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Review

Chiral separation of amino acids and peptides by capillary electrophoresis

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Abstract

Chiral separation of amino acids and peptides by capillary electrophoresis (CE) is reviewed regarding the separation principles of different approaches, advantages and limitations, chiral recognition mechanisms and applications. The direct approach details various chiral selectors with an emphasis on cyclodextrins and their derivatives, antibiotics and chiral surfactants as the chiral selectors. The indirect approach deals with various chiral reagents applied for diastereomer formation and types of separation media such as micelles and polymeric pseudo-stationary phases. Many derivatization reagents used for high sensitivity detection of amino acids and peptides are also discussed and their characteristics are summarized in tables. A large number of relevant examples is presented illustrating the current status of enantiomeric and diastereomeric separation of amino acids and peptides. Strategies to enhance the selectivity and optimize separation parameters by the application of experimental designs are described. The reversal of enantiomeric elution order and the effects of organic modifiers on the selectivity are illustrated in both direct and indirect methods. Some applications of chiral amino acid and peptide analysis, in particular, regarding the determination of trace enantiomeric impurities, are given. This review selects more than 200 articles published between 1988 and 1999. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Reviews; Diastereomer separation; Chiral selectors; Amino acids; Peptides

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1. Introduction

Amino acids are important biological compounds that are associated with peptides and proteins and are frequently found in food, feeds, body fluids and tissues. It is well known that in nature amino acids occur in L-forms and that they play an important role in the food and pharmaceutical industries. In the food industry, racemization of amino acids could occur during food processing, for example, in roasting or treatment of food protein under alkaline conditions [1]. Recently, D-forms have been discovered in species of lower animals [2,3], mammalian organs and blood [4,5]. Some D-form amino acids were also detected in various vegetables and fruits [6], 0.6–3.8% of D-alanine was thus found in freshly pressed plant juices. Moreover, the detection of D-amino acids could be used to control fermentation processes as markers of microbial contamination [7]. In the field of neuroscience, Okuma and Abe have found significant amounts of the D-forms in the nerve tissues and eyes of crustaceas [8], and Nagata et al. in rat cortex, hippocampus, striatum, cerebellum, and other brain regions [9]. Furthermore, some D-amino acids have been observed in mice [10], and in human plasma in relation to some diseases [11,12]. It has been recognized that the D- and L-stereoisomers differ in their biological or physiological activities, therefore chiral analysis of D,L-amino acids is of great interest and of increasing importance in life science and many other related fields.

Chiral separation of amino acids has, for many years, been extensively studied using chiral stationary phases in GC and HPLC. Currently established chiral chromatographic separation methods are able to resolve most of the protein amino acids, but there is still a need for a rapid, highly efficient method for the analysis of amino acids in complex matrixes. Capillary electrophoresis (CE) has recently received extensive attention for the separation of a variety of compounds including many substances of biological origin and of enantiomers and it is nowadays considered as an important technique complementary to chromatographic separation techniques. The CE technique can offer very high separation efficiency and resolution power, and enantiomers, which are not resolved by high-performance liquid chromatography (HPLC), may often be separated by a simple CE method. Also, baseline resolution of two compounds having a small separation factor (e.g. $\alpha=1.01$) is possible in CE because of the high separation efficiency, while for HPLC a separation factor larger than 1.05 ($\alpha>1.05$) is usually required. Further, CE separation is performed on a miniaturized simple system utilizing only small amounts of sample and small volumes of buffers. Moreover, the

technique is particularly attractive for chiral separation since expensive chiral selectors or not commercially available selectors can be employed in different separation modes on the same capillary. All together, this allows rapid development of a new chiral method at a low cost. So far, more than 200 papers dealing with chiral separation of amino acids and peptides by CE have been published and a variety of chiral CE modes and approaches have been developed, such as CZE with cyclodextrin (CD-CZE), CD modified micellar electrokinetic chromatography (CD-MEKC), chiral surfactants, chiral polymers, gel filled chiral CE, chiral capillary electrochromatography (CEC), diastereomeric separation and so on. In addition, numerous reviews on chiral CE separations have been presented [13–23], highlighting continuing new developments in this area. Chankvetadze has published an interesting book on capillary electrophoresis in chiral analysis [24]. Terabe et al. have discussed the fundamental principles of different chiral CE modes in detail [14]. Issaq and Chan [18] and Smith [21] have presented thematic reviews on amino acid analysis including the enantiomeric separations. However, a critical review focusing on chiral separation of amino acids and peptides has not been presented before.

In this review, we summarize the chiral separations of amino acids and of small peptides published in the past decade with detailed discussions and emphasis on optimization and analyte structure–selector function relationship. A number of application examples are selected showing the present state-of-art of chiral separation of amino acids and peptides. Some specific separation conditions and the number of amino acids resolved are tabulated for comparisons of different approaches. The potentials and limitations of different approaches are addressed. With this review, we hope not only to give readers fairly complete references in this area, but also to provide a general guideline for the selection of chiral CE method and an insight into the area of chiral recognition for different separation approaches.

2. Derivatization

As CE is performed on miniaturized systems with typical separation capillary diameters ranging from 25 to 75 μm and only nanoliters of sample injection, the concentration detection limit with a conventional UV detector is restricted because of the short optical light path employed for on-column detection. This is considered as a major disadvantage of CE. On the other hand, most protein amino acids lack strong UV absorbance, except for the three aromatic amino acids, Phe, Trp and Tyr. Thus, derivatization in many cases becomes necessary for sensitive detection of amino acids. Such a derivatization can lead to a considerable increase in sensitivity. In addition to sensitivity enhancement, the derivatization is expected to improve selectivity.

Several different derivatization approaches, e.g. pre-column, on-column and post-column have been summarized in review articles [25–27]. For chiral separation of amino acids, however, the derivatization of amino acids is, in general, performed by pre-column or prior to the introduction of sample into the separation capillary. This is because, in addition to considering sensitivity, a strong interaction is anticipated between chiral selector and the labeling moiety. The choice of a derivatization reagent is of crucial importance and high demands are therefore put on the properties of the derivatization reagents. An ideal reagent should thus fulfill several requirements [28]. First, it should be stable and give rapid reaction in high yield at low temperature and the corresponding derivatives should be sufficiently stable. Excess reagent and by-products from the reaction should not disturb the separation. Further, the reagent should be commercially available and inexpensive. For indirect separation, an additional requirement must be met for the optical purity of chiral reagent applied and this will be discussed in a separate section.

Although a large number of different derivatization reagents has been applied for amino acid analysis in HPLC and CE [25–27], only some of them have so far been employed for enantiomeric separations in CE. Table 1 summarizes those derivatization reagents, reaction conditions and spectral properties [29–44]. The derivatization schemes are shown in Fig. 1. Most of the reagents provide the means for fluorescence detection which leads to highly sensitive detection of amino acids by the application of laser induced fluorescence (LIF). Only the phenylthiohydantoin (PTH) is non-fluorescent, it

Table 1
Derivatization reagents, reaction conditions and spectral properties

Reagents	Abbreviation	Reaction conditions	$\lambda_{ex}/\lambda_{em}$ (nm)[a] λ_{max} (UV)[b]	LOD (*M*)	Ref.
2-(9-Anthryl)ethyl chloroformate	AEOC	RT[c]/5 min	348/366 256	$1\cdot10^{-10}$ $1.5\cdot10^{-7}$	[29]
6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate	AQC	50°C/10 min	360/395 214 or 254	$3\cdot10^{-7}$	[30,31]
Cyanine	Cy5	RT/60 min	635/670	$6\cdot10^{-8}$	[32,42]
Naphthalene-2,3-dicarboxaldehyde	NDA (CBI-amino acids)	RT/30 min	442/490 254	$5\cdot10^{-10}$	[33–35]
5-Dimethylaminonaphthalene-1-sulphonyl chloride	Dns	RT/120 min	360/570 254	–	[36,37]
2,4-Dinitrophenyl fluoride	DNP	–	254	–	[38]
Fluorescein isothiocyanate	FITC	RT/overnight	488/520	10^{-11}	[43,44]
9-Fluorenylmethyl chloroformate	FMOC	RT/45 s	265/310 256	$3.4\cdot10^{-8}$	[38–40]
o-Phthalaldehyde	OPA	RT/1 min	340/475 260	$2.1\cdot10^{-7}$	[41]
Phenylthiohydantoin	PTH	RT/5 min	254	–	[36,37]

[a] Laser-induced fluorescence (LIF).
[b] UV detection.
[c] RT: Room temperature.

displays a maximum in UV absorbance at 254 nm [36,37]. The main advantage of the PTH may be its high polarity and the good solubility of the derivatives in aqueous solutions. 5-Dimethylaminonaphthalene-1-sulphonyl chloride (Dns) is the most widely used derivatization reagent; it has maximum UV absorbance at 254 nm. Although the Dns-amino acids are fluorescent in nature, the aqueous environment of CE produces high reaction quantum yields. Further, the Dns-derivatives are photosensitive [36,37]. *o*-Phthalaldehyde (OPA) reacts with amino acids in the presence of a reducing agent, such as ethanethiol, to form a substituted isoindole ring, Fig. 1. The OPA-amino acids not only have a strong absorbance at 260 and 340 nm, but also strong fluorescence at 475 nm. Most importantly, this reagent reacts with amino acids rapidly and the excess OPA reagent is non-fluorescent and has virtually no interference with the electrophoretic separation, which is favorable for on-column and post-column derivatization. A disadvantage of this reagent is the relative instability of the derivatives. At room temperature decomposition can be detected after a few hours [36]. Similarly to OPA, naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide rapidly reacts with amino acids, and the resulting CBI derivatives display increased stability [33]. Another advantage of CBI-derivatives is that the excitation maximum wavelength coincides closely with the 442 nm output wavelength of the He–Cd laser. Highly sensitive detection of $2.5\cdot10^{-9}$ *M* CBI-amino acids was obtained using this laser by Ueda et al. [33,34]. It should be noted that due to the formation of an isoindole ring, Fig. 1, the OPA and NDA are only suitable for primary amino acids, thus, proline cannot be derivatized with these two reagents. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) is a relatively new fluorescent derivatization reagent that has demonstrated good stability and excellent sensitivity [30]. A limit of detection (LOD) of 0.2 μg/ml (equivalent to $3\cdot10^{-7}$ *M*) for AQC-tryptophan was achieved on a conventional UV detector. However, the reaction requires heating at 55°C for 10 min. 2-(9-Anthryl)ethyl chloroformate (AEOC) and 9-fluorenylmethyl chloroformate (FMOC) are chloroformate types of fluorescent derivatization reagents. The reaction conditions for those two reagents are simple and the

AEOC + RNH_2 →

AQC + RNH_2 →

DNS + RNH_2 →

DNP + RNH_2 →

FMOC + RNH_2 →

NDA + RNH_2 + CN^- → CBI-AAs

OPA + RNH_2 + HS-CH_2-CH_2-OH →

PTH + R-CH(NH_2)-COO^- →

Cyanine (Cy5)

FITC

Fig. 1. Derivatization schemes for amino acids.

resulting derivatives are highly stable. Both reagents provide excellent spectral properties, but the AEOC offers stronger UV absorbance and fluorescent characteristics than the FMOC [29]. About 5-fold higher sensitivity for AEOC-amino acids was observed as compared to the FMOC-amino acids and LODs for AEOC-amino acids down to n*M* level ($1\cdot10^{-10}$ *M*) was obtained with an Ar-ion laser by Engström et al. [29]. In some cases, the excess of reagents and hydrolysis products like FMOC–OH and AEOC–OH may interfere with detection and separation, but the hydrolysis products can be removed by extraction. In addition, Albin et al. compared OPA, FMOC and other fluorescent reagents and found that the FMOC-amino acids resulted in 5-fold higher sensitivity than the OPA-amino acids [41]. Recently, Kaneta et al. applied another fluorescence derivatization reagent, cyanine (Cy5), Fig. 1, for enantiomeric resolution of amino acids [32]. The main advantage of this reagent is its high emission wavelength in the deep-red and infrared region, in which almost all the biological substances, in a complex sample matrix, have no absorption band. Furthermore, the cyanine-labeled amino acids have highly negative charges so that they can migrate against the electroosmotic flow (EOF). A LOD of 10^{-8} *M* was obtained with an inexpensive diode laser [32]; however, owing to light scattering, this detection limit was not better than what was obtained with the CBI-amino acids. The potential of Cy5-derivatization for high sensitive detection of a Cy5-labeled oligonucleotide primer (10^{-10} *M*) has been shown earlier by Chen et al. [42]. More recently, Jin et al. have reported the use of fluorescein isothiocyanate (FITC) as derivatization reagent for enantiomeric separation of amino acids [43]. This reagent is a commonly used fluorescent labeling reagent for high sensitive detection. For example, Cheng and Dovichi reported the detection range from $5\cdot10^{-12}$ *M* to $\cdot\ 10^{-11}$ for FITC-amino acids when using Ar-ion LIF detection [44].

Another important feature of derivatization reagents is their reaction concentration limit; e.g., how low a concentration of the amino acids can be reproducibly derivatized with high yield [45]. Chan et al. have demonstrated that an alanine concentration as low as 10^{-7} μ*M* (n*M* level) could be derivatized with the FMOC [39]. In addition, the derivatization of amino acids at low concentration has been achieved with other fluorescent reagents for non-chiral separations. Camilleri et al. could derivatize amino acids at 10^{-7} *M* with 3-(4-tetrazolebenzoyl)-2-quinolinecarboxaldehyde (TBQCA) [46]. Arriaga and coworkers also demonstrated the derivatization of amino acids at 10^{-7} *M* level with another fluorescent reagent, 3-(*p*-carboxybenzoyl)quinoline-2-carboxyaldehyde (CBQ) [47]. It should be pointed out that the real reaction concentration of amino acids required for reaction in a matrix sample might be higher than that in a standard solution. Timperman et al. achieved a LOD of 10^{-13} *M* (around 100 molecules) labeled amino acids using an Ar–Kr mixed-gas ion laser and charge-coupled-device detector system [48], which is probably the lowest LOD reported for amino acid-derivatives so far. However, the limit for reaction concentration, as for many other reagents, was unknown. In our opinion, it seems that direct derivatization of amino acids below μ*M* level in complex matrices is not reliable. On-column derivatization may make reliable derivatizaton of lower concentrations possible, see Section 4.2.

3. Direct (enantiomeric) separation

Direct chiral separation is performed in a chiral environment by the interaction of enantiomers with a chiral selector added to the background electrolyte (BGE). When a chiral selector is added to the BGE, the enantiomers and the selector form complexes or diastereomeric pairs in a dynamic equilibrium process, which may result in different stability constants and thus different effective mobilities for two enantiomers. Therefore, the choice of chiral selector and consecutive optimization of relevant separation parameters are indispensable steps to achieve direct separation. So far, a variety of chiral selectors and approaches has been applied for enantiomeric separation of amino acids and of small peptides and these issues will be summarized in the following sections.

3.1. Chiral ligand-exchange complexation

On the basis of a chiral ligand exchange mechanism introduced earlier by Davankov et al. for HPLC

[49], Gassmann et al. first demonstrated chiral capillary electrophoresis (CCE) separation [50]. Using this technique, they successfully separated 12 Dns-amino acids [50]. The separation mechanism involves the use of Cu(II) and L-histidine as chiral chelator to form a ternary complex of diastereomers with Dns-L- and D-amino acids. The different stability of the diastereomeric complexes allows separation of the enantiomers. They also observed that chiral resolution was lost when the Cu(II) was replaced with Co(II). However, Fanali et al. separated several Dns-amino acids by the formation of a stable Co(III)-amino acid complex using L-(+)-tartrate as the chiral ligand [51]. Gozel and coworkers improved the separation by means of the formation of a Cu(II)-aspartame complex and thus resolved 14 pairs of Dns-amino acids [52]. They found that the ratio of metal ion to chiral chelator was critical and the best resolution was obtained when the stoichiometric ratio of Cu(II) to asparatame was about 0.5. Yuan et al. recently reported the simultaneous separation of 6 Dns-amino acids using a L-arginine as the chiral chelator with Cu(II) complexes at pH 7 [53], Fig. 2.

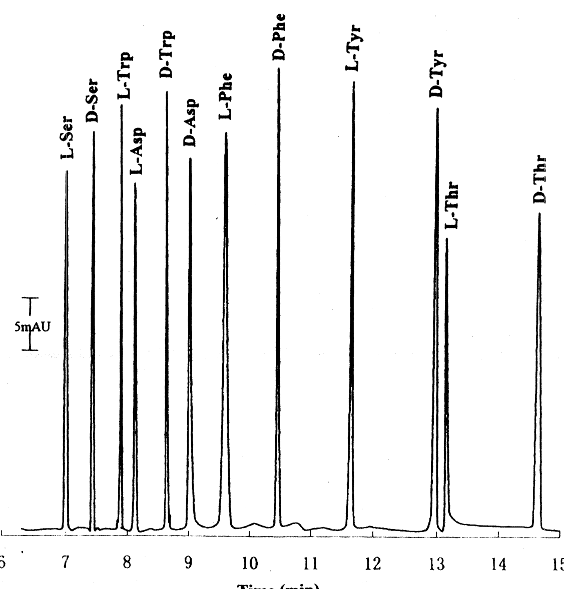

Fig. 2. Electropherogram of chiral Dns-amino acids by chiral ligand exchange CE. Conditions: 40 m*M* NH_4OAc (pH 4.0), 1 m*M* Cu(II)sulfate, 2 m*M* L-Arg. Capillary, 52 cm (38 cm to detector)×50 μm I.D. 25 kV; UV at 254 nm. (From Ref. [53] with permission.)

The authors also investigated other metal ions and ligands and observed that the substitutions of Cu(II) with Co(II), Zn(II) or Ni(II), and L-Arg with other ligands such as L-Asp, L-Glu, L-Ala resulted in a loss of chiral recognition in most cases. These results indicate that a central ion and ligand play an important role in the equilibrium. When utilizing L-4-hydroxyproline (L-Hypro) as a chiral chelator, Schmid and Gübitz were able to separate 11 underivatized aromatic amino acids in an electrolyte composed of 50 m*M* L-Hypro–25 m*M* Cu(II)–15 m*M* SDS–3 *M* urea at pH 4 [54]. Interestingly, the addition of SDS significantly improved the resolution and led not only to a reversal of the migration order of amino acids, but also to the reversal of enantiomeric migration order (EMO) for the amino acids. Moreover, a mixture of *N*,*N*,-di-decyl-L-alanine-Cu(II) and SDS was employed by Sundin et al. to resolve 11 Dns-amino acids [55]. Ligand exchange micellar electrokinetic capillary chromatography (LE-MEKC) has been applied for simultaneous separation of *o*-, *m*-, *p*-fluoro-DL-phenylalanine and *o*-, *m*-, *p*-hydroxy-DL-phenylalanine (tyrosine) [56,57]. More recently these authors have further investigated the effects of different types of micelles and organic modifiers on the EMO in LE-MEKC [58].

Recently, Végvári et al. reported a new chiral chelator, *N*-(2-hydroxyoctyl)-L-4-hydroxyproline (HO-L-Hypro) [59], and baseline separation of 12 underivatized amino acids was achieved by the formation of Cu(II) complex. More recently, Schmid et al. compared two synthesized *N*-alkyl-L-4-hydroxyproline derivatives, HO-L-Hypro and HP-L-Hypro [*N*-(2-hydroxypropyl)-L-4-hydroxyproline)] with the earlier reported L-Hypro [60]. They found that the modified *N*-alkyl-L-4-hydroxyprolines gave higher enantioselectivity towards amino acids and dipeptides than the L-Hypro. In total 13 underivatized aromatic and 6 aliphatic amino acids were successfully separated. However, only partial resolutions of 5 dipeptides were achieved among 10 examined dipeptides. The chemical structures of *N*-alkyl-L-4-hydroxyprolines and a possible structure of the copper complex are shown in Fig. 3. In addition, a correlation between the p*I* value of amino acid and the optimal pH was observed for the separation when using this type of chiral chelators [59,60].

Fig. 3. Chemical structures of *N*-alkyl-L-4-hydroxyprolines (A) and a possible structure of the copper complex (B). (Adapted from Ref. [60].)

Chiral ligand exchange using naturally occurring optical L-amino acids as the selector is a simple approach to achieve selectivity. However, the amino acids such as Arg and His can not be resolved when using L-Arg or L-His as the selectors. This drawback can be overcome by the use of modified *N*-alkyl hydroxyprolines as selectors as shown by Schmid et al. [60]. This technique, although it was the first approach used for chiral separation in CE, is limited to the resolution of enantiomers having two polar groups like amino acids that are likely to form a ligand-exchange complexation at a proper distance from each other. In addition, fluorescence detection is preferred to avoid the UV absorbance of Cu(II). Hence, this technique has not been as widely used as other types of chiral selectors such as cyclodextrins.

3.2. Host–guest inclusion complexation

3.2.1. Cyclodextrins and their derivatives as chiral selectors

Cyclodextrins (CD) are cyclic oligosaccharides consisting of six (α-CD), seven (β-CD), or eight (γ-CD) glucopyranose units. CD molecules possess hollow truncated cone structures with a hydrophobic interior cavity and an external hydrophilic surface containing many chiral centers. Further, CDs show excellent stability at a wide pH range (2–12) and the UV absorption is minimal. These unique characters make CDs outstanding as chiral selectors amenable to the formation of inclusion complexes with a large number of analytes. In fact, the CDs have become the most widely used chiral selectors in CE.

The utility of CD as the selector was first demonstrated by Snopek et al. [61], followed by Guttman et al. who succeeded to separate 12 Dns-D,L-amino acids utilizing polyacrylamide gel filled capillaries with incorporated β-CD as a selector [62]. Fanali demonstrated the feasibility of employing CD directly in free solution CE for chiral separation of sympathomimetic drugs [63]. This technique, CD-CZE, has since then become the most widely used approach for enantiomer separation. Unfortunately, chiral resolution of a complicated mixture is, in some cases, limited when using the CD-CZE. Terabe et al. [64,66] and Nishi and Matsuo [65] combined the CD with MEKC and developed a CD-MEKC technique, which has greatly widened the applicability for resolution of mixed analytes, e.g., amino acid mixtures. A variety of amino acids and peptides and their derivatives have been resolved using both native α-, β-, and γ-CDs and modified CDs [32–35,40,43,62,67–102]. Enantiomeric separation of di- and tri-peptides has also been reported recently by the application of CDs as the selectors [82,96–98]. Table 2 summarizes the amino acids and peptides resolved with the CDs and typical separation conditions applied.

As summarized in Table 2, the enantiomeric separations of the amino acids were performed mostly as Dns-derivatives [62,67,68,70–74,77,79,85–91]. Among these, the baseline resolu-

Table 2
DL-Amino acid derivatives and peptides resolved with cyclodextrins

Derivatization	Analyte	Chiral selectors	Separation buffer	Selectivity	Ref.
AEOC	12 AAs (a)	β-, γ-CD	(a) phosphate (pH=7.5), 40 mM SDS, 40 mM β-CD, 15% IPA, 1 M urea.	γ-CD>β-CD	[81]
	13 AAs (b)		(b) as in (a) but 10–25 mM γ-CD, without urea,		
AQC	14 AAs	α-,β-,γ-CD, DM-β(γ)-CD, HP-β(γ)-CD	20 mM borate (pH=9.2), 30 mM HP-β-CD, 20% methanol.	HP-β-CD > other CD	[78]
	14 AAs	β-,γ-CD, DM-β-CD, TM-β-CD, HP-βCD	10 mM BTP (pH=7.0), 2–5 mM HP-β-CD.	HP-β-CD > other CD	[83,84]
CBI	10 AAs	β-CD, γ-CD	100 mM borate (pH=9.0), 50 mM SDS, 10 mM CD of each.	γ->β-CD	[33,34]
	11 AAs	β-CD	30 mM phosphate/10 mM borate (pH=7–7.2), 20 mM β-CD+50 mM TODC.	β-CD +TODC> β-CD	[35,76]
	11 AAs	β-CD, HP-β-CD CM-β-CD	–		[75]
Cy5	6 AAs	γ-CD	10 mM phosphate (pH=4.55), 70 mM γ-CD, 1% PVP.		[32]
Dns	12 AAs	α-,β-,γ-CD, (gel filled column).	100 mM Tris/200 mM borate (pH=8.3), 7 M urea, 75 mM CD of each.	β>α, γ	[62]
	12 AAs	α-,β-,γ-CD; DM-α (β)-CD; TM-α (β)-CD	50 mM phosphate/100 mM borate (pH=9.0), 10 mM CD of each.	β-CD best	[67,68]
	5 AAs (a) 3 AAs (b)	α-, β-, γ-CD	(a) 100 mM phosphate (pH=2.6); (b) 100 mM borate (pH=9.5), 20 mM CD.	α-CD gave no resolution	[70]
	2 AAs (Phe, Trp)	G1α-CD HP-β-CD	10 mM phosphate/6 mM borate (pH=9.0), 10 mM CD of each.	HP-β-CD >G1α-CD	[71]
	10 AAs	β-CD γ-CD	100 mM borate (pH=8.3–8.6), 100 mM SDS, 60 mM CD of each.	γ-CD>β-CD	[72,73]
	12 AAs	β-CD, TM-β-CD 2,(3),(6)-MM-β-CD	50 mM phosphate/100 mM borate (pH=9.0), 10 mM CD, except for 6-MM-β-CD, 2 mM.	β-CD>other CDs	[77]
	12 AAs	β-,γ-CD, DM-γ-CD TM-α-(β)-CD	50 mM phosphate/100 mM borate (pH=9.0), 10 mM CD of each, 7 M urea.	β-CD >other CD	[79]
	12 AAs	β-CD	50 mM phosphate (pH=9.0), 10 mM β-CD, 20% methanol.	β-CD+methanol> β-CD	[85]
	8 AAs	β-CD	250 mM borate (pH=9.50), 200 mM SDS, 75 mM β-CD		[74]
	8 AAs	AM-β-CD	50 mM phosphate (pH=6.0), 10 mM AM-β-CD.		[86]
	12 AAs	CD-hm, CD-mh	100 mM phosphate (pH=5.0), 1 mM CD of each.	CD-mh>CD-hm	[87]
	2 AAs (Asp, Glu)	β-CD-EA	50 mM phosphate (pH=6.0), 3 mM CD.		[88]
	6 AAs	SBE-β-CD	10 mM Tris/HCl (pH=8.0), 3–20 mg/ml CD.		[89]
	7 AAs	SBE-γ-CD γ-CD	(a) 30 mM phosphate (pH=7.0), 5 mM SBE-γ-CD, 10% methanol. (b) 20 mM γ-CD+50 mM SDS.	No trend.	[91]
DNP	13 AAs	HP-β-CD	20 mM borate (pH=9.2), 30 mM HP-β-CD, 20 methanol.		[78]
	12 AAs	β-CD, γ-CD, ACD, HACD	50 mM phosphate (pH=7.0), 15 mM ACD.	ACD>β-CD	[92]

(Continued on next page)

Table 2 (*continued*)

Derivatization	Analyte	Chiral selectors	Separation buffer	Selectivity	Ref.
	5 AAs	β-CD	20 m*M* phosphate (pH=7.1), or 50 mm carbonate (pH=9.6), 8.8 m*M* β-CD.		[95]
FITC	21 AAs	β-,γ-CD	100 m*M* borate (pH=9.5), 30 m*M* SDS, 10 m*M* γ-CD.		[43]
FMOC	7 AAs (a)	(a) HP-β-CD	(a) 100 m*M* phosphate (pH=6.0), 2.5 m*M* HP-β-CD.		[40]
	20 AAs (b)	(b) β-,γ-CD	(b) 50 m*M* phosphate (pH=7.5), 50 m*M* SDS, 12 m*M* CD, 15% IPA.	β-CD> γ-CD	[80]
	9 peptides	β-,γ-CD	50 m*M* phosphate (pH=7.5), 40 m*M* SDS, 12 m*M* CD of each, 15% of IPA.	γ-CD>β-CD	[82]
PTH	7 AAs	TM-β-CD	(a) 10 m*M* formic acid/50 m*M* phosphate (pH=3.0), 35 m*M* TM-β-CD.		[93]
	15 AAs		(b) (a) mixed with digitonin, β-escin, SDS.	(b) Most pairs partially separated.	[94]
N-t-BOC	15 AAs	HP-γ-CD	50 m*M* phosphate (pH=7.0), 10 m*M* HP-γ-CD.		[99]
α- and β-naphthalene-sulfonyl	6 AAs	β-CD, γ-CD methylated CDs	100 m*M* borate–50 m*M* phosphate (pH=9.0), 2–10 m*M* of each CD.		[100]
Underivatized	1 tripeptide	CM-β-CD	(a) 50 m*M* phosphate (pH=5.25), 60 mg/ml CM-β-CD.		[96]
	1 dipeptide	CM-β-CD, DM-β-CD, TM-β-CD	(b) pH=3.3, 1.5% (w/v) CM-β-CD. (c) pH=1.7, 2–2.5% (w/v), TM-β-CD.	CM-β-CD>TM-β-CD>DM-β-CD	[97]
	2 dipeptides	β-CD	70 m*M* phosphate (pH=2.7), 36 m*M* β-CD, 2 *M* urea.		[98]
	Phe, Try, Tyr	α-CD	50 m*M* phosphate (pH=2.5), 30–50 m*M* α-CD.		[69]
	Phe, Try, Tyr	α-CD	100 m*M* phosphate/$HClO_4$ (pH=2.5), 40 mm α-CD		[101]
	Phe, Try, Tyr	TM-β-CD	40 m*M* Trizma phosphate (pH=?), 60 m*M* TM-β-CD.		[102]

AAs: amino acids.

tion of 12 Dns-amino acids was achieved [62,67,68,77,79,85,87]. The use of β-CD resulted in higher enantioselectivity than α-CD and γ-CD in the CD-CZE mode [62,67,68,77]. In contrast, Terabe et al. obtained better enantioselectivity with the γ-CD than with the β-CD in the presence of micelles, i.e., in CD-MEKC mode [72,73]. The application of α-CD led to a lack of chiral recognition for Dns-amino acids [62,70], but α-CD was found to be suitable for chiral resolution of three underivatized aromatic amino acids, Phe, Try and Tyr [69,101]. Ueda et al. achieved the enantioseparation of 10 CBI-amino acids in the CD-MEKC mode and observed that the γ-CD led to better enantioselectivity than the β-CD [33,34]. Okafo et al. also separated 11 CBI-amino acids using β-CD as the selector [35,76]. They found that the combination of β-CD and taurodeoxycholic acid (TODC) resulted in improved separation as compared to the use of β-CD alone. Lindner et al. evaluated structure–resolution relationship between CD types and different derivatives in the CD-CZE system and resolved 13 DNP-amino acids and 14 AQC-amino acids using 2-hydroxypropyl-β-CD (HP-β-CD) [78]. The authors showed that the γ-CDs, in general, gave best resolution for voluminous fluorescent derivatives Dns and FMOC, while the β-CD led to the best selectivity for the smaller chromophore groups of DNP, DNB, CBZ and AQC, indicating the importance of size-fit between CD cavity and derivative moieties for chiral recognition. When comparing the enanatioselectivity of methylated β- and γ-CDs for six α- and β- naphthalenesulfonyl-amino acids [100], Miura et al. observed that the methylated γ-CDs led to better chiral recognition than corresponding methylated β-CDs and the selective methylation of the secondary hydroxyl groups on the rim of CDs produced a remarkable enantioselectivity. The chiral recognition in aqueous media is, in many cases, based on inclusion complexation by means of the interaction

of the hydrophobic cavity and the chiral environments at the entrance of the cavity. However, besides the formation of inclusion complexes with the cavity, interactions like hydrogen bonds, dipole–dipole interaction also have to be taken into account. An example of this is the enantioseparation of 6 Dns-amino acids using an acyclic oligosaccharide, dextrin 10, as chiral buffer additive [103].

Although the CD-CZE is the most frequently applied chiral CE technique for the resolution of different types of amino acid derivatives and peptides (Table 2), the CD-MEKC offers obvious advantages over the CD-CZE in the resolution of a large number of mixed amino acids. We have successfully achieved chiral separation of 20 protein amino acids derivatized with FMOC using CD-MEKC containing 50 m*M* SDS and 10 m*M* β-CD in combination with an organic modifier [80]. Fig. 4 illustrates the enantioseparation of a mixture of 13 D,L-FMOC-amino acids under the optimized conditions. It was observed that presence of micelles resulted in enhanced enantioselectivity for FMOC-amino acids [80] and AEOC-amino acids [81] when employing β-CD and γ-CD as selectors. The use of β-CD resulted in the resolution of 13 AEOC-amino acids [81], but the application of γ-CD led to better enantioselectivity and relatively higher separation efficiency as well. In Fig. 5 is shown an example of chiral separation of 10 AEOC-amino acids. In addition, we applied similar conditions for chiral separations of FMOC-peptides [82]. Of 15 peptides examined, the major part were baseline separated with the γ-CD as the selector, while the use of the β-CD resulted in only partial or no enantioselectivity at all. More recently, Jin et al. have demonstrated enantioseparation of 21 individual FITC-amino acids and a mixture of 7 FITC-amino acids by CD-MEKC [43]. The FITC-amino acids had a relatively large size, c.a. 10.15·12.3 Å, consequently higher enantioselectivity was obtained with the γ-CD as selector. The chiral recognition mechanisms might be complicated due to the presence of the micelles and organic solvent modifier. Nevertheless, an enhanced enantioselectivity achieved in the organic modified CD-MEKC system has made it possible to resolve not only all protein amino acids but also a majority of the compounds in an amino acid mixture in one run [80,81].

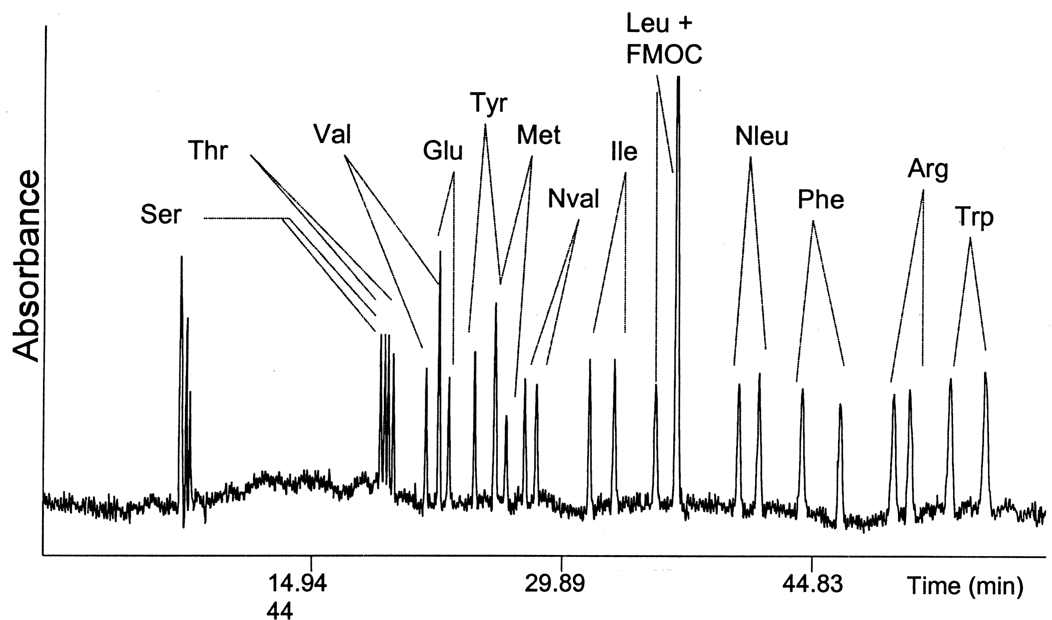

Fig. 4. Micellar electrokinetic chromatogram of FMOC-amino acids. Conditions: 50 m*M* phosphate (pH 7.5), 50 m*M* SDS, 12 m*M* β-CD, 15% (v/v) IPA. Capillary, 67 cm (45 cm to detector)×25 μm I.D.; 25 kV; current, 11 μA; temperature, 25°C. (From Ref. [80], with permission.)

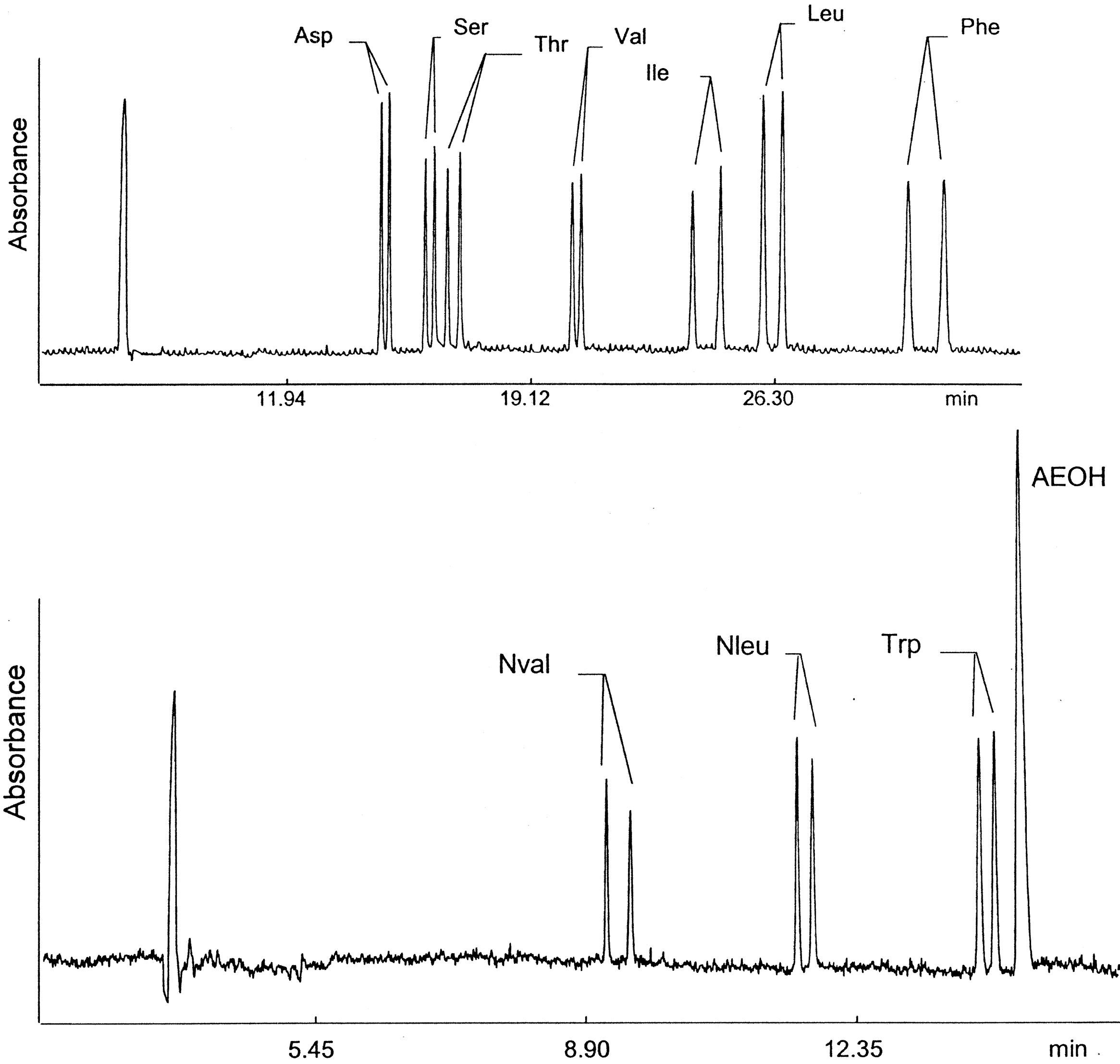

Fig. 5. Enantioseparation of 10 AEOC-amino acids by CD-MEKC. Conditions: (a) 50 m*M* phosphate (pH 7.5), 40 m*M* SDS, 10 m*M* γ-CD, 15% (v/v) IPA. Capillary, 67 cm (46 cm to detector)×25 μm I.D.; 30 kV; current, 12 μA; temperature, 25°C. (b) 20 m*M* γ-CD, 2-propanol, 0% IPA (v/v). 20 μA, other conditions as in (a). AEOH=AEOC hydrolysis product. (From Ref. [81], with permission.)

Recently, several new modified chargeable CDs have been applied for enantioseparation of amino acids and peptides [86–92,96,97], this currently represents a new interesting area. It has been recognized that the charged CDs exhibit superior solubility in aqueous media and provide improved chiral recognition by ion-pairing interactions with a charged analyte [104]. Fig. 6 shows the structures of typical modified CDs employed in enantiomer separation.

Terabe first showed the application of a cationic β-CD, mono-(6-β-aminoethylamino-6-deoxy)-β-CD (CDen), for resolution of 6 Dns-amino acids [105]. The charged CD, which acts like a carrier or quasi-stationary phase named as electrokinetic chromatography (EKC), can separate both neutral and charged

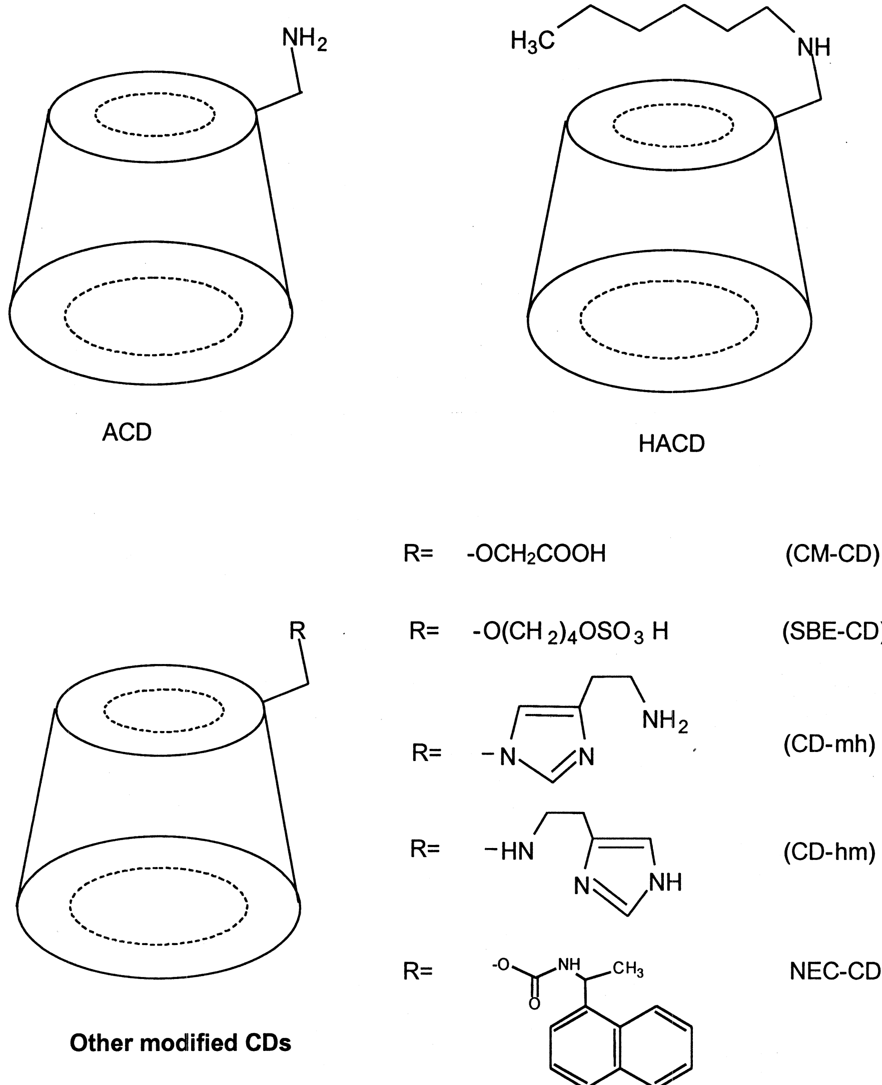

Fig. 6. Structures of modified CDs.

compounds [105]. Recently, Tanaka and Terabe demonstrated the separation of a mixture of 8 Dns-amino acids using a commercially available amphoteric-β-CD (AM-β-CD) [86]. Galaverna et al. applied two synthesized histamine-modified β-CDs, 6-deoxy-6-*N*-histamino-β-CD (CD-hm) and 6-deoxy-6-[4-(2-aminoethyl)imidazolyl]-β-CD (CD-mh) for resolution of Dns-amino acids [87]. These mono substituted CDs exhibit basic sites at a different distance from the cavity, Fig. 6. It was noted that the use of CD-mh resulted in higher resolution than that of CD-hm. This is probably the consequence of steric effects due to the proximity of the imidazole ring up the cavity. Baseline separation of 10 Dns-amino acids was achieved using CD-mh and chiral separation of Asp and Glu was also possible when using a short capillary. Further, the reversal of EMO was observed between the CD-hm and CD-mh. Egashira et al. utilized other types of positively charged CDs, 6-amino-6-deoxy-β-CD (ACD), 6-

deoxy-6-hexylamino-β-CD (HACD) for resolution of 12 DNP-amino acids [92]. The ACD and HACD generated similar selectivity, but the migration times resulting from the HACD were relatively shorter, suggesting that the hydrophobic hexyl chain might, to some extent, suppress the adsorption of HACD at the capillary surface. Moreover, higher enantioselectivity was observed for DNP derivatives than for the Dns derivatives. In addition, O'Keeffe et al. reported a new synthesized persubstituted β-CD {heptakis[6-hydroxyethyl(amino-6-dexoxy-β-CD)]} (β-CD-EA) [88]. This positively charged CD has been shown to be effective for resolution of Asp and Glu and mixed acidic racemates. However, the separation efficiency was rather poor as compared to the use of other positively charged CDs, probably due to too strong adsorption of the β-CD-EA at the capillary surface.

Some negatively charged CDs have been employed for chiral resolution of amino acids and peptides [89–91,96,97]. Desiderio and Fanali employed a sulfobutyl ether β-CD (SBE-β-CD) as the selector for resolution of Dns-amino acids [89]. Among 11 examined amino acids, 6 Dns-amino acids were individually separated in a Tris–HCl buffer containing 20 mg/ml SBE-β-CD at pH 6.0. Note that Asp and Glu were not resolved under this condition. When applying a coated column and a lower pH (3.1), Janini et al. demonstrated the separation of a mixture of 9 Dns-amino acids using the SBE-β-CD as the selector [90]. At the low pH, the Dns-amino acids are either neutral or weakly negatively charged. This facilitated their approach to the CD and their incorporation into the hydrophobic interior. Evidently, electrostatic repulsion was minimized at the lower pH, which allowed the chiral resolution of more Dns-amino acids including the Asp and Glu, while those amino acids were not resolved at a higher pH [89]. Jung and Francotte compared the enantioselectivity of γ-CD and SBE-γ-CD as the chiral selector for resolution of several Dns-amino acids [91]. Although distinctly different characters in enantioselectivity were noted between the γ-CD and SBE-γ-CD, no general trend was observed for resolution of amino acids and drug racemates. Furthermore, the reversal of EMO was obtained between these two CDs. More recently, Sabah and Scriba utilized a negatively charged CD, carboxymethyl-β-CD (CM-β-CD), for enantioseparation of di- and tripeptide isomers [96,97], and baseline separation of 8 stereoisomers of tripeptide Gly–α–β-DL-Asp–DL-PheNH$_2$ was demonstrated using this selector, Fig. 7 [96].

A charged resolving agent migration model, CHARM, facilitating optimization of buffer pH for separation of enantiomers using charged selectors

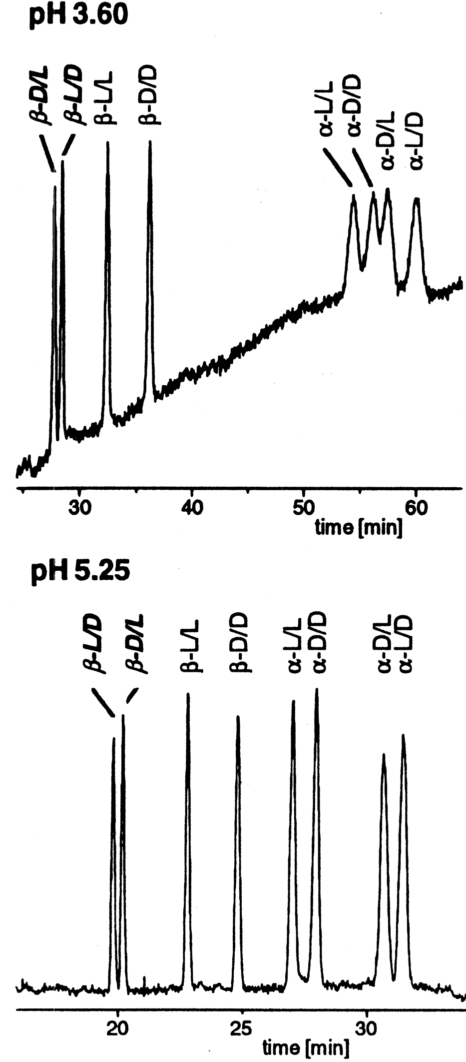

Fig. 7. Separation of the stereoisomers of the isomeric tripeptides Gly-α–β-D–L-Asp-D–L-PheNH$_2$. Conditions: 50 m*M* phosphate (pH 3.60, 5.25), 60 m*M*/ml CM-β-CD. Polyacrylamide-coated capillary, 47 cm (40 cm to detector)×50 μm I.D.; −20 kV. (From Ref. [96], with permission.)

has been presented by Vigh and coworkers [106,107].

The modified CDs, in general, provide higher enantioselectivity than native CDs. The modification of CDs at different positions can produce distinguished differences in enantioselectivity. An example of this has been shown by Gahm and Stalcup who demonstrated chiral resolution of 3 DNB-amino acids by use of regiospecifically monosubstituted 1-(1-naphthyl)ethylcarbamoylated β-CDs (NEC-β-CDs) [108]. Many modified CDs are actually in mixed form with different degrees of substitutions and this makes it more difficult to understand separation mechanisms. Also, low separation efficiency was generated from the positively charged CDs in most cases, thus a coated capillary is required to minimize the strong interaction between the capillary surface and this type of CDs. However, the main drawback of the mixtures of differently substituted CDs concerns their lack of batch-to-batch reproducibility. An example of this has been shown by Sänger-van de Griend and coworkers who obtained different selectivity from different suppliers when using DM-β-CD as the selector [109]. The application of chemically defined CDs represents a major advancement in this respect [110].

3.2.2. Crown ether as selector

Crown ethers having macrocyclic polyether rings are known to form stable inclusion complexes, e.g., host–guest inclusion with alkali metals, ammonium ion or protonated amines. On the basis of this principle, Kuhn et al. utilized a crown ether, (+)-18-crown-6-tetracarboxylic acid ($18C6H_4$), as a selector for chiral separation of underivatized amino acids, drug racemates and peptides [69,111]. They resolved 60% of a set of enantiomers including 8 amino acids and found that the best resolutions were achieved when the chiral center was adjacent to the amine groups. Using similar conditions, chiral resolution of 4 dipeptides and 5 tripeptides was also achieved [112]. Further, the resolution of all 8 stereoisomers of Tyr–Lys–Trp was demonstrated with a Tris–citric acid buffer containing 10 m*M* $18C6H_4$ at pH 2.0 [113]. Walbroehl and Wagner reported enantioseparation of analogues of DOPA and γ-amino butyric acid (GABA) [114]. Schmid and Gübitz applied the crown ether for enantiomeric separation of 12 dipeptides and most of them were baseline separated [115]. Interestingly, these authors also observed a relationship between the molecular mass of a glycyldipeptide and its separation factor α (t'_2/t'_1). More recently, Verleysen et al. have presented a review concerning the applications of crown ethers as selectors for enantiomeric separation of amino acids and peptides and chiral drugs [116]. They showed that buffer pH and composition had a pronounced effect on selectivity, Fig. 8.

The separation mechanism of the crown ether is similar to that of CDs based on host–guest complexation, which forms diastereomers with disparate complex formation constants. However, the separation has to be performed at a low pH (2–3) in order to form $C^*–NH_3^+$ complexes with the primary amino groups. The method has thus been limited to resolve only underivatized amino acids. Secondary amines do not form complexes with the crown ether and resolution of Pro and of peptides beginning with a Pro residual is impossible. Moreover, the Asp and Glu could not be chirally separated due mainly to strong electrostatic interactions. This effect has clearly been illustrated by an example where high enantioselective separation of a DL-aspartic acid β-benzyl ester was obtained after the Asp was altered to ester by blocking a carboxyl group [116].

The potential and limitations of crown ether as a chiral additive have been shown by Kuhn et al. [117,118] and by Verleysen et al. [116]. Resolution as high as 16 has been shown using the crown ether as the selector, for example, for separation of tryptophan hydroxamate, Fig. 8 [116]. Further, the combination of the crown ether and CD resulted in a dramatically improved resolution [117], enantioselectivity was not observed with either crown ether or CD alone [118]. Armstrong et al. recently demonstrated that addition of achiral 18-crown-6 to a CD based buffer system for the CE enantioseparation of primary amine containing compounds frequently enhances resolution [119]. A main drawback using crown ether as the selector is low separation efficiency, about 5-fold lower than that obtained from the CD [69]. The low separation efficiency may be attributed to the slow dynamic exchange and to adsorption resulting from the low pH which is required for chiral recognition. An additional disadvantage of using the crown ether as the selector is

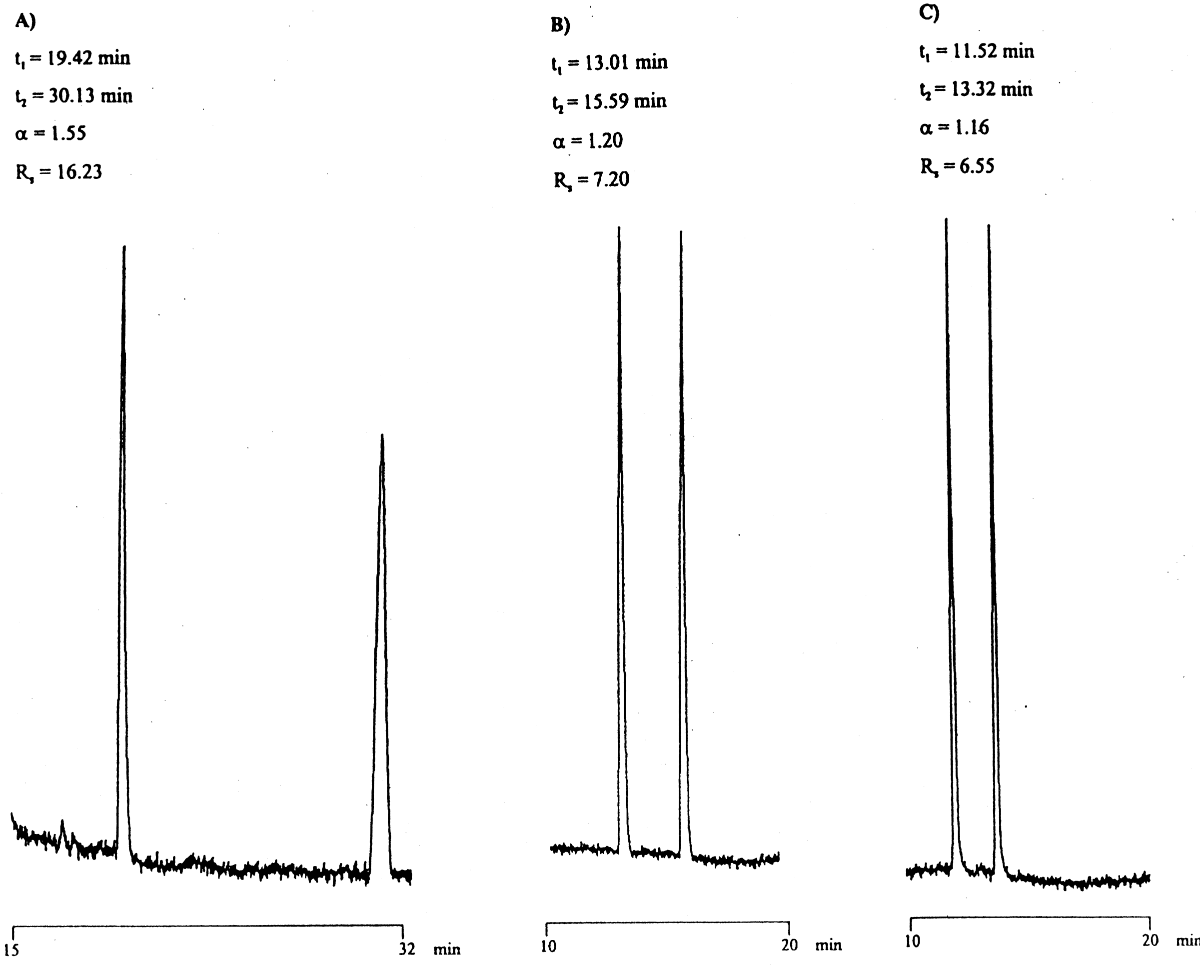

Fig. 8. Influence of buffer composition on the separation of tryptophan hydroxamate. Buffer composition: (A) 10 m*M* Tris–10 m*M* $18C6H_4$ (pH 2.4); (B) 10 m*M* NaH_2PO_4–10 m*M* $18C6H_4$ (pH 2.5); (C) 10 m*M* NaH_2PO_4–10 m*M* $18C6H_4$ (pH 2.7). (From Ref. [116], with permission.)

that buffers with potassium or ammonium components should be avoided due to their competitive binding to the crown ether [120].

3.3. Chiral surfactant based micellar electrokinetic chromatography

Based on micellar electrokinetic chromatography (MEKC) introduced by Terabe et al. [121,122], two classes of chiral surfactants have been used for the enantiomeric separation of amino acids and other enantiomers as well. One is naturally occurring surfactants such as bile salts, digitonin and saponins, while the other is synthesized optical amino acid derivatives with long hydrophobic alkyl-chains and modified glucopyranosides. Terabe et al. first employed bile salts, sodium deoxycholate (SDC) and sodium taurocholate (STC), for the enantioseparation of Dns-amino acids [123]. Although only two Dns-amino acids, Phe and Met, were baseline separated under acidic conditions (pH 3.0) with relatively long migration times; this chiral micellar approach has provided another possibility to resolve enantiomers in CE. When applying digitonin as a chiral micelle, Otsuka and Terabe separated 6 PTH-amino acids employing a long analysis time, 90 min [124]. Since

the digitonin is electrically neutral and largely insoluble in water, it was mixed with SDS or STDC [124,125]. Using another naturally occurring chiral surfactant, glycyrrhizic acid (GRA) and β-escin [126], 9 PTH-amino acids were nearly baseline resolved, but the efficiency was relatively low. Highly efficient separation of some NDA-derivatized amino acids has been achieved with sodium taurodeoxycholate (STDC) in conjunction with β-CD and an organic modifier [127]. The STDC seems to be the most effective among above-mentioned steroidal surfactants. The chiral recognition mechanism of natural surfactants is currently not well known.

In addition to the natural surfactants, some optically active amino acid derived synthetic surfactants have been utilized for MEKC enantioseparation of amino acids. Dobashi and coworkers employed a synthetic chiral surfactant, sodium *N*-dodecanoyl-L-valinate (SDVal) for chiral separation of amino acids as their *N*-(3,5-dinitrobenzoyl) *O*-isopropyl esters [128,129]. The resolution of four amino acid derivatives was demonstrated with chiral mixed micelles consisting of SDVal, SDS and methanol, but the resolution and peak shapes were poor. Otsuka et al. improved the selectivity and peak shape by the addition of SDS, urea and methanol to SDVal and resolved 6 PTH-amino acids [130]. Other similar chiral surfactants, sodium *N*-dodecanoyl-L-glutamate (SDGlu) and sodium *N*-dodecanoyl-L-serine (SDSer) were investigated [131,132], but separation characteristics were similar to the SDVal and only some of amino acids were resolved. The separation mechanisms of these chiral surfactants are based on the hydrophobic entanglement of the amino acid derivative with the micellar interior core and the hydrogen bonding affinity of the chiral amide functionality [133]. It seems that the enantioselectivity of these chiral surfactants has been limited in terms of selectivity and separation efficiency.

Recently, several novel chiral surfactants have been synthesized and utilized for the enantiomeric separation of different chiral compounds [134–138]. Table 3 shows the chemical structures of typical chiral surfactants. Mazzeo and coworkers have shown enantioseparation of drugs [134] and amino acid derivatives [135] using commercially available (*S*)- and (*R*)-*N*-dodecoxycarbonylvaline (DDCV). These surfactants have structures that are closely related to the earlier used SDVal but the amide linkage was replaced by a carbamate group (Table 3). This has several advantages in terms of enantioselectivity, separation efficiency and applicability. The separation of 13 AQC-amino acids including a mixture of 7 AQC-amino acids was demonstrated using 25 m*M* (*S*)- and (*R*)-DDCV at a pH 8. Tickle et al. reported another type of chiral glucopyranoside-based surfactants, dodecyl β-D-glucopyranoside monophosphate and monosulfate [136]. These new modified chiral surfactants, which have low pK_a, 1–2, and possess low CMC values from 0.5 to 1 m*M*, have been shown to be effective for the resolution of different drug enantiomers and Dns-amino acids as well. Fig. 9 illustrates the separation of a mixture of 6 Dns-amino acids with high separation efficiency. In addition, neutral alkyl-β-D-glucopyranoside based surfactants have been used for chiral resolution of amino acids. Desbène and Fulchic compared n-nonyl-β-D-glucopyranoside (NG) and *n*-octyl-β-D-glucopyranoside (OG) and observed that the best result was obtained with *n*-nonyl-β-D-glucopyranoside for enantiomeric separation of 5 amino acids derivatized as carbamates [137]. Mechref and El Rassi investigated the selectivity of NG, OG and OM (*n*-octyl-β-D-maltopyranoside) surfactants for Dns-amino acids and nearly baseline resolution of 4 Dns-amino acids was achieved with all these surfactants [138]. By use of in-situ charged micelles combining *N*,*N*-bis(3-D-gluconamidopropyl)-cholamide (Big CHAP) and -deoxycholamide (deoxy Big CHAP) and borate [139], they also resolved 4 Dns-amino acids. Recently, Ju and El Rassi presented an evaluation of a new surfactant, cyclohexyl-pentyl-β-D-maltoside as a chiral selector for Dns-amino acids [140]. Also, Ding and Fritz have synthesized a series of chiral carbamate surfactants, which are similar to the DDCV [141]. They compared the enantioselectivity of these surfactants with different hydrophobic chains of C_4 to C_{12} and found that a chain length of C_8 or C_{10} was the most effective. Also, a shift of the chiral part amino acid from, for example, leucine to valine had a major effect on chiral selectivity. In addition, chiral selectivity was enhanced using a duo-chiral system consisting of 100 m*M* (*S*)-(+)-*N*-octoxycarbonylleucine and 10 m*M* β-CD. As a result of this,

Table 3
Structures and CMC of some chiral surfactants

Name	Abbreviation	Structures	CMC (m*M*)	Ref.
Sodium taurodeoxycholate	STDC		8.5	[124,125] [254]
Sodium *N*-dodecanoyl-L-valinate	SDval			[128,129]
(*S*)-and (*R*)-*N*-Dodecoxycarbonyl valine	DDCV		3.5	[134,135]
Dodecyl β-D-glucopyranoside monophosphate			0.5	[136]
Dodecyl β-D-glucopyranoside monosulfate			1.0	[136]

baseline resolution of 8 Dns-amino acids was achieved, whereas no or partial selectivity was observed when using either of these selectors alone.

The advantage of chiral micelles is that a large number of mixed amino acids may be separated on the basis of simultaneous chiral solubilzation and non-chiral partitioning. Moreover, the availability of both (*R*)- and (*S*)- surfactants enables the reversal of EMO. In summary, the recent reports, discussed above, have provided useful information for the design and development of new types of chiral surfactants in this area. It seems that a more rational design requiring molecular modeling techniques and advances in stereoselective synthesis may lead to the development of a universal chiral surfactant [142].

3.4. Chiral polymer-mediated capillary electrophoresis

Hydrophilic chiral polymers such as polymerized surfactants, polysaccharides and cyclodextrin polymers as selectors have been recently introduced to CE, applications have been focused on the chiral separations of synthetic intermediates and pharmaceutically related compounds as summarized in

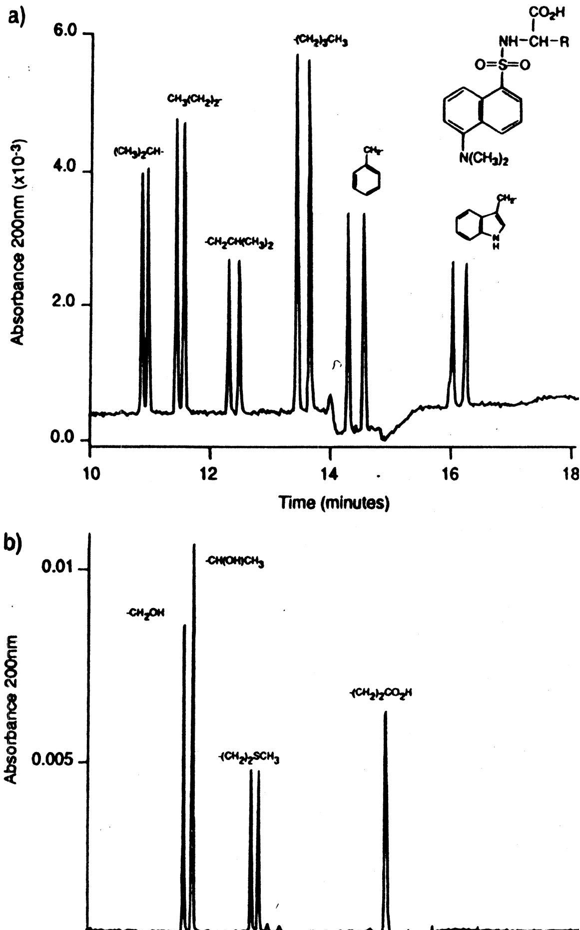

Fig. 9. Electropherograms of racemic mixture of Dns-amino acids using a novel chiral surfactant as the micelles. Conditions: 30 m*M* phosphate–10 m*M* borate (pH 8.0), 45 m*M* dodecyl β-D-glucopyranoside monophosphate. Capillary, 57 cm (50 cm to detector)×50 μm I.D.; 20 kV; temperature, 25°C. (From Ref. [136], with permission.)

recent review articles [20,143]. Only a few polymers have been employed for the enantioseparation of amino acids. Wang and Warner first reported the use of poly(sodium *N*-undecylenyl-L-valinate) ([Poly(L-SUV)] for enantioseparation [144]. By comparing the separation on polymerized surfactant and on non-polymerized micelles for the separation of (±)-1,1′ bi-2-naphthol, they showed that the polymerized surfactant gave better chiral separation. Moreover, the authors demonstrated that the combination of polymeric chiral micelles and γ-CD resulted in far better selectivity than when using either of Poly(D-SUV) or γ-CD alone [145]. Since surfactant monomers do not exist in the covalently bonded polymeric micelles, the interference of surfactant monomers on the enantioselectivity of γ-CD is virtually eliminated. This mixed approach has been shown to be a promising method for resolution of mixtures of enantiomers. In two recent publications, polymeric dipeptide chiral surfactants were used as selectors [146,147]. Unfortunately, the separation of amino acid mixtures was not reported by Warner and coworkers. When applying the Poly(L-SUV), Dobashi et al. demonstrated the resolution of amino acids derivatized with (3,5-dintrobenzoyl) amino acid isopropyl esters (DNB-amino acid isopropyl esters) [148]. Baseline separation of 4 DNB-amino acid isopropyl esters was shown using this polymerized surfactant, which produced higher selectivity than the non-polymerized surfactant SDVal. In addition, Sun and coworkers applied dextran polymers for the separation of 7 Dns-amino acids [149]. They showed that the use of dextran in combination with β-CD resulted in improved selectivity as compared to that of the dextran alone. Quang and Khaledi reported the separation of 6 Dns-amino acids using dextrin as the selector [103]. A CM-β-CD polymer has also been employed for separation of 4 amino acids [78]. In a study of enantioseparation of several basic drug racemates and tryptophan esters using a neutral β-CD polymer [150], it was observed that the β-CD polymer gave a higher enantioselectivity than the parent β-CD.

The chiral polymerized surfactant has shown chromatographic resolution behavior similar or superior to that of chiral micelles in MEKC, indicating that chiral recognition is possible independent of the dynamic association–dissociation equilibrium of surfactants in the bulk water phase. The benefit of using chiral surfactant polymers is that the effect of micellar polydispersion can be minimized as compared to conventional MEKC separation where the micellar polydispersion is considered as a major factor causing band broadening. Further, the critical micellar concentration (CMC) of polymerized surfactant is essentially zero; consequently, high organic

modifier concentrations can be applied in the separation buffer.

3.5. Macrocyclic antibiotics as chiral selectors

Macrocyclic antibiotics as a new class of chiral selectors have been utilized for enantioseparation of amino acids recently. This class includes vancomycin, ristocetin A, teicoplanin etc. Vancomycin, one of the most frequently used selectors, was first used as chiral stationary phase in HPLC and chiral mobile additive in TLC by Armstrong et al. [151,152], and it was then introduced in CE as a chiral additive [153]. The vancomycin has a characteristic basket shape with three internal macrocyclic rings (cavities) and two side chains. It possesses 18 asymmetric chiral centers and several functional groups such as carboxyl, hydroxyl, amino and amido groups and aromatic rings, which offer abundant chiral environments for π–π interactions, hydrogen bonding, and peptide and carbohydrate bonding [154]. The zero mobility (p*I* value) of the vancomycin was about 7.2 in a 100 m*M* sodium phosphate buffer [153], 7.5 in 20 m*M* sodium phosphate buffer [155], or 7.8 in a 15 m*M* univalent buffer [31]. In fact, the p*I* depends on the buffer composition [156]. Armstrong et al. demonstrated the wide applicabilities of vancomycin as selector for chiral resolution of over 100 racemates including many drugs and a large number of amino acids [153]. High resolution of different types of *N*-derivatized amino acids was achieved using pHs from 4.9 to 7.0. These authors also investigated another antibiotic, ristocetin A, as the selector [157], and a similar enantioselectivity was observed as compared to the vancomycin. Ristocetin A has a similar structure as the vancomycin and a slightly higher p*I* value (7.5) than the vancomycin.

A limiting factor of using macrocyclic antibiotics concerns its adsorption at the capillary surface, which has resulted in relatively lower separation efficiencies and irreproducible migration times. Several strategies have been made to minimize the adsorption effect. By adding SDS to the vancomycin buffer, Rundlett and Armstrong improved the separation efficiency by more than one order of magnitude; however, the selectivity was dramatically decreased [158]. Vespalec et al. utilized a zwitterionic biological buffer, 20 m*M* MOPS–Tris at pH 7.0, for resolution of some selenium- and sulfur-containing AQC-amino acids [31]. This buffer was supposed to have positive charge preventing the adsorption of vancomycin and higher separation efficiency ($2.5 \cdot 10^5$ theoretical plates/m) was obtained. The authors showed that the chemical composition and concentration of the buffer had a significant impact on the mobility difference for two enantiomers, i.e., on the vancomycin–analyte interaction. Further, by using a polyacrylamide coated column under the same conditions, fast and highly efficient enantioseparation of AQC-amino acids was achieved with a reversal of EMO [159]. Conversely, we have employed a pH (7.5) just above the zero mobility of vancomycin in combination with an organic modifier to minimize adsorption. Accordingly, high separation efficiencies, $2–5 \cdot 10^5$ theoretical plates/m, were achieved for the resolution of 20 FMOC-amino acids and 15 peptides [156,160]. It was also noted that higher efficiency was obtained for the second eluting peak (D-form) than for the first peak (L-form); for example, a 10 times difference in the number of theoretical plates/m was observed between D- and L-enantiomers for some amino acids and peptides [156,160]. More recently, Kang et al. demonstrated a significant improvement of separation efficiency ($3.2 \cdot 10^5$/m) by adding hexadimethrine bromide (HDB) to the background electrolyte to reduce the adsorption of the vancomycin; baseline separation of 12 individual FMOC-amino acids was achieved in less than 4.5 min [161]. In addition, Ward et al. reported an approach to reverse the migration of the vancomycin towards the injection side of the separation capillary using a coated capillary, thus avoiding the relatively high UV absorbance background from the vancomycin [162]. The relatively high UV absorbance was also considered as one of the drawbacks when using this type of selector for enantiomeric separation. These authors also reported chiral resolution of several Dns-amino acids using rifamycin SV as the selector by means of the indirect UV detection approach [163].

With respect to vancomycin and ristocetin A, teicoplanin has somewhat similar but unique structure containing mixed analogues. Its side hydrophobic chain enables the formation of micellar aggregates with a CMC of 0.18 m*M* [164]. As a

consequence of its molecular structure, the teicoplanin shows a slightly lower solubility in aqueous buffers than the other macrocyclic antibiotics. Further, the teicoplanin holds a p*I* value around 3.8 [164]. Rundlett et al. separated a wide range of acidic compounds including *N*-derivatized amino acids at pH 6. We achieved resolution of 15 FMOC-peptides using an optimized condition consisting of 25 m*M* phosphate–Tris, 1 m*M* tecoplanin and 40% ACN at pH 6.25 [165]. Furthermore, we noted that the teicoplanin had specific bindings only to the D-form peptides. Fig. 10 shows the enantioselective interaction of the teicoplanin between D- and L-peptides. In studies of the affinity of the vancomycin to peptides, it was observed that the interaction of the vancomycin with D-enantiomers was much stronger than with L-enantiomers [155,166,167].

Chiral recognition of macrocyclic antibiotics has not been well understood so far. High enantioselectivity generated from this type of selector is probably the consequence of multiple interactions. Gasper et al. have made a comparison and modeling study of vancomycin, ristocetin A, and teicoplanin for CE enantioseparations of racemates including different types of amino acid derivatives [168]. The authors showed that the similar but not identical structures of the three glycopeptides produce similar but not identical enantioselectivity. We compared enantioselectivity on vancomycin for two pairs of enantiomers having similar structures and found that no chiral resolution was obtained for FLEC-Gly which most likely lacks a chiral hydrogen bonding, while high enantioselectivity was achieved for FMOC-Ala ($R_s = 8.85$) under identical conditions [169]. When resolving another, but similar type of derivatives, AEOC and APOC, the use of vancomycin resulted in a resolution of 12.8 for AEOC-Ala, whereas only a partial separation of APOC-Gly was observed. These

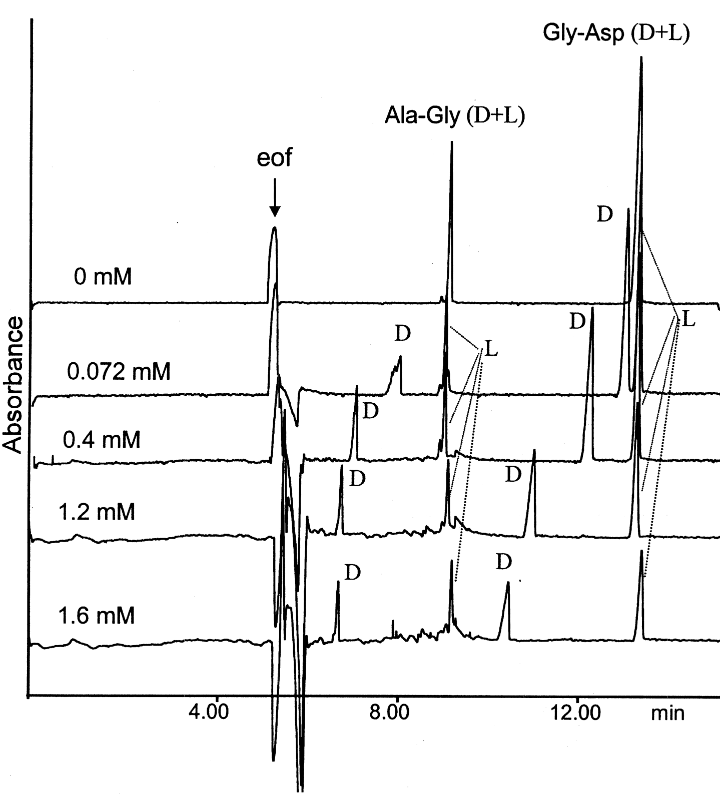

Fig. 10. Enantioselective interaction between teicoplanin and D- and L-peptides. Conditions: 25 m*M* phosphate–Tris (pH 6.25), 40% (v/v) ACN, 0~1.6 m*M* teicoplanin. Capillary, 62.5 cm (41.5 cm to detector)×25 μm I.D.; 25 kV; current, 17 μA; temperature, 25°C. (From Ref. [165], with permission.)

results suggest that a chiral hydrogen bonding may play an important role in chiral recognition. Moreover, we compared enantioselectivity for different amino acid derivatives and found that the enantioselectivity was, in general, in the order Dns>AEOC>FMOC, which is opposite to their hydrophobicity [160]. However, for peptide derivatives, the AEOC and FMOC derivatives facilitated chiral recognition. Fig. 11 illustrates the effect of different derivatives on the separation of a dipeptide having two chiral centers. In addition, a large difference in migration time for Asp and Glu was obtained as compared to the other amino acid-derivatives [156]. A similar behavior was also observed for the peptide Gly–Asp. Evidently, besides the chiral hydrogen bonding, hydrophobic and steric interaction, the electrostatic interaction between the vancomycin and amino acid-derivatives could contribute to chiral recognition.

Besides the vancomycin, ristocetin A, rifamycin SV and teicoplanin, some other macrocyclic antibiotics, kanamycin, fradiomycin [170], MDL 63246 (Hepta-Tyr) antibiotics [171,172], two vancomycin analogues [154,173], rifamycin B and rifamycin SV [163], have been used as chiral selectors for resolution of drug racemates, but few applications to amino acids have been reported [163]. Recently, enantiomeric separations of Dns-amino acids using

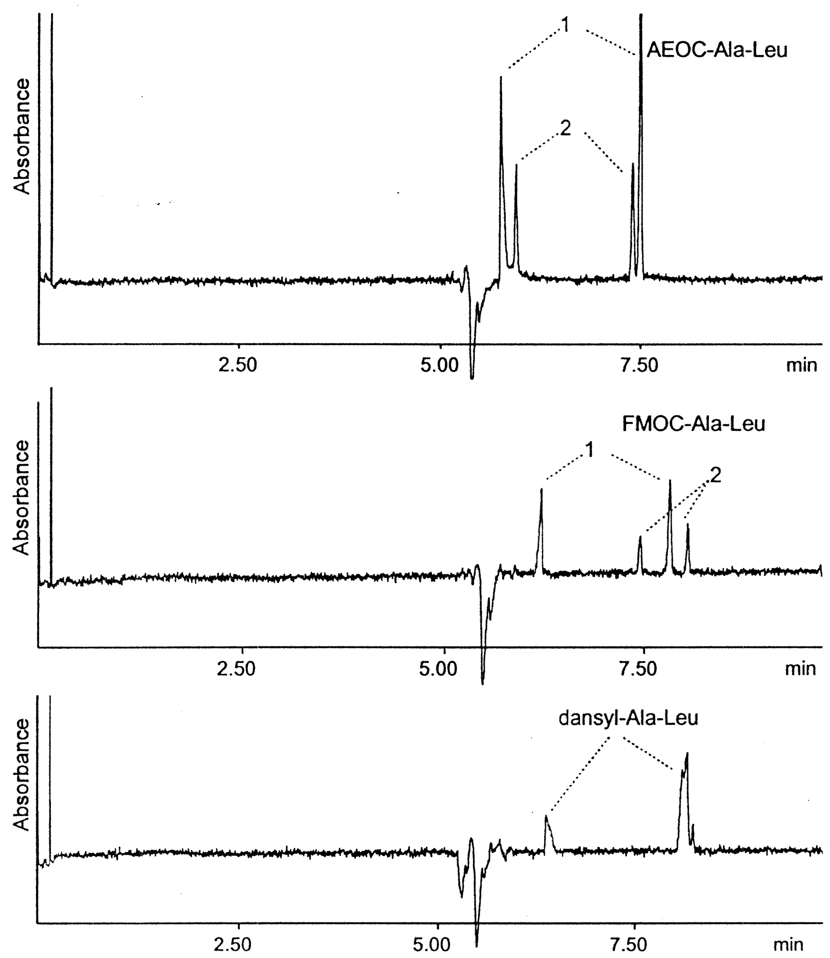

Fig. 11. Comparison of chiral separation of a two chiral center dipeptide, Ala–Leu, using different derivatization. Conditions: 50 m*M* phosphate (pH 7.5), 1 m*M* vancomycin. Capillary, 67 cm (45 cm to detector)×25 μm I.D.; 25 kV; current, 20 μA; temperature, 25°C. (From Ref. [160], with permission.)

some new types of macrocyclic antibiotics as chiral selectors were reported [174,175].

Compared to other types of selectors, the macrocyclic antibiotics exhibited remarkably high enantioselectivity towards amino acids and peptides. However, some drawbacks such as high UV background absorbance, adsorption at the capillary surface and instability have to be considered. The adsorption of analytes at the capillary wall can be reduced and the UV absorbance from the selectors can be avoided when using a coated capillary and applying a countercurrent process (partial filling technique) [176,177]. Using this technique, the EMO for some enantiomers can be reversed. For the applications of antibiotics as the selectors for resolution of other enantiomers, we refer to recent reviews presented by Ward and Oswald [178] and by Desiderio and Fanali [179].

3.6. Non-aqueous buffer systems

Non-aqueous capillary electrophoresis (NACE) is an alternative approach to achieve separation where organic solvents are used as BGE instead of the commonly used aqueous buffers. The NACE has advantages in several respects, such as increasing the solubility of analyte and chiral selector, altering selectivity and straightforward compatibility with mass spectrometric detection (MS). Further, it has been noted that the use of NACE media can considerably increase the stability for those compounds that are unstable under aqueous conditions [180]. Therefore, this approach has received increased attention recently. Two overviews concerning different NACE media and related applications have been presented by Tomlinson et al. [181] and Valkó et al. [182] and some chiral NACE using different organic solvents and chiral selectors for enantioseparation of amino acids have been reported [183–185]. Valkó et al. compared the chiral resolution of Dns-amino acids with two NACE media, formamide and *N*-methylformamide (NMF) using β-CD as the selector [183]. They obtained higher selectivity with the formamide but the efficiency was relatively poor, and vice versa with NMF. Chiral baseline resolution of 12 Dns-amino acids was achieved and separation efficiency over $5 \cdot 10^5$ theoretical plates/m was obtained in the NMF media. Further, they demonstrated the possibility to enhance the enantioselectivity by the increase of the β-CD concentration in the NACE [184]. The use of NMF permits the solubility of high β-CD concentrations, up to 200 m*M*, which led to an increased selectivity. Moreover, these authors determined the association constants of Dns-amino acids and β-CD in NMF and observed that the association constant values in the NACE were lower than that obtained in aqueous buffers, e.g., a phosphate buffer, thus indicating a weak host–guest interaction between Dns-amino acids and β-CD in NACE media [185]. This result implies that the reduction of binding constants in organic solvent could provide an alternative separation selectivity for those compounds that strongly bind to a chiral selector in aqueous buffers. Stalcup and Gahm reported chiral separation of acidic compounds and *N*-3,5-dinitrobenzoylated amino acids using quinine as a chiral selector with methanol containing ammonium acetate and acetic acid as the solvents [186]. The separation was based on ion pairing and π–π interaction. Piette et al. also used quinine or *tert*.-butylcarbamoylated quinine as selector for *N*-protected amino acids [187]. Mori et al. demonstrated chiral resolution of some primary amines and 3 amino acids employing crown ether as a selector in NACE using formamide as solvent [188]. They found that the addition of tetra-*n*-butyl ammonium perchlorate (TBAP) led to improved separation efficiency. More recently, Wang and Khaledi evaluated chiral separation using different solvents as NACE media with quarternary ammonium β-CD (QA-β-CD) as a chiral selector [189]. They showed that the use of formamide resulted in better chiral recognition for Dns-amino acids than that of the other examined solvents, NMF, methanol and dimethyl sulfoxide. Chiral baseline resolution of 11 Dns-amino acids was achieved with formamide containing 20 m*M* ammonium acetate, 1% acetic acid and 20 m*M* QA-β-CD. However, lower selectivity was observed for FMOC-amino acids as compared to the Dns-amino acids under the same conditions.

The chiral NACE approach has extended the scope of chiral CE and the possibilities to alter selectivity and coupling to MS have been shown. This technique is, in particular, useful for those compounds

that exhibit limited solubility and suffer from the instability in aqueous media.

3.7. Chiral selector modified capillary gel electrophoresis

Since Guttman et al. demonstrated chiral separation of Dns-amino acids by incorporation of CDs into polyacrylamide gel filled columns [62], only a few papers dealing with chiral amino acid separation using gel filled CE have been reported [190–194]. Cruzado and Vigh showed the chiral resolution of Dns-amino acids by means of allylcarbamoylated β-CD and acrylamide mixed gel [190]. Birnbaum and Nilsson separated tryptophan with high enantioselectivity on a bovine serum albumin (BSA) gel filled column [191]. Barker et al. also achieved good separation of tryptophan enantiomers with a BSA-glutaraldehyde gel [192]. Lin et al. have shown chiral resolution of 13 Dns-amino acids by the incorporation of a crown ether with β-CD in capillary agarose gel electrophoresis [193]. They also developed an approach using molecular imprinted polymer as chiral selector in a gel filled column for enantiomeric separation of some amino acids [194]. Using this approach, the selectivity can be predictable, i.e., the chiral recognition of compounds having similar structures is highly specific, thus chemical structures that do not fit can not be distinguished at all. This is a good example applying gel filled CE for chiral separation; otherwise, this technique, owing to inflexibility, has not received wide attention.

3.8. Chiral capillary electrochromatography

Capillary electrochromatography (CEC) is an emerging new separation technique, and its present status of technology and applications can be found in review articles [195–198]. Some applications of chiral amino acid separation have been reported [199–203]. Li and Lloyd evaluated the use of β-CD chiral stationary phase to resolve some Dns- and DNP-amino acids [199]. However, separation efficiency was lower than that obtained in free solution CE as reported in many cases. More recently, Lämmerhofer and Lindner reported the chiral CEC enantioseparation of DNZ-amino acids and FMOC-amino acid employing a capillary column packed with a weak anion-exchange type chiral stationary phase based on a quinine-derived chiral selector [200]. Impressingly high enantioselectivity, about 60 min between two enantiomers, was obtained at the expense of very long retention times. These authors also compared the results from CEC and HPLC and showed that the enantioselectivity values from CEC were as high as in HPLC and efficiency was typically a factor of 2–3 higher in CEC than in HPLC for a given column. More recently, they have shown rapid high efficiency enantioseparation of *N*-derived amino acids by means of packed CEC using ODS silica and the quinine-derived selector as ion-pairing agent [201]. The authors elucidated two separation modes, i.e., electrophoretic elution mode and chromatographic elution mode. In the electrophoretic elution mode, the ion-pairing agent was added to the BGE at a relatively high concentration (>10 m*M*), which resulted in high separation efficiency, e.g., $1.7 \cdot 10^5$/m, but the selectivity was moderate. In contrast, in the chromatographic elution mode, the chiral ion-pairing agent itself served as BGE at a low concentration (<10 m*M*) and EOF dominated transport, lower separation efficiency ($\sim 10^4$/m) and higher enantioselectivity were obtained.

Carter-Finch and Smith recently demonstrated rapid chiral CEC separation of DBZ-derivatized leucine and tryptophan by means of covalently bonded teicoplanin on 5 μm silica particles packed in a 100 μm I.D. capillary. The authors showed excellent repeatabilities of migration times and resolutions as well as good reproducibility of packing procedures. However, the separation efficiencies were relatively low, in the range of about $3 \cdot 10^4$/m [202]. In addition, Hofstetter et al. have shown chiral separation of 4 DNP-amino acids with resolution ranging from 1.5 to 12.8 using BSA as chiral stationary phase in affinity open tubular electrochromatography [203]. The authors stated that coatings were stable for up to one year if stored at 4°C and the method was considered more convenient than the previously applied addition of BSA to the buffer.

With increasing maturity of CEC column technology, more chiral CEC applications can be expected.

This technique might be advantageous in coupling to MS and finding conditions for HPLC.

3.9. Other chiral additives

Several types of natural proteins such as BSA [204–205], α-acidic glycoprotein (AGP) [95], ovomucoid [206], cellulase [207] and human serum albumin (HSA) [208] have been employed as buffer additives for chiral separation of various enantiomers in CE. However, only a few applications for separations of amino acids have been reported. Yang and Hage applied HSA as a buffer additive for resolution of tryptophan [208]. Wistuba et al. directly resolved 6 DNP-amino acids with four types of proteins as chiral additives [95]. Comparing BSA, AGP, ovomucoid and casein, the BSA was found to be the most effective selector for DNP-amino acids.

Proteins as chiral additives in CE, owing to simple handling and low cost, may be a practical alternative as compared to the immobilized protein based phases in HPLC for enantioseparation. However, some drawbacks should be mentioned. Since proteins have UV absorbance, only a low concentration of proteins can be applied in the BGE. Further, the separation efficiency when using proteins as chiral selectors was much lower than that using CDs as additives. This is largely due to the adsorption of proteins at the capillary wall; consequently, some other modifiers and coated columns are necessary to obtain the desired efficiency. A review regarding the use of proteins as selectors in CE has been presented recently by Lloyd et al. [209].

In addition, some other types of chiral additives have been examined for separation of amino acids. Ingelse et al. utilized an ergot alkaloid, 1-allyl derivative of (5*R*,8*S*,10*R*)-terguride (allyl-TER) as chiral selector for resolution of racemic hydroxy organic acids including *N*-formylphenylalanine and *N*-acetylphenylalanine [210]. Since the solubility of allyl-TER is limited in 100% water, the separation was performed in a BGE containing 50% MeOH and 62.5 m*M* allyl-TER at pH 5.5. Stalcup and Agyei employed heparin, a naturally occurring polyanionic glycosaminoglycan, as the selector for resolution of antihistamines and tryptophan [211]. Chiral separation of tryptophan methyl ester was achieved, but no enantioselectivity was obtained for tryptophan without esterification.

4. Indirect (diastereomeric) separation

Indirect separation is an approach to achieve chiral analysis without the presence of a chiral selector in the background electrolyte. This approach involves derivatization of the enantiomers with an optically pure reagent prior to analysis and the following separation of the diastereomers in an achiral environment by means of interaction with a pseudo-stationary phase. Thus, diastereomers differing in their electrophoretic or hydrophobic properties may be separated in an achiral separation system. This method is typically suitable for enantiomers, like amino acids, which contain an amino group connected to an asymmetric carbon and which can be easily derivatized with a suitable chiral reagent. Furthermore, the derivatization of the amino acids is oftentimes required to improve the limit of detection. Therefore, the indirect method becomes attractive since it may serve two purposes: the enhancement of selectivity and sensitivity.

4.1. Chiral reagents for diastereomer formation

To date, a variety of chiral reagents have been employed for the formation of diastereomers of amino acids and peptides for subsequent separation by means of CE [40,82,212–227,229,230]. These chiral reagents and their general properties are summarized in Table 4. The formation of amino acid diastereomers using a chiral reagent may proceed as follows.

$$\text{(D,L)R}-\overset{\text{NH}_2}{\underset{*}{\text{CH}}}-\text{COOH} \quad + \quad \text{(+)-CR'} \longrightarrow$$

(D,L – Amino acids) (Chiral reagent)

$$\text{((+)-D)R}-\overset{\text{NH-CR'}}{\underset{*}{\text{CH}}}-\text{COOH} \quad + \quad \text{((+)-L)R}-\overset{\text{NH-CR'}}{\underset{*}{\text{CH}}}-\text{COOH}$$

(Mixture of diastereomers)

Table 4
Chiral reagents used for diastereomer formation and their properties

Chiral reagents	Abbreviation	Structure	Optical purity	Reaction conditions	λ_{max} (UV) $\lambda_{ex}/\lambda_{em}$ (nm)	Ref.
D or L-1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide	Marfey's reagent		NS	35°C/90 min 60°C/15 min	340	[212]
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosylisothiocyanate	GITC		NS	RT/15 min	210	[213]
o-Phthalaldehyde (OPA) and *N*-*tert*.-butoxycarbonyl-L-cysteine	OPA + BocC		NS	RT/10 min	230 340	[214]
(+)-Diacetyl-L-tartaric anhydride	DAT	DAT: R=COCH3	NS	50°C/20 h	233	[222]
(+)-Dibenzoyl-L-tartaric anhydride	DBT	DBT: R=COPh	>99.5%	50°C/20 h	233	[223–225]
(+) or (−)-1-(9-Fluorenyl)ethyl chloroformate	FLEC		>99.9%	RT/5 min	265/310[a]	[40,82, 219,229]
			>99.99%		244/330[a]	[230]
OPA and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose	OPA + TATG		NS	RT/6 min	340 350/415[a]	[216]
R-(−) or *S*(+)-4-(3-Isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole	NBD-PyNCS		99.5%	60°C/10 min	488/520[a]	[221]

Table 4 (*continued*)

Chiral reagents	Abbreviation	Structure	Optical purity	Reaction conditions	λ_{max} (UV) $\lambda_{ex}/\lambda_{em}$ (nm)	Ref.
(+) or (−)-1-(9-Anthryl)-2-propyl chloroformate	APOC		99.91%	RT/10 min	256 351/412[a]	[220]
((1*R*,2*R*) or (1*S*,2*S*)-*N*-[(2-Isothiocyanate)cyclohexyl]-6-methoxy-4-quinolinylamide	CDITC		>99.9%	60°C/120 min	254 333/430[a]	[226]
(*S*)-1-(1-Naphthyl)-ethyl isothiocyanate	SNEIT		NS	60°C/30 min	210	[227]
(*S*)-(+)-α-Methylbenzyl isothiocyanate	SAMBI		NS	60°C/30 min	254	[227]

NS: Not stated; RT: Room temperature; [a] Fluorescence detection possible.

As mentioned above, Section 2, several essential requirements should be met when derivatizing amino acids. These requirements basically include the presence of a strong chromophore or fluorophore in the reagent, simple reaction conditions and stable derivatives. An additional requirement, as compared to the non-chiral derivatizing reagents, is that the chiral reagent applied should be of high optical purity. This is especially important when aiming at the determination of trace enantiomeric impurity. For instance, if a level of 0.5% enantiomeric impurity exists in the sample, the optical purity of the chiral reagent should be better than 99.5%. Unfortunately, the optical purity of chiral reagents has not been specified in many applications. On the other hand, if the optical purity of the reagent is known, the impurity of analyte can be corrected according to the method proposed by Engström et al. [228], where the enantiomeric purity of the analyte was given by:

$$Y = \frac{1}{2}\left[\frac{M_A - I_A}{(2\chi - 1)(M_A + I_A)} + 1\right] \quad (1)$$

where Y is defined as the molar fraction of one enantiomer $[Y = R/(R+S)]$ of the analyte, and χ is the optical purity of the reagent (defined in the same way as the molar fraction of one form of the reagent $\chi = [R/(R+S)]$; M_A and I_A the peak areas (i.e., corrected peak area) of the main and impurity peak of the analyte, respectively. Thus, the impurity of interest can be calculated as:

$$Y_I = (1 - Y) = \frac{1}{2}\left[1 - \frac{M_A - I_A}{(2\chi - 1)(M_A + I_A)}\right] \quad (2)$$

where Y_I is the impurity of the analyte to be investigated. In the case where $\chi = 1$ (100% optical purity of the reagent), Eq. (1) is written as:

$$Y_I = (1 - Y) = \frac{1}{2}\left[1 - \frac{M_A - I_A}{(M_A + I_A)}\right] = \frac{I_A}{M_A} \quad (3)$$

4.2. Micelle based pseudo-stationary phases

On the basis of partitioning of the diastereomers between aqueous and micellar phases, a number of

amino acids and peptides has been resolved using SDS micelles in MEKC. Tran et al. utilized Marfey's reagent for diastereomeric separation of 7 amino acids and 2 peptides using 200 m*M* SDS [212]. Upon derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), Nishi et al. were able to separate 17 D,L-amino acids [213]; this reagent has been widely used in reversed-phase HPLC for chiral separation of amine and amino compounds. Kang and Buck derivatized amino acids with *o*-phthalaldehyde (OPA) and *N*-*tert*.-butoxycarbonyl-L-cysteine (BocC) [214]. Of 17 examined amino acids, 13 were resolved and the separation of a mixture of 6 amino acids was shown in a run using 150 m*M* SDS. Similarly, Houben et al. prepared diastereomers by derivatization of valine with OPA and *N*-acetylcysteine for the determination of D-valine in an excess of L-valine [215]. With OPA and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (TATG), a chiral reagent similar to GITC, Tivesten and Folestad demonstrated simultaneous rapid separation of 17 amino acids in less than 5 min with only a few peaks co-eluting [216]. They accomplished high resolution (average $R_s = 14.3$) of most of the TATG-amino acids at pH 9.55 using 45 m*M* SDS and 4% ACN as the modifier. Further, Tivesten et al. demonstrated on-column chiral derivatization of D- and L-amino acids using OPA–TATG chiral reagent [217,218]. The amino acids were derivatized within a few seconds by zone mixing and subsequently separated in a single step in MEKC, Fig. 12. This method is considered to be highly useful for microchemical analytical applications, i.e., labeling of

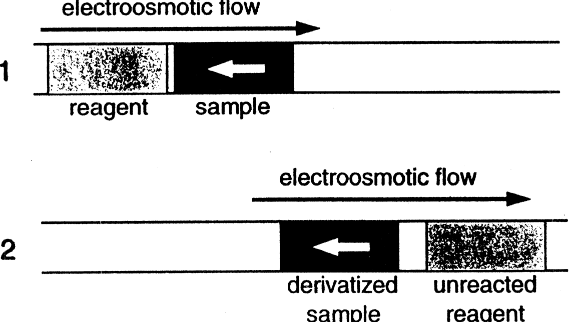

Fig. 12. Schematic representation of the on-column derivatization procedure. (From Ref. [218], with permission.)

nano- to picoliter samples since the dilution of the original sample is minimized as compared to pre-column derivatization [217,218]. In our opinion, this approach may be applicable to the derivatization of sub-μ*M* concentrations of amino acids in complex matrices; this is because two advantages can be anticipated. First, the effect of the matrix can be minimized due to different mobilities of amino acids as compared to other substances in the matrix. Second, electrostacking results in an enrichment of amino acids. This can be attractive for fully automated analysis of trace amino acids on commercial instruments. However, the technique is limited to a few reagents such as OPA and NDA that are able to react rapidly enough to produce quantitative yields. Development of new reagents for on-column derivatization and high sensitive detection of amino acids is important in the future application of automated analysis.

Wan et al. applied (+)- or (−)-1-(9-fluorenyl)ethyl chloroformate (FLEC) for the formation of diastereomers and resolved a large number of D,L-amino acids and di- and tripeptides [40,82]. The MEKC separation was performed at pH 9.2 using a low SDS concentration (10–20 m*M*). An example showing highly efficient separation of 10 FLEC-amino acids is shown in Fig. 13. The diastereomeric separation of 10 FLEC-amino acids was reported by Chan et al. [219]. The resolution of 3 FLEC-amino acids, Asp, Glu and Pro was also achieved under an acidic buffer condition. Thorsén et al. have recently shown separation of 17 amino acids and 4 dipeptides after derivatization with a new fluorescent chiral reagent, (+) or (−)-1-(9-anthryl)-2-propyl chloroformate (APOC) [220]. This reagent displayed similar chemical structure as FLEC; accordingly, similar separation conditions as for FLEC diastereomers were applied for the APOC diastereomers in MEKC. An advantage of the APOC is that this reagent can offer high UV molar absorptivity and 10 times higher UV absorbance was observed for the APOC derivative than for FLEC derivatives [220]. Fig. 14 illustrates the diastereomeric separation of a mixture of APOC-amino acids under a simple MEKC condition.

More recently, Liu et al. separated 6 amino acids and 1 dipeptide derivatized with R-(−) or S-(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-PyNCS) by means of a non-ionic

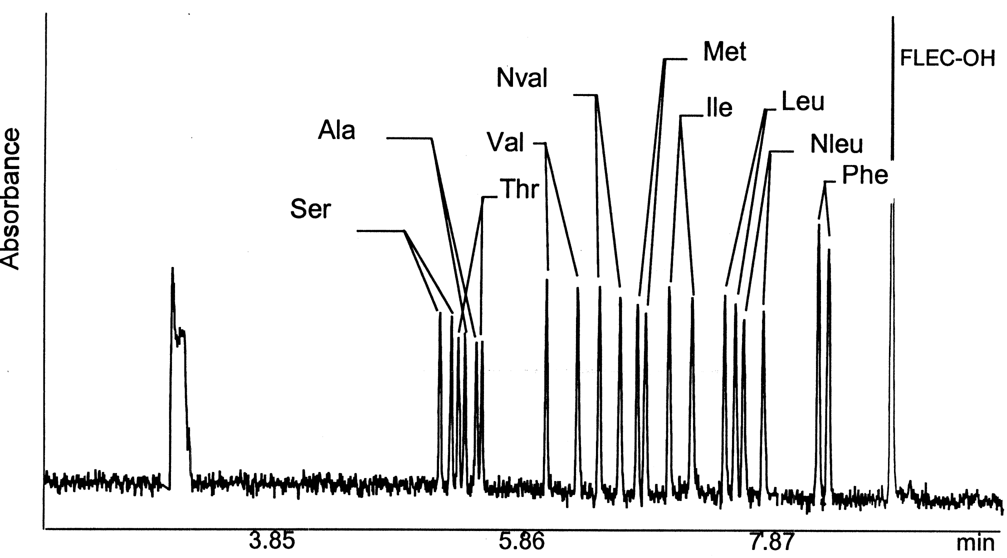

Fig. 13. Chromatogram of 10 (−)-FLEC-amino acids by MEKC. Conditions: 20 m*M* borate–15 m*M* phosphate (pH 9.20), 20 m*M* SDS. Capillary, 62.5 cm (45 cm to detector)×25 μm I.D.; 25 kV; 9.0 μA; temperature, 25°C. (From Ref. [40], with permission.)

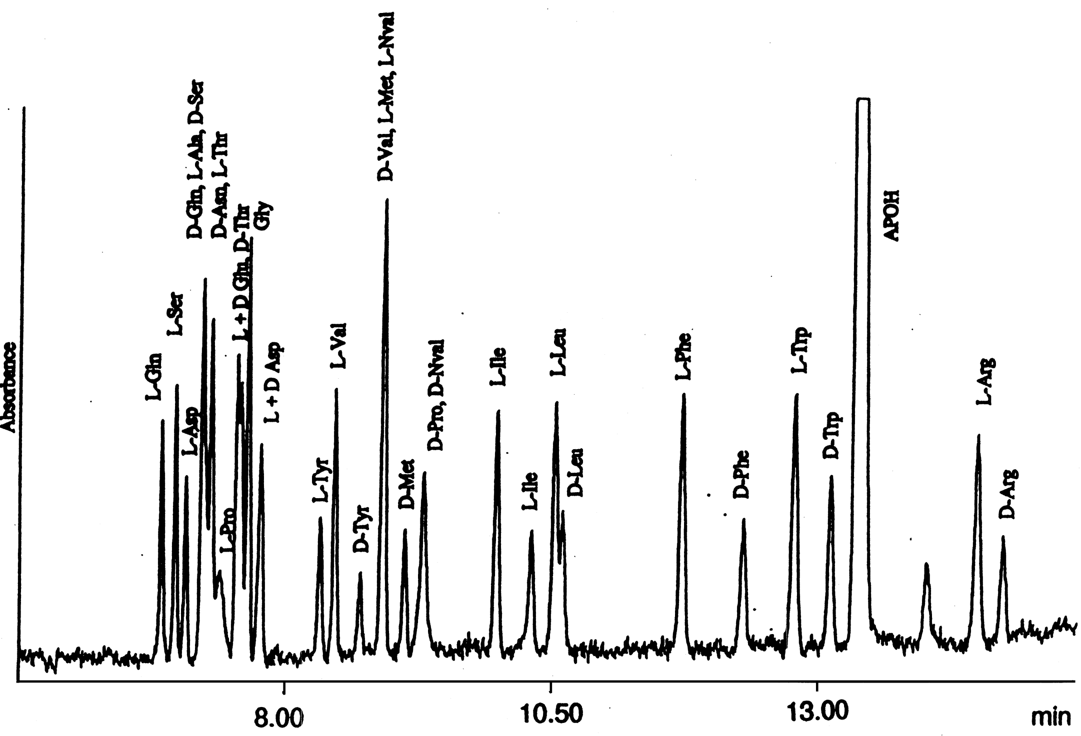

Fig. 14. Diastereomeric separation of a mixture of APOC-amino acids by MEKC. Conditions: 28.75 m*M* borate (pH 9.95), 20 m*M* SDS. Capillary, 70 cm (57 cm to detector)×50 μm I.D.; 30 kV. (From Ref. [220], with permission.)

surfactant at a low pH [221]. This benzofurazan fluorescence label reagent has excellent spectroscopic properties and a LOD of 50 n*M* was reported using an Ar-ion laser at 488 nm. However, this reagent (99.5% purity) is not optically pure enough for the application to trace impurity determinations. Moreover, heating was required for the reaction, which may cause racemization [36]. Note that heating was needed also for Marfey's reagent [212], NBD-PyNCS [221], DBT [223,224] and CDITC [226].

4.3. Polymer-mediated pseudo-stationary phases

Schützner et al. showed the separation of one amino acid, tryptophan, derivatized with (+)-diacetyl-L-tartaric anhydride (DAT) by hydrophobic or dipole interaction with polyvinyl pyrrolidone added to the BGE as a polymeric pseudo-stationary phase [222]. Later when derivatizing amino acids with (+)-dibenzoyl-L-tartaric anhydride (DBT) [223–225], Schützner et al. found that the use of DBT derivatives led to improved selectivity. Three types of linear polymers, poly(vinylpyrrolidine) (PVP), poly(ethylene glycol) (PEG) or poly(acrylamide) (PPA), were examined in their study and the best selectivity was observed with the PVP. They attributed this to an enhanced π–π interaction between PVP and DBT derivatives. More recently, Kleidernigg and Lindner achieved the separation of 19 amino acids when using another fluorescent derivatization reagent, {(1*R*,2*R*)- or (1*S*,2*S*)-*N*-[(2--isothiocyanate)cyclohexyl] - 6 - methoxy - 4 - quinolinylamide} (CDITC) [226]. The diastereomeric separation was performed at a low pH (3.0) using PVP in combination with an organic modifier and a resolution as high as 16 was obtained for Tyr. In Fig. 15 is shown diastereomeric separation of 6 (*R*,*S*) CDITC-amino acids by a polymeric pseudo-stationary phase. An obvious advantage of using a non-ionic polymer instead of SDS as pseudo-stationary phase is that less Joule heat is formed during the separation; however, the separation window may be relatively narrow as compared to conventional MEKC. Nevertheless, the polymeric phase provided an alternative approach for diastereomeric separation of amino acids and peptides. This approach may also be applicable to diastereomers formed by reaction with other types of chiral reagents such as FLEC, APOC etc, in which abundant π–π or hydrophobic interactions between the moieties are possible.

4.4. Selectivity based on hydrophobic interaction

Table 5 summarizes separation conditions and amino acids resolved after derivatization with differ-

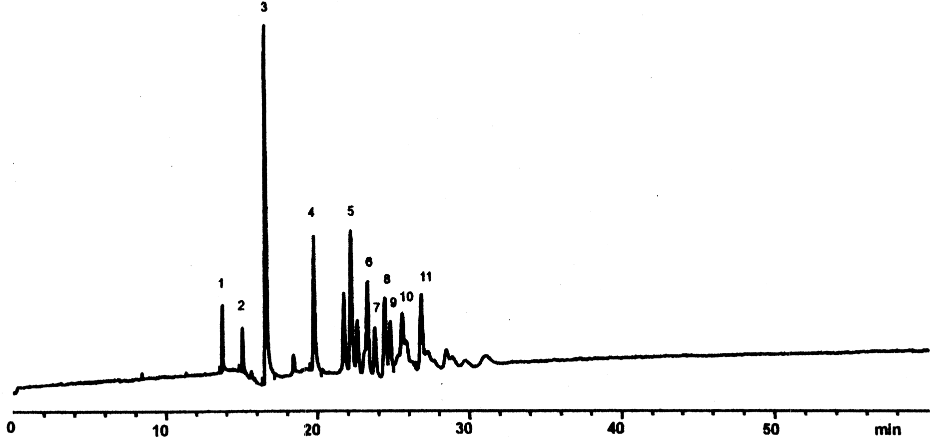

Fig. 15. Diastereomeric separation of 6 (*R*,*S*) CDITC-amino acids by polymer-mediated pseudo-stationary phase. Conditions: 20 m*M* citrate (apparent pH 3.2), 0.5% (w/v) PVP, 9% 2-propanol, 1% TBME. Capillary, 62 cm (54 cm to detector)×75 μm I.D.; 30 kV. UV 254 nm; temperature, 20°C. Peak identification: 1=(*R*)-His, 2=(*S*)-His, 3=(*R*,*R*)-CDITC, 4=(*R*)-Pro, 5=(*S*)-Pro, 6=(*R*)-Phe, 7=(*S*)-Leu, 8=(*R*)-Val, 9=(*R*)-Met, 10=(*S*)-Leu, 11=(*S*)-Phe. (From Ref. [226], with permission.)

Table 5
Chiral reagents applied for diastereomenic separation of amino acids and peptides in CE

Chiral reagents	Amino acids separated	Separation buffer	Migration time (min)	Resolution range	Ref.
Marfey's reagent	7 AAs[a] 1 DP[b], 1 TP[c]	100 m*M* borate, pH 8.5; 100 m*M* SDS.	8~30	–	[212]
GITC	17 2 DP[b]	20 m*M* phosphate–borate, pH 9.2, 200 m*M* SDS.	10~36	–	[213]
OPA+BocC	13 AAs[a]	7.5 m*M* borate, pH 9.6, 150 m*M* SDS, 5.5% (v/v) methanol.	13~30	–	[214]
DAT	Trp	16 m*M* phosphate, pH 6.35, 6% (w/v) PVP.	12	–	[222]
DBT	5 AAs[a] 1 DP[b]	30 m*M* phosphate, pH 5.8, 2.5% (w/v) PVP.	8~16		[223] [224]
FLEC	15 AAs[a]	20 m*M* borate–15 m*M* phosphate, pH 9.2, 20 m*M* SDS, 10% ACN.	5~13	1.2~9.5	[40]
	7DP[b], 2TP[c]	40 m*M* borate, 15 m*M* SDS, 15% 2-propanol.	5~11	2.5~11	[82]
	7 AAs[a]	10 m*M* phosphate, pH 6.8 or 5 m*M* borate, pH 9.2, 25 m*M* SDS, 10–15% ACN.	10−18	–	[219]
OPA+TATG	17 AAs[a]	40 m*M* borate, pH 9.55, 45 m*M* SDS, 4% ACN.	5	0.8~24	[216]
NBD-PyNCS	6 AAs[a] 1 DP[b]	25 m*M* acetate (pH 4.0), 10 m*M* Triton X-100	10~36	–	[221]
APOC	17 AAs[a] 4 DP[b]	28.75 borate, pH 9.95, 15 m*M* SDS 10 borate–7.5 m*M* phosphate, pH 9.2, 15 m*M* SDS.	5~14	–	[220]
CDITC	19 AAs[a]	20 m*M* sodium citrate (pH 3), 0.5% (w/v) PVP, 10% 2-propanol, 1% TBME.	15~28	1.4~16	[226]
SNEIT/SAMBI	3 AAs[a]	50 m*M* borate, (pH 10), 20 m*M* β-CD or TM-β-CD.	15	0.9~3.1	[227]

[a] AAs: amino acids.
[b] DP: dipeptide.
[c] TP: tripepetide.

ent chiral reagents. As shown in Table 5, most of these separations were performed by MEKC [40,82,212–214,216,219–221]. Although Tran et al. have achieved the diastereomeric separation of some amino acids derivatized with Marfey's reagent in CZE without the presence of any pseudo-stationary phase [212], the MEKC separation obviously offered better selectivity than the CZE. This is because the additional selectivity that is provided by the hydrophobic interactions with the micelles. Another diastereomeric separation of amino acids without the use of micelles was the application of (*S*)-1-(1-naphthyl) ethyl isothiocyanate (SNEIT) or (*S*)-(+)-α-methylbenzyl isothiocyanate (SAMBI) as the chiral derivatization reagents by Bonfichi et al. [227]. Owing to the limited solubility of these derivatives in aqueous buffer, cyclodextrins were employed as additives in the buffers to improve the solubility and achieve separation. Using this technique, the separation of three amino acids was demonstrated.

Using a chemometric experimental design and optimization approach, Wan et al. obtained optimal conditions for different FLEC-amino acids and peptides [40,82]. It was observed that optimal conditions are strongly dependent on the hydrophobic properties of the analyte and the more hydrophobic the amino acids and peptides, the lower SDS concentrations are required for selectivity. For example, the early eluting Thr required a relatively high SDS concentration; conversely, the last-eluted compound, Phe, had much lower optimal SDS concentration [40]. Similar effects were also observed for dia-

stereomeric separation of a group of FLEC-peptides [82]. This indicates that it would be difficult to separate all amino acids in one run due to their wide range of hydrophobicity of the side chains. For the separation of such samples, the SDS concentration has to be adopted to the least hydrophobic analyte, first eluting peak in the system used, retention time for the most hydrophobic analyte, last eluting peak, will then become quite long.

On the other hand, the different types of chiral reagents differ in their hydrophobicity as can be seen from their structures, Table 4. As a consequence of the relatively larger polarity or lower hydrophobicity, quite high SDS concentrations were required for Marfey's reagent and GITC's diastereomers [212,213]. In contrast, the opposite results were obtained for FLEC and APOC diastereomers [40,82,219,220]. Increasing SDS concentrations lead to increasing buffer conductivity which in turn leads to increased Joule heating and decreased efficiency. Thus, as a general principle, SDS concentrations should be kept as low as possible.

In addition to the hydrophobic interaction, electrostatic interactions play an important role in the resolution of analytes such as Asp and Glu. These two amino acids were the most difficult to separate with anionic micelles such as SDS, which is a consequence of the stronger electrostatic repulsion resulting from double negative charges occurring at high pH. The use of low pHs minimized this effect and these two amino acids could thus be separated, for example, as FLEC and Marfey's derivatives [212,219]. Alternatively, high resolution of Asp and Glu was achieved with a nonionic surfactant Triton X-100 after derivatization with the chiral NBD-PyNCS [221].

4.5. Potentials and limitations of the indirect method

Comparing the overall performances of amino acids derivatized with different chiral reagents such as Marfey's reagent, GITC, OPA/TATG, APOC and FLEC etc, Table 5, the FLEC reagent possesses several advantages. First, the derivatization reaction is rapid and simple and the derivatives are sufficiently stable for analysis [40]. Second, FLEC derivatives exhibit relatively higher hydrophobic properties and consequently quite low SDS concentrations (10~20 m*M*) are required for selectivity in MEKC separation [40,82]. The use of a lower SDS concentration can reduce conductivity and also the adsorption of SDS at the capillary wall, which altogether leads to high separation efficiencies. Finally, both (+) and (−) FLEC are commercially available in highly optical purity (>99.9%) [229] and the label provides good UV absorbance and high-sensitivity fluorescence detection. A recent detailed study has confirmed that the optical purity of the FLEC is greater than 99.99% [230]. One disadvantage of FLEC might be the high background fluorescence emanating from buffer additives and contaminants from sample matrix [220,221]. This is due to its excitation wavelength (265 nm) close to UV maximum absorbance at 256 nm. Chan et al. have reported the LOD of 50 n*M* for FLEC-amino acids using a pulsed KrF laser with the excitation wavelength at 248 mm [219]. Using a frequency doubled Ar-ion laser (244 nm), Wan et al. have successfully achieved the detection of 40 n*M* FLEC-Gly in a mixed electrolyte background consisting of HEPES, SDS, γ-CD and IPA [230]; it was noted that these additives contributed to a somewhat high background fluorescence.

The APOC exhibits quite similar properties as the FLEC, but it has higher UV absorbance than the FLEC. However, the optical purity reported for this reagent, 99.91 [220], may not be enough for the direct determination of trace enantiomeric impurity lower than 0.1%. A correction has to be made to subtract the effect of enantiomeric impurity present in the chiral reagent.

The OPA with TATG has shown promising properties as chiral reagent for diastereomeric separation in terms of fast separation, high resolution and simple reaction conditions [216]. The reaction kinetics may have to be taken into consideration in reactions with OPA [231]. It is uncertain whether all of the chiral reagents when reacting with two enantiomers produce the same yield of the two diastereomers. It can be anticipated that the D- and L-form of two enantiomers with identical physical and chemical properties (only different optical rotation) give the same reaction kinetics, UV absorbance properties and fluorescence yield. However, in a study on the reaction kinetics of diastereomeric

amino acids with OPA, Meyer et al. [231] have observed that L-isoleucine reacts faster than its D-epimer whereas L-threonine reacts slightly slower than its D-epimer. Although the reaction rates of the amino acid epimers were different, their UV absorbance characteristics were found to be identical. The effect may also be present in the direct separation method when a derivatization procedure is performed. However, no other data have been published so far and further investigations are required regarding this issue.

The advantages of diastereomeric separation are that optimization of separation conditions is relatively simple and rapid and that the MEKC technique, in general, provides higher selectivity and separation efficiency than direct chiral separation where complexation with the chiral selector may result in relatively slow kinetics. As shown in Table 5, most of the protein amino acids could be resolved as diastereomers when using chiral derivatization reagents such as FLEC, APOC, OPA/TATG, GITC and CDITC. An additional advantage of indirect separation is that the elution order of diastereomers can be readily reversed by using the (+) or (−) chiral reagent (when available). Such reversal offers an unequivocal means for quantitative analysis of enantiomeric excess where the minor peak is best eluted first especially when the resolution is not large.

A prerequisite for successful application of the indirect method is that the purity of the chiral reagent applied must be ascertained or known; this is, in particular, necessary for trace enantiomeric impurity determination. Further, an inherent limitation of the indirect method is the requirement that the chiral center of the chiral reagent and that of analyte must be in rather close proximity [37,232] if separation is to be achieved in a non-chiral system. Thus, peptides beginning with glycine could not be separated when applying the indirect method [82].

5. Optimization and selectivity enhancement

5.1. Application of chemometric experimental designs and optimization to enhance selectivity

It has been shown in a large number of CE applications that chiral separations are influenced by many factors, these include pH, chiral selector concentration, voltage, temperature, concentration of BGE, ionic strength and the concentration of additives such as SDS, organic modifier, polymer etc. The optimization of these factors and the application of optimal conditions can improve selectivity significantly. Traditional optimization methods involve the systematic alteration of one variable while maintaining the other factors at constant levels. Such a univariate approach can be time-consuming, especially when a number of factors are involved. Further, mutual interaction among the factors could easily be overlooked with this approach. Consequently, the optimal conditions could be missed when mutual interactions between parameters take place. However, by means of chemometric experimental designs, it is possible to optimize all parameters simultaneously and find out optimal conditions more efficiently. In addition, non-linear models from the optimization can describe resolution as a function of separation variables, which can facilitate the understanding of chiral recognition mechanisms. In general, when a large number of variables are considered in the separation, a scouting is initially made to find out which factors are the most influencing. This can be done by Plackett-Burman design, fractional factorial design and other screening designs with reduced experiments. Once the key factors are found, a following optimization is performed by means of full factorial design or central composite design (CCD).

The functional relation between the experimental variables and the obtained results is approximated as a truncated Taylor's series expansion [233]:

$$y = \beta_0 + \Sigma\beta_i x_i + \Sigma\Sigma\beta_{ij} x_i x_j + \beta_{ii} x_i^2 + e \tag{4}$$

where the coefficients β are the parameters of the model and e is the overall error term. The estimation of the parameters is done using multiple linear regression, matrix least squares, a polynomial model is fitted to clarify the experimental results. The linear coefficient for the experimental variables, β_i, describes their quantitative influence on the model. The cross-product β_{ij} will measure the interaction effect between the variables, and the square term $\beta_{ii} x_i^2$ will describe the non-linear effect of the response.

A number of publications on the use of experimental designs to optimize separation parameters in CE has been reported. These dealt with the use of Plackett-Burman design [234–237], fractional factorial design [238], full fractional factorial design and CCD [239–242]. A review concerning the application of chemometric experimental designs in CE has been presented by Altria et al. [243]. Moreover, Corstjens et al. presented a review on optimization of selectivity in CE and highly recommended the use of a systematic optimization strategy in the development of CE methods rather than trial and error [244]. However, the use of chemometric experimental designs for chiral analysis of amino acids and peptides is limited to papers [40,80–82,156,160,165,228].

We have applied factorial designs for chiral resolution of amino acids and peptides for direct as well as for indirect methods and resolved 20 protein amino acids in the direct separation mode utilizing CDs as the chiral selectors and also a number of amino acids and peptides in the indirect separation mode [40,80–82]. We compared CD-CZE and CD-MEKC modes and showed that the CD-MEKC is superior to CD-CZE mode for the resolution of a majority of amino acids and peptides derivatized with chloroformates FMOC and AEOC [80,81]. It was thus found that the presence of SDS resulted in increased selectivity. The selectivity was more affected by the change of SDS concentration than of γ-CD concentration. However, in the case of β-CD as the selector, the β-CD concentration rather than the SDS concentration to a great extent influenced the selectivity. A similar effect was also observed for the resolution of FMOC-peptides. The enhanced selectivity may be explained according to the theoretical discussion presented by Terabe et al. [14], where the selectivity in the CD-MEKC was given by [14]:

$$\alpha = \frac{1 + \phi_{\mathrm{CD}} K_{\mathrm{CD},1}}{1 + \phi_{\mathrm{CD}} K_{\mathrm{CD},2}} \tag{5}$$

A combination of Eqs. (5) and R_s in MEKC [121,122] gives the R_s of CD-MEKC as:

$$R_s = \frac{N^{1/2}}{4} \cdot \left(\frac{K_{\mathrm{CD},1} - K_{\mathrm{CD},2}}{1/\phi_{\mathrm{CD}} + K_{\mathrm{CD},1}} \right) \cdot \left(\frac{k_2'}{1 + k_2'} \right) \cdot \left[\frac{1 - t_0/t_{mc}}{1 + (t_0/t_{mc})k_1'} \right] \tag{6}$$

where α is separation factor, i.e. selectivity; ϕ_{CD} is the ratio of the volume of CD to that of the aqueous phase; $K_{\mathrm{CD},1}$, $K_{\mathrm{CD},2}$ are the complexation formation constants of CD with enantiomers 1 and 2, respectively; N is number of theoretical plates; k_2' and k_1' are the capacity factors of enantiomer 1 and 2 and t_0 and t_m are migration times of EOF and micellar phase, respectively. This equation suggests that the resolution is affected by several factors, e.g. voltage (the efficiency N), the concentration of surfactant (the capacity factor k'), pH (EOF t_0), CD concentration (k', $K_{\mathrm{CD},1}$, $K_{\mathrm{CD},2}$). Since both the CD and SDS concentrations have a large impact on selectivity, a simultaneous optimization of SDS and CD can result in an optimal selectivity. Terabe et al. suggested that it is more effective to change the concentration of the surfactant than that of the CD, in order to adjust the capacity factor to the optimal value [14]. Our results showed that the selectivity is more affected by the change of concentration of SDS than of the CD concentration when using γ-CD as the selector. In contrast, the change of CD concentration had a larger impact on the selectivity than that of SDS when employing β-CD as the selector [40,80,82]. Often, the optimization curve is relatively flat around optimal conditions, and then the effect of a small change in the variable becomes strongly dependent on how far from optimum you are. The optimal SDS concentration for maximum resolution could be too high according to the optimizations, which would practically lead to unnecessarily long migration times. Maximum resolution is not always needed. Further, a decrease in separation efficiency was obtained at higher SDS concentration [82]. Therefore, a compromise was made regarding the SDS concentration. Selectivity, migration times and efficiency all had to be taken into account in this context. In general, an average value of 40–50 m*M* SDS was utilized in our separations. Moreover, strong interactions between SDS and CDs occurred [80,81], indicating that a change of one parameter would cause the change of other parameters. This implies that a traditional one-by-one parameter optimization would most likely have missed the real optimal conditions.

In addition, it should be mentioned that Guttman et al. proposed an approach to optimize chiral separation parameters and selectivity [245]. This approach, CD array chiral analysis, is a step-by-step

method development based on the systematic univariate optimization of parameters. Thus, it is not only time-consuming and less straightforward but also suffers from the lack of model description and parameter interaction as compared to chemometric experimental designs.

5.2. Relation between optimal conditions and analyte structure

In the CD-CZE mode, it was observed that the optimal concentrations varied for different AEOC-amino acids [81]. As shown in Fig. 16, for example, an optimal γ-CD concentration for AEOC-Phe is at higher than 30 m*M* while for AEOC-Met the optimum is below 5 m*M* [246]. Moreover, an optimal concentration of 20 m*M* γ-CD was obtained for AEOC-Asp (optimization not shown). In addition, the optimal pH was different for different amino acids.

Interestingly, in the CD-MEKC system, similar optimal conditions were observed for the different FMOC-amino acids [40,80] and for the AEOC-amino acids [81]. For instance, all examined FMOC-derivatives of Phe, Leu, Met, Thr, Asp and Glu had similar optimal conditions when three factors, organic modifier, β-CD and SDS concentration were optimized [40,80]; the 4 AEOC-amino acids, Leu, Met, Phe and Val, displayed similar conditions when two factors, the organic modifier and the β-CD concentration, were optimized [81]. This was also observed for the chiral separation of AEOC-amino acids when employing γ-CD as chiral selector. In Fig. 17 is shown the optimization of 4 AEOC-amino acids in CD-MEKC, in this case, the results are completely different from those obtained in CD-CZE. In addition, in a recent study of chiral analysis of (±)-FLEC reagents derivatized with non-chiral amino acids, e.g., glycine, β-alanine and GABA, similar optimal conditions were obtained for the different types of derivatives in CD-MEKC mode when using β-CD and γ-CD as the selectors [230].

On the basis of these optimizations, a conclusion can be drawn that the derivative moieties, FMOC and AEOC, may have a dominating role in the retention and chiral recognition of these amino acids in the CD-MEKC mode. As a result of similar optimal conditions obtainable from the CD-MEKC mode, we have successfully resolved a large number of AEOC-amino acids and FMOC-amino acids and baseline separation of 20 FMOC-amino acids has been achieved, regardless of their differences in hydrophobicity, structure and pK_a values. As is known, the prediction of optimum conditions based on the chemical structures of the analytes is an interesting and important objective but it remains rather difficult due to multiple interactions. However, with the aid of chemometric experimental designs, more information can be attained.

5.3. Influence of organic modifiers

The use of different organic solvents as modifiers to improve separation has been demonstrated in CE

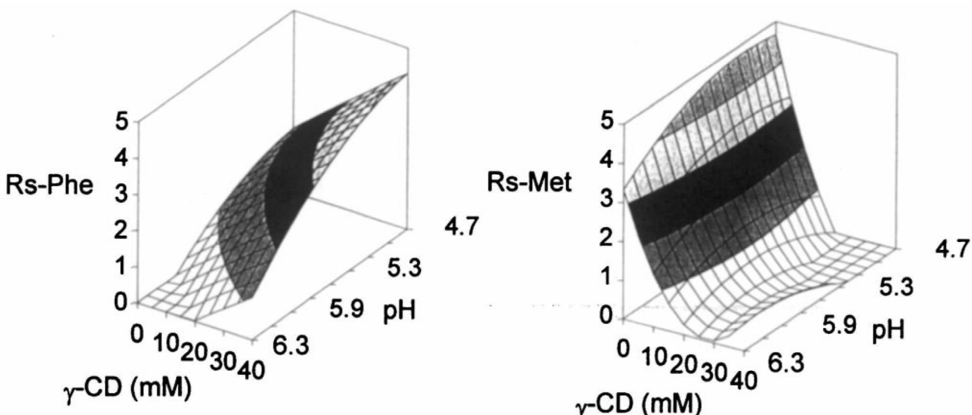

Fig. 16. Optimization of AEOC-Phe and Met; R_s as a function of pH and γ-CD concentration in CD-CZE. Conditions: 25 m*M* phosphate–25 m*M* borate. Capillary, 61 cm (45 cm to detector)×25 μm I.D.; 30 kV. (From Ref. [246].)

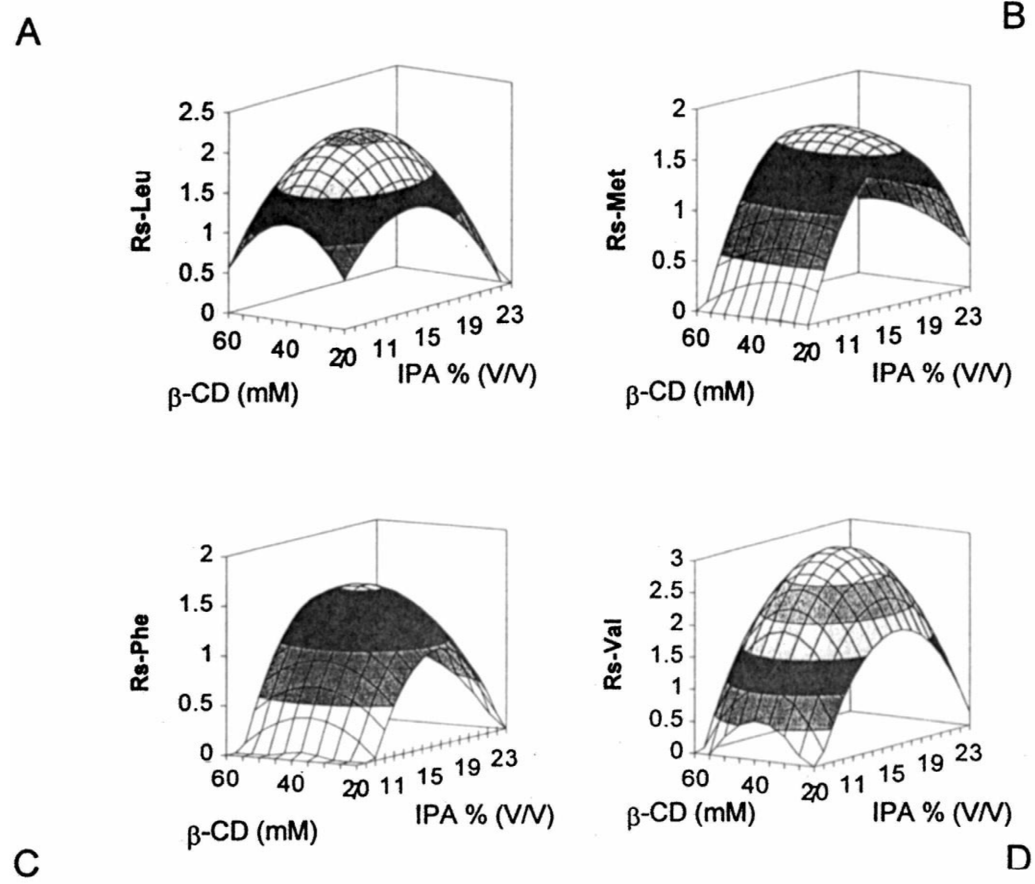

Fig. 17. Optimization of 4 AEOC-amino acids; R_s as a function of β-CD and 2-propanol concentration in CD-MEKC. Conditions: 50 m*M* phosphate (pH 7.50), 40 m*M* SDS, 1 *M* urea. Capillary, 67 cm (46 cm to detector)×25 μm I.D.; 30 kV. (From Ref. [81], with permission.)

[85,199,247–251]. A theoretical model describing the effects of organic modifier on optimal CD concentration was given by Wren and Rowe [252]. Recently, Sarmini and Kenndler have presented a review on the influence of organic modifiers on the separation selectivity [253]. Commonly used organic modifiers include acetonitrile (ACN), methanol and 2-propanol (IPA). These modifiers generally influence the separation in the following ways: (i) to decrease the EOF and thus improve the separation; (ii) to reduce the conductivity, resulting in improved efficiency by suppressing Joule heating; (iii) to minimize the adsorption of solutes or chiral selectors at the capillary surface; (iv) to extend the separation window (increased t_m/t_0 value) in MEKC and (v) to alter the partitioning. In CD-MEKC, the effect of organic modifiers on separation may be more complicated due to the complexity of the system. In addition, the micelle formation, shape, aggregation numbers, and CMC may be changed even by the addition of small concentrations of organic modifiers to an aqueous micellar system [254]. We have demonstrated the benefits of using organic modifiers for chiral separation of different amino acids and peptide derivatives in the direct as well as in the indirect method [40,80–82,156,160,165,169].

5.3.1. Effect of organic modifier on enantiomeric separation

In the both CD-CZE and CD-MEKC mode, enantioselectivity was not observed for FMOC-amino acids when β-CD was used as chiral selector without

the presence of organic modifiers [40,80]. However, the addition of an organic modifier resulted in an enantioselectivity for chiral resolution of FMOC-amino acids. Thus, the presence of IPA was found to be a prerequisite for chiral recognition of most FMOC-amino acids in either CZE or CD-MEKC. Other organic modifiers, methanol, ACN and THF were less effective for chiral separation of FMOC-amino acids and peptides. Fig. 18 shows the effects of different organic modifiers on the selectivity of peptides using γ-CD as the selector [82]. For the chiral separation of AEOC-amino acids in MEKC, the IPA was found indispensable for most AEOC-amino acids using β-CD as the chiral selector [81]. The presence of IPA also led to improved chiral selectivity for most of the AEOC-amino acids when using γ-CD as the chiral selector. Generally, the addition of 15% (v/v) of IPA resulted in the highest selectivity and efficiency. The selectivity observed in the presence of IPA can be mainly attributed to the decreased polarity of the buffer media. For the organic modifiers investigated, the polarity of the separation systems decreased as MeOH>ACN>THF>IPA [82]. It was suggested by Zukowski et al. for the separation of FMOC-amino acids and peptides in HPLC that the FMOC moiety might be forced into the cavity of CDs in an aqueous phase, which resulted in a lack of selectivity [255]. It may be anticipated that FMOC and AEOC moieties tightly fit the cavity of CDs in aqueous media. The addition of IPA may modify the interaction between the CD cavity and FMOC-amino acids and AEOC-amino acids (larger differences in k'_{CD1}- and k'_{CD2}) by reducing binding constants. It has been shown that most organic solvents in buffers decrease binding constants between the CD cavity and the analytes [184,256]. Consequently, a decrease in buffer polarity facilitates chiral recognition with the addition of a less polar organic solvent such as IPA. It has also been suggested that an organic solvent may aid the desorption of analytes from the CD cavity [257].

Furthermore, a reversal of EMO was observed for some AEOC-amino acids by means of the addition of IPA from 0 to 15% (v/v), Fig. 19 [81]. This clearly illustrates the important role that the organic modifier plays in chiral discrimination.

When vancomycin was employed as the chiral selector, the addition of 10% (v/v) IPA led to

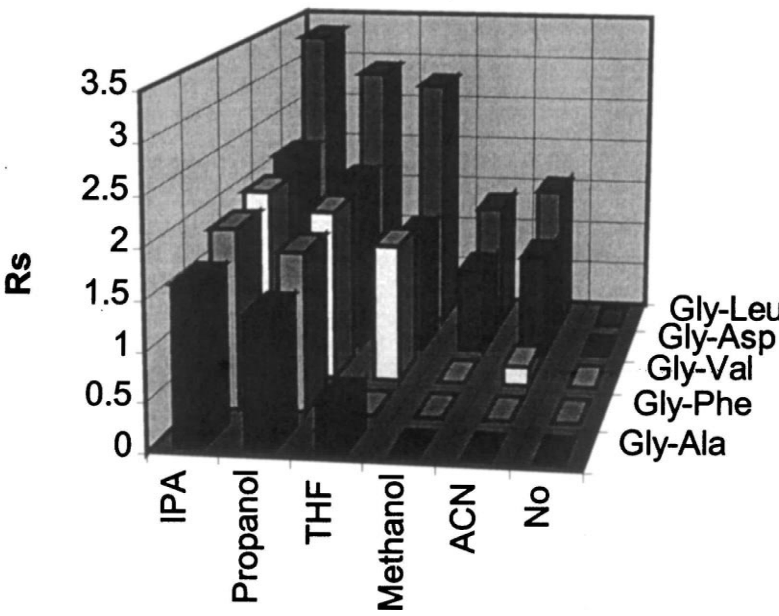

Fig. 18. Effects of different organic modifiers on chiral resolution of FMOC-dipeptides. Conditions: 40 m*M* phosphate (pH 7.50), 40 m*M* SDS, 12 m*M* γ-CD, 15% (v/v) IPA. Capillary, 68 cm (46 cm to detector)×25 μm I.D.; 25 kV. No=without organic modifier. (From Ref. [82], with permission.)

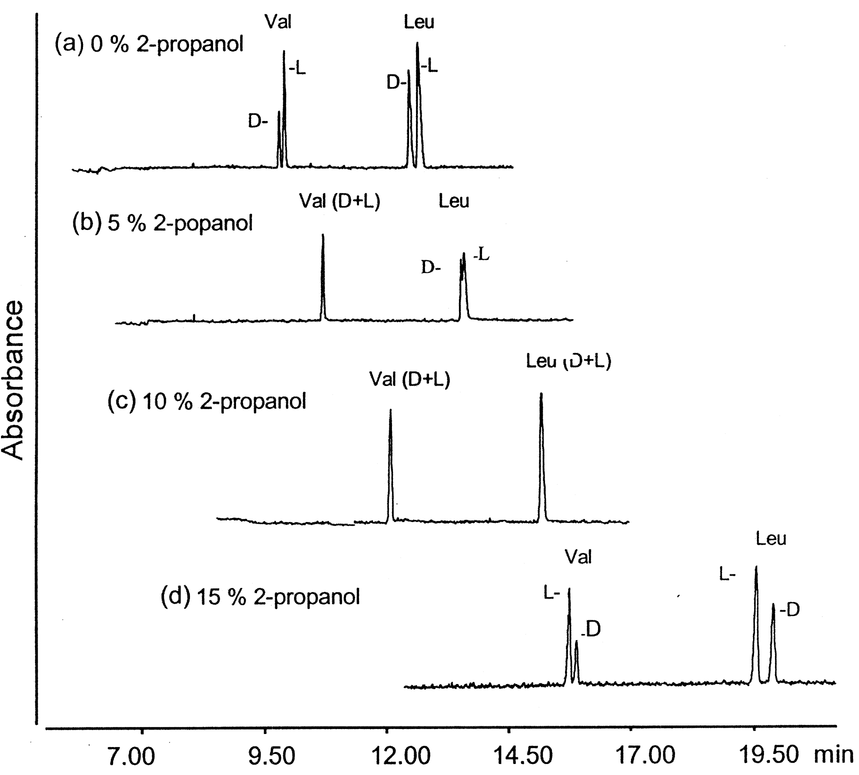

Fig. 19. Effect of organic modifier on enantiomeric elution order of AEOC-amino acids. Conditions: 50 m*M* phosphate (pH 7.50), 40 m*M* SDS, 20 m*M* γ-CD. Capillary, 67 cm (46 cm to detector)×25 μm I.D.; 30 kV. (From Ref. [81], with permission.)

improved efficiency by reducing the adsorption of vancomycin at the capillary wall [156,160]. Further, we have demonstrated that an increase in selectivity can be achieved for the resolution of FMOC-peptides by utilizing as high as 40% ACN in the BGE when employing teicoplanin as the selector [165].

5.3.2. Effect of organic modifier on diastereomeric separation

As for the resolution of FMOC- and AEOC-amino acids in CD-MEKC, 2-propanol was found to be most effective for diasteromeric separation of FLEC-peptides. It was observed that the presence of 15% IPA led not only to enhanced selectivity, but also to improved separation efficiency for all FLEC-peptides [82]. This dipeptide, which exhibits stronger hydrophobicity than other peptides, was not resolved in the absence of an organic modifier.

6. Applications

A number of applications has been presented. In this section, some typical examples are selected showing the enantiomeric impurity determination or related issues.

6.1. Determination of enantiomeric impurity

Ruyters and Van der Wal reported a fully automated method for the determination of the enantiomeric purity of amino acids [258]. This method was based on automated pre-derivatization of amino acids with 4-fluoro-7-nitrobenz-2,1,3-oxadiazole (NBD-F) and separation of these derivatives in CZE with β-CD and dimethyl-β-CD as the chiral selectors, detection was by an Ar-ion laser detection at 488 nm excitation. The authors demonstrated the possibility to determine 0.5% levels of either D-phenylglycine or

L-phenylglycine with the precision of about 3% RSD. Further, a calibration curve was made for L-Phe in D-Phe by standard-addition to determine the LOD. It was stated that linearity over more than three orders of magnitude could be reached and 0.05% of L-Phe could be determined with a calculated LOD 0.014% by this approach. Guttman et al. have demonstrated determination of 0.064% of D-Phe in L-Phe derivatized with Dns [245]. The separation was carried out in CD-CZE mode using HP-β-CD as the selector with a conventional UV detection at 214 nm. Moreover, Assi et al. recently demonstrated the determination of trace enantiomeric impurities of tryptophan using α-CD as the selector with an uncoated capillary and with surface modified capillaries [259]. LODs achieved with UV for the determination of D-impurity in L-tryptophan were 0.5% and 0.35% (mol/mol) for the uncoated and neutral capillaries. Moreover, the use of a polyamine coated capillary improved the LOD of the minor D-impurity to 0.03% while a LOD of 0.05% for the L-impurity was reported.

In addition, applications of indirect methods by employing a chiral reagent for the determination of optical impurities have been reported. Thorsén et al. recently have shown the determination of enantiomeric impurities in commercially available peptides by MEKC after the derivatization with a chiral reagent APOC [220]. The authors demonstrated simultaneous determinations of L-impurities in 4 different D-peptides in one run. With this approach, 0.12–0.28% of L-impurities in D-peptides were determined, detection was with LIF. Owing to the presence of a small optical impurity in the APOC reagent, the results had to be corrected. Consequently, 0.06–0.22% impurities were obtained. Tivesten et al. showed the determination of chiral Asp and Glu in biological samples from beers, urine, yogurt and serums [260]. After pretreatment, the samples were derivatized with a chiral reagent OPA–TATG and analyzed using a neutral surfactant, octylglycoside as micelles and a He–Cd laser was used for the detection. Both D- and L-forms of Asp and Glu were found in all tested food samples with different D/L-ratios, but the D-forms of these amino acids were not detected in serum samples. The authors reported good repeatabilities with RSD from 0.33 to 2.56% for Asp and 0.3–2.21% for Glu.

In some cases, response factors may vary between antipodes. Hempel and Blaschke demonstrated that analyte complexation with CD may enhance fluorescence intensity [261]. Thus, analytes forming strong complexes may have higher response factors than less strongly complexed analytes. On the other hand, the above-mentioned effects are not always the major source of incorrectness in the quantification of separation results [262].

6.2. Analysis of D- and L-residues and racemization study

Liu applied two different approaches for the determination of enantiomeric forms of amino acids derived from novel depsipeptide antitumor antibiotics [263]. The amino acids after hydrolysis were derivatized with either Dns or FITC and then chirally separated by chiral ligand exchange and CD-MEKC, respectively. The presence of D-serine and L-β-hydroxyl-*N*-methylvaline enantiomeric forms was identified by two approaches, which gave good correlation. Another interesting application is the chiral analysis of D-/L-Phe and D-/L-Arg residues in the sequences of bradykinin antagonists by Brown et al. [127]. After acid hydrolysis of these peptides, a MEKC buffer containing taurodeoxycholic acid (STCA) and β-CD was utilized for determination of the ratios of D-/L-Phe and D-/L-Arg. It was found that the ratio of D-/L-Arg was close to the expected ratio of 2:1 in all cases, while the expected ratio of 1:1 for D-/L-Phe was not obtained. Their result indicates that the racemization of L-Phe was intermolecularly catalyzed by the unprotected free thiol group on the cysteine. This illustrates that protection of the thiol group of cysteine residues is essential prior to the acid hydrolysis of peptides containing both D- and L-amino acid residues. Furthermore, the MEKC results were confirmed by a reversed-phase HPLC method using isobutyryl-L-cysteine *ortho*-phthalaldehyde as derivatization reagent. The methods were found to be in good agreement. Liu et al. demonstrated the determination of D- and L-amino acids residues in peptides after hydrolysis and also in biological matrices (rabbit serum and homogenate of Aplysia californica buccal ganglion) by the use of a chiral derivatization reagent (−)-NBD-PyNCS [221]. Moreover, Kurosu et al. reported the identification of

the PTH-D-Ala residue from the protein sequencer for [D-Ala^2]-methionine enkephalin using a mixed system containing 17.5 m*M* TM-β-CD, 12.5 m*M* digitonin, 12.5 m*M* β-escin and 50 m*M* SDS [94].

Riester et al. applied a chiral CE approach to study the racemization of amino acids in solid-phase peptide synthesis (SPPS) [113]. A crown ether was employed as the selector to resolve a tripeptide Tyr–Lys–Trp. A LOD of 0.05% impurity was obtained. It was also shown that the determined stereoisomeric impurities were in good agreement with theoretical calculations. Furthermore, Nouadje et al. investigated the racemization of L-serine by the application of CD-MEKC and laser-induced fluorescence detection [264]. The racemization rate of 10 n*M* L-serine was measured at 100°C in water. The CD-MEKC was performed using a separation buffer consisting of 100 m*M* SDS–60 m*M* β-cyclodextrin–100 m*M* borate at pH 9.2 and detection was with a 488 nm wavelength laser. Electropherograms taken at 0–55 h showed the growth of the D-serine peak with time, which gave a calculated racemization half-life of L-serine, e.g., 40 days at 100°C.

7. Conclusions

A variety of chiral CE approaches has been successfully employed for the resolution of different amino acids and peptides. In fact, a large number of amino acids and peptides, even in complex mixtures, has been chirally resolved by either the direct method using CDs or antibiotics as the chiral selectors or by the indirect method applying optical reagents such as FLEC and APOC, etc.

The indirect method has many advantages over the direct method such as fewer parameters to be optimized, high selectivity, suitability for resolution of complex amino acid mixtures and the possibility to select EMO. If a chiral reagent, like FLEC, is of high optical purity, the indirect method can be the best solution. This technique should receive more attention in the development of new methods for chiral amino acid analyses. In principle, the chiral reagents used in HPLC are applicable to chiral CE separation. However, the high demand on optical purity of the reagents may limit this approach.

The CD, as the most widely used selector, has demonstrated its power and suitability for the resolution of most amino acids. Selectivity can be improved by mixed approaches such as CD combining with chiral polymer, CD with crown ether, CD-MEKC with organic modifiers etc. Those combined approaches have further increased the CD's applicability. Moreover, the simultaneous optimization of key parameters by the application of chemometric experimental designs can enhance selectivity significantly, enabling chiral resolution of a majority of amino acids under the same conditions. In addition, other selectors such as antibiotics, chiral surfactants etc. can be complementary.

Owing to minute sample and buffer requirements, a diversity of approaches available and simple performance and rapid method development, the chiral CE has become a powerful separation technique complementary or alternative to HPLC, in particular, for resolution of complex sample matrices.

8. Abbreviations

ACD	6-Amino-6-deoxy-β-CD
ACN	Acetonitrile
AEOC	2-(9-Anthryl)ethyl chloroformate
AGP	α-Acidic glycoprotein
Allyl-TER	1-Allyl derivative of (5*R*,8*S*,10*R*)-terguride
AM-β-CD	Amphoteric-β-CD
APOC	(+) or (−)-1-(9-Anthryl)-2-propyl chloroformate
AQC	6-Aminoquinolyl-*N*-hydroxy-succinimidyl carbamate
BGE	Background electrolyte
Big CHAP	*N*,*N*-bis(3-D-gluconamidopropyl)-cholamide
BocC	*N*-*tert*.-butoxycarbonyl-L-cysteine
BSA	Bovine serum albumin
CBI	1-Cyano-2-substituted-benz[L]isoindole
CBZ	Carboxybenzyl
CBQ	3-(*p*-Carboxybenzoyl)quinoline-2-carboxyaldehyde
CCD	Central composite design
CD	Cyclodextrin
CD-hm	6-Deoxy-6-*N*-histamino-β-CD

CD-mh	6-Deoxy-6-[4-(2-aminoethyl)-imidazolyl]-β-CD
β-CD-EA	Heptakis(6-hydroxyethyl(amino-6-dexoxy-β-CD))
CDen	Mono-(6-β-aminoethylamino-6-deoxy)-β-CD
CDITC	(1*R*,2*R*) or (1*S*,2*S*)-*N*-[(2-Isothiocyanate)cyclohexyl]-6-methoxy-4-quinolinylamide
CD-CZE	CD capillary zone electrophoresis
CD-MEKC	CD-modified micellar electrokinetic chromatography
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
$18C6H_4$	(+)-18-Crown-6-tetracarboxylic acid
CMC	Critical micellar concentration
CM-β-CD	Carboxymethyl-β-CD
Cy5	Cyanine derivative
CZE	Capillary zone electrophoresis
DAT	(+)-Diacetyl-L-tartaric anhydride
DBT	(+)-Dibenzoyl-L-tartaric anhydride
DBZ	3,5-Dintrobenzoyl
DDCV	(*S*)-and (*R*)-*N*-Dodecoxycarbonylvaline
Deoxy Big CHAP	*N*,*N*-bis(3-D-Gluconamidopropyl)-deoxycholamide
DM-β-CD	Heptakis(2,6-di-*O*-methyl)-β-CD
DNB	3,5-Dintrobenzoyl
Dns	5-Dimethylaminonaphthalene-1-sulphonyl chloride
DNP	2,4-Dinitrophenyl fluoride
DNZ	3,5-Dintrobenzyloxycarbonyl
DOPA	3,4-Dihydroxyphenylalanine
EKC	Electrokinetic chromatography
EMO	Enantiomeric migration order
EOF	Electroosmotic flow
FITC	Fluorescein isothiocyanate
FLEC	(+) or (−)-1-(9-Fluorenyl)ethyl chloroformate
FMOC	9-Fluorenylmethyl chloroformate
GABA	γ-Aminobutyric acid
GITC	2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate
GRA	Glycyrrhizic acid
HACD	6-Deoxy-6-hexylamino-β-CD
HDB	Hexadimethrine bromide
HO-L-Hypro	*N*-(2-Hydroxyoctyl)-L-4-hydroxyproline
HEPES	*N*-2-Hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
HPLC	High-performance liquid chromatography
HP-L-Hypro	*N*-(2-hydroxypropyl)-L-4-hydroxy proline
HP-β-CD	2-Hydroxypropyl-β-CD
HSA	Human serum albumin
L-Hypro	L-4-Hydroxyproline
GITC	2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosylisothiocyanate
IPA	2-Propanol
LE-MEKC	Ligand-exchange-micellar electrokinetic capillary chromatography
LIF	Laser-induced fluorescence
LOD	Limit of detection
Marfey's reagent	D or L-1-Fluoro-2,4-dinitrophenyl-5-L-alanineamide
MeOH	Methanol
MOPS	Morpholinpropanesulfonic acid
MS	Mass spectrometric detection
MEKC	Micellar electrokinetic chromatography
MG	*n*-Octyl-β-D-maltopyranoside
NACE	Non-aqueous capillary electrophoresis
NDA	Naphthalene-2,3-dicarboxaldehyde
NBD-F	4-Fluoro-7-nitrobenz-2,1,3-oxadiazole
NBD-PyNCS	*R*-(−) or *S*-(+)-4-(3-Isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole
NEC-β-CDs	1-(1-Naphthyl)ethylcarbamoylated β-CDs
NG	*n*-Nonyl-β-D-glucopyranoside
NMF	*N*-Methylformamide
OG	*n*-Octyl-β-D-glucopyranoside
ODS	Octadecylsilyl-
OM	*n*-Octyl-β-D-maltopyranoside
OPA	*o*-Phthalaldehyde
OPA+BocC	*o*-Phthalaldehyde (OPA) and *N*-*tert*.-butoxycarbonyl-L-cysteine
OPA+TATG	OPA and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose
Pamino acid	Poly(acrylamide)
PEG	Poly(ethylene glycol)

Poly(L-SUV)	Poly(sodium *N*-undecylenyl-L-valinate)
PTH	Phenylthiohydantoin
PVP	Poly(vinylpyrrolidine)
QA-β-CD	Quarternary ammonium β-CD
R_s	Resolution
SAMBI	(*S*)-(+)-α-methylbenzyl isothiocyanate
SBE-β-CD	Sulfobutyl ether β-CD
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SDGlu	Sodium *N*-dodecanoyl-L-glutamate
SDSer	Sodium *N*-dodecanoyl-L-serine
SDVal	Sodium *N*-dodecanoyl-L-valinate
SNEIT	(*S*)-1-(1-Naphthyl)ethyl isothiocyanate
RSD	Relative standard deviation
SPPS	Solid-phase peptide synthesis
STC	Sodium taurocholate
STCA	Taurodeoxycholic acid
STDC	Sodium taurodeoxycholate
TATG	2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose
TBAP	Tetra-*n*-butylammonium perchlorate
TBQCA	3-(4-Tetrazolebenzoyl)-2-quinolinecarboxaldehyde
THF	Tetrahydrofuran
TM-β-CD	Heptakis(2,3,6-tri-*O*-methyl)-β-CD
TODC	Taurodeoxycholic acid
Tris	Tris(hydroxymethyl)aminomethane

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