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Journal of Chromatography A, 906 (2001) 309–363

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

Enantioseparations in capillary electromigration techniques: recent developments and future trends

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Abstract

This review summarizes the current status of enantioseparations using capillary electromigration techniques and gives the authors insights on the selected fundamental aspects and future trends in this field. The most recent developments in the field of chiral separations using capillary electrophoresis (CE) and capillary electrochromatography (CEC) are summarized. The status of chiral electromigration techniques is evaluated tacking into account the most recent developments in related techniques such as chiral HPLC, GC and SFC.

Keywords: Reviews; Enantioseparations; Chiral selectors; Chiral stationary phases

Contents

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0021-9673/01/$ – see front matter
PII: S0021-9673(00)01124-9

1. Introduction

Many recent review papers [1–7], book chapters [8–10] and one book [11] summarize the developments in enantioseparations using capillary electromigration techniques which are only 15 years old. The overviews published in early 1990s covered general aspects of the field with many examples of new chiral selectors, separations, and few applications for real problem solving, whereas several review papers on particular types of chiral selectors appeared during the last years [3,5,9,12–16]. Other special aspects of enantioseparations using electromigration techniques which are covered in recent reviews include enantioseparations in capillary electrochromatography (CEC) [17], enantiomer migration order [18], partial filling technique [19], dual and multiple chiral selectors [20], method development strategy [21], application to chiral drugs [22] and pesticide analysis [23], and clinical analysis [24].

Taking into account the large number of already published review papers with a comprehensive coverage of the field, it is not an easy task to write one more review paper which can be of real use for both, the newcomers and experienced scientists in the field. Just summarizing even in the most complete way all the papers published and the chiral analytes involved does not seem to be useful because something like this is also offered by computer databases which are available for most workers in the field. Rather critical treatment of already established viewpoints and tracing of the most actual future trends seems to be rather important. For this reason, discussing one or another aspect of chiral separations using electromigration techniques, the reader may find in this overview some personal, sometimes disputable insights by the authors which they want to present for an extensive discussion.

Some heterogeneity of the viewpoints by different groups seems to be logical for a relatively new field with intensive research activity. In one group of published papers for example, the electroosmotic flow (EOF) is considered to be nonselective driving force in chiral capillary electrophoresis (CE) compared to electrophoretic mobility of chiral analyte, in other group of the works principal difference is made between chiral separations with charged and uncharged chiral analytes, in the third group of published works a formation of micellar phase is considered as a necessary prerequisite for separation, etc.

In our recent papers [2,16,22] selected fundamental aspects of chiral CE were discussed, in part. Some of these concepts are discussed below rather comprehensively using the examples from the most recent literature.

2. Fundamental aspects

2.1. Separation principle in chiral CE: electrophoretic or chromatographic?

One important conceptual point in chiral CE is to realize that the enantioseparation is commonly not based on an electrophoretic separation principle which postulates the separation as a result of different migration velocities caused by different charge densities of analyte components. The enantiomers of a chiral compound possess the same charge densities. Therefore, none of the potential migration forces in CE, such as the electrophoretic mobility of the analyte, the EOF, their combination or a transport by a nonenantioselective carrier is, in principle, able to differentiate between the enantiomers.

Enantiomers may be recognized stereoselectively only on the stage of their interaction with a chiral selector. This is a chromatographic separation principle. The opponents of this idea may use the argument that according to the IUPAC nomenclature the separation principle may be called chromatographic when it is based on a distribution of an analyte between two immiscible phases [25]. The more important point for a chromatographic enantioseparation is that these immiscible phases should possess different mobilities. In chromatographic techniques, one of these phases is commonly mobile and another one stationary. In chiral CE, there are not two immiscible phases present but pseudophases in the best case, or even only one monophasic, homogenous system in the direct meaning of the term ''phase''. However, chiral recognition occurs on the molecular level and not on the macroscopic level of the phases. Therefore, a technique must allow to transform this molecular level event (in this case chiral recognition) to a macroscopic phenomenon which is the different retention times of the enantiomers in chromatography and the effective mobility difference between the enantiomers in electrophoretic techniques, respectively. The immiscibility of the involved phases becomes a prerequisite in chromatographic separations due to the fact that the pressure as a driving force can not selectively drive a given component from several species residing in the same phase. However, the electrically-driven mobility under certain circumstances can be selective for the one or several species residing in the same phase. For this reason the immiscibility requirement of the two phases falls in CE separations. The phenomenon responsible for enantioseparation is the same in chromatographic and electrophoretic techniques and this is the enantioselective interaction between the analyte enantiomers and a chiral selector. It is not critical whether there are two immiscible phases present in the system or just molecular entities with discrete properties which are able to migrate differently under the effect of the applied voltage in the monophasic, homogenous system. Thus, the principal difference between chromatographic techniques and CE is that pressure as the driving force can not differentiate between the different molecular components residing in a monophasic system whereas the electrically-driven mobility which is the driving force in CE is able to do this under certain circumstances.

The same applies to the classification of enantioseparations into groups such as capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), etc. The separation principle in CZE is a distribution of the analyte components according to their charge density which is equal for the enantiomers. This means that it is impossible to resolve enantiomers based on the separation principle of CZE. The separation principle in gel electrophoresis is a sieving effect of the charged analyte molecules depending on their size. Again, the enantiomers do not differ in their size and therefore are unresolvable with achiral gel. The separation principle in CIEF is based on the pK_a difference between the analytes which is also the same for the enantiomers. Thus, the enantiomer separation in all of these techniques relies on enantioselective noncovalent/intermolecular interactions between the analyte and a chiral selector which may be expressed as the effective

mobility difference (CZE and CGE), stereoselective shift of the acid–base equilibrium (CIEF), etc. Thus, all enantioseparations in chiral CE may be unified under the term capillary electrokinetic chromatography (CEKC). This term was introduced by Terabe and coworkers in 1985 [26]. The micellar electrokinetic chromatography (MEKC) represents one particular mode of CEKC.

2.2. Enantioseparations with charged and uncharged chiral selectors: is there any principal difference?

In many published papers enantioseparations of charged analytes with neutral chiral selectors are attributed to CZE and the enantioseparations of neutral analytes with charged chiral selectors to CEKC. However, from the mechanistic point of view there is no principal difference whether an analyte or a chiral selector is charged or vice versa. Actually, it is the subject of convention which counterpart of chiral recognition process will be named selectand and which one chiral selector. The reciprocal chiral recognition strategy for a design of effective chiral selectors proposed by Pirkle and co-workers in HPLC is based on this philosophy [27].

Recently, the possibility of interchanging the roles between a chiral selector and a selectand in CE has been nicely illustrated by Lindner and co-workers [28]. In this example the enantiomers of *N*-derivatized amino acids (among them (*R,S*)-DNB-leucine) were resolved in nonaqueous CE using *tert.*-butylcarbamoylquinine as a chiral selector (Fig. 1a). Alternatively, the quasi enantiomers of quinine and quinidine carbamates were resolved using (*S*)- or (*R*)-DNB-Leu as a chiral selector (Fig. 1b, c). This example clearly illustrates that it is beyond the ''ability'' of a chiral separation system to make any difference between a selector and a selectand. This means that the underling mechanisms of the separation of charged enantiomers with neutral chiral selector and vice versa is absolutely the same and both of these cases should be classified as CEKC.

2.3. Enantioselective and nonselective phenomena in chiral CE

The role of electrophoretic and electroosmotic mobilities, μ_{el} and μ_{EOF}, respectively, in enantioseparations represents the object of confusion in chiral CE. In many published works μ_{el} is considered to be a selective transport able to make a difference between the enantiomers while μ_{EOF} is considered to be a non-selective transport. This idea does not seem to be correct. What are the most likely reasons for this misunderstanding? (a) The main

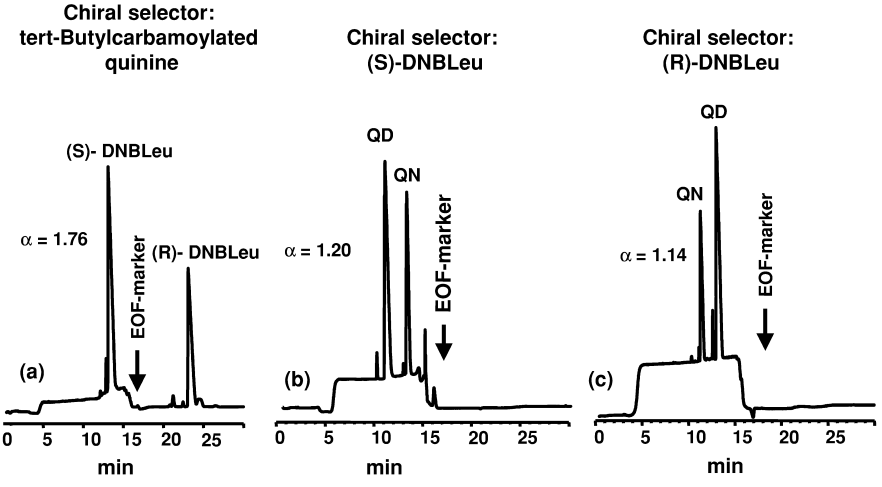

Fig. 1. Enantioseparation of (RS)-DNBLeu using *tert.*-butylcarbymoylated quinine as chiral selector (a) and enantioseparation of quasi-enantiomers of *tert.*-butylcarbamoylated quinine and quinidine using (*S*)-DNBLeu (b) and (*R*)-DNBLeu (c) as the chiral selectors. (Reproduced with permission from Ref. [28].)

reason is that the above-mentioned statement applies without any limitation for true electrophoretic separations, i.e. for the separations which are based on a different electric charge density of the sample components. The analyte specific quantities such as the effective charge (q) and mass (M) enter Eq. (1) for the calculation of μ_{el} [29]:

$$\mu_{el} = kqM^{-2/3} \quad (1)$$

This means that the μ_{el} is an analyte-specific property. On the other hand, μ_{EOF} which can be calculated according to Eq. (2):

$$\mu_{EOF} = \frac{\varepsilon E \xi}{4\pi\eta} \quad (2)$$

depends on the dielectric constant of the medium (ε), the applied electric field strength (E), the zeta potential on the solid–liquid interface (ξ) and the viscosity of the medium (η). The terms entering Eq. (2) are system specific but neither of them is explicitly analyte-specific. For this reason μ_{el} is selective and μ_{EOF} is a non-selective transport in true electrophoretic separations. Enantioseparations in CE are based on the chromatographic separation principle as mentioned in Section 2.1. The quantities entering Eq. (1) although may be different for other charged analytes, they are the same for the enantiomers. For this reason μ_{el} is as non-selective transport for the enantiomers as μ_{EOF} is [2,16].

Other reason for the above-mentioned misunderstanding on the role of μ_{el} and μ_{EOF} on enantioseparations in CE seems to be the fact that in early studies it was possible to observe a significant improvement of enantioseparations in CE under the conditions when the EOF was suppressed. However, no attention was paid to the fact that the same apparent effect may, in principle, be observed when one suppresses the μ_{el} instead of μ_{EOF} under appropriate conditions. In the latter studies it was shown by Kenndler that even in virtually electrophoretic separations the EOF, depending on its magnitude and direction, may not only negatively but also positively affect a separation [30,31].

Thus, both migrating forces in CE, μ_{el} and μ_{EOF} lack inherently the enantiomer differentiating ability. The factor implementing enantioselectivity to a chiral CE separation system is enantioselective non-covalent intermolecular interactions between a chiral analyte and a chiral selector.

A principal difference between μ_{el} and μ_{EOF} is that the former is a substance-specific transport whereas the latter is a system-specific transport. In addition, it does not make any difference whether the substance is the analyte or the selector.

The conceptual point in chiral CE is that the analyte in its free and complexed form shall possess different mobilities. For this, it may be required that the analyte and the chiral selector possess different effective mobilities. Whether they reside in the same or different phase or pseudophases is again not critical for electrophoretic separations because μ_{el} possess the distinguished property to be selective for the species residing in the same phase. Pressure-driven flow as well as μ_{EOF} lack this property and are ''phase-selective'' transports.

2.4. Similarities and differences between enantioseparations by chromatographic and capillary electrophoretic techniques

As mentioned above, enantioseparations in CE rely on a chromatographic separation principle. Despite this fact, there are significant differences between these techniques. Responsible for all differences between chromatographic and electrophoretic enantioseparations is the above-mentioned property of the electrophoretic mobility, in particular, its ability to be selective for the analytes residing in the same phase. Another important point is that in chromatographic techniques, except that with a chiral mobile phase additive (CMPA) the analyte is virtually immobile when associated with a chiral selector. In CE the analyte selector complex is commonly mobile.

Basic differences between chromatographic and electrophoretic enantioseparations can be derived analyzing the equation proposed for the calculation of the electrophoretic mobility difference $\Delta\mu$ between enantiomers [32]:

$$\Delta\mu = \mu_1 - \mu_2 = \frac{\mu_f + \mu_{C_1} K_1[C]}{1 + K_1[C]} - \frac{\mu_f + \mu_{C_2} K_2[C]}{1 + K_2[C]} \quad (3)$$

where μ_1 and μ_2 are the mobilities of the first and

the second migrating enantiomer, respectively. K_1 and K_2 are the binding constants between enantiomer 1 and 2 and the chiral selector, μ_f and μ_c are the mobilities of the free and complexed analyte and [C] is the concentration of a chiral selector.

One important point obviously seen from Eq. (3) is a crucial role of the mobilities in enantioseparations in CE. This parameter is absent in the major chromatographic techniques except above-mentioned mode with CMPA. The contribution of the mobilities in chiral CE separations may allow the observation of following distinguished effects:

1. It is feasible in chiral CE but not in chromatographic techniques that the selectivity of enantioseparation exceeds the thermodynamic selectivity of chiral recognition;
2. It is possible in chiral CE to adjust the enantiomer migration order without reverting the affinity pattern between the enantiomers of the analyte and a chiral selector. This is impossible in chromatographic techniques at least in the mode when the chiral selector is immobilized and not used as a CMPA.
3. Although not yet unambiguously proven experimentally, the most striking difference between these two techniques seems to be the fact that CE allows, in principle, the enantioseparation in the absence of chiral recognition in the classical meaning of this term.

Bellow, these differences between CE and chromatographic enantioseparations are illustrated using recent examples from the literature.

As already mentioned, in chromatographic techniques the selectivity of enantioseparation is entirely defined by the chiral recognition, i.e. by the difference between the affinity of enantiomers towards the chiral selector. Therefore, the selectivity of enantioseparations in common chromatographic techniques may in the best case approach to the thermodynamic selectivity of the chiral recognition but will never exceed it. In contrast to this in CE the separation selectivity may easily exceed the thermodynamic selectivity of the recognition. This is experimentally illustrated in Fig. 2 [33]. In all separations of the chlorpheniramine enantiomers with CM-β-CD shown in this figure, the components involved in chiral recognition on the molecular level are invariant. This means that chiral recognition itself does

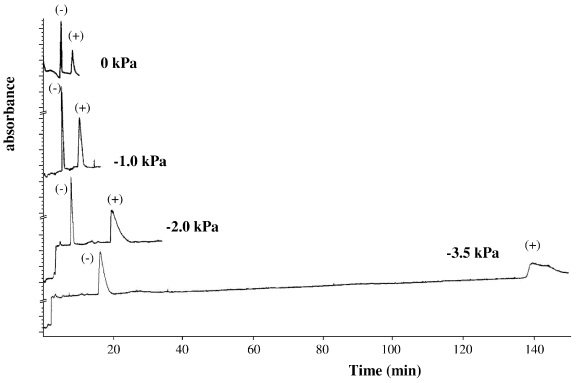

Fig. 2. Effect of increasing counterpressure on the separation of (±)-chlorpheniramine in the presence of 2 mg/ml CM-β-CD. (Reproduced with permission from Ref. [33].)

not change significantly. However, an enormous (in principle unlimited) enhancement of the separation selectivity becomes possible on the step of transforming the chiral recognition into a chiral separation. In this particular example this was achieved by applying a counterbalancing pressure to the separation capillary in the opposite direction to the analyte migration.

The principle of separation factor enhancement without any change in recognition selectivity is a decrease of the effective averaged mobility term $\mu_{av} = 1/2(\mu_1 + \mu_2)$ while retaining the mobility difference ($\Delta\mu = \mu_1 - \mu_2$) constant in Eq. (4) [34]:

$$R_S = \frac{1}{4}\sqrt{N}\frac{\Delta\mu}{\mu_{av}} \quad (4)$$

As shown schematically in Fig. 3, this concept

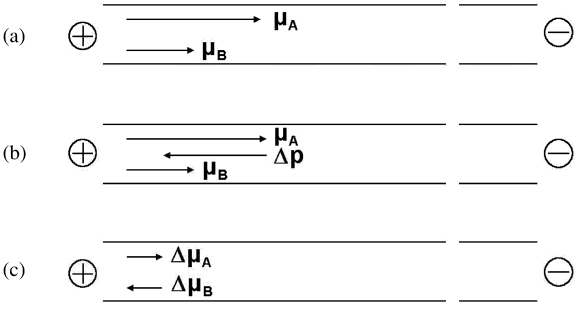

Fig. 3. Schematic representation of flow-counterbalanced separation principle in CE: (a) without counterbalanced flow; (b) with counterbalanced flow; (c) resulting mobilities. (Reproduced with permission from Ref. [33].)

may allow to design a separation system in a way that two enantiomers certainly possessing the electric charge of the same sign will migrate towards opposite electrodes which means that the enantioseparation factor becomes infinitely large. In analogy to electrolysis this phenomenon was named ''enantiolysis'' [33]. The technique proposed in [33] certainly may be applied also for micropreparative purposes as well as for separations of achiral analytes not only in a binary but also in multicomponent mixtures.

Another important point is that a manipulation of the mobility terms in CE allows not only an adjustment of the selectivity of enantioseparation but also a reversal of the enantiomer migration order without changing the affinity pattern of the enantiomers towards chiral selector. This is again somewhat impossible in chromatographic techniques. This significant difference between chromatographic and electrophoretic separations from the viewpoint of the enantiomer migration order has been noted in previous studies [18,35].

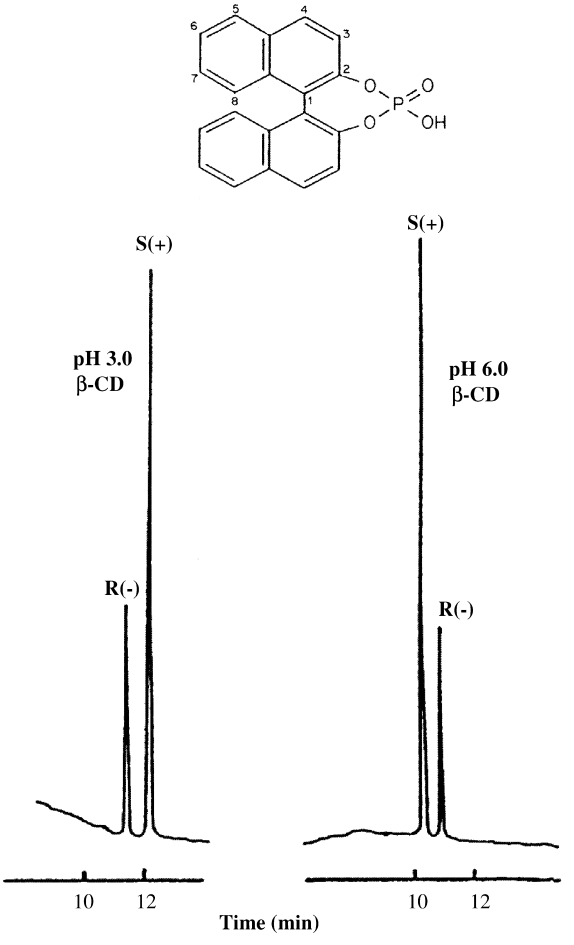

Fig. 4. Reversal of the enantiomer migration order in CE without principal modification of chiral recognition. (Reproduced with permission from Ref. [18].)

Thus, taking into account that the mobility in CE is a vectorial quantity one can imagine that just reverting the sign of $\Delta\mu$ even without any change of the chiral recognition will result in a reversal of the enantiomer migration order [35]. Similar to the case shown in Fig. 2, the components of a separation system immediately involved in chiral recognition are not significantly modified in the separation depicted in Fig. 4 [18]. However, the pH of the separation buffer is changed by a designed way to allow the detection of the analyte on the anodic or cathodic ends of the separation capillary alternatively. This means the reversal of the direction of the vector $\Delta\mu$ and consequently, a reversal of the enantiomer migration order. The idea of the experiment shown in Fig. 4 is schematically described in Refs. [16,18,35].

A simplified form of Eq. (3) can be obtained when one supposes that the diastereomeric complexes of both enantiomers with a given chiral selector possess equal mobilities ($\mu_{C1} = \mu_{C2} = \mu_C$) [32].

$$\Delta\mu = \frac{C(\mu_f - \mu_c)(K_1 - K_2)}{1 + C[K_1 + K_2] + C^2 K_1 K_2} \quad (5)$$

This equation indicates some ways of affecting the direction of $\Delta\mu$ without affecting the affinity characteristics between a chiral analyte and a selector. In particular, from Eq. (5) it is obvious that not only a reversal of the algebraic sign of $(K_1 - K_2)$ term but also that of $(\mu_f - \mu_c)$ term may result in a change of the algebraic sign of $\Delta\mu$. This means, reversal of the enantiomer migration order. Changing the algebraic sign of $\mu_f - \mu_c$ may be achieved by affecting the effective mobility of the analyte or the chiral selector or both of them. The earlier examples of the reversal of the enantiomer migration order based on mobility adjustments have been summarized in [18]. Several new examples have been published in the recent literature (see Section 5.5).

Eq. (5) does not explicitly contain separation parameters such as electric charge and concentration of the chiral selector, pH of the background electrolyte, the direction and magnitude of the EOF. However, all of these parameters may affect the mobility term ($\mu_f - \mu_c$) in Eq. (5). Therefore, all of them may affect the enantiomer migration order implicitly.

It should be noted that not the intrinsic mobility of the free and complexed analyte but effective mobilities determine the enantiomer migration order in CE. Thus, if one can manage (even using external parameters such as pressure) the separation conditions requiring the change the polarity of the high voltage supply for a detection of the analyte, then the reversal of the enantiomer migration order may be observed [35].

Another difference between enantioseparations in CE and HPLC is the fact that an enantioseparation even in the absence of chiral recognition is, in principle, feasible in CE. This conclusion can be derived from Eq. (3). According to this equation, for the generation of a mobility difference between the enantiomers, e.g. enantioseparation in CE, the following is required:

(a) Formation of transient diastereomeric complexes between the analyte and chiral selector; this means that the enantioseparation is impossible in CE without chiral selector;

(b) Effective mobilities must be different for the free and complexed analyte ($\mu_f \neq \mu_c$). If both abovementioned prerequisites apply then enantiomers may be resolved with equal success by following two alternative mechanisms:

(1) For a given overall migration time the residence time in the free and complexed forms is not equal for both enantiomers. The time which the enantiomers reside in the free and complexed form is defined by the binding constants, e.g. in this case a difference in binding constants is required. This means that the enantioseparation will be based on the same principle as in chromatographic techniques.

(2) Alternatively, both enantiomers may reside the same time in a free and complexed form, e.g. $K_1 = K_2 = K$. Chiral recognition but not necessarily chiral separation is absent in this case. Eq. (3) under these conditions may be rewritten in following form:

$$\Delta\mu = \mu_1 - \mu_2 = \frac{K[C](\mu_{C1} - \mu_{C2})}{1 + K[C]} \quad (6)$$

From Eq. (6) it is clear that the prerequisite for the enantioseparation in this case is a formation of the transient diastereomeric complexes of both enantiomers with different mobilities μ_{c1} and μ_{c2}, e.g. $\mu_{C1} \neq \mu_{C2}$.

Thus both, either the binding constant difference (chiral recognition) or mobility difference of the corresponding diastereomeric complexes may result in enantioseparations in CE. Rather common is the former case ($K_1 \neq K_2$) or combination of both. To our knowledge no enantioseparation has been reported yet in CE entirely based on the second mechanism ($K_1 = K_2$ and $\mu_{C1} \neq \mu_{C2}$, simultaneously). However, significant differences between the mobilities of transient diastereomeric complexes has been reported in several studies [36–38]. In addition, separations of covalent diastereomeric complexes have been reported in free zone capillary electrophoresis without any chiral additives [39]. This means that one may expect enantioseparations without chiral recognition under optimized conditions in CE.

Thus, as summarized in this section, there are significant differences between enantioseparations in pressure-driven and electrically-driven systems. On one hand, these differences make the techniques complimentary. This is an advantage. On the other hand, the rules and dependencies observed in one technique should be applied to the other with some care in order to avoid serious mistakes in the interpretations of the experimental results.

3. Modes of chiral CE separations

The most traditional mode of enantioseparations in CE is when a separation capillary and both, inlet and outlet vials are filled with a buffer solution containing a chiral selector and an analyte migrates with its own electrophoretic mobility, EOF or their combination from the inlet vial towards the outlet vial passing a detector. Enantioselective interactions between the analyte and a chiral selector selectively affect the mobility of the enantiomers and this is the most common phenomenon responsible for enantioseparations in CE. Together with this mode some alternative modes of CE enantioseparations have been proposed which are briefly summarized below.

3.1. Partial-filling and counter-current techniques

The partial filling technique has been proposed by Hjerten and co-workers [40] and involves the filling of a separation capillary only in part with a chiral selector. Later it has been found that in the case when a chiral selector possesses sufficient self electrophoretic mobility in the opposite direction to the chiral analyte, it is possible to fill the entire capillary with a chiral selector [41]. This technique was named counter-current separations. In both of above-mentioned techniques the outlet vial is free of chiral selector. The advantages of these techniques are the following: (a) Chiral selectors to which given detector possesses a significant response may be used for chiral separations. This relates not only to UV-absorbing chiral selectors as commonly described in the literature [42,43] but also allows on-line CE–MS coupling [44–46] and, in principle, may allow to use chiroptical detectors also in chiral CE; (b) Some expensive and exotic chiral selectors may be used in lower amounts in this mode; (c) Binding constants between an analyte and a chiral selector can be calculated by variation of plug length and concentration of a chiral selector in a plug [47,48]. More comprehensive description of partial filling and counter-current migration principles are given in a recent review by Amini and Westerlund [19].

3.2. Mobility counterbalanced mode

Another promising mode of chiral CE separations seems to be the mobility counterbalanced mode. Counterbalancing of analyte electrophoretic mobility by pressure has been applied by Culbertson and Jorgenson for the enhancement of the detection sensitivity in achiral CE [49]. Later the same technique was used for the separation of isotopomers of phenylalanine [50]. The potential advantage of flow counterbalanced capillary electrophoresis (FCCE) can be seen from Eq. (7) [51]:

$$R_S = (\mu_1 - \mu_2)\frac{E\sqrt{t}}{4\sqrt{2D}} \quad (7)$$

where E is the electric field strength, D is the average effective diffusion coefficient of two analytes, and t is the electrophoretic migration time. In FCCE the sample is driven forward by electromigration and then backward by pressure induced flow. The samples travel back and forth in the capillary until sufficient separation is obtained [51]. In the mode of FCCE as proposed by Culbertson and Jorgenson [49] the electric field and the pressure are applied alternatively as the driving forces.

In other mode of FCCE the counterbalancing driving force such as pressure may be applied to the separation chamber continuously during the entire time of separation [33]. An enormous increase of separation factor in chiral and achiral CE separations may be achieved using this technique as already mentioned above [33].

The difference between counter-current [41] and flow-counterbalancing CE [33] techniques is that in the latter case not a chiral selector and a chiral analyte migrate in the opposite direction to each other but the bulk flow moves with a definite velocity in the opposite direction to the effective mobility of the analyte zone. The principle of this technique is schematically shown in Fig. 3 [33].

The advantages of mobility counterbalancing technique include the following: (a) Enormous, in principle unlimited, enhancement of the separation factor may be achieved. (b) This technique allows to easily transform a discontinuous zonal separation of a binary mixture into a continuous separation with stepwise migration of the sample components from the inlet towards the outlet vial (Fig. 5) [33]. (c) This technique may be used for micropreparative purposes and offers significantly higher sample capacity compared to discontinuous separations. Other potential advantages of mobility counterbalancing techniques are discussed in Ref. [33].

The mobility counterbalancing technique is certainly not limited to binary mixtures and it can easily be applied in a stepwise mode for the separation of multicomponent samples.

The pressure/vacuum, the EOF, hydrodynamic pressure (leveling of the inlet and outlet vials), etc. may be used as a driving force for countermobilities in this technique [33].

3.3. Synchronous cyclic capillary electrophoresis

Synchronous cyclic capillary electrophoresis (SCCE) was proposed by Jorgenson's group as a technique which allows to overcome the dispersion problems in FCCE caused by the parabolic counter-

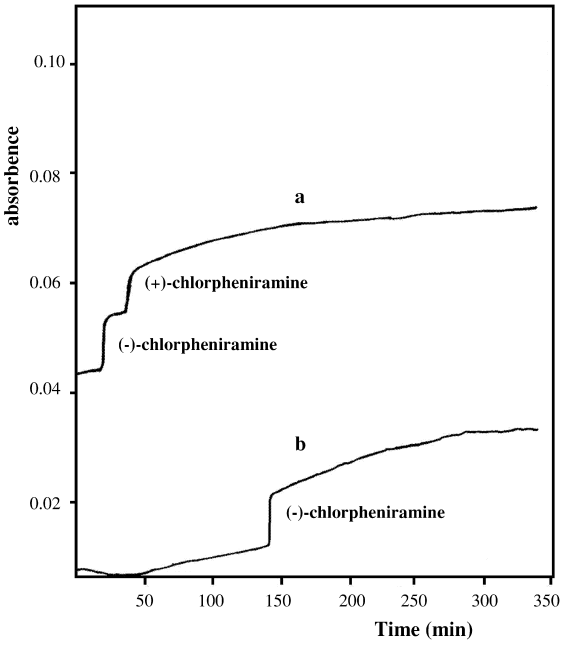

Fig. 5. Continuous CE separation of (±)-chlorpheniramine with 3.5 mg/ml CM-β-CD (a) in the absence and (b) in the presence of 689-6 Pa counterpressure. (Reproduced with permission from Ref. [33].)

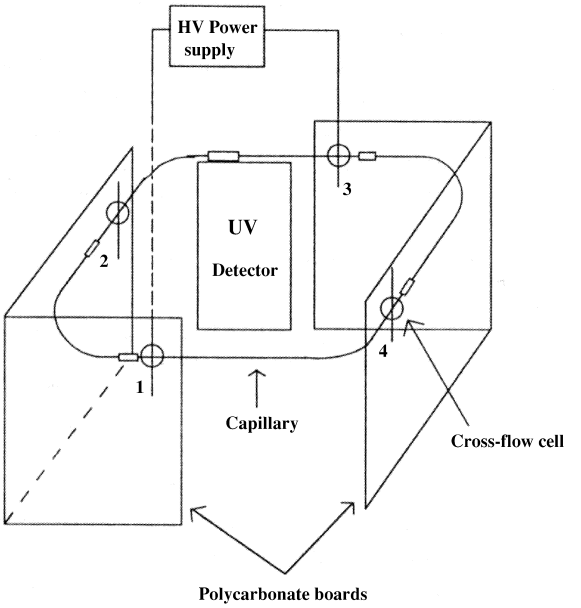

Fig. 6. Schematic diagram of SCCE system setup. (Reproduced with permission from Ref. [51].)

flow profile [51,52]. High resolution is achieved in SCCE by driving the samples in a virtually closed loop until desired resolutions are achieved. According to the authors the system operates analogous to a CE system with direct current voltage over a very long capillary.

A SCCE system is schematically represented in Fig. 6. In this experimental setup four identical sections of capillary, typically 50 μm I.D. and 50 cm long, are connected by joints and controlled by such a way that it makes possible to apply the high voltage gradient alternatively on the routes $1 \rightarrow 3$ or $3 \rightarrow 1$ which allows the sample to circulate inside the virtually closed loop until adequate separation is obtained. Each time the sample passes through the detector on the second capillary, the sample peak will be recorded in order to observe the progress of resolution improvement.

This technique was applied for isotopic and chiral separations. The dynamic of the chiral separation of a mixture of (α-hydroxybenzyl)methyltrimethylammonium and (2-hydroxy-1-phenyl)ethyltrimethylammonium in SCCE clearly illustrated the power of SCCE for solving complex separation problems. In the third cycle, the enantiomers of (α-hydroxybenzyl)methyltrimethylammonium with selectivity of 1.0078 were almost baseline separated in 3.5 h. As mentioned by the authors similar separations with a multicycle LC system as described in [53] would be also possible but would have taken 43 h.

3.4. Carrier-mode separations

In the carrier mode a chiral selector is not only responsible for the enantioselectivity in the separation system but also transports the resolved analytes to a detector. The most important advantage of this mode is that the analyte migrates to the detector only when associated with a chiral selector, i.e. when participating in the chiral recognition process. The uncomplexed analyte remains immobile or in certain circumstances may possess a self mobility directed towards the inlet vial. Among the chiral selectors charged ones possessing a self electrophoretic mobility may be used as the carriers in CE as noted by Terabe in 1989 [54].

Carrier mode chiral separations offer significant advantages and may become a very useful technique for biomedical applications where structurally similar analytes, such as chiral drugs and their metabolites must be separated and enantioseparated simultaneously. Such, rather complex separation problem implies higher enantio- and chemoselectivity requirements to a separation system (Fig. 7) [55].

Actually there is no principal difference between carrier mode enantioseparations and the enantioseparations of weakly acidic anionic analytes in an uncoated capillary when the analytes are detected at the cathode. In the latter case a neutral or cationic chiral selector (even an anionic chiral selector with a lower self mobility compared to the analyte) also accelerates the analyte towards the detector. The difference between these two modes is that in the latter case the carrier ability of the selector is ''assisted'' by the EOF which in this particular mode may represent a ''parasitic'', nonselective migration towards the detector. The carrier mode allows to easily revert the enantiomer migration order in CE as illustrated previously [2,18,56–58].

Carrier mode separations might be realized beside charged CDs also with other chiral selectors possessing a self-electrophoretic mobility as reported earlier [58]. With increasing application of combined chiral selectors the carrier ability of a chiral selector is often employed in this mode which is briefly summarized in the next section.

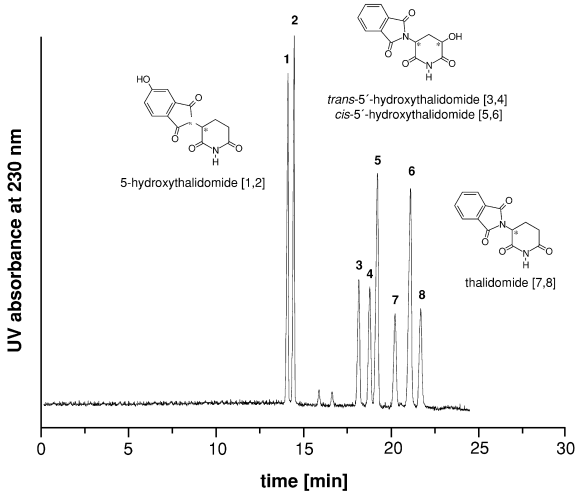

Fig. 7. Simultaneous carrier mode CE separation and enantioseparation of thalidomide and its hydroxylated metabolites. (Reproduced with permission from Ref. [55].)

3.5. Combination of chiral selectors

Combination of chiral selectors is a well-know approach in chiral CE and has been summarized in several reviews [20,59]. Our insights on the optimization of this technique have been summarized in the recent review [2] and research [60] papers. Therefore, below just one example from our ongoing studies is discussed in order to illustrate some of our previously published ideas.

A rational design of a chiral CE separation system containing more than two chiral selectors [61] seems to be extremely difficult and is not covered in this section.

As mentioned above, a chiral separation in CE may be decoupled in two basic steps: (a) chiral recognition which occurs on the molecular level and (b) transformation of chiral recognition to a chiral separation. According to our approach in order to design a dual chiral separation system it is useful to analyze both of abovementioned steps separately. When two chiral selectors cooperate in the first step, a design of the dual system becomes almost impossible without involving additional techniques and can not be optimized according below described simplified approach [2,60].

An additional point is that both, mobility and affinity effects must be considered and a conclusion about a favorable affinity pattern for an enhancement of a chiral separation may be drawn depending on the effect of both chiral selectors on the mobility of the analyte [2,60]. Some of these points are illustrated below using warfarin (WF) as a chiral analyte [78].

The enantiomers of WF have been resolved using different chiral selectors in CE [62–76]. However, the enantiomer migration order has been addressed only in few of them [72,76]. Before designing a dual chiral recognition system the affinity pattern of the enantiomers under study must be determined for each chiral selector. The enantiomer migration order of WF when using various cyclodextrins as chiral selectors is summarized in Table 1. The following seems noteworthy when discussing the results shown in Table 1. Although the enantiomer migration order

Table 1
Migration time and the migration order of WF in presence of CDs [78]

Cyclodextrin	Concentration, (mg/ml)	Migration time, (min) t_1	t_2	Migration order
Without CD		29.796	29.796	–
α-CD	20	41.047	41.047	–
	40	51.130	52.039	(−) before (+)
	60	60.834	62.838	(−) before (+)
β-CD	3	50.589	50.589	–
	6	51.934	51.934	–
	12	73.838	75.575	(+) before (−)
	15	74.431	76.504	(+) before (−)
γ-CD	2	33.765	33.765	–
	5	38.415	38.415	–
	10	42.609	44.012	(+) before (−)
DM-β-CD	6	113.343	128.539	(+) before (−)
TM-β-CD	1	32.208	32.208	–
	5	34.987	36.037	(+) before (−)
	10	40.196	42.700	(+) before (−)
HS-β-CD	5	22.417	23.075	(−) before (+)
	10	22.058	22.746	(−) before (+)
	15	19.458	19.971	(−) before (+)
	25	16.975	17.396	(−) before (+)
	50	14.550	14.642	(−) before (+)
DMS-β-CD	20	32.993	32.993	–
	40	32.346	33.008	(−) before (+)
	60	31.112	31.900	(−) before (+)
HDAS-β-CD	20	27.917	30.575	(+) before (−)

is the same when using the neutral CDs (except α-CD) and heptakis(2,3-diacetyl-6-sulfate)-β-CD (HDAS-β-CD) [77] actually the affinity pattern is opposite [78]. The reason why the CDs with opposite affinity pattern result the same enantiomer migration order is their opposite effect on the mobility of analyte. Neutral CDs decelerate the WF towards the detector whereas charged HDAS-β-CD in the given experimental conditions accelerates it.

Based on the results summarized in Table 1 and previous considerations [2,60] a combination of neutral CDs (except α-CD) and HDAS-β-CD may be beneficial for the enhancement of the separation selectivity, whereas a combination of HDAS-β-CD and the other sulfated β-CD derivative, heptakis(6-sulfato)-β-CD (HS-β-CD) [79] may disfavor the enantioseparation. This was actually observed (Fig. 8) [78].

A mathematical model describing the enantioseparation in dual chiral selector systems has been proposed by several groups [80–82]. In selected cases an application of dual chiral separation system allows to observe extremely high selectivities of enantioseparation [75]. This technique bears certain potential also for practical biomedical problem solving as shown above in Fig. 8 [55] as well as for a better understanding of fine mechanisms of chiral separations which are sometimes difficult to observe in a single selector system [60,80,83,84].

In the papers dealing with a combination of chiral selectors in CE basically multiple CD systems are discussed. However, it shall be noted that CDs may be combined with other types of chiral selectors or a combined chiral selector system without a CD component can also be used.

Extending the scope of combined chiral selector systems beyond the CDs one may note that combined chiral selectors are used in CE for a rather long

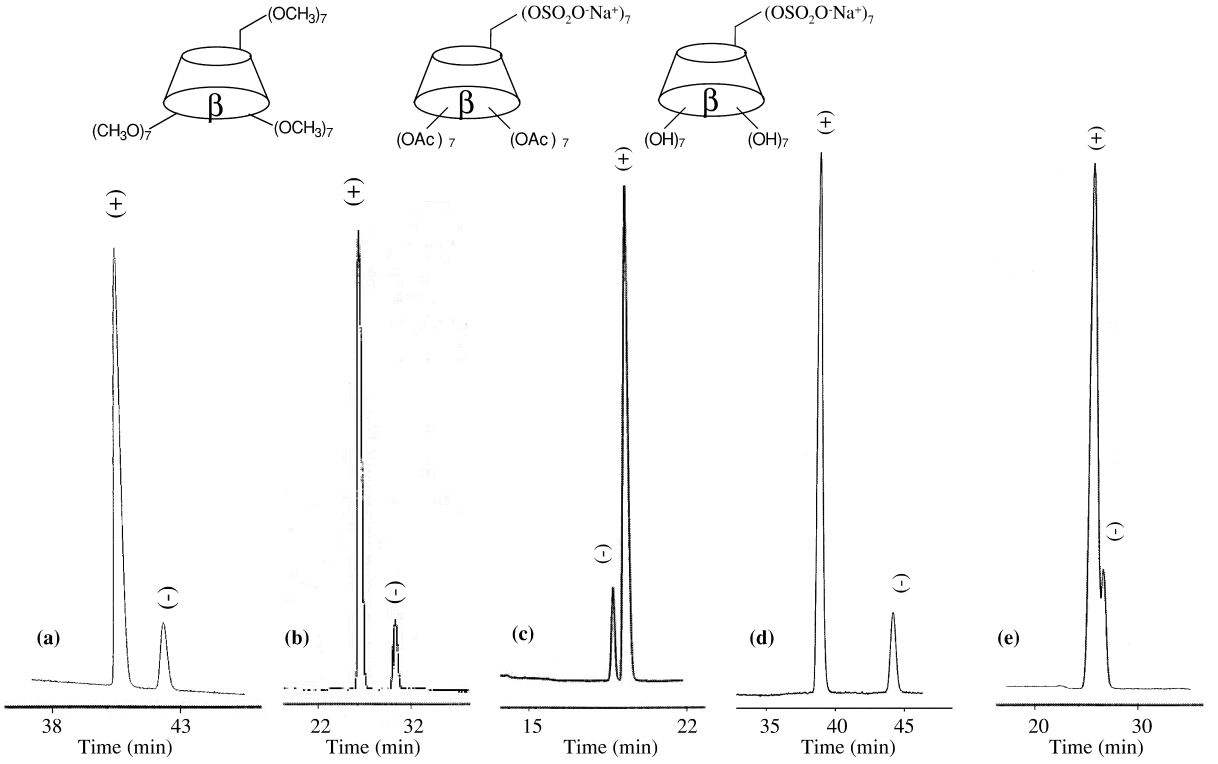

Fig. 8. Enantioseparation of warfarin using TM-β-CD (a), HDAS-β-CD (b), HS-β-CD (c), combination of TM-β-CD with HDAS-β-CD (d) and HS-β-CD (c) as chiral selectors. (Reproduced with permission from Ref. [78].)

time [85–88]. The very first example of combined chiral selectors in CE seems to be that reported by Fanali et al. [85] in 1989 when 15 m*M* L-(+)-tartaric acid buffer was used in combination with 15 m*M* β-CD in order to resolve the enantiomers of chiral cobalt complexes. CDs have been also combined with chiral surfactants such as cholic acids [86,87] or synthetic micelle-forming agents [88]. In recent years several studies were published on the combination of CDs with chiral [89–92] and achiral [83,93–95] crown others. The latter studies [83,93–95] where the achiral crown-ether can not contribute to enantioseparations independently, clearly illustrate that the simplified approach described in Refs. [2,60] may not universally applied to all dual chiral separation systems in CE.

Other interesting works on dual chiral separation systems are those involving chiral (and achiral) ion-pairing [96,97] and other low-molecular weight additives [98,99] in combination with CDs.

D'Hulst and Verbeke [99] reported a very similar approach to that described in [85]. In particular, one of the chiral selectors served at the same time as buffer anion and assisted the linear oligosaccharide-type chiral selector Glucidex6. It seems important to note that similar to other chiral selector combinations, the lower-molecular weight counterpart may not only co-operate but also counteract the other component of a separation system. For example, Glucidex6 afforded the separation of the enantiomers of aminopromazine in the presence of D-(−)-tartrate as a buffer anion, a complete loss of enantioselectivity was observed in the presence of L-(+)-tartrate and the racemic tartrate produced an intermediate resolution. In contrast to this observation, Jira et al. noted that the chirality of camphorsulfonic acids (CSA) did not play any important role in the separations studied because the enantiomeric separations have also achieved with (+)- and (−) CSA in the molar ratio 1:1 [101]. The same group also

reported the significant effect of achiral ion-pair reagents on enantioseparations [97].

Horimai et al. [98] reported an interesting example of a combination of ligand-exchange and inclusion-complexation mechanisms in order to achieve the enantioseparation of some new quinolone drugs. The separation of ofloxacine enantiomers was impossible when either the metal complex of optically pure phenylalanine or γ-CD was absent in the separation system. A crucial role of the phenylalanine metal complex was illustrated by the reversal of the enantiomer migration order of ofloxacin when using alternatively D- or L-phenylalanine as a ligand for the Zn cations (Fig. 9).

Examples of enantioseparations using combined chiral selector systems in CE are summarized in Table 2.

3.6. Enantioseparations in nonaqueous CE

Nonaqueous enantioseparations in CE have been reported since 1994 [114]. Nonaqueous buffers offer certain advantages compared to aqueous ones from the viewpoints of the alternative chiral recognition mechanisms involved in the separation [115], lower electric current and Joule heat generation, higher solubility and stability of certain analytes and chiral selectors in nonaqueous buffers [116–119], easier on-line coupling to mass spectrometer, etc.

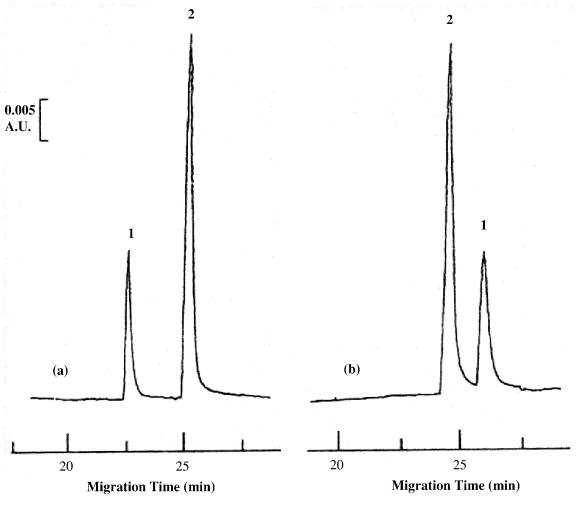

Fig. 9. Effect of configuration of Phe on the enantiomer migration order of DU-6859 in the presence of 20 m*M* γ-CD and 10 m*M* $ZnSO_4$. (Reproduced with permission from Ref. [98].)

Among the nonaqueous solvents *N*-methylformamide (NMF), *N,N′*-dimethylformamide (DMF), dimethylacetamide (DMA) and lower alcohols represent certain interest as separation media in nonaqueous chiral CE. The protic organic solvents such as NMF, DMF, etc. possess advantages for on-line chiral CE–MS coupling because they do not necessarily require electrolytes as conductive additives. However, these solvents strongly inhibit an inclusion complex formation and hydrogen-bonding interactions which are often essential contributors to chiral recognition. For this reason the concentrations of chiral selectors required for enantioseparation in these organic solvents is relatively high and sometimes it becomes questionable whether nonaqueous CE is really beneficial compared to the enantioseparation in aqueous buffer with the same chiral selector.

It has been considered that pure water, alcohols and many organic solvents require some ionizable additives such as ammonium formate, ammonium acetate, etc. These may create some problems for on-line coupling of chiral CE with MS. However, many chiral selectors work rather effectively in the solvent such as alcohols [116–119] compared to more polar solvents (DMF, NMF, etc.). Recently, Valko et al. reported significant EOF in methanol, ethanol, acetone, acetonitrile and several other organic solvents without the addition of electrolytes [120].

Nonaqueous CE enantioseparations using the chiral selectors which are impossible to use in aqueous solvents are more interesting [115–118]. It could be obviously of some interest to compare the same chiral selector in both aqueous and nonaqueous buffers from the mechanistic point of view (enantiomer migration order, intermolecular forces involved in complex formation and chiral recognition, structure of complexes, etc.). Enantioseparations of many chiral analytes have already been described with the same chiral selector in aqueous and nonaqueous buffers [118,119,121,122]. However, to the best of our knowledge until now no comparative study has been published which clearly illustrates that alternative chiral recognition mechanisms are involved when performing chiral separation with the same

Table 2
Examples of applications of combined chiral selector for enantioseparation in CE

Chiral selectors	Analytes	Ref.
L-(+) Tartaric acid + β-CD	Chiral cobalt complexes	[85]
Sodium d-camphor-10-sulphonate l-Menthoxyacetic acid + β-CD	barbiturates, atropisomeric binaphthyls, chiral alcohols	[96]
Sodium taurodeoxycholic acid + β-CD	Dns-DL-norvaline	[86]
Sodium taurodeoxycholic acid + γ-CD	Dns-DL-amino acids	[87]
SBE-β-CD + neutral CDs (β-CD, DM-β-CD)	Amphetamines	[102]
β-CD + CM-β-CD	Aminoglutethimide	[103]
Chiral crown ether + CDs	Primary amines	[89–91]
Sodium taurodeoxycholic acid + β-CD	DL-Baclofen + aromatic DL-amino-phosphates	[104]
Neutral and anionic CDs	Chiral drugs	[75,105–107]
β-CD + TM-β-CD	Chiral phenoxy acid herbicides (after derivatization with 7-aminonapthalene-1,3-disulfonic acid)	[108]
Sulfated-β-CD + α-CD	Monoterpens	[109]
Sulfated-β-CD + γ-CD	Inden, tetralin and benzosuberan derivatives	[110]
Neutral (TM-β-CD) and cationic CD (6-monoamino-6-deoxy-β-CD)	Chiral drugs (arylpropionic acid derivatives), benzoin and benzoin methyl ether	[80]
α-CD + TM-β-CD (multiple CD systems)	Atropisomeric binaphthyl derivatives	[61]
β-CD + γ-CD	Polychlorinated biphenyls	[111]
Sodium taurodeoxycholate + hydroxyalkyl (ethyl, propyl) β-CD	Chiral drug intermediate (aminotetralin derivative)	[112]
Sulfated β-CD + neutral CDs (α, β, γ, HP-α-CD, HP-β-CD, HP-γ-CD)	Basic and acidic chiral drugs	[113]
CM-β-CD + SBE-β-CD CM-β-CD + TM-β-CD CM-β-CD + β-CD	Atropisomeric binaphthyls, chiral drugs (dimethindene, chlorpheniramine, verapamil, brompheniramine, ephedrine)	[60]
Sulfated-β-CD + DM-β-CD	Chiral indole derivative (proprietary pharmaceutical)	[100]
Chiral crown ether + β-CD	*O*-, *m*-, and *p*-fluoro-DL-phenylalanine, amino acids, primary amines	[92]
Cationic and anionic CDs in combination with wall-coated open tulular capillary	Hexobarbital	[84]
Chiral and achiral ion-pairing reagents + CDs	Chiral basic and acidic drugs	[97,100]
D-(−) and L-(+) tartaric acid + linear oligosaccharides	Chiral drugs	[99]
D- and L-phenylalanine complexes, γ-CD	Quinoline drugs (ofloxacin + analogs)	[98]

chiral selector either in aqueous or nonaqueous buffers. Nevertheless, Wang and Khaledi have shown that performing enantioseparations in nonaqueous media in some cases may allow to avoid severe electrodispersion resulting from the mobility mismatch between a charged CD-analyte complex and co-ions of the running buffer (Fig. 10) [123].

Further details of enantiomeric separations by nonaqueous CE are summarized in the recently published review by Wang and Khaledi [124].

3.7. Enantioseparations by capillary isoelectric focusing (CIEF)

A special section is devoted to this topic because despite to the early examples of enantioseparations

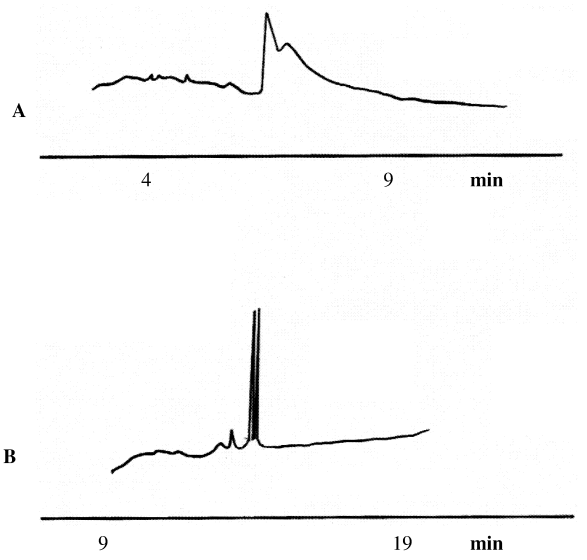

Fig. 10. Comparison of separation of thioridazine enantiomers with sulfated-β-CD (degree of substitution (DS)=4) in aqueous and nonaqueous media. (Reproduced with permission from Ref. [123].)

described by Righetti and co-workers based on the isoelectric focusing principle in the slab-gel format [125] for a long time capillary isoelectric focusing (CIEF) was considered to be only the mode of CE in which an enantioseparation was impossible. The enantiomers possess the same pK_a values and apparent isoelectric point. Due to this reason the enantiomers have been considered to be unresolvable in CEIF. In contrary to this opinion, although enantiomers also do not possess either different charge density or different size which are required for separation in CZE and CGE, respectively, the enantiomers were considered to be resolvable using these techniques. As stated above (see Section 2.1) enantiomers are virtually unresolvable using the separation principles of CZE, CGE, CIEF or capillary isotachophoresis (CITP). However, if the chiral analyte is involved in multiple interdependent equilibria and at least one of these equilibrium is enantioselective then the entire process may appear enantioselective.

Although not clearly emphasized in original paper the first application of interdependent multiple equilibria for enantioseparations in the CE format seems to be that described by Vigh and co-workers [126]. These authors reported the enantioseparation of fenoprofen and ibuprofen with neutral β-CD at pH 4.50. It was found that only the neutral forms of these chiral compounds are stereoselectively recognized by β-CD. However, this can not generate a measurable mobility difference between the enantiomers because $\mu_f = \mu_c$ in Eq. (5). The phenomenon responsible for a generation of a mobility difference between the enantiomers in this system seems to be following: the neutral forms (as well as the ionic forms) of these compounds are involved simultaneously in two interdependent equilibria: the acid–base equilibrium and an interaction with β-CD. When the latter is stereoselective, the effective concentration of the neutral forms of the *R*- and *S*-enantiomers which are available for participation in the acid–base equilibrium becomes different, and this turns the inherently non-stereoselective dissociation process into apparently stereoselective one. Thus, the *R*- and *S*-enantiomers will be ionized in different degrees, which results in a difference between their effective mobilities, e.g. enantioseparation [2].

Due to the above-mentioned reason the effective pK_a value (apparent isoelectric point) of the enantiomers in analogy to their effective mobilities in CZE may become different. The difference in the (effective) pK_a values between the analyte components is responsible for a separation in CIEF.

The feasibility of enantioseparations in the CIEF mode has been experimentally shown recently by two groups. Glukhovskiy and Vigh described analytical-scale enantioseparations in the CIEF mode (Fig. 11) as well as micropreparative separations using the commercially available continuous free-flow electrophoretic unit Octopus (Fig. 12) and proposed a mathematical model considering the simultaneous multiple equilibria involved in the chiral recognition process [127]. Using the model it was predicted that the Δp*I* vs. CD concentration curves approach limiting Δp*I* values which can be as large as 0.1, even when the binding constants of the enantiomers differ only by 10%.

Fundamental treatment of pK_a-shift associated effects in enantioseparations by CD-mediated CE was given by Rizzi and Kremser [128]. The authors

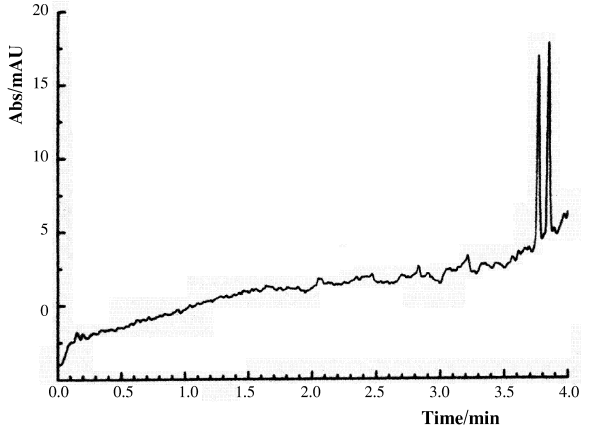

Fig. 11. EOF-mobilized CIEF separation of the enantiomers of DNS-Phe in 20% ampholyte pH 3–5, 30 m*M* HP-β-CD background electrolyte. (Reproduced by permission from Ref. [127].)

used this effect for enantioseparations by CIEF and noted a preparative potential of the technique [129]. These studies are of unambiguous practical significance. However, their importance for understanding of certain ''confusing'' effects in chiral CE is even higher. One example of this kind [130,131] is discussed in detail in Section 5.5.

3.8. Enantioseparations on microfabricated devices

Microfabricated devices offer important advantages from the viewpoint of analysis time, costs and throughput capacity. The problems with pressure-driven microchip technologies are basically connected to the management of fluid flow with acceptable characteristics through the miniaturized channels. Electrokinetically driven flow can be precisely controlled by regulating the applied potentials at the terminus of each microchannel. Using electric fields to direct and control fluid flow eliminates the need for micro-moving parts such as pumps and valves allowing for a convenient integration process of complete assays. Due to the flat cross section of the channel and the large thermal mass of the glass chip, temperature dissipation on chips is greatly improved compared to conventional capillaries. This allows to apply higher electric fields, which in combination with short separation lengths enable fast separations [132,133]. Offering these advantages achiral CE separations using microfabricated devices is extensively developing during last few years [134–138]. Recently, two papers appeared on chiral CE separations on microchips which are briefly summarized below [132,133].

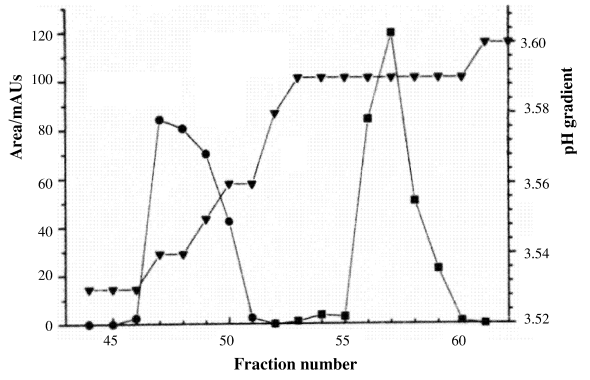

Fig. 12. Preparative continuous free-flow isoelectrofocusing separation of the enantiomers of DNS-Phe using Bier's serine-propionic acid, 0.1% HPMC, 30 m*M* HP-β-CD background electrolyte in the Octopus unit (Reproduced by permission from Ref. [127].)

The microfabricated device used by Hutt et al. [133] is schematically shown in Fig. 13. The system consists of a folded electrophoresis channel (19.0 cm long × 150 μm wide × 20 μm deep) that was photolithographically fabricated in a 10-cm-diameter glass wafer sandwich coupled to a laser-excited confocal fluorescence detection apparatus providing subatomole sensitivity. Using a sodium dodecyl sulfate/γ-CD carbonate buffer at pH 10.0 and a separation voltage of 550 V/cm at 10°C, baseline resolution was observed for Val, Ala, Glu, and Asp enantiomers and Gly in only 4 min (Fig. 14). This technique was applied for the analysis of hot water extracts taken from interior and exterior samples of the Murchinson meteorite (Fig. 15). The results obtained with this technique were comparable to those previously obtained using GC–MS and HPLC techniques. The more racemic values were found in the interior versus the exterior samples. According the authors this finding supports the hypothesis that there was contamination by terrestrial L-amino acids after the meteorites fall to Earth in 1969 [133].

In the study by Rodriguez et al. [132], electrophoretic separation conditions were similar to those used by Hutt et al. [133] although a detection system and chip design were slightly different. Fluorescein isothiocyanate (FITC)-labeled amino acid enantio-

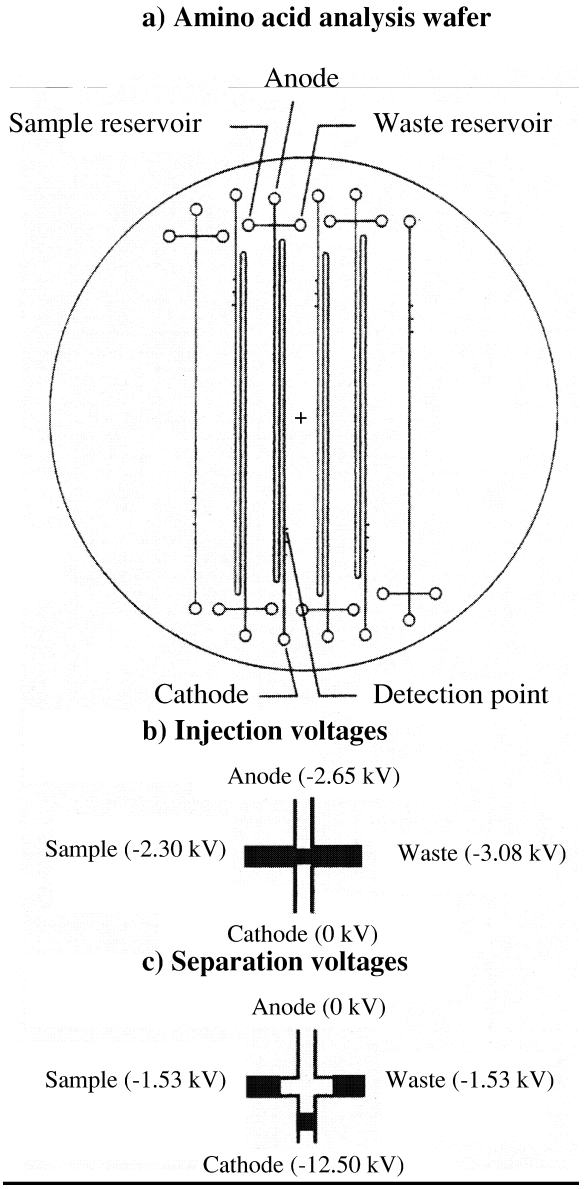

Fig. 13. Schematic representation of the amino acid analysis wafer used in Ref. [133].

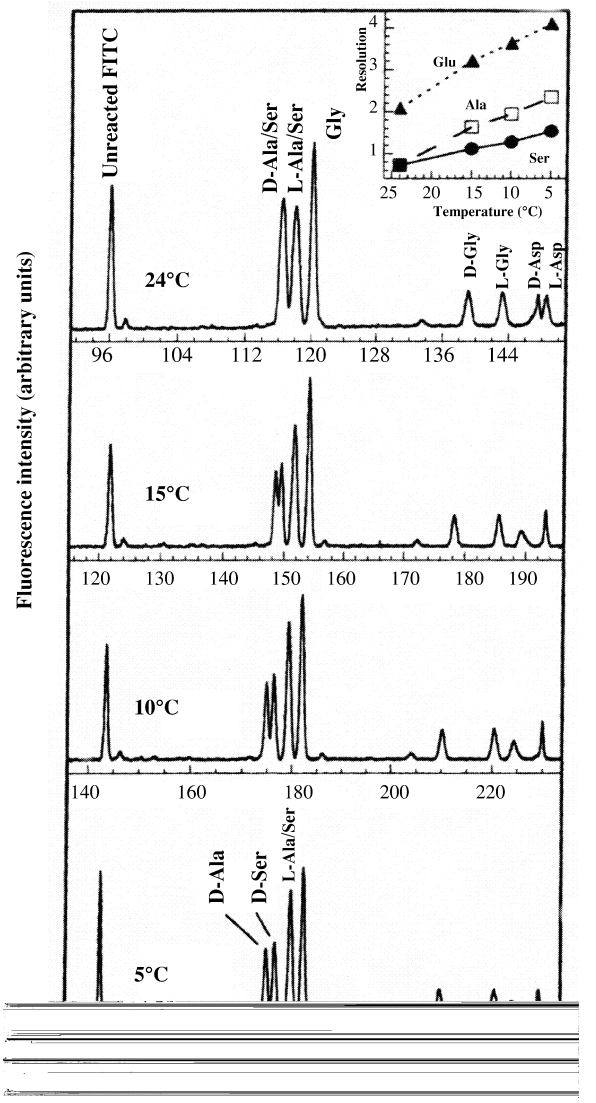

Fig. 14. Electropherograms of a FITC-labeled standard mixture of racemic amino acids obtained using weafer described in Fig. 13. (Reproduced with permission from Ref. [133].)

mers were resolved in the analysis time ranging from 75 to 160 s with efficiencies from 100 000 up to 395 000 counts per meter [132]. As the authors concluded microchip-electrophoresis could be a useful tool for high throughput chiral analysis in the pharmaceutical industry [132].

3.9. Micropreparative enantioseparations

Chiral CE is considered basically to be a technique suitable for analytical-scale enantioseparations. However, as recent studies show this technique possesses a certain potential for micropreparative and preparative scale enantioseparations [33,127–129,139–142]. Some examples of micropreparative scale enantio-

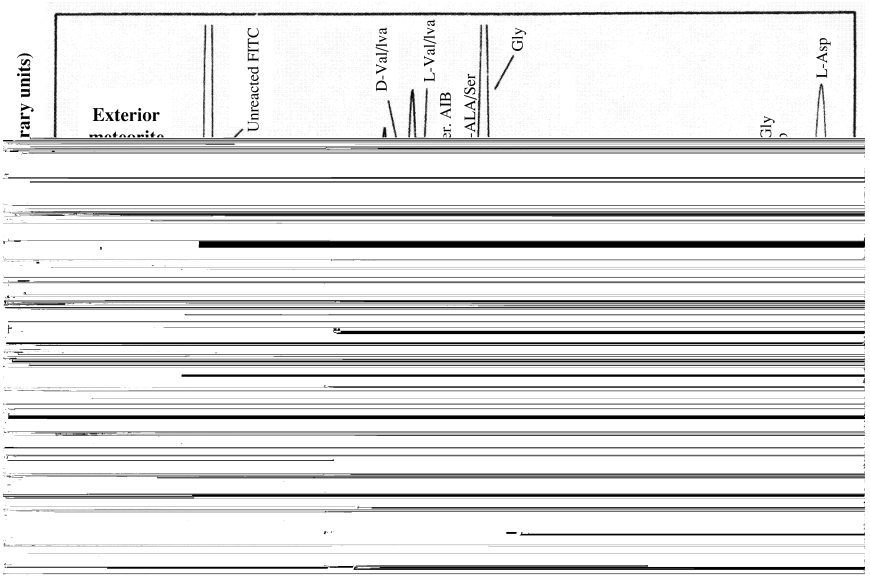

Fig. 15. Electropherograms of FITC-labeled amino acid extracts of samples taken from the interior and exterior of the Murchinson meteorite. (Reproduced with permission from Ref. [133].)

separations using the mobility counterbalancing [33] and isoelectric focusing [127] techniques were already mentioned above. Additional examples of preparative scale enantioseparations using electrophoretic techniques are described in Refs. [139–142].

In the studies by Stalcup and co-workers [139,140] similar to [127], CE has been used basically in order to optimize enantioseparation in classical gel electrophoresis and further to study the optical purity of collected fractions. This group has been the first who on the example of a short-acting β_2-adrenergic agonist terbutaline demonstrated that classical gel electrophoresis is indeed viable as a method for the separation of milligram quantities of chiral compounds [139]. In another study by the same group the enantiomers of the chiral drug piperoxan were resolved on the micropreparative scale using commercially available ''Mini Prep Cell'' from Bio-Rad (Hercules, CA, USA) (Fig. 16). This unit allowed under optimized conditions to resolve in a single run (Fig. 17) 0.5 mg of racemic piperoxan in the run time 4.5–5.0 h. The electropherograms of fractions collected from the Prep Cell run, indicated that the fractions were almost enantiomerically pure.

Lanz et al. recently reported the enantiomeric separation of methadone by a CD-based capillary and recycling isotachophoresis [141]. The latter technique was also applied for micropreparative purposes using two commercially available units, the model RF3 (Protein Technologies, Tucson, AZ, USA) and MiniPhor (Protein Technologies, distributed through Rainin Instrument Co., Woburn, MA, USA). Both of these units allowed to achieve a

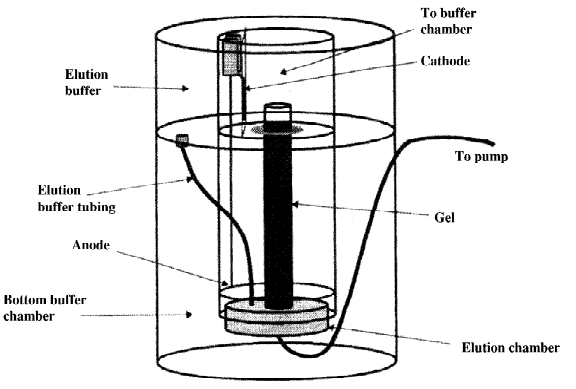

Fig. 16. The Bio-Rad Prep Cell. (Reproduced with permission from Ref. [140].)

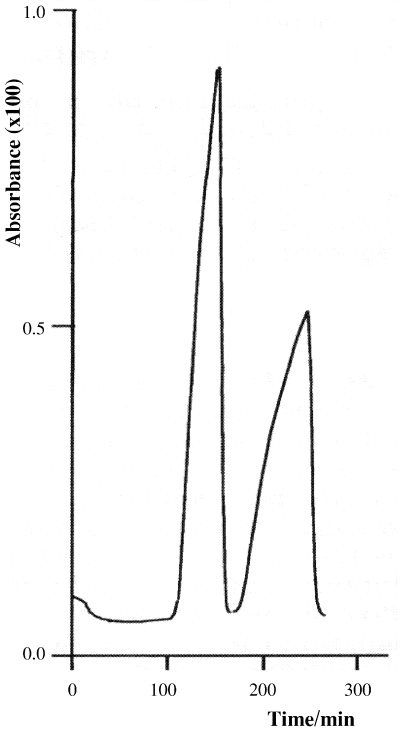

Fig. 17. Enantioseparation of piperoxan using the Prep Cell. (Reproduced with permission from Ref. [140].)

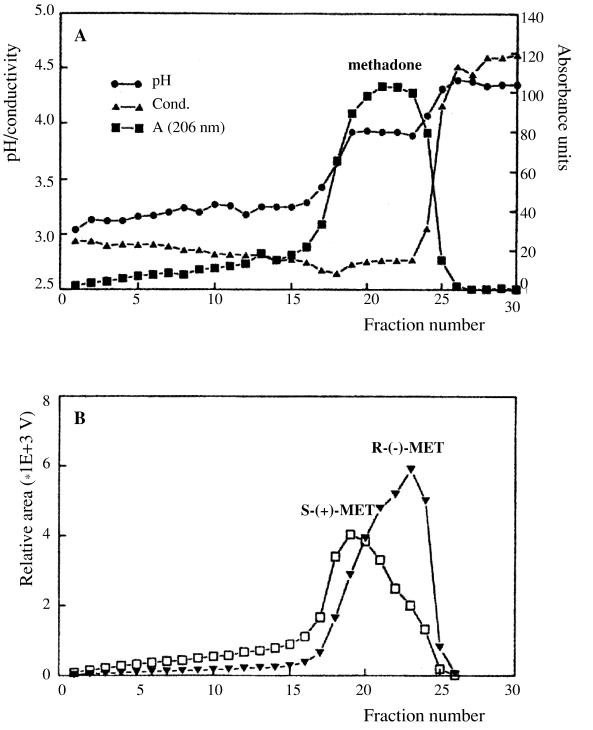

Fig. 18. Chiral recycling isotachophoretic data of 45 mg methadone obtained with the RF3 unit. The total processing time was 179 min. (Reproduced with permission from Ref. [141].)

partial separation of *R*-(−)-methadone and *S*-(+)-methadone being significantly enriched at the front and back side (Fig. 18). Indeed, this technique which is based on the use of counterflows has been applied earlier by the same group also for the achiral fractionation of compounds [143,144]. According the authors in the study [141] it was impossible to obtain optically pure fractions of (*S*)- and (*R*)-methadone.

Kaniansky et al. [142] have shown that similar to [33], electrophoresis in the capillary format may be directly applied for micropreparative purposes. After the introduction of commercial CE equipments with multiple capillary bundles (several hundreds of capillaries), the techniques described in Refs. [33] and [142] may gain more actuality. In the latter work the authors initially showed on the theoretical basis that the sample capacity may be several orders higher when performing separations in the isotachophoretic mode compared to the CZE mode. Further, in the experimental setup in which a combination of 1.0 mm I.D. and 0.8 mm I.D. fluorinated ethylene–propylene copolymer capillaries were used microgram amounts of racemic 2,4-dinitrophenyl-DL-norleucine were separated with high enantiomeric purity and a recovery of about 75% in 20 min.

Very recently, the counter flow migration principle reported by Chankvetadze et al. for capillary format [33] has been applied by Glukhovskiy and Vigh [145] in the continuous free-flow electrophoretic system, the Octopus. The production rates 2.8 mg/h were achieved for the enantiomers of terbutaline when using heptakis-6-sulfato β-CD as a chiral resolving agent.

As noted almost in all papers on the micropreparative scale enantioseparations using electrophoretic techniques, there are still many practical problems to be solved before these become a viable alternative to other techniques for obtaining enantiomericaly pure compounds. However, progress is rather fast and significant developments may be expected in the very near future.

4. Newly developed chiral selectors

A short overview on the trends regarding new chiral selectors for CE is given in Ref. [22] which describes the application of chiral CE in drug analysis. As mentioned in [22], although CDs and their derivatives remain the most widely used chiral selectors in CE, new chiral selectors are intensively proposed. Not all of the novel chiral selectors become widely used due to different reasons such as availability, costs, compatibility and competitivity with established chiral selectors, etc. However, some of them are interesting from the mechanistic point of view. In other examples, the recent developments in this field clearly illustrate the power of chiral CE which allows to detect a chiral recognition in those cases of selector-selectand pairs where other techniques are ineffective due to lower efficiency or enantiorecognition power.

4.1. Cyclodextrin derivatives

Several new randomly and selectively substituted CD derivatives have been used as chiral selectors in CE in last 2–3 years. The techniques for a detailed characterization of the substitution pattern of randomly modified CD derivatives are known [66,146–155]. In addition, some randomly modified CD-derivatives can be separated into the fractions as shown in an elegant way in [153–155]. Moreover, the monosubstituted fraction has been resolved into the components containing individual regioisomers. The suppliers of randomly substituted CD derivatives also try to provide their products in reproducible quality and well characterized form. Nevertheless, the CD derivatives with a known derivatization pattern are recommended to use for more or less deeply going mechanistic studies as well as for a development of validated chiral CE assays. Derivatization of a CD in a designed way by selective activation and protection of the hydroxyl groups on the CD rim is a well-known technique in the carbohydrate chemistry [156]. The application of selectively substituted CDs in achiral CE has also a relatively long history. The first charged CD derivative used for enantioseparation by Terabe was also a single isomer 6-β-monoaminoethylamino-6-monodeoxy-β-CD [54]. Later, Nardi et al. [157] also used two single-isomer positively charged β-CD derivatives, 6-monomethylamino-6-deoxy-β-CD and $6,^{A}6^{D}$-dimethylamino-dideoxy-β-CD for the enantioseparation of chiral 2-hydroxy acids. Some examples on the use of single-isomer positively charged β-CD derivatives as chiral selectors in CE have been published in literature [158–161]. When these earlier studies were performed, chiral CE was rather new field and only few randomly derivatized CDs were available as chiral selectors. Therefore, potential disadvantages of multicomponent mixtures, such as additional band broadening and poor reproducibility of experimental results have been not realized that time. Consequently, in the above-mentioned studies [156–161], not much attention was paid to a single-component composition of the applied cyclodextrins.

This topic attracts enhanced attention after describing the syntheses and applications of the negatively charged single-isomer CD sulfates by Vigh's group [77,79,162]. The first three members of this family, namely, heptakis-(2,3-diacetyl-6-sulfato)-β-CD [77], heptakis-6-sulfato-β-CD [79] and heptakis-(2,3-dimethyl-6-sulfato)-β-CD [162] have been commercialized. Although useful for enantioseparation of chiral cationic, anionic, neutral and zwitterionic analytes, the single isomer CD sulfates do not seem to be superior from the viewpoint of chiral separation power compared to randomly substituted analogs such as commercially available β-CD-sulfate or sulfobutyl-β-CD. However, unambiguous advantages of the single-isomer CD-sulfates are better reproducibility of separation results achieved with this kind of chiral selector. Other, still less recognized advantage of these CDs is their perfect suitability for the mechanistic studies. This relates not only to the reliable thermodynamic quantities obtained in the binding studies or developing mathematical models but also to clear, well resolved signals in ^{1}H- and ^{13}C-NMR spectra. The latter makes single-isomer CD sulfates especially well suitable probes for studies of structure of CD-analyte complexes in solutions using nuclear Overhauser enhancement (NOE) in NMR-spectroscopy. One additional, again yet not well studied, advantage of the CD derivatives described in [77,79,162], especially of heptakis-(2,3-diacetyl-6-sulfato)-β-CD, seems to be its opposite enantiomer binding pattern for many racemates compared to other CDs (see for example Fig. 8 and

Refs. [163,164]). This property is interesting from both a practical and a mechanistic points of view. Recently, the same group reported the synthesis and application of oktakis (2,3-diacetyl-6-sulfato)-γ-cyclodextrin [165]. It is difficult to believe that the charged CD-derivatives with the maximal possible charge is favorable for chiral separations. Nevertheless, several interesting applications were described recently with 14-fold charged single-isomer β-CD derivatives [166].

The syntheses and applications of single-isomer cationic derivatives of CDs have been also reported [80,167–173]. In addition, the first two members of the family of single-isomer cationic CDs, namely, 6-monodeoxy-6-monoamino-β-CD and 6-monodeoxy-6-monoamino-heptakis (6-*O*-methyl)-heptakis (2,3-di-*O*-methyl)-β-CD became commercially available from Cyclolab Ltd. (Budapest, Hungary). Although both of these derivatives are rather expensive, the minute amounts of chiral selectors required in CE may facilitate a more extensive study of at least the commercially available single isomer cationic CD derivatives as chiral selectors in CE.

Together with the above-mentioned cationic and anionic CD derivatives, few other representatives of this family such as the sodium salts of β-CD-6-monophosphate, the sodium salts of β-CD-6-monocarboxylic acid and mono- and di-carboxymethyl β-CDs were used for mechanistic studies in Ref. [174].

Several papers by Fu et al. [175] reported the use of 2,6-di-*O*-carboxymethyl-β-CDs for CE enantioseparation of few basic analytes. Although not providing any information about the synthesis, commercial availability or even if all seven glucose unites or only one of them is derivatized, these works may represent some interest. Among other charged derivatives of CDs randomly substituted succinyl-β-CD and CD phosphates are commercially available and have been used as chiral CE selectors in several studies [61,176]. However, no significant advantages of these derivatives compared to other members of this family have been shown and it seems less probably that they will gain significant use in the field.

Zwitterionic CD derivatives may represent some interest as chiral CE selectors. Several interesting applications have been reported [177,178]. In these CDs anionic, neutral and cationic forms may be switched pH-dependent and this property seems to be attractive. However, it remains still to be proved that these derivatives may successfully cover the wide spectrum of cationic, anionic, zwitterionic and neutral chiral analytes resolvable with the anionic or cationic CDs available. It seems noteworthy that zwitterionic mono-(6-*δ*-glutamylamino-6-deoxy)-β-CD described in [177] also represents a single-isomer chiral selector.

Several papers were published in the last few years reporting the application of well-known commercially available neutral CD derivatives, such as heptakis-(2,3-di-*O*-methyl)-β-CD (DM-β-CD) and heptakis-(2,3,6-tri-*O*-methyl)-β-CD (TM-β-CD) [5,179] as chiral selectors in CE. Together with these derivatives, other well characterized single-component neutral CDs such as variously methylated α, β and γ-CDs [180], heptakis-(2,3-diacetiyl)-β-CD [181–183] were used as chiral CE selectors. Cyclolab provides several single-component methylated, ethylated and acetylated derivatives of α-, β- and γ-CD. Recently, Chiari et al. [184] reported the use of one additional member of neutral CD polymer family for enantioseparations of cationic chiral analytes.

Summarizing this subsection it can be noted that there is no more urgent need to introduce new chiral selectors of CD family. In contrast, despite of rather long-time studies it seems that the binding and chiral recognition mechanisms of CDs are still not well understood. Therefore, the designed synthesis of CD derivatives with desired properties and the study of their interaction with chiral analytes using other instrumental and computation techniques in combination with CE seems to be rather important. These aspects are discussed below in Section 5.4.

4.2. Non-cyclic oligosaccharides and polysaccharides

Among the most recent developments in this field it seems interesting to note that even molecules as small as monosachcarides may, in principle, be used as chiral CE selectors [185]. For some disaccharide molecules it becomes possible to study the effect of the characteristics such as the nature of the monosaccharide unites, linkage position and the linkage type

Table 3
Enantioseparation of BDHP enantiomers with various di- and trisaccharides as Chiral selectors[a] (data from Ref. [186])

Saccharide		Migration time (min)		Separation factor (α)	First-eluted enantiomer
		t_1	t_2		
Cellobiose	β-D-Glc-[1→4]-D-Glc	22.30	–	1.00	–
Gentiobiose	β-D-Glc-[1→6]-D-Glc	11.30	11.60	1.02	(*S*)-(+)
Isomaltose	α-D-Glc-[1→6]-D-Glc	16.12	16.33	1.01	(*S*)-(+)
Isomaltulose	α-D-Glc-[1→6]-D-Fru	24.39	–	1.00	–
Lactose	β-D-Gal-[1→4]-D-Glc	23.91	24.14	1.01	(*R*)-(−)
Lactulose	β-D-Gal-[1→4]-D-Fru	26.25	26.55	1.01	(*R*)-(−)
Maltose	α-D-Glc-[1→4]-D-Glc	23.00	24.10	1.05	(*S*)-(+)
Melibiose	α-D-Gal-[1→6]-$_D$-Glc	20.80	–	1.00	–
Trehalose	α-D-Glc-[1→1]-α-D-Glc	22.66		1.00	–
Trehalose[b]	α-D-Glc-[1→1]-α-D-Glc	24.24	25.13	<1.01	(*R*)-(−)
Sucrose	β-D-Fru-[1→4]-α-D-Glc	28.00	–	1.00	–
Turanose	α-D-Glc-[1→3]-D-Fru	25.17	25.39	1.01	(*R*)-(−)
Cellotriose[c]	(β-D-Glc-[1→4])$_2$-D-Glc	23.50	24.00	1.02	(*R*)-(−)
Maltotriose[c]	(α-D-Glc-[1→4])$_2$-D-Glc	26.60	27.20	1.02	(*S*)-(+)

on the chiral recognition ability (Table 3) [186]. Interestingly, the enantiomer migration order of atropisomeric 1,1′-binaphtyl-2,2′-diyl hydrogen phosphate (BDHP) is opposite when using malto- and cellotriose as chiral selectors. These two trisaccharides differ from each other only in the linkage pattern between the glucose units which is α-(1,4) for malto- and β-(1,4) for cellotriose. Similar effect of the type of linkage on the enantiomer recognition pattern was observed also for polysaccharide derivatives. Thus, the enantiomers of the same atropisomeric compound BDHP exhibited the opposite affinity pattern towards water-soluble amylose (α-1,4 linkage) and alkylated celluloses (β-1,4 linkage) [187]. Significant chiral recognition ability of polysaccharide alkylderivatives which are sometimes used as ''inert'' dynamic EOF modifiers in chiral CE separations needs to be reconsidered at least for racemic compounds having a structure similar to those mentioned in these studies [186,187].

Together with neutral non-cyclic carbohydrate derivatives many charged analogs were further studied which are summarized in Table 4 [188–194].

4.3. Chiral surfactants

Recent developments in the field of CE enantioseparations using chiral surfactants have been summarized in several review papers [195–197]. In these reviews the reader may find fundamentals, effect of various separation parameters and applications to chiral drugs, environmental pollutants and chiral chemicals. Newly developed chiral surfactants are summarized in Table 5 [196–208].

4.4. Macrocyclic antibiotics

The application of macrocyclic antibiotics in chiral CE was summarized in the review papers by Armstrong and Nair [13] and by Desiderio and Fanali [14]. In the last two years the applications of macrocyclic antibiotics, in particular vancomycin, has been demonstrated for many enantioseparations of practical importance [209–211]. Several macrocyclic antibiotics such as avoparcin [13], tubocurarin [210], etc. have been introduced as chiral selectors in CE [211–215]. Although few complementary enantioseparations were demonstrated with the recent members of this family, it seems that the best three members have been found out in the pioneering studies by Armstrong's group [216–218].

4.5. Proteins, peptides and peptide libraries

Peptides have been used further as for enantioseparations in CE [219–231] as well as for drug binding studies to biologically relevant proteins [232]. Together with previously used bovine serum

Table 4
Enantioseparations using noncyclic carbohydrates in CE

Carbohydrate	Analyte	Buffer	Ref.
Dextran, Dextrin	Atropisomeric binaphthyl derivatives, chiral cationic and anionic drugs	20 m*M* Phosphate buffer, pH 2.5 or 7.0	[188]
Diethylaminoethyl dextran	Atropisomeric binaphthyl derivatives	20 m*M* Phosphate buffer, pH 7.5–9.0	[189]
Dextrin sulfopropyl ether	Atropisomeric binaphthyl derivatives, chiral anionic and cationic drugs	Phosphate buffers at various pH adjusted by Tris	[190]
Heparin	Basic chiral drugs (pheniramine, chlorpheniramine, brompheniramine, carbinoxamine, doxylamine)	30 m*M* Phosphate buffer, pH 3.5	[191]
Dermatan sulfate	Basic chiral drugs	25 m*M* Citrate buffer at pH 3.0, 4.5 and 6.5	[192]
Semisynthetic chondroitins	Basic chiral drugs	30 m*M* Potassium phosphate at pH 2.5	[193]
Neutral and charged polysaccharides (methylcellulose, hydroxypropylcellulose, laminaran, pullulan, amylose, carboxymethylamylose)	Atropisomeric binaphthyls, chiral basic and acidic drugs	Triethanolamine phosphate buffer (100 m*M*) at pH 3.0 and 6.0	[187]
Di- and oligosaccharides	1,1′-Binaphthyl-2,2′-hydrogen phosphate, *cis*-diltiazem	100 m*M* Triethanolamine phosphate at pH 3.0	[186]

Table 5
Recently developed chiral surfactants for MEKC enantioseparations

Surfactant	Analyte	Buffer	Ref.
Alkylglucosides	Atropisomeric binaphthyl derivatives, Tröger's base, chiral pesticides (arylpropionic acid derivatives), chiral drugs	Sodium phosphate at various pH. Borate buffer in some special applications	[196–203]
Alkylmaltoside			
Cyclohexyl-alkyl-β-D-maltosides			
Poly(sodium undecanoyl-L-valinate) (Poly L-SUV)	Atropisomeric binaphthyl derivatives, chiral alcohols, diazepines and some drugs, derivatized amino acids	Borate buffer at higher pH (≥ 7.0)	[195,204–208]
N-Undecanoyl-L-valianate (L-SUV)			
Polymeric dipeptide surfactants [Poly(sodium undecanoyl L,L-valyl-valinate), Poly(sodium undecanoyl L,L-threonyl-valinate) Poly(sodium undecanoyl L,L-seryl-valinate) Poly(sodium undecanoyl L,L-alyl-valinate)			[195]

albumin (BSA), human serum albumin (HSA), α-acid glycoprotein (α-AGP), etc. several additional proteins have been applied as chiral CE selectors [219–233]. The most innovative development in this field seems to be the earlier reported application of cyclopeptide-libraries as chiral selectors by Jung et al. [230] which was later followed by the interesting study of Chiari et al. [231]. In both of these works the potential of combinatorial libraries for a discovery of new chiral selectors has been illustrated.

4.6. Miscellaneous chiral selectors

From other types of chiral selectors few new ligand-exchange materials and synthetic macrocyclic compounds were examined as chiral selectors [234–237]. Synthetic macrocycles, such as calixarens and resorcarens may, in principle, allow to extend the applicability of chiral crown ethers beyond the compounds containing a primary amino group [236,237]. However, the problems associated with the strong UV-absorbance of aromatic synthetic macrocycles must be resolved by applying the partial-filling or countercurrent selector selectand migration principle [40,41]. In addition, these new groups of chiral selectors need to be more competitive to other well established chiral selectors in CE.

5. Design and adjustment of enantioseparations in CE

Rational design of a separation experiment is as complex as important. At an early stage of method development a decision must be made which one among several instrumental techniques available for enantioseparation, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC) and CE can be used in an optimal way for a particular separation problem. The discussion of the strategies for making this decision, as well as the consideration of other techniques such as diastereomeric crystallization, asymmetric synthesis, kinetic resolutions, etc. are beyond the scope of this review.

Once a decision has been made to perform an enantioseparation by CE, then the questions concerning the most suitable mode, chiral selector and separation conditions need to be answered. For the latter it is possible to use as more traditional univariate as well as multivariate approaches.

5.1. Univariate approach

Most commonly, the enantioseparations in CE have been optimized using the univariate approach. According to this strategy the effect of a selected parameter will studied on the separation parameters when other parameters are fixed at a defined constant value. To the parameters in chiral CE belong the nature and concentration of a chiral selector, the ionic strength, the pH and achiral additives to a separation buffer, the separation temperature, the material, dimensions and the inner surface of the capillary, etc. In many research papers enantioseparations are studied depending on the above-listed or some additional variables. These studies as well as simple screening of chiral selectors for a wide variety of chiral analytes become currently somewhat less actual because large material of this kind has already been accumulated. It seems rather interesting to rationalize our knowledge in chiral CE by studying structure-binding and structure-chiral recognition dependencies in more detail. For a particular separation problem, however, an adjustment of the separation by a variation of the separation conditions will still remain actual. To this group of problems may belong a particular drug and its metabolites, environmental pollutants and their degradation products, important synthetic chemicals in combination with their precursors and possible side and/or degradation products. This review does not summarize the effect of particular variables on the enantioseparation in CE because this topic has been extensively discussed in many recent [5,6,179] as well as earlier published reviews [1,4,7,9,11,13,15].

As mentioned above, many papers are published concerning the effect of different variables on chiral CE separations. However, only few of them represent a systematic approach to cost and time effective method development [21,238,239] based on the univariate approach. The subject has been recently reviewed by Fillet et al. [21] who, in analogy to other published works [238,239] also suggested a method development chart (Fig. 19) and illustrated its suitability for achieving the enantioseparation of

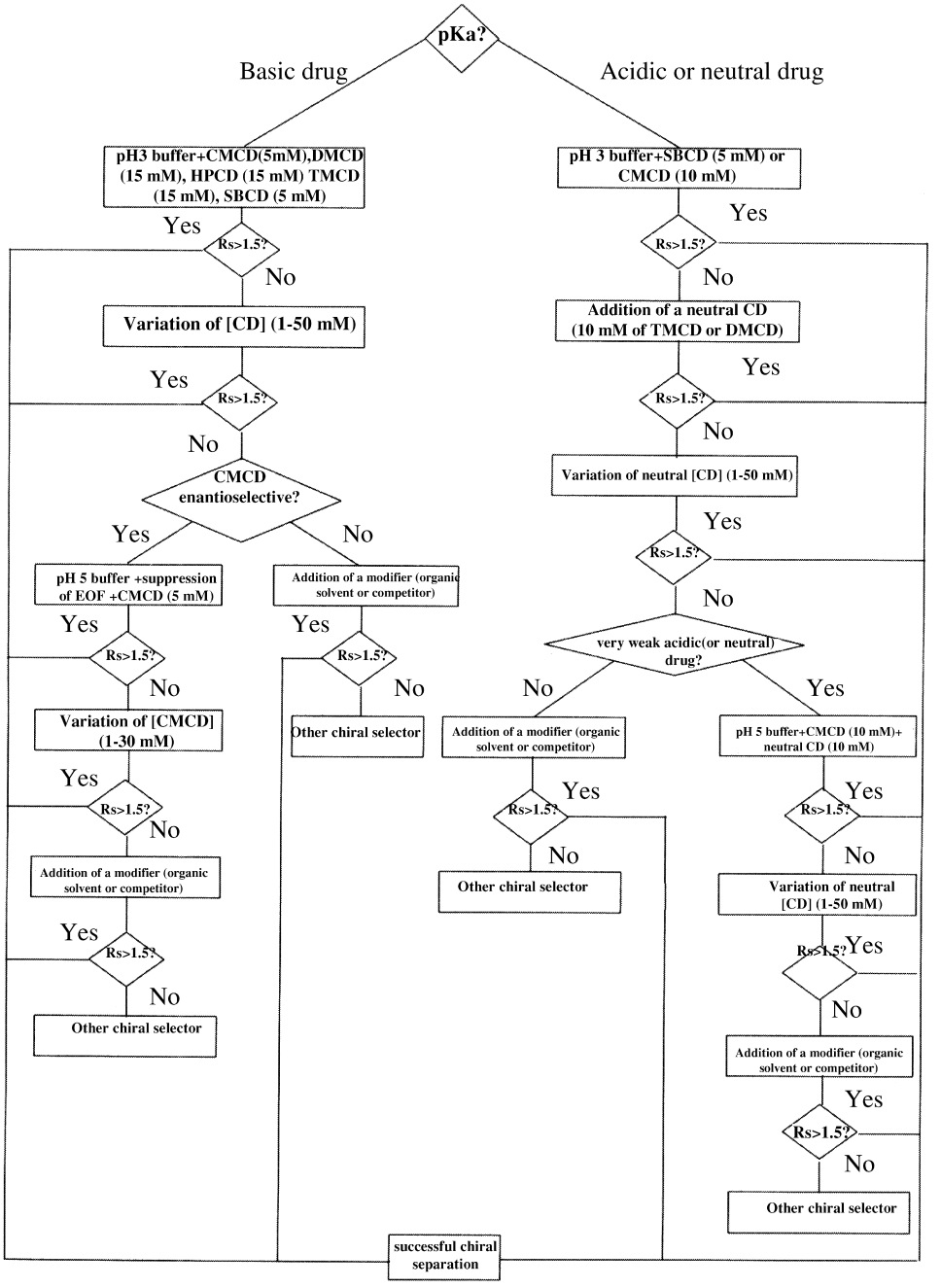

Fig. 19. Method development scheme for the CE enantioseparation. (Reproduced with permission from Ref. [21].)

48 of 50 chiral analytes tested. There is no need in discussion about the usefulness of these schemes. However, one has to keep in mind that every chart is somewhat intuitive and based on empirical knowledge, even on the personal experience of the authors. Because the charts are a priori based on a limited number of experiments its predictivity may be limited. For instance, the scheme proposed in [238]

suggests that the enantiomers of phenprecoumon as an analyte containing a condensed aromatic groups can be resolved with γ-CD, and not resolved with α-CD. This suggestion is based on the idea that the analytes which fit better into the cavity of a CD are better enantioselectively recognized. However, as recent mechanistic studies indicate (see Section 5.4) the complete inclusion of the analyte into the cavity of CD is not a necessary prerequisite for enantioseparations. A partial inclusion or external intermolecular interaction may also be sufficient. Thus, when contrary to the above-mentioned suggestion no enantioseparation of phenprecoumon in the presence of γ-CD but baseline enantioseparation with α-CD was observed (Fig. 20), the authors explained this effect by an interaction of the side-chain of phenprocoumon with α-CD cavity [238]. Thus, the contradiction was apparently correctly solved but, unfortunately, this does not improve the predictivity of the scheme. The predictivity of such charts may significantly be improved when they will based on the rules of mathematical statistics and large databases (for instance, Chirbase CE [240]).

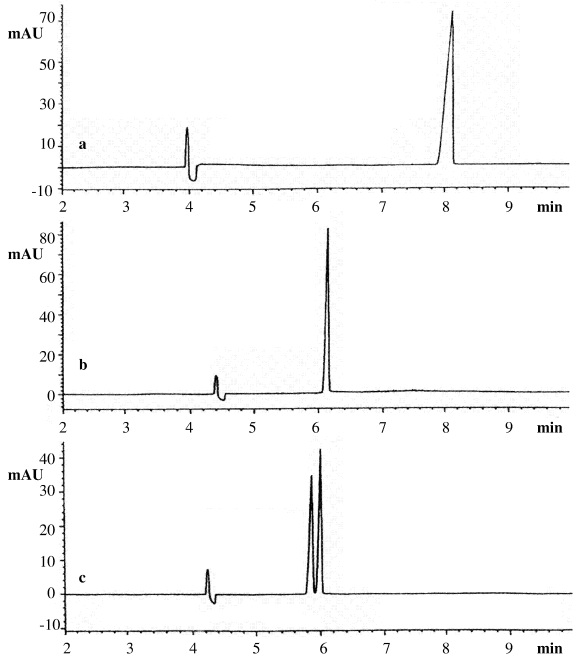

Fig. 20. Electropherograms of phenprecoumon in the absence (a) and in the presence of 15 m*M* γ-CD (b) and α-CD (c). (Reproduced with permission from Ref. [238].)

The univariate approach requires a large number of independent experiments because it typically involves keeping all parameters constant while varying one parameter at a time and measuring the method responses of interest (separation factor, peak efficiency, resolution, analysis time, etc.). This approach is experimentally reliable and when properly designed provides beside the optimization of the separation conditions some direct or indirect information about the separation mechanism. However, the univariate approach is not ideal from the viewpoints of method development with respect to time and costs (personal and equipment time, reagents, etc.).

In latter years several papers were published applying the chemometric experimental designs to the development of chiral CE methods. These studies are briefly reviewed in the next section.

5.2. Chemometric experimental designs

The goal of statistical design techniques is the reduction of the number of experiments required for optimization and to consider the possible interdependence of parameters [241]. This is very important in CE because the separation variables are usually interdependent in this technique.

Several types of designs are available [241–256], and the choice of design depends on the number of variables and how detailed the information has to be. A full factorial design is a good choice when the number of variables is four or less. When more than four variables are of interest, a fractional design is applicable. With a large number of variables a Plackett-Burman design (PBD) [242,243] is the preferred choice.

A factorial design is a statistical method in which all possible factor combinations are considered, allowing the calculation of the single effects of each factor and any factor interactions. The number of experiments required in this technique can be calculated using the following equation:

$$N_{\text{exp}} = m^n \tag{8}$$

where n is the number of factors and m is the number of levels (number of values of each factor). The number of experiments required for factorial

designs increases quickly with an increasing number of factors to be optimized. If, for example, one needs to optimize the concentration of the chiral selector, the ionic strength and pH of the buffer, the applied voltage and the amount of organic modifier in the background electrolyte (five parameters), each one at three levels, the number of experiments required will be $N_{exp}=3^5=243$.

Statistical techniques such as central composite designs (CCD) developed by Box and Wilson [244] and the PBD [242] require a smaller number of experiments and lead in general to the same result.

The number of experiments required in CCD is

$$N_{exp} = 2^n + 2n + 1 \quad (9)$$

where n is the number of factors to be optimized [245].

CCD provides data which are sufficient for the fitting of a linear polynomial model to a set of data. Regression analysis can be used for such a model, which enables one to predict the response at levels of the variables within the factor space not investigated in the design. The response in the case of CE can be peak resolution or selectivity and factors can be the concentration of the chiral selector, pH and ionic strength of the buffer, etc.

Small et al. [246] achieved a rapid optimization of the chiral CE separation of amlodipine using the CCD technique (Fig. 21). In this study the response surface was modeled for three factors by fitting a second-order polynomial in four dimensions. The number of experiments was $N_{exp}=2^3+(2\times3)+1=15$ by taking the pH of the background electrolyte, the separation temperature and α-CD concentration as factors.

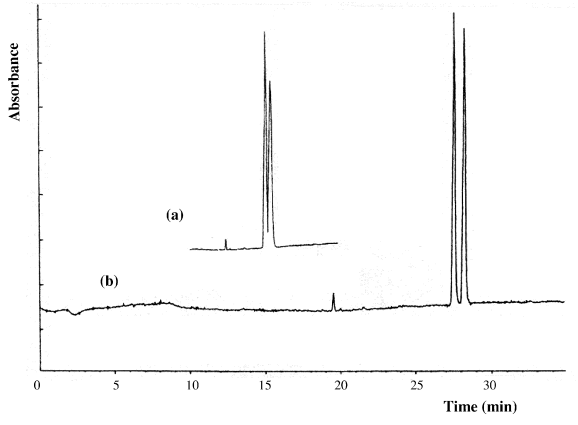

Fig. 21. Electropherograms of enantioseparation of amlodipine (a) before and (b) after optimization. (Reproduced with permission from Ref. [246].)

The factor space defined for each CCD parameter is illustrated in Table 6. The central point of the design corresponded approximately to the median value of each of these parameters. The ''star points'' in the design were located in factor space symmetrically about the central point, at points corresponding to 50% greater than the value assigned to each factor. The parameters used as response criteria in assessing the resulting electropherograms were the Kaiser's peak separation function P_1 and the resolution R_s.

The data acquired from this CCD were analyzed to model a four-dimensional response surface (Fig. 22). The optimum conditions predicted by CCD were used experimentally and resulted in a baseline separation of the enantiomers (Fig. 21). The experimental results observed for P_i and R_s are in excellent agreement with the predicted values. Although the response surface is four-dimensional, it can be readily visualized as a three-dimensional graphic by presenting the response to two factors, while the third is kept constant at its optimum value (Fig. 22).

Wan et al. [247] reported the use of a full factorial

Table 6
Experiments for a three-factor CCD optimization the separation of amlodipine by CE with α-CD. (Reproduced with permission from Ref. [246])

Experiment No.	pH	T (°C)	[α-CD] (m*M*)
1	2.75	15	10
2	2.75	15	20
3	2.75	25	10
4	2.75	25	20
5	4.25	15	10
6	4.25	25	20
7	4.25	15	10
8	4.25	25	20
9	3.50	20	5
10	3.50	20	25
11	3.50	10	15
12	3.50	30	15
13	2.00	20	15
14	4.00	20	15
15	3.50	20	15

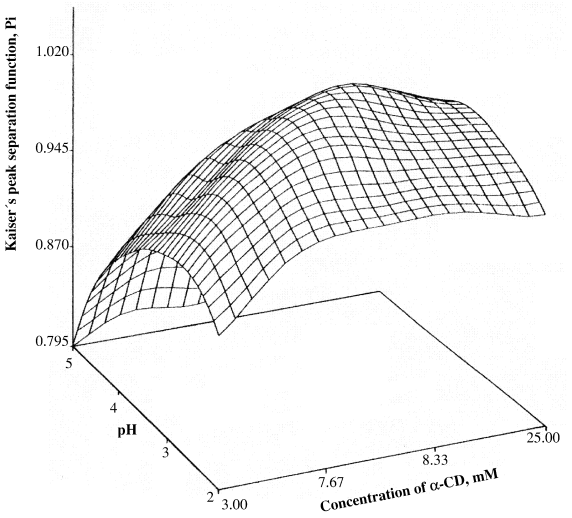

Fig. 22. Response surface for Pi of amlodipine enantiomers. Temperature 17.2°C; pH (X_1) vs[α-CD] (X_3) (m*M*). (Reproduced with permission from Ref. [246].)

design for the optimization of pH and sodium dodecyl sulfate (SDS) concentration in the separation of amino acids (AA) which were derivatized with (−)-1-(9-fluorenyl)ethyl chloroformate (FLEC). For the design, 10 experiments, including two centre points were performed utilizing a mixture of four different FLEC-AAs [threonine (Thr), isoleucine (Ile), valine (Val) and phenylalanine (Phe)]. The results obtained from the optimization indicated that a high pH was necessary for the chiral resolution of these four FLEC-AAs and that the different AAs showed optimal chiral separation at different SDS concentrations. For example, the first-eluted analyte, Thr, has a relatively high optimal SDS concentration, of about 60 m*M*. In contrast, for the last-eluted analyte, Phe, the SDS concentration should be as low as possible. This result was explained by the higher hydrophobicity of Phe compared to Thr. A buffer containing an intermediate concentration of SDS, 20 m*M*, and with a high pH, 9.2, was therefore selected in order to facilitate the separation of a maximum number of FLEC-AAs. As a result, 11 of the 19 FLEC-AAs examined were baseline separated.

The same technique was used for the optimization of β-CD and propan-2-ol concentrations for the separation of four achirally derivatized racemic amino acids [leucine (Leu), methionine (Met), Phe and Val] [248]. 2-(9-Anthryl)ethyl chroroformate was used as an achiral derivatizing reagent.

The propan-2-ol concentration was optimized in the range 8–22% (v/v) and β-CD concentration in the range 25–54 m*M*. The four amino acids have similar optimal separation conditions: β-CD 42–47 m*M* and propan-2-ol 14–17% (v/v). No interaction effects of β-CD and propan-2-ol on the resolution were observed. Under the optimal conditions, enantiomers of 12 from the 19 examined amino acids were separated.

The important advantage of full factorial design for the optimization of CE separations is that it considers non-linear changes of parameters and the interrelation between them.

The most critical point of this technique is the selection of the low and high limits of the designed parameters. A good knowledge of the separation system is generally required to do this properly. In a case where the high and low limits have been incorrectly selected, the experiment will be misleading as to the direction in which the optimum will be found [247]. CCD was shown to be useful approach for optimizing CE enantioseparation conditions of a mixture of five chiral amphetamines [249]. The same group illustrated that experimental designs offer an efficient test for the robustness of the analytical method. Recently, Zerbinati et al. [250] used a full factorial design in order to optimize the separation of the enantiomers of phenoxyacid herbicides.

Several papers have described the use of PBD [242] for the optimization of CE enantioseparations [251–254]. This technique was used by Rogan et al. [251] for chiral CE analysis of clenbuterol. It has been shown that a PBD is very useful for the optimization of separation parameters.

A simultaneous application of a fractional factorial design and a central composite design for the optimization of the CE separation of epinephrine enantiomers has also been reported [253]. In this study a compromise between conflicting goals, such as maximization of resolution and minimization of analysis time, was achieved by introducing a desirability function D. Balancing these goals, the most acceptable solution to the problem was found and the optimized method gave a fast separation with baseline resolution of the epinephrine enantiomers.

The PBD focuses on the main effect of the factors

and is especially effective when the number of variables is high. The limitation of this technique compared to full factorial designs is that the former does not allow to consider easily the interdependence between the parameters. In addition, similar to all first-degree factorial designs, the Plackett-Burman design also assumes a linearity of the estimated variables over the whole range of the experiment. In chiral CE non-linear dependencies are quite common.

5.3. Mathematical models of CE enantioseparations

The mathematical models have the advantage that in an ideal case they not only describe a separation as an entire process but also may allow to find out the effects which are difficult to prevail based either on the intuitive or empirical approach. The first mathematical model proposed for an optimization of the selector concentration in chiral CE was proposed by Wren and Rowe [32,257]. This model is based on the same equation as an earlier developed model by Stepanova and Stepanov [258] for the electrophoretic separation of cations.

The model described in [32,257] allows to optimize the concentration of a chiral selector which may result in a maximum mobility difference between the enantiomers. Although the model proposed by Wren and Rowe allows to optimize only one separation parameter, in particular the concentration of a chiral selector, among many variables, it attracts much attention by the researchers perhaps most likely due to its relative simplicity. The second reason for a wide acceptance of this model seems to be the fact, that the chiral selector concentration which this model allows to optimize, represents one of the major variables in chiral CE.

Two critical points should be mentioned when applying the aforementioned model for optimization purposes in chiral CE: (1) The maximal mobility difference between the enantiomers does not a priori mean the maximal resolution; and (2) The model does not cover several major parameters affecting chiral CE separations. However, this model without any doubt markedly contributed to the development of chiral CE and good correlations between optimal chiral selector concentrations calculated based on this model [32,257] and observed experimentally have been reported [259–263].

The duoselective chiral separation model proposed by Vigh and co-workers [126,264–269] in the initial form covers besides the selector–selectand interactions also the acid–base equilibrium of the analyte and thus, allows to optimize also pH of a buffer solution. This is a rather advanced model compared to that described in [32,257]. The authors of the duoselective separation model made interesting predictions about the reversal of the enantiomer migration order based on the calculations. Although this effect in most cases may be predicted intuitively without any cumbersome calculations, the above-mentioned prediction proves the correctness and the power of the model. Later the duoselective separation model was extended to other separation parameters [268], among them the inclusion of the effect of the EOF seems to be the most important point. The authors of Refs. [265–269] consider the EOF as a nonenantioselective transport in chiral CE. Although the mathematics is elegant as well as the examples are illustrative in the papers [265–269], we consider in general the EOF and the electrophoretic mobility of the analyte as equal transport phenomena from the viewpoint of enantioseparations (see Section 2.3).

Further advancement of mathematical models of chiral CE was the development of a chiral charged resolving agent migration model (CHARM) by the same group [269]. This model allows to predict several nontrivial phenomena. Many of these predictions were experimentally verified by the authors group as well as by other research groups [270–272].

One conclusion of the CHARM model seems worth to be discussed in more detail. As concluded in several papers in this series a chiral selector possessing the highest possible charge will be advantageous for chiral CE separations [270]. It is true that highly charged analytes in CE commonly (but not always) migrate faster and allow less time for band-broadening due to diffusion. With some approximation one may also suggest that a chiral selector with a high charge when injected discontinuously, (which is commonly not the case), will migrate fast and result in a less diffused zone which may favor higher peak efficiency of the analyte. However, this discussion relates only to the dynamic part of a

separation process. It does not address the chiral recognition part of the entire separation process. How does the charge of the chiral selector affect the selector–selectand interactions (thermodynamics and kinetics)? This remains beyond the scope of the model. However, this is an important point because there is no way to observe a chiral separation in CE without selector–selectand interactions.

Recently Zhu and Vigh extended the CHARM model by introducing three parameters as follow [273]:

$$b = K_{RCD}/K_{SCD} \quad (10)$$

$$s = \mu^0_{RCD}/\mu^0_{SCD} \quad (11)$$

$$a = \mu^0_{RCD}/\mu^0 \quad (12)$$

where the coefficient *b* represent a binding selectivity, the parameter *s* is named the size selectivity and actually represent a mobility ratio of two diastereomeric complexes, and the parameter *a* describes how the analyte mobility is altered by complexation with a chiral selector. Analyzing Eqs. (10)–(12) together with above-mentioned Eqs. (5) and (6) one may easily find the similarities between them. In particular, Eqs. (10)–(12) use the ratio of the parameters instead of their differences in Eqs. (5) and (6). Thus, the coefficient *b* in Eq. (10) closely relates to the term $K_2 - K_1$ in Eq. (5), the coefficient *s* [Eq. (11)] relates to $\mu_{c1} - \mu_{c2}$ in Eq. (5) and the coefficient *a* [Eq. (12)] relates to $\mu_f - \mu_c$ in Eq. (5). Both, the analysis of Eqs. (5) and (6) and Eqs. (10)–(12) allow to separately evaluate the contribution of different factors to the overall separation factor. Although both of these approaches lead to the same principal results, Eqs. (10)–(12) seem to be more convenient for mathematical treatment.

Gareil et al. [80], Surapaneni et al. [81], and Crommen and co-workers [82] have proposed mathematical models optimizing dual chiral separation systems (see Section 3.5). All of these models allow, in principle, a more or less complete description of the separation system if one suggests that the boundary conditions are correctly selected. However, the last is the bottleneck of any (mathematical) model which is a product of our imagination. When developing a model, a criteria must be found allowing an estimation of the model approaching reality. A model in an ideal case should allow to predict effects which are nontrivial and impossible to be derived by a simple logistic or intuitive way. One of the most important requirements to a model is to be elegant, simple and understandable for most of the researchers working in the field. Thus, the scientists involved in the development of models have to consider the old wisdom: ''One of the principal objects of theoretical research in any department of knowledge is to find the point of view from which the subject appears in its greatest simplicity'' [274].

5.4. Selector–selectand interactions in chiral CE

As already mentioned in this overview the enantioseparation in CE can be decoupled in two processes: chiral recognition and the transformation of a chiral recognition into a chiral separation, e.g. the generation of enantioseparation from enantiorecognition.

A correlation between a recognition and a separation is more simple in pressure-driven techniques compared to electrically driven techniques. As stated in our previous papers [16,275], in CE, in contrast to chromatographic techniques, a chiral separation is possible even in the absence of a chiral recognition in the classical meaning of this definition (see also Section 2.4). On the other hand, a chiral recognition does not always lead to chiral separation in CE. Thus, enantioseparation in CE may be a result of stereoselective selector–selectand interactions, as well as nonstereoselective selector–selectand interactions but different mobility of the diastereomeric complexes formed, or the combination of both. Independent of which of the aforementioned three mechanisms is involved in a particular case, selector–selectand interactions are an unavoidable part of any chiral CE separation. In extension to our previous review paper on the subject of selector–selectand interactions related to chiral CE [275] below the techniques suitable for this purpose and new examples from the most recent literature are described.

5.4.1. Determinations of stereoselective selector–selectand binding constants using CE

CE offers significant advantages for the determination of stereoselective selector–selectand binding

constants in the way that the constants can be calculated under the same or very similar to the separation conditions. This means that the binding constants determined in CE may correlate best with separation.

The equation for the description of steady state in electrophoresis was introduced by Tiselius [276]. Recently, Rundlett and Armstrong [277,278] summarized this topic in the review paper [278]. A critical treatment of the subject is given and together with advantages of CE for the binding studies, such as high efficiency, ease of automation, short analysis time, small sample size and buffer volume, limitations of this technique and possible error sources in CE binding constant determinations were also addressed. In order to avoid duplications with the above-mentioned papers which are advised for additional reading, some fundamental aspects of the subject are omitted in this section.

One important advantage of CE which does not seem to be adequately addressed in earlier studies is the possibility to study the binding of a given solute to multiple hosts and vice versa, the competitive binding of multiple guests to a single host or even the combination of both. This potential seems especially challenging with the increasing activity in the fields of combinatorial synthesis and high throughput screening.

The dependence of a solute mobility on the concentration of a selector represents basic information for the calculation of the binding constants. Therefore, the experimental data must be refined in the way that only the effect of a binding with a chiral selector on the mobility of analyte enantiomers are included in the final plot. Thus, the effects of an increasing viscosity of the BGE with increasing concentrations of a chiral selector, a possible selector adsorption on the capillary wall, an increasing ionic strength of the BGE, a variation of conductivity, etc. must be eliminated from the observed overall mobility. In addition, the equilibrium time scale must be faster than the CE separation time scale and the concentration of the chiral selector must be varied in a wide range in order to adequately cover binding isotherm [278]. Two additional points seem worthy of mention: As in most other techniques, concentrations are used instead of activities in CE. Therefore, the binding constants are not true thermodynamic equilibrium constants but apparent constants. Second, almost all equations used for equilibrium constant determinations in CE assume 1:1 stoichiometry while actually the complexes with other stoichiometry are also common.

Several interesting studies on the determination of stereoselective binding constants using chiral CE have been published in last two years [128,129,279,280]. A critical discussion on the general aspects of this technique was recently published by Vespalec and Bocek [281]. One important trend that can be noted is the increasing number of research groups using binding constant determination for a method optimization and explanation of diverse effects observed in chiral CE.

5.4.2. UV-, NMR and ESI-MS studies of the stoichiometry of selector–selectand complexes

UV–Vis spectrometry has been established for a long time as the useful technique in studies of intermolecular noncovalent interactions. Both the stoichiometry and the equilibrium constants of selector–selectand complexes may be determined using this technique. Although the shift of the absorbance maximum of a selectand (or selector) is usually affected by complexation, the more direct information on the involvement of both counterparts in the complex formation may be obtained from the changes of the molar absorption coefficient (e). Experimental data are commonly treated according to the continuous variation plot (Job's plot) [275,282] for obtaining the information on the stoichiometry of the complexes and according to Scott's technique for the determination of the equilibrium constants [275,283]. The advantage of UV–Vis spectrometry is the relative simplicity, availability and universality. The disadvantage of this technique is that it commonly does not provide different signals for the diastereomeric complexes between the enantiomers and a chiral selector. For this reason, either pure enantiomers are required to be used in the experiment or the information obtained will be not enantioselective. This fact may be one of the reasons limiting the use of UV–Vis spectrometry for the description of selector–selectand complexes in chiral CE [183].

The most distinct advantage of NMR spectrometry compared to UV- and many other spectrometric

techniques is that the former may, in principle, provide two sets of the resonance signals for noncovalent diastereomeric complexes between the selector and the selectand enantiomers. Thus, the NMR spectrometry may allow the application of racemic samples or nonracemic mixtures of enantiomers for the stereoselective determination of the stoichiometry and equilibrium binding constants of selector–selectand complexes. Besides the easier availability of racemic analytes, NMR spectrometry offers the possibility of competitive binding studies. This means that the interaction of one of the enantiomers of an analyte with a chiral selector may be studied in the presence of the opposite enantiomer which approaches well to the real conditions in chiral CE separations. An additional advantage of NMR-spectrometry is that it provides a multiple set of data based on a single set of experiments. UV–Vis spectrometry and most other instrumental techniques provide a change of averaged molecular characteristics (specific/molar absorbance, shift of absorption maximum, solubility, etc.) while NMR spectrometry provides distinct signals for each proton, carbon atom, etc. involved in different fragments and functional groups of a selectand and a selector molecule. Thus, NMR spectrometry may provide statistically more reliable data for the characterization of selector–selectand complexes.

Technical aspects of stoichiometry determination of selector–selectand complexes based on NMR spectroscopy have been summarized in several previous works [11,174,181–183,284–294]. The most convenient way seems to be a preparation of equimolar solutions of a selector and a selectand and mixing them in the ratio 10:0; 9:1; 8:2; . . . ..0:10 and measurement of the NMR spectra of these samples. A dependence of

$$\Delta\delta \; x \; \frac{[selector]}{[selector] + [selectand]} \text{ vs. } - \frac{[selector]}{[selector] + [selectand]}$$

results a continuous variation plot (Fig. 23) [174]. A maximum of this plot indicates the stoichiometry of the complex. Although a construction of Job's plot does not represent a problem, certain experience is required in order to give a correct interpretation to

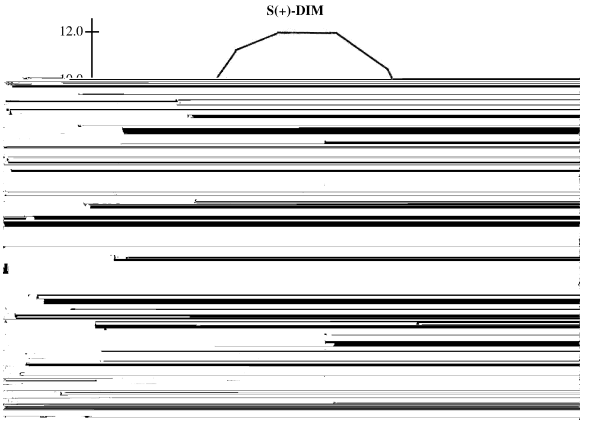

Fig. 23. Job's plot for (±)-DIM/β-CD complex. (Reproduced with permission from Ref. [174].)

the observed dependencies. Special care must be taken in order to differentiate between uniform and multiple complex formation. The experimental data cannot be treated according a continuous variation plot in the latter case because the chemical shifts of the resonance signals due to complex formation, so-called complexation-induced chemical shifts (CICS) are not additive in this case. Additional care must be taken when a maximum is not sharp on a continuous variation plot. This may be the indication for the formation of a very week complex, as well as multiple complexes. For instance, the Job's plot shown in Fig. 23 indicates that the stoichiometry of the complex between dimethindene (DIM) and β-CD is favorable 1:1. However, the plot does not have a characteristic sharp maximum. Further studies using ESI-MS spectrometry confirmed the formation of a minor amount of the complex with 1:2 stoichiometry besides a complex of 1:1 stoichiometry (Fig. 24) [287]. Thus, as this example shows, NMR spectrometry which is in general very powerful technique may not always be applicable in studies of selector–selectand complexes. One additional example of this kind is shown in Fig. 25 [174]. The data shown in this figure indicate that the continuous variation plot cannot be constructed due to multiple complexation between the DIM and CM-β-CD. Despite this failure, the data shown in Fig. 25 are very informative. Beside aforementioned multiple complex formation, these data indicate that the complexes formed have a different stoichiometry and in addition, the chiral

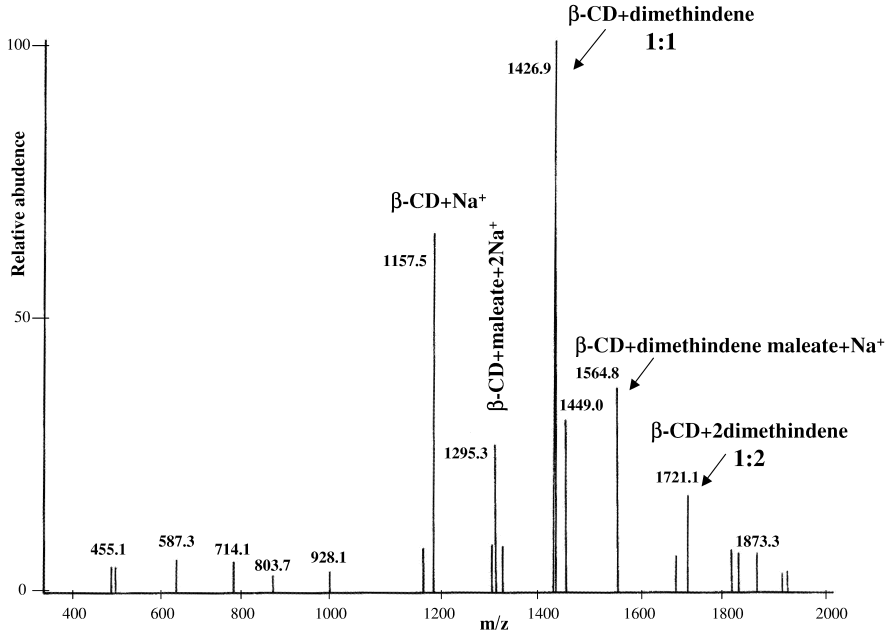

Fig. 24. ESI-MS spectrum of (±)-DIM/β-CD mixture. (Reproduced with permission from Ref. [287].)

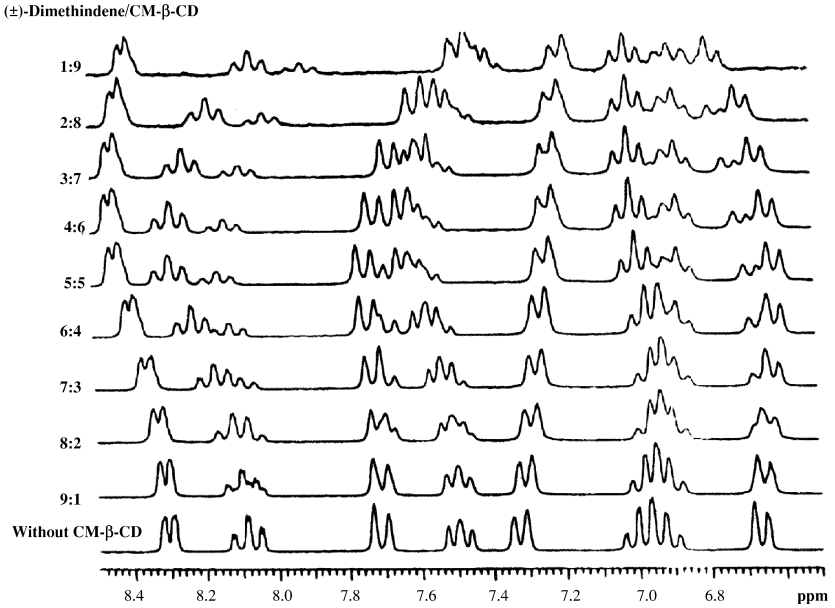

Fig. 25. 1H-NMR spectra recorded in order to construct the Job's plot for (±)-DIM/CM-β-CD complex. (Reproduced with permission from Ref. [174].)

recognition pattern in the complexes with different stoichiometry is opposite to each other. The latter seems to be the unique and the most interesting result of this experiment.

In certain cases ESI-MS experiments can complement data obtained using NMR spectrometry. One example of this kind was mentioned above (see Fig. 24). Other examples may be found in Refs. [288,291–294]. The advantage of ESI-MS is that this technique provides the information about the M/Z ratio of the complex and this way the stoichiometry from a single experiment. This is impossible using other techniques mentioned above which require a set of experiments and therefore, are time-consuming and expensive. In the ESI-MS studies care must be taken due to the possible formation of ''false-peaks'' [295]. The new generation of time-of-flight (TOF) ESI-MS equipments with orthogonal interface design allow to somewhat avoid experimental artifacts of this kind. In addition, a variation of the experimental conditions and the addition of some standard compounds can be used to solve this problem [294].

Together with ESI-MS other soft-ionization MS techniques such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and fast atom bombardment (FAB) MS may be used for the determination of the stoichiometry of selector–selectand complexes.

5.4.3. NMR spectrometric studies of the enantioselective selector–selectand binding constants

Various techniques which are suitable for the determination of equilibrium constants in noncovalent interactions [296,297] may be applied also to chiral CE related studies. The applications of fluorescence [298] and circular-dichroism spectrometry [299] have been reported. The advantage of the latter is that it is a chiroptical technique. The application of other techniques, such as microcalorimetry, electron-spin resonance, etc. although possibly useful, have not yet been reported in the studies related to chiral CE.

At present, NMR spectrometry remains the major technique used for the determination of selector–selectand binding constants related to chiral CE. Techniques and treatment of experimental data have been described in several studies [174,286–288,291–294]. The effect of the increasing amount of a selector on the CICS of NMR-active nucleus of a selectand such as ^{1}H, ^{13}C, ^{19}F, etc. (or vice versa) is measured. It is important to cover the widest available concentration range of a chiral selector. Providing that CICS is stereoselective, then a racemic or optically impure selectand can be used and the competitive binding constants between enantiomers and the chiral selector may be determined.

Experimental data may be treated according to Scott's modification [283] of the Benesi-Hildebrand equation [300]. Technically easier and more exact, may appear other methods described especially for the treatment of NMR data [301]. The known stoichiometry of the complex is a prerequisite for obtaining correct binding data. Almost all techniques described in the literature are suitable for 1:1 complexes. The formation of complexes of other stoichiometry may significantly complicate the treatment of the data or introduce in the calculations a significant source of error. Nonlinear curve fitting techniques may allow to avoid the problems of this kind.

Among NMR-binding studies performed in order to explain the results observed in chiral CE the example of chiral Ca^{2+} blocking drug verapamil (VP) seems to be rather illustrative [292]. The migration times of the enantiomers of VP in CE were much longer in the presence of β-CD compared to equimolar amounts of heptakis-(2,3,6-tri-*O*-methyl)-β-CD (TM-β-CD). However, the enantioseparation factor was higher in the latter case and the migration order of the enantiomers was opposite to each other in the presence of these two CDs [292]. The binding studies performed using NMR spectrometry clearly indicated that the enantiomers of VP possess a higher affinity towards β-CD ($K_{(+)}=272\pm34\ M^{-1}$, $K_{(-)}=207\pm59$) compared to TM-β-CD ($K_{(+)}=6\pm1\ M^{-1}$, $K_{(-)}=30\pm7$). The enantiomers are more enantioselectively recognized by TM-β-CD (the enantioselectivities of the binding were 1.3 and 5.0, towards β-CD and TM-β-CD, respectively). In addition, (+)-VP possesses a higher affinity towards β-CD whereas more tightly bound enantiomer in the case of TM-β-CD is (−)-VP. Thus, the binding studies performed using NMR spectrometry allowed in this particular case to explain rather unusual effects observed in chiral CE.

Another example illustrating the usefulness of

NMR spectrometry in chiral CE related studies is discussed below. Opposite migration order of the enantiomers was observed for the atropisomeric compound 1,1′-binaphthyl-2,2′-diyl hydrogen phosphate (BDHP) in chiral CE in the presence of several neutral and negatively charged sulfoalkyl CD derivatives. In a first approximation based only on CE data one could assume that the enantiomers of BDHP possess the opposite affinity pattern towards the neutral and negatively charged sulfoalkyl CDs. However, the binding studies performed using NMR spectrometry clearly indicated that the affinity of the enantiomers of BDHP was the same towards all CDs studied [293]. Further careful examination of the entire separation system allowed to find a reasonable explanation for the opposite migration order observed in these studies. The reason for this effect was the relative mobilities of the selector and the selectand and not the binding interactions between them [18,293].

Although it is suitable, NMR spectrometry is not recommended to be used just for screening of chiral selectors due to following reasons: (a) The NMR spectrometric experiment is commonly more time-consuming and expensive compared to CE. (b) Enantioselective CICS observed in NMR spectrometry do not a priori translate to an enantioseparation in CE. The possible reasons for this discrepancy between the two techniques have been discussed earlier [159]. (c) Although both CE and NMR spectrometry are liquid-phase techniques and are performed in a similar or even in the same medium, some technical problems may arise on this step.

In general, for a given pair of a chiral selector and selectand, one may expect a higher enantioseparation power in CE compared to the enantiorecognition ability in NMR spectrometry. In particular cases however, NMR spectrometry may provide an indication for a chiral recognition in those selector–selectand pairs which have been considered to be unsuccessful based on the CE experiment alone. For instance, native β-CD has been suggested as not to be a suitable chiral selector for the enantioseparation of the cationic form of chiral cholinergic drug aminoglutethimide (AGT). However, two other native CDs (α and γ) allowed baseline enantioseparation of AGT [103,302]. In contrast to the CE data, NMR studies indicated the most pronounced interactions between AGT and β-CD among three native CDs (Fig. 26). The indications for the strongest interactions between AGT and β-CD compared to α- and γ-CDs are the most significant upfield shifts observed on the aromatic protons of AGT as well as on H-3 and H-5 protons of β-CD located inside the cavity. These data were also supported by ESI MS studies of comparative affinity of AGT enantiomers towards these CDs. Careful optimization of the

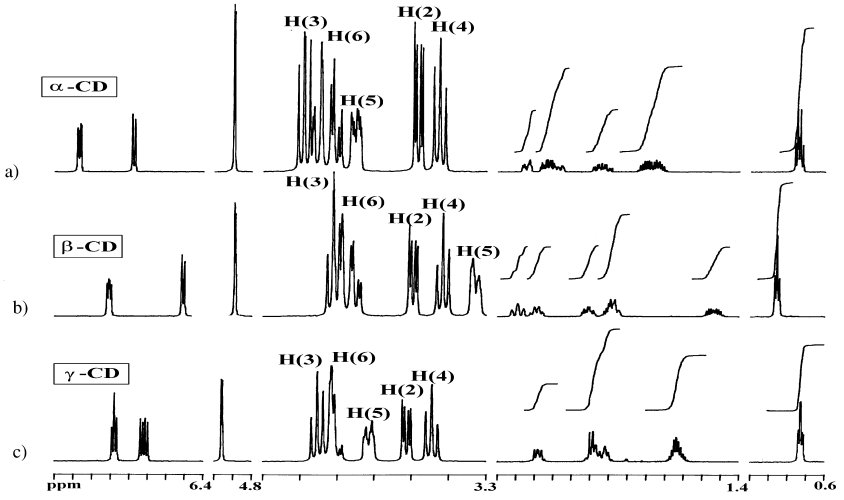

Fig. 26. The 600 MHz ^{1}H-NMR spectra of (±)-AGT in the presence of two equivalents of α-CD (a), β-CD (b) and γ-CD (c). (Reproduced with permission from Ref. [294].)

separation in CE allowed to resolve the enantiomers of AGT also with β-CD. The migration times were longest in the presence of β-CD and in addition, the enantiomer migration order was opposite compared to two other native CDs (Fig. 27). Thus, in this particular case NMR and ESI MS studies allowed to optimize the enantioseparation in CE and to find an example of opposite affinity of the AGT enantiomers towards native CDs. The examples of an affinity reversal of enantiomers depending just on the size of the CD cavity are rather few.

5.4.4. NMR spectrometric studies of structure of selector–selectand complexes in solution

NMR spectrometry has been well established as one of the major techniques suitable for studies of noncovalent intermolecular complexes in solution [303]. The chemical shift pattern, line-shape analysis and nuclear Overhauser effect (NOE) may be used in order to obtain information on the structure and the dynamics of the complexes. The data obtained using NOE are easier to interpret and seem to be more direct. Several studies illustrate the feasibility of this technique for structure elucidation of complexes relevant to chiral CE [171,182,287–289,294]. It does not seem reasonable to perform an NOE-experiment in a routine way for any selector–selectand complex because this experiment requires measurements with rather strong magnets and may be expensive and time consuming. However, in certain cases NOE studies provide unique information which is impossible to obtain using any alternative technique.

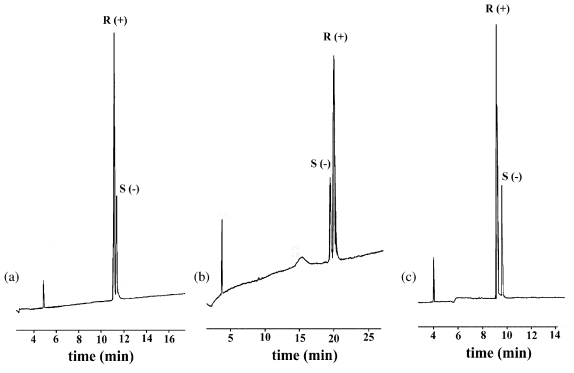

Fig. 27. Electropherograms of AGT enantiomers [(+)/(−)=2/1] in the presence of 10 mg/ml α-CD (a), β-CD (b) and γ-CD (c). (Reproduced with permission from Ref. [294].)

One example of NOE studies related to chiral CE is shown in Fig. 28 [294]. The possible structural reasons of above-mentioned opposite affinity of the enantiomers of AGT towards native β- and γ-CD has been investigated in this study. The NOE data depicted in Fig. 28 allow to deduce the structure of the complexes shown in Fig. 29. Thus, by selective saturation of the aromatic protons in the ortho position of (±)-AGT equally strong intermolecular NOE-effects were observed for both H-3 and H-5 protons of β-CD (Fig. 28a). However, by irradiation of the aromatic protons in the meta position only a minor effect was observed for the H-3 protons of β-CD and the NOE-effect appeared instead on the H-6 protons. These data support a deep inclusion of

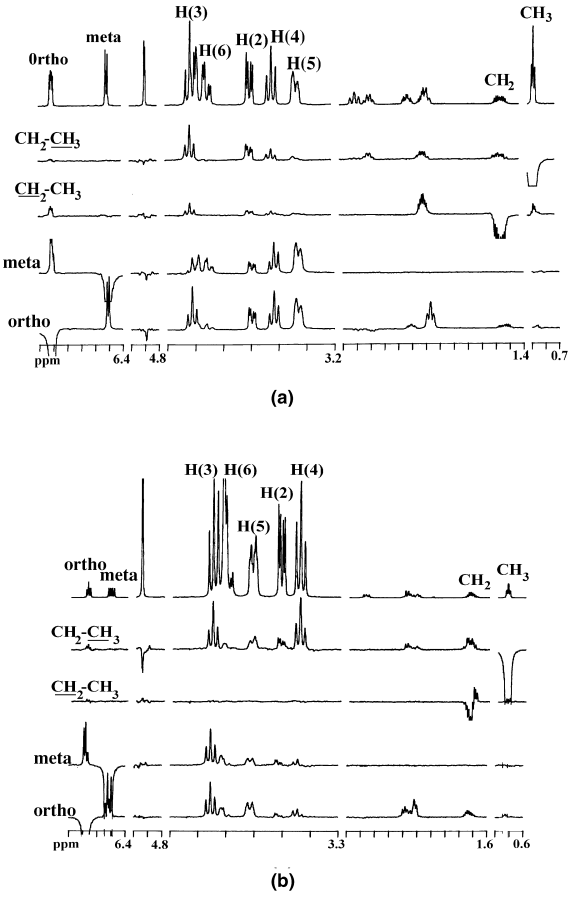

Fig. 28. 1D-ROESY spectra of (±)-AGT and two equivalents of β-CD (a) and γ-CD (b).(Reproduced with permission from Ref. [294].)

the *p*-aminophenyl moiety of the AGT molecule into the cavity of β-CD entering it from the wider secondary side (Fig. 29a). The deep inclusion of the aromatic moiety of the AGT molecule into the cavity of β-CD on the secondary side is supported also by a significant NOE effect observed between the H-3 protons of CD and the ethyl moiety of AGT. Rather strong ''NOE-like'' effects observed on the external CD protons in this experiment make it questionable whether the structure represented in Fig. 29a is the only possible structural element of this complex or if the alternative structures are also present.

In contrast to the AGT/β-CD complex, the NOE effect decreased for the H-5 protons and remained almost unchanged for H-3 protons when irradiating the protons in the meta position instead of the protons in the ortho position of the aromatic ring of AGT in the (±)-AGT/γ-CD complex (Fig. 28b). These data support a complex formation from the narrower primary side of γ-CD with amino group ahead (Fig. 29b). The glutarimide ring is apparently less involved in the complex formation in this case. However, the involvement of the methyl group in complex formation by a still unknown mechanism cannot be completely excluded. The structure of the (±)-AGT/γ-CD complex depicted in Fig. 29b was derived by saturation the aromatic protons of AGT. The structure was also supported by the data which were obtained when the γ-CD protons were saturated and the response was observed for the aromatic protons of AGT. Thus, when saturating the H-5 protons of γ-CD the response for the protons in the ortho position of the aromatic ring was more pronounced compared to the protons in the meta position (Fig. 30). However, a similar response was observed for both, ortho and meta protons of the aromatic ring when γ-CD protons in the position 3 were saturated (Fig. 30).

Thus, as illustrated above, one-dimensional rotating frame nuclear Overhouser and exchange spectroscopy (ID-ROESY) experiments may provide a reasonable explanation for significant qualitative and

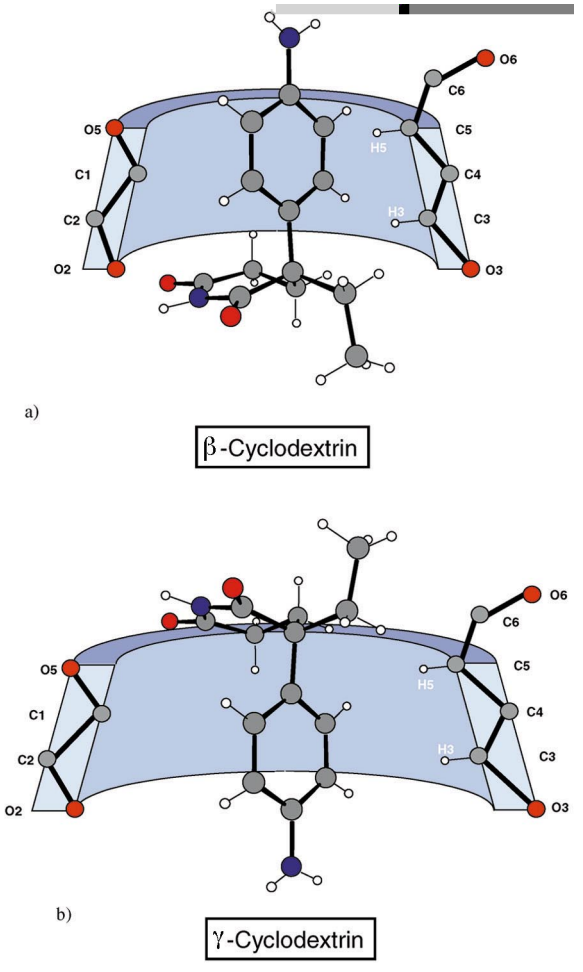

Fig. 29. Structure of AGT complexes with β-CD (a) and γ-CD (b) derived based on the 1D-ROESY spectra depicted in Fig. 30. (Reproduced with permission from Ref. [294].)

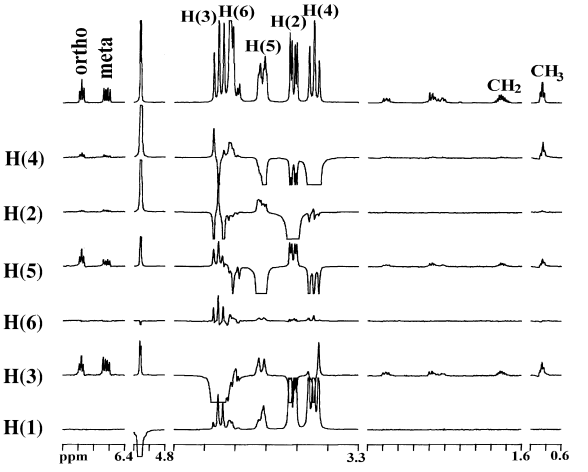

Fig. 30. 1D-ROESY spectrum of (±)-AGT and two equivalents of γ-CD (the protons of γ-CD were saturated). (Reproduced with permission from Ref. [294].)

quantitative differences observed in selector–selectand interactions related to chiral CE. This information becomes even more important when the alternative techniques for structure elucidation fail. This was the case with the AGT/CD complexes which formed monocrystals but of a twin-type which were not suitable for X-ray crystallographic studies. Although ID-ROESY seems to be a powerful technique for structural investigation of noncovalent complexes in a liquid phase the experimental data need to be interpreted very carefully. Thus, a detailed analysis of above-described ID-ROESY spectra of the AGT/CD complexes may indicate that the structures shown in Fig. 29 are just fragments of rather complex supramolecular aggregates. This seems even more likely when considering a certain tendency of AGT to form dimers in aqueous solution which has been also confirmed from the X-ray crystallographic data in the solid-state.

Another ''confusing'' example of ID-ROESY studies is described in Ref. [288]. In this study, the structure of the complex between antihistaminic drug brompheniramine (BrPh) and β-CD and TM-β-CD was studied by 1D-ROESY experiments in solution. For the complexes of (+)-BrPh with both CDs the unambiguous confirmation was obtained indicating the inclusion of the 4-bromophenyl moiety of the analyte into the cavity of the CD. In addition, in the case of the (+)-BrPh complex with β-CD, a week but positive NOE effect was observed also for the protons of the maleate counteranion when saturating the CD protons H-3 and H-5 located inside the cavity. This observation may indicate the simultaneous inclusion of the 4-bropmophenyl moiety and maleate counteranion into the cavity of β-CD but this contradicts to simple geometric considerations and the assumption the stoichiometry of the complex to be 1:1. Thus, the involvement of alternative techniques may become sometimes necessary for the unambiguous interpretation of ID-ROESY data.

5.4.5. X-ray crystallographic studies of the structure of selector–selectand complexes in the solid state

X-Ray crystallography has a long history as a powerful technique for structure investigation of CDs and their complexes in the solid state. The first experimental evidence of the inclusion complex formation ability of CDs in the solid state was obtained by Hybl et al. in 1965 using this technique [304]. X-Ray studies of CD complexes have been summarized in several recent reviews [305,306]. However, studies relevant to chiral CE have been scarcely published [287,288]. This may have the following reasons: (a) X-Ray crystallography is a solid state technique and a separation in CE is performed in solution. Therefore, these two techniques may not ideally correlate with each other. (b) Growing the monocrystals of suitable size containing both counterparts (a selector and a selectand) requires experience. (c) High quality X-ray crystallographic experiments on monocrystals is time consuming and rather expensive. (d) The structure generation from the experimental data requires powerful computer-softwares and is not always trivial. Despite the aforementioned, X-ray crystallography may appear sometimes very useful for structural studies related to enantioseparations in CE.

As mentioned above, the ID-ROESY studies performed on the complex between (+)-BrPh and β-CD in solution did not allow to explain the NOE-effect observed on the protons of the maleate counteranion [288]. X-Ray crystallographic study performed on the monocrystals obtained from a 1:1 aqueous solution of (+)-BrPh maleate and β-CD (Fig. 31) provides a plausible explanation for above-mentioned contradiction. In particular, as shown in Fig. 31 (+)-BrPh forms with β-CD, at least in the solid state not a 1:1 complex but a complex with 1:2 stoichiometry. In this complex the (+)-BrPh molecule is sandwiched between two molecules of β-CD. The 4-bromophenyl moiety of (+)-BrPh enters the cavity of one of the β-CD molecules whereas the cavity of another β-CD molecule is occupied by the maleate counteranion. Thus, X-ray crystallography may provide useful information on the supramolecular structure of the selector–selectand complexes and this way complement well 1D-ROESY data. However, the aforementioned possible differences between the structure of the complexes in solution and in the solid phase must be considered.

One additional application for X-ray crystallography in chiral CE related studies may be the determination of the structure of various selectors and selectands. These data may appear very useful as thermodynamically most stable starting structures for

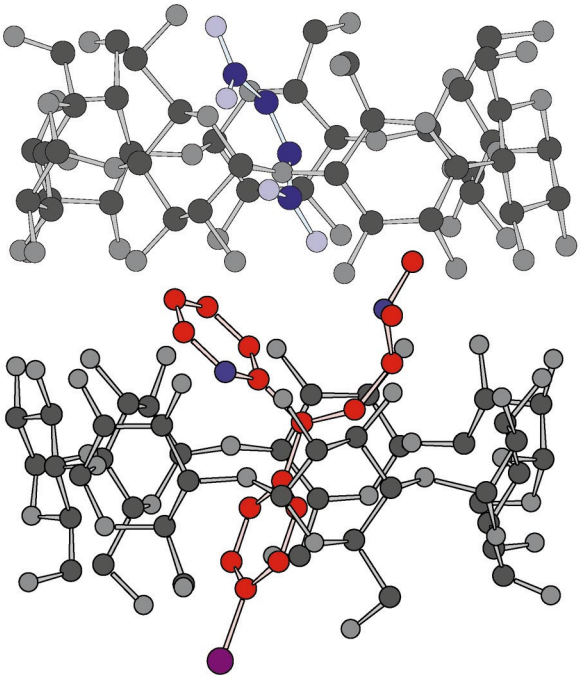

Fig. 31. Structure of (+)-BrPh maleate β-CD complex in the solid state determining by X-ray crystallography. (Reproduced with permission from Ref. [288].)

further optimization based on the molecular mechanics and molecular dynamics calculations.

5.4.6. Molecular modeling of selector–selectand interactions in chiral CE

Molecular modeling calculations may allow in the ideal case to compute in a reasonable time and rather precisely the energy and structure of intermolecular complexes of biomedical, pharmaceutical and chemical relevance.

In the early 1990s, several studies were published about the computation of selector–selectand interactions in chiral CE. This relates basically to the interactions between CDs and their chiral guests which seems to be caused by the fact that CDs are rather rigid molecules of medium size and therefore calculations for these molecules are easier, faster and may be precise. In addition, many of CDs are well studied by alternative techniques for structure elucidation. Among these, X-ray crystallographic data are of the highest interest.

The major difference between the mathematical models of chiral CE (Section 5.3) and molecular modeling studies discussed in this section is that the former models describe the entire separation process and do not pay any significant attention to the selector–selectand interactions. In contrast, the molecular modeling techniques summarized in this section try to describe the selector–selectand interactions and do not estimate their role in the overall separation process.

A separation factor observed in any instrumental technique is defined by the difference between the free energy of formation of transient diastereomeric complexes between enantiomers and a chiral selector. Therefore, the exact calculation of the absolute energy values is not required in molecular modeling studies related to enantioseparations. This simplifies the calculations. On the other hand, due to extremely high efficiency of CE this technique allows to observe the enantioseparation even in those selector–selectand pairs where the above-mentioned difference between the free energy of formation of diastereomeric complexes is extremely small. The precise calculation of very small energy differences remains a challenging task even for very sophisticated state of the art energy minimization softwares. Additional care must be taken in order to maximally approach a model to the real separation conditions. Thus, for instance, the molecular modeling calculations are often performed in vacuum without taking into account the effect of the medium. However, the aqueous medium commonly used in CE, dramatically affects the hydrophobic and hydrogen bonding interactions. Moreover, the ionic strength of the buffer plays a decisive role for electrostatic intermolecular interactions. Another important point is a correct selection of the starting and the boundary conditions for energy minimization. Incorrectly defined conditions may totally confuse the calculations. For instance, when performing the molecular modeling calculations for the complex between TM-β-CD and (+)-BrPh in a neutral form the energy values were obtained which indicated that the complex formation with the alkylamino moiety included into the cavity of TM-β-CD would be energetically favorable. The structure with the alkylamino moiety included into the cavity was also observed in X-ray experiment performed on the monocrystals obtained

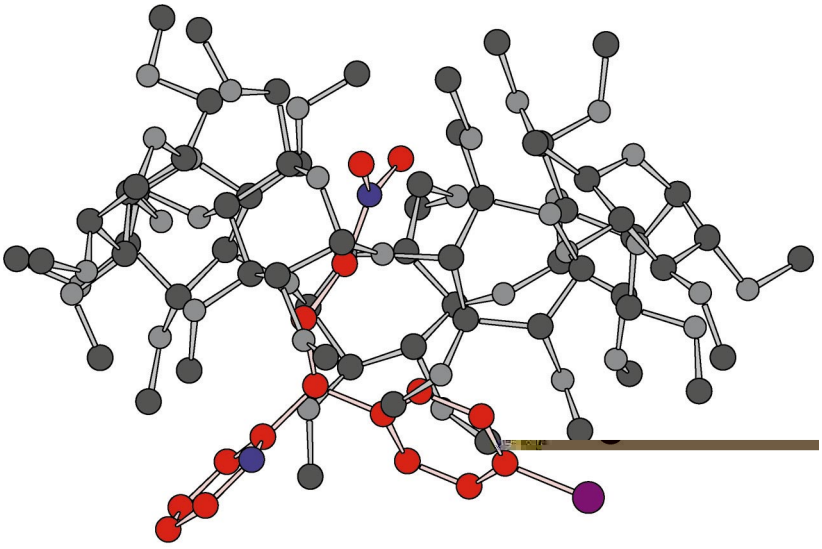

Fig. 32. Structure of (+)-BrPh maleate TM-β-CD complex in the solid state determined by X-ray crystallography. (Reproduced with permission from Ref. [288].)

from the mixture of aqueous suspension of deprotonated (+)-BrPh as a free base and TM-β-CD (Fig. 32). These results are contradictory to the structure derived from the 1D-ROESY experiment in solution. The intermolecular NOE-effects observed in this experiment clearly indicated the inclusion of the 4-bromophenyl moiety into the cavity of TM-β-CD (Fig. 33) [288]. Taking into considerations that the (+)-BrPh maleate, e.g. the protonated form of (+)-BrPh molecule was applied for the 1D-ROESY studies in solutions the force-field calculations were performed again for interactions of a single positively charged (+)-BrPh with TM-β-CD. The energy values obtained in this case clearly indicate that the complex formation with the 4-bromophenyl moiety of the (+)-BrPh molecule included into the cavity of TM-β-CD is energetically favorable which is in agreement with the structure observed using 1D-ROESY studies in solution (Fig. 33).

A general overview of the molecular modeling techniques applied to chiral separations can be found in the papers by Lipkowitz [307–309]. Some special aspects of molecular modeling techniques related to chiral CE and earlier studies on the subject are summarized in Ref. [11,37,38,299,310].

In summary, molecular modeling when used in combination with instrumental techniques, especially with 1D-ROESY and X-ray crystallography may significantly contribute to the understanding the nature of the intermolecular forces responsible for

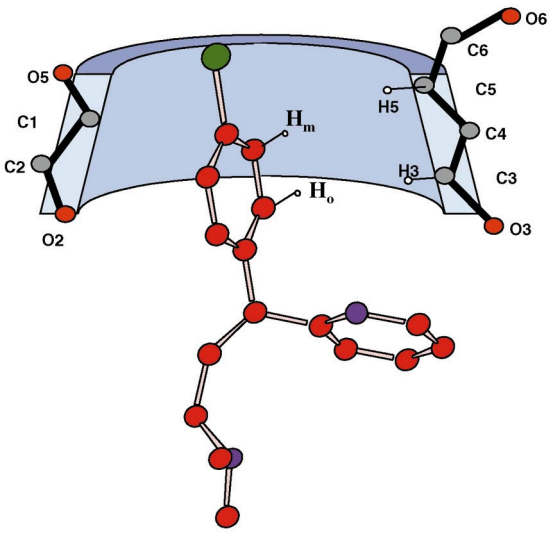

Fig. 33. Structure of (+)-BrPh maleate β-CD complex in solution derived from 1D-ROESY experiment. (Reproduced with permission from Ref. [288].)

selector–selectand interactions and chiral recognition.

5.5. Enantiomer migration order

Enantiomer migration order (EMO) represents an important issue in chiral CE from the viewpoint of both practical analysis and mechanistic studies.

State of the art synthetic and resolution techniques for obtaining pure enantiomers allow to achieve rather high degrees of enantiomeric purity. This means that only small amounts of the enantiomeric impurities may be present. In addition, the regulatory agencies are introducing continuously increasing purity requirements to the compounds used as pharmaceuticals, food additives, agrochemicals, etc. This relates also to the enantiomeric purity of chiral compounds. Thus, methods are required which allow to detect and precisely quantify increasingly small enantiomeric impurities. In contrary to chromatographic techniques where a common effect is peak tailing both, peak tailing and peak fronting are possible in chiral CE. For the determination of small enantiomeric impurities relatively high amounts of a sample will be commonly injected onto the capillary. This leads to overloading effects which are associated with disturbance of a peak symmetry. In the case of peak tailing it is desirable to elute the minor enantiomer in front of the major one. Most of the effective chiral selectors used in CE are compounds of natural origin available in one configuration only (CDs, macrocyclic antibiotics, proteins, polysaccharides, etc.). Therefore, a trivial chromatographic approach of a reversal of the EMO by reverting the configuration of chiral selector is not always applicable in CE and alternative techniques must be developed [18].

The role of the EMO seems to be underestimated in chiral CE. Thus, in many published papers one may find a discussion of minor quantitative differences (for instance, small differences in separation factor and peak resolution) between the chiral selectors whereas a rather principal qualitative difference such a chiral recognition pattern, is not addressed. In studies of this kind it is easily to consider very different chiral selectors to be similar. The aforementioned status may have the following reasons: a) Enantiomerically pure reference standards of many chiral substances are not available. It is technically more difficult to elucidate on-line the EMO in CE compared to HPLC or SFC because the optical pathlength is very short in the former technique and, in addition, the signals of a chiral selector may interfere with signals of the analyte.

Somewhat misleading seems also the aforementioned fact that most chiral selectors are available only in one stereochemical configuration and therefore intuitively, an opposite affinity of the enantiomer is less probably towards these chiral selectors. Many examples opposing to this opinion have been published not only for CDs but also for other types of chiral selectors which contain the chiral building blocks of the same configuration [18,35,56,71,78,163,186,187,287,288,291–294].

Almost all previously described and most of the recent examples of the reversal of EMO [84,98,311–315] may be predicted and explained based on the Eqs. 5 and 6 [18]. Thus, based on these equations the following three principal possibilities for a reversal of EMO become evident: (a) Reversal of the algebraic sign of $\Delta\mu$; (b) Reversal of the algebraic sign of the term $\mu_f - \mu_c$, or alternatively, (c) reversal of the algebraic sign of the term $K_R - K_S$. Among these three effects only the last one is possible in chromatographic techniques. All three aforementioned effects may be realized in CE by different technical ways which are summarized in Ref. [18] and in many research papers [35,56–60,71, 78,84,98,165,186,187,287,288,291–294,311–314].

Together with well predictable examples of the reversal of EMO one example remained confusing for a long time. Thus, several years ago Schmitt and Engelhardt described an interesting phenomenon of the reversal of the enantiomer migration order depending on the concentration of a chiral selector [130]. The recent studies by Rizzi and Kremser [128,129] allowed to find an elegant explanation of this example as well as to extend this phenomenon to other chiral compounds and selectors. Although the authors of Ref. [130] provided intuitively the correct explanation for the observed effect assuming faster mobility of the diastereomeric complex of the more strongly bonded enantiomer they did not experimentally prove this assumption. Later Wren published experimental data which did not support the above-

mentioned suggestion by Schmitt and Engelhardt [131]. The rather precise treatment of the experimental points considering pH-dependent effects by Rizzi and Kremser clearly indicate [129] that the explanation given in [130] is, in principle correct for DNS-Phe at pH 2.5 but may not be necessarily valid at pH 6.0. [128]. As shown in this study, at pH 2.5 D-DNS-Phe is the preferentially bound enantiomer to HP-β-CD ($K_L = 117$ and $K_D = 147$ M^{-1}) and the complex D-DNS-Phe/HP-β-CD also possesses higher mobility at this pH ($\mu_L^{complex} = 3.40 \times 10^{-5}$ and $\mu_D^{complex} = 4.28 \times 10^{-5}$ cm^2 V^{-1} S^{-1}, respectively. This is not the case at pH 6.0 [128].

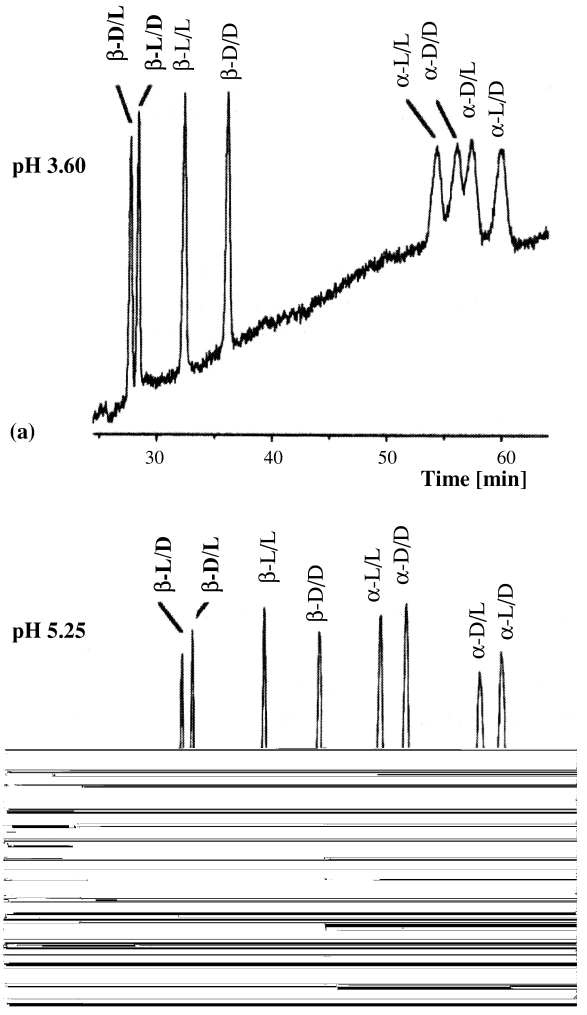

Fig. 34. Separation of the stereoisomers of the isomeric tripeptides Gly-α/β-D/L-Asp-D/L-PheNH$_2$ in polyacrylamide-coated capillaries at pH 3.60 (a) and 5.25 (b). (Reproduced with permission from Ref. [279].)

Sabah and Scriba investigated the separation of the stereoisomers of aspartyl dipeptides and tripeptides in a series of studies [279,280,316–319]. These authors described several examples of a reversal of the EMO when using different CDs as chiral selectors, applying for example the carrier ability of a chiral selector [318,319]. One very interesting example from these studies is discussed in detail below [279,280]. When studying the separation of the stereoisomers of tripeptides in polyacrylamide coated capillary at pH 3.60 and 5.25 the authors observed the reversal of the EMO for the enantiomers of glycine-β-L-aspartyl-D-phenylalanine/glycine-β-D-aspartyl-L-phenylalanine while the migration order of other enantiomeric pairs remained unchanged (Fig. 34). The more detailed study of the pH dependence of the EMO (Fig. 35) confirmed this unique finding. Further binding studies performed in CE indicated the pH-dependent affinity reversal of above-mentioned pair of tripeptide enantiomers towards CM-β-CD [279]. Analyzing the aforementioned works by Rizzi and Kremser [128,129] together with studies by Sabah and Scriba [279,280,319] one may find apparently similar underlying mechanisms of the observed phenomena.

6. Enantioseparations in capillary electrochromatography (CEC)

In the early 1990s when the potential of CE was still intensively exploring for chiral separations the fascination by this minute-scale, versatile and highly efficient technique was clearly overbalancing few potential bottlenecks already noted for that time. The research groups involved in enantioseparations using chromatographic techniques easily found one or more significant advantage of a new technique and were expanding their research activities taking in their arsenal also chiral CE.

With the maturation of chiral CE it became obvious that this is really a very effective extention of the existing chromatographic techniques for enantioseparations on the analytical scale. The comments like: ‘‘CE continues to find new applications, and it is beginning to become a competitive tech-

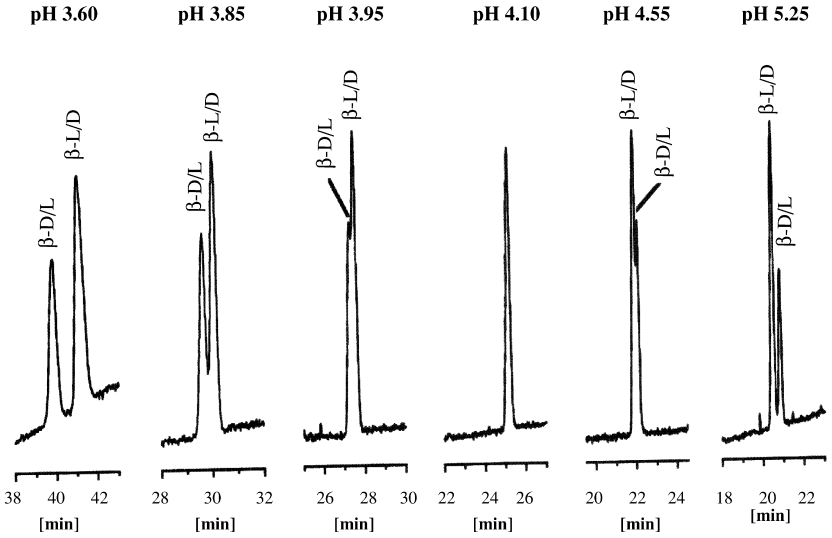

Fig. 35. Effect of the pH of the BGE on the migration order of the tripeptide enantiomers Gly-β-L-Asp-D-Phe NH_2 and Gly-β-D-Asp-L-Phe NH_2. (Reproduced with permission from Ref. [279].)

nique for certain applications that traditionally were HPLC's domain such as chiral . . . '' [320] may be found in the recent literature.

On the other hand, with time it became clear that CE, similar to any other instrumental technique, also suffers from certain disadvantages and it would be ideal to combine the advantages of HPLC and CE also for the enantioseparations. Capillary electrochromatography (CEC) as a hybrid technique of HPLC and CE may offer significant advantages for enantioseparations. A pseudostationary chiral selector that is responsible for the high flexibility and versatility of CE sometimes may also cause problems. In particular, a dissolved chiral selector is not desirable in on-line coupling of chiral CE to polarimetric, circular dichroism, mass spectrometric, etc. detectors. In addition, it is impossible to find a suitable solvent for every chiral selector that may dissolve it without diminishing its chiral recognition ability and, in addition, serves as a good BGE for CE or at least is compatible with it. Mobile chiral selectors must be replaced or renewed after each analysis that is not optimal from an economic point of view and, in addition, may lead to certain problems for run to run reproducibility of the analytical characteristics of a method. These problems may be eliminated in chiral CEC which additionally may allow to combine high separation selectivity of HPLC chiral stationary phases (CSP) with a high separation efficiency of CE.

The history of CEC apparently goes back to early 1950s when Mould and Singe noted the potential of the electroosmotic flow (EOF) as a driving force in separation techniques [321,322]. Later, Pretorius et al. [323] paid attention to some advantages of the EOF, such as the plug-like profile, independence of the EOF on the particle size and geometry, etc., as a potential driving force in chromatographic separations. In 1980s Jorgenson and Lukacs [324] and Knox's group [325–327] contributed significantly to the development of the capillary format of electrochromatography. Many review papers and several special thematic issues of journals appeared in the last few years dedicated to CEC [11,328–331]. In this section major attention is paid to the development of chiral CEC during the last 3 years. Earlier studies on chiral CEC are summarized in Ref. [11].

Enantioseparations are in principle possible in three different modes of CEC: 9a) In the capillaries packed with achiral stationary phases while a chiral selector is added to the mobile phase (or BGE); 9b) In open tubular capillaries containing a chiral selec-

tor coated onto its inner wall; and (c) in capillaries packed with CSP. These modes are summarized in following subsections.

6.1. Enantioseparations in wall-coated open tubular capillaries

Capillary electrochromatographic enantoseparations in wall-coated open tubular (WCOT) capillaries have been pioneered by Mayer and Schurig in 1992 [332]. Recently, this subject has been summarized by the inventors [333]. Therefore, the discussion bellow focuses only on some special aspects of this technique.

A critical treatment of the potential of achiral chromatographic and electrochromatographic separations in WCOT capillaries was given by Tsuda and co-workers in the early 1980s [334]. The theoretical calculations showed that a capillary column with an I.D. 2.5 and 10 μm might be suitable for open-tubular capillary chromatography with laminar flow. A parabolic profile of the laminar flow causes substantial band-broadening. Tsuda et al. [334] noted that the use of the EOF with a much flatter flow profile than the laminar flow, may allow the use of open-tubular capillary columns with a slightly greater I.D. The authors have shown experimentally that in Pyrex and soda-lime glass capillaries with I.D. 30 μm modified with octadecylsilane (C-18) the band-broadening was substantially lower when the EOF was used as a driving-force than that in the case of a laminar flow. According to the authors ''The potential for the use of electroosmotic flow in capillary LC is great. A good ODS capillary column of 10 μm I.D. under electroosmotic flow would correspond to a column of 2 μm I.D. under laminar flow'' [334].

Thus, theory does not predict very successful separations even in the CEC mode in the capillaries with I.D. higher than 30 μm. Despite to this pessimistic prediction the studies performed by Schurig and co-workers [332,333,335–340] as well as by several other research groups [341–354] proved the principal feasibility of highly efficient CEC enantioseparations in WCOT capillaries with an I.D. of 50 μm. On the other hand, it has to be noted that the theoretical predictions by Tsuda et al. [334] have been confirmed and some limitations of CEC in WCOT capillaries have been prevailed. For instance, Schurig and Wistuba noted in the recent review that ''the enantiomer separation by open-tubular CEC on Chirasil-Dex appears to be limited to polar compounds such as carboxylic acids and alcohols and related compounds'' [333].

The basics of CEC enantioseparations in WCOT capillaries was discussed by Vindevogel and Sandra [354]. In CZE the separation occurs throughout the interior of the column and the main source of in-column band-broadening is axial diffusion. In CEC, similarly to pressure-driven chromatography, the separation occurs on the mobile phase/stationary phase interface and the exchange kinetics between the mobile and stationary phases are important. Radial diffusion imposes severe limits on the column diameters in the latter technique.

In order to illustrate the effect of the capillary diameter and flow profile on the separation efficiency, the Golay equation was used [355].

$$H = \frac{2D_m}{u} + \frac{C_m r^2 u}{D_m} \tag{13}$$

where H is the plate height, D_m is the diffusion coefficient, u is the linear flow rate, C_m is the resistance to mass transfer factor and depends on the capacity factor (k') and the flow profile, and r is the capillary radius.

The first term on the right-hand side of Eq. (13) describes the axial diffusion and the second term the radial diffusion. There is no difference between the axial diffusion terms for pressure-driven and electrokinetically-driven flows but there is a difference in the term of radial diffusion. This difference is caused by the difference in the resistance to mass transfer factors (C_m), which can be expressed as follows:

$$C_{m1flat} = \frac{(k')^2}{4(1+k')^2} \tag{14}$$

$$C_{m1parabolic} = \frac{11(k')^2 + 6k' + 1}{24(1+k)^2} \tag{15}$$

where k' is the capacity factor.

The difference expressed by Eqs. (14) and (15) is the essence of the difference between electrochromatography and pressure-driven chromatography.

Some typical curves for capillaries of different diameters and different modes of migration are

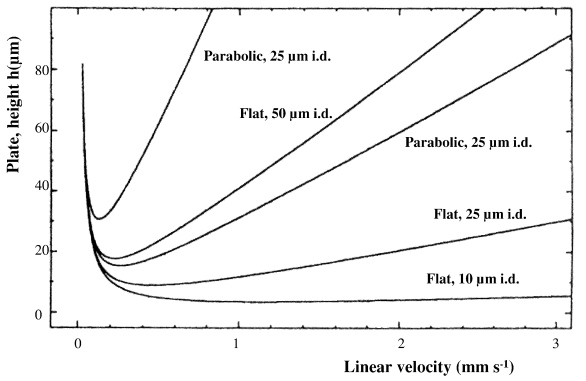

Fig. 36. Effect of column diameter and flow profile on plate height for $k'=1$ according to Eq. (13) ($D_m=10^{-9}m^2\ s^{-1}$). (Reproduced with permission from Ref. [354].)

shown in Fig. 36. The significant advantage of electroosmotic propulsion of an analyte and a decrease in capillary I.D. is clear from this figure. For instance, for a given pressure-driven separation system, the reduction of the capillary column radius by a factor of 2 will have a more pronounced effect on the efficiency than reducing C_m by replacing the pump with a high-voltage source. This is especially true at flow velocities higher than the optimum.

The plot of the combined function of the capacity factor (k') and the peak efficiency (N) vs. linear flow velocity (u) showed that a selectivity factor of ca. 1.1 can be sufficient for achieving baseline peak resolution in CEC when using WCOT capillary, whereas this value would be closer to 1.2 in a pressure-driven system. It was also demonstrated that in CEC mode the complete resolution of the pairs with smaller selectivities ($\alpha=1.05$) is also possible but at lower linear velocities and with larger capacity factors.

CEC in WCOT capillaries suffers from the following inherent controversy: For a separation which occurs on the mobile phase/stationary phase interface and not throughout the interior of the capillary column, retention of the analyte on the capillary wall (chiral stationary phase) is a necessary requirement for achieving a separation. On the other hand, any retentive analyte-capillary wall interactions are associated with a drastic decrease in peak efficiency in the capillary electromigration techniques.

In principle, it is not difficult to find a possibility for increasing the capacity factors even in capillaries having a diameter of 50 μm. This can be achieved by increasing the film thickness of a chiral selector [353] or increasing a capillary inner surface/volume ratio by etching the surface [350,351] or by other special treatments of the capillary inner wall. However, this does not seem to be a solution for the problem due to the above-mentioned controversy. The problem can be solved only in the way of using a CSP with highest possible enantiomer recognition ability. This may allow to perform successful enantoseparations with the lowest possible capacity factors (k').

6.2. Enantioseparations in capillaries packed with achiral stationary phases in combination with chiral buffer additives

Enantioseparations with achiral stationary phases in combination with chiral mobile phase additives was rather popular in high-performance liquid chromatography (HPLC) in early days when highly effective CSPs were not yet available. This mode in CEC may offer some alternative possibilities compared to a mode with CSPs [356–361]. For example, a chiral additive and its concentration in the mobile phase may be varied easier compared to a CSP packed into the capillary. One capillary with a standard packing material may be used in combination with many different additives to the background electrolyte (BGE). Thus, it is somewhat possible to find some niches for chiral CEC with achiral stationary phase in combination with chiral additives of a BGE compared to chiral CEC with CSPs. It is rather difficult to do this when comparing this technique to chiral CE because the chiral selectors which are soluble and exhibit chiral recognition ability in a given BGE is easier and in general more effective to be used in the empty capillary compared to the capillary packed with achiral packing material [11]. However, even in this case some particular applications may be found for the technique described in this subsection because the chiral selector preadsorbed on the achiral stationary phase certainly behaves differently compared to that residing in free solution.

Two interesting works on the effect of rather low applied voltage on the behaviour of a chiral mobile

phase additive in the HPLC mode have been published in the last years [357,358]. Porter and coworkers created a dynamically controlled separation system consisting of a porous graphitic carbon stationary phase and β-CD. These authors have found that the applied voltage affects the amount of β-CD electrosorpted on the surface of a conductive support. This was undoubtedly confirmed by switching the enantiomer elution order of mephenytoin depending on the applied voltage (Fig. 37). At the lower voltage the amount of the chiral selector residing in the mobile phase was higher and this determined the enantiomer elution order. In contrast, at higher voltage the amount of electrosorped β-CD on the stationary surface was higher and the enantiomer elution order was determined by this fraction. The voltage used in these studies was low and basically not used as a driving-force for the analytes. Therefore, the authors named this technique as electrochemically modulated liquid chromatography (EMLQ). A combination of this technique with CEC driving mechanism appears to be promising also from the viewpoint of mechanistic studies.

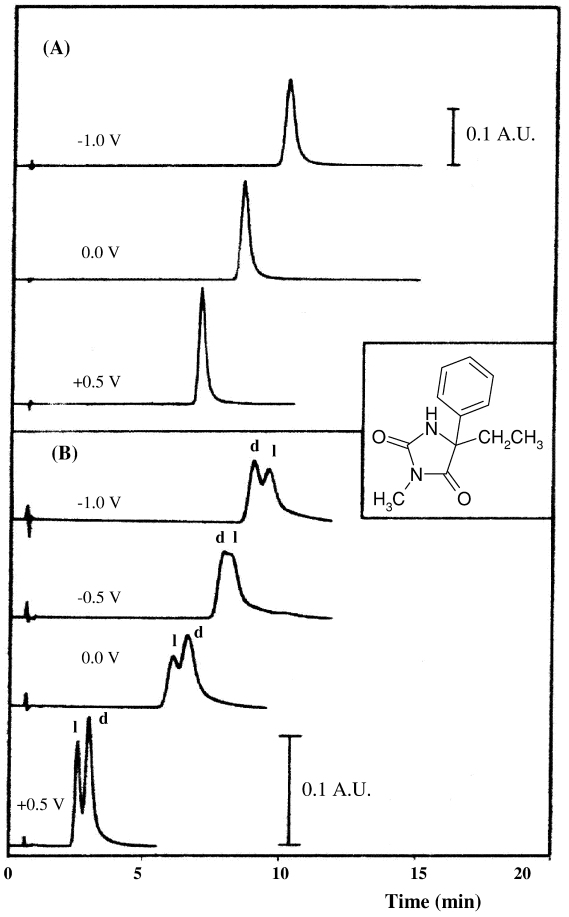

Fig. 37. Electrically modulated liquid chromatography separations of mephenytoin. (A) Separations in mobile phase devoid of β-CD at −1.0, 0.0 and 0.5 V. (B) Separations with 15 m*M* β-CD as a mobile-phase additive at −1.0, 0.5, 0.0 and +0.5 V. (Reproduced with permission from Ref. [357].)

6.3. Enantioseparations in capillaries packed with chiral stationary phases (CSP)

Enantioseparations in capillaries packed with CSPs is the most intensively developing mode of chiral CEC [362–385]. Many of CSPs which have earlier proved to be useful for HPLC enantioseparations have been adapted to the CEC mode. To these belong proteins [362,364], cyclodextrins and their derivatives [363,365,381], Pirkle-type CSPs [366,377], macrocyclic antibiotics [367,378,379], chiral acrylamides [368] and methacrylates [371,374], anion and cation exchangers [370,375,376] and polysaccharide derivatives [368,372,373,380,382–384]. Together with traditional approaches for CSP preparation, Peters et al. reported the enantioseparations using a ''moulded'' rigid monolithic capillary column [369]. Although this kind of capillary column may in principle offer improved dynamic characteristics, the plate numbers reported in this study (61 000 plates m^{-1}) were not exceptionally high.

In the early studies on chiral CEC in packed capillaries just illustrating the applicability of one or another type of the CSPs useful in HPLC also for the CEC mode, preparation of the frits, generation of the EOF and just illustrating a few enantioseparations was considered to be an impressive result. However, in several earlier studies, a significant advantage of the electrokinetically-driven mode compared to the pressure-driven mode in the same capillaries could not be always unambiguously demonstrated [362–364,368,372]. Most recently however, a better understanding of the separation mechanisms and appropriate optimization of the preparation and application conditions of CEC capillaries allowed to achieve

plate numbers in the range of 150–200 thousand plate per meter [366,370,375–378,383,385]. This was achieved by an optimization of the nature and loading of a chiral selector onto the surface of silica [370–376,383], the particle size [366,377,382,384] and the pore size [382] of silica, the separation conditions [362–384], etc.

Together with CEC enantioseparations in packed capillaries with aqueous and aqueous–organic BGEs, nonaqueous CEC enantioseparations are developing [371–373,375,382,383]. This technique may allow to further extend the applicability of chiral CEC with packed capillaries also for some problems of biomedical relevance [372]. Very recently, significantly higher plate numbers were reported in this technique compared to capillary LC in the same capillary (Fig. 38) [383].

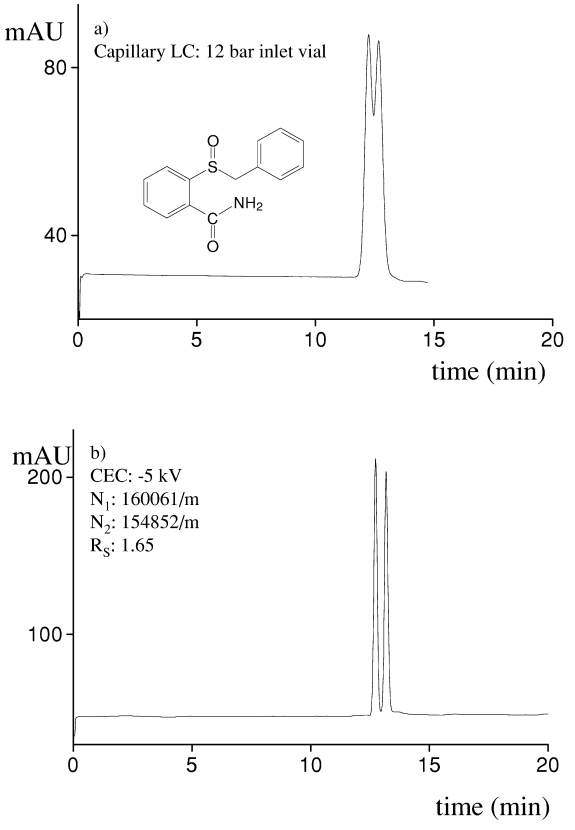

Fig. 38. Enantioseparation of 2-(benzylsulfinyl)benzamide in capillary LC (a) and CEC (b) mode. (Reproduced with permission from Ref. [383].)

The present status of chiral CEC may be described in the following way: Various modes of this technique work in principle, some technical and few mechanistic aspects of the EOF generation and its effect on the separation are understood. However, CEC in general and chiral CEC in particular seems still to be far from the maturation stage. Many technical and major mechanistic aspects have still to be better understood which will be apparently followed by impressive applications of this undoubtedly promising technique.

7. Nomenclature

AA	Amino acids
AGT	Aminoglutethimide
BDHP	1,1′-Binaphthyl-2,2′-diyl hydrogen phosphate
BGE	Background electrolyte
BrPh	Brompheniramine
CCD	Central composite design
CD	Cyclodextrin
CDS	Circular dichroism spectrometry
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CE–MS	Capillary electrophoresis–mass spectrometry
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CM-β-CD	Carboxymethyl β-CD
CMPA	Chiral mobile phase additive
CSA	Camphorsulfonic acid
CZE	Capillary zone electrophoresis
DIM	Dimethindene
DMA	*N,N′*-Dimethylacetamide
DMF	*N,N′*-Dimethylformamide
DM-β-CD	Heptakis-(2,6-di-*O*-methyl)-β-CD
DNBLeu	3,5-Dinitrobenzoylleucine
DNS	Dansyl
DS	Degree of substitution
1D ROESY	One dimensional rotating frame nuclear Overhauser and exchange spectroscopy
EMLC	Electrochemically modulated liquid chromatography
EMO	Enantiomer migration order

EOF	Electroosmotic flow
ESI MS	Electrospray ionization mass spectrometry
FCCE	Flow counterbalanced capillary electrophoresis
FITC	Fluorescein isothiocyanate
FLEC	1-(9-Fluorenyl)ethyl chloroformate
HDAS-β-CD	Heptakis-(2,3-diacetyl-6-sulfato)-β-CD
HDMS-β-CD	Heptakis-(2,3-dimethyl-6-sulfato)-β-CD
GC	Gas chromatography
HP-β-CD	Hydroxypropyl β-CD
HPLC	High-performance liquid chromatography
HPMC	Hydroxypropylmethylcellulose
HS-β-CD	Heptakis-(6-sulfato)-β-CD
Ile	Isoleucine
Leu	Leucine
Met	Methionine
NMF	N-Methylformamide
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
PBD	Plackett-Burman design
Phe	Phenylalanine
SBE-β-CD	Sulfobutyl β-CD
SCCE	Synchronous cyclic capillary electrophoresis
SDS	Sodium dodecyl sulfate
SFC	Super(sub)critical fluid chromatography
Thr	Threonine
TM-β-CD	Heptakis-(2,3,6-tri-*O*-methyl-β-CD)
UV–Vis	Ultraviolet–visible spectrometry
Val	Valine
VP	Verapamil
WCOT	Wall-coated open tubular
WF	Warfarin

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