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Review

Separation of enantiomers: needs, challenges, perspectives☆

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Abstract

Chiral drugs, agrochemicals, food additives and fragrances represent classes of compounds with high economic and scientific potential. First the present implications of their chiral nature and necessity of separating enantiomers are summarised in this article. In the following a brief overview of the actual approaches to perform enantioseparations at analytical and preparative scale is given. Challenging aspects of these strategies, such as problems associated with data management, choice of suitable chiral selectors for given enantioseparations and enhanced understanding of the underlying chiral recognition principles, are discussed. Alternatives capable of meeting the requirements of industrial processes, in terms of productivity, cost-effectiveness and environmental issues (e.g., enantioselective membranes) are critically reviewed. The impact of combinatorial methodologies on faster and more effective development and optimisation of novel chiral selectors is outlined. Finally, the merits and limitations of most recent trends in discrimination of enantiomers, including advances in the fields of sensors, microanalysis systems, chiroptical methods and chemical force microscopy are evaluated.

Keywords: Reviews; Enantiomer separation; Chiral resolution; Chiral selectors; Membranes, enantioselective

Contents

☆Dedicated to Professor Y. Okamoto on the occasion of his 60th birthday.

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1. Prolog

Being selected to contribute the first chapter to a special volume dedicated to honour one of the most successful players in the big game of enantioseparations is a pleasure and privilege, but at the same time, a challenging task. The art of enantioseparation has seen a most dramatic development and progress over the last two decades, maturing from a speciality field of a few experts to an area of major scientific and economic interest. The number of scientific contributions dealing with one or the other aspect of enantioseparation are literally exploding, and holding closely track with these developments almost exceeds the possibilities of any expert. In this sense, the intention of this comprehensive chapter is not to review the historical development or the actual state-of-the-art in a detailed fashion, but to give a personally flavoured selective overview of current needs, trends and future perspectives of enantioseparation sciences.

2. Chirality and its consequences

The chiral nature of living systems has evident implications on biologically active compounds interacting with them. On a molecular level, chirality represents an intrinsic property of the ''building blocks of life'', such as amino acids and sugars, and therefore, of peptides, proteins and polysaccharides. As a consequence, metabolic and regulatory processes mediated by biological systems are sensitive to stereochemistry and different responses can be often observed when comparing the activities of a pair of enantiomers. Stereoselectivity is often a characteristic feature of enzymatic reactions, messenger–receptor interactions and metabolic processes; it can vary interspecifically and even from one individual to the other [1–4]. Therefore, stereochemistry has to be considered when studying xenobiotics, such as drugs, agrochemicals, food additives, flavours or fragrances.

The interest in chirality and its consequences is not a new phenomenon. However, during the last decade it has raised increasing expectations due to scientific and economic reasons, being the pharmaceutical industry the main contributor and driving force.

2.1. Pharmacological implications

Drug action is the result of pharmacological and pharmacokinetic processes, by which it enters, interacts and leaves the body. There is a broad range of examples where the stereoisomers of drugs show differences in terms of their bioavailability, distribution, metabolic and excretion behaviour and where stereochemical parameters have a fundamental significance in their action and disposition in biological systems. Thus, the β-blocker propranolol or the cardiotonic agent verapamil have different efficiency when administered as racemates intravenously or orally. The reason for this effect was shown to be the stereoselective first-pass metabolism, which is selective for the more active enantiomer of verapamil and the less active one of propranolol [4]. Moreover, genetic polymorphisms affecting the metabolism of certain drugs can be frequently encountered. Poor metaboliser phenotypes for certain chiral drugs, such as debrisoquine, mephenytoin, mephobarbital and diazepam, have a relative high incidence in certain geographic zones [3]. This exquisite stereoselectivity which can occur in many of the metabolic routes of a given drug or xenobiotic has to be carefully considered when its development is envisaged or its safety is evaluated.

2.2. Scientific implications

Strong emphasis has been concentrated upon the search of therapeutic benefits with the goal of developing safer and more effective drugs. The high degree of stereoselectivity of many biological processes implies that when a given racemic mixture is administered as a drug both enantiomers should not have to be equally potent. In fact, very often one of them represents the more active isomer for a given action (eutomer), while the other one (distomer) might be even active in a different way, contributing to side-effects, displaying toxicity, or acting as antagonist [1,5].

Despite the fact that scientists are fully aware of these differences since more than two decades, the main advances in the development of enantiomerically pure compounds have been accomplished in the last years with the prosperity of new asymmetric synthesis methodologies, and powerful analytical and preparative separation techniques. Until the beginning of the present decade, the commercialisation of enantiomerically pure drugs could just be recommended as a desirable challenge with many practical limitations [6]. In this sense, regulatory authorities could just encourage the pharmaceutical industries to provide single enantiomer of drugs, although most of them were commercialised as racemates [7]. Nowadays the situation has definitely changed as technical advances permit production of many single enantiomers on a commercial scale. Many enantiomerically pure drugs have successfully reached the market. Therefore, health and regulatory authorities, such as the US Food and Drug Administration (FDA), have defined more strict requirements to patent new racemic drugs, demanding a full documentation of the separate pharmacological and pharmacokinetic profiles of the individual enantiomers, as well as their combination [2,7–11]. This can be also extended to the field of agrochemicals and crop protection, where the presence of a non-active or less active stereoisomer just contributes to increase the levels of pollution without any benefit on the desired action.

The development of asymmetric synthesis strategies in combination with the advances in analysis of enantiomeric mixtures facilitated the investigation of stereochemical problems in pheromone chemistry. The limited availability of these compounds from their natural sources was a definite drawback for these studies. Due to the importance of chirality in pheromone perception, establishing absolute configurations of the active forms is an essential precondition. This information contributes to understand the different biological processes in which pheromones are involved and to design analogues which could be potentially used to control the reproduction of insects. In this context, highly sensitive processes have been described for sex pheromones, where Japonilure (the eutomeric female pheromone of the Japanese beetle) with a 1% enantiomeric excess (ee) of the distomer was found to be 2/3 as active as the enantiomerically pure pheromone [12].

Furthermore, the analysis of chiral compounds is finding interesting applications in food and clinical research. The detection of D-amino acids in food is needed to assess the factors that influence their formation, biological function, safety and role, since they are formed during food processing and also originated from microbiological sources, and may become a part of our diet [13]. Their presence can lead to nutritionally poorer and less safe products by generating nonmetabolisable and biologically nonutilisable forms of amino acids. Moreover, analysis of other chiral food components may be a method to confirm their supposed quality. Thus, the detection of filbertone (*E*-5-methyl-hept-2-en-4-one), the principal flavour component of hazelnuts, can be a clear sign of adulteration when found in expensive edible oils, such as extra virgin olive oil [14–17].

In the same sense, clinical applications of chiral markers can be found in the literature. Just as an illustration, the analysis of 2-hydroxy acids in urine would be essential for the diagnosis of the Maple Syrup Urine Disease (MSUD), an inherited metabolic disorder which causes neurological damage and mental retardation by accumulation of branched-chain amino acids. The determination of the enantiomeric ratios of the relevant metabolites is supportive in the definition of models concerning metabolic enzyme activities in MSUD [18].

In the field of archeology, measurements of the degree of racemisation of specific amino acids are used to date the age of human remains such as bones and teeth [19]. Another curious application of enantioseparation concerns the determination of amino acid enantiomeric excesses in meteorites [20].

2.3. Economic consequences for bioactive compound development

Economic interests are obvious and essential driving forces in the spectacular development of new chiral substances and technological improvements related with the subject. Single-enantiomer drug sales show a continuous growth worldwide (21% sales increment from 1997 over 1996) and many of the top-selling drugs are marketed as single enantiomer (269 of the top 500 drugs) [21–23] (Fig. 1). The focus of drug discovery research has been shifting from acute to chronic illnesses such as Alzheimer's, cancer, obesity, AIDS, asthma, arthritis and neurologic diseases and a large number of the drugs used for their treatment contain one or more stereogenic centres. The therapeutic groups of drugs where more advances with regards to enantiomerically pure ones have been described in the last years are chiral

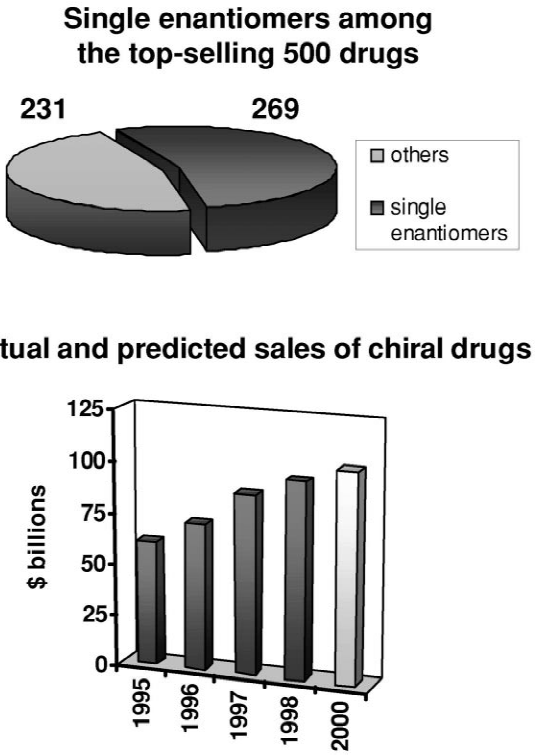

Fig. 1. Proportion of single enantiomeric drugs among the top-selling drugs and chiral drug sales worldwide until 1998 and expected for the year 2000 [21–23].

cardiovascular, central nervous system, antiviral and anticancer drugs [22].

The economic significance of the world drug market is outstanding (about US$300 billion in 1997) [22,24] (Fig. 1). The time frames and costs of developing new products have continued to rise [24]. Competition between pharmaceutical firms is very strong and to drive company growth they invest great efforts in aggressive productivity goals. Along this line chiral drugs are a challenging field for investment and research (the sales of stereochemically pure drugs represented about 28% of the total number of sales in 1997 [22]).

Despite of the fact that the development of chiral drugs implies to screen three products from the early stages up to clinical trials (the racemate and the two single-enantiomers), the figures of benefits successful single enantiomer type products might bring to pharmaceutical firms are often convincing reasons to follow all these exercises.

Furthermore, the debate ''racemate-versus-enantiomer'' has opened a new market strategy, the so-called *racemic switch*. A racemic switch stands for the development in single-enantiomer form of a drug that was first approved as a racemate. This means that a company can get in this way a patent on an individual enantiomer. Although in some of the cases such a switch would not give a genuine therapeutic benefit, the number of examples of new stereochemically pure drugs patented in the last years is increasing. The bronchodilator levalbuterol, the antidepressant (*S*)-citalopram and the gastrointestinal drug (+)-norcisapride are some of the most recent examples (for chemical structures see Fig. 2). The introduction of the new single-enantiomer form often implies an improvement in the effectiveness of the drug (e.g., the D-*threo*-methylphenidate, Ritalin, which is used for treatment of attention deficit disorder in children is 13-times more potent than its L-enantiomer [25]) or the suppression of side-effects related to the other enantiomeric form (e.g., levoalbuterol avoids the irregular heartbeat and the worsening of the asthma observed with the racemic albuterol). Moreover, in some cases the separate study of an enantiomer activity can reveal new biological effects. This is the case for (*S*)-fluoxetine which shows remarkable therapeutic effects in preventing migraines, while the racemic drug (the antidepressant

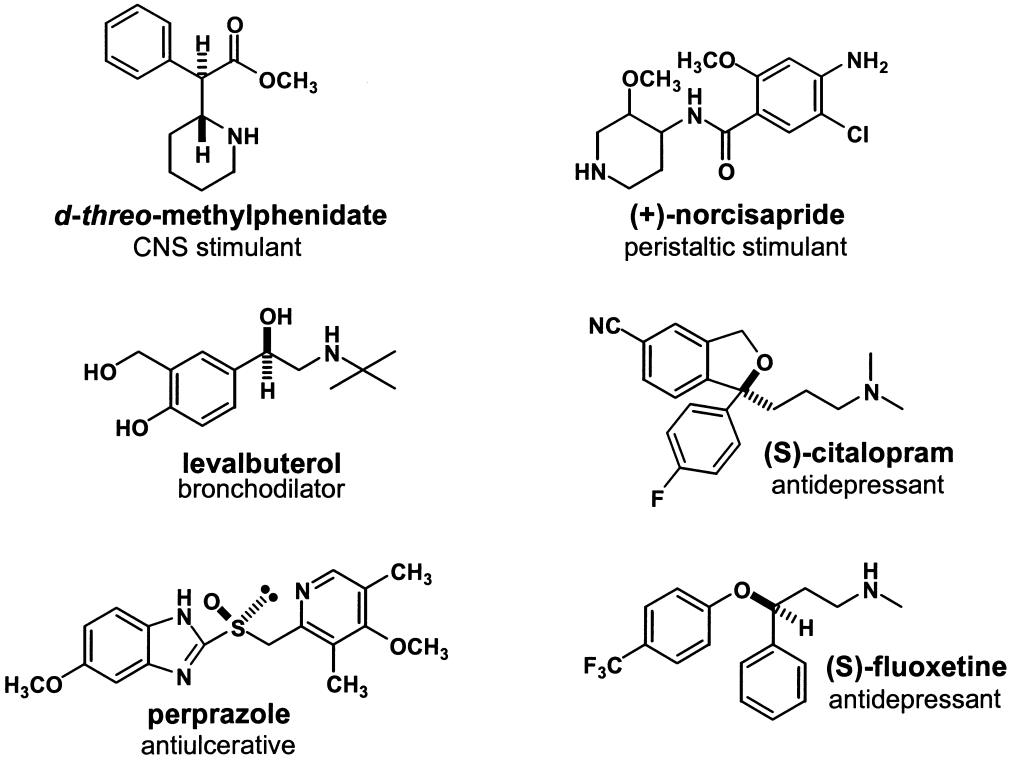

Fig. 2. Chemical structures of several stereochemically pure drugs as single enantiomers patented in the last few years.

Prozac) has no effect [26]. Therefore, ''highly justified'' candidates for a racemic switch would be those drugs, whose administration as racemates would imply a potential risk for patients, either due to toxicity or side-effects of one of the stereoisomers, or whose efficacy would be significantly enhanced by administration as a single enantiomeric form.

However, pharmacological indications seem to be sometimes difficult to rationalise independently from the figures of benefits, specially when top selling drugs are concerned. Thus, AstraZeneca is expected to file a new drug application in 1999 for perprazole, the (*S*)-isomer of omeprazole (Fig. 2), used for the treatment of stomach ulcers, which was the world's biggest selling drug in 1997 with US$5 billion sales [22]. This new patent will give the company several additional years of market exploitation, which will expire for the racemate in 2001. In the herbicide business, the development of a large-scale enantioselective catalytic process allowed the market introduction of (*S*)-metolachlor in 1997 in the USA [27].

The increasing demand of chiral compounds in the pharmaceutical industry has also stimulated the development of new and more specialised companies in asymmetric synthesis providing enantiomerically pure substances, synthetic intermediates and catalysts or even libraries of chiral compounds with application in combinatorial chemistry [28–30]. Simultaneously biotechnologies and biocatalysis are rapidly expanding fields to produce and to purify chiral intermediates [31].

3. Separation of enantiomers: needs

The scientific and economic relevance of chiral substances has favoured the outstanding developments in separation techniques in the last two decades. Although a number of stereoselective syn-

theses have been described and applied to the production of single-enantiomeric substances, relatively few are selected for large-scale preparations, particularly at the early stages of development of new drugs. Time constraints to have some amounts of pure enantiomers for the first pharmacological tests are usually crucial before a manufacture route (protocol) has to be selected. At these early stages, the development of an asymmetric synthesis would be both expensive and time consuming and thus, preparative techniques for the separation of enantiomers have an interesting potential.

The induction of chirality or the resolution of racemic compounds necessitates the presence of a chiral environment. For this purpose, chiral auxiliaries, catalysts or selectors are necessary. The formation of the corresponding diastereomeric species implies an energetic difference between them which allows in many cases their enantiodiscrimination [32]. These diastereomers can also be covalently formed and thus their separation can be achieved taking advantage of their different chemical or physical properties by crystallisation, non-stereoselective chromatography or distillation. Then the pure enantiomeric substance are isolated *indirectly* after release of the so-called chiral auxiliary. Indirect separation methods are frequently used, especially at the large-scale level [33,34], although the nature, enantiomeric purity, availability, costs and ease of cleavage of the chiral handle derived from the chiral derivatising agent, are sometimes limiting issues of this strategy.

Alternatively, *direct* methodologies are usually based on the formation of non-covalent diastereomeric pairs of molecule associates. Forces such as electrostatic, hydrogen bonding, repulsive/attractive van der Waals and $\pi-\pi$ or dipolar interactions and inclusion phenomena, contribute to the recognition process (for more details see further chapters of this issue). These separation methods yield, without any further sophisticated cleavage process, the enantiomerically pure substances and this approach is frequently the most advantageous one. Direct resolutions of enantiomers can be achieved by means of the interaction of the enantiomeric mixture with a so-called chiral selector (SO), either being part of a chiral stationary phase (CSP), or as a chiral mobile phase additive (CMPA). Investigations about the behaviour of chiral selectors and their potential and underlying enantiorecognition abilities are essential issues in the field and still many studies have to be carried out for a better understanding and exploitation of their applicabilities.

Chiral selectors can be obtained from natural sources or can be generated from natural or synthetic building blocks. The main types of selectors used in enantioseparations, along with the separation techniques where they can be applied, their suitability for analytical or preparative scale and some bibliographic information are included in Table 1.

Almost the entire spectrum of separation techniques can be employed as potential tools for the resolution of enantiomers. In the light of the target molecule and the scale of the separation, the requirements to be fulfilled will be different, thus directing the choice of the SOs.

Analytical methods are necessary to control the enantiomeric purity of starting materials and products. Moreover, the requirements of the regulatory authorities made compulsory the availability of enantioselective techniques to assess the stereoisomeric composition of chiral substances. Nevertheless, it is evident that the limits of detection which would be required might be different in the pharmaceutical industry, where the safety of drugs is the major concern, compared to other chiral compound applications. Highly sensitive immunological methods have been described for the determination of enantiomeric purity with antibodies which stereospecifically bind to D- or L-α-amino acids [113]. These antibodies were successfully applied in an enzyme-linked immunosorbent assay (ELISA) format for detection of the presence of various amino acids in a 100 000-fold excess of the respective enantiomers [114].

The control is also interesting in the case of chiral reagents, auxiliaries or catalysts [115], because the quality of these compounds limits the enantiomeric purity of the resulting products. Accuracy in the determination of extreme enantiomeric ratios and detection of impurities are often critical points in any of the techniques available and should be considered. Readers can find further interesting observations and examples about this issue regarding to different

Table 1
Main groups of chiral selectors arranged according to their origin and applicable separation techniques

Source	Type	Chiral selector	Techniques	Scale[a]	Refs.
Natural	Proteins	Serum albumin		A	
		Orosomucoid		A	
		(α_1-acid glycoprotein)	LC, CE, CEC,		
		Ovomucoid	Membranes,	A	
		Cellobiohydrolase I	extraction	A	[35–43]
		Avidin		A	
		Chymotrypsin		A	
		Ovotransferrin		A	
	Oligosaccharides	α-, β- and γ-Cyclodextrins	LC, CE, CEC, GC, TLC, cryst.	A/P	[44–51]
		Disaccharides	CE	A	[52]
		Maltodextrins	CE	A	
	Polysaccharides	Cellulose		A	
		Amylose	CE	A	[53]
		Starch		A	
		Dextran		A	
		Heparin	CE	A	[54]
		Pectins	CE	A	[55]
	Antibiotics	Vancomycin			
		Teicoplanin	LC, CE, GC,	A/P	[48,56–67]
		Ristocetin	SFC		
		Avoparcin			
	Low M_r molecules	Amino acids	LC, CE, SFC,		
		Cholic acids/bile salts	cryst.		[68,69]
		Alkaloids			
		Tartaric acids			
Semisynthetic	Modified oligosaccharides	Derivatised cyclodextrins	LC, CE, CEC, GC, TLC	A/P	[44–51]
		Cyclodextrin polymers	CE	A	[53]
	Modified polysaccharides	Polysaccharide carbamates	LC, CE, SFC, TLC, membranes, extraction	A/P	[70–80]
		Polysaccharide esters	LC, CE, SFC, TLC, membranes, extraction	A/P	[70–81]
	Polysaccharide sulfates	Dextran sulfate	CE		[53]
		λ-Carrageenan	CE		[53]
		Chondroitin derivatives	CE		[53,54]
	Modified low M_r molecules	Ion-exchange selectors	LC, CE, CEC, SFC, extraction	A/P	[82–91]
Synthetic	Synthetic low M_r molecules	Pirkle type selectors	LC, CE, GC, TLC, SFC, membranes	A/P	[92–98]
		Receptor molecules	LC, CE, extraction, cryst.	A	
	LEC selectors	Crown ethers	LC, CE, extraction,	A/P	[48,99–101]
		Proline derivatives	LC, CE, TLC, extraction, membranes	A/P	[102–104]
	Helical synthetic polymers	Polyacrylamides	LC, CE, SFC,	A/P	[105,106]
		Polyacrylates		A/P	[107,108]
		Crosslinked tartaramides	LC	A/P	[109]
		MIPs	LC, CE, SFC, membranes	A	[110–112]

[a] A: Analytical scale; P: preparative scale.

separation methodologies in the following references: liquid chromatography (LC) [116,117], gas chromatography (GC) [118,119] or capillary electrophoresis (CE) [48,120,121].

In any case, at analytical level, run time, sensitivity and selectivity should be enhanced in order to improve the limits of detection and the overall analysis time. Taking into account these criteria, several techniques should be mentioned as being more adapted for analytical purposes: GC, high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), CE, capillary electrochromatography (CEC), thin-layer chromatography (TLC) and sensor devices.

At the *preparative scale* different aims and targets are envisaged [74]. Apart from appropriate enantioselectivity, a high loading capacity is an important prerequirement, together with robustness, chemical inertness and thermal stability of the CSP, but also of the enantiomers to be resolved. Additionally, ideal chiral selectors for preparative applications should be readily available and/or synthesised, in order to be competitive in economic aspects with currently established techniques. Other parameters, such as the solubility of the sample to resolve in the media where the separation might take place should be seriously considered with regards to productivity, in contrast with the analytical resolutions where this would not be usually a problem. Chromatographic methodologies, in any of their different approaches (see Fig. 3) [74–76,122–124], are the most utilised techniques and several chapters of this volume will be devoted to this topic. However, when talking about the preparative scale, one should not forget crystallisation [125,126] and kinetic resolution procedures, which are very commonly described for industrial processes, either as single methods to obtain the desired enantiomerically pure compounds or in combination with some chromatographic techniques, such as simulated moving bed (SMB) [127,128]. Other emerging techniques, such as enantioselective membranes and liquid–liquid extraction, have also a high potential for preparative purposes, although some technical problems have to be overcome to make them more efficient. Some of these points would be discussed in the following section.

It should be pointed out that biotransformations take a prominent position among the industrial scale technologies for enantioseparation and will gain more importance in the near future [31]. Currently, most of the industrially implemented processes capitalise on the exquisitely enantioselective properties of lipases, acylases and hydrolases [23]. Recently, many of the intrinsic problems associated with enzyme catalysis under ''real world'' process conditions have been addressed by the introduction of cross-linked enzyme crystals (CLECs) [129]. The crucial step of these technology is a treatment of enzyme crystals with glutaraldehyde, leading to preparations characterised by highly improved mechanical and thermal stability, enhanced specific reactivities, excellent tolerance towards organic solvents and protease resistance [130]. Apart from enzymes, progress in asymmetric synthesis, asymmetric catalysis and kinetic resolution will enrich the technically useful repertoire of enantiomer preparation techniques in the near future. To date, enantioselective hydrogenation, asymmetric epoxidation, dihydroxylation, aminohydroxylation and ring opening reactions have reached almost maturity in terms of technical requirements [21–23].

When talking about the separation of kilogram to ton amounts of chiral compounds, ecological/environmental considerations have also to be taken into account, in order to reduce toxicity and hazard potential of the process. Thus, the large amounts of solvents used in chromatography are not so convenient and, therefore, methods to recycle would be needed. Furthermore, alternative techniques, with other type of mobile phase (SFC, with CO_2 and small amounts of alcoholic modifiers) [131], using the so-called ''green solvents'' appear to be attractive. When the crystallisation is the technique of use, methods of racemisation of the ''non-desired'' enantiomer, which represents the 50% of the material (or any other ratio), would be advantageous, for ecological, but also for economical reasons.

The main groups of techniques for the separation of enantiomers are schematised in Fig. 3, considering their use at analytical or preparative scale. Special emphasis has been devoted to chromatographic techniques which, together with CE [48,132–134], are major fields of development in the resolution of enantiomers.

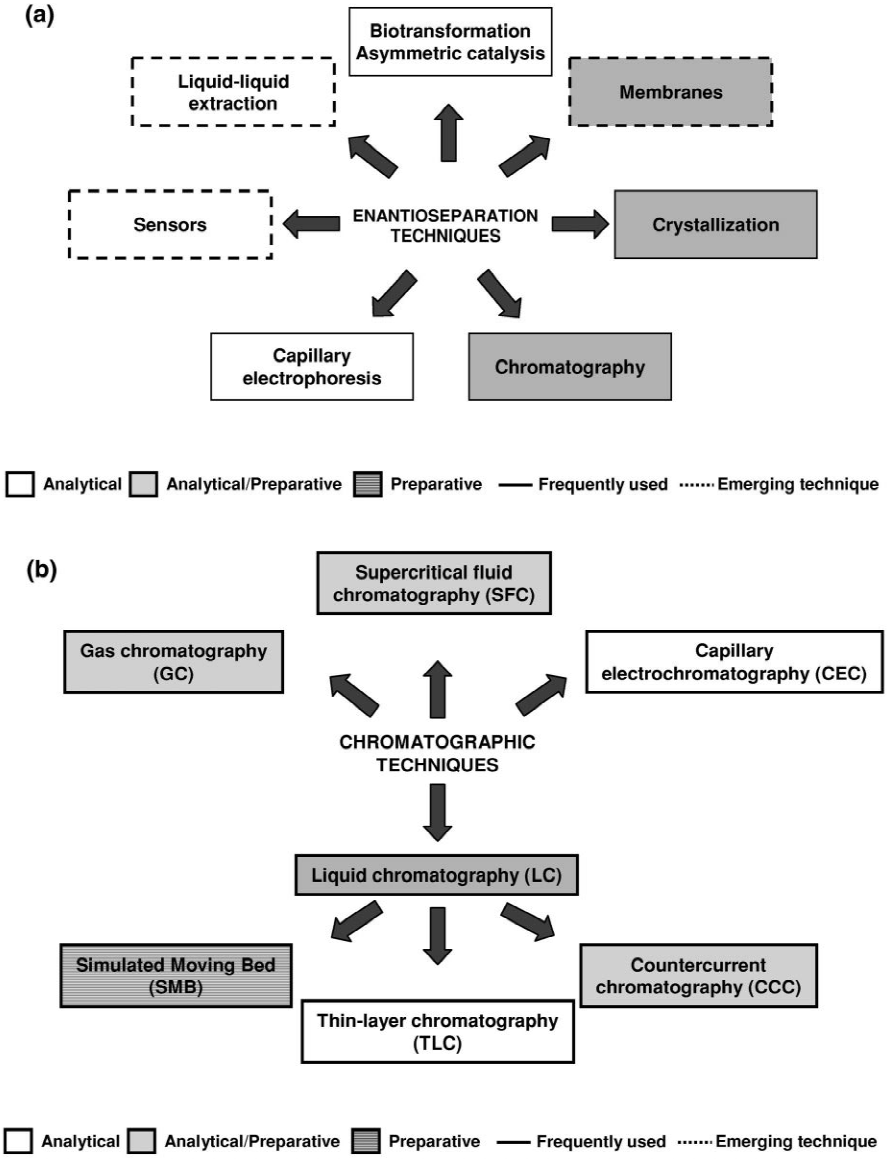

Fig. 3. (a) Techniques used for the separation of enantiomers; (b) main chromatographic techniques.

Recent references of preparative scale separations can be found using the different chromatographic techniques: GC [74]; LC [135] and particularly in HPLC [74,136], SMB [74,127,136–148], TLC [149–151], or countercurrent chromatography–centrifugal partition chromatography (CCC–CPC) [152–159]; SFC [74,131,160–163].

4. Separation of enantiomers: challenges

4.1. Data management and choice of chiral selectors for given enantioseparation problems

The separation of enantiomers is an important analytical operation in many fields of academic,

industrial and pharmaceutical research. Almost any enantioseparation can be achieved with at least one of the about 200 commercially available chiral selectors in combination with well-established GC, LC, SFC and CE separation techniques [164]. Problems, however, arise with regard to the selection of appropriate systems from the constantly growing repertoire of chiral selectors. Unfortunately, only for a limited number of selector–selectand combinations reliable chiral recognition models have been developed allowing predictions with respect of separability, magnitude of enantioselectivity, elution order and suitable chromatographic conditions [92]. Frequently, the identification of suitable selectors for a specific pair of enantiomers requires considerable experimentation and might be, therefore, highly demanding with respect to time, material and labour. Clearly, there is a need for empirical and/or rational strategies to facilitate this tedious selection procedure and to avoid to some extent the ''trial-and-error'' approach.

To address these needs, several companies producing CSPs even offer the development of enantioseparations at an analytical and preparative scale as a customer service and free accessible application guides and databases summarising separations of enantiomers possible with their products [165–168]. Commercial molecular databases compiling literature on LC and GC enantioseparation data with two-dimensional (2D) structures, chromatographic data, experimental conditions and bibliographic source are available. The most popular among them, CHIRBASE, is a LC database currently providing information on more than 61 000 separations of about 40 000 entries (unique sample–CSP combinations) [169]. Integrated search facilities allow to use the 2D structure, substructures and/or 2D descriptors (e.g., functional groups) of the chiral compound of interest as search statements to identify rapidly chiral selectors capable of separating its enantiomers and/or those of related compounds. For enantiomers with complex architectures, however, 2D structures and descriptors provide only a poor representation of the stereochemical requirements. These limitations were recognised and addressed most recently by introducing a modified, more sophisticated version of CHIRBASE (CHIRSOURCE), including three-dimensional (3D) structures of the selectors and selectands [170,171]. The researchers maintaining CHIRBASE have also announced to add relevant physico–chemical parameters (dipole, lipophilicity, surface area and volume, HOMO-LUMO, Verloop parameters, molar refractivity) and molecular indices (describing connectivity, shape, topology and electrotopology, atom ring and group counts) for the 3D models. In this context, it would be highly desirable to include in this up-date also dispersed data of numerous quantitative structure–property relationship (QSPR) studies performed with different classes of analytes on well-established chiral selectors [172]. Incorporation of these data would allow a more precise and reliable comparison and prediction of LC systems.

Currently, to the best of our knowledge, there is just one database available providing information on the rapidly growing body of enantioseparation achieved with CE, developed by Koppenhöfer et al. [173]. The spectrum of selectors used as chiral background buffer additives or background electrolyte (BGE) in CE comprises natural and derivatised cyclodextrins, crown ethers, surfactants, alkaloids, metal complexes [132] (see Table 1) and identifying a suitable chiral selector for a given pair of enantiomers might be still challenging.

For the intensively employed cyclodextrins, screening protocols based on ^{1}H nuclear magnetic resonance (NMR) spectroscopy have been suggested as a promising tool to identify rapidly optimal CE selector systems. Thus, in a series of papers, Blaschke and co-workers demonstrated the predictive character of NMR data for the optimisation of CE enantioseparation conditions [174–177]. In a similar study, Owens et al. performed ^{1}H-NMR screening experiments of 1:1 mixtures of nine different cyclodextrins and racemic oxamniquine (anti-schistosomasis drug). Shift non-equivalence for the diagnostic protons of the drug could only be observed with anionic cyclodextrins, indicating only in these cases efficient chiral recognition [178]. Subsequent CE experiments confirmed that the corresponding anionic cyclodextrins were efficient chiral selectors for oxamniquine, while in presence of neutral cyclodextrins no enantioseparation could be achieved. Similarly, suitable conditions for LC enantioseparation might be identified by microscale batch adsorption experiments with different CSPs. Welch et

al. demonstrated that the most efficient systems of combinatorial libraries of novel silica-bound π-donor/acceptor type selectors can be easily selected by equilibrating small amounts of CSPs with the corresponding analytes in appropriate solvents and analysing the enantiomeric excess (ee) of the supernatant [179]. This strategy of selector screening might be of broad applicability as demonstrated for a series of silica bound anion-exchange type selectors [180,181] and batch cellulose derivatives [182].

4.2. Mechanistic aspects of enantioseparation

Understanding how and where chiral recognition by a chiral selector molecule occurs may provide valuable information with respect to estimate the qualitative magnitude of enantioseparation; types of analytes separable on a given selector; predictability of elution order; and appropriate chromatographic conditions. Moreover, it is desirable to obtain structural models that explain the binding forces operating in concert recognising the more favourable enantiomers and to understand qualitatively the reasons why the less favourable enantiomer is actually less effectively bound.

Compared with the number of chiral selectors available, relatively few detailed studies on enantioseparation mechanisms are available. Among them, most investigations have been performed on low-molecular-mass selectors, including π-donor/acceptors, crown ethers and cyclodextrins. The most popular strategy to establish chiral recognition models for a given selector involves the collection of a representative body of chromatographic enantioseparation data with a series of analytes displaying incremental structural modifications. A systematic interpretation of these data may provide some mechanistic information on the contributions of the individual structure elements to enantioselective selector-analyte binding.

A more sophisticated strategy for the development of chemometrically driven predictions of retention and enantioselectivity is the construction of quantitative structure–enantioselective retention relationships (QSERRs). They combine quantitatively comparable retention data for a set of solutes and of molecular descriptors reflecting the structural features of these solutes. The focus of the studies is to develop statistically significant equations, which might be meaningful in the understanding of solute and CSP interactions. Several investigations have been performed in this direction dealing with protein-derived CSPs [183], DACH-DNB [*N*,*N*′-(3,5-dinitrobenzoyl)-*trans*-1,2-diaminocyclohexane] CSPs [184], cellulose triacetate [185] and amylose tris-(3,5-dimethylphenyl)carbamate [186,187]. More sophisticated statistical algorithms, such as multivariate regression analysis and neural networks, have been successfully applied to model the enantioselective chromatographic behaviour as a function of non-empirical descriptors [188].

For the elucidation of chiral recognition mechanisms on a molecular level, spectroscopic and X-ray structure analysis have been used. Spectroscopic evidence is most valuable, as it provides information on chiral recognition events in solution, which is closely related to the situation found in liquid phase (CE and LC) separation processes. Among spectroscopic techniques, NMR spectroscopy represents the most powerful tool. It allows one to assess complexation stoichiometry, association constants ($\Delta\Delta G$ values, correlate to the enantioseparation factor) and information on time-averaged complex structures via complexation-induced shifts. Nuclear Overhauser enhancement (NOE) effects of intermolecular and intramolecular nature allow to estimate distances between protons of selectors and selectand, elucidating often conformational details and preferences and association mode between chiral selector and selectand. Spin-lattice relaxation times (T_1) are sensitive probes for the mobility of subunits of the selector and selectand, revealing often which part of the selector and selectand are affected by association. Infrared (IR) spectroscopy, although less frequently used, may serve as a convenient tool to detect hydrogen bonding interaction occurring in course of chiral discrimination [189]. Circular dichroism (CD) spectra are highly sensitive to conformational changes that might be induced by enantioselective binding [190,191]. X-Ray crystal structures of selector–selectand complexes are generally accepted as strong support of chiral recognition mechanisms [90,192,193]. However, care must be exercised as the solution state situation might deviate considerably from that reflected in the crystalline state. Packing forces might lead to distortion of the

geometry, especially for small energetic differences (i.e., small α values) between the favourable and less favourable complex. In this situation, the corresponding X-ray structures might provide poor or even misleading information [194]. Relative well-established recognition models with predictive character have been developed for low-molecular-mass selectors, e.g., π-donor/acceptor type selectors [90,195–197] and cyclodextrins [174–178].

However, macromolecular and polymeric selectors seem more problematic to be studied in detail. Especially polymeric selectors, e.g., polysaccharide (cellulose and amylose) based selectors and proteins are challenging systems to study. High-molecular-mass selector type molecules, including aggregates thereof, may generate different binding sites with different affinities to the analytes. Generally, these systems are reluctant to form crystals allowing X-ray structure analysis. Moreover, limited solubility require the use of solvents that may preclude efficient chiral recognition. In many cases, it is difficult to produce high quality spectra due to intensive overlap of signals and poor band shapes, as a consequence of slow time averaging of many similar conformational states.

As example the investigation of chiral recognition of binaphthols on cellulose carbamates has been described [198]. The authors succeeded to produce a chloroform soluble cellulose tris-(5-fluoro-2-methylcarbamate), which enabled them to study the chiral recognition between this selector and binaphthol derivatives by NMR spectroscopy. The data obtained by continuous variation type titration with the more strongly bound (*S*)-enantiomer allowed them to establish 1:1 complexation stoichiometry between the polymer and the analyte, indicating that each individual derivatised glucose provides a single binding site. The presence of the polymeric selector led to pronounced complexation induced shifts for the more strongly bound enantiomer, with diagnostic aromatic protons of the binaphthol showing upfield shifts characteristic for interaction with aromatic moieties. On the other hand, the hydroxy protons shifted upfield, indicating their participation in hydrogen bonding interactions. Measurement of ^{1}H spin-lattice relaxation times in presence of the cellulose carbamate showed reduced values for some protons of the (*S*)-enantiomer. This finding was interpreted as a consequence of partial insertion of the analyte into the binding site located in the groove formed by the arylcarbamates moieties attached to the polymeric glucose unit. The observation of a significant upfield shift, detected during titration of the cellulose derivative with the better recognised enantiomer, provided additional support for this assumption. Throughout these experiments, the presence of the cellulose derived selector had only small effects on the NMR shifts of the less strongly bound (*R*)-binaphthol. For both enantiomers the association constants with the cellulose carbamate type selector were determined based on complexation induced shifts and the corresponding $\Delta\Delta G$ value was calculated. Comparison with $\Delta\Delta G$ values derived from HPLC measurements, however, revealed that the $\Delta\Delta G$ value obtained by NMR was substantially larger. The authors reasoned that this deviation might reflect the combined influences of immobilisation of the selector and of different solvents used for NMR and HPLC investigations.

The elucidation of the chiral recognition mechanisms of protein-type chiral selectors may be even more challenging than with cellulose polymers. If no X-ray crystal data are available, the high structural complexity of proteins makes both identification of the enantioselective binding domain(s) and assessment of the non-covalent interaction sites contributing to chiral recognition difficult. Moreover, proteins express their chiral recognition properties preferably in aqueous medium in which ligand binding is governed by a highly delicate balance of electrostatic and hydrophobic interactions. In many cases the contributions of hydrophobic forces to molecular recognition processes are considered to be important and, unfortunately, they are poorly understood [199]. Often only slight changes in pH and ionic strength may have tremendous influence on the enantioselectivity. Especially for immobilised proteins used as CSPs the ratio of the number of binding sites to molecular mass is highly unfavourable. Interactions with the solid support and the non-selective sites at the protein result in mixed retention mechanisms. Consequently, the enantioseparation data are severely ''obscured'' by contribution of non-selective interactions and their mechanistic interpretation might lead to erroneous conclusions. In order to derive data allowing for a detailed mechanistic

analysis of the ''intrinsic'' chiral recognition properties, enantioselective and non-selective contributions must be segregated.

In studies directed towards understanding temperature and pH influences on enantioseparation of propranolol on cellobiohydrolase I (CBH I), Fornstedt et al. have demonstrated a methodology to deconvolute the contributions of the mixed retention mechanisms [200,201]. The procedure involves the measurements of the adsorption data of the two enantiomers on the corresponding CSP, followed by the modelling of the resultant data by a bi-Langmuir isotherm, which in turn allowed the determination of the non-selective and the enantioselective components of retention. An elaborate analysis of the ''intrinsic'' thermodynamic parameters revealed that increasing enantioselectivity at higher column temperature must be due to perturbation of the highly organised water structure at the chiral recognition site [201]. The pH dependence of the enantioselectivity was explained based on a careful assessment of the number of binding sites and monolayer capacities of non-selective/enantioselective sites in combination with the binding constants for the individual enantiomers. The non-selective adsorption was found to be almost independent from the pH of the mobile phase, while the enantioselective adsorption of the more strongly retained enantiomer was enhanced with increasing pH. Based on these observations, the authors concluded that ionic interactions are the main component to chiral recognition, which was in agreement with independent studies based on the X-ray structure of the protein.

4.3. Modelling chiral recognition with computational tools

Atomistic chiral recognition models derived from experimental observations provide a valuable basis to make prediction with regard to separability of special classes of enantiomers, the elution order and the relative magnitude of enantioselectivity. However, the type of information is qualitative in nature and unambiguous predictions are restricted to a limited set of structurally closely related analytes. Experimentally derived models often do consider the intermolecular interactions between selector and enantiomers in the light of a few low-energy conformations only, neglecting the realistic situation in which all energy-allowed conformations may contribute to the chiral recognition event. Assessing the relative energetic contributions of individual binding sites to the chiral recognition process with empirically established recognition models can be extremely difficult. Moreover, in numerous cases enantioseparation factors are too small to allow unambiguous mechanistic models to be established by experimental techniques.

In these cases, molecular modelling might be used as a complementing and supportive tool to enhance our understanding of chiral recognition. The computational tools employed in molecular modelling (quantum mechanics, empirical force fields as used in molecular mechanics, molecular dynamics, Monte Carlo simulations and computer graphics) have reached high levels of sophistication, allowing to reproduce and even predict intermolecular binding scenarios between relatively small molecules with high reliability. In context with chiral recognition, the challenges in molecular modelling are the correct prediction of the free energy of the diastereomeric association equilibria ($\Delta\Delta G = -RT\ln \alpha$) between a chiral selector and a given pair of enantiomers and the elution order. In addition, molecular modelling should provide information that is relevant to the chiral recognition process but inaccessible by experimental techniques. Several excellent reviews documenting the progress of molecular modelling in chiral discrimination have been published [172,202,203]. In the following, some examples of molecular modelling studies relevant to chiral recognition should briefly discussed to demonstrate the applicability, merits and limitations of this methodology [204].

Lipkowitz et al. studied the enantioselective binding of 2,2,2-trifluoro-1-(9-anthryl)ethanol and a truncated version of Pirkle's dinitrobenzoylphenylglycine CSP using molecular mechanics. For this purpose, the lowest-energy binding regions and the most stable orientation of the interacting molecules were identified by ''rolling'' the individual enantiomers over the van der Waals surface of the selector. To obtain a free energy value directly comparable with the experimental data, a statistical mechanics averaged interaction energy from all important shapes of the enantiomers, selector and orientations of the

molecules was computed. This procedure permitted to locate all the minima on the complex's intermolecular surface and thus to obtain a good representation of the macroscopic free interaction energy. The enantioseparation factor found by the molecular modelling approach was in excellent agreement with the experimental value.

The same group investigated enantioselective binding of tryptophan enantiomers to α-cyclodextrin using the CHARMM force field for molecular dynamics simulation [205]. Both the elution order and the enantioseparation factor could be reproduced correctly, and the results were in accordance with experimental NMR data. Partitioning of the total averaged energies into component terms provided interesting information inaccessible by experimental techniques. Thus, electrostatic contributions were similar for both diastereomeric complexes. The more strongly bound (*R*)-enantiomer was favoured because the complex had less torsional strain and because the non-bonded interactions were more favourable. Structurally, the calculated diastereomeric complex showed that the enantiomers were highly localised in the interior of the cyclodextrin, binding to different sides of the rim. The authors also evaluated the number and type of intermolecular hydrogen bonds between the selector and the enantiomers. They found that the more strongly bound enantiomer forms twice as many hydrogen bonds than the (*S*)-enantiomer, and that the hydrogen bonds of the (*R*)-enantiomer with the selector are usually of the multi-contact type. Interestingly, the calculation indicated that primarily the carboxylate and the indole NH are involved in hydrogen bonding and not the ammonium group of the tryptophan.

Similar to investigations of chiral recognition processes with experimental techniques, molecular modelling of enantioselective binding processes of polymeric selectors are extremely difficult. Apart from the fact that molecular modelling of large molecules is extremely time-consuming and may strain the limits of the currently available computational facilities, there is little structural information available for many polymeric phases. Nevertheless, pioneering studies in this direction were performed by Yamamoto et al. [206]. These investigations were carried out to enhance the mechanistic understanding of enantioselective interactions between *trans*-stilbene oxide and cellulose triphenylcarbamate (CTPC). As a truncated model for CTPC, the authors used the corresponding octamer, which was created computationally from a monomeric unit using molecular mechanics (MM) calculation and molecular dynamics (MD) simulations. The octameric CTPC was fairly good in agreement with the X-ray structure of CTPC, representing the polymer as a left-handed threefold helix. To search the lowest interaction energies the individual enantiomers were sampled systematically around all carbamate NH groups of the four central glucose units of the octamer. Depending on the glucose unit and the carbamate unit investigated, quite different values for the interaction energy were obtained. The distribution of the individual interaction energy indicated, in agreement with the HPLC experiment, that the (*S,S*)-enantiomer of the stilbene oxide was more strongly bound. The interaction energy calculated based on the lowest values, however, gave an enantioseparation factor significantly larger than that observed by chromatography.

When comparing experimental enantioselectivity data with those derived from molecular modelling, one should be aware that the current level of calculations generally do not account for the presence of modifiers, ions, effects of differential solvation of the diastereomeric complexes, the influences of immobilisation and the underlying support. Nevertheless, pooling information on mechanistic aspects derived from experimental observation with results of molecular modelling studies has great potential to advance our still limited knowledge on chiral discrimination processes and phenomena.

4.4. Alternative preparative techniques for enantioseparation: membrane technologies

The increasing need for single enantiomers as key intermediates in chemical and pharmaceutical industry has stimulated a significant demand for efficient processes to resolve racemic mixtures. In context to potential industrial applications, the focus is on technologies allowing to perform enantioseparation in continuous fashion. Apart from the promising concept of SMB chromatography with chiral stationary phases [127], considerable efforts have been

concentrated also on the development of enantioseparation procedures based on membrane processes [207]. Technically, membrane separation processes are particularly suited for large-scale applications as they combine the following attractive features: continuous operation mode, easy adaptation to different production-relevant process configurations, convenient up-scaling and, in most cases, ambient temperature processing [208]. Membrane processes for enantiomer separation may be categorised into two general types: either direct separation on an ''intrinsic'' enantioselective membrane (e.g., enantioselective polymer or liquid), or separation in which a non-selective membrane assists an enantioselective process (as support for a chiral carrier in supported liquid membranes, liquid–liquid extraction). Since the demonstration of highly efficient enantioseparation of amino acid derivatives by Cram's ''catalytic resolving machine'' (Fig. 4, [209]), a rich literature on membrane-mediated enantioseparation has been accumulated, which has been critically reviewed recently [207]. In the following, a few selected examples should be discussed to give an impression of the standards reached in this field.

Impressively enantioselective membranes based on modified poly-[(*S*)-glutamates] were developed by Maruyama et al. [210]. Poly-[(*S*)-glutamate] was derivatised with (*n*-nonylphenoxy)-oligo(oxyethylene) side chains, which led to the formation of helical structures, which are selectively permeable for amino acids. In laboratory-scale permeation experiments performed with this membrane the complete resolution of tryptophan enantiomers could be observed (see Fig. 5). Pirkle and Bowen described the separation of *N*-(3,5-dinitrobenzoyl)leucine through a liquid membrane containing fatty acid esters or amides of (*S*)-*N*-(1-naphthyl)leucine as highly enantioselective carriers [211]. The separation was performed in a bulk liquid membrane system (Fig. 6), in which the carrier was circulated between an adsorption and desorption hollow-fibre unit. The formation and dissociation of the chiral complex took place at different location, which allows to adjust appropriate conditions for enantioselective binding and release of the transported enantiomer. Using this configuration, an enantioselectivity >95% could be achieved.

A strategically different concept was developed by Keurentjes and co-workers [207,212], who combined enantioselective countercurrent liquid–liquid extraction and non-selective membrane technology (Fig. 7). In a completely symmetric system separated by a

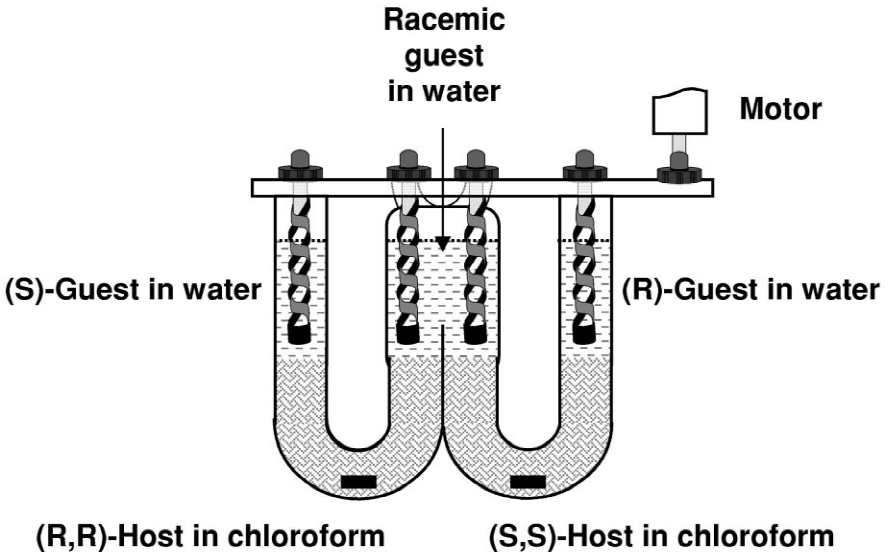

Fig. 4. Scheme of Cram's resolving machine reproduced from Ref. [209].

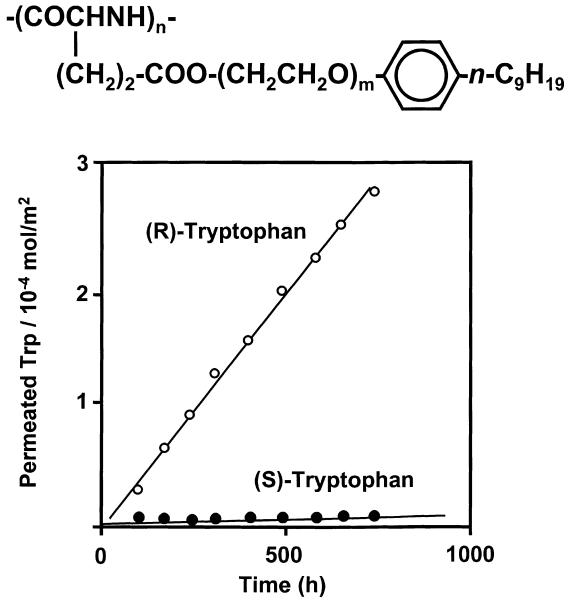

Fig. 5. Permeation behaviour of D- and L-tryptophan from a racemic mixture at 34°C adapted from Ref. [210].

liquid membrane two solutions containing opposing chiral selectors were transported countercurrently to each other. The liquid membrane was chosen to be insoluble for both the carriers as well as the solvents used. The racemic mixture to separate was introduced in the centre of the system. Employing this set-up in an hollow-fibre membrane configuration, the authors could achieve the complete enantioseparation of norephedrine using both enantiomers of dihexyl tartrate (0.25 *M*) as carriers. This technology is remarkable in that relatively low enantioselectivities ($\alpha = 1.05–1.2$) are sufficient to separate enantiomers completely provided the length of the apparatus can be adjusted appropriately (2–5 m).

Lakshmi and Martin [213] demonstrated a general strategy to prepare enantioselective composite membranes based on physically entrapped enzymes (Fig. 8). As host membrane a 10-μm thick polycarbonate filter with 400-nm pores was used, as single-sided modified with a gold/polypyrrole film. The modified filter was charged by vacuum filtration with a D-amino acid oxidase apoenzyme solution and subsequently sealed with a polypyrrole film by electropolymerisation. In diffusion experiment, a facilitated transport of D-phenylalanine could be observed leading with relative moderate enantioselectivity. Another promising approach to generate enantioselective membranes by immobilisation of proteins was demonstrated by Nakamura et al. [214]

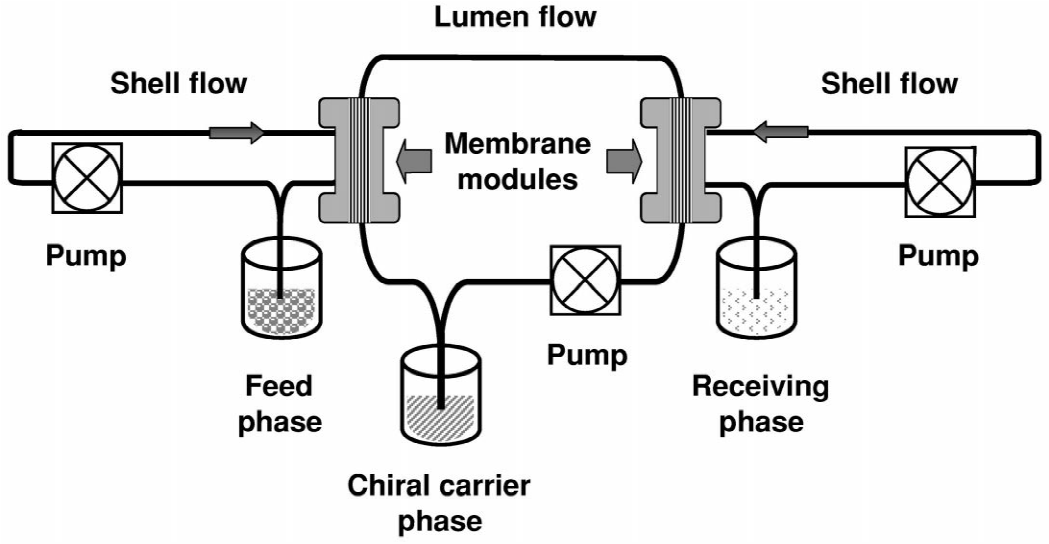

Fig. 6. Version of the diagram of Sepracor MSX-750 membrane solvent extraction system presented in Ref. [211]. The system uses three independent circulating solvent loops. The water immiscible carrier phase flows in a closed loop (lumen), in the two modules and back to the reservoir. The aqueous feed and receiving phases circulate independently, each through the shell of a module and back to the reservoir.

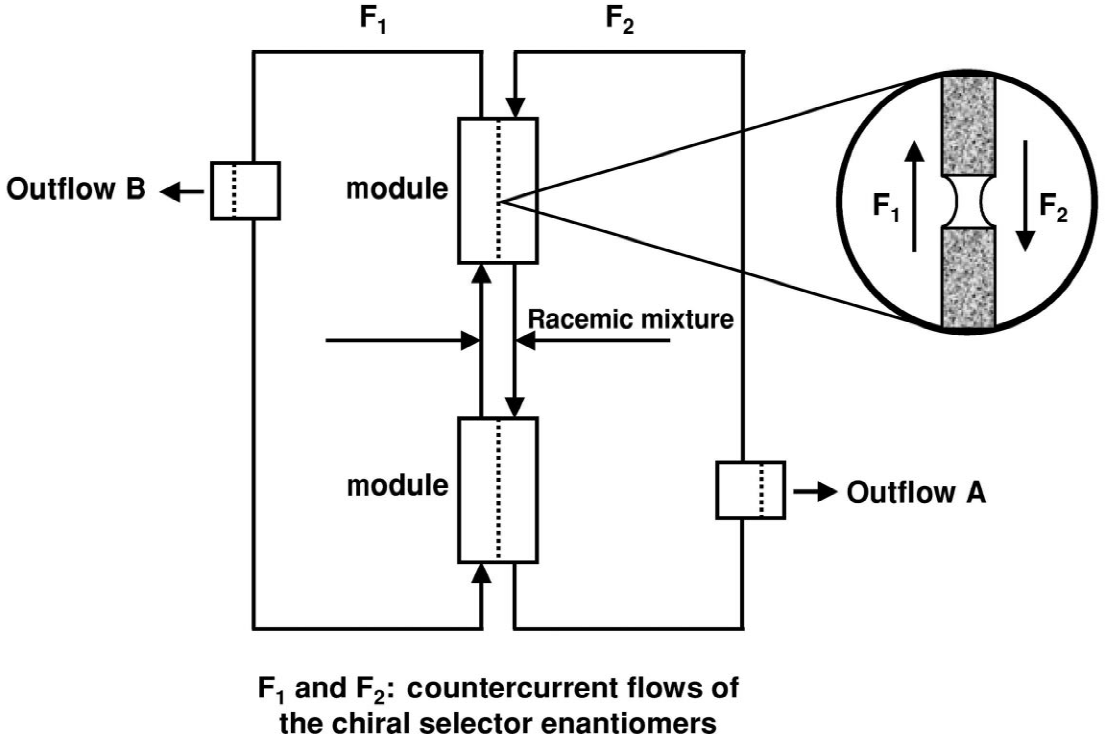

Fig. 7. Schematic representation of a countercurrent liquid–liquid fractionation system using membrane technologies reproduced from Refs. [207,212]. Two liquids containing the opposing enantiomers of the chiral selector (F_1 and F_2) are flowing countercurrently through the column and are kept separated by a liquid membrane (ampliated figure). The racemic mixture is added to the middle of the system and the separated enantiomers are recovered from the outflows A and B, respectively.

most recently (Fig. 9). A porous polyethylene hollow-fibre was activated by electron beam irradiation to produce reactive groups on its surface. Glycidyl methacrylate was grafted on the fibre, which in turn was treated with amines to generate basic groups on the surface. Onto this fibre, a high amount of bovine serum albumin (BSA, 160 mg/g) could be adsorbed ionically under controlled conditions. The presence of an homogeneous multilayer of protein molecules through the membrane was demonstrated by X-ray investigations. Leaching of the protein was efficiently suppressed by cross-linking with glutaraldehyde. Evaluation of this modified, hollow-fibre membrane for tryptophan under chromatographic conditions produced an excellent enantioseparation factor of 12.

These results make evident the potential of enantioseparations by membrane technologies. Nevertheless, several limitations associated with the currently studied systems should be pointed out. Generally, the mass transport through membranes under dialysis is quite low, while the use of external driving forces, such as pressure, leads often to a significant reduction in enantioselectivity. Furthermore, membranes containing chiral selectors in physically immobilised-fashion (*supported liquid membranes*) suffer from instability. The solubility of membrane components in the feed and strip media and emulsification phenomena due to lateral shear forces are some of the main drawbacks [215]. However, current progress in the field of membrane materials and engineering promises to address these limitations in near future. The development of chiral selectors with receptor-like enantioselectivities and efficient protocols for the preparation of composite membranes allowing the use of external driving forces might be instrumental to establish membrane separation as a competitive process in technical production environment.

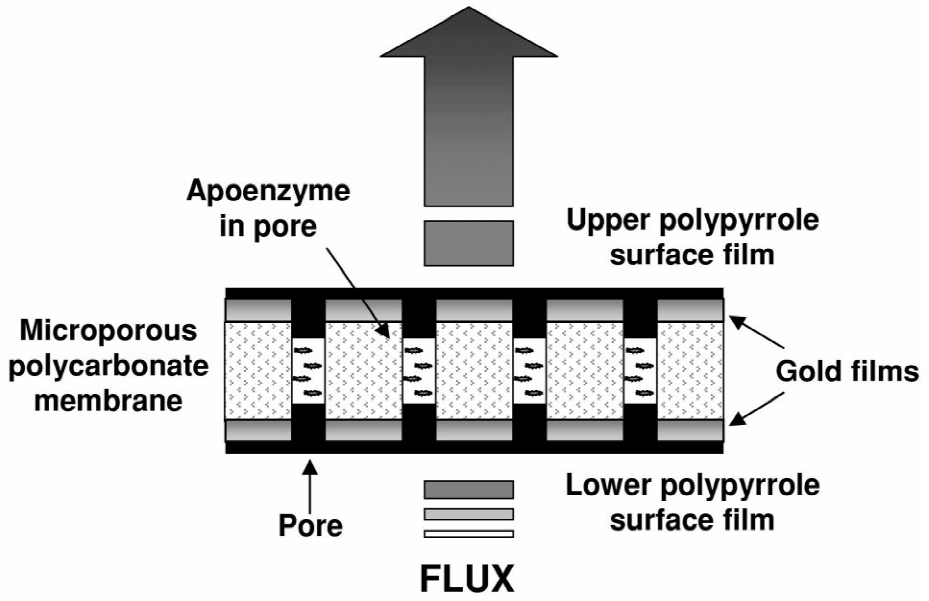

Fig. 8. Adapted schematic cross-section of an enantioselective membrane containing apoenzyme entrapped in the pores [213].

4.5. Combinatorial strategies in the development and optimisation of chiral selectors

As pointed out above, future needs in enantioseparation may focus on two categories: the development of robust chiral selector systems for applications in process environment, combining the features of appropriate enantioselectivity, mechanical stability, resistance against acids, bases and ions, wide range of solvents and accessibility from inexpensive starting materials [74,216]. On the other hand, selector systems with receptor-like enantioselectivities to serve in the development of newly emerging ''single-plate'' enantioseparation techniques, including membrane separation, batch-adsorptions and sensor devices. However, although significant progress has been made in understanding the molecular principles of chiral recognition, the possibility for ''de-novo'' directed design of chiral selectors is still limited. Moreover, the established synthetic strategies for the development of chiral selectors involve multiple cycles of time-consuming and laborious steps which need to be improved in terms of efficiency and productivity.

To meet these challenges, the implementation of combinatorial strategies is highly desirable. Combinatorial synthesis protocols, e.g., parallel and/or mix-and-split strategies, allow economic and rapid generation of large libraries of potential selectors, from which the systems with desirable properties may be extracted by appropriate screening methods. The application of combinatorial strategies may be particularly attractive for the development and optimisation of the properties of polymeric CSPs. The increasing recognition of the utility of combinatorial strategies in selector development is reflected in most recent publications.

4.5.1. Low-molecular-mass chiral selectors

Thus, the consequent application of the reciprocity principle of chiral recognition in Pirkle's group should be recognised as an early ''combinatorial chemistry approach'', as dedicated screening procedures of potential selector candidates on ''analyte-type'' CSPs were systematically used as supporting tool in chiral selector development [93]. Jung et al. have demonstrated the use of cyclohexapeptide libraries as chiral selectors in CE to separate amino acid derivatives [217]. Welch et al. reported parallel synthesis of DNB-oligopeptide libraries on amino-functionalised silica [179,218]. To identify promising selector structures for a π-basic analyte, an attrac-

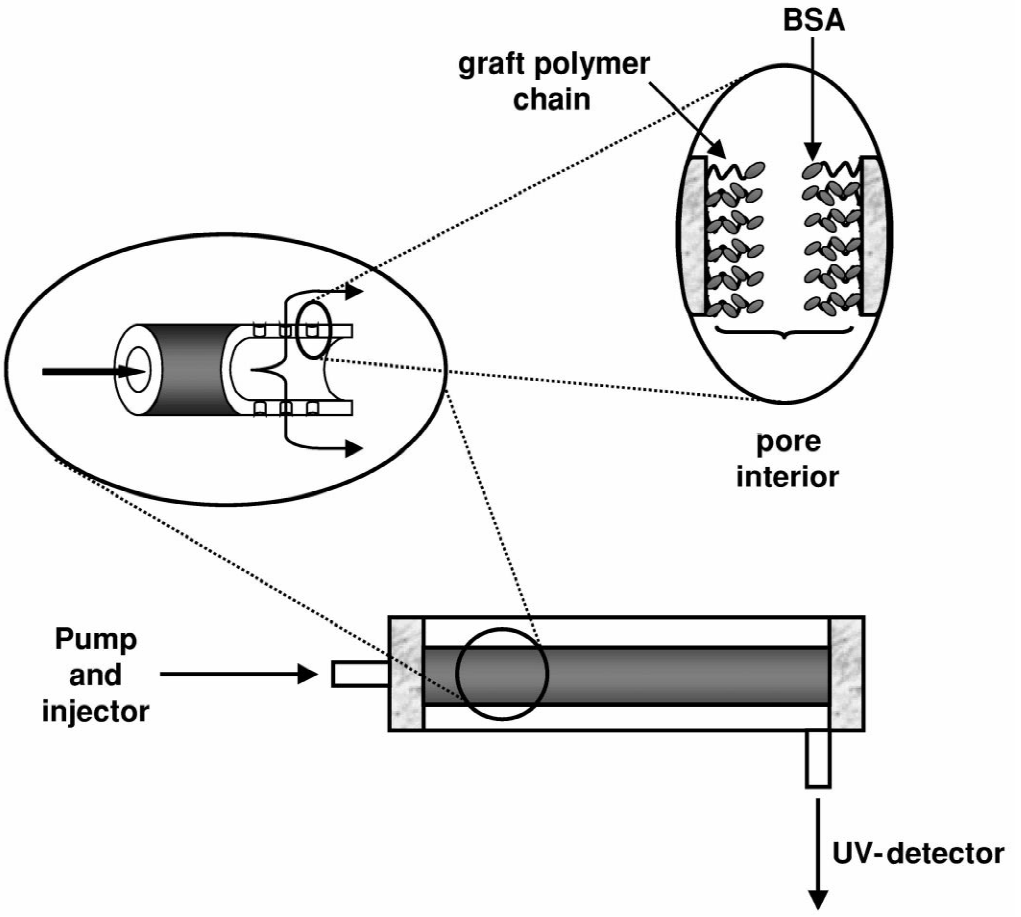

Fig. 9. Experimental apparatus for chromatography with a single hollow-fibre module, containing cross-linked BSA captured by the graft chains reproduced from Ref. [214].

tively simple screening protocol based on solid–liquid extraction was established. In a subsequent report, the most promising structural motifs were further optimised by creating two more focused libraries, from which a selector with excellent enantioselectivity and preparative capacity for the target compound could be selected [218]. The feasibility of extracting efficiently optimised selector systems from large libraries was proved by Chiari et al. [219]. They created a library containing more than 8000 cyclohexapeptides, and chose CE as a screening tool to evaluate the enantioselective properties for *N*-(2,4-dinitrophenyl)amino acids (DNP-amino acids). Stepwise deconvolution of the library was performed using a combination of HPLC fractionation of the original library and resynthesis of sublibraries, leading ultimately to the identification of a single structure with excellent CE enantioseparation properties for DNP-amino acids. Lewandowski et al. exploited the reciprocity principle of chiral recognition to select the most efficient enantioselective candidate for DNB-amino acids in a library of dihydropyrimidines [220]. In a related paper, the same group reported an interesting approach of deconvoluting a mix-and-split bead-bound 36 member libraries by consecutive chromatographic screening of sublibraries [221].

A demonstration that the combinatorial search for new selectors can be extended beyond the world of amino acids was given by Weingarten et al. [222]. In

this study, they presented an attractive modular approach to create novel materials for kinetic resolution of racemates. A chemically encoded, solid-supported 60-member library of chiral amines (Fig. 10) was generated by a mix-and-split protocol employing three classes of chiral building blocks comprising macrocycles, a diamine and different amino acids. For screening of the kinetic resolution properties, the bead-bound library was incubated with active esters of amino acids, the enantiomers of which were tagged with different dyes. Consequently, the enantioselective acylating properties of the selectors could be evaluated by visual inspection of differently coloured beads. Chemical encoding of the most intensively coloured beads made it possible to identify the most enantioselective candidate.

4.5.2. Enantioselective imprinted polymers

Combinatorial strategies may also be efficient tools to optimised the enantioselective binding properties of polymers accessible by molecular imprinting technology. Molecular imprinting, introduced by Wulff [223], is an appealing strategy to create specific enantioselective binding sites with well-defined shape and functionalities in prearranged fashion [224,225]. To this end, the corresponding template enantiomer is equilibrated with polymerisable functionalities to form non-covalent complexes. Subsequently, these complexes are copolymerised in presence of a high content of cross-linker and a porogenic solvent to form a rigid polymeric matrix. After extraction of the template enantiomer, polymers with cavities are obtained, which have shape and arrangement of the functionalities complementary to the imprint enantiomer. Consequently, under chromatographic conditions, imprinted polymers show good to excellent recognition properties for the template enantiomer and can be used the separate the corresponding racemate. Chirally imprinted polymers exhibit several attractive features: the elution order of this material is predefined; the enantiomer originally used as template is always more strongly retained; no sophisticated design and laborious synthetic protocols have to be elaborated to generate

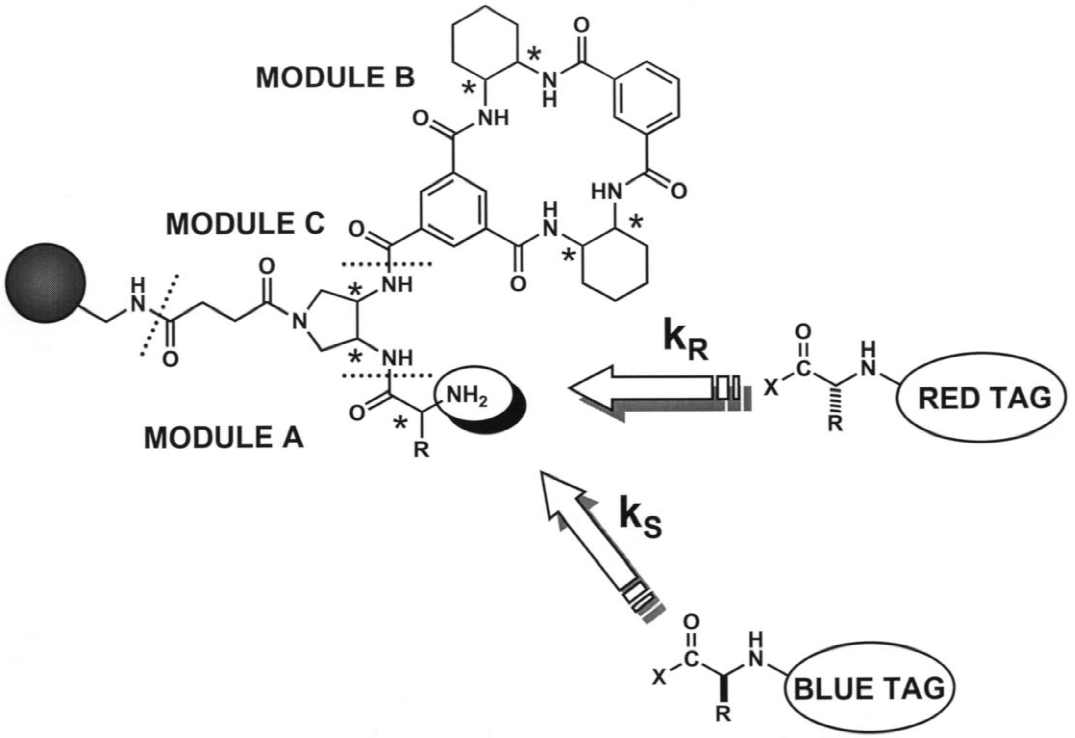

Fig. 10. Kinetic resolution of differently dye-tagged solid-supported amino acids using a 60-member library of ‘‘chiral selectors’’ [222].

enantioselective interaction sites and the required components are readily available and inexpensive bulk chemicals. The polymers themselves show beneficial properties such as remarkable stability against mechanical stress, elevated temperature, high pressure, acids, bases, metal ions and a wide range of solvents in combination with a long shelf life, making them attractive for application in process environment.

The general applicability of molecular imprinting technology to produce CSPs based on enantioselective polymers has been shown for amino acids [226,227], amino acid derivatives, peptides, profens, amino alcohols [223,225,228]. Their application for the selective removal of enantiomeric impurities (''enantiopolishing'') has been suggested [229]. As a variation of this theme, the incorporation of enantioselectively imprinted polymers into supported membranes has been investigated [230,231].

There are also several disadvantages with imprinted polymers. Generally, only about 10–15% of the loaded template results in efficient binding site formation and, therefore, in relative low loading capacity. Imprinted polymers also suffer from binding site heterogeneity and, thus, give rise to extreme peak tailing for the imprinted molecule in chromatography [232]. However, promising approaches to improve both capacity as well as efficiency of molecular imprints have been suggested. The formation of more stable template–functional polymer complexes might have favourable effects on capacity and binding site homogeneity. To this end, the currently available repertoire of ''diversity'' of functional monomers needs to be enhanced. In this line, the development of dedicated chiral functional monomers [228] might be advantageous. The application of multifunctional cross-linker systems might be also a promising strategy to stabilise interaction sites [233].

Unfortunately, the numerous factors that affect the outcome of molecular imprinting are not yet fully understood and for the development of industrial applications continual series of trial-and-error experiments are necessary. To meet this challenge, combinatorial technologies appear to be most suitable approaches. Promising results of pioneering efforts by Takeuchi et al. [234] and Lanza and Sellergren [235] to improve the properties of non-chirally imprinted polymers by such combinatorial strategies have been published most recently (for other references see Table 1).

4.5.3. Crystallisation

The introduction of combinatorial strategies has proven also advantageous for ''classical'' type of enantioseparation methods, e.g., resolution of enantiomers by crystallisation of diastereomers. Despite considerable research efforts invested to create rational concepts for diastereomeric salt crystallisation, the possibilities of predicting ideal combinations of resolution agents for a given racemic mixture are still very limited. Therefore, identifying appropriate resolution agents and conditions often requires substantial ''trial-and-error'' type experimentation and might be expensive in time, labour and material.

Most recently, Vries et al. [236] reported an attractive ''combinatorial approach'', which promises to facilitate classical resolution procedures greatly. This technology capitalises on the use of ''families'' of resolution agents in the separation of enantiomers. For this purpose, the researchers employed ''tool kits'' of single enantiomer mixtures of structurally closely related acids for the resolution of racemic bases and vice versa. Under these conditions, the resolution was much more efficient in terms of productivity and enantiomeric purity than with single resolution agents. In many cases, only a single recrystallisation was essential to obtain salts containing the target compound in 99% ee. Almost in all cases, the diastereomeric salts derived from crystallisation with ''reagent families'' showed a non-stoichiometric incorporation of the single components.

In an attempt to explain this behaviour, Collet suggested that the presence of structurally closely related resolution agents might facilitate the formation of solid solution, displaying reduced solubility and higher densities. The closer packing in these crystals, in turn, may be the reason for improved selectivities towards incorporated analytes [237].

5. Separation of enantiomers: perspectives

Currently, progress in drug design, catalyst tech-

nology and material sciences is strongly driven by the implementation of combinatorial strategies providing the possibility to generate a large number of novel compounds and materials within short periods of time. In context with this development, there is an increasing need for appropriate analytical technologies for characterising new materials and compounds and to evaluate the outcome of high throughput screenings. Although the established techniques for analytical separation of enantiomers have reached high standards, they often fail to meet actual challenges in the changed research and development environment. Therefore, it is to be expected that currently available enantioseparation tools will be adapted and extended in favour of analytic technologies that combine the attributes of higher flexibility, speed, parallelism and low costs.

5.1. Enantioselective sensors

Enantioselective sensor systems, allowing fast qualitative/quantitative determination of enantiomeric purity may be a promising alternative. In this context, solution assays giving an immediate assessment of enantiomeric purity due to pronounced changes in colour or fluorescence might be extremely useful in screening comprehensive combinatorial libraries, quality control in pharmaceutical industry and related fields. On the other hand, established selector systems may be interfaced with appropriate transducers to convert chemical into electronic information to provide enantioselective sensors for on-line monitoring of enantiomeric purity in technical process environment as biotransformation and asymmetric syntheses. The development of enantioselective sensor systems may represent a major branch of future research. The feasibility of enantioselective sensors has been demonstrated recently by the results of several groups [238].

Pioneering efforts to create a ''color indicator to judge the absolute configuration of chiral amines'' have been described by Kaneda et al. As sensing systems chiral azophenolic acerands were employed (Fig. 11A) [239]. These chiral hosts were shown to be capable of discriminating between the enantiomers of chiral amines by shift of bands in the visible spectra. In a more recent contribution, Kubo and co-workers described an exquisitely enantioselective chromogenic sensor based on a calix[4]arene scaffold (Fig. 11B) [240,241]. In this molecular sensor, two indophenol units attached to the upper rim of the calixarene served as chromophores, while the chiral information was introduced by a crown ether attachment integrating a binaphthol unit at the lower rim. The use of different spacer length for the crown ether allowed to position the chiral binaphthyl unit closer to one of the indophenols than to the other, rendering the chromophores distinct. On binding of a chiral substrate, the chromophores should be affected to different extents to create a strong chromogenic response. In fact, addition of (*R*)-phenylglycinol to an ethanol solution of the sensor led to an immediate colour change from purple to blue–violet. The visible spectrum indicated significant changes on complexation. A spectral shift of the sensor band at 515 to 538 nm and the formation of a new band at 652 nm reflected the different behaviour of the indophenol chromophores on enantioselective complexation. In contrast, the addition of the corresponding enantiomer did neither lead to a colour change nor to noticeable spectral shifts. With a competition experiment the authors demonstrated that even low concentration of (*R*)-phenylglycinol can be reliably detected even in presence of 500 equivalents of (*S*)-enantiomer.

Another conceptually different approach was chosen by James et al. to design a solution based enantioselective fluorescence sensor for monosaccharides (Fig. 11C) [242]. The sensor capitalised on a fluorescent binaphthol unit, modified with side chains containing tertiary amino groups and boronic acid functions in close proximity. The molecular principle of sensing is based on the interaction of the boronic acid with the amine, the latter acting as a intramolecular quencher of the binaphthol fluorescence. The authors could show that the binding of monosaccharides to the sensor in aqueous buffer gave rise to a significant increase of the fluorescence intensity, allowing chiral discrimination between the enantiomers of fructose, mannose, glucose and galactose. The change in fluorescence intensity was explained as a consequence of two interrelated mechanisms: (1) formation of the 1:1 complex with the monosaccharide, which leads to a stronger nitrogen–boron interaction and, thus fixes the nitrogen in a certain orientation relative to the aromatic system,

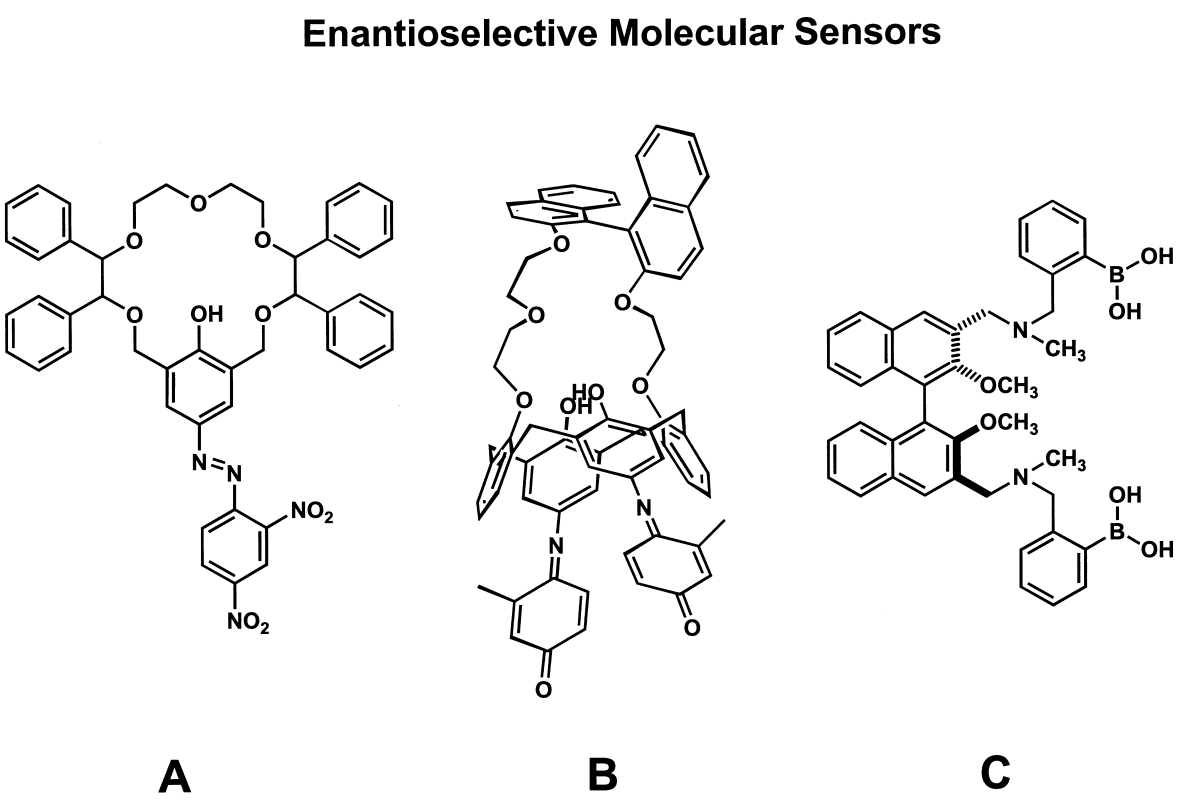

Fig. 11. Chemical structures of chiral molecular sensors: (A) azophenolic acerands showing chromogenic enantioselective response for chiral amines [238]; (B) chromogenic sensor discriminating the enantiomers of phenylglycinol [239–241]; and (C) fluorescent sensor showing enantioselectivity for monosaccharides [242].

determining its quenching efficiency; (2) additional modulation of the quenching efficiency, which is induced due to enantioselective steric interaction enforcing a twist around the binaphthyl bond on binding of the D- or L-monosaccharides.

Apart from these solution based enantioselective sensors, conventional selector systems have been coupled with established transducer set-ups to evaluate their utility in discriminating between enantiomers. Successful chiral recognition of methyl lactate, methyl-2-chloropropionate, and the inhalation anaesthetics enflurane, isoflurane and desflurane with a gas sensor using perpentylated γ-cyclodextrin was reported recently by Bodenhöfer et al. [243]. The gas sensors were prepared by coating the cyclodextrin in polysiloxane solution on thickness shear mode resonators (TSMRs), which served as transducer elements to convert chemical in electronic information. TSMRs act as quartz microbalances, which allow to detect highly sensitively changes in mass deposited or removed from their surface via shifts in their operating frequency. The preferential adsorption of the better recognised enantiomers and the resulting changes in mass on the TSMR elements permitted chiral discrimination of the tested compounds at low concentration levels (5–45 μg/l). The low detection limits in combination with the unique advantage of on-line monitoring of enantiomer composition makes this system attractive for a broad range of analytical applications.

In an extension of this work, Bodenhöfer et al. investigated the possibility of quantitative measurements of the enantiomeric ratio of racemic mixtures with gas sensors [244]. Two different transducer systems, TSMRs and reflectometric interference spectroscopy (RIFS) were employed for these gas

sensors. RIFS is a optical technique which enables the detection of changes in the optical thickness of substrates coated on a glass surfaces. In this case, both enantiomers of the octyl-Chirasil-Val were employed as chiral selectors to allow ''cross checking''. As both enantiomers are simultaneously exposed to the analyte, higher and lower sensor response can be observed at the same time. Experiments performed with this sensor array using non-racemic test mixtures of methyl lactate (α=1.13 under GC conditions on the respective selectors) showed systematic and consistent signal increases or decreases with changing enantiomeric composition of the analyte. Both sensor systems allowed fast, real-time and in situ determination of enantiomeric excess with 10% resolution.

The most impressive results in terms of enantioselectivity were reported by Hofstetter et al. with an immunosensor for amino acids [245]. The biosensor capitalised on surface plasmon resonance transducer technology to assess competitive binding of amino acids and immobilised haptens to enantioselective antibodies with relaxed side chain specificity. Under optimised conditions, this device was capable of detecting one part of D-enantiomer in presence of 2500 parts of the respective L-enantiomer. Compared with the performance of the most sensitive enantioselective analytics currently available (GC), this result represents an improvement in the detection level by one order of magnitude.

To summarise, enantioselective sensors might represent a most promising alternative to traditional and often instrumentally demanding enantioseparation techniques. In particular, inexpensive and disposable sensor devices may replace conventional ''off-line'' methodologies in process control, clinical diagnostics and high throughput screening procedures of chiral compounds. Nevertheless, the development of generally applicable strategies to create molecular sensors, combining the benefits of high substrate specificity and enantioselectivity interlinked with readily detectable changes in properties (UV adsorption, fluorescence, etc.), will definitely require considerable research efforts. In similar fashion, the optimisation and adaptation of existing transducer technologies, with respect to the requirements of enantioselective sensor systems, might be another challenging task.

5.2. Miniaturisation

As outlined above, the enormous interest in single enantiomer compounds in many field of academic and industrial research has generated the demand to assess the enantiomeric purity of large bodies of samples. Evidently, the throughput rate achievable with conventional techniques and instrumentation is limited due to relatively long processing and run times, costs and personal resources. Therefore, the development of alternative technologies allowing more efficient processing of sample pretreatments and enantioselective analytics represents currently an extreme active area of research.

One of the promising approaches to achieve these objectives focuses on the generation of miniaturised devices, such as micro total analysis systems (μ-TAS) [246]. Compared to conventional analytical technologies, micromethods allow to increase the analytical throughputs dramatically. Other obvious advantages include the reduction of solvent, reagent and sample consumption, manufacturing, and maintenance costs, along with the improvement of the efficiency and the possibility of automation. However, the benefits of miniaturisation are complicated by problems associated with detection, dead volumes and how to couple capillaries to detectors and injectors. In this context, capillary techniques, such as CE, are especially promising as transport of liquids, pumping and mixing can be achieved using electroosmotic phenomena rather than pressure-driven forces. Recently, several successful applications of CE coupled with detection on glass and quartz chips (laser-induced fluorescence, LIF; chemoluminiscence, CL; derivatisation) have been reported [247–252], demonstrating the feasibility of microanalytical separations.

Among these experiments, one was devoted to ''chiral fingerprinting'' of biogenic amino acids in samples of the Murchison meteorite [252]. Four pairs of enantiomers of fluoresciene-labelled amino acids were resolved with excellent efficiency, using γ-cyclodextrin as chiral selector within 4 min on a chip prototype schematised in Fig. 12. The enantiomeric ratios found by these investigations were in excellent agreement with those established by conventional HPLC and GC–MS techniques, however, run times were 8- and 20-times shorter, respectively.

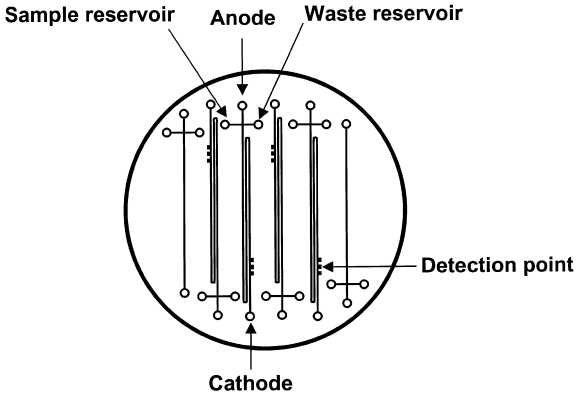

Fig. 12. Schematic drawing of a micro total analysis system (μ-TAS) for CE enantioseparation of fluorescent labelled amino acids reproduced from Ref. [252]. The wafer (10 cm diameter) is produced from quartz (capillary: 19.0 cm×150 μm×20 μm). The doubly folded channels are used for the separation of enantiomers.

5.3. Chiroptical methods – a renaissance?

The implementation of combinatorial core technologies in the fields of drug development, catalyst technology and material science has generated the demand of determining the enantiomeric excess of large libraries of compounds in short time frames.

Established ''off-line'' enantioseparation techniques based on HPLC, CE and GC are less suited as baseline separation of enantiomers requires often substantial run times leading to serious bottle-necks within the combinatorial high-throughput machinery. In order to maintain the intrinsic advantages of combinatorics, efforts have been focused on strategies allowing assessment of enantiomeric purities without the necessity of physical separation of enantiomers. In this context, chiroptical technologies seem to be most promising candidates to meet these challenges.

Most recently, preliminary results on an adaptation of polarimetry for high throughput screening of chiral compounds were disclosed. Gibbs et al. have developed and evaluated a laboratory-made ''imaging polarimetry'' device allowing to determine the optical rotation of 37 samples simultaneously [253]. Additional to conventional optical components, the polarimetric apparatus was equipped with a multichannel cell to accommodate the samples and a charged coupled device camera to record averaged pixel intensities for each individual channel of the sample array. Calibration was performed with varying concentration of a compound of known optical rotation. The authors reported a resolution <0.05 degrees based on the observations made during real time monitoring of an enantioselective enzymatic reaction.

Ding et al. reported a ''super-high-throughput-screening'' protocol for a specific chiral arylalcohol formed in course of combinatorial optimisation studies of asymmetric catalyst systems [254]. To identify the most effective catalytic cocktails within the investigated libraries, the enantiomeric enrichment of the model product was assessed using reversed-phase chromatography in conjunction with a commercial CD detection system. The CD detector provided simultaneously CD signal ($\Delta\epsilon$), the absorption (ϵ) and, consequently, the corresponding anisotropy factor ($g=\Delta\epsilon/\epsilon$) at a given wavelength. Attractively, the anisotropy factor represents a concentration-independent parameter, but is correlated linearly with the enantiomeric excess of the corresponding compounds [255]. The combination of ''achiral'' chromatography and CD detection permitted to evaluate the stereochemical outcome of a single catalytic reaction within 3 min. Thus, complete screening of the 36 member arrays of reactions employed in this study could be performed in less than 2 h.

Chiroptical methods can be also expected to play a more prominent role as supportive tools in the investigations of chiral recognition processes and the assignment of the absolute configuration of chiral molecules. Apart from the features which have been already mentioned for electronic circular dichroism (ECD), an emerging technique capable of measuring the CD of vibrational transitions (VCD), is attracting the attention of scientists in recent times [256]. Although the first measurements were already described in the 1970s, the absence of suitable instrumentation and practical methodology for properly predicting VCD intensities were major limitations. Recent developments in ab initio Density Functional Theory (DFT) and Gauge-Invariant Atomic Orbitals (GIAOs) have generated a reliable basis to predict conformational behaviour and absolute configuration even for large organic molecules. VCD presents several advantages over ECD [257,258]. On the one

hand, it can be measured for many transitions, usually narrower than the electronic ones and, therefore, leading to highly resolved spectra. On the other hand, the calculation of VCD intensities are easier and more reliable since only knowledge of the molecules electronic ground state is required and not of the excited ones. Moreover, DFT/GIAO methodology for predicting VCD is general and of high accuracy. In view of these combined advantages, VCD appears to complement or even replace some other more classically used technologies for the determination of absolute configurations and enantiomeric purity.

5.4. Chemical force microscopy – assessing enantioselectivity at a molecular level

Elucidation of chiral recognition phenomena means to understand intermolecular forces stabilising selectively the more favourable over the less favourable diastereomeric complex between chiral selector and the respective enantiomers. This can be achieved, as pointed out earlier, by a rich repertoire of experimental and theoretical techniques. Nevertheless, direct measurements of discriminating forces between single molecules would represent a much more appealing approach.

Chemical force microscopy (CFM) might be an efficient tool to achieve this desirable goal. CFM is a technique combining chemical discrimination with atomic force microscopy by chemical modification of the scanning probe. Most recently, McKendry et al. demonstrated the fascinating possibility of measuring the stereodiscriminating forces between π-donor–acceptor type selector molecules and mandelic acid derivatives by this methodology [259]. For this purpose, the researcher modified the scanning tip of the FM with a few flexibly tethered DNB-phenylglycine molecules. This ''selector probe'' was subsequently used to evaluate the binding energies with sample surfaces exposing monolayers of immobilised mandelic acid derivatives. The authors were able to reproduce sample surfaces displaying a genuine pattern of alternating strips of the individual enantiomers by the corresponding friction maps, providing unambiguous evidence for chiral recognition at molecular level. Further improvements of this intriguing methodology might provide a powerful supporting and complementing tool to study theoretical aspects of chiral recognition. Apart from this obvious potential, CFM may serve as a fast ''single molecule'' screening technology for identification of promising chiral selectors in arrayed combinatorial libraries.

6. Conclusions

The impact of chirality on almost any pharmacological and biological process is well recognised and is finding strong repercussion on many fields of economic interest, such as the development of drugs, agrochemicals, food additives, fragrances, new materials and catalysts. In context with the increasing demand of enantiomerically pure compounds, efficient strategies for analytical and preparative separation of enantiomers are required. For industrial scale production, resolution still dominates over asymmetric synthesis, in particular at the early stages of development of bioactive compounds, where time constraints are a major issue. Generally, the set-up and optimisation of an asymmetric synthesis process requires considerable experimentation, expensive catalysts and auxiliaries, and qualified scientific personnel, often yielding products with insufficient enantiomeric purity. Nevertheless, the recent progress in this area of research might establish asymmetric synthesis as a serious competitor for conventional resolution techniques in the near future.

Currently changing philosophies for the development, production and analytics of chiral compounds, being dominated by the concepts of combinatorial synthesis and high throughput screening technologies, might lead to the need of adapting established enantioseparation techniques. Further advances in the preparative sector will obviously favour environmentally benign protocols for technical processes. In this sense, reduction of waste streams and recycling of solvents and materials will be highly appropriate.

For analytical applications, enantioseparation will keep and even gain a higher importance. Improvements of existing technologies to meet the future demands in terms of speed, parallelism and low costs can be envisaged. Sensor devices, miniaturised systems, chiroptical methods and hyphenated techniques

with highly sensitive detection systems, such as MS might be instrumental in fulfilling the actual expectations. Finally, an enhancement of our still limited knowledge of the molecular principles/mechanisms governing chiral recognition represents a major challenge for the future to choose and create suitable chiral selectors for given enantiomers. The implementation of novel strategies allowing a synergistic interaction between experimental data and molecular modelling may lead to further progress in this field.

In any case, the challenge of understanding chirality, a key to the major secrets of life, will continue fascinating and stimulating future generations of scientists.

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