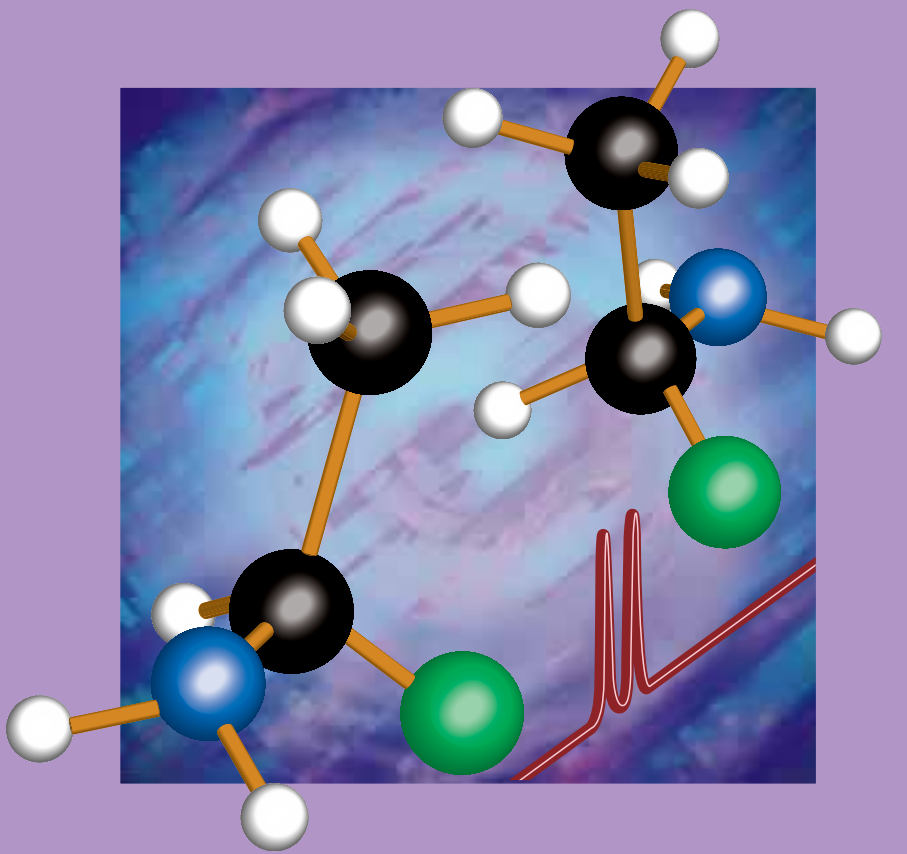
An Introduction to Chiral Analysis by Capillary Electrophoresis



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An Introduction to Chiral Analysis by Capillary Electrophoresis

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About the Author

Dr. Salvatore Fanali is a researcher at the Istituto di Cromatografia del Consiglio Nazionale delle Ricerche in Montelibretti (Rome) Italy. After experience in high performance paper electrophoresis and paper chromatography, his research concerned the study of capillary isotachophoresis in the pharmaceutical field. Since 1988 his research has focused on electromigration methods, mainly applied to the separation of enantiomers. He is author or co-author of seventy publications, two booklets, and chapters in two books. He is a member of the advisory editorial boards of Bollettino Chimico Farmaceutico, Chromatographia, Electrophoresis, Journal of Capillary Electrophoresis, Journal of Chromatography, and Journal of High Resolution Chromatography.

Section 1
Introduction

1.1 Abbreviations Used in the Text

APA	aryl propionic acid
BGE	background electrolyte
CD	cyclodextrin
CGE	capillary gel electrophoresis
CM-β-CD	carboxymethylated-β-cyclodextrin
CM-β-CD pol	carboxymethylated-β-cyclodextrin polymer
CMC	critical micellar concentration
Crown-ether	18-crown-6-ether tetra-carboxylic acid
CSP	chiral stationary phase
CZE	capillary zone electrophoresis
DAT	(+)-O,O'-diacetyl-L-tartaric anhydride
DBT	(+)-O,O'-dibenzoyl-L-tartaric anhydride
di-OMe-β-CD	2,6-di-O-methyl-β-cyclodextrin
EHC	electro-chromatography
eof	electro-osmotic flow
GC	gas chromatography
HEC	hydroxyethylcellulose
HP-β-CD	hydroxypropyl-β-cyclodextrin
HPLC	high performance liquid chromatography
HPMC	hydroxypropylmethylcellulose
ITP	isotachophoresis
LE	leading electrolyte
LEC	ligand exchange chromatography
MEKC	micellar electrokinetic chromatography
NDA	naphthalene-2,3-dicarboxyaldehyde
NSAIDs	non-steroidal anti-inflammatory drugs
PhL	phenyl lactic acid
PVA	polyvinyl alcohol
PVP	polyvinyl pyrrolidone
SBE-β-CD	sulfobutyl-ether(IV)-β-cyclodextrin
SDAla	sodium N-dodecanoyl-L-alaninate
SDS	sodium dodecyl sulfate
SDVal	sodium N-dodecanoyl-L-valinate
STC	sodium taurocholate
STDC	sodium taurodeoxycholate
TLC	thin layer chromatography
tri-OMe-β-CD	2,3,6-tri-O-methyl-β-cyclodextrin

1.2 Introduction to Chiral Analysis

The separation of enantiomers is widely studied in analytical chemistry, especially in the pharmaceutical and biological fields, since chiral drugs are administered either as enantiomers or as racemic mixtures. Very often two enantiomers of the same racemic drug possess different pharmacological effects. For example, S(-)-propranolol is considerably more active than its enantiomers. The anesthetic ketamine is administered as racemate, and the S(+)-ketamine form is more potent than the R(-) form. In addition, the R(-) form may cause post-operative effects. Because of the side effects that could be caused by the presence of an undesirable component in a racemic drug, the current tendency in the pharmaceutical industry is to prepare drugs with one enantiomer only. However, the production of such drugs through stereoselective reaction or preparative enantiomeric separation can provide impure material. Thus rapid, sensitive analytical methods of high resolving power are required to control the synthetic chiral process of the pharmaceutical.

Analytical methods which have been used for the separation of chiral compounds include high performance liquid chromatography (HPLC),[1–3] gas chromatography (GC),[4,5] thin layer chromatography (TLC),[6] and recently, capillary electrophoresis (CE).[7–15] CE is especially useful for the analysis of different classes of compounds, including organic and inorganic ions, peptides, proteins, saccharides, drugs, optical isomers, and others. In CE the separation will take place if the analytes, under the influence of an applied electric field, move toward the detector with different velocities (different effective mobilities). Thin capillaries, with internal diameters of 10–100 μm, allow application of relatively high electric fields (200–1,000 V/cm) permitting analytes to be separated in short times with high resolution and high efficiency, because the Joule heat, the main cause of zone dispersion, is easily controlled.

The different separation modes available in CE, namely zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), gel electrophoresis (CGE), and isotachophoresis (ITP), combined with the high resolving power and high efficiency, make the CE technique competitive with others.[16] CE offers additional advantages over other techniques such as HPLC, including

- A relatively small volume of sample and buffer (nl and μl, respectively) is required
- Expensive chiral columns can be avoided because the chiral selector can be easily added to the BGE

- The separation is highly reproducible because the buffer with the chiral selector is replenished after each run

The use of CE for analysis of chiral compounds is well documented and, considering the enormous increase in publications, it can be expected that in the near future chiral analysis by CE will be widely applied to pharmaceutical, clinical, and environmental samples.

Published reviews,[17–24] and the special issue edited by us[25] dealing with the separation of optical isomers by CE, are available for further reading. The article by Fanali *et al*. provides a list of enantiomeric compounds which have been separated using capillary electrophoresis.[24]

Section 2
Chirality and Optical Isomers Separation

When four different ligands are bound to a tetravalent carbon, an asymmetric molecule is generated in which the carbon is the asymmetric center. As shown in Figure 1, two optical isomers can be generated due to the different spatial orientations of the ligands around the chiral center.

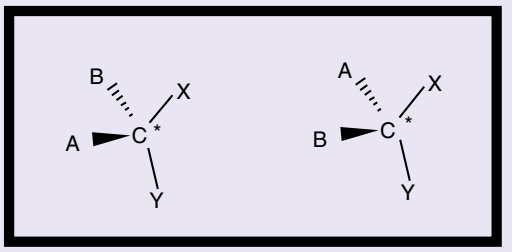


Fig. 1. Mirror image of two optical isomers.

Enantiomers are two stereoisomers which exhibit non-superimposable mirror images. Diastereomers generally possess at least two asymmetric centers (one of them has the same configuration), and are not mirror images. The most common chiral center is represented by tetrahedral carbon, although other atoms, like nitrogen, sulfur, and phosphorous, can be found in stereoisomers. Compounds possessing at least two enantiomers are chiral compounds.

The main property of stereoisomers is represented by the rotation of polarized light in different directions, counter-clockwise (levo-rotatory) and clockwise (dextro-rotatory). They are l(-)- and d(+)-isomers, respectively. The optical rotation of two enantiomers (in a racemic mixture) is of the same magnitude, while in the case of diastereomers the two parameters may not be the same due to different physico-chemical properties. Unlike enantiomers, diastereomers may have different melting points, boiling points, and solubility.

Although the d, or (+), and l, or (-), symbols show a very important physical property of the molecule, they unfortunately do not give any information concerning the spatial arrangement of the chiral center. The Fisher convention, widely employed for sugars and amino acids, makes use of D and L symbols and is based on the comparison of the substituents of the chiral center of the compound under investigation with that of (+)-glyceraldehyde. However, the D and L convention can be confused with d and l terminology, and thus the currently recognized convention is the Cahn-Ingold-Prelog.[26] Here the priority of the ligand to the asymmetric center based on the atomic number is controlled, and the group with lower priority is positioned far away from the observation point. Then the priority of the other substituents is examined. If it decreases clockwise, the R configuration is assigned, otherwise the S configuration is given.

Section 3
Separation of Diastereoisomers by Indirect Methods

Because two diastereoisomers of the same compound have different physico-chemical properties, they can, in principle, be separated from each other by CE using a non-chiral electrophoretic system. As an example, consider the chemical structure of nor-ephedrine and ψ-nor-ephedrine (Figure 2). The two compounds possess the same molecular weight and the same substituent groups at the two asymmetric carbons, and the configuration of only one of the asymmetric centers is the same; thus they are diastereoisomers. Each diastereoisomer exists as a pair of enantiomers (d- and l-norephedrine and d- and l-ψ-norephedrine, respectively).

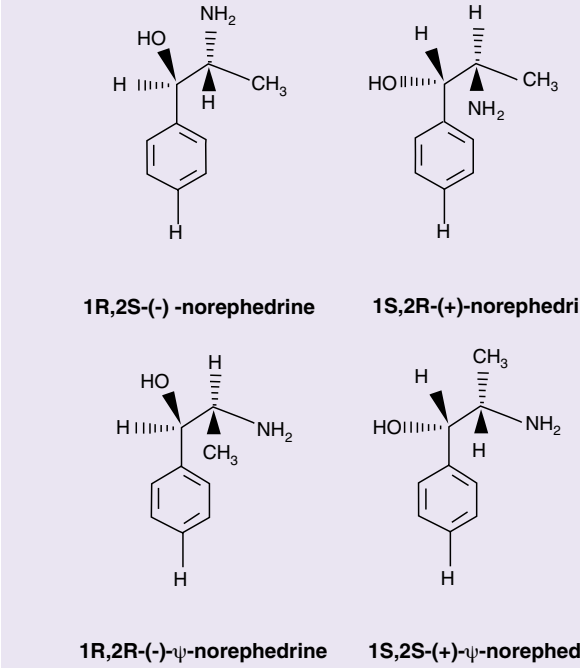


Fig. 2. Chemical structure of nor-ephedrine and ψ-nor-ephedrine.

When a mixture of the two compounds is injected for an electrophoretic run using a non-chiral background electrolyte (phosphate buffer at pH 2.5, see Figure 3), two peaks, corresponding to the two diastereoisomers, are obtained; each peak represents a couple of enantiomers, but the electrophoretic system is unable to separate them.

The above principle can be advantageously used for the resolution of a pair of enantiomers. Thus it is necessary to use a chiral environment that will modify the physico-chemical properties of the two analytes, transforming enantiomers into diastereoisomers. By adjusting the selectivity of the separation, the resolution can be improved.

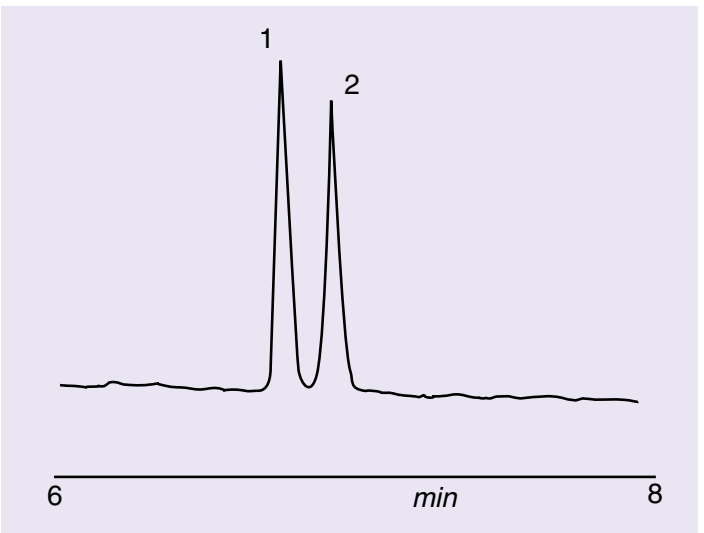


Fig. 3. Electropherogram of the separation of 1) ψ-norephedrine and 2) norephedrine. Apparatus, BioFocus® 3000 system; capillary, (coated) 35 cm x 0.05 mm ID; background electrolyte, 50 mM phosphate buffer, pH 2.5; applied voltage 15 kV; injection, electrokinetic 7 kV, 7 s of 2 x 10^{-5} M of each compound; carousel and capillary temperature 25 ¡C; detection at 206 nm.

The indirect separation method is based on the reaction, before the analysis, of a racemic mixture with a chiral reagent (R or S), producing a mixture of two diastereoisomers that can be resolved using a non-chiral electrophoretic system. The product of the reaction is a mixture of two stable compounds where relatively strong bonds (covalent) are involved in the process.

The derivatization of α-amino- or α-hydroxyacids with (+)-O,O'-diacetyl-L-tartaric anhydride (DAT) or (+)-O,O'-dibenzoyl-L-tartaric anhydride (DBT) can serve as an example. Figure 4 shows the chemical structure of the two diastereoisomers formed between DBT and L- and D-amino acids. The configuration of the two carbons of the tartrate is the same, and that of the amino acid chain is different.

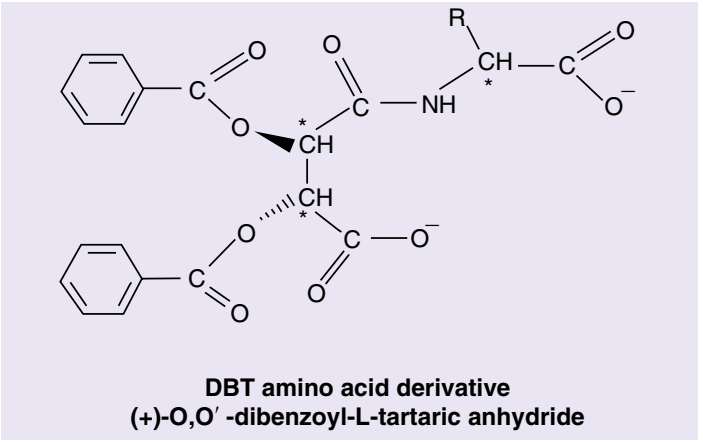


Fig. 4. Chemical structure of the two diastereoisomers formed in the reaction between (+)-O,O'-dibenzoyl-L-tartaric anhydride (DBT) and L-, D-amino acid.

The separation of such derivatives was obtained by CE using a BGE at pH 5.8 containing polyvinyl pyrrolidone (PVP) as a physical network.[27] The effect of the concentration of PVP added to the BGE for the separation of mandelic acid and tryptophan after derivatization with DBT[28] is shown in Figure 5.

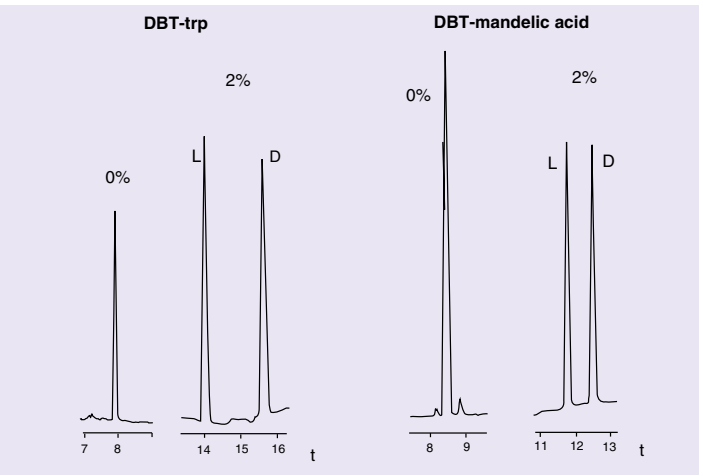


Fig. 5. Effect of the concentration of polyvinylpyrrolidone (PVP) on the separation of diastereoisomers of DPT-D,L-mandelic acid and DBT-D,L-tryptophan. Capillary, (coated) 56 cm x 0.1 mm ID; background electrolyte, 25 mM phosphate buffer, pH 5.8; applied voltage 12 kV; injection, hydrodynamic (5 s at a height distance of 10 cm) of 10^{-4} M of sample; detection at 233 nm (modified from reference [28]).

Although the indirect separation method using the appropriate chiral reagent (R or S) can be used advantageously when an inversion of migration order is required, the method presents several drawbacks. It is time consuming, it requires the use of very pure chiral reagent, the enantiomers must contain reacting groups (hydroxyl, amino), and the kinetics of the reaction can lead to different peak areas for the two diastereomers formed. Probably due to these and other drawbacks of the indirect separation method, the direct separation method is becoming very popular for enantiomeric separation by CE.

Section 4
Direct Method for Chiral Separation in Capillary Electrophoresis

In the direct separation method the chiral selector can be added to the BGE, bound to the capillary wall, or included in a gel matrix. It interacts with the two enantiomers during the electrophoretic process, forming labile diastereoisomeric complexes. Relatively weak bonds are involved, *e.g.*, hydrogen, π-π, or hydrophobic.

The separation of two enantiomers can take place only if the two diastereoisomers formed possess different stability constants, causing the two analytes to move with different velocities. The effective mobility of the most complexed enantiomer is lower than that of its isomer (this is not true if the chiral selector is negatively charged). For example, Figure 6 shows the separation of the four optical isomers analyzed in Figure 3, using the same background electrolyte supplemented with a chiral selector (2,6-di-O-methyl-β-CD) that allows the separation not only of the diastereoisomers but also of the enantiomers present in the mixture.

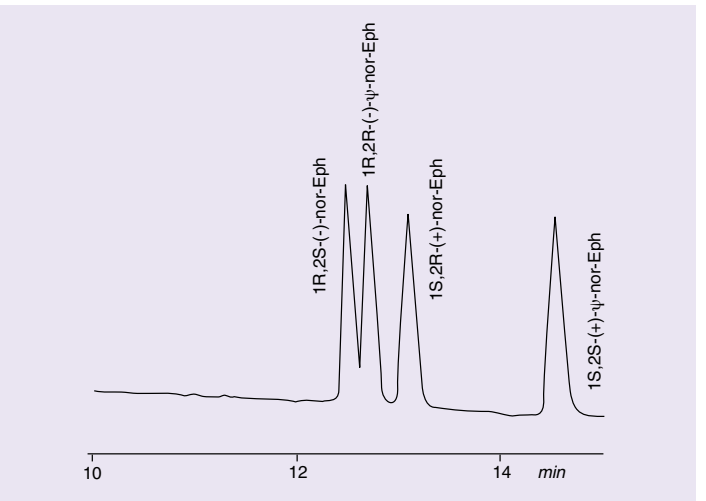


Fig. 6. Electropherogram of the separation of the four optical isomers of norephedrine and ψ-norephedrine. Apparatus, BioFocus 3000 system; background electrolyte, 50 mM phosphate buffer, pH 2.5, and 20 mM of 2,6-di-O-methyl-β-cyclodextrin. For other experimental conditions, see Figure 3.

The direct separation of enantiomers by CE is easier to perform than the indirect separation. This is demonstrated by the increasing number of publications reported in the literature (see references 24 and 29). The direct method is less time consuming since derivatization and purification are not required, a wide number of chiral selectors are commercially available, and small amounts of chiral selectors can be used. Furthermore the chiral purity of the system is not of paramount importance. In fact, the presence of impure chiral selectors will only cause a reduction of resolution of the two enantiomers in the direct method. In the indirect method, the presence of impure chiral selectors will produce four diastereoisomers (more difficult to separate). This is demonstrated in Figure 7, where a mixture of (+)-$Co(en)_3^{3+}$ and (-)-$Co(en)_3^{3+}$ is separated using sodium L(+)-tartrate; when the BGE contained the same chiral selector with 10% D(-)-tartrate, the resolution of the two enantiomers was reduced, while use of 100% D(-)-tartrate allowed baseline resolution with inversion of migration order.

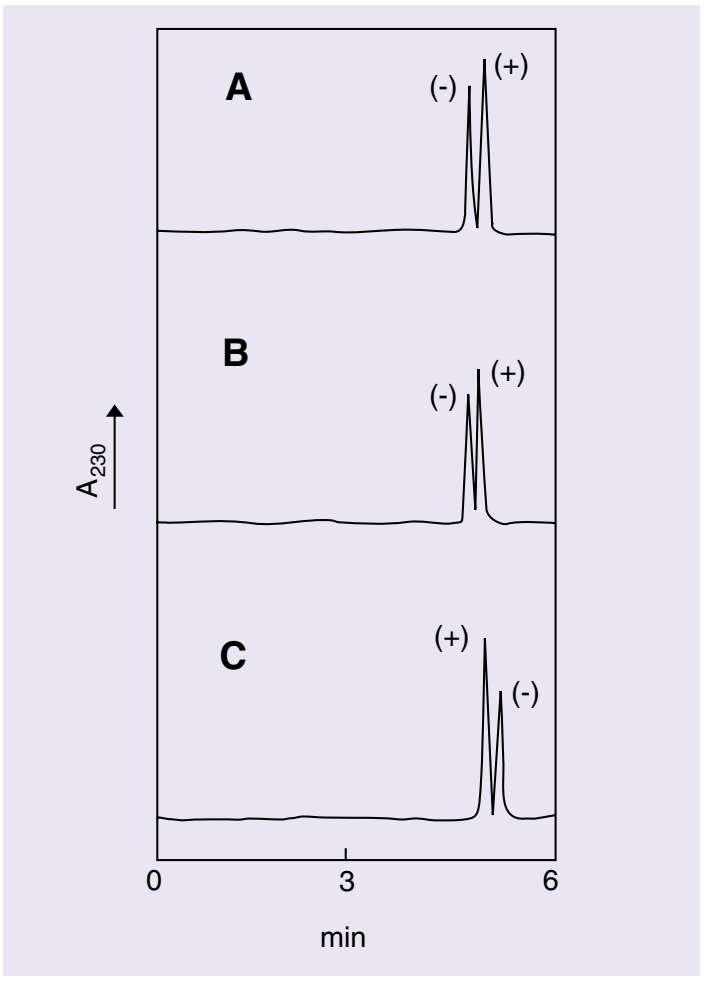


Fig. 7. Electropherograms of the separation of (+/-)-Co(en$_3$)$^{3+}$. Apparatus, BioFocus 3000 system; capillary, (coated) 24 cm x 0.05 mm ID; background electrolyte, 50 mM sodium tartrate, pH 5.1 [A= 100% L(+), B= 90% L(+) and 10% D(-), C= 100% D(-)]; applied voltage 5 kV, 9.4 A; injection, 5 psi*s 35 mg racemic Co-complex + 9 mg (+)-antipode in 100 ml; detection at 230 nm.

Table 1 summarizes the main chiral selectors used in CE for the direct separation of enantiomers, as well as the different CE modes used and the main mechanisms involved in the separation process.

Table 1. Chiral Inclusion-Complexing Agents Used In Capillary Electrophoresis.

Compound	CE Type	Mechanism	References
Uncharged Compounds			
α-cyclodextrin (α-CD)	CZE, ITP, CGE, MEKC	inclusion	14, 30–32
β-cyclodextrin (β-CD)	CZE, ITP, CGE, MEKC	inclusion	15, 33–35
γ-cyclodextrin (γ-CD)	CZE, ITP	inclusion	14, 36–38
glycosylated-α-cyclodextrin (G-α-CD)	CZE	inclusion	39
heptakis-2,6-di-O-methyl-β-cyclodextrin (di-OMe-β-CD)	CZE, ITP	inclusion	13, 15, 30, 40
heptakis-2,3,6-tri-O-methyl-β-cyclodextrin (tri-OMe-β-CD)	CZE, ECH	inclusion	40–43
hydroxyethyl-β-cyclodextrin (HE-β-CD)	CZE	inclusion	41
hydroxypropyl-β-cyclodextrin (HP-β-CD)	CZE	inclusion	33, 39, 41, 44
allyl derivatized-β-CD (Al-β-CD)	CGE	inclusion	45
Uncharged-β-cyclodextrin polymer	CZE	inclusion	46
Chargeable Compounds			
6A-methylamino-β-cyclodextrin (6A-NH-β-CD)	CZE	inclusion	47
6A,6D-dimethylamino-β-cyclodextrin (6A,D-di-NH-β-CD)	CZE	inclusion	47
4-sulfobutyl-ether-β-cyclodextrin (SBE-β-CD)	CZE	inclusion	33, 48–52
mono-(6-β-aminoethylamino-6-deoxy)-β-cyclodextrin (β-CDen)	CZE	inclusion	53
carboxymethyl-β-cyclodextrin (CM-β-CD)	CZE	inclusion	9, 54
carboxymethyl-β-cyclodextrin polymer	CZE	inclusion	55
18-crown-6-ether tetracarboxylic acid (18-crown ether)	CZE	inclusion	32, 56
copper(II)/L-proline or L-hydroxyproline or aspartame	CZE, MEKC	ligand exchange	57–59
maltodextins	CZE	affinity	60
bile salts	MEKC	affinity-MEKC	7, 61, 62
proteins	CZE	affinity	63–65
antibiotics	CZE	inclusion	66, 67
L-tartrate	CZE	outer-sphere	68

4.1 Inclusion Complexation

The resolution of enantiomers has been obtained in CE through inclusion-complexation mechanisms using either cyclodextrins or crown-ether derivatives. Here the two analytes (guest compounds) fit the cavity of the chiral selector (host compound), forming host-guest or inclusion-complexes. The separation of the two enantiomers can take place only if the two diastereomeric complexes, formed during the
electrophoretic process, possess different stability constants. The chiral separation is obtained due to the formation of secondary bonds between the substituent groups on the chiral center of the analytes and those of the chiral selectors positioned outside the cavity (hydroxyl or modified hydroxyl and carboxylic groups for cyclodextrins and crown-ethers, respectively). To clarify the recognition mechanism of chiral resolution using inclusion-complexation, it is necessary to describe the structure and the main properties of the chiral selectors involved in the electrophoretic process.

Cyclodextrins and their Derivatives in Capillary Zone Electrophoresis

Cyclodextrins (CD) are neutral and natural cyclic oligosaccharides composed of several glucopyranose units. They are chiral due to the presence of asymmetric carbons on the glucose units. In spite of the fact that cyclodextrins containing 6–12 D(+)-glucopyranose units are reported in the literature, only 6, 7, and 8 unit-formed molecules, named with the Greek alphabet letters α, β, and γ, respectively, are in frequent use in analytical chemistry.[69,70]

The shape of cyclodextrins is similar to that of a truncated cone with a cavity of different dimensions depending on the CD type (the number of the glucose units and the substituent groups). Their cavity is relatively hydrophobic and able to accept guest compounds of different types, particularly those with non-polar groups. The outside is relatively hydrophilic due to the presence of hydroxyl groups (primary and secondary).

Figure 8 shows the chemical structure of α-cyclodextrin, while Table 2 gives the main properties of the most commonly used CDs.

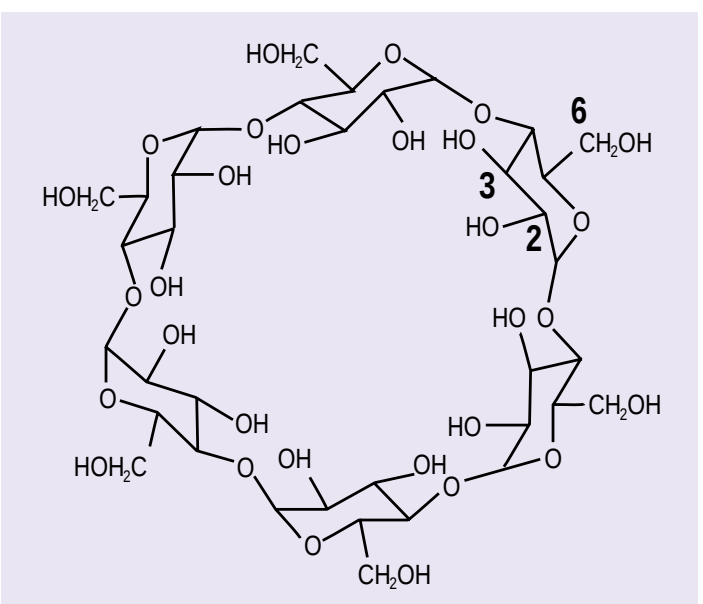


Fig. 8. Chemical structure of α-cyclodextrin.

Table 2. The Main Properties of Native Cyclodextrins [69,71]

Cyclodextrin type	α	β	ψ
Number of glucopyranose	6	7	8
Molecular weight	973	1,135	1,297
Internal diameter (nm)	0.47–0.52	0.60–0.64	0.75–0.83
Depth (nm)	0.79–0.80	0.79–0.80	0.79–0.80
Specific rotation [α]25 D	150.5	162.5	177.4
Melting point (K)	551	572	540
Solubility in water g/100 ml 25 °C	14.50	1.85	23.20

The solubility of β-CD is relatively low when compared to that of α and γ; this could be a problem when using the chiral selector as an additive to the background electrolyte. When higher concentrations of β-CD must be used, water-methanol, -ethanol, or -urea mixtures can be successfully used. In fact the solubility of β-CD in aqueous solutions of 4 and 8 M urea increases up to 0.089 and 0.226 M, respectively.[72]

The hydroxyl groups present on the two entrances of CD at position 2, 3, and 6 of each glucopyranose can be modified, by chemical reactions, to produce CD derivatives with different properties than those of the parent ones. By manipulating the reaction type, the operational reaction conditions, and the ratio of the reagents, the modification can be obtained in only one or more than one hydroxyl group. Reaction with hydroxyl groups alters the properties of the CD, allowing

- improved solubility of the CD
- formation of different bonds with analytes that can improve the inclusion-complexation
- analysis of uncharged optical isomers

For example, the solubility of 2,6-di-O-methyl-β-CD and of the negatively charged β-cyclodextrin polymer in water were found to be 57 g/100 ml[73] and >40 g/100 ml, respectively, relatively high in comparison to that of β-CD (1.85 g/100 ml). Furthermore, the cavity of di-OMe-β-CD is deeper than its parent due to the presence of methoxy groups at the entrances, and thus is more hydrophobic. Considering other derivatives like carboxymethyl, methyl amino, phosphate, and sulfate, it is easy to recognize the advantages in analytical chemistry, especially in CE. In fact, such types of CDs can be charged to allow the optimization of the separation, *e.g.*, migration in the opposite direction of the analyte, or introduction of charge to neutral compounds through complexation (for a comprehensive review of CD derivatives, see reference 74).

Inclusion complexation and stereoselectivity are influenced by several experimental parameters, such as CD type and concentration, applied voltage, capillary temperature and length, ionic strength, organic solvent, electro-osmotic flow, and polymeric additives. Some effects of these parameters are discussed in this section, with the aim of describing the appropriate experimental conditions when enantiomers must be separated using cyclodextrins.

CD Type and Concentration

The first requirement for inclusion complexation is fitting the analyte into the CD cavity, and thus CD selection must be made considering the shape of the analyte. As previously discussed, the internal diameters of native CDs increase by increasing the number of glucose units γ>β>α. Thus, selecting the appropriate CD is related to the shape and dimensions of the analyte. As an example,

consider the chemical structure of terbutaline and nicergoline (see Figure 9a and 9b). Terbutaline is currently used in the treatment of asthma diseases, and nicergoline is used as a vasodilator in acute myocardial infarction with diastolic hypertension. Terbutaline contains only one aromatic ring and one asymmetric carbon at the α position, while nicergoline is formed by more than one aromatic ring and possesses several chiral centers.

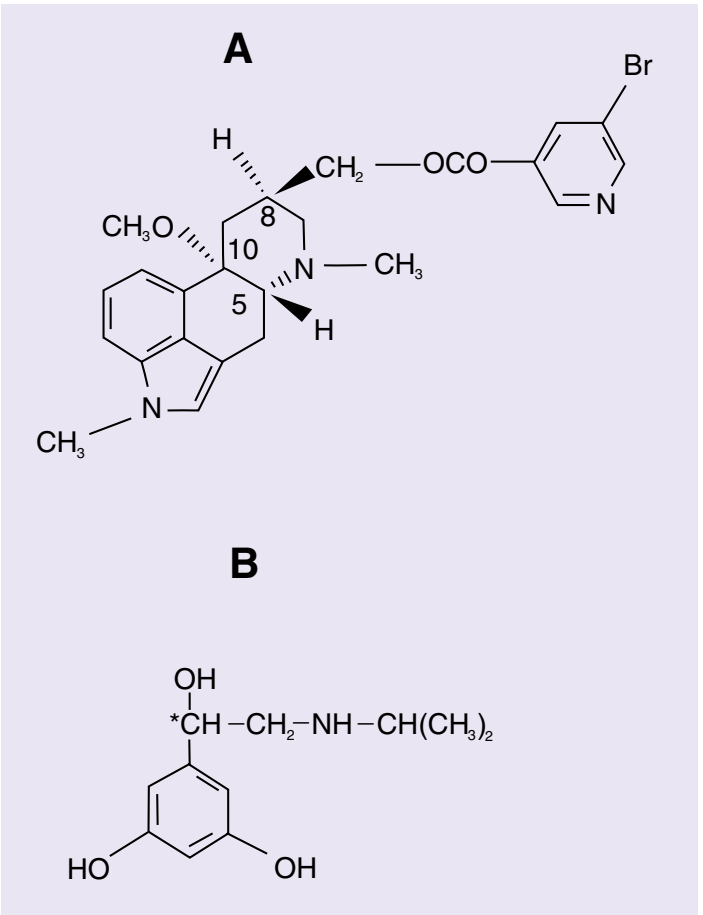


Fig. 9. Chemical structure of (A) nicergoline and (B) terbutaline.

Figures 10a, 10b, and 10c show the electropherograms of the separation of terbutaline and nicergoline into their enantiomers by capillary zone electrophoresis when α, β, and γ-CD were separately added to a BGE at pH 2.5.

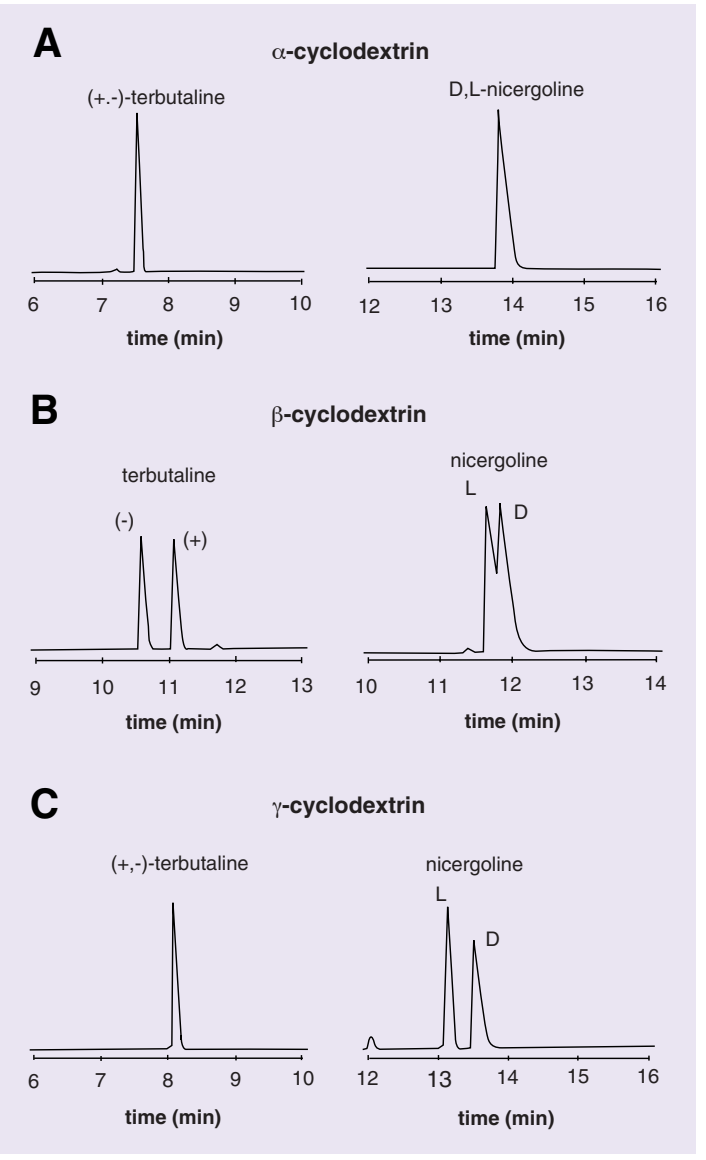


Fig. 10. Electropherograms of the enantiomeric separation of racemic terbutaline and nicergoline using different types of cyclodextrins as chiral selectors by CE. Apparatus, BioFocus 3000 system; capillary, (coated) 35 cm x 0.05 mm ID; background electrolyte, 0.1 M phosphate buffer +a) 30 mM α-CD, b) 20 mM β-CD and c) 30 mM γ-CD; applied voltage 15 kV; injection, electrokinetic 7 kV, 7 s of 5 x 10^{-5} M of racemic terbutaline or nicergoline.

Clearly α-CD was not able to separate both racemic mixtures into their enantiomers because its cavity is too small to guest the two compounds. β-CD allowed the resolution of terbutaline, while nicergoline was partly resolved, and γ-CD was not able to guest terbutaline (the molecule is too small) but was very effective for nicergoline.

In a previous study it has been shown that for the enantiomeric separation of terbutaline, either β-CD and dimethylated-β-CD can be successfully used in CE but the latter forms the strongest inclusion complexes and is more stereoselective than the former. The complexation of terbutaline increased with increasing concentration of both β-CD and its derivative, while the resolution showed a maximum at 15 mM and 5 mM for β-CD and dimethylated-β-CD, respectively.[15] Figure 11 shows the effect of the concentration of β-CD and di-O-methyl-β-CD on the resolution of (+) and (-)-terbutaline.

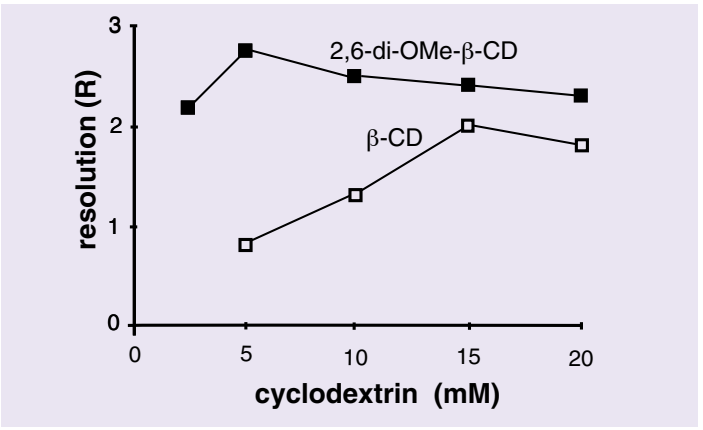


Fig. 11. Effect of the concentration of β-cyclodextrin and di-O-methyl-β-cyclodextrin on the resolution of racemic terbutaline into their enantiomers.

It is important to consider the degree of substitution (DS) of the cyclodextrin used. The effect of DS was demonstrated for the chiral separation of some hydroxyacids using different β-CDs modified with methyl amino[47] or hydroxypropyl groups[75] where the presence of more than one substituent can strongly affect the chiral recognition. The CD type can also influence the migration order of the two enantiomers; this effect was observed for the separation of dansyl-norvaline enantiomers using trimethylated-β-CD and β-CD.[76] Other authors have discussed the influence of the concentration of CD in CZE, indicating that the concentration of the CD can play a very important role for chiral resolution by CE, and thus this parameter should be

carefully controlled in order to find the optimal experimental conditions.[8,34,38] Wren and Rowe discussed the optimum concentration of CD for enantiomeric separation and found that it is dependent upon the formation constants of the diastereoisomers formed during the electrophoresis (equation 1).[77]

$$\textbf{(1)} \quad [C_{CD}] = \frac{1}{[K_{(+)} K_{(-)}]^{1/2}}$$

where C_{CD} is the optimum concentration of the CD used, and K is the formation constants of the two enantiomers (+) and (-). The mathematical model was verified for the enantiomeric separation of propranolol using β-CD or its dimethylated derivative.

Analyte shape

The shape of the analytes is another important parameter to be considered for inclusion-complexation with cyclodextrin. This effect was studied for the resolution of several tryptophan derivatives (methyl, hydroxyl) where the position of the substituent group on the indole ring strongly influenced the stability of the complex formed, and thus the chiral recognition.[30] The shape of the enantiomeric pairs can be modified, for example, by derivatizing with hydrophobic achiral compounds that can be included into the CD cavity. Amino acids as dansyl-, naphthalene-2,3-dicarboxaldhyde- (NDA), and naphthylamide derivatives, and monosaccharides derivatized as naphtalene sulfonate, were resolved into their enantiomers using cyclodextrins.[78–81]

Charge of CD

The charge of the cyclodextrin can play an important role in the resolution mechanism when the electrophoretic separation of enantiomers has to be carried out. In this case the electrostatic interaction with the analytes, the movement of the chiral selector in the opposite direction of the two enantiomers, and the possibility for separating uncharged compounds (inducing the charge) are the main advantages for using such chiral selectors.

The use of charged cyclodextrins was first demonstrated by Terabe,[53] who separated several racemic dansyl-amino acids using a positively charged β-cyclodextrin. In our laboratory we used a methylamino-β-cyclodextrin for the enantiomeric resolution of several α-hydroxyacids[47] and a chargeable β-cyclodextrin polymer (carboxymethylated-β-CD polymer, CM-β-CD pol)[55] for the separation of racemic basic compounds into their enantiomers.

The inversion of migration order of studied enantiomers was demonstrated[55,82] using a charged chiral selector in the presence of electro-osmotic flow; this approach can be advantageously employed when the enantiomeric impurity migrates behind the main isomer. In this case, it was necessary to increase the injected amount of the sample in order to detect the impurity (< 1%), and a loss of resolution occurred.

Electro-osmotic flow, concentration of the chargeable cyclodextrin, and the pH of the BGE have to be controlled to optimize the separation method.

When carboxymethylated-β-CD polymer is used in CE, the weakly acidic carboxylic groups can be charged or uncharged, depending upon the pH of the BGE. At low pH, 2–3, the carboxylic groups of the CD are protonated, the chiral selector behaves as a quasi stationary phase, and the groups form hydrogen bonds with analytes. At pH >3.5, the CD is charged due to the dissociation of the carboxylic substituents and migrates with its own mobility, while forming inclusion complexes and allowing ion-pair interactions with analytes.

Recently a new modified β-cyclodextrin (sulfobutyl-ether(IV)-β-cyclodextrin, SBE-β-CD) has been synthesized and characterized by CE with indirect UV detection.[83] This modified β-CD has been used in CE for the enantiomeric separation of several classes of compounds (positively charged, negatively charged, or uncharged). As shown in Figure 12, the SBE-β-CD contains four modified primary hydroxyl groups (position 6 of four glucose units) with a butyl chain and sulfonic groups. Due to its chemical properties, the modified CD is negatively charged at any commonly used pH in CE. These features allow its use in a charged mode over a wide pH (2–11) range.

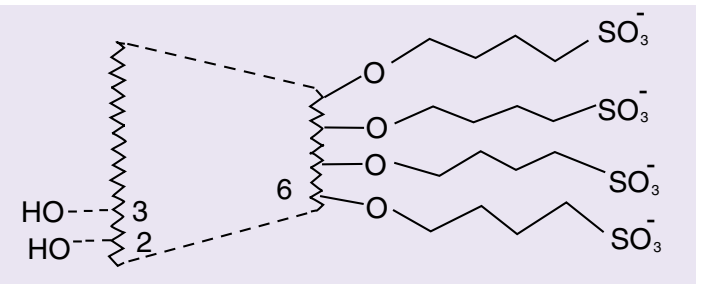


Fig. 12. Chemical structure of sulfobutyl-ether(IV)-β-cyclodextrin (SBE-β-CD).

The chiral selector was used at pH 10, close to the pK of several ephedrines, to obtain good enantiomeric resolution.[48] Operation at pH 2.5 allowed the enantiomeric separation of several basic compounds of pharmaceutical interest, including thalidomide, dimethindene, and mefloquine.[49] Recently we showed the usefulness of this new chiral selector for the enantiomeric separation of several positively and negatively charged compounds of pharmaceutical interest as well as uncharged phenyl-alcohols and dansyl-amino acids.[52] In the case of acidic compounds, ion-pair interactions are not involved in the chiral resolution mechanism, but electrostatic repulsion can be responsible for the different stability constants of the two diastereoisomers formed during the electrophoretic process. Figure 13 shows the electropherograms of the enantiomeric separation of warfarin, bupivacaine, 2-phenyl-2-butanol and dansyl-phenylalanine.

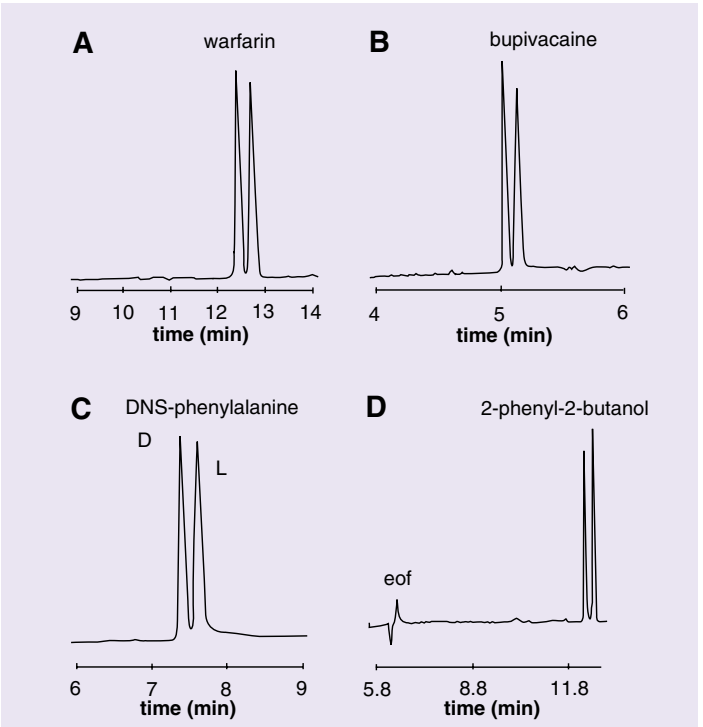


Fig. 13. Electropherograms of the enantiomeric separation of warfarin, bupivacaine, 2-dansyl-phenylalanine, and phenyl-2-butanol. Experimental conditions: apparatus, BioFocus 3000 system; capillary, 50 cm x 0.05 mm ID; applied voltage, 15 kV. Background electrolyte: A and B, 50 mM phosphate buffer (pH 6) and SBE-β-CD 6 and 10 mg/ml, respectively; C, 50 mM Tris/HCl, pH 8, with 3 mg/ml of SBE-β-CD; D, 50 mM borate buffer, pH 9, and 20 mg/ml SBE-β-CD; injection, 5 psi*s 10^{-4} M of each racemic compound.

Composition of the Background Electrolyte

Chiral resolution can be strongly influenced by the composition of the BGE, and thus the selection of the appropriate buffer system, its concentration, its ionic strength, its pH, the presence of polymeric additives, and the content of the organic additive should be carefully considered. Decreasing the ionic strength of the BGE generally causes a reduction of migration time and resolution; peak tailing has also been observed.[34] The negative effect related to the low ionic strength of the buffer is due to electromigration dispersion that can be avoided using a BGE at a concentration 10^2 times higher than that of the sample. The peak shape can be controlled by selecting a co-ion with similar electrophoretic mobility to that of the analyte.

The addition of an organic solvent to the BGE can produce a negative effect on the binding constant of the inclusion-complex with cyclodextrins due to the competition between the organic additive and the analyte. The affinity of the analytes for the organic additive must be considered. However, organic solvents can improve the selectivity of the enantiomeric separation by differential influence on the binding constants of the two enantiomers. Propranolol enantiomers were not separated even using 40 mM of β-CD (urea was added to the BGE in order to increase the solubility of the CD), while separation was obtained when 30% (v/v) methanol was employed as a component of the buffer.[15] Recently we demonstrated that the use of methanol can greatly improve the stereoselectivity of the separation of racemic non-steroidal anti-inflammatory drugs (NSAIDs). Racemic flurbiprofen was baseline resolved into its enantiomers at pH 5 using 30 mM of 2,3,6-tri-O-methyl-β-cyclodextrin, but the drug was poorly resolved when the CD concentration was lowered to 5 mM. The addition of methanol to the BGE containing 5 mM of chiral selector caused an increase in migration time as well as an increase in resolution, allowing baseline enantiomeric separation when the buffer contained 20% organic additive. The effect of methanol on the enantiomeric separation of propranolol and flurbiprofen is shown in Figure 14.

A theoretical model of the effect of organic solvent in the BGE when cyclodextrins are used as chiral selector in CE has been discussed by Wren.[84] When the CD concentration was at or below the optimum value (maximum resolution), the addition of organic solvent (methanol or acetonitrile) caused a reduction of resolution. This effect was due to the change in the formation constants of the inclusion-complexes, modifying the optimum CD concentration.

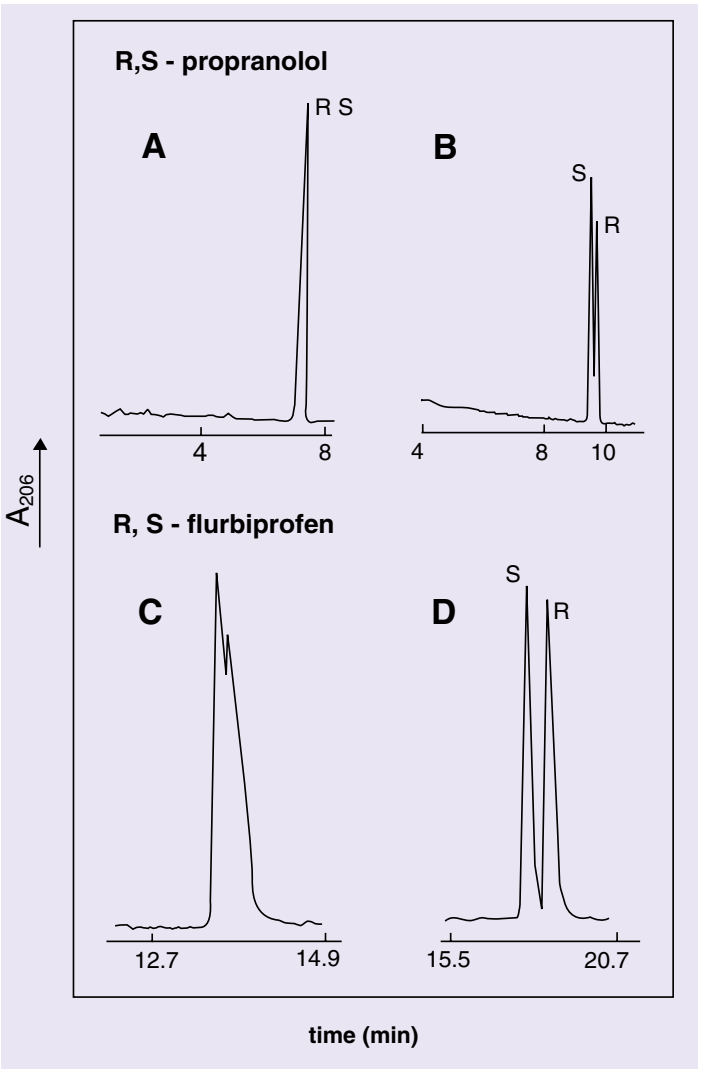


Fig. 14. Effect of methanol added to the BGE on the resolution of R,S-propranolol and R,S-flurbiprofen. A, (without methanol) and **B**, (with 30% methanol); apparatus HPE 100 system, capillary, 20 cm x 0.025 mm ID; background electrolyte, 0.1 M phosphate pH 2.5, 4 M urea and 40 mM β-CD. **C**, (without methanol) and **D**, (with 20 % methanol); apparatus BioFocus 3000 system; capillary, (coated) 36 cm x 0.05 mm ID; 0.1 M MES, pH 5, and 5 mM tri-OMe-β-CD; applied voltage 20 kV.

The influence of the pH of BGE was discussed by several authors and a theoretical model has also been described.[85]

Strong electro-osmotic flow is not recommended for good enantiomeric separation by CE, because the time spent by the analyte for inclusion-complexation equilibrium is reduced. A coated capillary of short length can be used to improve the resolution or to perform the analysis in a reasonable time.

For many applications in the separation of enantiomers of basic compounds, acidic pH values have been used. At low pH the electro-osmotic flow is reduced, increasing the resolution.[29] Similar effects can be obtained by adding polymers such as hydroxyethylcellulose (HEC), hydroxypropylmethylcellulose (HPMC), or polyvinylalcohol (PVA) to the BGE, or by using capillaries coated internally, for example with polyacrylamide. The addition of polymers will reduce the eof and increase the viscosity. In all cases, the adsorption of analytes is also avoided or reduced.

The pH of the BGE not only influences the electro-osmotic flow but can also affect the charge of the analytes, and thus their effective mobility. When the analysis of weakly acidic compounds must be carried out in the presence of electro-osmotic flow and a native CD is used, both CD and analytes (uncharged and charged, respectively) are transported to the detector by the electro-osmotic flow. The migration time of the analyte in the presence of the CD is shorter than in its absence due to inclusion-complexation. Increasing the pH will cause an increase in migration time due to increased analyte charge, and this phenomenon can help the resolution. Of course, for the optimum experimental conditions, the magnitude of the electro-osmotic flow must be considered.

Capillary Temperature

Efficient temperature control is recommended to prevent loss in resolution when cyclodextrins are used as chiral selectors for enantiomeric separations. This can be easily obtained using a BioFocus electrophoresis system, which provides a system for circulating liquid coolant into the cartridge containing the capillary.

Temperature increases cause a decrease in buffer viscosity, and thus a decrease in migration time. Furthermore, a change in the capillary temperature can strongly influence the stability of the inclusion complex formed between analyte and cyclodextrin (generally an increase in temperature causes a decrease in the binding constant).[86] This effect is shown in Figure 15, where the separation of norepinephrine, epinephrine, and isoproterenol enantiomers was obtained using a dimethylated-β-CD.[87]

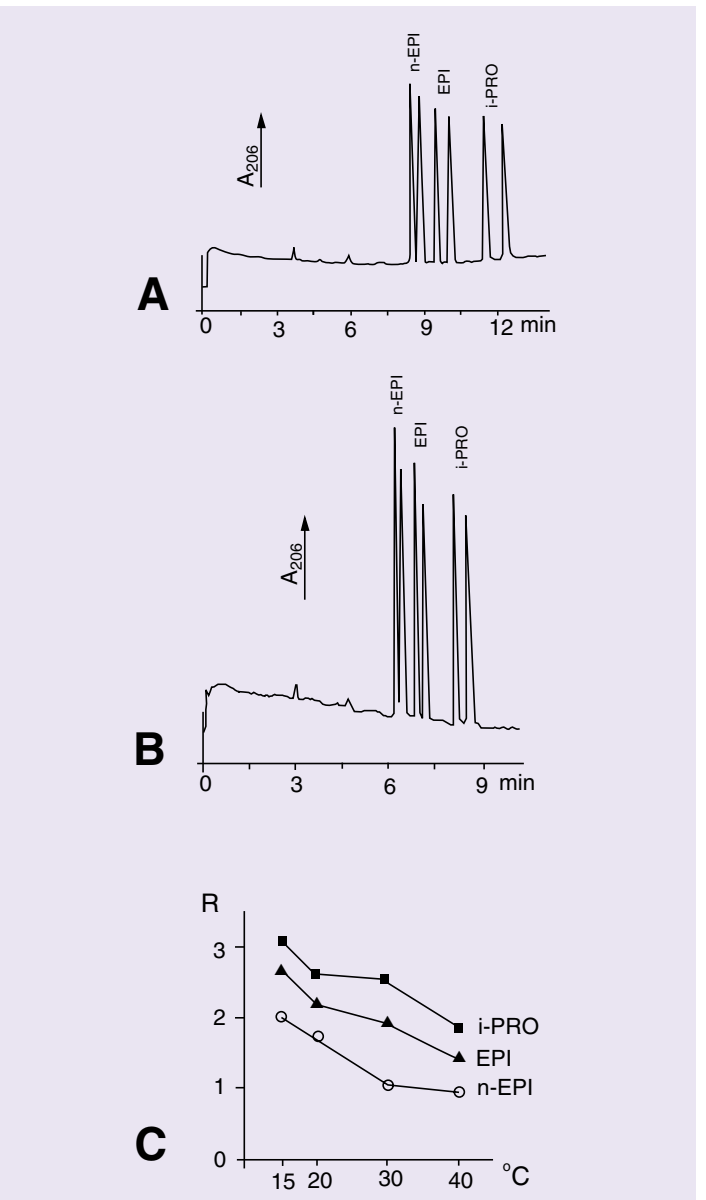


Fig. 15. Influence of the capillary temperature on the resolution of racemic norepinephrine (n-EPI), epinephrine (EPI), and isoproterenol (i-PRO) into their enantiomers. Apparatus, BioFocus 3000 system; capillary, (coated) 30 cm x 0.05 mm ID; background electrolyte, 50 mM phosphate buffer, pH 2.5, and 20 mM of di-OMe-β-cyclodextrin; applied voltage 10 kV; column temperature, **A**) 20 ¡C, **B**) 40 ¡C. **C**) resolution (R) vs. column temperature (modified from reference 87).

Cyclodextrins or their Derivatives in Capillary Gel Electrophoresis, Isotachophoresis, and Electrochromatography

Cyclodextrins or their derivatives have also been used as chiral selectors for enantiomeric separations with other capillary electrophoretic techniques, although there are fewer publications on this than on CZE and micellar electrokinetic chromatography. The use of capillary gel electrophoresis (CGE) for the separation of enantiomers was first described by Guttman et al.[8] A capillary, filled with crosslinked polyacrylamide gel and also containing unbound α- or β- or γ-cyclodextrin, was used for the enantiomeric separation of several dansyl-amino acids. The electro-osmotic flow was suppressed by coating the capillary wall, and the separations were performed at pH 8.3. The CD type and its concentration, as well as the column temperature, influenced the migration time and the resolution of the amino acid derivatives studied. The addition of 10% methanol improved the separation of enantiomers. After this pioneering work, gel filled capillaries were not employed for chiral separations in CE until 1992, when Cruzado[45] used a modified β-CD (allyl carbamoylated-β-CD) copolymerized with acrylamide for the enantiomeric separation of several dansyl-amino acids and homatropine. The gel-modified cyclodextrin presented several drawbacks, including short lifetime and poor reproducibility.

The feasibility of enantiomeric separation using a chiral stationary phase (CSP) in CE has been shown by Mayer and Schurig.[43] The CSP was bound to the capillary wall after static thermal coating with Chirasil-Dex containing monokis-6-O-octamethylene-per-methyl-β or γ-cyclodextrin. The coating stability was tested by gas chromatography and by CE.

The separation of several racemic mixtures into their enantiomers was obtained with a borate/phosphate buffer at pH 7 in presence of electro-osmotic flow. Recently, the enantiomeric separation of both neutral and charged analytes, *e.g.*, benzoin, hexobarbital, and several amino acid derivatives, has been achieved using a β-CD bonded chiral stationary phase (CSP with 5 μm particle diameter employed in HPLC).[88] The type of BGE influenced the direction of the electro-osmotic flow. The results obtained suggested perspectives on the use of a wide number of chiral phases.

Enantiomeric separations have also been performed by capillary isotachophoresis (ITP) employing cyclodextrins or their derivatives as chiral additives to the leading electrolyte (LE). The first paper by Smolkova-Keulemansova's group[89] dealt with the separation of some

pseudo ephedrine alkaloid enantiomers using β-CD or di-OMe-β-CD as a chiral additive to the LE. In this study, the CD type and concentration, and the pH of the LE, influenced the efficiency of the separation. After this pioneering study the same group showed the enantiomeric resolution of some phenothiazines with β- or γ-CD and discussed the importance of the counter-ion type on the ITP separation.[90] Ketotifen and its intermediate enantiomers[91] have also been separated by this group using ITP. Furthermore the possibility of using a coupled column system, in which the two capillaries contained two different cyclodextrins, for a two-step separation has been demonstrated. The authors called the system two-dimensional separation. Unfortunately, this technique did not become popular, even though it offered several advantages over CZE, including a concentrating effect, analysis of compounds in traces, and the possibility of working with larger volumes of sample. Considering the advantages, we are convinced that in the near future ITP will be widely used, at least in combination with CZE, in order to improve the sensitivity of the separation method.

Chiral Crown-Ether

Another class of compounds used for enantiomeric resolution by the inclusion-complexation mechanism is represented by crown-ethers. These macromolecules, discovered in 1967 by Pederson,[93] are able to form inclusion complexes with several inorganic and organic ions in which the guest compound fits the cavity, forming weak bonds (ion-dipole) with the etheroatoms (O, S) of the crown. Here the inclusion-complexation is completely different than that of CDs; the hydrophilic part of the analyte is included, while with CDs it is the hydrophobic part. Although the inclusion of the amino group is necessary for enantiomeric separation, other interactions are required in order to perform chiral recognition. Figure 16 shows the chemical structure of the chiral 18-crown-6-ether tetra carboxylic acid used in capillary zone electrophoresis.

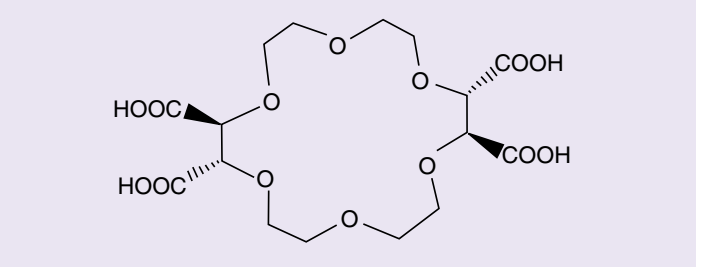


Fig. 16. Chemical structure of 18-Crown-6-ether tetracarboxylic acid.

Two different chiral resolution mechanisms have been proposed by Kuhn[94] in which the four carboxylic groups of the crown were involved in the chiral recognition process, either forming electrostatic interactions or causing a steric barrier effect with the included molecule.

The chemical structure of the analyte plays a very important role for chiral recognition with the modified crown-ether; in fact the presence of a primary amino group is fundamental for inclusion-complexation. For the barrier mechanism, nonpolar substituents on the asymmetric center have a strong influence. For example, naphthylethylamine was resolved, while phenylethylamine was not at all. Enantiomers of aliphatic amino acids with branched chains were better separated than those with linear chains (valine > norvaline). Finally the distance of the amino group from the chiral center has to be considered. Better resolution is obtained with short distances.

Other parameters which must be controlled for the optimization of the chiral separation using chiral crown ether by CZE include the pH of the BGE, the organic additive, and the buffer composition (K^+, NH_4^+, Na^+ ions should be avoided because they can compete with analytes in the inclusion process while Tris seems to be the most effective cation).[94] Applications using 18-crown-6-ether tetra carboxylic acid as chiral selector in capillary zone electrophoresis cover a wide range of amino derivatives, including racemic amino acids,[32] amines and peptides,[56] and amino alcohols and sympathomimetic drugs.[95]

4.2 Ligand Exchange

The ligand-exchange mechanism, first introduced by Davankov in chromatography,[96] was successfully applied in CE for the enantiomeric separation of dansyl amino acids[57,58] and α-hydroxy acids.[59] The separation is based on the interaction between a metal complex with a chiral ligand, added to the BGE, and the two enantiomers to be resolved. L- or D-proline, L- or D-hydroxyproline and aspartame are the ligands which have been employed in CE.

Figure 17 shows the separation of D and L-phenyllactic (3-PhL) acid when Cu(II)-L-hydroxyproline complexes were used as the chiral selector. The two analytes migrate as anions, toward the anode in the coated capillary, while the positively charged metal chelate migrates in the opposite direction. The 3-PhL forms a ternary complex with Cu(II), causing a reduction of the effective mobility of both enantiomers; the two complexes possess different stability constants and thus are separated by the end of the electrophoretic run. The optimization

of the separation method requires the control of several parameters, namely the metal and chiral ligand type, their ratio and concentration, the pH, the ionic strength, the organic additive and the column temperature.

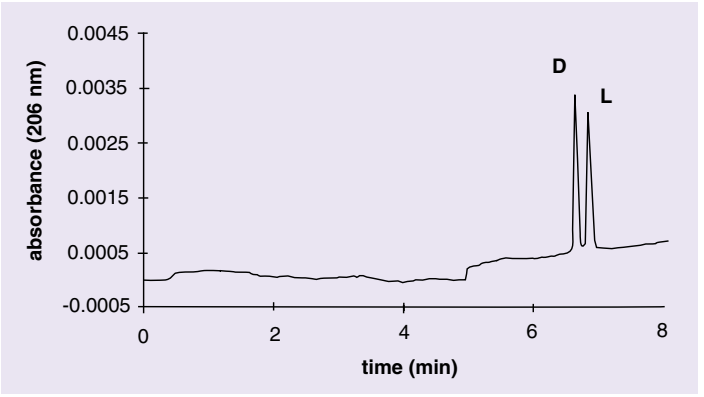


Fig. 17. Electropherograms of the separation of D- and L-phenyllactic acid using a ligand-exchange mechanism by CE. Apparatus, BioFocus 3000 system; capillary, (coated) 35 cm x 0.05 mm ID; background electrolyte, 0.02 M phosphate buffer, pH 4.4, and copper(II) acetate (2 mM)/ L-hydroxyproline (4 mM); applied voltage 15 kV, 15 A; injection 10 psi*s of 10^{-4} M of racemic 3-phenyllactic acid; carousel and capillary temperature, 25 ¡C.

One drawback that can be expected when ligand exchange is used in CE is the UV absorption of the metal complex, which reduces the sensitivity of the method. This drawback can be negated by using different approaches, *e.g.*, by filling only part of the capillary or by using a different detector such as laser induced fluorescence[57] or conductivity.

4.3 Chiral Micelles

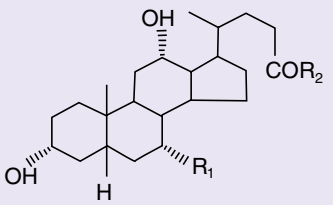
The use of micelles in capillary electrophoresis was first introduced by Terabe *et al*. for the separation of neutral compounds. They called this technique micellar electrokinetic chromatography (MEKC).[97] Surfactants such as sodium dodecyl sulfate (SDS) were added to the aqueous solution in order to improve the selectivity of the separation of neutral analytes. With neutral-alkaline solutions, the electro-osmotic flow is relatively high and the bulk solution (analytes and micelles) moves toward the cathode. The migration of the negatively-charged micelles is retarded relative to neutral compounds. The analyte interacts with the two phases (aqueous and micellar) and thus its

migration is also retarded. The extent of the retardation is a function of the percentage of analyte distributed into the micellar phase.

Chiral recognition can be achieved in MEKC by including a chiral selector in the micellar system, either bound to the micelles or added to the electrolyte.

Sodium N-dodecanoyl-L-valinate (SDVal) and sodium N-dodecanoyl-L-alaninate (SDAla) were applied as chiral micelle selectors for the separation of several amino acid derivatives, namely 3,5-dinitrobenzoyl, 4-nitrobenzoyl and benzoyl-O-isopropyl esters.[98] SDVal was also used for the enantiomeric separation of phenylthiohydantoin amino acids by Terabe's group. Addition of methanol to the SDVal micellar solution as an organic modifier improved resolution, and the addition of urea significantly improved peak shapes.[99,100]

Other very interesting chiral micelles are represented by bile salts, introduced for the first time in MEKC by Terabe *et al*.[7] These surfactants, which possess a steroid nucleus and a side chain carrying a carboxylic group that can be conjugated with taurine, have both hydrophobic and hydrophilic properties. When taurine is conjugated, due to the presence of sulfonate groups, the chiral selector is charged even at low pH. Figure 18 shows the chemical structure of the main bile acid salts used in CE.



Bile acids (sodium salts)	Abbreviation	R_1	R_2
Cholate	SC	OH	ONa
Taurocholate	STC	OH	$NHC_2H_4SO_3Na$
Deoxycholate	SDC	H	ONa
Taurodeoxycholate	STDC	H	$NHC_2H_4SO_3Na$

Fig. 18. Chemical structure of bile acid salts.

Sodium taurocholate (STC) and taurodeoxycholate have been used successfully as chiral selectors for the separation of several dansyl amino acids into their enantiomers.[7] The reduction of the electro-osmotic flow by decreasing the pH to 3 produced good separations not obtainable under other experimental conditions. The same class of chiral surfactants proved to be useful chiral selectors for the separation of several racemic drugs into their enantiomers, *e.g.*, diltiazem and trimetoquinol,[61,101] fonoldopam, 4-hydroxymephenytoin,[102] laudanosine,[101] tetrahydropapaveroline,[103] naphtol and binaphthyl derivatives.[62] Digitonin,[100] glycirrhizic acid, and β-escin[104] are natural surfactants which have also been used for chiral separations at a relatively low pH, and also combined with SDS in mixed micelle systems.

A very promising approach in MEKC is the use of chiral micelle polymers. Poly(sodium N-undecylenyl-L-valinate) has been shown to be a good chiral selector for the separation of 1-1'-bi-2-naphtol and laudanosine into their enantiomers. The advantages of such polymers over the monomers are their enhanced stability and rigidity. Furthermore, since the polymicelles have no CMC, they can be used at any concentration. Resolution was influenced by the polymer concentration and the pH. At low pH the charged polymerized micelles have a compact conformation, while at higher pH they lose it.[105]

The addition of cyclodextrins to the micellar phase (CD-modified MEKC) enables the enantiomeric separation of uncharged compounds which could also be analyzed with charged cyclodextrins or with electrochromatography.

4.4 Affinity Interactions

Proteins as Chiral Selectors

Proteins are natural biopolymers with helical conformation able to interact selectively with a wide number of compounds of small size, such as pharmaceuticals. They have been used successfully as chiral selectors in capillary electrophoresis. The enantiomeric separation is based on stereoselective interactions between the protein and the two analytes, with the formation of two labile diastereoisomeric complexes during the electrophoretic run. Tanaka *et al.*[64] discussed a theoretical model for enantiomeric separation with proteins where the electrophoretic mobility of the free analyte (μ_s) and of the protein (μ_p), and the concentration of the protein (P), are correlated by the following equation:

$$\mathbf{(2)} \quad \mu_{app} = \mu_{eo} + \frac{\mu_s + \mu_p K[P]}{1 + K[P]}$$

where μ_{app}, μ_{eo}, and K are the apparent mobilities of the analyte, electro-osmotic flow, and binding constant, respectively.

Calculating $\Delta\mu_{app} = \mu_{app1} - \mu_{app2}$, the following equation provides the optimum concentration of protein for the best chiral resolution (see equation 3)

$$\mathbf{(3)} \quad [P]_{op} = (K_1 K_2)^{-1/2}$$

The theoretical model is similar to that discussed by Wren, where an uncharged chiral selector was used.[77] The Tanaka model was verified for the enantiomeric separation of several acidic compounds using avidin as a chiral selector. Avidin, a basic protein with a pI of 10.0–10.5, is positively charged at acidic/neutral pH and migrates in a direction opposite that of the analytes.

When proteins are used as chiral selectors in CE, several drawbacks have to be expected. The protein may adsorb on the capillary wall, the sensitivity in the low UV region can be reduced due to strong absorbtion of the chiral selector, and the protein may not be stable under the operating conditions. Coated capillaries were used in order to reduce the adsorption of the protein to the wall and to reduce the electroosmotic flow.

Figure 19 shows schematically the separation principle of affinity EKC for analysis of an acidic compound with avidin at acidic pH.

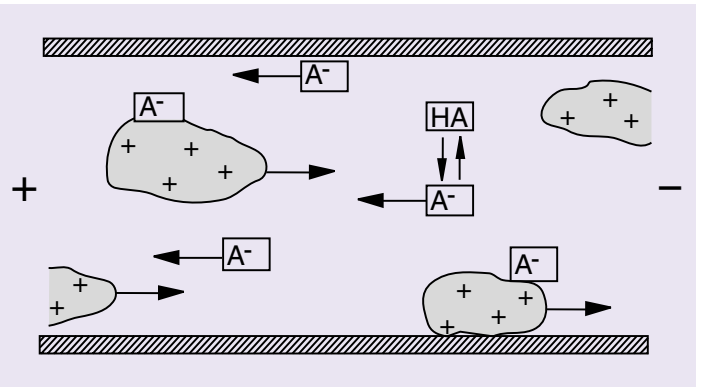


Fig. 19. Electrophoretic separation scheme for anions using avidin as a chiral selector (with permission from reference 48).

The separated enantiomeric compounds were vanilmandelic acid, warfarin, ibuprofen, ketoprofen, flurbiprofen, and the two diastereoisomers of folinic acid. Several parameters were investigated, *e.g.*, the concentration of the protein, the pH of the BGE, the content of the organic solvent and the capillary temperature. As shown in Figure 20, increasing the concentration of avidin from 0 to 25 μM gave better resolution of the enantiomeric pair of vanilmandelic acid at pH 4.

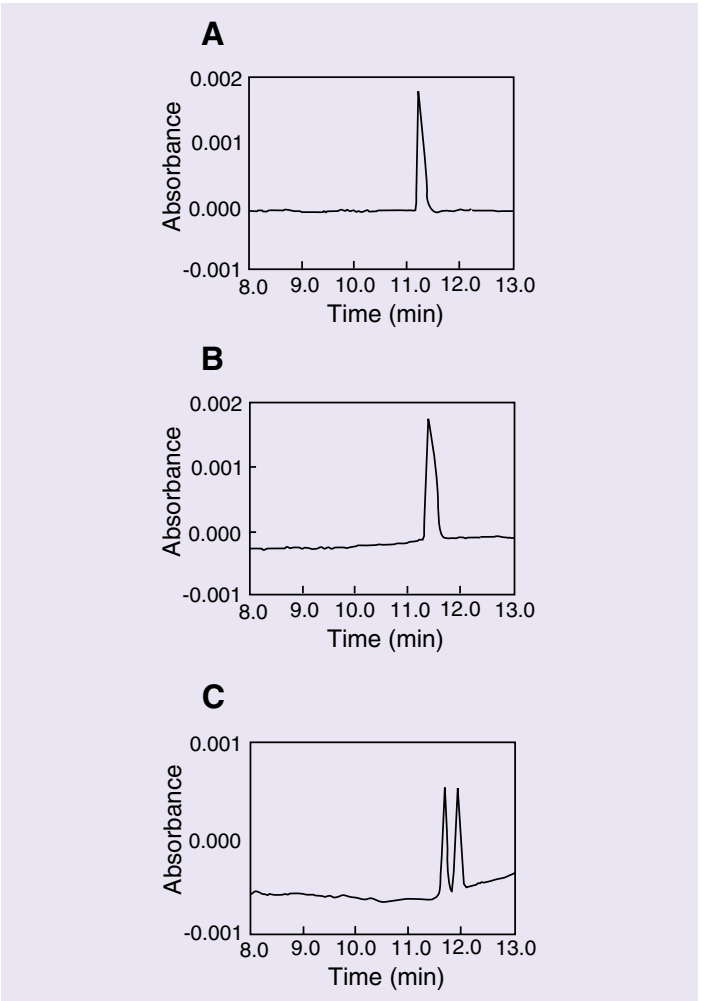


Fig. 20. Effect of the concentration of avidin on the enantiomeric separation of racemic vanilmandelic acid. Apparatus, BioFocus 3000 system; capillary, (coated) 36 cm x 0.05 mm ID; background electrolyte, 0.05 M phosphate buffer, pH 4, and avidin **A)** 0 M; **B)** 10 M and **C)** 25 M; applied voltage -12 kV; detection at 240 nm; injection, 1 psi*2s (sample 0.4 mg/ml five fold dilution) (with permission from reference 48).

It is worthwhile to mention here an interesting approach used by Valtcheva et al., employing the enzyme cellulase for the enantiomeric separation of several pharmaceutical compounds such as propranolol, pindolol, metoprolol, and labetalol.[10] The chiral selector was negatively charged at the operating pH (pH=5.1), and the analytes migrated in the direction opposite to the enzyme. To optimize the method, the detector window was chiral selector-free, improving the detection limit and allowing use of a relatively high concentration of buffer (0.4 M) supplemented with 2-propanol.

Interactions with Saccharides (Linear Polymers) and Antibiotics

Linear polymers of α-(1–4)-linked D-glucose, termed maltodextrins, have proven to be good chiral selectors in CZE for the enantiomeric separation of non-steroidal anti-inflammatory drugs 2-APA NSAIDs (flurbiprofen, ibuprofen, carprofen, suprofen, and indoprofen) as well as coumarinic anticoagulant drugs.[60,106]

An anionic biopolymer, heparin, was studied for the enantiomeric separation of several drugs including antimalarials and antihistamines. Heparin is a di-, tetra-, or hexasaccharide composed of uronic acid and glucosamine with a helical conformation in aqueous solutions and a molecular weight of 10–30,000. The enantiomeric separations were obtained at pH 4 and 5 and the chiral recognition was influenced by the size of the analyte and by the electrostatic interactions.[107]

Another interesting class of compounds that can be used in CE as chiral selectors for enantiomeric separations is antibiotics. Vancomycin, a glycopeptide, was effective for the separation of enantiomeric dipeptides D-Ala-D-Ala/L-Ala-L-Ala and their acetyl derivatives[108] and for the separation of racemic derivatized tri- and tetrapeptides;[10] in both studies the binding constants have been calculated. A recent paper demonstrated the applicability of the macrocyclic antibiotic rifamycin b for the separation of chiral compounds by affinity electrophoresis.[110] Indirect UV detection was used because the antibiotic had strong absorption at low wavelengths.

4.5 Combination of Chiral/Chiral or Chiral/Achiral Selectors

Improvement in the selectivity of an enantiomeric separation can be obtained in CE by employing mixed selector systems. This approach consists of the use of a mixture of either chiral/achiral or chiral/chiral compounds. In a wide number of chiral separations, the combination of cyclodextrins with micelles was effective for the resolution of several uncharged compounds. Figure 21 shows schematically the electrophoretic separation when CD-MEKC is employed.

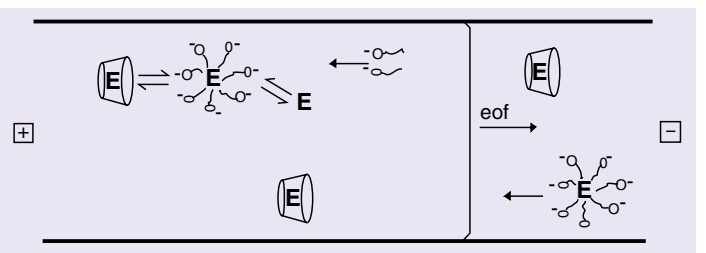


Fig. 21. Scheme of the electrophoretic separation when CD-modified MEKC is used.

= cyclodextrin = SDS **E** = analyte = micelles

The system is composed of an ionic micelle (usually SDS) and an aqueous phase (buffer + cyclodextrin). The analytes move with the eof, and are distributed between the micellar and the aqueous phase. The stereoselectivity is due to the interactions with CDs. The higher the interaction of the analyte with the chiral selector, the lower the migration time.

The selectivity can be modified by controlling several parameters, including surfactant type and concentration, CD type and concentration, organic additive, buffer type and concentration, and pH.

CD-MEKC allows the separation of enantiomers of amino acids, as dansyl-[78] or naphthalene-2,3-dicarboxaldehyde (NDA) derivatives,[111] using β- and/or γ-CD, as well as compounds of pharmaceutical interest, such as mephenytoin and its hydroxy derivative in urine.[112]

Figure 22 shows the electropherogram of an extracted undeglucoronidated urine sample from an extensive metabolyzer (phenotyping). The presence of S-4-hydroxymephenytoin and R-mephenytoin was confirmed by spiking the sample mixture with the racemic

compounds (Figure 22 A and B) and comparing the UV spectra of the pure enantiomers with the separated peaks (Figure 22 D and E).

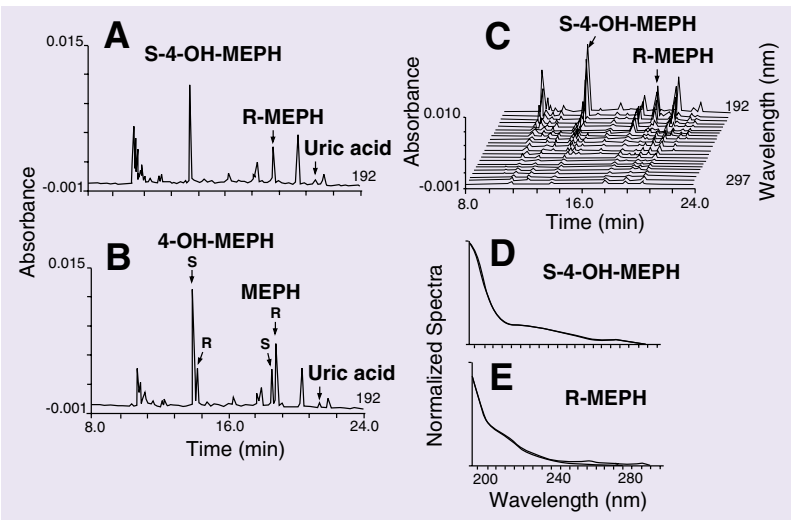
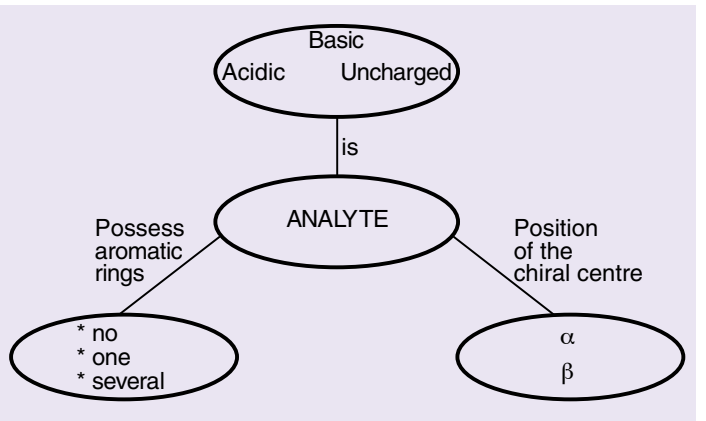


Fig. 22. Electrophoretic separation and characterization of S-4-hydroxymephenytoin (S-4-OH-MEPH) and R-mephenytoin (R-MEPH). Undeglucoronidated urine sample from an extensive metabolizer. Single and multiwavelength detection (**A** and **C**, respectively); in **B** the sample from **A** was spiked with the racemic compounds, while in **D** and **E** the spectra of the two detected compounds in the sample are compared with those of the pure enantiomers. (with permission from reference 112).

Another example of combining two chiral selectors, namely β-CD and L-tartrate, has been shown by us for the separation of several stereoisomers of cobalt(III) complexes with ethylenediamine and amino acids like Gly, Pro, and Phe. The L(+)-tartrate alone did not allow the resolution of the enantiomers of $Co[(en)_2D\text{-}Phe]^{2+}$ and $Co[(en)_2L\text{-}Phe]^{2+}$ and the addition of β-CD provided satisfactory results. In this separation both inclusion- and outer-sphere complexation are involved in the separation mechanism.[68]

Section 5
Optimization of the Method

When a racemic mixture has to be separated into its enantiomers, it is necessary to consider several parameters in order to select the optimum electrophoretic experimental conditions. First the chemical structure has to be inspected to determine the following information:



When basic compounds must be analyzed (this case represents a wide number of pharmaceutical compounds), an acidic pH of the BGE is selected and the separation can be performed either in a coated or uncoated capillary. The recommended buffers are phosphate or citrate at pH 2.5 and 3.5, respectively (50–100 mM). In some cases a pH one unit lower than the pK_a of the enantiomers allows the migration of analytes as cations, and a pH higher than 2.5–3.5 could be considered.

The use of a pH >4–5 can be helpful because it enables the enantiomeric separation and allows sufficient electro-osmotic flow for the analysis of achiral impurities in the sample. Moreover the electro-osmotic flow should be controlled because it reduces the migration time and thus minimizes the time spent by the analytes in contact with the chiral selector.

Increasing the BGE concentration generally improves the resolution of enantiomers, but increases the migration times due to ion-pairing effects and reduction of electro-osmotic flow. Furthermore, peak-tailing and electromigration dispersion can be avoided. An upper limit for buffer concentration is dictated by the increase in current and resulting Joule heating which reduces the efficiency of the separation. Use of a zwitterionic buffer can be considered as a good solution to this problem.

When the enantiomeric separation of negatively charged compounds must be performed, the general rules for basic compounds must be considered. Selecting a pH for the BGE in the range 4.5–8 will charge (negatively) the two enantiomers and produce a sufficient electro-osmotic flow for movement of the analytes toward the cathode. The use of a coated capillary permits operation in a reversed polarity mode, and inversion of migration can be obtained. This

approach can be advantageously used when a minor component in a mixture of enantiomers has to be quantified.

The separation of uncharged enantiomers does not represent a problem in CE, because different electrophoretic mechanisms and chiral selector types are available. With uncharged analytes, a relatively strong electro-osmotic flow is necessary to perform the analysis within a reasonable time, and a charged chiral selector should be employed. Carboxymethylated or ethylated-β-cyclodextrin, sulfobutyl-ether-β-cyclodextrin, or a charged β-cyclodextrin polymer could be effective for chiral recognition. By inducing a negative charge, a selective increase in the migration times allows separation of the enantiomers.

Another approach for the enantiomeric separation of uncharged compounds is the use of surfactants with chiral selectors, *e.g.*, SDS-cyclodextrins. The partition of the analytes into the micellar phase causes a selective migration of neutral compounds, while the chiral selector allows chiral resolution. A buffer at a pH in the range 6–9 containing 25–100 mM of SDS supplemented with variable amounts of cyclodextrins is recommended. The effective mobility should be controlled by changing the composition of the buffer, to select the appropriate experimental conditions. The electro-osmotic flow should be controlled, and in some cases the addition of organic solvents can help the separation of enantiomers. If the uncharged compounds cannot be resolved into their enantiomers, different chiral selectors should be employed, *e.g.*, bile salts.

The capillary temperature should also be controlled when performing enantiomeric separations because, in general, an increase in temperature will result in a reduction of resolution. This parameter influences both the viscosity of the BGE (reduction of migration time) and mass transfer kinetics of analytes and chiral selectors. Thus preliminary studies should be carried out at 20–25 °C, and then a lower temperature should be used to optimize the chiral separation.

The capillary length can also influence the resolution of two enantiomers, and thus its selection should be done considering parameters such as applied voltage and capillary type. The use of longer capillaries can increase the resolution as well as the analysis time. When coated capillaries are used and the electro-osmotic flow is reduced or eliminated, good enantiomeric separations can be obtained even with capillaries of 20 cm length. For coated capillaries, lengths in the range of 20–35 cm are recommended, while with uncoated capillaries 50–70 cm lengths should be tried.

Selection of the Chiral Selector

The most simple chiral selectors that can be used for enantiomeric separation by CE are represented by native cyclodextrins because they are commercially available, not expensive, and several applications have been published.

If the analyzed compound does not contain aromatic rings, α-cyclodextrin or its derivatives can be selected. The same choice could be made if the analyte contains only one aromatic ring, unless substituents in ortho or meta position are present. Such substituents will cause steric hindrance effects, responsible for poor inclusion complexation.

When the compounds possess in their structure two aromatic rings (*e.g.* condensed), β-CD has to be selected, while for more than two aromatic groups γ-CD could be the appropriate chiral selector.

If successful enantiomeric separation cannot be achieved using native cyclodextrins, the wide number of modified ones (*e.g.*, dimethylated, trimethylated, hydroxypropylated) should be considered. Charged cyclodextrins may also be investigated, selecting the appropriate electrophoretic mechanism and experimental parameters.

As a general suggestion, run the compound initially in the absence of CDs and calculate the effective mobility. Then perform the run with the same BGE, containing a relatively low amount of CD (2.5–5 mM) in order to verify a decrease in effective mobility. If complexation is observed but insufficient resolution is obtained, increase the concentration of the chiral selector until satisfactory separation is achieved. In the case of β-CD, concentration screening is limited by its solubility (< 20 mM); solubility can be increased by addition of urea (4–8 M) or methanol or ethanol (<30% v/v) to the BGE. Alternatively, modified cyclodextrins can be selected to improve the solubility or to introduce different secondary stereoselective bonds.

When compounds containing primary amines in their chemical structures, (*e.g.*, amino acids, peptides, drugs) have to be separated into their enantiomers, 18-crown-6-ether tetracarboxylic acid can be used advantageously. In this case attention should be paid to the selection of the BGE (pH and cation type, the latter should not compete with analytes in the inclusion-complex mechanism).

Bile salts with planar structures, namely sodium cholate, taurocholate, deoxycholate, and taurodeoxycholate, are recommended for chiral compounds with rigid skeletons. Optimization of the enantiomeric separation can be performed by modifying those parameters

involved in micellar systems, *e.g.*, buffer type and concentration, concentration of the bile salt, pH, and organic solvent.

For the enantiomeric separation of amino acids or hydroxy carboxylic acids, cyclodextrins are the simplest chiral selector to be used; however a ligand exchange electrophoretic system can also be considered. Copper(II)-amino acid or aspartame complexes could be selected, but unfortunately the detection limit is reduced due to the absorption of the chiral BGE.

Section 6
Quantitation

Quantitative analysis of chiral compounds is a very interesting and important topic that, although it has been shown to be possible by CE, unfortunately up to now is not yet well established. However, considering the good results obtained by several authors, the applicability of CE will be extended.

When a racemic mixture of a certain compound is analyzed by CE using an appropriate chiral selector, it should be separated into two peaks whose area ratio should be 1.00. The electrophoretic analysis of a racemic mixture of epinephrine[14] showed two peaks with two different areas (49% of (-)-epinephrine and 51% of its antipode). In order to explain the observed differences, we have to consider that during the electrophoretic process the formation of two diastereoisomers takes place, and due to the different association constants, a different UV absorbance co-efficient could be expected.[14] Furthermore, the analyte residence time in the detection path is different, the second enantiomer spending a longer time than the first one because it possesses a lower velocity.[113] In order to solve this problem, it has been suggested that the measured areas be normalized to migration time,[14,113] and/or that an internal standard (I.S.) be used.[14]

It has been shown that CE can be successfully used for quantitation of chiral impurities of drugs with good precision and accuracy. Quantitative analysis of trimetoquinol enantiomers by MEKC has been carried out with STDC as a chiral selector. The calibration graph was linear for concentrations of the R-antipode in the range 2.5–15% and less than 1% of this compound could be detected.[101]

Peterson and Trowbridge performed quantitative determination of (-)-epinephrine/(+)-epinephrine with and without an internal standard (pseudopehedrine). The correlation coefficient and the RSD were found to be 0.9998 and 1.3%, respectively using the I.S., while

without I.S. they found 0.9992 and 3.3%, respectively. The authors analyzed a pharmaceutical formulation with different ages (5, 10, and 29 months) and found the presence of (+)-epinephrine in the range 1.2–2.3% (this antipode should not be present at all).

The analysis of the antiviral compound 2'-deoxy-3'-thiacytidine (BCH189), a nucleoside analog containing 0.3% of the undesired (-)-antipode, was done using a BGE at pH 2.5 and 50 mM of di-OMe-β-CD with a detection limit < 0.1%.[114]

A capillary electrophoretic method has been validated for the determination of the chiral purity of fluparoxan. Detector linearity was quite good in the studied range 1.5–125% of a target concentration (1.25 mg/ml) and the limit of quantitation and detection were found to be 1% and 0.3%, respectively. The concentration of β-CD used for the method was relatively high (150 mM) and thus urea and isopropanol were added to the BGE.[115]

Section 7
Applications

Chiral capillary electrophoresis has been applied, until now, mainly for enantiomeric separation of compounds of pharmaceutical interest. Resolution mechanisms, binding constants, and parameters affecting chiral analysis have been widely discussed. Several applications dealing with biological fluids are also available, as well as those dealing with quantitative analysis. The former topic is only at the beginning, and we are convinced that in the near future will be extensively developed.

For simplicity and to avoid repetition of some information given previously, Table 2 presents selected applications for enantiomeric separation of compounds of pharmaceutical interest.

Table 2. Selected Applications on the Enantiomeric Separation of Drugs

Compound	Chiral selector	CE type	References
adrenaline	di-OMe-β-CD	CZE	14, 116
atenolol	di-OMe-β-CD tri-OMe-β-CD	CZE	41, 42
basic drugs (terbutaline, isoproterenol, metaproterenol, synephrine, metanephrine, salbutamol, epinephrine, norphenylephrine, ephedrine, ψ-ephedrine, octopamine,)	macrocyclic antibiotic (rifamycin B)	CZE	67
basic drugs (nor-phenylephrine, ketamine, nor-ephedrine, ephedrine, epinephrine, terbutaline, propranolol)	negative β-CD polymer	CZE	55
basic drugs (atenolol, bupivacaine, ephedrine, homatropine, ketamine, metoprolol, N-methylpseudoephedrine, norephedrine, octopamine, pindolol, terbutaline)	(S)-N-dodecanoylvaline (S)-N-dodecoxy-carbonylvaline	MEKC	117
bupivacaine	di-OMe-β-CD	CZE/MEKC	40
carvediol	di-OMe-β-CD	CZE	40
chloramphenicol	di-OMe-β-CD	CZE	38
chlorpheniramine	β-CD	CZE or MEKC	118, 119
cicletanine	γ-CD	MEKC	120
clenbuterol	β-CD	CZE	34
clenbuterol	SBE-β-CD	CZE	49
cyclophosphamide	glycoprotein	affinity	121
diltiazem	STDC	MEKC	103
dimethindene	SBE-β-CD	CZE	49
ephedrine	di-OMe-β-CD	CZE	13
epinephrine	di-OMe-β-CD	CZE	13, 116
ergot alkaloids (lisuride, meluol, nicergoline,terguride)	γ-CD	CZE	36
herbicides (phenoxy acids)	α-, β-, γ-, di-OMe-β-CD	MEKC	122
hexobarbital	Chirasil-β-CD	EHC	123
mefloquine	SBE-β-CD	CZE	49
mephenytoin hydroxy metabolite	SDS-β-CD β-CD-STDC	MEKC	102, 112
methotrexate	vancomycin	CZE	66
mianserine	SBE-β-CD	CZE	49

(continued on the next page)

Table 2. *(continued)*

Compound	Chiral selector	CE type	References
naproxen	HP-β-CD	CZE	124
NSAIDs (APA) (flurbiprofen, indoprofen, carprofen, ketoprofen, suprofen, fenoprofen, ibuprofen, naproxen)	vancomycin	CZE	66
NSAIDs (APA) (flurbiprofen, ketoprofen, fenoprofen, ibuprofen, suprofen)	tri-OMe-β-CD	CZE	125
NSAIDs (APA) (ibuprofen, cicloprofen, flurbiprofen, carprofen)	Chirasil-β-CD	EHC	123
NSAIDs (APA) (warfarin, ibuprofen, ketoprofen, flurbiprofen)	avidin	affinity	64
octopamine	di-OMe-β-CD	CZE	87
quinagolide	crown-ether	CZE	94
racemethorphan, racemorphan	SDS-β-CD	MEKC	126
salbutamol (and its chiral and achiral impurities)	di-OMe-β-CD	CZE	127
thalidomide	SBE-β-CD	CZE	33, 49
trimetoquinol and its isomers	dextran sulfate	affinity MEKC	128
trimetoquinol	STDC	MEKC	103
warfarin	BSA	affinity	63
warfarin	SD-Val	MEKC	129
warfarin	SBE-β-CD	CZE	52
warfarin	di-OMe-β-CD	CZE	130

Section 8 Conclusions

The enantiomeric separation of different classes of compounds can be obtained easily and rapidly by CE techniques, with good reproducibility and at a relatively low cost. Direct and indirect separation methods can be successfully applied, but the former seems to be the most popular due to its simplicity.

Cyclodextrins have become the most popular chiral selectors in CE, since they are stable and quite soluble in the buffers commonly

used in CE, and because the faster equilibration of the CD-solute complex provides high efficiency and good peak symmetry. The hydroxyl groups of the CD (primary and secondary) can be easily modified, using chemical reactions, to obtain CDs with different properties, thus extending the application range by allowing different electrophoretic separation mechanisms. However, several other chiral selectors are available, *e.g.*, chiral crown ethers for compounds containing amino groups, proteins, chiral surfactants, saccharides, and recently, antibiotics. It has been shown that the enantiomeric resolution is strongly influenced by several parameters, including chiral selector type and concentration, composition of the BGE (ionic strength, ion type and concentration, pH, organic solvent), polymeric additives in the BGE, applied voltage, and capillary temperature.

The advantages of CE over other separation methods, *e.g.* HPLC, include higher efficiency, shorter analysis time, and lower costs (only a few μL of chiral buffer are required). Poor detection limits and limited preparative capability are the main disadvantages not yet resolved; in fact the loading of relatively high amounts of sample is not possible in CE. Some solutions to this problem have been shown, *e.g.*, the combination of ITP and CE.

Section 9
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