Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1068 (2005) 3–30
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Review

Chiral separations by capillary electromigration techniques in nonaqueous media
I. Enantioselective nonaqueous capillary electrophoresis

Michael Lämmerhofer*

Christian Doppler Laboratory for Molecular Recognition Materials, Institute of Analytical Chemistry, University of Vienna, Währingerstrasse 38, A-1090 Vienna, Austria

Available online 19 December 2004

Abstract

Enantiomer separations by CE employing nonaqueous conditions are reviewed. The general focus of this article is directed towards solvent effects on chiral recognition and the separation mechanism. After a general discussion of solvent effects on the individual processes involved in CE enantiomer separation, specifics for various selector classes are discussed together with a few applications of enantioselective nonaqueous CE.

Keywords: Electromigration; Electrophoresis; Separation mechanism; Chiral recognition; Solvent effects; Binding constants; Chiral selector; Cyclodextrin; Cyclodextrin derivatives; Single isomer charged cyclodextrins; Chiral counter-ions; Ion-pairing selectors; Dual selector system; Electrolyte additives

Contents

1. Introduction

In the past decade, CE has become an attractive microscale separation technique and has proven its exceptional

* Tel.: +43 1 4277 52323; fax: +43 1 4277 9523.
E-mail address: Michael.Laemmerhofer@univie.ac.at

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.11.091

potential for enantiomer separation [1]. For the separation of the solute enantiomers, diastereomers (indirect approach) or diastereomeric associates (direct approach) are created by reaction with chiral derivatizing agents and the addition of chiral selectors to the background electrolyte (BGE), respectively, which eventually leads to mobility differences of the individual solute stereoisomers. Several benefits such as high efficiency, flexibility of method development, separation speed, minimized organic waste, low costs, capability to be coupled to mass spectrometry are frequently encountered as key advantages of CE enantiomer separation technique and have contributed that it is nowadays introduced in both research and quality control laboratories in the chemical and pharmaceutical industry.

Water of course has been and will be the solvent of first choice, with few exemptions, in CE enantiomer separation due to reasonable solubility of electrolytes and ionized analytes as well, and because it is cheap, non-toxic and non-hazardous, has a high boiling point that is favorable in terms of method precision, and so forth. Moreover, the most prominent selectors, native cyclodextrins (CDs) as well as their neutral and charged derivatives, possess reasonable to excellent solubility in water.

However, it is obvious that a single solvent system will not permit to cover the entire selectivity space principally available for a given solute (selectand, SA)–chiral selector (SO) combination. A wider parameter space might be desirable in many instances. This has been attributed partially by use of hydro-organic mode which certainly expanded the capabilities of CE. But still the dominance of the water properties cannot be completely overcome. Therefore, nonaqueous CE (NACE) attracted some attention since its first introduction by Waldbrohl and Jorgenson [2]. However, it lasted until the mid of the 1990s to be discovered for its utility for enantioselective CE. The first publications on chiral separation by NACE appeared in the literature in 1996 [3–6] and on nonaqueous capillary electrochromatography (NACEC) a few years later in 1999 [7,8] (see Part II of this review).

In general, however, the studies on enantioselective NACE remained limited in absolute numbers in the following years as is underlined by Fig. 1. Not more than four to seven papers per year appeared in the literature dealing with this subject, except of 2000 when the number of enantioselective NACE studies published reached an apex of 11 (Fig. 1a). The relative modest role becomes evident if these numbers are related to the total number of publications on enantioselective CE (Fig. 1b). As can be seen, enantioselective NACE typically contributes not more than 2% to the total chiral CE studies and even in 2000 it was still less than 4%. Overall this situation has its origin in the dominant role of CD selectors and CD-based CE systems in enantioselective CE, and it is not expected that the number of NACE studies will 'explode' in the future. It may also be argued that the lack of studies and knowledge on theory of mobilities and dissociation equilibria in nonaqueous media has discouraged researchers from wider use of nonaqueous conditions. Since such theory has been explored recently in more depth specifically with respect to CE for a variety of polar organic solvents, fundamentals of CE in nonaqueous media are now well described [9–11] and provide a profound basis for proper use of nonaqueous solvents in CE.

Nevertheless, despite the modest numbers of published papers NACE has been convincingly shown to have impact as it certainly expands the feasibilities and potential of CE enantiomer separation for reasons that have been discussed in numerous papers and reviews [12–18]. Analytes not soluble in aqueous or hydro-organic buffers can be analyzed by NACE. Various chiral selectors are poorly soluble in aqueous systems so that the optimal concentrations yielding the highest separation factors cannot be reached. A variety of chiral selectors prefer nonaqueous environment for effective chiral recognition and distinction, and on contrary, do not

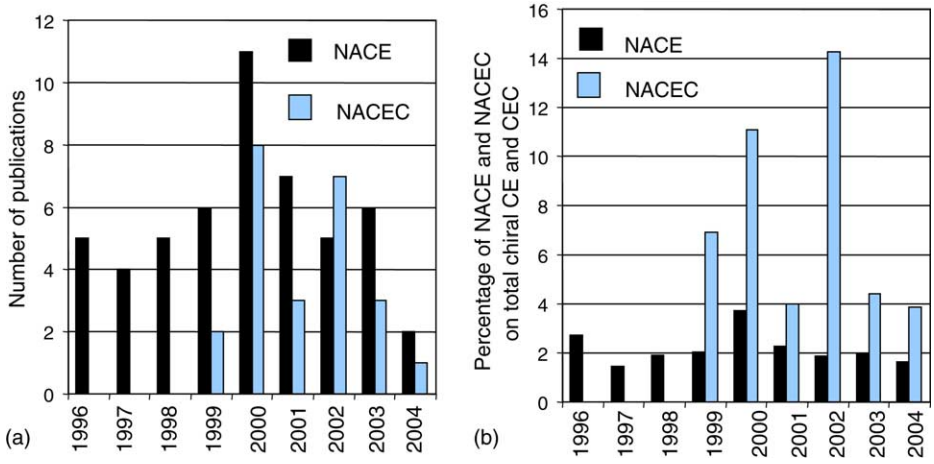

Fig. 1. Number of publications on enantioselective nonaqueous CE (NACE) and CEC (NACEC): (a) absolute numbers, and (b) percentage related to total publications on the respective topics including both aqueous and nonaqueous media. (*Note*: Numbers of publication as referred in the SciFinder Scholar database. This statistics was intended to be comprehensive. However, one or the other nonaqueous separation may be hidden in papers dealing with aqueous CE, which have been performed for reason of comparison or in the course of optimization studies.)

properly work in aqueous media. Such selectors are therefore not common in CE and nonaqueous BGEs could broaden the choice of chiral selectors. Since the spectrum of applicable solvents with distinct physical and chemical properties is tremendously extended in NACE, a drastic gain in versatility is the result. There are considerably more choices for fine-tuning of SO–SA interactions and intrinsic enantioselectivities, for adjusting ionic and effective mobilities as well as minimizing or maximizing electroosmotic flow (EOF) depending on what the given separation requires. As a result there are many more options available for optimization of mobilities, separation factors (enantioselectivities), efficiencies, resolutions and run times. The resulting enhanced flexibility provides a better chance of finding attractive, tailor-made and useful conditions for many analytical problems. Often, low conductivities and resultant low currents are invoked as significant advantages, which enable the use of higher field strengths without Joule heating having a positive effect on run times and efficiencies. The latter argument however has to be assessed critically (see below) [19].

The present article follows a number of previous review articles on enantioselective NACE [15,20] and NACEC [17]. Moreover, a variety of reviews that dealt with the much broader topic of NACE in general included a chapter on chiral separations by NACE as a part of their article [13,14,16,18,21]. Although the primary focus of this publication is more on recent studies in enantioselective NACE and NACEC, some redundancies to the previous review articles may evolve, in order to cover the topic representatively and comprehensively.

2. Concepts of enantioselective nonaqueous capillary electrophoresis

Like in other enantiomer separation technologies two principal concepts are available for the creation of distinctive migration of enantiomers [1]: the indirect and the direct approach. The indirect methodology, in which the SA enantiomers are transformed to diastereomers by derivatization with an enantiomeric chiral derivatizing agent, is less common in CE due to practical disadvantages and occurrence of problems (e.g. enantiomeric impurities of chiral derivatizing agents, kinetic resolution, potential for racemization during derivatization) on the one hand, and simplicity and success of the direct approach on the other hand. Moreover, the perception that diastereomers have identical nominal charge-to-mass ratio and thus will be hardly resolvable by CE may have contributed too to the limited popularity of this approach. However, it has been well documented that the electron distribution in diastereomers and thus p*K* values may be significantly different. In addition, conformational differences of the both diastereomers may result in distinct shape and consequently hydrodynamic radii facilitating their differential migration. For example, the indirect concept has been utilized by Tivesten et al. [22] for the NACE separation of aspartic acid and glutamic acid enantiomers. Asp and Glu were derivatized with *o*-phthaldialdehyde (OPA) and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (TATG) to yield fluorescing diastereomeric isoindole derivatives. Nonaqueous CE was compared to aqueous CE, and it was found that NACE showed different separation behavior compared to aqueous CE, e.g. both diastereomeric pairs of Glu and Asp could be separated by NACE with NMF and tetramethylammonium chloride as supporting electrolyte (R_S for Glu = 0.9, R_S for Asp = 1.6), while there was no separation observed with aqueous conditions. The mobility difference between the diastereomers in this study turned out to be only moderate. If, however, distinct conformational arrangements of the both diastereoisomers are stabilized by stereoselective intramolecular interactions, remarkable mobility differences between diastereomers can be achieved [23]. The intramolecular interactions can be stabilized or destabilized by distinct solvents (see below), and solvent effects are therefore an effective tool to be considered for optimization.

More common, less prone to errors, and more straightforward is the direct approach. In the direct approach a chiral selector is added as additive to the background electrolyte and forms diastereomeric selector–selectand associates. The SO–SA association is driven by intermolecular interactions forming non-covalent bonds (e.g. electrostatic ion–ion, ion–dipole, and dipole–dipole interactions, hydrogen-bonds, π–π interactions, π–cation interactions and so forth) as well as solvophobic interactions. Moreover, entropic contributions such as loss of rotational, translational and conformational degrees of freedom upon complexation need to be considered too. If this complexation occurs stereoselectively, a difference of net migration velocities of the both enantiomers will be created.

Mathematical models such as the mobility difference model (MDM) [24] and the charged resolving agent migration model (CHARM) [25] have been presented to describe on the basis of the underlying equilibria the mobilities and mobility differences or separation factors as a function of the most important experimental variables such as selector concentration (MDM, CHARM) and pH of the BGE (CHARM). Thus, the mobility of an analyte in the electric field can be obtained by the linear combination of the mobilities of each analyte species present in the BGE weighted by their respective molar fractions.

If protonation equilibria are not taken into account and, for sake of simplicity, a fully ionized analyte and 1:1 stoichiometry of complexation is assumed, the effective electrophoretic mobility (μ_{eff}) of the analyte can be expressed as

$$\mu_{\text{eff},(R)} = \frac{\mu_{\text{f}} + \mu_{\text{c}(R)} K_{(R)}[\text{SO}]}{1 + K_{(R)}[\text{SO}]} \quad (1)$$

where μ_{f} and μ_{c} are the mobilities of free and complexed solute and $K_{(R)}$ the equilibrium constant of the comlexation reaction for the (*R*)-enantiomer. The same relationship holds for the (*S*)-enantiomer so that the dependence of the separation factor (α) on the SO concentration [SO] can be

written as

$$\alpha = \frac{\mu_{\mathrm{eff},(R)}}{\mu_{\mathrm{eff},(S)}} = \frac{(\mu_\mathrm{f} + \mu_{\mathrm{c}(R)}K_{(R)}[\mathrm{SO}])}{(\mu_\mathrm{f} + \mu_{\mathrm{c}(S)}K_{(S)}[\mathrm{SO}])}\frac{(1 + K_{(S)}[\mathrm{SO}])}{(1 + K_{(R)}[\mathrm{SO}])} \tag{2a}$$

where $K_{(S)} > K_{(R)}$ and $\mu_{\mathrm{eff},(R)} > \mu_{\mathrm{eff},(S)}$. [*Note*: Electrophoretic mobilities of non-complexed analyte μ_f are identical for (*R*)- and (*S*)-enantiomers.]

The mobility difference model by Wren and Rowe, which was applied to neutral CDs, also assumes identical electrophoretic mobilities for (*R*)- and (*S*)-complexes with CD. Thus the mobility difference $\Delta\mu$ can be expressed as

$$\Delta\mu = \frac{(\mu_\mathrm{f} - \mu_\mathrm{c})(K_{(R)} - K_{(S)})[\mathrm{SO}]}{1 + (K_{(R)} + K_{(S)})[\mathrm{SO}] + K_{(R)}K_{(S)}[\mathrm{SO}]^2} \tag{2b}$$

Both Eqs. (2a) and (2b) clearly emphasize that the concentration of the selector is a primary experimental variable to be optimized, in order to achieve maximal selectivity. Eq. (2a) also indicates that by theory enantiomer separation can be even achieved if the binding constants of (*R*)- and (*S*)-enantiomers are identical e.g. when the mobilities of the both distereomeric associates are significantly different, i.e. $\mu_{\mathrm{C(R)}} \neq \mu_{\mathrm{C(S)}}$. Such a scenario together with literature examples have been recently discussed by Chankvetadze et al. [26]. The optimal selector concentration can be estimated from the magnitude of the binding constants by Eq. (3), and vice versa

$$[\mathrm{SO}]_\mathrm{opt} = \frac{1}{\sqrt{K_{(R)}K_{(S)}}} \tag{3}$$

From the above discussion it becomes also quite clear that one must distinguish between intrinsic enantioselectivities (thermodynamic enantioselectivities, binding selectivities) that can be calculated from the ratio of the binding constants of the (*R*)- and (*S*)-enantiomers as measured in a given BGE for a specific selector–selectand pair, and separation factors (conditional enantioselectivities) that are usually calculated as ratio of effective mobilities (or sometimes also from apparent mobilities) and thus also depend on the employed selector concentration. A more thorough discussion on the fundamentals of CE enantiomer separation is, besides above cited original papers, also given in a recent review by Rizzi [27].

From a practical point of view, resolution (R_S) is the key parameter that needs to be optimized and the common equation of R_S in CZE is given by Eq. (4) [20].

$$R_\mathrm{S} = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\Delta K[\mathrm{SO}]}{1 + (K_{(R)} + K_{(S)})[\mathrm{SO}] + K_{(R)}K_{(S)}[\mathrm{SO}]^2}\right) \times \left(\frac{\mu_\mathrm{f} - \mu_\mathrm{c}}{\mu_\mathrm{avg} + \mu_\mathrm{eo}}\right) \tag{4}$$

wherein μ_avg is the mean of the electrophoretic mobilities of the first and second migrating enantiomer. It shows that besides maximization of mobility difference also a proper design of the electroosmotic flow may have a positive effect on the afforded R_S value. For example, if a codirectional separation process is established (μ_avg and μ_eo having the same sign), elimination of the EOF result in an improved resolution. On contrary, in a counterdirectional separation (opposite sign of μ_avg and μ_eo), the EOF may enhance resolution. The EOF will also exert a profound influence on R_S in experiments, where the capillary is only partially filled with the selector (separation zone shorter than the effective length). In the optimal case, the separation zone should be stationary, while the length of the separation zone will be shortened in case of counterdirectional separation (EOF directed towards inlet end of capillary), which is accompanied by a loss of R_S. If dispersion from inappropriate experimental conditions such as electrokinetic dispersion, Joule heating, wall adsorption, and so forth can be excluded, resolution will increase with the square root of the plate numbers (*N*), which is directly proportional to the ratio of mobility and diffusion coefficient (μ/D) in the respective solvent (it represents the peak dispersion contribution due to longitudinal diffusion) [19]. A maximum of *N* is obtained at infinite dilution, but *N* decreases with increasing ionic strengths. Unfortunately, as was shown by Kenndler and coworkers the decrease is more pronounced for MeOH and ACN than water so that at a given finite ionic strength the both organic solvents always showed stronger peak broadening from longitudinal diffusion and thus lower plate numbers than a corresponding aqueous BGE.

Hence a positive solvent effect in NACE compared to aqueous CE will mainly be observed when the mobility difference (i.e. the selectivity) term is favorably altered in the nonaqueous media. This can be achieved by the delicate influence of selector–solute interactions (see below).

3. Solvents and solvent effects

A brief summary of the physical and chemical properties of the solvents discussed in this article is given in Table 1 [28]. It can be seen that the compiled solvents vary their properties in a wide range leading to the expectation that they exert significantly different effects on the processes being of relevance in enantioselective NACE.

Most frequently used solvents in NACE include amide type solvents (such as FA, NMF, DMF), alcohols (MeOH, EtOH, 2-PrOH) and acetonitrile (ACN) or mixtures thereof. Some of these solvents are already sufficiently conductive per se, even without addition of electrolytes, e.g. amide type solvents like FA due to its bisprotic nature and autoprotolysis and/or electrolytic impurities stemming from decomposition. They can be used without supporting electrolyte. The other solvents mentioned, on the other hand, readily dissolve electrolytes, buffers or simply organic acids and bases, which support ion conduction in the nonaqueous media. At this point it must be mentioned that the concept of buffers in NACE is critical and often ill-defined. Shifts in pK_a and pH scale as a result of altered solubilization of ionic species in distinct organic solvents and thus changed dissociation

behavior in nonaqueous BGEs are resulting. Therefore, the concept of buffer and pH adjustment in NACE needs reconsideration, as was pointed out by Porras and Kenndler recently, and some guidelines have been suggested [9,11]. It is self-explaining that altered protonation equilibria will have a decisive influence on electrophoretic parameters and often also analyte–selector interactions.

Recently also the addition of more uncommon solvents like 1,2-dichloroethane (DCE) and dichloromethane (DCM) has been tested, the latter though being of limited value due to its low boiling point. Such solvents of course can only be used in NACE as mixture with more polar solvents, because of their low dielectric constants. The dielectric constant is the critical solvent parameter in NACE and it must be sufficiently high to allow ionic dissociation to a large or full extent [28]. Solvents with $\varepsilon > 30$ are supposed to permit complete dissociation of electrolytes, while below ε of 10 (like DCM) no dissociation occurs. In the intermediate range (e.g. EtOH, 2-PrOH) partial dissociation of electrolytes may be accompanied by extensive ion-pairing with, e.g. electrolytes, which reduces mobilities, or ionic selectors, which would be ideal conditions for ion-pairing selectors.

The understanding of solvent-induced alterations of the various processes involved in NACE enantiomer separation, viz. the solvent effect on electrophoretic and electroosmotic mobilities and underlying complexation equilibria (analyte–selector interactions), is of prime importance for the proper design of NACE enantiomer separation methods. Through the intricate relationships of these individual processes a complex interplay of effects upon solvent exchange will be the result which is hard to predict and troublesome to deconvolute. The difficulty to forecast solvent effects in CE enantiomer separation arises mainly from the fact that normally only diffuse knowledge of chiral recognition mechanisms exists, which holds in particular for the forces that are active in a stereoselective manner. On contrary, the theory and experimental behavior of electrophoretic mobilities [10] and EOF [29–31] in nonaqueous solvents is now well established and easier to estimate.

Electrophoretic mobilities (μ_{ep}) and electroosmotic mobilities (μ_{eo}) are given by Eqs. (5) and (6), respectively [32].

$$\mu_{ep} = \frac{z_i e}{6\pi\eta r} = \frac{2\varepsilon_0\varepsilon_r\zeta_{ion}}{3\eta} \quad (5)$$

$$\mu_{eo} = \frac{\varepsilon_0\varepsilon_r\zeta_{wall}}{\eta} \quad (6)$$

where z_i is the charge number of the ion, e the electron charge, r the hydrodynamic radius of the ion (i.e. the radius of the solvated ion), η the viscosity of the medium, ε_0 the permittivity of vacuum, ε_r the relative permittivity, ζ_{ion} and ζ_{wall} the electrokinetic potentials (ζ-potentials) of the ion and the capillary wall, respectively. Hence it is seen that the dielectric constant and viscosity, i.e. the ε/η ratio, are the main solvent parameters determining mobilities in distinct solvents.

From Eq. (6) and Table 1 it is seen that NMF and ACN are the solvents that generate the strongest electroosmotic flow, because of their high ε/η ratio. While this would be advantageous with regards to fast NACE analysis if a codirectional separation process is materialized (i.e. when electroosmotic and electrophoretic mobilities are directed towards the same electrode), Eq. (4) predicts a lower resolution for such a case. In a counterdirectional NACE enantiomer separation it is worthwhile or even mandatory to minimize or suppress the EOF, e.g. either by use of solvents with low ε/η ratio [33], additives to the BGE such

Table 1
Characteristics and properties of solvents used for nonaqueous enantiomer separation by CE and CEC and discussed in this report [28]

Solvent	Abbreviation	Dielectric constant (ε) (dimensionless)	Viscosity (η) (mPa s)	ε/η	Polarity (π^*)[a]	$\Delta_V U/V$ (J/cm^3)	DN[b] (kcal mol^{-1})	AN[c] (dimensionless)	α[d]	β[e]
Water	H_2O	78.4	0.89	88	1.09	2294	18.0	54.8	1.17	0.47
Formamide	FA	109.5	3.30	33	0.97	1568	24.0	39.8	0.71	0.48
N-Methylformamide	NMF	182.4	1.65	111	0.90	910	27.0	32.1	0.62	0.80
N,*N*-Dimethylformamide	DMF	36.7	0.80	46	0.88	581	26.6	16.0	0.00	0.69
Methanol	MeOH	32.7	0.55	59	0.60	858	30.0	41.5	0.98	0.66
Ethanol	EtOH	24.6	1.08	23	0.54	676	32.0	37.1	0.86	0.75
1-Propanol	1-PrOH	20.5	1.94	11	0.52	595	30.0	33.7	0.84	0.90
2-Propanol	2-PrOH	19.9	2.04	10	0.48	558	36.0	33.5	0.76	0.84
Acetonitrile	ACN	35.9	0.34	105	0.66	581	14.1	18.9	0.19	0.40
Dimethylsulfoxide	DMSO	46.5	1.99	23	1.00	708	29.8	19.3	0.00	0.76
Dichloromethane	DCM	8.9	0.41	22	0.82	414	1.0	20.4	0.13	0.10
1,2-Dichloroethane	DCE	10.4	0.78	13	0.73	400	0.0	16.7	0.00	0.10
n-Hexane	*n*-Hex	1.9	0.29	6	−0.11	225	0.0	0.0	0.00	0.00

[a] Chemically the polarity is characterized by the sum of all the molecular properties responsible for all the interactions forces between solvent and solute molecules and can be assessed e.g. by the empirical Kamlet and Taft's π^* parameter.
[b] DN, donor number: characterizes the electron pair donicity of a solvent; empirical value determined by Gutmann from the complex formation reaction of the given solvent with antimony pentachloride. 1 cal = 4.184 J.
[c] AN, acceptor number: characterizes the ability of a solvent to form a hydrogen bond by accepting an electron-pair of a donor (Mayer, Gutmann and Gerger).
[d] α: H-bond acidity (Kamlet, Taft): measure of H-bond ability and H-donor properties, respectively (correlated to electron acceptor property AN; $r^2 = 0.9395$).
[e] β: H-bond basicity (Kamlet, Taft): measure of H-bond acceptor property (correlated to electron-pair donor property DN; $r^2 = 0.9415$).

as quaternary ammonium compounds [3,34] or permanently coated capillaries (polyacrylamide [35] or PVA coated capillaries [36]), in order to benefit from a faster analysis.

In a first approximation, the solvent effect on electrophoretic mobilities can be roughly estimated by the empirical Walden's rule which states that the product of absolute mobility (i.e. ion mobility at infinite dilution) and viscosity is constant, if the solvent effect on the size of the solvated ion is negligible [10]. Consequently, ACN and MeOH are expected to yield higher and FA lower mobilities than water. Another important dependency relates the actual mobility (i.e. ion mobility at finite ionic strength I) to the square root of the ionic strength ($\sqrt{I}$). With increase of the ionic strength the mobility will decrease. The slope for the decrease of the mobility with increasing ionic strength is different for the distinct solvents and by theory is expected to be larger in the order ACN > MeOH > water > FA [10]. This means that the decrease of mobilities with ionic strength should be much lower for FA than, e.g. ACN.

A favorable effect of a solvent exchange could be accomplished through maximization of the term $\mu_f - \mu_c$. Since both μ_f and μ_c are subjected to the same trends of mobility variations, it might be argued that a significant maximization of $\mu_f - \mu_c$ will not be attainable. However, if the solvent induces a significant change on the binding mechanism and overall conformation of the selector–analyte complex while the hydrodynamic radius of the free analyte remains unchanged, a significant alteration of $\mu_f - \mu_c$ might result. Such a scenario seems very likely, because different solvents may selectively stabilize or destabilize inter- and intramolecular interactions (see below). Such alterations of inter- and intramolecular interactions upon a solvent exchange may exert considerable changes on the three-dimensional structure of the involved species through conformational changes and thus on their size and shape, but also on electron distributions and thus pK values. The same applies for the (size) selectivity term $\mu_{c,(R)}/\mu_{c,(S)}$. If mobility differences of the both diastereomeric complexes significantly contribute to enantioselectivity or are the only source for enantioselectivity [26] in NACE, a solvent exchange is supposed to affect selectivity through its weakening or strengthening influence on stereoselectively occurring intra- and intermolecular interactions and thus differential conformation, size, shape, and electronic effects on (R)- and (S)-complexes. Current literature, however, lacks profound experimental data that support the above hypothesized solvent effects on $\mu_f - \mu_c$ and $\mu_{c,(R)}/\mu_{c,(S)}$.

Certainly, the most strongly responding influential parameter upon a solvent exchange in NACE will be the binding selectivity term $K_{(R)} - K_{(S)}$ or $K_{(R)}/K_{(S)}$. The binding constant K is related to the free energy of binding (by the well-known relationship), which decides whether and to what extent association occurs and whether this process is endothermic or exothermic. This of course is largely solvent dependent, because the energy balance over several incremental contributions is the determining factor, i.e. solute–selector interactions but also solute–solvent, selector–solvent, as well as internal changes in the solute, the selector and the solvent have to be considered. Obviously solvation processes of selector, solute and selector–solute associates play a major role, and they are characterized by the free energy of solvation, which is essentially a result of the three processes: cavitation, dispersion–repulsion (Van der Waals forces), electrostatics [28,37]. Cavitation is related to the formation of a cavity large enough to accommodate the solute. Since cohesive forces between solvent molecules must be broken, it is typically energetically unfavorable and must be compensated for by the other contributions. Once a cavity is formed dispersive interactions between the solute and the solvent come into force, while repulsion between solvent–solute is weaker so that a positive contribution to solvation is resulting. Finally electrostatic interactions are activated. The net energy balance over these partial processes determines the Gibbs free energy of solvation and eventually also binding strength.

From a more practical point of view, the effect of solvents on the binding and chiral recognition properties may be explained by the strength of the individual solute–selector interactions in the distinct solvents and their interference with the involved intermolecular interactions, respectively. Bowser et al. [21] presented a thorough discussion on the strength of solute–additive interactions in NACE, (mainly based on the linear solvation energy relationship). A distinction was made between the effects on solvophobic interactions, electrostatic interactions (including ion–ion, ion–dipole and dipole–dipole interactions), and donor–acceptor interactions (including hydrogen bonding). The strength of solvophobic interactions is directly related to the strength of solvent–solvent interactions. They are strong in the polar solvents such as water and FA and can be characterized by the cohesive energy density $\Delta_V U/V$ [28], i.e. the energy that has to be put into a system per unit volume to transfer all solvent molecules in this volume into the gas state. It represents the energy that is required to disrupt all the solvent–solvent interactions. If they are strong, solvation of a solute that does not interact with the solvent is energetically unfavorable. In such a case, association of the solute with a selector having appropriate binding sites for the solute is energetically favorable resulting in a considerable binding strength. The interaction of hydrophobic solutes with cyclodextrin in aqueous system is an example for strong solvophobic interactions. As can be seen from the cohesive energy density $\Delta_V U/V$ values in Table 1, solvophobic interactions are strongest in water, while they are significantly weaker in FA. Electrolytes, e.g. such as phosphate may exert a kind of salting out effect and thereby strengthen such solvophobic interactions. Weak solvophobic interactions may also exist in NMF, while they are even weaker or will not take place in other nonaqueous solvents.

On contrary, the strength of electrostatic interactions including ion–ion, ion–dipole and dipole–dipole interactions is inversely related to the dielectric constant ε of the medium. Hence these interactions are weak in polar solvents such as NMF, FA and water, and are strengthened when the dielectric constant gets lower (see Table 1). Favorable solvents for

electrostatic interactions are DCM, DCE and alcohols. The electrolytes dissolved in the BGE on the other hand may also influence the strength of electrostatic interactions. They exert a shielding or competitive effect so that with increasing ionic strength electrostatic interactions in particular ionic interactions become actually weaker.

The effect of solvents on donor–acceptor interactions such as hydrogen bonding, metal chelation and so forth can be explained by competition of the donor or acceptor functions of the solvents with donor–acceptor interaction between solute and selector, and are often characterized by the donor number DN and acceptor number AN, respectively (Table 1). Protic solvents such as H_2O, MeOH, FA, EtOH have strong acceptor qualities (AN declines from H_2O to EtOH) and therefore unfavorably interfere with e-donor–acceptor interactions between selector and analyte. They act in particular (due to their strong H-bond acidity α) as competitive H-bond donors, whereby the competitive effect of MeOH, FA, and EtOH is less than that of water. On the other hand, alcohols like 2-PrOH, EtOH, MeOH (DN drops in this order) have pronounced e-donor properties which are much stronger than those in water and therefore may serve as stronger competitors with regards to H-bond basicity β. Likewise they interfere with donor–acceptor interactions between solute and selectors. Overall, the H-bonding strength or propensity in various solvents appears to rise approximately in the order H_2O < MeOH < EtOH < 2-PrOH < FA < NMF, and thus the interfering competitive effect will decline in the reversed order (see Table 1).

While the strength of individual interaction forces in various solvents may provide a rough guess of the available binding strength which results as the sum of all contributions, information on the stereoselectivity cannot be derived directly therefrom. Strong binding does not necessarily implicate high enantiorecognition capability. If the primary interaction force becomes too strong, it has most often a negative effect on stereoselectivity. This may be related to non-stereoselective occurrence of non-balanced dominating primary interactions. A proper balance of the involved intermolecular SO–SA interactions seems to be favorable in any case, and due to broadening of the spectrum of available solvents NACE certainly offers more options in this respect so that the probability of a successful enantiomer separation may rise considerably compared to aqueous CE. In reality, however, the broad range of solvents' properties has usually not been exploited.

Aside of these solvents effects on the separation process itself, also other analysis parameters such as detection or injection demand certain requirements on the employed media. For example, it is obvious and self-explaining that for UV detection the transmittance of the solvent at the detection wavelength employed has to be considered, in order to reach the detection limits necessary for a sensitive analysis. In this context, it has to be borne in mind that in enantioselective analysis the quantitation of a minor enantiomeric purity present in the sample, e.g. at the concentration level of 1% or even as low as 0.1% represents the real case. This may be challenging if for example amide type solvents are used that are far beyond optimum with regards to detection sensitivity at low wavelength. ACN, MeOH and water behave much better in this respect. Moreover, it has also to be pointed out that a variety of buffers used in NACE such as acetate or formate buffers exhibit reasonable UV absorbance at low wavelengths. Their UV cut-off is about 230 nm. On contrary, phosphate buffer has a lower UV cut-off but lacks reasonable solubility in nonaqueous media such as ACN or EtOH. However, Vigh and coworkers could demonstrate that phosphate buffer displays satisfactory solubility in MeOH at least up to concentrations of about 25 mM (see below). For on-line electrospray ionisation mass spectrometry (ESI-MS) detection high volatility of the BGE components and low surface tension are desired properties to guarantee a stable electrospray and high ionization efficiency as well as to avoid blockage of the needle. Typical BGEs used in NACE such as organic acids/bases in alcohols or ACN possess satisfactory characteristics in this respect. However, the argument is to some extent devaluated or weakened, because a sheath liquid interface that supports a stable current is admixed which can be composed such that the appropriate spray properties are obtained. Therefore, also the argument of beneficial surface tension of many organic solvents as compared to aqueous BGEs is largely set off.

Another practical aspect is related to injection. If it comes to the analysis of enantiomeric impurities in the 0.1% level, sample stacking for enrichment and focussing is often required [38]. A high pre-concentration factor by sample stacking is however more difficult to realize in NACE due to the relatively lower conductivity difference of the more conductive nonaqueous BGE and the lower conducting sample (matrix) zone. Therefore, the stacking effect is supposed to be less pronounced in NACE compared to aqueous CE so that the stacking capabilities can be really limited in NACE. Another problem may appear during method validation. The lower boiling point and higher vapor pressure of most nonaqueous solvents as compared to aqueous media may lead to worse repeatabilities and intra-assay and inter-day precisions, respectively. Thermostatization (cooling) of the sample tray and of the capillary can easily help to solve this problem and is therefore recommended in such cases.

4. Selectors

Many of the aspects and characteristics of NACE are related to the class of selectors used. In the following we discuss therefore specific issues that concern a given class of selector. From a molecular recognition point of view, the selectors that are most frequently used in enantioselective NACE may be classified into the following distinct categories: (i) selectors with inclusion complexation capabilities (mainly native cyclodextrins, CDs, and neutral derivatives), (ii) selectors for ion-pair formation (chiral counter-ions), and (iii) selectors acting by a combination of both (charged cyclodextrin deriva-

tives and chiral crown ether with carboxylic functions). In addition, (iv) chiral chelating agents have been utilized for NACE (although this concept owing to solubility limitations of metal ions is of minor importance for NACE) as well as (v) a combination of various selectors (charged CDs as well as ion-pair selectors). In the subsequent discussion we largely adhere to this classification, however, for didactic reasons we partly changed the order of appearance. A collection of the NACE enantiomer separations using these selectors is given in Table 2.

4.1. Native cyclodextrins

Cyclodextrins (CDs) as the most prominent selectors in enantioselective CE have been early investigated for their enantiomer separation capabilities in NACE, and such NACE studies have been performed with native β-CD and γ-CD, but not with α-CDs yet. This can be conveniently explained by the common understanding of the size-fit concept of inclusion complexation which predicts higher affinity and also more effective chiral recognition capability for the host–guest pairs with the best match of the size of hydrophobic portions of the solute and the cavity dimension of the employed CD which for most drugs is usually afforded with β-CDs and γ-CDs, respectively, depending on the extensions and volume of the included hydrophobic moieties.

As can be seen from Table 2, amide-type solvents such as formamide (FA), *N*-methylformamide (NMF) and dimethylformamide (DMF) are preferred in NACE with native CD selectors [3,4,34,39,40]. These solvents combine a number of characteristics that make them attractive for CD based enantiomer separations in NACE. While the solubility of CDs and in particular β-CD in aqueous solution is really limited (e.g. β-CD in water 16.3 mM at 25 °C and γ-CD by a factor of 10 higher [72]), the amide type solvents such as FA, NMF, DMF exhibit much better solubility. For example, β-CD can be readily dissolved in NMF at concentrations >700 mM which is about 40× better soluble than in water [4]. This fact may in some instances help to reach the optimal selector concentration and therefore the best separation (see below).

NMF and FA have been used either without electrolytes [4], with citrate/Tris buffer [3,34] or using salts such as NaCl [39,40] that do not interact with the CD at low ionic strength. Along this line, the salt free use of NMF has been comparatively investigated with NaCl-doped NMF-based BGE by Valko et al. [4]. Stronger EOF and faster migration were observed without electrolytes, but (baseline) stability and reproducibilities of the system were greatly improved with 10 mM NaCl. The generated current of both the BGE without electrolyte and the one composed of 10 mM NaCl in NMF was significantly lower (5–9 μA for the latter) than of a aqueous BGE consisting of 50 mM phosphate–100 mM borate (pH 9.0) that produced a current of 115 μA and was typically used to achieve comparable separations. It is obvious that such a high current is not optimal, because it may hamper efficiencies as a result of non-dissipated heat and temperature gradients across the capillary. Moreover, the NMF-based salt-free and salt-supported BGEs produced extremely fast separations.

As indicated above and well known, the typical preferential molecular recognition mechanism of hydrophobic guest molecules by CDs in common polar media such as aqueous solutions involves inclusion complexation driven by weak interactions. A two-step mechanism has been proposed [73]: (i) penetration of the hydrophobic part of the guest molecule into the CD cavity, and (ii) release of solvent (water) molecules from guest and CD molecules (entropic effect). The combined effect is called the hydrophobic effect, while it is common perception that the process of (i) is contributed or driven by pure van der Waals interaction. It is also quite clear that (iii) hydrophilic interactions with hydroxyl groups at the upper an lower rim (hydrogen bonding, dipole–dipole interactions) of complementary polar moieties in the solute that are properly exposed and spatially oriented may take place and positively contribute to complex stabilities. These combined effects and in addition others like conformational changes upon complexation (e.g. induced fit) all contribute to sometimes extraordinary complex stabilities even in excess of 10^3 L/mol in aqueous solution. Group contributions to complexation thermodynamics especially for aqueous media have been discussed previously in detail, while only limited information in this respect is available for nonaqueous solvents [73].

This general molecular recognition mechanism driven by solvophobic interactions between hydrophobic part of the guest molecule and the internal surface of the CD cavity may be prevailing also in a variety of organic solvents in particular those with high polarity such as FA and NMF. Nevertheless, solvophobic interactions will be weakened in such media due to less strong solvent–solvent interactions compared to water (accompanied by less unfavorable solvation energy of hydrophobic solvents, and thus less energy gain upon association of the solute with CD), as is indicated by their lower cohesion energy densities $\Delta_V U/V$ (Table 1). In more apolar solvents with lower dielectric constants and lower cohesion energies on the other hand polar interactions such as hydrogen bonding and/or dipole–dipole interactions may become the driving forces for association and inclusion complexation may loose its importance. In such cases, binding may occur at the polar outside of the CD at the lower and upper rims. Such molecular recognition mechanism was proposed by Armstrong et al. [74] for normal-phase liquid chromatography with CD-based chiral stationary phases and hexane or heptane based eluents, but such a binding is most likely not strong enough with polar organic solvents typically employed in NACE.

Weakened solvophobic interactions are supposed to translate into lower binding strengths. This has been verified by Wang and Khaledi, who investigated systematically the change of the association constants of some basic pharmaceuticals (mianserin, trimipramine, thioridazine) with neutral native β-CD in various media using a mixture of 50 mM citric acid and 25 mM Tris as electrolytes. Binding constants for

Table 2
Summary of chiral separations by NACE

Selector (comments)	Solvents	Electrolytes	Selectands	Reference
Macrocyclic selectors with inclusion complexation capabilities (cyclodextrin selectors, crown ethers)				
β-Cyclodextrin (β-CD) (80 mM)	NMF		Dns-amino acids	[4]
β-CD (1–10 mM in FA; 5–100 mM in NMF)	FA, NMF	10 mM NaCl	Dns-amino acids	[39]
β-CD	NMF	10 mM NaCl	Dns-amiono acids	[40]
β-CD	FA	100 mM Tris, 150 mM citric acid, 5–15% (v/v) TEA	Bepridil, ondansetron, pinacidil	[34]
β-CD, γ-CD, methyl-β-CD (Me-β-CD), hydroxypropyl-β-CD (HP-β-CD), sulfated β-CD (S-β-CD)	FA, NMF, DMF, water, 6 M urea in water	50–200 mM citric acid and 25–50 mM Tris	Trimipramine, mianserin, thioridazine, and other basic compounds	[3]
Hydroxyethyl-β-CD (HE-β-CD), HP-β-CD, Me-β-CD (200 mM)	MeOH, FA, FA-ACN (1:2, v/v)	25 mM ammonium acetate/1 M acetic acid and 25 mM citric acid/12.5 mM Tris	1,3,4-Thiadiazine and 1,3,4-selenadiazine derivatives	[41]
Monomers and oligomers ($n = 2$–4) of norbornene-derivatized β-CDs (up to 4%, w/v)	NMF	35 mM sodium bicarbonate in NMF	Dns-amino acids	[42]
Carboxymethyl-β-CD (CM-β-CD) (ds ∼ 3) (75 mM)	FA		Ephedrine, amphetamine, metoprolol, dipipanone, methadone, and propranolol	[13]
S-β–CD	FA, NMF	100 mM Tris–150 mM citric acid	Various basic drugs	[43]
Quaternary ammonium β-CD (QA-β-CD)	FA, NMF, MeOH, DMSO	100 mM Tris–100 mM acetic acid	Profens (NSAIDs), Dns-amino acids, FMOC-amino acids, 1,1′-binaphthyl-2,2′-diyl hydrogen phosphate	[44]
Heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-CD (HDMS-β-CD)	MeOH	25 mM phosphoric acid and 12.5 mM NaOH	Epinephrine, isoproterenol, metaproterenol, oxyphencyclimine, propranolol	[45]
HDMS-β-CD (0–40 mM)	MeOH	20 mM phosphoric acid and 10 mM NaOH	Various basic pharmaceuticals	[46]
Heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-CD (HDAS-β-CD)	MeOH	Dichloroacetic acid/triethylamine, 50/25 mM (acidic) and 25/50 mM (basic)	Weak bases	[47]
Heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-CD (HDAS-β-CD)	MeOH	10 mM ammonium formate/0.75 M formic acid	Salbutamol (in urine)	[48]
Octakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-γ-CD (ODAS-γ-CD) (variable concentrations; 10 mM)	MeOH	25 mM H_3PO_4/12.5 mM NaOH	Weak bases (alprenolol, salbutamol, metoprolol, methadone)	[49]
ODAS-γ-CD (variable concentrations; 0–45 mM)	MeOH	25 mM H_3PO_4/12.5 mM NaOH	Weakly basic pharmaceuticals	[50]
Octakis(2,3-di-*O*-methyl-6-*O*-sulfo)-γ-CD (ODMS-γ-CD) (0–40 mM)	MeOH	20 mM phosphoric acid and 10 mM NaOH	Various basic pharmaceuticals	[51]
HDAS-β-CD (as heptakis tetrabutylammonium salt) (2–10 mM)	ACN	50 mM methanesulfonic acid/21 mM TEA	Various basic pharmaceuticals	[52]
(+)-18-Crown-6-tetracarboxylic acid	FA	With and without tetra(*n*-butyl)ammoniumperchlorate as supporting electrolyte	8 Primary amines (aromatic amines, amino acids, aminoalcohols)	[53]
Ion-pair NACE				
Acidic counter-ions				
Camphorsulfonic acid (CSA), (*R*) or (*S*)	ACN	NaOH	β-Blockers, salbutamol, ephedrine (1,2-aminoalcohols)	[5]
(−)-2,3:4,6-Di-*O*-isopropylidene-2-keto-L-gulonic acid (DIKGA)	MeOH	NaOH	Amino alcohols, e.g., pronethalol, labetalol and bambuterol	[35]
DIKGA (100 mM)	ACN, Water, 2-PrOH	NaOH, NH_4Ac (various conc.)	Amino alcohols (β-blockers, β-sympathomimetics, ephedrine)	[54]
N-3,5-Dinitrobenzoyl-leucine (DNB-Leu) (10 mM) (*R*) and (*S*)	ACN–MeOH (70:30, v/v)	100 mM AcOH, 12.5 mM TEA	*O-tert*-Butylcarbamoyl-mefloquine, pseudoenantiomeric quinine (QN) and quinidine (QD), as well as QN and QD *tert*-butylcarbamates, pseudoenantiomeric and diastereomeric amphoteric amino acid derivatives of QN and QD	[23]

Table 2 (*Continued*)

Selector (comments)	Solvents	Electrolytes	Selectands	Reference
N-Benzoxycarbonylglycyl-(*S*)-proline (ZGP) (50–250 mM)	MeOH and methanol mixed with different proportions of dichloromethane, 1,2-dichloroethane or 2-propanol	Ammonium acetate	Local anesthetic bupivacaine and the β-adrenoceptor blocking agent pindolol	[33]
Basic counter-ions				
Native cinchona alkaloids				
Quinine (2.4 mM)	MeOH	Ammonium acetate (13 mM)	*N*-3,5-Dinitrobenzoyl amino acids, 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate, phthalic acid 1-(1-naphthyl)ethylamide	[6]
Quinine (1–2.5 mM; counter-current technique, CCT)	MeOH, MeOH–EtOH, MeOH–ACN (various ratios)	Acetic acid, octanoic acid, and other organic acids; ammonia	DNB-amino acids	[55]
Quinine, quinidine, cinchonine, cinchonidine (10 mM; CCT)	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 12.5 mM ammonia	DNB-amino acids, DNZ-amino acids, Bz-amino acids	[56,57]
Quinine and/or quinidine derivatives				
1-Methylquininium iodide (10 mM; CCT)	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 12.5 mM ammonia	DNB-amino acids, DNZ-amino acids, Bz-amino acids	[58]
O-tert-Butylcarbamoyl quinine (tBuCQN) and quinidine (tBuCQD) (2.5–10 mM; CCT)	MeOH, MeOH–EtOH, MeOH–ACN (various ratios)	Acetic acid, octanoic acid, and other organic acids; ammonia	DNB-amino acids	[55,56]
tBuCQN (10 mM; CCT)	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 12.5 mM ammonia	DNB-amino acids, DNZ-amino acids, Bz-amino acids	[57]
tBuCQN (10 mM; partial filling technique, PFT)	EtOH–MeOH (60:40, v/v)	100 mM octanoic or acetic acid, 12.5 mM TEA	Amino acids and 1-aminoethanephosphonic acid as DNB, DNP, DNZ, FMOC, Bz derivatives	[59]
tBuCQN (10 mM; PFT)	EtOH–MeOH (60:40, v/v)	100 mM acetic acid, 12.5 mM TEA	Phosphaserine and phosphaisoserine as DNP derivatives	[60]
tBuCQN or tBuCQD (10 mM; PFT)	EtOH–MeOH (60:40, v/v)	100 mM acetic acid, 12.5 mM TEA	1-Amino-2-hydroxypropane phosphonic acid and 2-amino-1-hydroxypropane phosphonic acid as DNP derivatives	[61,62]
tBuCQN (various conc.; PFT)	Various EtOH–MeOH ratios	Various acetic acid and TEA concentrations	Peptides (all-*R*)/(all-*S*) $Ala_{1\text{-}6}$ enantiomers and diastereomers as DNP, DNZ, and DNB derivatives	[36]
tBuCQN (10 mM; on-line FT-IR detection)	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 22 mM NH_4OH	DNB-Leu	[63]
1-Adamantyl carbamoylated quinine (10 mM)	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 12.5 mM ammonia	DNB-amino acids, DNZ-amino acids, Bz-amino acids	[58]
3,4-Dichlorophenylcarbamoylated quinidine (10 mM)	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 12.5 mM ammonia	DNB-amino acids, DNZ-amino acids, Bz-amino acids	[58]
Allyl carbamoylated dihydroquinine and -quinidine (10 mM)	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 12.5 mM ammonia	DNB-amino acids, DNZ-amino acids, Bz-amino acids	[58]
Cyclohexyl and 2,4-dinitrophenyl carbamoylated quinine (10 mM)	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 12.5 mM ammonia	DNB-amino acids (Phe, Leu)	[56]
Cyclohexyl and 2,4-dinitrophenyl carbamoylated quinine (10 mM)	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 12.5 mM ammonia	DNB-amino acids, DNZ-amino acids, Bz-amino acids	[57]
Bis-quinine and bis-quinidine carbamates (dimeric selectors)	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 12.5 mM ammonia	DNB-amino acids, DNZ-amino acids, Bz-amino acids	[64]
Bis-quinine (QN) carbamate, bis-quinidine (QD) carbamate, quinine-quinidine-bis-carbamate (dimeric selectors)			DNB-amino acids, DNZ-amino acids, Bz-amino acids, dichlorprop	[65]
Various quinine and quinidine carbamates	EtOH–MeOH (60:40, v/v)	100 mM octanoic acid, 12.5 mM ammonia	DNB-amino acids, DNZ-amino acids, Bz-amino acids	[66,67]
(*S*)-Atenolol (40 mM)	ACN–MeOH (80:20, v/v)	50 mM AcOH, 0.5 mM TEA	*N*-3,5-Dichlorobenzoyl-, *N*-3,5-dinitrobenzoyl-, and *N*-(4-allyloxy-3,5-dichlorobenzoyl)-1-amino-3-methylbutanephosphonic acid	[68]
Others				
Copper(II) with (*S*)-proline or (*S*)-isoleucine	MeOH	25 mM ammonium acetate and 1 M acetic acid	Eight unmodified amino acids	[69]
OPA/TATG (indirect) (TATG: 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose	NMF	20 mM tetramethylammonium chloride	Asp, Glu	[22]
Combination of selectors				
HDMS-β-CD (10–20 mM) and (*S*)-(+)-camphorsulfonate (10–40 mM)	MeOH	1 M formic acid, or 1 M formic acid and 40 mM NH_4Cl	Various basic pharmaceuticals	[70]
HDMS-β-CD (5–30 mM) and potassium (*S*)-(+)-camphorsulfonate (10–30 mM)	MeOH	0.75 M formic acid	Basic pharmaceuticals (β-blockers, local anesthetics, sympathomimetics)	[71]

these solutes were determined in water, water containing 6 M urea, FA, NMF, and DMF from corrected mobilities (considering the changes of viscosities upon variation of the CD concentrations to eliminate nonlinearities from the dependencies of mobilities on selector concentration). As expected, binding constants (L/mol) dropped several orders of magnitude, e.g. for thioridazine upon change from water ($\sim 10^4$), to 10^3 (in water-urea), to $\sim 10^1$ (in FA), to $\sim 10^0$ (in NMF), to $\sim 10^{-2}$ (in DMF) (the latter value must be assessed critically as it is outside of the range of values that can be measured accurately with CE and hence it appears to be more a rough estimation). It is seen that the trend of decreasing binding strengths coincides largely with decreasing polarity (π^*, see Table 1) and cohesion energy densities, respectively, and indicates that the solvophobic interactions are (partially) disrupted by FA, NMF, and in particular NMF. The behavior of urea can be readily explained by its chaotropic effect, akin to a 'salting in' effect, and its reduction of surface tension, respectively, yielding a weaker solvophobic effect. A similar trend was observed for the other two test solutes. On contrary, intrinsic enantioselectivities (as calculated from the ratio of the binding constants of both enantiomers) did not vary parallel to the trend of binding strengths. While NMF showed no enantioselectivity for thioridazine, it was a favorable solvent for mianserin and trimipramine. On the other side, FA exhibited fairly good enantioselectivities for thioridazine and trimipramine while chiral recognition for mianserin was insufficient. DMF did not at all exhibit enantioselectivity for any of the three test compounds. From these subtle solvent effects on selector–analyte interactions it becomes evident that there is no direct strict relationship between the binding strength and stereoselectivity clearly emphasizing the potential and necessity of exploiting solvent effects to achieve satisfactory separation.

Altered SO–SA interactions and binding strengths have direct implications on observed mobilities and mobility differences, as was thoroughly discussed by Wang and Khaledi [3,20]. Upon complexation of the cationic solutes with the neutral CD the mobility is reduced (due to a larger size of the SO–SA complex); the stronger the binding the steeper the slope of the mobility drop when the selector concentration in the BGE is increased. More important, with the altered binding strength upon a solvent exchange the maximum of the mobility difference is, according to Eq. (2b), shifted. Since separation factors with neutral CDs as selectors are often very small in CE, it is mandatory to optimize the selector concentration of the BGE, in order to find the optimum and afford sufficient resolution between enantiomer peaks. This holds in particular for compounds with elevated binding constants such as obtained, e.g. for thioridazine in water. In such a case, a sharp maximum of $\Delta\mu$ is observed at very low selector concentration (Fig. 2, curve A) and a trial and error optimization may easily fail to find the optimum [3]. The position of the optimum is shifted to higher selector concentrations when media that lead to weaker SO–SA binding are adopted as shown for 6 M urea in aqueous buffer (Fig. 2, curve B) and

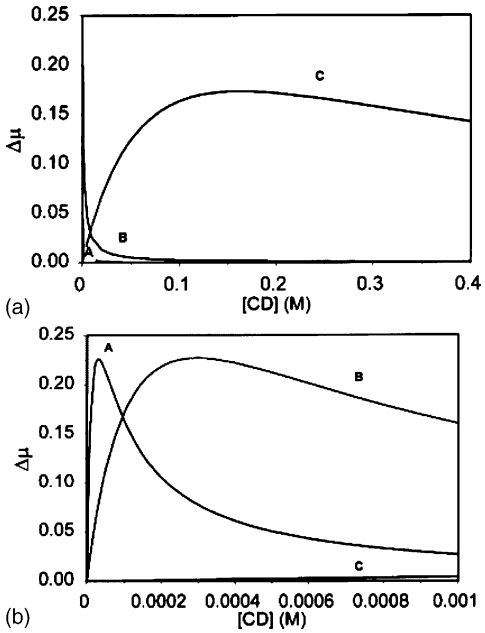

Fig. 2. Dependence of mobility difference $\Delta\mu$ of thioridazine on β-CD selector concentration in different solvent systems. Curve (A) aqueous buffer, (B) 6 M urea in aqueous buffer, and (C) FA. Electrolytes: 50 mM citric acid and 25 mM Tris. Reprinted with permission from [3].

FA (Fig. 2, curve C). For example, in FA average binding constants of 6.5 L/mol for thioridazine yield an optimum selector concentration at 150 mM CD as a flat plateau-like optimum. It is seen that more or less maximal selectivity is obtained over a wide range of selector concentrations. As a consequence, minor deviations from the absolute optimum are not as critical with FA media as compared to aqueous media, and the flater optimum is expected to provide a more robust method.

Dynamic wall coatings with long alkyl-chain quaternary ammonium surfactants or tertiary amine has been found to be a viable, simple and cheap means for manipulation and minimization of electroosmotic flow in aqueous CE, and avoid wall adsorption of the cationic solute species. It has been demonstrated by Wang and Khaledi [3] that the EOF can be controlled to some extent also under nonaqueous conditions (i.e. FA-based BGEs) with tetrabutylammonium (TBA) and tetramethylammonium (TMA) cations. TMA (100 mM) was more effective in the control of EOF than TBA and allowed baseline separation of trimipramine that was only partially separated without TMA and with TBA, which competitively interfered with trimipramine inclusion into the β-CD cavity. Unlike with aqueous conditions, EOF could not be reversed with either one of the quaternary ammonium ions. One hundred millimolar tetramethylammonium salt showed the best result, but β-CD needed to be added in excess (250 mM), since also the tetramethylammonium ion apparently competed for binding to the cavity. With the same goal triethylamine (5–15%, v/v) has been used as additive for reduction of EOF and adsorption of the basic solutes to the capillary wall [34]. In both studies, the separation slowed down but a

favorable effect on R_S was achieved, as expected, with the dynamic coating.

β-CD was successful for NACE enantiomer separation of cationic solutes and anionic analytes likewise. At about the same time as Wang and Khaledi, Valko et al. focussed their studies on the resolution and investigation of complexation of anionic dansyl (Dns) amino acids with β-CD as selector in 10 mM NaCl-doped NMF as electrolyte [4,39,40]. They found a similar trend regarding binding strengths in non-aqueous media. While optimum selector concentrations can be hardly reached under aqueous conditions due to limited β-CD solubility, they were due to its excellent solubilization capacity for β-CD easily reached with NMF, despite its complex destabilizing effect relative to aqueous BGEs and resultant shift of the optimum to higher concentrations. In NMF, association constants between 2 and 13 L/mol were determined for nine Dns-amino acids [40], well in the range that was predicted from the optimum selector concentrations (∼175 mM) deduced from the mobility difference versus β-CD concentration dependencies of a previous study [4]. Conversely, they were reported to be one to two orders of magnitude higher, when they were measured in aqueous solution [40]. The decline of binding strengths in the nonaqueous medium compared to aqueous BGE is again in line with the common understanding of weaker solvophobic interactions that drive inclusion complexation of the dansyl group into the β-CD cavity. Intrinsic enantioselectivities ($K_{(R)}/K_{(S)}$) were between 1.03 (Dns-Trp) and 1.70 (Dns-Glu).

Nice baseline separations of all 12 investigated compounds were accomplished in the counterdirectional separation with the positive polarity mode in which the free anionic SA species show countermigration, i.e. in opposite direction to the EOF [39]. In any case, the (*R*)-enantiomer that was stronger bound and spent less time as free form was therefore migrating less in opposite direction and hence appeared first in the detector. The counterdirectional electrophoretic mobilities of Dns-amino acids were by a factor of 2 higher in the aqueous system owing to the enhanced viscosity of NMF (which conforms roughly with the Waldens rule). Overall, elution of the anionic solutes was driven by EOF, and hence a high electroosmotic flow (note the favorable ε/η ratio of NMF; see Table 1) accelerated the analysis in this separation arrangement (positive polarity mode). Since electroosmotic mobilities were strong and nearly identical in both of the media and significantly higher than the countercurrent electrophoretic mobilites [4], relatively fast separations could be achieved in both aqueous and also nonaqueous NMF-based BGEs.

Valko et al. [39] compared also the effect of different amide solvents, viz. FA and NMF and mixtures thereof, on the separation of Dns-amino acids. The ratio of electroosmotic mobilities in NMF and FA was 3.287 (indicating a lower dielectric/viscosity ratio in FA than NMF) which corresponds well to the ratio of $(\varepsilon_{NMF}/\eta_{NMF})/(\varepsilon_{FA}/\eta_{FA})$. This also shows that the solvent effect of FA and NMF on the ζ-potential of the FS wall is negligible or comparable. As a consequence

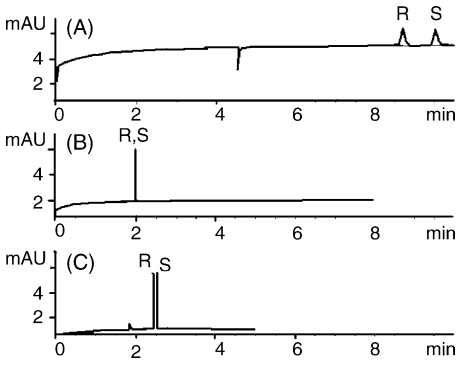

Fig. 3. Enantiomer separation of Dns-Leu with (A) 10 mM β-CD in FA, (B) 10 mM β-CD in NMF, and (C) 100 mM β-CD in NMF. Supporting electrolyte: always 10 mM NaCl. Reprinted with permission from [39].

migration times were significantly shorter with NMF than FA, but the decreased binding strength in the less polar NMF required increased β-CD concentrations (100 mM) as compared to FA (10 mM β-CD provided baseline resolutions) (Fig. 3). Remarkably fast baseline separations within 5 min could be achieved with NMF and 100 mM β-CD despite the counterdirectional setup. Aromatic amino acids' side chain competes for inclusion and hence lower selectivities and resolutions were afforded for these solutes. The acidic side chain, on the other side, resulted in longer migration times due to increase of counter-migration towards the anode. The higher ionic strength of the nonaqueous NMF-based BGE (10 mM NaCl) enabled exploitation of sample stacking phenomena when the sample matrix contained only 1 mM NaCl improving efficiencies up to ∼500,000 m^{-1}.

4.2. Neutral derivatized cyclodextrins

NACE studies with neutral CD derivatives are scarce and only few studies have been reported in the literature employing hydroxypropyl-β-CD (HP-β-CD) and methyl-β-CD (Me-β-CD) [3], or hydroxyethyl-β-CD (HE-β-CD), HP-β-CD and Me-β-CD [41], as well as norbornene-derivatized β-CD based monomers and oligomers [42]. By the derivatization of the hydroxyl groups at the upper and/or lower rim the depth of the CD cavity as well as the quality of the supportive secondary interactions and thus binding may be influenced substantially. The derivatization pattern at the rim may alter the cavity dimensions, introduce steric barriers that support stereoisomer distinction and/or provide additional interaction sites (like H-bond donor–acceptor groups, dipole–dipole interaction sites). Moreover, by the derivatization the flexibility of the CD may be affected as well which was found to be a major determinant factor for the complexation thermodynamics.

Nonaqueous conditions turned out to be beneficial for the separation of 1,3,4-thiadiazine and 1,3,4-selenadiazine derivatives (which were synthesized as potential antituberculostatic agents) using HE-β-CD, HP-β-CD, and Me-β-CD (200 mM) [41]. The limited aqueous solubilities of these solutes necessitated nonaqueous BGEs. As solvents MeOH, FA,

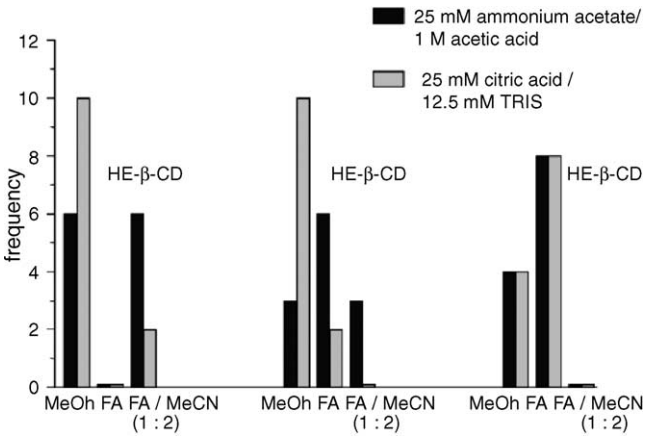

Fig. 4. Frequency of the greatest separation factors of chiral 1,3,4-thiadiazine and 1,3,4-selenadiazine derivatives achieved in each solvent. Conditions: 200 mM of the respective CD derivative. Reprinted with permission from [41].

and FA-ACN mixtures (1:2, v/v) containing 25 mM ammonium acetate/1M acetic acid or 25 mM citric acid–12.5 mM Tris as electrolytes were investigated to optimize separations and find conditions that give satisfactory resolution of all of the 12 target test racemates. In particular, it was tested which of the above specified solvents and solvent mixture, respectively, yields more often the greatest separation factor for the 12 investigated compounds (Figs. 4 and 5). Thereby, it was found out that different CD derivatives prefer different solvents as BGE constituents and also the selection of the electrolytes was critical. The supporting electrolyte (both BGEs charged with excess of acid) appeared to profoundly determine whether a compound is separated into enantiomers or not, suggesting its implication in the chiral recognition mechanism.

None of the individual solvents/CD-derivative/BGE combinations resolved all of the 12 test compounds. Although it is difficult and maybe precarious to draw sound conclusions about chiral recognition mechanisms from data obtained with randomly substituted CD derivatives, the results seem to obey a trend in importance of solvophobic and polar interactions, respectively, for enantiomer separation capability of the respective β-CD derivative. Using Me-β-CD as selector FA yielded more often the greatest separation factor than MeOH or FA/ACN. Solvophobic interactions seem to dominate chiral recognition. In contrast, the preference was clearly shifted to MeOH (in particular with citric/Tris buffer) when the more polar HE-β-CD and HP-β-CD selectors were employed indicating that polar interactions may become important for chiral recognition too. Overall, the more polar selectors seemed to be a better choice for the investigated solutes except for derivatives with voluminous lipophilic residues, where Me-β-CD provided better separations. Since inclusion complexation is presumably still active also for the HE-β-CD and HP-β-CD selectors, it may be inferred from this study that the preference for a particular solvent may vary with a minor change of the chiral recognition mechanism.

Entirely new derivatives of CD have been presented as potential chiral selectors for NACE by Eder et al. [42]. Oligomers (prepared by ring-opening methathesis polymerization, ROMP, degree of polymerization of 2–4) and monomers of norbornen-5-yl carboxylic acid ester based β-CD and norbornen-5-ylmethylsilyl ether-based β-CD derivatives (with up to three norbornene ester and up to five norbornene silylether units) were evaluated for their separation capabilities of Dns-amino acids in NACE with 35 mM sodium hydrogencarbonate in NMF. ROMP allowed the control over the degree of polymerization which resulted in narrower polydispersity than is typically obtained by radical addition reaction so that formation of insoluble polymers could be avoided. Nonaqueous media were required due to insolubility of monomers and oligomers of the selectors in water and other solvents, which were however reasonably soluble in NMF (maximum selector concentration 4%, w/v). Results showed that norbornene silylether derivatives were more effective selectors than norbornene ester derivatives and pentakis(norbornene-5-ylmethylhydroxysiloxyl)-β-CD turned out to be superior to mono(norbornene-5-ylmethylhydroxysiloxyl)-β-CD. This indicates a favorable steric effect or additional solvophobic interactions of the introduced voluminous bulky groups. On the other hand, oligomers were superior or at least equally effective selectors and maximal resolution was found with oligo[pentakis(norbornene-5-ylmethylhydroxysiloxyl)-β-CD]. Through the larger size of the neutral oligomeric selector the mobility difference between free and complexed solutes was maximized which afforded higher mobility difference and therefore better separations (typically a significant improvement of enantioresolution of about 46% could be realized). Although nonaqueous conditions were required owing to solubility constraints, the addition of 5% water in the BGE stabilized the separation system so that R.S.D. of migration times were between 0.7 and 3% R.S.D. Since in such a system the anionic solutes migrate against the cathodic EOF, for all investigated Dns amino acids the (*R*)-enantiomers that show stronger complexation passed the detector first and resolutions up to about 2.5 were obtained. While under comparable conditions all the monomers of the new CD derivatives had R_S in the range of native β-CD, the oligomeric silylether derivatives with five monomer units reportedly showed a significantly improved separation nearly throughout.

4.3. Charged cyclodextrins

Charged cyclodextrins in general and in particular sulfated CDs play an emerging role in CE enantiomer separations. Over the time, they have demonstrated remarkable universality with respect to their application spectrum which includes basic, acidic, amphoteric and neutral compounds as well. For neutral solutes, the charged CDs serve as carrier so that owing to differential binding of the both enantiomers to the CD a mobility difference may arise. For NACE both randomly substituted anionic sulfated β-CD [3,43] and cationic

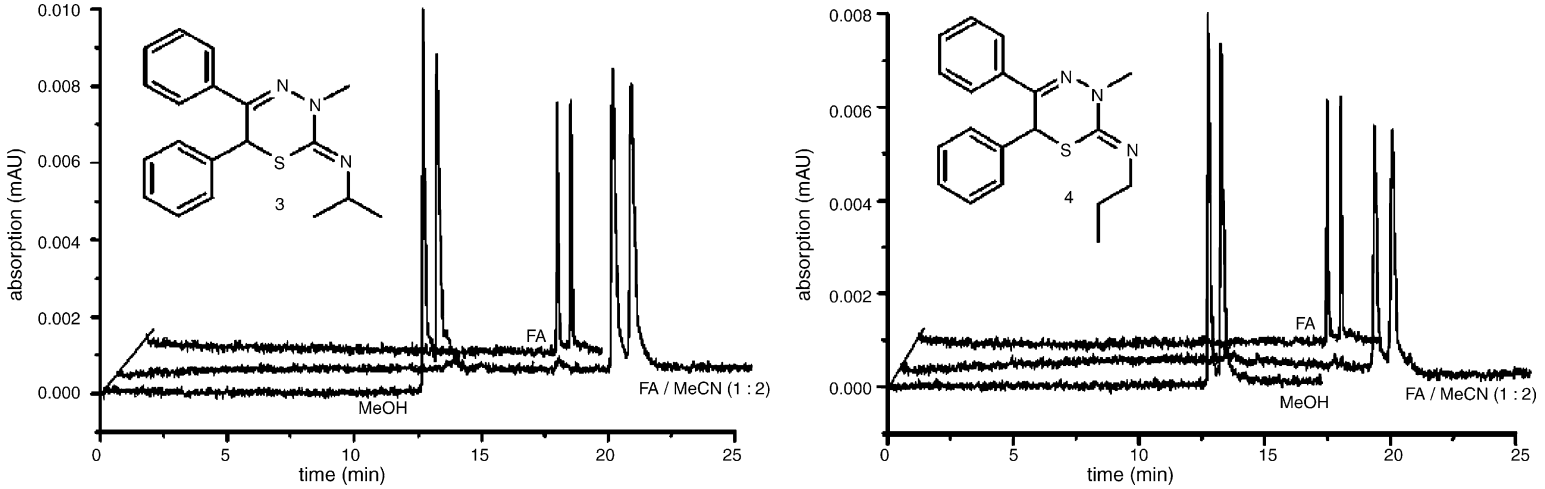

Fig. 5. NACE enantiomer separations of 1,3,4-thiadiazine derivatives with different electrophoretic media. Conditions: 200 mM HE-β-CD in 25 mM citric acid–12.5 mM Tris; 20 kV (MeOH), 30 kV (FA and FA–ACN). Reprinted with permission from [41].

quaternary ammonium-β-CD [44] as well as single isomer CDs [45–47,49–51] have been tested. Studies with randomly substituted sulfated and quaternary ammonium β-CDs in NACE have been thoroughly reviewed previously [20] and are therefore only briefly discussed herein. The latter class of selectors is discussed separately (see below).

These permanently charged selectors (fully ionized also under nonaqueous conditions) may combine inclusion complexation with ion-pairing mechanisms if the solutes carry oppositely charged groups. In fact, owing to the tremendously weakened inclusion complexation in NACE the use of permanently charged CDs so far seems to be reserved for the separation of the enantiomers of oppositely charged solutes for which inclusion complexation is supported by the additional ion-pairing. Thus sulfated β-CDs have been adopted for cationic solutes [3,43] and quaternary ammonium β-CD for anionic solutes [44]. For both randomly substituted S-β-CD (average degree of substitution, ds = 4) and QA-β-CD (ds = 3.8) FA turned out to be the best choice as solvent, while a decrease or loss of selectivity was observed for NMF and DMF as well as some other solvents (e.g. MeOH, ACN). This behavior again points towards a dominance of the inclusion complexation for chiral recognition, while the ionic interaction at the surface of the CD most likely occurs non-stereoselectively. FA possesses reasonable solubility for the permanently charged β-CD derivatives. The maximal concentrations employed were 3.8% (w/v) of S-β-CD and 4.28% (w/v) of QA-β-CD (both corresponding to ∼25 mM). High concentrations of electrolytes which were readily soluble in the highly dielectric medium were typically utilized (such as 100 mM Tris–150 mM citric acid for the S-β-CD system [43] and 100 mM Tris–100 mM acetic acid for the QA-β-CD system [44]), in order to minimize electrokinetic dispersion that might originate from a mismatch of conductivities of sample zone and surrounding BGE.

The risk for occurrence of such an electrokinetic dispersion has been demonstrated to be greater with highly charged β-CDs such as S-β-CD and QA-β-CD. The additional ionic interactions support the inclusion complexation of hydrophobic moieties and lead to a enormous enhancement of binding strength in both aqueous and nonaqueous media as well. Concomitantly, the optimal selector concentration is shifted by orders of magnitude to much lower values, and the optimal selector concentration may be found even at μM concentration levels. While this shift minimizes S-β-CD consumption and thus may help to spare precious selector, it may be accompanied by problems such as electrokinetic dispersion. For example, an aqueous BGE system containing 1.54×10^{-4}% (w/v) sulfated β-CD (∼1 μM) provided fair mobility difference for thioridazine, however, severe peak tailing due to electrodispersion that was attributed to a mobility mismatch of the anionic S-β-CD/thioridazine complex and BGE co-ions [43]. Limitations with regards of generated currents in the aqueous BGE system did not allow to adjust the ionic strength for matching the conductivities. In FA, both binding strength was much lower because of destabilized inclusion complexation and a much lower conductivity was observed, which minimizes the risk of both bandspreading due to electrodispersion and due to Joule heating. In fact, when a FA-based BGE containing 1.54% (w/v) sulfated β-CD (∼10 mM) was tested for the separation of the thioridazine enantiomers, increased electrolyte concentrations could be used as well as a higher voltage was tolerated, which together provided perfect peak shapes as well as vastly improved separation efficiencies, at expense of only slightly longer run times. In addition, sample stacking effects could be exploited by the high ionic strength nonaqueous electrolyte with potential beneficial impact on achieved efficiencies. Both the presence of a higher selector concentration in FA BGE as well as the feasibility for sample stacking are assumed to facilitate the analysis of minor enantiomeric impurities in single enantiomer drugs. Sample stacking enables pre-concentration to reach low quantitation limits for the minor enantiomer peak and the enhanced selector concentration avoids easy overloading and loss of resolution upon injection of elevated sample masses to reach the detection sensitivity required for the minor enantiomer peak [75].

Another favorable aspect of charged CDs has been frequently emphasized. The selectivity term $\mu_f - \mu_c$, representing the mobility alteration of the solute upon complexation, is a proper tool for the maximization of the mobility difference (see Eq. (2b)). In principle, extraordinary mobility differences may be obtained when μ_f and μ_c have opposite signs and this widens the separation window. This possibility of course exists for both separation systems discussed, sulfated β-CDs for cationic solutes and quaternary ammonium β-CD for anionic solutes. Although in principle amenable, a reversal of net migration (compared to free solutes) that may be predicted for elevated selector concentrations and/or strongly binding solutes could not be reached in NACE over the investigated concentration range, neither with S-β-CD (0.154–3.85%, w/v; pH^* 5.1) (mobilities of basic solutes stayed cationic; positive polarity mode) [43] nor with QA-β-CD (up to 4.28%; pH^* 7.5) (mobilities of acids remained anionic; negative polarity mode) [44].

A critical factor shown, e.g. for S-β-CD, to influence the separation was, as expected, the degree of substitution, which may impair quality of enantiomer resolutions. Better selectivities in FA media were mostly obtained with S-β-CD with ds = 4 compared to S-β-CD with ds = 7–11 from a different supplier, which generated also a higher current. Care has therefore to be taken when the source of the selector is changed, because it might lead to poor reproducibility [43]. In contrast, lot-to-lot reproducibility of a given manufacturer is usually sufficiently good. These issues however are not restricted to NACE but were previously demonstrated for aqueous systems too [1].

Twenty-four basic SAs were (partially) separated with anionic S-β-CD (with typical concentrations of the selector between 0.154 and 3.85% (w/v) in FA. The range of applicability was reported to be broadened compared to separations with native β-CD in the nonaqueous mode indicating the beneficial effect of the additional ionic interaction on

chiral recognition and/or improvements of the selectivity term $\mu_f - \mu_c$ [43].

Some specifics which concern the EOF behavior emerged in the course of the study of QA-β-CD as selector [44]. With QA-β-CD the electroosmotic mobilities turned anionic in both aqueous BGE (50 mM Tris–25 mM acetic acid, pH 8) and nonaqueous FA media (100 mM Tris–100 mM acetic acid, pH* 7.5) around concentrations of ∼50 μM and ∼100 μM, and then leveled off at −22 and -3.57×10^{-5} cm^2 V^{-1} s^{-1} under aqueous and nonaqueous conditions, respectively. Obviously, QA-β-CD is coated onto the wall more effectively in water than in FA which, according to the authors, competes as basic protic solvent with the QA-β-CD selector for wall adsorption. The behavior in terms of maximal electroosmotic mobilities achieved, largely follows theory: EOF is larger in water than in FA according to its higher dielectric/viscosity ratio. When the selector concentrations were increased, electrophoretic mobilities of the acidic solutes decreased, but as already mentioned remained anionic over the investigated concentration range. Mobility differences for the acidic test solutes increased continuously with increasing concentration of the selector and leveled off at about 2–4% (w/v) depending on the type of test solutes (Dns-, FMOC-amino acids and profens). Baseline separations of fenoprofen and other profens could be achieved in nonaqueous FA media (100 mM Tris–100 mM acetic acid, pH* 7.5) with QA-β-CD, while no separations were observed with water, NMF, MeOH, DMSO.

4.4. Single isomer charged cyclodextrins

It has already been discussed that for randomly substituted cyclodextrin derivatives the degree of substitution plays a major role on the afforded enantiomer separations [43]. Hence, stringent demands are put on batch-to-batch reproducibility of the degree of substitution and the distribution of the distinct species. The regiospecific reactivity of the different hydroxyls facilitate this so that substitutional heterogeneities are minimized as long as the same synthesis procedure is maintained leading to sufficient batch-to-batch reproducibility of a specific supplier. In contrast, when the synthesis protocol is changed the degree of substitution will most likely vary and reproducibility of the enantiomer separations is no longer guaranteed which may happen when the supplier has been changed [1]. It has also to be considered that when working with a mixture of different selector species the observed effect is the result of the weighted average of each individual selector species. This makes theoretical studies impossible and/or meaningless.

In order to overcome these problems and limitations, single isomer charged CD derivatives, which represent single molecular species and have been synthesized involving selective protection/deprotection and dedicated derivatization of hydroxyls, were developed by Vigh and co-workers [76]. They are fully charged on the lower narrower rim, because all of the primary hydroxy groups have been sulfated leading to permanently multiply charged CD derivatives. The secondary hydroxyls have been used to introduce specific potentially interacting residues and/or modulate solubilities. One of the major goals of the work by Vigh was also to present a unified theory for the migration of various solutes utilizing charged resolving agents and apply such a charged resolving agent migration model (CHARM) [25] to simulate migration, selectivity and resolution for various solutes. Although the predictive power of the CHARM model should not be overestimated, it certainly represented a major advance in the understanding and modeling of enantiomer separations in particular of charged CDs.

Opposed to the randomly substituted CD derivatives described above, the single isomer charged CD derivatives are well defined chiral selectors, now commercially available at isomeric purities >95% with excellent batch-to-batch reproducibility as various derivatives ensuring a broad spectrum of application. Of all the available single isomer charged CD derivatives so far 2,3-dimethylated and 2,3-diacetylated 6-sulfated β- and γ-CDs were investigated in nonaqueous CE, i.e. heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-CD (HDMS-β-CD) [45,46], heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-CD (HDAS-β-CD) [47] as well as their γ-CD analogs octakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-γ-CD (ODAS-γ-CD) [49,50] and octakis(2,3-di-*O*-methyl-6-sulfo)-γ-CD (ODMS-γ-CD) [51]. (*Note*: The nomenclature was sometimes inconsistent, and sulfato and sulfo was used synonymously. We adhere herein to the descriptor sulfo.)

Until recently, in all presented NACE studies with single isomer sulfated CDs [45–47,49–51] MeOH has been employed to prepare the BGEs. Although the solubilities of single isomer sulfated CDs (and their utilized sodium salts, respectively) are lower in acetonitrile and methanol than in FA, NMF and DMF, their better UV-transparency at 216 nm made them, in particular MeOH, the first choice. While randomly sulfated CDs are poorly soluble in this solvent (owing to the presence of a number of primary and secondary hydroxyls) the single isomer sulfated CD derivatives could be readily dissolved at concentrations up to 40 mM (HDMS-β-CD, HDAS-β-CD, and ODAS-γ-CD) [45–47,49,50] and 60 mM (ODMS-γ-CD) [51], respectively. Phosphoric acid (20–25 mM) and NaOH (10–12.5 mM) which were utilized as electrolytes showed apparently decent solubility in methanol and satisfactory buffering capacity. Alternatively, dichloroacetic acid (50 mM) and triethylamine (25 mM) (used for HDAS-β-CD) likewise yielded acidic low pH* BGEs. These electrolyte solutions turned out to be useful for the separation of strongly basic and weakly basic chiral pharmaceuticals which were supposed to be fully protonated under these conditions. The traces of water that were produced during the acid–base equilibrium reaction or were introduced with the concentrated *ortho*-phosphoric acid could even have a favorable effect on the solubilities of the electrolytes and charged selectors as well as on system stability. Within the investigated concentration range of the CD derivatives (<0.06 M), conductivities with the above

specified phosphate-electrolyte was typically below 5 μA and viscosity changes were almost negligible (e.g. within 1% for HDMS-β-CD) [45].

Only the latest work explored acetonitrile as solvent [52], but required to play the trick of using the heptakis tetrabutylammonium salt of the resolving agent, HDAS-β-CD, for solubility reasons. The pH* shift in ACN-based BGE required a modification of the electrolyte system, in order to sufficiently protonate also weakly basic analytes: above specified dichloroacetic acid buffer needed to be substituted by 50 mM methanesulfonic acid–21 mM triethylamine. Under these conditions, the effective mobilities were higher than with corresponding aqueous and methanolic BGEs indicating that the bases were protonated and giving rise to the assumption that due to the low viscosity in this system a fast analysis should be possible.

Like for randomly sulfated β-CD the spectrum of applicability of the single isomer sulfated CD derivatives in NACE was more or less restricted to chiral bases, because of too weak binding of neutral and acidic compounds under adopted nonaqueous conditions (with acidic and basic BGEs as well) and detrimental high normalized, i.e. relative EOF mobilities ($\mu_{eo}/\mu_{eff,2}$) under basic conditions [47]. This matter simplifies the following discussion which always will refer to separation of chiral basic analytes with acidic BGE (strong bases and fully protonated weak bases). Hence, the general equations of CHARM [25] can be simplified to obtain Eqs. (1) and (2a) for the modeling of the effective mobilities and separation factors in dependence of the selector concentration. Effective mobility curves for both enantiomers as simulated by the CHARM model utilizing reasonable values for the various parameters are graphically displayed in Fig. 6a. It is seen that with increasing concentration of the charged CD selector cationic mobilities decrease and become then anionic, first for the stronger binding enantiomer and at higher selector concentration also for the weaker binding enantiomer [45]. Fig. 6b depicts the corresponding representative selectivity curve, which has been divided into five segments by Vincent and Vigh [47]: When the charged CD concentration in the BGE is raised, the α value first slowly and then rapidly increases (segment 1), develops into a discontinuity when the stronger binding enantiomer approaches the CD concentration at which its effective mobility is more or less 0 (segment 2). In this case, the cationic mobilities of the free base are compensated by the anionic mobilities of the complexed base. On contrary, the mobility of the weaker binding enantiomer is still cationic. Then, the α value crosses over to the other side of the discontinuity (segment 3) after the effective mobility of the stronger binding enantiomer has become negative. Once the effective mobility of the weaker binding enantiomer 1 approximates 0, α first approaches 0 from the negative side and then α becomes zero when the effective mobility of enantiomer 1 is 0 (segment 4). Finally, at higher CD concentrations α approximates its limiting value (segment 5) (note the reversed elution order of the enantiomers compared to segment 1).

The behavior was then studied experimentally for various basic pharmaceuticals and the different charged CD derivatives [45–47,50,51]. Experimentally, in NACE with the different dimethylated and diacetylated sulfated CD derivatives only few solutes were identified that displayed all segments, while most of the investigated analytes remained in segments 1 and 2 within the accessible and investigated selector concentration ranges (<0.05 M). For example, in the study of Zhu and Vigh with octakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-γ-cyclodextrin (ODAS-γ-CD) [50] three groups of solutes have been distinguished:

(1) *Weakly binding bases*: The effective mobilities of weakly binding bases remained cationic throughout the investigated selector concentration range (0–45 mM) and consequently selectivities constantly increased with the selector concentration (Fig. 7a and b, curves for chlophedianol).
(2) *Moderately binding bases*: They displayed the entire mobility and selectivity pattern discussed above (Fig. 7, curves for tetrahydropapaveroline). Thus effective mobilities of moderately binding bases exhibited the cross-over from cationic to anionic mobilities. As a consequence, the selectivity curve shows a discontinuity, the location of which obviously depends on the binding strength: the stronger complexation the lower the CD concentration where the discontinuity is observed. In general, α increased as μ_{eff} approached zero, then decreased again as the SO was further increased (Fig. 7b). It is evident that the highest separation factors may be achieved with CD concentrations close to the discontinuity. However, such conditions are obviously unfavorable in terms of separation speed and robustness as well.
(3) *Strongly binding bases*: They reached negative effective mobilities already at extremely low selector concentrations, e.g. 2.5 mM. Except at this very low CD concentration, only segment 5 was obtained, i.e. separation selectivities decreased with higher selector concentrations (Fig. 7, curves for quinine/quinidine). The stronger complexed enantiomer elutes first.

Later, a new selectivity pattern was found in nonaqueous CE with ODAS-γ-CD [49], not seen before in CE with single isomer sulfated CDs neither with aqueous nor aqueous–organic media. The new pattern was exemplified by four weak basic pharmaceuticals in acidic methanolic BGE (see Fig. 8). Characteristic for this pattern is that the selectivity curves go through a maximum before it develops into the discontinuity, which however was not reached by the examples shown in Fig. 8. In this respect, NACE contributed to expand the applicability, the understanding and the theory of CE enantiomer separation. Representative electropherograms are depicted in Fig. 9.

Overall, CE enantiomer separation by the single isomer charged CDs developed into a success story and nonaqueous conditions broadened the scope. Although it is difficult to

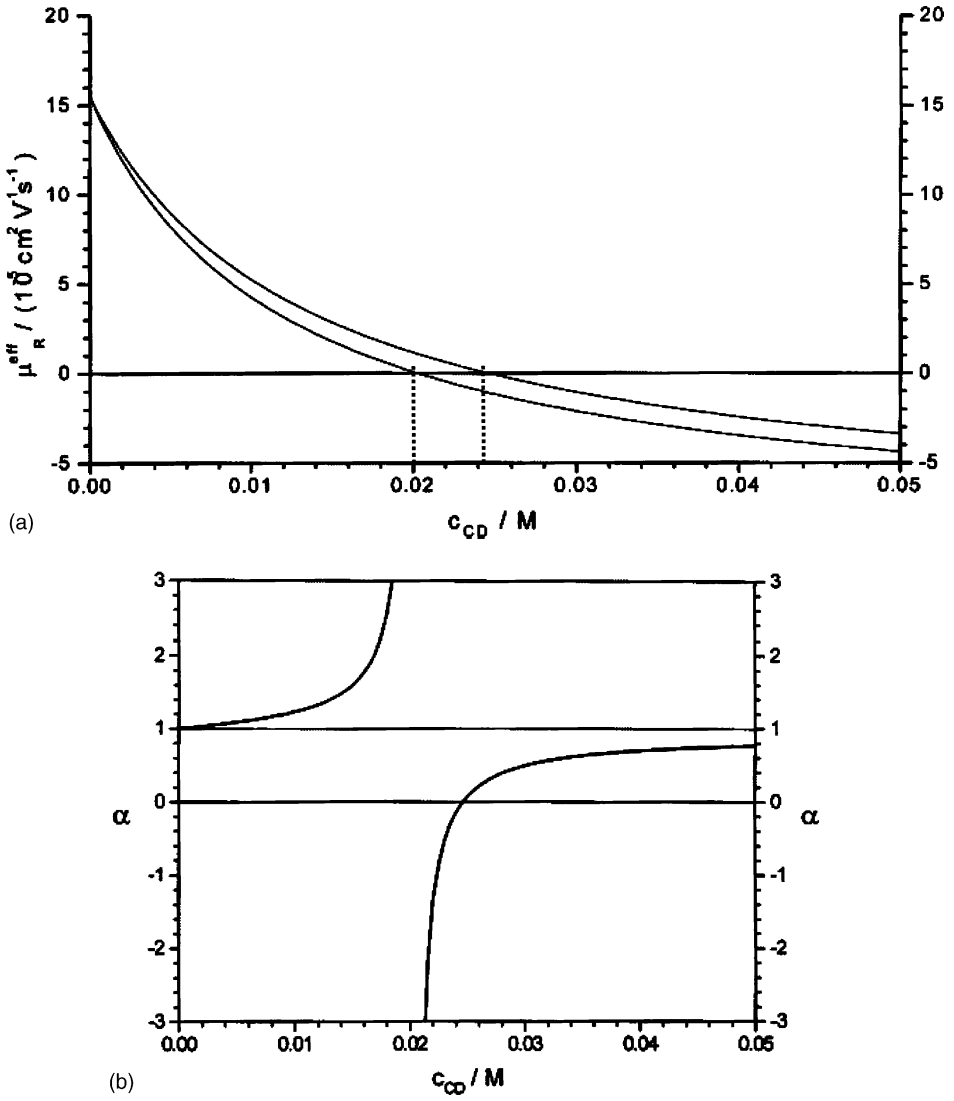

Fig. 6. Effective mobility curves (a) and separation selectivity curves (b) in dependence of the selector concentration for a fully protonated weakly basic enantiomer pair with HDMS-β-CD as resolving agent, calculated according to the CHARM model (Eq. (29) of ref. [25]) using the following parameters: $\mu^0_R = \mu^0_S = 15.5 \times 10^{-5}$ cm^2 V^{-1} s^{-1}, $\mu^0_{RCD} = -8.4 \times 10^{-5}$ cm^2 V^{-1} s^{-1}, $\mu^0_{SCD} = -9.1 \times 10^{-5}$ cm^2 V^{-1} s^{-1}, $K_{RCD} = 75$, $K_{SCD} = 84$. μ^0_R, μ^0_S are the mobilities of free (*R*)- and (*S*)-enantiomers, μ^0_{RCD}, μ^0_{SCD} are the mobilities of the corresponding enantiomers complexed with the CD-derivative, and K_{RCD} and K_{SCD} are the equilibrium constants of the complexation reaction of the both enantiomers with the CD-derivative. Reprinted with permission from [45].

generalize, it seems that for a variety of test solutes NACE holds great promise for faster analysis and often also provides higher resolutions than the aqueous counterpart. The latest work with ACN-based acidic BGE and chiral basic analytes (e.g. atenolol and HDAS-β-CD) showed that, if binding of the base is weak in methanolic BGE and separation fails for that reason, its substitution by ACN may strengthen electrostatic interactions and thus analyte–selector binding in general, leading eventually to successful enantiomer resolution [52]. With the three different solvents H_2O, MeOH, and ACN it is now possible to play with the strength of the involved solvophobic and electrostatic interactions. Hence more variables are offered to suitably balance the involved interactions what may be necessary to achieve reasonable enantioselectivity and fast separation at the same time.

The applicability and advantage of NACE for a real life problem that was previously reserved to normal-phase HPLC because it requires nonaqueous conditions was illustrated by Tacker et al. [46]. The enantiomeric purity of a very hydrophobic intermediate of mitomycin, an azidopyrrolidine derivative which was insoluble even in MeOH–water (1:1), could be determined in the acidic methanolic BGE with HDMS-β-CD within 6 min and the quantity of minor enantiomer amounted to 4.2% in the analyzed batch.

Very recently, NACE using heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-CD (HDAS-β-CD) was adopted for a bioanalytical study, i.e. the determination of salbutamol enantiomers in human urine [48]. Salbutamol is a potent β_2-adrenoceptor agonist that is administered as bronchodilator for the treatment of respiratory diseases. It is partly eliminated renally in native

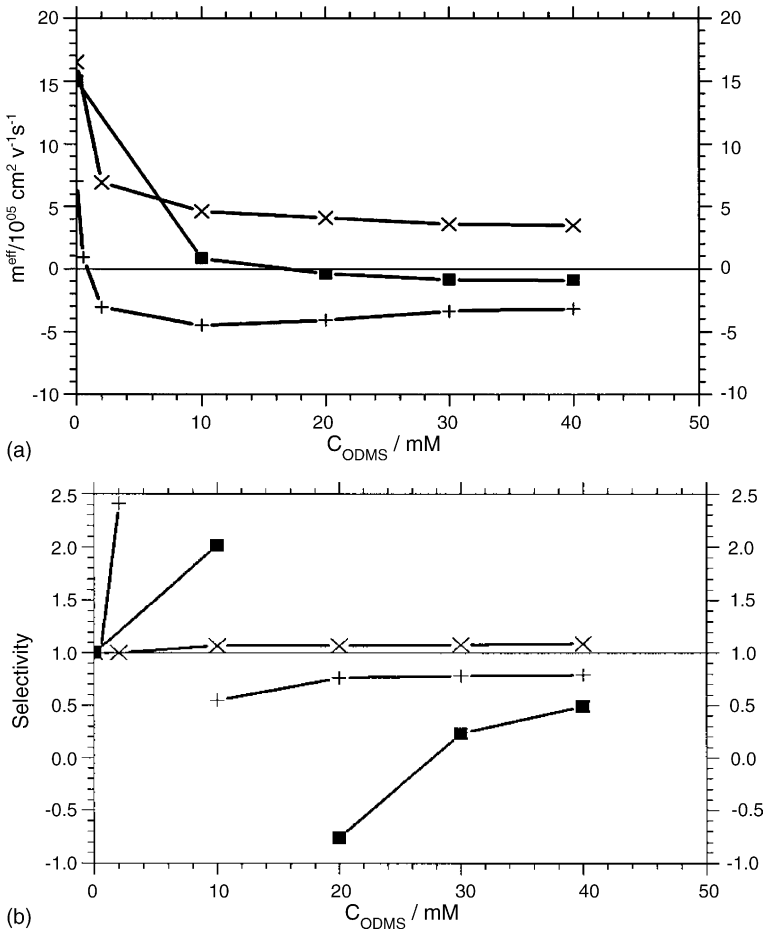

Fig. 7. Typical effective mobility and separation selectivity plots with ODMS-γ-CD as chiral resolving agent. Markers: (×) chlophedianol, (■) tetrahydropapaveroline, (+) quinine/quinidine. Reprinted with permission from [51].

form and partly as 4-*O*-sulfo conjugate. Solid-phase extraction (SPE) was selected as sample preparation technology, because it allows simultaneous extraction, pre-concentration, removal of inorganic ions and other (endogeneous) disturbing interferences of the urine and last but not least a solvent exchange (elution with MeOH containing 2% ammonia). Since traces of water turned out to severely disturb the NACE run (abrupt voltage drops were observed), the eluate was evaporated and reconstituted with MeOH. The application solvent as well as the capacity of the SPE sorbent (which was a mixed-mode reversed-phase/cation-exchange material HCX-3 from Isolute) were found to be extremely critical and therefore optimized. In order to provide a high recovery (75%), 130 mg sorbent needed to be employed for a 1 mL urine sample aliquot and the application solvent was a 0.25 M sodium formate (pH 6). The optimized analysis method was successfully validated in the concentration range between 375 and 7500 ng/mL including linearity ($r^2 > 0.9963$), trueness (relative bias $<\pm 2.6\%$), precision (R.S.D. of repeatability and intermediate precision between 2.6 and 7.7%), accuracy (tested by limits of confidence of bias which did not exceed the acceptance limits for all tested concentration levels), LOD (125 ng/mL), LOQ (375 ng/mL). The validated method was finally applied to a real urine sample collected from an asthmatic subject which contained salbutamol at a concentration close to LOQ.

4.5. Ion-pairing selectors (low-molecular-mass chiral counter-ions)

Exploitation of ion-pairing phenomena in separation science with classical low-molecular-mass counter-ions such as long chain sulfonic acids or quaternary ammonium compounds has a long tradition. Chiral counter-ions on the other hand have already been utilized in the early 1980s for enantiomer separation by enantioselective ion-pair liquid chromatography, e.g. by Pettersson and Schill [77]. More than 15 years later, the first ion-pair CE studies were published by Terabe and coworkers [5] as well as Stalcup and Gahm [6]. From the beginning, nonaqueous conditions turned out to be more suitable because they provide a more friendly environment for the apolar ion-pair that is formed by the selector–analyte association reaction. For sake of clarity we make here a distinction between methods that use acidic chiral counter-ions (**1**–**4**) (see Fig. 10) for the separation of enantiomers of chiral bases [5,23,33,35,54] and basic chiral

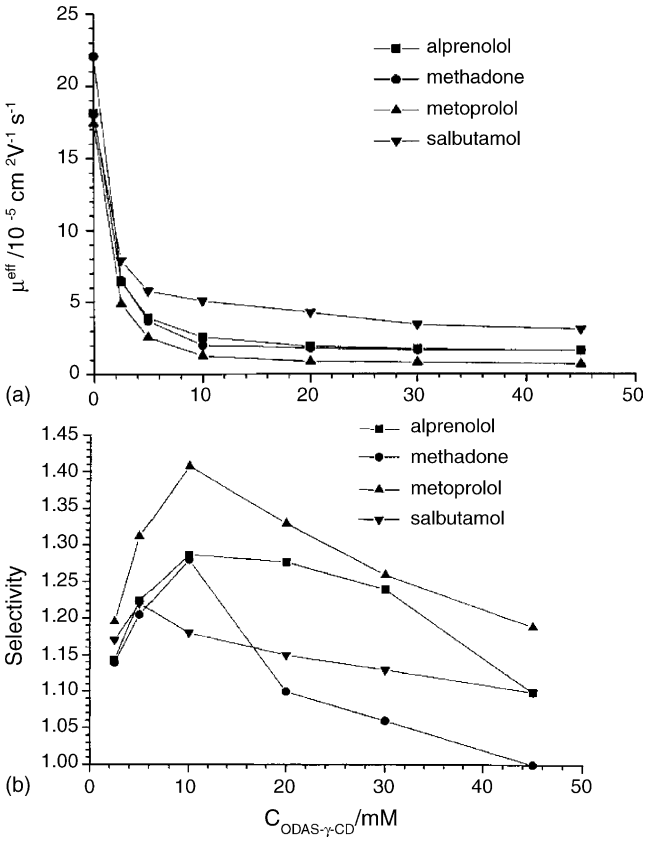

Fig. 8. Effective mobilities (a) and separation factors (b) of weak base analytes with ODAS-γ-CD in methanolic BGE. Reprinted with permission from [49].

counter-ions (**5**–**6**) (Fig. 10) for the separation of chiral acids [6,36,55–59,61–67] (see also Table 2). At this point, it is however noted that from a molecular recognition point of view the roles of analyte and counter-ion are interchangeable so that a single enantiomer of an analyte resolved by a given counter-ion may serve as potential chiral selector to resolve the racemate of the counter-ion. The validity of this reciprocity principle of chiral recognition for ion-pair CE has been demonstrated, e.g. by Zarbl et al. [23,68] and may help in the development of new ion-pair CE separation methods.

Electroneutrality of the formed ion-pairs results in zero electrophoretic mobility of the complexed (ion-paired) species (which move solely with the EOF) so that Eqs. (1) and (2a) can be simplified to [5]:

$$\mu_{\mathrm{eff},(R)} = \frac{\mu_\mathrm{f}}{1 + K_{(R)}[\mathrm{SO}]} \quad \text{and} \quad \mu_{\mathrm{eff},(S)} = \frac{\mu_\mathrm{f}}{1 + K_{(S)}[\mathrm{SO}]} \tag{7}$$

and

$$\alpha = \frac{\mu_{\mathrm{eff},(R)}}{\mu_{\mathrm{eff},(S)}} = \frac{1 + K_{(S)}[\mathrm{SO}]}{1 + K_{(R)}[\mathrm{SO}]} \tag{8}$$

where $K_{(S)} > K_{(R)}$ and $\mu_{\mathrm{eff},(R)} > \mu_{\mathrm{eff},(S)}$. It is noted that due to opposite charge of free SO and uncomplexed analytes their electrophoretic migrations will take place in opposite directions. From Eq. (8) it can be derived that inequality of thermodynamic binding constants of the both enantiomers ($K_{(R)}$ and $K_{(S)}$) (binding selectivity term) is the only source for enantioselectivity in the present systems, while stereoselectivity contributions arising from mobility differences of diastereomeric associates (size selectivity term) are absent due to their zero mobility.

With few exceptions [78,79], enantioselective ion-pair CE has been carried out in nonaqueous media which due to their low dielectric constant and less interference with electrostatic interactions provide a better environment for ion-pair formation than water. Aprotic solvents such as ACN, THF, dioxane, DCM, DCE hence appear to be favorable. However, out of this group solvents only ACN exhibits sufficient solubility for electrolytes. From this viewpoint protic organic solvents such as alcohols seem to be a better choice, and in fact, protic solvents in particular methanol with addition of a certain percentage of a more apolar solvent turned out as the preferred media for ion-pair NACE. For example, Carlsson et al. [35] used 25% 2-propanol in methanol as optimal solvent mixture as compromise between resolution and migration speed. Hedeland et al. [33] investigated also more uncommon solvents for CE such as DCM and DCE with ammonium acetate as electrolyte. The addition of these solvents as well as 2-propanol in 20% gave improved separations. The poor repeatability observed with the MeOH–DCM mixture due to the high volatility of DCM could be improved when DCM was substituted for DCE (2.2% R.S.D. of migration times). The latter solvent mixture permitted also higher voltages due to low conductivity and lower currents. For basic counter-ions, Piette et al. [55] optimized the solvent of the BGE and found out that a mixture of MeOH–EtOH typically 40:60 (v/v) ratio revealed the best results. Thereby, the addition of EtOH supported ion-pair formation. ACN was less useful in this particular separation system due to its high EOF [36] (see below).

Since the type of co-ion and competing electrolyte in the BGE enters into competitive equilibria with the solute cation for ion-pair formation, a number of studies tried to eliminate additional supporting electrolytes and thus competitive achiral ion-pairing. To do so, the salt (sodium or potassium) of the chiral counter-ion was used as sole electrolytes merely [5,35]. In other studies, the addition of supporting electrolytes turned out to be advantageous with regards to the conductivity of the BGE and has led to elimination of electrokinetic dispersion [55]. This also yielded more stable and robust CE methods.

For the stereoisomer separation of chiral bases including β-blockers, β-sympathomimetics, local anesthetics, and cinchona alkaloids and derivatives, acidic chiral compounds in their enantiomeric form such as (*R*)- and (*S*)-camphorsulfonic acid (CSA) (**1**) [5], (−)-2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid (DIKGA) (**2**) [35,54], the amino acid derivatives (*R*)- and (*S*)-*N*-(3,5-dinitrobenzoyl)-leucine (DNB-Leu) (**3**) [23] as well as the dipeptidic *N*-benzyloxycarbonylglycyl-(*S*)-proline (ZGP) (**4**) [33] have been utilized as chiral counter-ions.

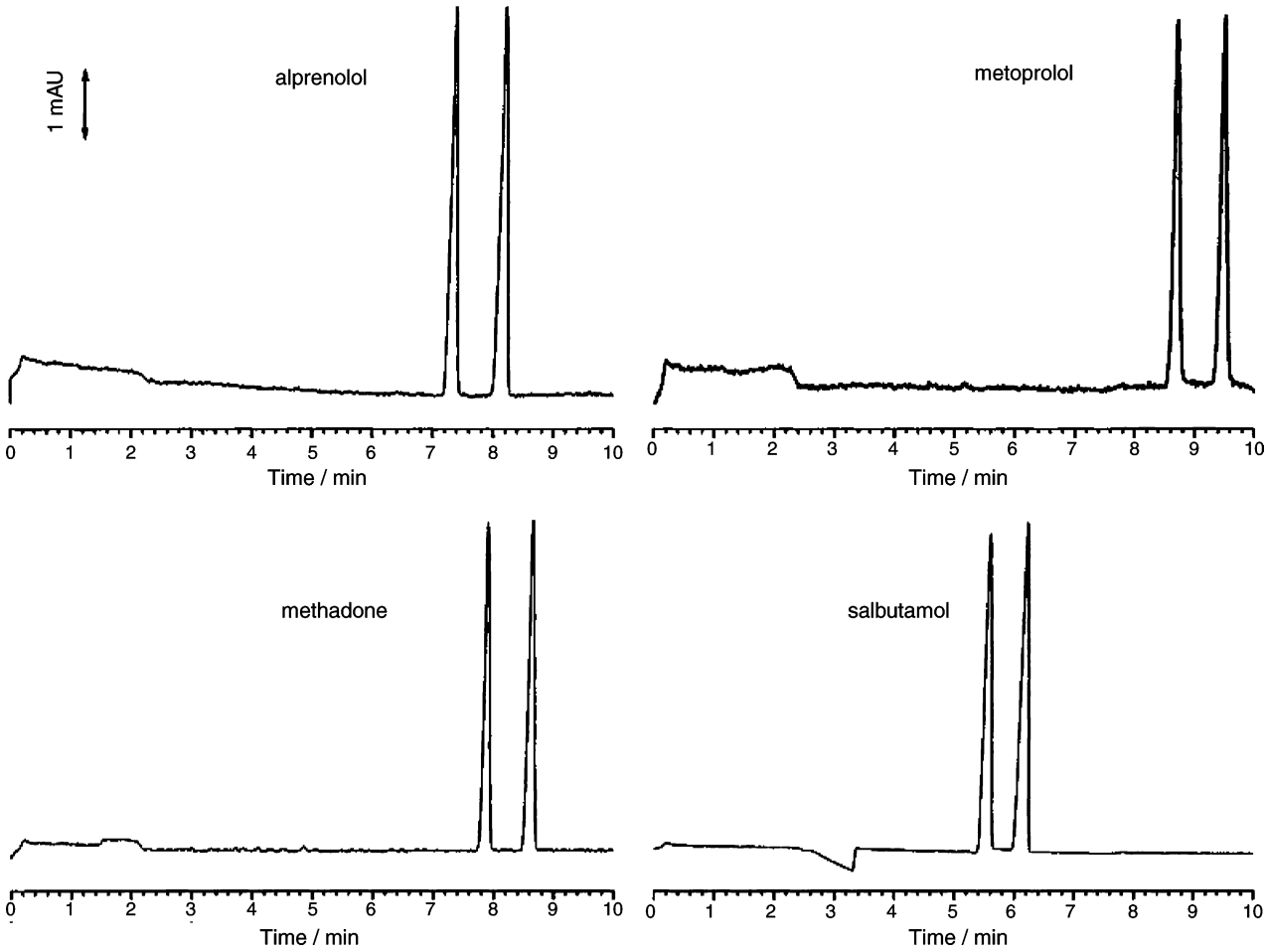

Fig. 9. Electropherograms of weak bases in 10 mM ODAS-γ-CD in acidic methanolic BGE. Reprinted with permission from [49].

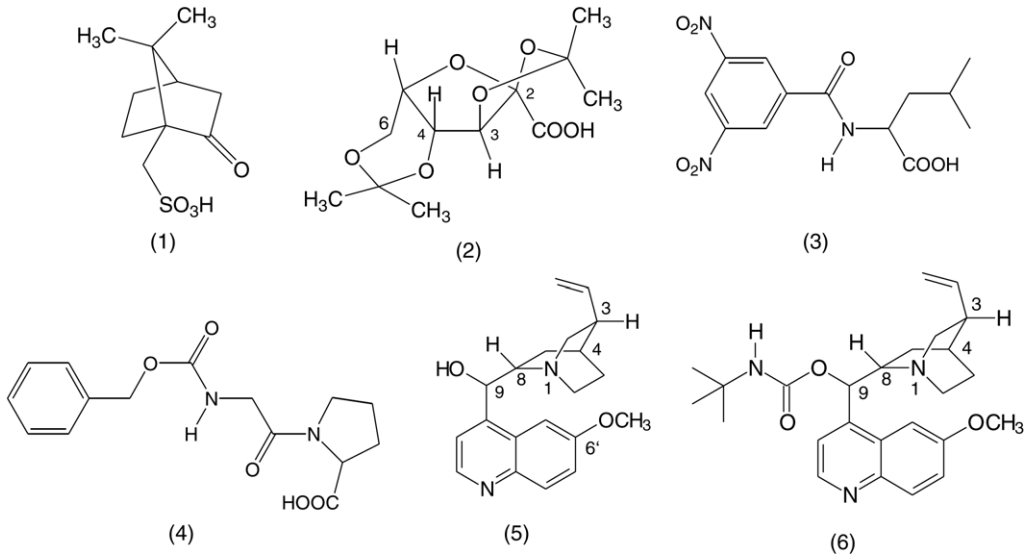

Fig. 10. Structures of chiral counter-ions utilized in NACE enantiomer separations.

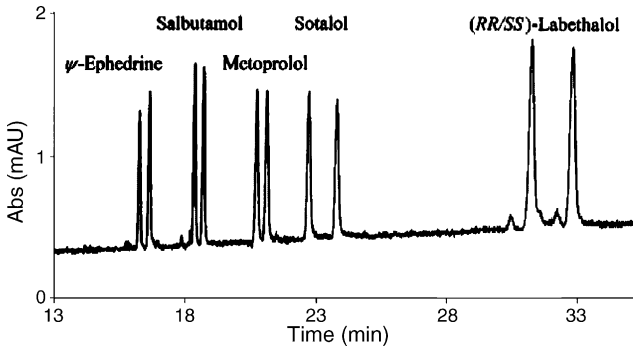

Fig. 11. NACE enantiomer separation of basic drugs using 80 mM DIKGA in MeOH–2-PrOH (75:25, v/v) containing 40 mM ammonium acetate.

Camphorsulfonic acid (**1**) [5] and (−)-2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid (**2**) (Fig. 11) [35] have low UV absorbance and could therefore be utilized in a conventional experimental setup, i.e. with the counter-ion as additive to the BGE in both the inlet and outlet electrolyte vessels so that the total capillary is filled with the selector-BGE solution over the entire length ensuring a maximal separation zone length. Both papers also attempted to deal with the detrimental effect of the electroosmotic flow on resolution which is to be considered when it has the same migration direction as the solutes (Eq. (4)). Bjørnsdottir et al. [5] tried to eliminate the EOF by a dynamic wall coating with addition of Tween 20, while the permanently coated capillaries tested led to noisy baselines and/or poor reproducibility of the separations or had still a high EOF. Carlsson compared different permanently coated capillaries such as in-house prepared polyacrylamide as well as 3-aminopropylsilane (APS) coated capillaries with untreated FS capillaries using 100 mM (−)-DIKGA and 40 mM NaOH as chiral counter-ion and electrolytes, respectively, in methanol [35]. As reported also by Bjørnsdottir et al. [5], there was still significant EOF also in the polyacrylamide coated capillary (μ_{eof} acrylamide = 2.17×10^{-5} cm^2 V^{-1} s^{-1}), which however was reduced by a factor of about 4 compared to the untreated capillary (μ_{eof} uncoated = 8.02×10^{-5} cm^2 V^{-1} s^{-1}). In the APS-coated capillary the EOF was, as expected, reversed (μ_{eof} aminopropyl = -2.84×10^{-5} cm^2 V^{-1} s^{-1}). Unfortunately, the expected favorable effect of the EOF decrease on resolution was partly set off by worse plate numbers in coated capillaries (untreated > acrylamide > APS).

In other instances, where the chiral counter-ion shows strong UV absorbance or when MS detection is employed, the partial filling technique (PFT) [80,81] or counter-current technique (CCT) [82,83] can be adopted to eliminate the selector from the detector thereby avoiding strong background noise and contamination of the MS interface or suppression of ionization due to ion-pairing, respectively [23,54]. In case of separation of chiral cationic compounds with acidic counter-ions and untreated capillaries in the positive polarity mode, care has to be taken to select proper conditions. If the selector zone is co-migrating with the cationic solutes, which happens when the electroosmotic mobility adopts larger absolute values than the anionic electrophoretic mobility of the counter-ion ($\mu_{eo} > -\mu_{eff,SO}$), it is recommended to reduce the mobility of the co-migrating selector zone as much as possible, thereby widening the elution window [54]. Addition of excess of acetic acid to suppress ionization of silanols of the FS wall as well as addition of 25% 2-PrOH to MeOH to lower the ε/η ratio had due to the EOF-reducing effect of both a positive influence. The appearance of the selector zone in the detector was delayed so that undisturbed detection also of slowly migration solutes ahead of the selector zone became possible. Ammonium acetate has been used as volatile electrolyte for MS detection. The PFT–NACE–MS method was utilized with success for the separation of pronethalol using a sheath-liquid electrospray ionization interface with a hydroorganic sheath liquid (MeOH–water in 1:1 ratio plus 0.25% acetic acid) [54]. Despite a counterpressure of 15 mbar applied from the injection end to stabilize the spray and current, a faster migration of the solute compared to the NACE–UV method was observed, which was ascribed to a suction effect triggered by the ESI and made re-optimization necessary. Unfortunately, resolution was lost upon coupling to the MS compared to PFT–NACE–UV, which was attributed to loss of efficiency originating from the suction effect inducted by the ESI and dilution or mixing by the sheath liquid. The principal feasibility of PFT–NACE–MS for enantiomer separation however could be demonstrated.

For the separation of chiral acidic compounds native cinchona alkaloids and derivatives thereof have exhibited partly exceptional stereoselective ion-pairing capabilities and have been exploited for capillary electrophoretic enantiomer separations of chiral acids [6,36,55–62,64–67]. The native cinchona alkaloid quinine (1S,3R,4S,8S,9R)-**5** (Fig. 10) has first been suggested as chiral counter-ion for non-aqueous ion-pair CE with methanolic background electrolytes by Stalcup and Gahm [6]. Piette et al. [56,57] compared the enantiomer separation capabilities of the native cinchona alkaloids quinine, quinidine (1*S*,3*R*,4*S*,8*R*,9*S*)-**5**, cinchonine (1*S*,3*R*,4*S*,8*R*,9*S*)-6′-demethoxy-**5**, and cinchonidine (1*S*,3*R*,4*S*,8*S*,9*R*)-6′-demethoxy-**5** (Fig. 10). The latter were throughout slightly less enantioselective for the enantiomer separation of N-derivatized amino acids and the stereoisomeric forms of quinine/quinidine as well as cinchonidine/cinchonine with opposite configurations at C8 and C9 exhibited reversed elution orders. Overall, separation factors achieved with these native cinchona alkaloids for chiral acids by all the aforementioned methods were quite moderate. By introduction of a carbamate group at the hydroxyl at C9, favorably in combination with a bulky carbamate residue such as *tert*-butyl (**6**) (see Fig. 10), an enormous gain of the enantiomer discrimination ability resulted for a wide variety of chiral acids, e.g. for *N*-benzoyl-β-phenylalanine from R_S of 0 with native quinine to R_S of 5.1 with *tert*-butylcarbamate of quinine as chiral counter-ion or for *N*-3,5-dinitrobenzoyl-leucine from 5.5 to 64.3 [57]. These significant improvements in enantiorecognition were mainly attributed to the more rigid

selector structure with a better defined binding pocket as well as the favorable hydrogen donor–acceptor properties of the carbamate group [57]. A variety of other carbamate derivatives such as cyclohexyl, 1-adamantyl, 3,4-dichlorophenyl, 3,5-dinitrophenyl carbamates and bis-(carbamoylquinine) derivatives were then synthesized and showed partly even higher enantioselectivities (e.g. 1-adamantyl carbamate) or to some extent complementary stereodiscrimination potential (e.g. aromatic carbamates) compared to the *tert*-butyl carbamate [55–58,64,65]. Quaternization of the quinuclidine, which appeared to be favorable in terms of applicable pH^* range and was envisaged for faster high pH^* separations, unfortunately turned out to be negative with respect to enantioselectivities. For example, the separation factor α for DNB-Leu dropped with the quaternary *O*-hexylcarbamoyl-1-methylquininium as counter-ion to 1.077 from 1.993 with the corresponding non-quaternized analog. On the molecular level this phenomenon may be interpreted by the loss of the favorable directed H-bond of the H-bond-mediated ionic interaction that is available in the tertiary amine counter-ion but not the quaternary analog, or alternatively, due to steric overcrowd in the binding site of the quaternary counter-ion originating from the methyl at the primary ionic interaction site.

Overall, the *tert*-butyl carbamates of quinine and quinidine turned out to be a very effective and easy accessible chiral counter-ion and their spectrum of applicability spans a wide variety of chiral bases including in particular N-derivatized amino acids [55–59,64,65], amino phosphonic acids [59–62], amino sulfonic acids, peptides (see Fig. 12) [36], aryloxycarboxylic acids (e.g. herbicides like dichloroprop), and aryl carboxylic acids. Like underivatized quinine and quinidine also the corresponding *tert*-butyl carbamates exhibit pseudo-enantiomeric behavior, which is manifested in reversed migration orders of (*R*)- and (*S*)-enantiomers.

The preferred nonaqueous media consisted of methanol or methanol–ethanol mixtures (the optimum EtOH content with regards to enantioselectivity was typically found at 60%

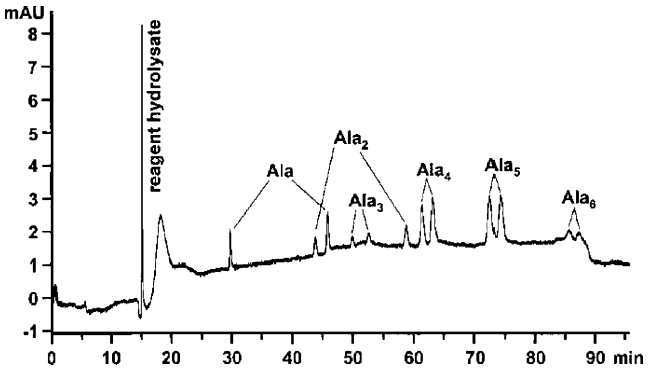

Fig. 12. Simultaneous separation of the (all-*R*)/(all-*S*)-enantiomers of *N*-3,5-dinitrobenzoyl-derivatized Ala_n-peptides (n = 1–6) by NACE using *tert*-butylcarbamoylquinine (**6**) (10 mM) as chiral counter-ion. PVA-coated capillary; BGE: MeOH–EtOH (80:20, v/v) containing 100 mM acetic acid and 12.5 mM triethylamine. Elution order: for all enantiomeric pairs the (all-*R*)-enantiomer is eluted before the (all-*S*)-enantiomer. Reprinted with permission from [36].

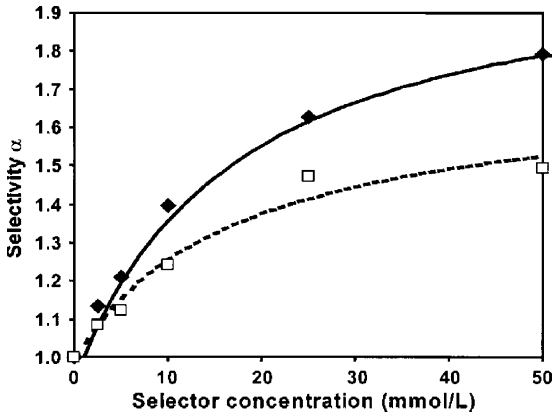

Fig. 13. Dependence of separation factors on the counter-ion concentration. Counter-ion: *tert*-butylcarbamoylquinine (**6**). Lines were fitted according to Eq. (8): (♦) DNB-Ala, (□) DNB-Ala–Ala. Reprinted with permission from [36].

[36,55]) containing organic acids such as acetic acid or octanoic acid and bases such as ammonia and triethylamine as electrolytes. These nonaqueous media ensure also sufficient solubility of the formed ion-pairs and of lipophilic counter-ion likewise, which need to be added to the BGE at concentrations between 2 and 100 mM. Fig. 13 depicts typical α versus selector concentration curves. It is seen that flat optima are attained and that even with 50 mM the optimum has not been surpassed. Since apparent mobilities are low with such high counter-ion concentrations (owing to zero self-electrophoretic mobility of ion-pair and counterdirectional EOF), usually suboptimal selector concentrations are applied (e.g. typically 10 mM). This is only made possible by the exceptional intrinsic stereoselectivities of these selectors for many chiral acids. On the other hand, with above specified EtOH-based media counterdirected electroosmotic mobilities are kept at a relatively tolerable velocity. For the same reason ACN is obviously not a suitable solvent due to its high EOF. This is of particular importance when owing to these strongly UV absorbing counter-ions the partial filling [36,59,66] or countercurrent techniques [55–58,64] are adopted. Besides of low separation speed, the rapid reduction of the separation zone, which migrates to the injection end of the capillary with the EOF as well as its self-electrophoretic mobility (cationic selector), is also highly adverse with respect to separation factors and enantiomer resolution [59]. PVA-coated capillaries, though still generating flow, have been utilized to overcome the adverse effect of the EOF, partially with success [36].

An uncommon detection scheme in NACE has been proposed recently for use of *tert*-butylcarbamoyl quinine counter-ion. A Fourier-transform infra-red spectroscopy study on-line hyphenated with the stereoselective ion-pair NACE method was presented to provide stereochemical information not acquirable by either UV or MS [63]. A home-made CE instrument was coupled to a FT-IR spectrometer via a micromachined flow cell consisting of two IR-transparent

CaF_2 plates (to overcome the total absorption of FS of on-capillary detection) separated by a polymer coating and a titanium layer, thus obtaining an IR detection window with 25 μm path length onto which the IR beam was focused. The connections between capillary and flow cell were made by O-rings of silicon rubber being resistant to the organic solvents. The spectral range between 1900 and 1100 cm^{-1} provided reasonable IR data in the on-line NACE–FT-IR experiment for the analysis of the model compound DNB-Leu under nonaqueous BGE conditions (below 1100 cm^{-1} the C–O vibration from the alcohols in the BGE caused a strong background noise). Thus, functional group information (symmetric and asymmetric NO_2 stretch, amide I C=O stretching vibration, C=O stretch of carboxylic acid) could be obtained. In the on-line experiments, two diastereomers, which may differ in their IR spectra, were actually detected when the both enantiomers were resolved due to the presence of the selector in the detection cell. A shift of the stretching vibration of the carboxylate of the stronger binding enantiomer for example was visible in the IR spectra extracted from the CE run, which allowed the on-line distinction between (*R*)- and (*S*)-enantiomers. Although the dissimilarities of the IR spectra of the (*R*)- and (*S*)-enantiomers of DNB-Leu were small, the proof of principle could be shown.

Besides of separating test racemates ion-pair NACE with cinchona alkaloid-derived counter-ions has proven its usefulness in real applications. For example, it was shown that satisfactory empirical correlations between separation factors of various selector–selectand pairs (all cinchona alkaloid derivatives and N-derivatized amino acids, respectively) obtained by NACE and HPLC under similar or identical mobile phase conditions do exist [66,67]. NACE was therefore helpful in the course of development and screening of new ion-pairing selectors which allowed a rough semi-quantitative estimation of the intrinsic enantiomer separation ability of new selectors avoiding time consuming immobilization and column packing procedures in the course of the development of new chiral stationary phases.

In another application the unknown stereochemistry of a fosfomycin biosynthesis side product could be unveiled [61,62]. For this purpose, the crude product of fosfomycin, *cis*-(1*R*,2*S*)-1,2-epoxypropylphosphonic acid, which is produced by various *Streptomyces* species and other bacteria by oxidative cyclization of (*S*)-2-hydroxypropylphosphonic acid, was converted to the corresponding aminohydroxypropylphosphonic acid by treatment with ammonia for sake of easier isolation from a fermentation broth (namely by cation-exchange) converted by treatment with ammonia to the corresponding amino-hydroxypropylphosphonic acid. In principle, eight isomers could be formed, viz. four stereoisomers of each 1-amino-2-hydroxypropane phosphonic acid **7** and 2-amino-1-hydroxypropane phosphonic acid **8**. NACE employing *O*-(*tert*-butylcarbamoyl) quinine and quinidine as chiral counter-ions allowed the simultaneous separation of all eight components after derivatization with Sanger's reagent to yield the strongly chromophoric *N*-2,4-dinitrophenyl derivatives using the partial filling mode (Fig. 14) [61]. Reference compounds of the individual isomers of **7** and **8** (Fig. 14) were prepared separately by stereoselective synthesis and their absolute configurations were assigned by the Mosher method and verified indirectly by their preferential binding strength towards quinine and quinidine carbamates. The reaction of biological fosfomycin samples with ammonia yielded (1*R*,2*R*)-2-amino-1-hydroxypropylphosphonic acid **8** as main product and (1*S*,2*S*)-1-amino-2-hydroxypropylphosphonic acid **7** as side product due to nucleophilic attack at either C_2 or C_1 of fosfomycin, respectively. In addition, the small amount of the unknown impurity (2%) was, supported by NMR for firm structure elucidation of the constitution, identified to be (1*S*,2*R*)-2-amino-1-hydroxypropylphosphonic acid **8**, stemming from *trans*-(1*S*,2*S*)-1,2-epoxypropylphosphonic acid, which thus was indirectly proven to be a co-metabolite of the biosynthesis of fosfomycin in *Streptomyces fradiae*.

4.6. Other selectors

Besides these more often used major selector classes a few other selector systems have been utilized by researchers to succeed in enantiomer separations by NACE.

NACE with chiral crown ether, (+)-18-crown-6-tetracarboxylic acid (typical selector concentrations between 2.5 and 50 mM) was used for the separation of eight primary amino compounds including aminoalcohols, amino acids, and aromatic amines [53]. The molecular recognition mechanism is based on inclusion complexation into the 18-crown-6 macrocycle driven by polar hydrogen bond interactions between ammonium hydrogens and ether oxygens of the crown serving as hydrogen acceptors. The carboxylic acids at the rim of the macrocycle form chiral barriers and act as lateral electrostatic interaction sites, which in the present situation are supposed to counteract inclusion complexation. Among ten solvents tested, FA turned out to be the best choice, although it is expected to weaken the donor acceptor interactions (hydrogen bonding) that drive inclusion complexation into the crown. Also the electrostatic interactions are supposed to be weaker in FA than water or alcohols which seems to be favorable in the present case. Thus, enantiomer separations were afforded for 1-(1-naphthyl)ethylamine, 1-(1-phenyl)ethylamine, DOPA, Phe, Trp, norephedrine, noradrenaline, 2-amino-1,2-diphenylethanol with a 10 mM selector concentration. Although additional electrolytes were not necessary, tetra(*n*-butyl)ammonium perchlorate (TBAP) (0–100 mM) in the BGE improved various of the separations. Presumably non-stereoselectively occurring ionic interactions of the ammonium solutes outside of the macrocycle were weakened by competitive ion-pairing with TBAP. This makes the inclusion complexation and the steric carboxylate barriers more effective, which eventually may have been responsible that enhanced enantioselectivities were mostly achieved.

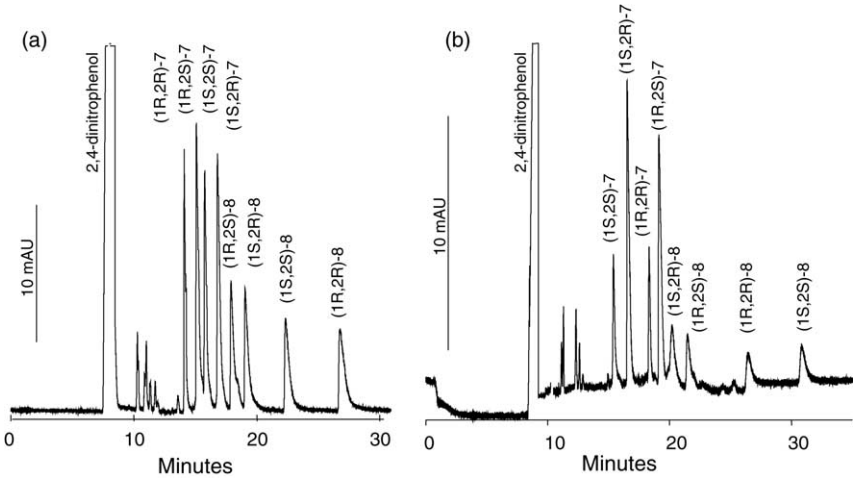

Fig. 14. Simultaneous separation of all isomers of 1-amino-2-hydroxypropane phosphonic acid **7** and of 2-amino-1-hydroxypropane phosphonic acid **8** as *N*-2,4-dinitrophenyl derivatives in a single run by PFT–NACE and *O*-(*tert*-butylcarbamoyl)quinine (a) and *O*-(*tert*-butylcarbamoyl)quinidine (b), respectively, as chiral counter-ions. Note the reversed elution orders of the enantiomeric forms upon exchange of the quinine selector by its quinidine analog. BGE: EtOH–MeOH (60:40, v/v) containing 10 mM of the chiral counter-ion, 100 mM acetic acid and 12.5 mM triethylamine; voltage: −25 kV. Reprinted in slightly modified form with permission from [61].

More uncommon, NACE was even applied for stereoselective ligand exchange with chelating selectors that separate on the basis of the formation of diastereomeric mixed ternary metal complexes between selector and analytes [69]. Free amino acids were separated by use of Cu(II) complexes with (*S*)-proline and (*S*)-isoleucine in methanolic BGE and 25 mM ammonium acetate and 1 M acetic acid as supporting electrolytes. In NACE, the optimal Cu(II) to (*S*)-proline ratio was changed to 1:3 compared to 1:2 with aqueous BGE, which clearly indicates that the complex stability is shifted with nonaqueous conditions. Moreover, the optimal concentration of the chelating agent $CuCl_2 \cdot 2H_2O$ at constant Cu(II)/(*S*)-proline ratio was reached at about 5 mM, where the obtained separation factors leveled off. As expected, changing the configuration of proline allowed the elution order to be reversed, a significant practical advantage of this and other low-molecular-mass selectors.

4.7. Mixtures of selectors (dual selector systems)

The combination of different types of selectors in one BGE has been shown to facilitate or improve enantiomer separations. For NACE, this was demonstrated by Crommen and coworkers [70,71]. A combination of a charged CD, heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-CD (HDMS-β-CD), and a chiral or achiral ion-pairing agent ((*S*)-camphorsulfonic acid and alkanesulfonic acids, respectively) have been utilized as selectors in NACE enantiomer separation of basic pharmaceuticals (β-blockers such as atenolol, metoprolol, propranolol, β-sympathomimetic salbutamol, localanesthetic bupivacaine). The both selectors obviously acted cooperatively. This synergistic effect was clearly demonstrated by the fact that omission of the ion-pairing agent from the BGE led typically to loss or at least reduction in separation selectivity. However, for some other compounds stereoselectivity was reduced with the additional ion-pairing selector. Hence, the effect seems to be compound specific and cannot be predicted a priory. Obviously, it is related to a shielding of ionic interactions of the solute with the charged moiety at the CD by competitive ion-pairing. If these ionic interactions at the charged CD derivative become too strong or dominant they may evolve non-stereoselectively and thus have a negative influence on enantioselectivity. In this case, the addition of the ion-pair selector and competitive ion-pairing improve resolution through balancing of the non-stereoselective ionic interactions with the charged CD. If the ionic interactions between solute and charged CD are weak or properly balanced, an interference with ionic interaction by competitors seems to be detrimental leading to a decrease of resolution. This explanation was supported by the fact that also achiral ion-pair agents (alkanesulfonic acids) acted in the same way. In a follow-up study, the enantiomer separation of a series of basic pharmaceuticals including β-blockers, local anesthetics, sympathomimetics was optimized by help of a face-centered central composite design [71]. With this experimental design optimal concentrations of the HDMS-β-CD and potassium camphorsulfonate selectors in the methanolic BGE containing 0.75 M formic acid could be found more efficiently with a minimum of experiments (11 for each analyte) yielding resolution values between 4 and 24 for the test analytes.

5. Conclusions

The potential of nonaqueous enantioselective CE as a complement to the more broader used aqueous counterpart has readily been explored for the most effective chiral selectors that are more frequently used in CE enantiomer separations. A reasonable number of studies has now been

compiled and was presented in the literature so that the field is covered in a representative way which allows sound conclusions to be drawn on the feasibilities, peculiarities, advantages and drawbacks of enantioselective NACE. In parallel, in the last few years a variety of theoretical studies have been published on NACE, offering now a deeper understanding on migration, resolution and buffer preparation in nonaqueous solvents. This provides the basis for the design of good NACE methods and will be also helpful for the development of enantioselective NACE separation methods. This may encourage more researchers to evaluate NACE and to use this peculiar technique more often, also for practical applications to solve real life problems. However, the knowledge of solvent effects on chiral recognition of a variety of selectors is still somewhat limited so that it will be difficult to predict the enantiomer recognition abilities of distinct solvents for many selectors. The same argument applies on the other hand also for aqueous conditions.

From a practical point of view, it may be concluded that NACE has gained importance and relevance when the solubility of the solute was insufficient in aqueous BGEs. In this particular case, NACE extended certainly the scope of CE enantiomer separation, previously reserved to normal-phase enantioselective HPLC or enantioselective HPLC in the polar organic mode. NACE may also become the method of choice, when with a given selector no separation can be achieved in the aqueous BGE, or on contrary, if a selector that is not soluble in aqueous BGEs is required to solve a given enantiomer separation problem. Due to the broad spectrum of applicability of CDs and their derivatives the latter case will seldom become reality. The potential of NACE for theoretical studies about solvent effects on physico-chemical parameters such as equilibrium constants was clearly demonstrated by early work with CDs. Sometimes, e.g. for enantiomeric impurity profiling at the low level which requires injection of high sample masses, it may be desirable to weaken selector–selectand interactions, in order to obtain a method with a higher optimal selector concentration and a flatter optimum. This is supposed to improve the sample loadability and method robustness. Of particular interest may also be the speed of NACE, which has in some instances, e.g. for sulfated CDs been proven. In such a case, NACE could offer distinct advantages. Overall, NACE has extended substantially the versatility and the scope of applicability of CE enantiomer separation. It should be regarded and treated as a complement rather than a competitive technique to aqueous CE enantiomer separation.

6. Abbreviations

ACN acetonitrile
AcOH acetic acid
Ala alanine
APS 3-aminopropylsilane
AN acceptor number
Asp aspartic acid
BGE background electrolyte
Bz *N*-benzoyl
CE capillary electrophoresis
CCT counter-current technique (outlet vial devoid of selector during run)
CD cyclodextrins
CHARM charged resolving agent migration model
CM-β-CD carboxymethyl-β-CD
CSA camphorsulfonic acid
D diffusion coefficient
DCE 1,2-dichloroethane
DCM dichloromethane
DIKGA (−)-2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid
DMF *N*,*N*-dimethylformamide
DMSO dimethylsulfoxide
DN donor number
DNB-Leu *N*-(3,5-dinitrobenzoyl)-leucine
DNP *N*-(2,4-dinitrophenyl)
Dns dansyl (5-dimethylaminonaphthalene-1-sulfonyl)
DNZ *N*-(3,5-dinitrobenzyloxycarbonyl)
DOPA 3,4-dihydroxyphenylalanine
ds degree of substitution
e electron charge
EOF electroosmotic flow
EtOH ethanol
ESI electrospray ionization
FA formamide
FMOC *N*-(9-fluorenylmethoxycarbonyl)
FS fused silica
FT-IR Fourier-transform infrared
Glu glutamic acid
HE-β-CD hydroxyethyl-β-cyclodextrin
HDAS-β-CD heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-cyclodextrin
HDMS-β-CD heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-cyclodextrin
HP-β-CD hydroxypropyl-β-cyclodextrin
I ionic strength
$K_{(R)}$, $K_{(S)}$ equilibrium constants of the complexation reactions for the (*R*)- and (*S*)-enantiomers
LOD limit of detection
LOQ limit of quantitation
MDM mobility difference model
Me-β-CD methyl-β-cyclodextrin
MeOH methanol
MS mass spectrometry
N theoretical plate number
NACE nonaqueous CE
NACEC nonaqueous capillary electrochromatography
NMF *N*-methylformamide
ODAS-γ-CD octakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-γ-cyclodextrin
ODMS-γ-CD octakis(2,3-di-*O*-methyl-6-*O*-sulfo)-γ-cyclodextrin

OPA *o*-phthaldialdehyde
PFT partial filling technique
Phe phenylalanine
2-PrOH 2-propanol
PVA poly(vinyl alcohol)
QA-β-CD quaternary ammonium β-cyclodextrin
QD quinidine
QN quinine
r hydrodynamic radius of the ion (i.e. the radius of the solvated ion)
ROMP ring-opening methathesis polymerization
R_S resolution
R.S.D. relative standard deviation
SA selectand (solute to be separated)
S-β-CD sulfated β-cyclodextrin
SO chiral selector
SPE solid-phase extraction
tBuCQD *O-tert*-butylcarbamoyl quinidine
tBuCQN *O-tert*-butylcarbamoyl quinine
TATG 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose
TBA tetrabutylammonium
TBAP tetra(*n*-butyl)ammonium perchlorate
THF tetrahydrofuran
TMA tetramethylammonium
TEA triethylamine
Trp tryptophan
$\Delta_V U/V$ cohesive energy density
z_i charge number of the ion
ZGP *N*-benzoxycarbonylglycyl-(*S*)-proline

Greek letters
α separation factor
$\Delta\mu$ mobility difference
ε dielectric constant
ε_0 permittivity of vacuum
ε_r relative permittivity
η viscosity of the medium
μ_{avg} mean of the electrophoretic mobilities of the first and second migrating enantiomer
μ_c effective electrophoretic mobility of complexed solute
μ_{eff} effective electrophoretic mobility
μ_{eo} electroosmotic mobility
μ_f effective electrophoretic mobility of free solute
ζ_{ion} and ζ_{wall} electrokinetic potentials (ζ-potentials) of the ion and the capillary wall, respectively

Acknowledgements

The financial support by the Austrian Christian Doppler Research Society and the industrial partners AstraZeneca (Södertälje, Sweden), Merck KGaA (Darmstadt, Germany) and piCHEM (Graz, Austria) is gratefully acknowledged.

References

[1] B. Chankvetadze, Capillary Electrophoresis in Chiral Analysis, Wiley, West Sussex, 1997.
[2] Y. Waldbroehl, J.W. Jorgenson, J. Chromatogr. 315 (1984) 135.
[3] F. Wang, M.G. Khaledi, Anal. Chem. 68 (1996) 3460.
[4] I.E. Valko, H. Siren, M.-L. Riekkola, J. Chromatogr. A 737 (1996) 263.
[5] I. Bjørnsdottir, S.H. Hansen, S. Terabe, J. Chromatogr. A 745 (1996) 37.
[6] A.M. Stalcup, K.H. Gahm, J. Microcol. Sep. 8 (1996) 145.
[7] M. Lämmerhofer, W. Lindner, J. Chromatogr. A 839 (1999) 167.
[8] K. Krause, B. Chankvetadze, Y. Okamoto, G. Blaschke, Electrophoresis 20 (1999) 2772.
[9] K. Sarmini, E. Kenndler, J. Biochem. Biophys. Methods 38 (1999) 123.
[10] S.P. Porras, M.-L. Riekkola, E. Kenndler, Electrophoresis 24 (2003) 1485.
[11] S.P. Porras, E. Kenndler, J. Chromatogr. A 1037 (2004) 455.
[12] M.-L. Riekkola, S.K. Wiedmer, I.E. Valko, H. Siren, J. Chromatogr. A 792 (1997) 13.
[13] M.-L. Riekkola, J. Chromatogr. A 892 (2000) 155.
[14] M.-L. Riekkola, Electrophoresis 23 (2002) 3865.
[15] M.-L. Riekkola, H. Siren, in: G. Gübitz, M.G. Schmid (Eds.), Chiral Separations: Methods and Protocols, in: J.M. Walker (Ed.), Methods in Molecular Biology, vol. 243, Totowa, NJ, 2004, p. 365.
[16] F. Steiner, M. Hassel, Electrophoresis 21 (2000) 3994.
[17] B. Chankvetadze, G. Blaschke, Electrophoresis 21 (2000) 4159.
[18] M. Fillet, A.-C. Servais, J. Crommen, Electrophoresis 24 (2003) 1499.
[19] J. Muzikar, T. Van de Goor, E. Kenndler, Anal. Chem. 74 (2002) 434.
[20] F. Wang, M.G. Khaledi, J. Chromatogr. A 875 (2000) 277.
[21] M.T. Bowser, A.R. Kranack, D.D.Y. Chen, Trends Anal. Chem. 17 (1998) 424.
[22] A. Tivesten, A. Lundqvist, S. Folestad, Chromatographia 44 (1997) 623.
[23] E. Zarbl, M. Lämmerhofer, P. Franco, M. Petracs, W. Lindner, Electrophoresis 22 (2001) 3297.
[24] S.A.C. Wren, R.C. Rowe, J. Chromatogr. 603 (1992) 235.
[25] B.A. Williams, G. Vigh, J. Chromatogr. A 777 (1997) 295.
[26] B. Chankvetadze, W. Lindner, G.K.E. Scriba, Anal. Chem. 76 (2004) 4256.
[27] A. Rizzi, Electrophoresis 22 (2001) 3079.
[28] Y. Marcus, The Properties of Solvents, Wiley, Chichester, 1998.
[29] P.B. Wright, A.S. Lister, J.G. Dorsey, Anal. Chem. 69 (1997) 3251.
[30] I.E. Valko, H. Siren, M.-L. Riekkola, J. Microcol. Sep. 11 (1999) 199.
[31] M. Grob, F. Steiner, Electrophoresis 23 (2002) 1853.
[32] M. Jansson, J. Roeraade, Chromatographia 40 (1995) 163.
[33] Y. Hedeland, M. Hedeland, U. Bondesson, C. Pettersson, J. Chromatogr. A 984 (2003) 261.
[34] X. Ren, A. Huang, T. Wang, Y. Sun, Z. Sun, Chromatographia 50 (1999) 625.
[35] Y. Carlsson, M. Hedeland, U. Bondesson, C. Pettersson, J. Chromatogr. A 922 (2001) 303.
[36] C. Czerwenka, M. Lämmerhofer, W. Lindner, Electrophoresis 23 (2002) 1887.
[37] M. Orozco, F.J. Luque, Chem. Rev. 100 (2000) 4187.
[38] H. Wikstrom, P.K. Owens, J. Sep. Sci. 25 (2002) 1167.
[39] I.E. Valko, H. Siren, L.-M. Riekkola, Chromatographia 43 (1996) 242.
[40] I.E. Valko, H. Siren, M.L. Riekkola, Electrophoresis 18 (1997) 919.
[41] A. Karbaum, T. Jira, J. Biochem. Biophys. Methods 48 (2001) 155.
[42] K. Eder, F. Sinner, M. Mupa, C.G. Huber, M.R. Buchmeiser, Electrophoresis 22 (2001) 109.
[43] F. Wang, M.G. Khaledi, J. Chromatogr. B 731 (1999) 187.

[44] F. Wang, M.G. Khaledi, J. Chromatogr. A 817 (1998) 121.
[45] H. Cai, G. Vigh, J. Pharm. Biomed. Anal. 18 (1998) 615.
[46] M. Tacker, P. Glukhovskiy, H. Cai, G. Vigh, Electrophoresis 20 (1999) 2794.
[47] J.B. Vincent, G. Vigh, J. Chromatogr. A 816 (1998) 233.
[48] A.-C. Servais, P. Chiap, P. Hubert, J. Crommen, M. Fillet, Electrophoresis 25 (2004) 1632.
[49] W. Zhu, G. Vigh, Electrophoresis 21 (2000) 2016.
[50] W. Zhu, G. Vigh, J. Chromatogr. A 892 (2000) 499.
[51] M.B. Busby, O. Maldonado, G. Vigh, Electrophoresis 23 (2002) 456.
[52] S. Sanchez-Vindas, G. Vigh, J. Chromatogr. A, submitted for publication.
[53] Y. Mori, K. Ueno, T. Umeda, J. Chromatogr. A 757 (1997) 328.
[54] H. Loden, Y. Hedeland, M. Hedeland, U. Bondesson, C. Pettersson, J. Chromatogr. A 986 (2003) 143.
[55] V. Piette, M. Lämmerhofer, W. Lindner, J. Crommen, Chirality 11 (1999) 622.
[56] V. Piette, M. Fillet, W. Lindner, J. Crommen, Biomed. Chromatogr. 14 (2000) 19.
[57] V. Piette, M. Fillet, W. Lindner, J. Crommen, J. Chromatogr. A 875 (2000) 353.
[58] V. Piette, W. Lindner, J. Crommen, J. Chromatogr. A 894 (2000) 63.
[59] M. Lämmerhofer, E. Zarbl, W. Lindner, J. Chromatogr. A 892 (2000) 509.
[60] F. Hammerschmidt, W. Lindner, F. Wuggenig, E. Zarbl, Tetrahedron: Asymmetry 11 (2000) 2955.
[61] M. Lämmerhofer, E. Zarbl, W. Lindner, B. Peric Simov, F. Hammerschmidt, Electrophoresis 22 (2001) 1182.
[62] B. Peric Simov, F. Wuggenig, M. Lämmerhofer, W. Lindner, E. Zarbl, F. Hammerschmidt, Eur. J. Org. Chem. (2002) 1139.
[63] P. Hinsmann, L. Arce, P. Svasek, M. Lämmerhofer, B. Lendl, Appl. Spectrosc. 58 (2004) 662.
[64] V. Piette, W. Lindner, J. Crommen, J. Chromatogr. A 948 (2002) 295.
[65] P. Franco, P.M. Klaus, C. Minguillon, W. Lindner, Chirality 13 (2001) 177.
[66] M. Lämmerhofer, E. Zarbl, V. Piette, J. Crommen, W. Lindner, J. Sep. Sci. 24 (2001) 706.
[67] V. Piette, M. Lämmerhofer, W. Lindner, J. Crommen, J. Chromatogr. A 987 (2003) 421.
[68] E. Tobler, M. Lämmerhofer, F. Wuggenig, F. Hammerschmidt, W. Lindner, Electrophoresis 23 (2002) 462.
[69] A. Karbaum, T. Jira, J. Chromatogr. A 874 (2000) 285.
[70] A.-C. Servais, M. Fillet, A.M. Abushoffa, P. Hubert, J. Crommen, Electrophoresis 24 (2003) 363.
[71] A.-C. Servais, M. Fillet, P. Chiap, W. Dewe, P. Hubert, J. Crommen, Electrophoresis 25 (2004) 2701.
[72] K.A. Connors, Chem. Rev. 97 (1997) 1325.
[73] M.V. Rekharsky, Y. Inoue, Chem. Rev. 98 (1998) 1875.
[74] D.W. Armstrong, L.W. Chang, S.C. Chang, X. Wang, H. Ibrahim, G.R. Reid, T.E. Beesley, J. Liq. Chromatogr. 20 (1997) 3279.
[75] M.A. Nussbaum, Electrophoresis 20 (1999) 2664.
[76] J.B. Vincent, A.D. Sokolowski, T.V. Nguyen, G. Vigh, Anal. Chem. 69 (1997) 4226.
[77] C. Pettersson, G. Schill, J. Chromatogr. 204 (1981) 179.
[78] W. Thormann, F. Prost, A. Prochazkova, J. Pharm. Biomed. Anal. 27 (2002) 555.
[79] P. Barták, P. Bednár, L. Kubácek, M. Lämmerhofer, W. Lindner, Z. Stránský, Anal. Chim. Acta 506 (2004) 105.
[80] L. Valtcheva, J. Mohammad, G. Pettersson, S. Hjerten, J. Chromatogr. 638 (1993) 263.
[81] A. Amini, U. Paulsen-Sörman, D. Westerlund, Chromatographia 50 (1999) 497.
[82] B. Chankvetadze, G. Endresz, G. Blaschke, Electrophoresis 15 (1994) 804.
[83] T.J. Ward, C. Dann III, A.P. Brown, Chirality 8 (1996) 77.