chromatographia*, 1982, **16**, 192. Copyright 1982. Fr. Vieweg & Sohn Verlagsgesellschaft mbH).

Structure: 

n	R_1	R_2	R_3	R_4	α
1	H	$CH_2CH_2OCH_3$	$CH(CH_3)_2$	OCH_2	1.09
2	H	$CH_2CH_2OCH_3$	$CH(CH_3)_2$	OCH_2	1.00
3	H	$CH_2CH_2OCH_3$	$CH(CH_3)_2$	OCH_2	1.00
1	H	$CH_2CH_2OCH_3$	$CH(CH_3)_2$	—	1.09
1	H	$OCH_2CH{=}CH_2$	$CH(CH_3)_2$	OCH_2	1.08
1	$CH_2CH{=}CH_2$	H	$CH(CH_3)_2$	OCH_2	1.08
1	$OCH_2CH{=}CH_2$	H	$CH(CH_3)_2$	OCH_2	1.00
1	H	OCH_3	$CH(CH_3)_2$	OCH_2	1.08
1	H	CH_2CH_3	$CH(CH_3)_2$	OCH_2	1.09
1	H	$OCH_2CH_2OCH_3$	$CH(CH_3)_2$	OCH_2	1.09
1	$CH_2CH{=}CH_2$	H	$CH_2CH_2OC_6H_4CONH_2$	OCH_2	1.11
1	Cl	H	$CH_2OC_6H_4CONH_2$	OCH_2	1.11
1	Br	$CH_2CH_2OCH_3$	$CH_2OC_6H_4CONH_2$	OCH_2	1.11
1	CH_3	H	$CH_2CH_2CH_2C_6H_5$	OCH_2	1.00
1	H	H	$CH_2CH_2C_6H_4CH_3$	OCH_2	1.00

Solid phase Lichrosorb Diol. Mobile phase: (+)-10-camphorsulphonate (2.2mM) in methylene chloride + 1-pentanol (199:1). $\alpha = k'(-)/k'(+)$

surface-modified silica with hydrophilic properties) is generally preferred because of its better performance.

(2) The water content of the mobile phase. This is critical owing to its pro-

nounced influence on retention (and resolution). A water content of 80–90 ppm has been recommended [222]. Higher concentrations of water are quite deleterious. The small amount of water is probably essential in order to deactivate the silica surface, which otherwise might adsorb polar components too strongly.

(3) The capacity ratios of the enantiomers. These usually decrease as the concentration of counter-ion increases. This has been interpreted as due to competition of the counter-ion and the ion-pairs for the same adsorption sites on the sorbent.

(4) The polar components of the mobile phase. These, e.g. 1-pentanol, cause a drastic decrease in retention of the solute, which is usually accompanied by a decrease in optical resolution. The latter effect is thought to be caused by a competition for hydrogen-bonding groups in the ion-pair components, leading to reduced stereoselectivity.

(5) The optical purity of the mobile phase additive. This will, of course, influence the separation factor. It can easily be shown that

$$\alpha_{obs} = \frac{\alpha P + (100 - P)}{\alpha(100 - P) + P} \tag{7.5}$$

where P is the fraction (%) of one enantiomer in the mobile phase additive, and α_{obs} is the observed separation factor [224]. Note that P is not equal to the optical purity, which is 0 for $P = 50$.

A quite different ion-pairing chromatographic technique, utilizing (+)-dibutyl tartrate (DBT) as chiral additive, has recently been described [225]. The principle of the technique originates from studies by Prelog *et al.* [226] of the liquid–liquid distribution of enantiomeric amino-alcohols as ion-pairs in the presence of tartaric acid esters; an unequal distribution of the enantiomers was observed.

In the chromatographic method DBT is used as a physically immobilized CSP, applied by adsorption from an aqueous phase onto a hydrophobic matrix (alkyl-silica). This system has permitted partial optical resolution of a series of amino-alcohols as their ion-pairs with hexafluorophosphate in a reversed-phase system employing a completely aqueous buffer. The mechanism of this enantiodifferentiation is not quite clear.

BIBLIOGRAPHY

R. Audebert, Direct Resolution of Enantiomers in Column Liquid Chromatography, *J. Liquid Chromatog.*, 1979, **2**, 1063.

G. Blaschke, Chromatographische Racemattrennung, *Angew. Chem.*, 1980, **92**, 14 (*Int. Ed.*, 1980, **19**, 13).

V. A. Davankov, Resolution of Racemates by Ligand-Exchange Chromatography, *Adv. Chromatog.*, 1980, **18**, 139.

W. Lindner, Trennung von Enantiomeren mittels moderner Flüssigkeits-Chromatographie, *Chimia*, 1981, **35**, 294.

W. Lindner, Resolution of Optical Isomers by Gas and Liquid Chromatography, in *Chemical Derivatization in Analytical Chemistry*, Vol. 2, R. W. Frei and J. F. Lawrence (eds.), Plenum, New York 1982, p. 145.

V. A. Davankov, A. A. Kurganov and A. S. Bochkov, Resolution of Racemates by High-Performance Liquid Chromatography, *Adv. Chromatog.*, 1983, **22**, 71.

W. H. Pirkle and J. Finn, Separation of Enantiomers by Liquid Chromatographic Methods, in *Asymmetric Synthesis*, Vol. I, J. D. Morrison (ed.), Academic Press, New York, 1983.
S. Allenmark, Recent Advances in Methods of Direct Optical Resolution, *J. Biochem. Biophys. Methods*, 1984, **9**, 1.
D. Armstrong, Chiral Stationary Phases for High Performance Liquid Chromatographic Separation of Enantiomers: A Mini-review, *J. Liquid Chromatog.*, 1984, **7** (Suppl. 2), 353.
R. W. Souter, *Chromatographic Separation of Stereoisomers*, CRC Press, Boca Raton, 1985.
D. W. Armstrong, Optical Isomer Separation by Liquid Chromatography, *Anal. Chem.*, 1987, **59**, 84A.
W. L. Hinze and D. W. Armstrong (eds.), *Ordered Media in Chemical Separations*, ACS, Washington DC, 1987.
W. Lindner, Recent Developments in HPLC Enantioseparation — A Selected Review, *Chromatographia*, 1987, **24**, 97.
Y. Okamoto, Separate Optical Isomers by Chiral HPLC, *Chemtech.*, 1987, 176.

REFERENCES

[1] G. M. Henderson and H. G. Rule, *Nature* 1938, **141**, 917.
[2] V. Prelog and P. Wieland, *Helv. Chim. Acta*, 1944, **27**, 1127.
[3] C. E. Dent, *Biochem. J.*, 1948, **43**, 169.
[4] M. Kotake, T. Sakan, N. Nakamura and S. Senoh, *J. Am. Chem. Soc.*, 1951, **73**, 2973.
[5] C. E. Dalgliesh, *Biochem. J.*, 1952, **52**, 3.
[6] C. E. Dalgliesh, *J. Chem. Soc.*, 1952, 3940.
[7] R. Weichert, *Arkiv Kemi*, 1970, **31**, 517.
[8] S. F. Contractor and J. Wragg, *Nature*, 1965, **208**, 71.
[9] T. Alebic-Kolbah, S. Rendic, Z. Fuks, V. Sunjic and F. Kajfez, *Acta Pharm. Jugoslav.*, 1979, **29**, 53.
[10] S. Allenmark, *Chem. Scr.*, 1982, **20**, 5.
[11] O. A. Battista, *Microcrystal Polymer Science*, McGraw-Hill, New York, 1975.
[12] S. V. Rogizhin and V. A. Davankov, *Russ. Chem. Rev.*, 1968, **37**, 565.
[13] K. Bach and J. Haas, *J. Chromatog.*, 1977, **136**, 186.
[14] S. Yuasa and A. Shimada, *Sci. Rep. Coll. Gen. Educ. Osaka Univ.*, 1982, **13**, Nos. 1–2, 13.
[15] G. Gübitz, W. Jellenz and D. Schönleber, *J. High Resol. Chromatog., Chromatog. Commun.*, 1980, **3**, 31.
[16] S. Yuasa, A. Shimada, K. Kameyama, M. Yasui and K. Adzuma, *J. Chromatog. Sci.*, 1980, **18**, 311.
[17] S. Yuasa, M. Itoh and S. Shimada, *J. Chromatog. Sci.*, 1984, **22**, 288.
[18] A. Chimiak and J. Polonski, *J. Chromatog.*, 1975, **115**, 635.
[19] B. Frank and G. Schlingloff, *Liebigs Ann. Chem.*, 1962, **659**, 123.
[20] B. Frank and G. Blaschke, *Liebigs Ann. Chem.*, 1966, **695**, 144.
[21] W. Mayer and F. Merger, *Liebigs Ann. Chem.*, 1961, **644**, 651.
[22] B. Philipp, J. Kunze and H.-P. Fink, in *The Structures of Cellulose*, R. H. Attala (ed.), ACS, Washington DC, 1987, p. 178.
[23] W. Steckelberg, M. Bloch and H. Musso, *Chem. Ber.*, 1968, **101**, 1519.
[24] R. K. Haynes, H. Hess and H. Musso, *Chem. Ber.*, 1974, **107**, 3733.
[25] H. Hess, G. Burger and H. Musso, *Angew. Chem.*, 1978, **90**, 645.
[26] H. Krebs, J. A. Wagner and J. Diewald, *Chem. Ber.*, 1956, **89**, 1875.
[27] H. Krebs and W. Schumacher, *Chem. Ber.*, 1966, **99**, 1341.
[28] F. Schardinger, *Zentralbl. Bakteriol. Parasitenk., Abt. II*, 1908, **22**, 98 (*Chem. Zentralbl.*, 1909, 68).
[29] M. L. Bender and M. Komiyama, *Cyclodextrin Chemistry*, Springer-Verlag, Berlin, 1978.
[30] W. L. Hinze, *Sep. Purif. Methods*, 1981, **10**, 159.
[31] D. W. Armstrong, *J. Liquid Chromatog.*, 1980, **3**, 895.
[32] W. L. Hinze and D. W. Armstrong, *Anal. Lett.*, 1980, **13**, 1093.
[33] N. Wiedenhof, *Stärke*, 1969, **21**, 119, 164.
[34] J. L. Hoffman, *Anal. Biochem.*, 1970, **33**, 209.
[35] A. Harada, M. Furue and S. Nozakura, *J. Polym. Sci.*, 1978, **16**, 187.
[36] B. Zsadon, L. Decsei, M. Szilasi and F. Tudös, *J. Chromatog.*, 1983, **270**, 127.
[37] K. Fujimura, T. Ueda and T. Ando, *Anal. Chem.*, 1983, **55**, 446.
[38] Y. Kawaguchi, M. Tanaka, M. Nakae, K. Funazo and T. Shono, *Anal. Chem.*, 1983, **55**, 1852.
[39] D. W. Armstrong and W. Demond, *J. Chromatog. Sci.*, 1984, **22**, 441.
[40] W. L. Hinze, T. E. Riehl, D. W. Armstrong, W. Demond, A. Alak and T. Ward, *Anal. Chem.*, 1985, **57**, 237.

[41] A. Lüttringhaus and K. C. Peters, *Angew. Chem.*, 1966, **78**, 603.
[42] A. Lüttringhaus, U. Hess and H. J. Rosenbaum, *Z. Naturforsch.*, 1967, **22b**, 1296.
[43] G. Hesse and R. Hagel, *Chromatographia*, 1973, **6**, 277.
[44] G. Hesse and R. Hagel, *Chromatographia*, 1976, **9**, 62.
[45] G. Hesse and R. Hagel, *Liebigs Ann. Chem.*, 1976, 996.
[46] H. Koller, K. H. Rimböck and A. Mannschreck, *J. Chromatog.*, 1983, **282**, 89.
[47] M. Mintas and A. Mannschreck, *Chem. Commun.*, 1979, 602.
[48] E. Francotte, R. M. Wolf, D. Lohmann and R. Mueller, *J. Chromatog.*, 1985, **347**, 25.
[49] T. Shibata, H. Nakamura, Y. Yuki and I. Okamoto, *Chem. Abstr.*, 1985, **102**, 97219p.
[50] Y. Yuki, I. Okamoto, T. Shibata and H. Nakamura, *Chem. Abstr.*, 1985, **102**, 26690z.
[51] T. Shibata, I. Okamoto and K. Ishii, *J. Liquid Chromatog.*, 1986, **9**, 313.
[52] H. Nakamura, H. Namikoshi, T. Shibata, I. Okamoto and K. Shimizu, 1985, *50th National Meeting Chemical Society of Japan*, Tokyo, April 1985, Abstr. 4Z08.
[53] A. Ichida, T. Shibata, I. Okamoto, Y. Yuki, H. Namikoshi and Y. Toga, *Chromatographia*, 1984, **19**, 280.
[54] I. Okamoto, M. Kawashima and K. Hatada, *Polymer Preprints*, Japan, 1984, **33**, 596, 597.
[55] R. Isaksson, J. Roschester, J. Sandström and L.-G. Wistrand, *J. Am. Chem. Soc.*, 1985, **107**, 4074.
[56] G. Blaschke, *Chem. Ber.*, 1974, **107**, 237, 2792.
[57] G. Blaschke and F. Donow, *Chem. Ber.*, 1975, **108**, 1188, 2792.
[58] G. Blaschke and A.-D. Schwanghart, *Chem. Ber.*, 1976, **109**, 1967.
[59] A.-D. Schwanghart, W. Backmann and G. Blaschke, *Chem. Ber.*, 1977, **110**, 778.
[60] G. Blaschke and H.-P. Kraft, *Makromol. Chem., Rapid Commun.*, 1980, **1**, 85.
[61] G. Blaschke and H. Markgraf, *Chem. Ber.*, 1980, **113**, 2031.
[62] G. Blaschke, H.-P. Kraft and H. Markgraf, *Chem. Ber.*, 1980, **113**, 2318.
[63] G. Blaschke, *J. Liquid Chromatog.*, 1986, **9**, 341.
[64] Y. Okamoto, K. Suzuki, K. Ohta, K. Hatada and H. Yuki, *J. Am. Chem. Soc.*, 1979, **101**, 4763.
[65] Y. Okamoto, K. Suzuki and H. Yuki, *J. Polym. Sci. Polym. Chem. Ed.*, 1980, **18**, 3043.
[66] Y. Okamoto, H. Shohi and H. Yuki, *J. Polym. Sci. Polym. Chem. Ed.*, 1983, **21**, 601.
[67] Y. Okamoto, I. Okamoto and H. Yuki, *Chem. Lett.*, 1981, 835.
[68] Y. Okamoto, S. Honda, I. Okamoto, H. Yuki, S. Murata, R. Noyori and H. Takaya, *J. Am. Chem. Soc.*, 1981, **103**, 6971.
[69] H. Yuki, Y. Okamoto and I. Okamoto, *J. Am. Chem. Soc.*, 1980, **102**, 6356.
[70] Y. Okamoto, I. Okamoto and H. Yuki, *J. Polym. Sci. Polym. Chem. Ed.*, 1981, **19**, 451.
[71] Y. Okamoto and K. Hatada, *J. Liquid Chromatog.*, 1986, **9**, 369.
[72] Y. Okamoto, M. Ishikura, K. Hatada and H. Yuki, *Polym. J.*, 1983, **15**, 851.
[73] Y. Okamoto, S. Honda, E. Yashima and H. Yuki, *Chem. Lett.*, 1981, 1221.
[74] Y. Okamoto, E. Yashima and K. Hatada, *Chem. Commun.*, 1984, 1051.
[75] L. Pauling, *Chem. Eng. News.*, 1949, **27**, 313.
[76] G. Wulff, W. Wesper, R. Grobe-Einsler and A. Sarhan, *Makromol. Chem.*, 1977, **178**, 2799.
[77] G. Wulff, R. Grobe-Einsler, W. Wesper and A. Sarhan, *Makromol. Chem.*, 1977, **178**, 2817.
[78] G. Wulff and W. Wesper, *J. Chromatog.*, 1978, **167**, 171.
[79] G. Wulff, R. Kemmerer, J. Vietmeir and H.-G. Poll, *Nouv. J. Chim.*, 1982, **6**, 681.
[80] G. Wulff, H.-G. Poll and M. Minarik, *J. Liquid Chromatog.*, 1986, **9**, 385.
[81] G. Wulff, W. Dederichs, R. Grotstollen and C. Jupe in *Affinity Chromatography and Related Techniques*, T. C. J. Gribnau, J. Vesser and R. J. F. Nivard (eds.), Elsevier, Amsterdam, 1982, p. 207.
[82] N. A. Brown, E. Jähnchen, W. E. Müller and U. Wollert, *Mol. Pharmacol.*, 1977, **13**, 70.
[83] W. E. Müller and U. Wollert, *Mol. Pharmacol.*, 1975, **11**, 52.
[84] K. K. Stewart and R. F. Doherty, *Proc. Natl. Acad. Sci. U.S.A.*, 1973, **70**, 2850.
[85] C. Lagercrantz, T. Larsson and H. Karlsson, *Anal. Biochem.*, 1979, **99**, 352.
[86] C. Lagercrantz, T. Larsson and I. Denfors, *Comp. Biochem. Physiol.*, 1981, **69C**, 375.
[87] S. Allenmark, B. Bomgren and H. Borén, *J. Chromatog.*, 1982, **237**, 473.
[88] S. Allenmark and B. Bomgren, *J. Chromatog.*, 1982, **252**, 297.
[89] S. Allenmark, B. Bomgren and H. Borén, *J. Chromatog.*, 1983, **264**, 63.
[90] S. Allenmark, B. Bomgren and H. Borén, *J. Chromatog.*, 1984, **316**, 617.
[91] S. Allenmark, *LC, Liquid Chromatog., HPLC Mag.*, 1985, **3**, 348.
[92] S. Allenmark, *J. Liquid Chromatog.*, 1986, **9**, 425.
[93] K. Schmid in *The Plasma Proteins*, F. W. Putnam (ed.), Academic Press, New York, 1975, p. 184.
[94] J. Hermansson, Eur. Pat. Appl. No. 84850169.8, Publ. No. EP0128886A2.
[95] E. Pike, B. Skuterud, D. Kierulf, S. M. Abdel Sayed and P. K. M. Lunde, *Clin. Pharmacokinet.*, 1981, **6**, 367.

[96] J. Hermansson, *J. Chromatog.*, 1984, **298**, 67.
[97] G. Schill, I. W. Wainer and S. A. Barkan, *J. Liquid Chromatog.*, 1986, **9**, 641.
[98] G. Schill, R. Modin, K.-O. Borg and B.-A. Persson, in *Handbook of Derivatives for Chromatography*, K. Blau and G. S. King (eds.), Heyden, London 1978, p. 550.
[99] A. Tilly-Melin, Y. Askemark, K. G. Wahlund and G. Schill, *Anal. Chem.*, 1979, **51**, 976.
[100] G. Schill, I. W. Wainer and S. A. Barkan, *J. Chromatog.*, 1986, **365**, 73.
[101] J. Hermansson and M. Eriksson, *J. Liquid Chromatog.*, 1986, **9**, 621.
[102] F. Vögtle and E. Weber (eds.), *Top. Curr. Chem.*, 1984, **121**, 1 and earlier volumes in the series.
[103] G. W. Gokel and H. D. Durst, *Aldrichim. Acta*, 1976, **9**, 3.
[104] W. D. Curtis, D. A. Laidler, J. F. Stoddart and G. H. Jones, *Chem. Commun.*, 1975, 833, 835.
[105] E. P. Kyba, G. W. Gokel, F. de Jong, K. Koga, L. R. Sousa, M. G. Siegel, L. Kaplan, G. D. Y. Sogah and D. J. Cram, *J. Org. Chem.*, 1977, **42**, 4173.
[106] E. P. Kyba, J. M. Timko, L. Kaplan, F. de Jong, G. W. Gokel and D. J. Cram, *J. Am. Chem. Soc.*, 1978, **100**, 4555.
[107] L. R. Sousa, G. D. Y. Sogah, D. H. Hoffman and D. J. Cram, *J. Am. Chem. Soc.*, 1978, **100**, 4569.
[108] S. V. Rogozhin and V. A. Davankov, *Dokl. Akad. Nauk SSSR*, 1970, **192**, 1288.
[109] S. V. Rogozhin and V. A. Davankov, *Chem. Commun.*, 1971, 490.
[110] V. A. Davankov, A. A. Kurganov and A. S. Bochov, *Adv. Chromatog.*, 1983, **22**, 71.
[111] V. A. Davankov and Y. A. Zolotarev, *J. Chromatog.*, 1978, **155**, 285.
[112] B. Lefebvre, R. Audebert and C. Quivoron, *J. Liquid Chromatog.*, 1978, **1**, 761.
[113] D. Müller, J. Jozefonvicz and M. A. Petit, *J. Inorg. Nucl. Chem.*, 1980, **42**, 1083.
[114] J. Boué, R. Audebert and C. Quivoron, *J. Chromatog.*, 1981, **204**, 185.
[115] Y. Zolotarev, N. Myasoedov, V. Penkina, O. Petrenik and V. Davankov, *J. Chromatog.*, 1981, **207**, 63.
[116] A. Foucault, M. Caude and L. Oliveros, *J. Chromatog.*, 1979, **185**, 345.
[117] G. Gübitz, W. Jellenz, G. Löffler and W. Santi, *J. High Resol. Chromatog., Chromatog. Commun.*, 1979, **2**, 145.
[118] H. Engelhardt and S. Kromidas, *Naturwiss.*, 1980, **67**, 353.
[119] W. Lindner, *Naturwiss.*, 1980, **67**, 354.
[120] K. Sugden, C. Hunter and G. Lloyd-Jones, *J. Chromatog.*, 1980, **192**, 228.
[121] G. Gübitz, W. Jellenz and W. Santi, *J. Liquid Chromatog.*, 1981, **4**, 701.
[122] G. Gübitz, W. Jellenz and W. Santi, *J. Chromatog.*, 1981, **203**, 377.
[123] G. Gübitz, F. Juffman and W. Jellenz, *Chromatographia*, 1982, **16**, 203.
[124] P. Roumeliotis, K. K. Unger, A. A. Kurganov and V. A. Davankov, *J. Chromatog.*, 1983, **255**, 51.
[125] P. Roumeliotis, A. A. Kurganov and V. A. Davankov, *J. Chromatog.*, 1983, **266**, 439.
[126] B. Feibush, M. J. Cohen and B. L. Karger, *J. Chromatog.*, 1983, **282**, 3.
[127] A. A. Kurganov, A. B. Telvin and V. A. Davankov, *J. Chromatog.*, 1983, **261**, 233.
[128] H. G. Kicinski and A. Kettrup, *Z. Anal. Chem.*, 1983, **316**, 39.
[129] G. Gübitz, *J. Liquid Chromatog.*, 1986, **9**, 519.
[130] V. A. Davankov, A. S. Bochkov, A. A. Kurganov, P. Roumeliotis and K. K. Unger, *Chromatographia*, 1980, **13**, 677.
[131] L. H. Klemm and D. Reed, *J. Chromatog.*, 1960, **3**, 364.
[132] L. H. Klemm, K. B. Desai and J. R. Spooner, *J. Chromatog.*, 1964, **14**, 300.
[133] M. S. Newman, W. B. Lutz and D. Lednicer, *J. Am. Chem. Soc.*, 1955, **77**, 3420.
[134] M. S. Newman and W. B. Lutz, *J. Am. Chem. Soc.*, 1956, **78**, 2469.
[135] P. Block, Jr. and M. S. Newman, *Org. Synth.*, 1968, **48**, 120.
[136] F. Mikeš, G. Boshart and E. Gil-Av, *Chem. Commun.*, 1976, 99.
[137] F. Mikeš, G. Boshart and E. Gil-Av, *J. Chromatog.*, 1976, **122**, 205.
[138] H. Numan, R. Helder and H. Wynberg, *Rec. Trav. Chim. Pays-Bas*, 1976, **95**, 211.
[139] B. Feringa and H. Wynberg, *Rec. Trav. Chim. Pays-Bas*, 1978, **97**, 249.
[140] Y. H. Kim, A. Tishbee and E. Gil-Av, *Chem. Commun.*, 1981, 75.
[141] H. Nakagawa, S. Ogashiwa, H. Tanaka, K. Yamada and H. Kawazura, *Bull. Chem. Soc. Japan*, 1981, **54**, 1903.
[142] W. J. C. Prinsen and W. H. Laarhoven, *J. Chromatog.*, 1987, **393**, 377.
[143] C. H. Lochmüller and R. Ryall, *J. Chromatog.*, 1978, **150**, 511.
[144] F. Mikeš and G. Boshart, *J. Chromatog.*, 1978, **149**, 455.
[145] F. Mikeš and G. Boshart, *Chem. Commun.*, 1978, 173.
[146] W. H. Pirkle and J. Finn, *J. Org. Chem.*, 1981, **46**, 2935.
[147] W. H. Pirkle, J. Finn, J. Schreiner and B. Hamper, *J. Am. Chem. Soc.*, 1981, **103**, 3964.
[148] W. H. Pirkle, J. M. Finn, B. C. Hamper, J. Schreiner and J. R. Pribish, in *Asymmetric Reactions and Processes in Chemistry*, E. L. Eliel and S. Otsuka (eds.), ACS, Washington DC, 1982, p. 245.

[149] W. H. Pirkle and D. L. Sikkenga, *J. Chromatog.*, 1976, **123**, 400.
[150] W. H. Pirkle and D. W. House, *J. Org. Chem.*, 1979, **44**, 1957.
[151] W. H. Pirkle, D. W. House and J. M. Finn, *J. Chromatog.*, 1980, **192**, 143.
[152] W. H. Pirkle and C. J. Welch, *J. Org. Chem.*, 1984, **49**, 148.
[153] W. H. Pirkle, T. C. Pochapsky, G. S. Mahler and R. E. Field, *J. Chromatog.*, 1985, **348**, 89.
[154] W. H. Pirkle and T. C. Pochapsky, *J. Am. Chem. Soc.*, 1986, **108**, 352.
[155] W. H. Pirkle, T. C. Pochapsky, G. S. Mahler, D. E. Corey, D. S. Reno and D. M. Alessi, *J. Org. Chem.*, 1986, **51**, 4991.
[156] W. H. Pirkle and M. H. Hyun, *J. Chromatog.*, 1985, **322**, 309.
[157] W. H. Pirkle and M. H. Hyun, *J. Org. Chem.*, 1984, **49**, 3043.
[158] W. H. Pirkle, M. H. Hyun and B. Bank, *J. Chromatog.*, 1984, **316**, 585.
[159] W. H. Pirkle and M. H. Hyun, *J. Chromatog.*, 1985, **322**, 287.
[160] W. H. Pirkle and M. H. Huyn, *J. Chromatog.*, 1985, **322**, 295.
[161] W. H. Pirkle, G. S. Mahler, T. C. Pochapsky and M. H. Hyun, *J. Chromatog.*, 1987, **388**, 307.
[162] W. Pirkle, D. M. Alessi, M. H. Huyn and T. C. Pochapsky, *J. Chromatog.*, 1987, **398**, 203.
[163] W. H. Pirkle and T. J. Sowin, *J. Chromatog.*, 1987, **396**, 83.
[164] W. H. Pirkle and R. Däppen, *J. Chromatog.*, 1987, **404**, 107.
[165] S. Hara and A. Dobashi, *J. High Resol. Chromatog., Chromatog. Commun.*, 1979, **2**, 531.
[166] S. Hara and A. Dobashi, *J. Liquid Chromatog.*, 1979, **2**, 883.
[167] S. Hara and A. Dobashi, *J. Chromatog.*, 1979, **186**, 543.
[168] R. Charles, U. Beitler, B. Feibush and E. Gil-Av, *J. Chromatog.*, 1975, **112**, 121.
[169] A. Dobashi, K. Oka and S. Hara, *J. Am. Chem. Soc.*, 1980, **102**, 7122.
[170] Y. H. Kim, A. Tishbee and E. Gil-Av, *J. Am. Chem. Soc.*, 1980, **102**, 5915.
[171] Y. H. Kim, A. Tishbee and E. Gil-Av., *Science,* 1981, **213**, 1379.
[172] A. Tambuti, A. Begos, M. Lienne, M. Caude and R. Rosset, *J. Chromatog.*, 1987, **396**, 65.
[173] J. Schultze and W. A. König, *J. Chromatog.*, 1986, **355**, 165.
[174] N. Oi and H. Kitahara, *J. Chromatog.*, 1983, **265**, 117.
[175] N. Oi, M. Nagase and T. Doi, *J. Chromatog.*, 1983, **257**, 111.
[176] N. Oi, M. Nagase, Y. Inda and T. Doi, *J. Chromatog.*, 1983, **259**, 487.
[177] N. Oi, M. Nagase, Y. Inda and T. Doi, *J. Chromatog.*, 1983, **265**, 111.
[178] N. Oi and H. Kitahara, *J. Chromatog.*, 1984, **285**, 198.
[179] N. Oi and H. Kitahara, *J. Liquid Chromatog.*, 1986, **9**, 511.
[180] J. LePage, W. Lindner, G. Davies, D. Seitz and B. L. Karger, *Anal. Chem.*, 1979, **51**, 433.
[181] W. Lindner, J. LePage, G. Davies, D. Seitz and B. L. Karger, *J. Chromatog.*, 1979, **185**, 323.
[182] Y. Taphui, N. Miller and B. L. Karger, *J. Chromatog.*, 1981, **205**, 325.
[183] V. A. Davankov, A. S. Bochkov and Y. P. Belov, *J. Chromatog.*, 1981, **218**, 547.
[184] E. Gil-Av, A. Tishbee and P. E. Hare, *J. Am. Chem. Soc.*, 1980, **102**, 5115.
[185] S. Lam and F. Chow, *J. Liquid Chromatog.*, 1980, **3**, 1579.
[186] S. Lam, F. Chow and A. Karmen, *J. Chromatog.*, 1980, **199**, 295.
[187] S. Lam, *J. Chromatog.*, 1982, **234**, 485.
[188] E. Oelrich, H. Preusch and E. Wilhelm, *J. High Resol. Chromatog., Chromatog. Commun.*, 1980, **3**, 269.
[189] P. E. Hare and E. Gil-Av, *Science*, 1979, **204**, 1226.
[190] S. Lam and A. Karmen, *J. Chromatog.*, 1982, **239**, 451.
[191] S. Lam and A. Karmen, *J. Chromatog.*, 1984, **289**, 339.
[192] S. Lam, *J. Chromatog. Sci.*, 1984, **22**, 416.
[193] W. Klemisch, A. von Hodenberg and K. Vollmer, *J. High Resol. Chromatog., Chromatog. Commun.*, 1981, **4**, 535.
[194] C. Gilon, R. Leshem and E. Grushka, *J. Chromatog.*, 1981, **203**, 365.
[195] C. Gilon, R. Leshem and E. Grushka, *Anal. Chem.*, 1980, **52**, 1206.
[196] E. Grushka, R. Leshem and C. Gilon, *J. Chromatog.*, 1983, **255**, 41.
[197] C. Gilon, R. Leshem, Y. Taphui and E. Grushka, *J. Am. Chem. Soc.*, 1979, **101**, 7612.
[198] G. Gundlach, E. Sattler and U. Wagenbach, *Z. Anal. Chem.*, 1982, **311**, 684.
[199] S. Weinstein, M. Engel and P. E. Hare, *Anal. Biochem.*, 1982, **121**, 370.
[200] S. Weinstein and S. Weiner, *J. Chromatog.*, 1984, **303**, 244.
[201] N. Grinberg and S. Weinstein, *J. Chromatog.*, 1984, **303**, 251.
[202] S. Weinstein, *Trends Anal. Chem.*, 1984, **3**, 16.
[203] S. Weinstein, *Angew. Chem.*, Suppl., 1982, 425.
[204] N. Nimura, A. Toyama and T. Kinoshita, *J. Chromatog.*, 1982, **234**, 482.
[205] N. Nimura, A. Toyama, Y. Kasahara and T. Kinoshita, *J. Chromatog.*, 1982, **239**, 671.
[206] R. Horikawa, H. Sakamoto and T. Tanimura, *J. Liquid Chromatog.*, 1986, **9**, 537.
[207] W. F. Lindner and I. Hirschböck, *J. Liquid Chromatog.*, 1986, **9**, 551.
[208] A. Dobashi, Y. Dobashi and S. Hara, *J. Liquid Chromatog.*, 1986, **9**, 243.

[209] A. Dobashi and S. Hara, *J. Chromatog.*, 1983, **267**, 11.
[210] Y. Dobashi, A. Dobashi and S. Hara, *Tetrahedron Lett.*, 1984, **25**, 329.
[211] Y. Dobashi and S. Hara, *J. Am. Chem. Soc.*, 1985, **107**, 3406.
[212] J. Debowski, D. Sybilska and J. Jurczak, *J. Chromatog.*, 1982, **237**, 303.
[213] J. Debowski, D. Sybilska and J. Jurczak, *Chromatographia*, 1982, **16**, 198.
[214] J. Debowski, J. Jurczak and D. Sybilska, *J. Chromatog.*, 1983, **282**, 83.
[215] D. Sybilska, J. Zukowski and J. Bojarski, *J. Liquid Chromatog.*, 1986, **9**, 591.
[216] J. H. Knox and J. Jurand, *J. Chromatog.*, 1981, **218**, 341.
[217] J. H. Knox and J. Jurand, *J. Chromatog.*, 1982, **234**, 222.
[218] C. Pettersson and G. Schill, *J. Chromatog.*, 1981, **204**, 179.
[219] C. Pettersson and G. Schill, *Chromatographia*, 1982, **16**, 192.
[220] C. Pettersson and M. Josefsson, *Chromatographia*, 1986, **21**, 321.
[221] C. Pettersson and K. No, *J. Chromatog.*, 1983, **282**, 671.
[222] C. Pettersson, *J. Chromatog.*, 1984, **316**, 553.
[223] C. Pettersson and G. Schill, *J. Liquid Chromatog.*, 1986, **9**, 269.
[224] C. Pettersson, A. Karlsson and C. Gioeli, *J. Chromatog.*, 1987, **407**, 217.
[225] C. Pettersson and H. W. Stuurman, *J. Chromatog. Sci.*, 1984, **22**, 441.
[226] V. Prelog, Ž. Stojanac and K. Konačević, *Helv. Chim. Acta*, 1982, **65**, 377.