16
Chiral Separations by Capillary Electrophoresis

16.1 Introduction

The potential of CE to achieve chiral separations is described in this chapter. With this aim, the principles of chiral recognition in CE, the most employed CE modes for chiral analysis, the specific strategies to be successful in the CE–MS coupling, and some of the most recent and relevant applications developed in different representative fields are discussed together with the expected future perspectives of chiral analysis by CE.

16.1.1 Chirality and its Consequences

Enantiomers are molecules that are identical in their atomic composition and bonds but differ in the three-dimensional arrangement of their atoms and are nonsuperimposable mirror images. An equimolar mixture of enantiomers is called a racemate. These compounds have essentially identical physical (except optical rotation) and chemical (except in a chiral environment) properties. Their structure usually consists of an atom (either carbon, nitrogen, or sulfur) surrounded by at least four different functional groups, which is the asymmetric or chiral center. For chemists, the R/S system is the most important nomenclature for denoting enantiomers (IUPAC systematic nomenclature). It labels each chiral center as R or S according to a system that assigns its substituents a priority according to the Cahn–Ingold–Prelog priority rules based on atomic number [1]. If the center is oriented so that the lowest priority of the four is pointed away from a viewer, the viewer will then see two possibilities: if the priority of the remaining three substituents decreases in clockwise direction, it is labeled R (for Rectus, Latin for straight), if it decreases in the counterclockwise direction, it is S (for Sinister, Latin for left). In the case of biomolecules such as amino acids and sugars, they are commonly described as D- and L-isomers with reference to the spatial configuration of their atoms (named after Latin Dexter and Laevus, right and left). Natural amino acids are L-isomers which follow an S configuration,
while the natural sugars are D-isomers which follow an R configuration. Finally, an enantiomer can also be named by the direction in which it rotates the plane of polarized light. If it rotates the light clockwise, that enantiomer is labeled as (+). Its mirror image is labeled as (−). Note that there is no relationship between the rotation sign and the sequence of the substituents, these being (R, +), (R, −), (S, +), and (S, −).

Chiral recognition phenomena play an important role in living systems because chiral bioactive compounds interact in a stereospecific way with each other. On a molecular level, chirality represents an intrinsic property of the “building blocks of life,” such as amino acids and sugars, and therefore, of peptides, proteins, and polysaccharides. As a consequence, metabolic and regulatory processes mediated by biological systems are sensitive to stereochemistry and different responses can be often observed when comparing the activities of a pair of enantiomers. Thus, enantiomers are considered different compounds because they can have different properties in a biological context.

The interest in chirality and its consequences due to scientific reasons is not a new phenomenon. However, during the last two decades economic interests have been the essential driving force in the spectacular development of new chiral substances, being important contributors to the pharmaceutical industry. There is a broad range of examples where the enantiomers of drugs show differences in terms of their bioavailability, distribution, metabolic, and excretion behavior. Thus, enantiomers of a drug can differ markedly in potency, toxicity, and may have distinct biological interactions and, consequently, different pharmacological or toxicological activities [2]. In fact, very often one of them produces the desired therapeutic activities (eutomer), while the other one (distomer) might be inactive, active in a different way, acting as antagonist, producing unwanted side effects, and even displaying toxicity. In view of this, since 1992 the Food and Drug Administration (FDA) in USA and the European Committee for Proprietary Medicinal Products require that the properties of each enantiomer are studied separately before decisions are taken to market the drug as one of the enantiomers or as a racemate [3], and in the case of drug substances developed as single-enantiomeric forms, the guidelines of the International Conference on Harmonization (ICH) require that the distomers are considered in the same manner as the other impurities [4]. Recently, the FDA in USA, and regulatory authorities in Europe, China, and Japan provided guidelines indicating that preferably only the active enantiomer of a chiral drug should be brought to market, because in the best case, when side effects are not serious, the inactive enantiomer must be metabolized and thus represents an unnecessary burden for the organism. Thus, the debate “racemate versus enantiomer” has opened a new market strategy, the so-called racemic switch. A racemic switch stands for the development in single-enantiomer form of a drug that was first approved as a racemate. This means that a company can in this way get a patent on a new single-enantiomer form, which implies an improvement in the effectiveness of the drug or the suppression of side effects related to the other
enantiomeric form. Moreover, in some cases the separate study of an enantiomer activity can reveal new biological effects.

Now, not only chiral drugs, but also agrochemicals, food additives, flavors, or fragrances, new materials, and catalysts represent classes of compounds with high economic and scientific potential, and have an important role in many aspects of life sciences, environment, food chemistry, synthetic chemistry, and many other fields of economic interest. Therefore, everything mentioned above for chiral drugs can also be extended to the field of other xenobiotics (food additives, flavors, or fragrances), even to the field of agrochemicals and crop protection, where the presence of a nonactive or less active stereoisomer just contributes to increase the levels of pollution without any benefit on the desired action.

In summary, there are obvious benefits derived from the study of the properties of the enantiomers of a chiral molecule with respect to efficacy and safety, and therefore since more than two decades the separation of enantiomers is a fundamental analytical task in many fields of academic, industrial, and research activities, which have clear guidelines recommending that the enantiomers of all chiral bioactive molecules have to be separated and tested. Due to the increasing demand of enantiomerically pure compounds, efficient strategies for analytical separation capable of sensitively differentiating enantiomers are required. In this context, there is no doubt that CE plays a very important role nowadays.

16.1.2 Chiral Recognition by Separation Techniques

Discriminating enantiomers by a separation technique requires the presence of a chiral environment. For this purpose, chiral reagents or chiral selectors are necessary, and the formation of the corresponding diastereomeric species which implies an energetic difference between them, allows in many cases their enantioseparation.

Enantioseparations can be carried out by indirect or direct methods. In the indirect approach, the analyte enantiomers react with a chiral reagent that must be an enantiopure compound to form a pair of diastereomers via covalent bonds. The diastereomers can be subsequently separated under achiral conditions on the basis of their different chemical or physical properties. Today, indirect separation methods are used scarcely due to the limitations of this strategy which requires the use of a chiral reagent with high enantiomeric purity that has a very high cost and very limited commercial availability. Alternatively, the most commonly used in chiral separations are the direct methodologies, which are based on the formation of noncovalent and temporary diastereomeric associates. In this case, the separation of enantiomers is in a chiral environment where the resolutions of enantiomers can be achieved by means of the interaction between the enantiomeric mixture and a so-called “chiral selector,” either bounded to an immobile support (stationary phase) or as an additive in the background electrolyte (mobile phase).
In order to rationalize the interactions at the molecular level, it is considered that the chiral recognition generally depends on a minimum of three simultaneous interactions to form the temporary diastereomeric associates between the chiral selector and each enantiomer, the so-called “three points interaction model” of Dalgliesh [5]. Figure 16.1 shows how according to this model, the R enantiomer of the molecule has an optimum fit and presents three substituents to match the chiral selector’s three-point site while its mirror image (S enantiomer) is limited to a single contact point (B–B, A–A, or C–C). Thus, the binding constant to the chiral selector is higher for the R enantiomer than for the S enantiomer, enabling chiral separation of both enantiomers [6]. In addition, at least one of the interactions has to be attractive to allow the formation of one of the two possible temporary diastereomeric associates [7]. Ionic interactions are strong and may be primarily involved in the establishment of the “first contact” due to their long-range nature. However, as both enantiomers of an ionized solute are able to form these interactions, they may not be stereoselective. In contrast, ion–dipole or dipole–dipole interactions, hydrogen bonds, van der Waals interactions, and π–π interactions are short-range directional forces so that these may be primarily responsible for stereoselective interactions, that is, stereoselectivity. Furthermore, steric factors, that is, fit or nonfit of an enantiomer in a cavity or cleft of the selector, contribute to the chiral recognition.

In conclusion, enantiomers may be stereoselectively recognized only when they are interacting with a chiral selector. This interaction is an analog to the differential partition of analytes between different phases in chromatographic techniques. Although, practice has shown that the chiral recognition of a selector may differ, whether it is fixed to a stationary phase or added to the mobile phase (which contains the analytes), this is not a conceptual difference. The most relevant conceptual difference between chromatographic techniques and CE is the contribution of the electrophoretic mobility in CE separations. The electrophoretic mobility allows the species residing in the same phase to migrate.
at different velocities, being separated one from another in CE. However, in chromatographic techniques, the immiscibility of two media is a fundamental prerequisite to achieve separation [8], because the pressure, as a migration force, does not allow different velocities of miscible phases or different velocities of the species residing in the same phase. Therefore, the enantioseparation principle in CE is chromatographic but based on the selective distribution of the analyte enantiomers between at least two constituents of the same liquid phase having a different mobility [9].

As a consequence, the most striking difference between chromatographic and electrophoretic techniques is the fact that the enantiomers can be resolved in CE not only based on the difference of their association constants with the chiral selector but also based on the different electrophoretic mobilities of their corresponding temporary diastereomeric associates [9–13]. Even the electrophoretic mobility difference between the temporary diastereomeric associates may be (solely) responsible for the separation of enantiomers in CE [14]. However, in chromatographic techniques the selectivity of enantioseparations is entirely defined by the difference between the affinities of the enantiomers toward the chiral selector.

In summary, chiral separation in CE relies on a chromatographic separation principle, but there are significant differences. The property of electrophoretic mobility in chiral CE in particular, and its ability to be selective for the analytes residing in the same phase, is responsible for all the differences. Another important point is that in chromatographic techniques, except with a chiral mobile phase additive, the analyte is virtually immobile when associated with a chiral selector. By contrast, in CE the analyte–selector complex is commonly mobile. These differences are advantageous as they make these techniques complementary.

16.1.3 Potential of CE for Chiral Separations

The main techniques used for chiral separations are Gas Chromatography (GC), Supercritical Fluid Chromatography (SFC), High performance Liquid Chromatography (HPLC), and Capillary Electrophoresis (CE). HPLC remains the dominant technique for chiral separation in industry for reasons of robustness and familiarity of analysts with this technique. However, 30 years after the first chiral separation by CE [15], this technique appears as a well-developed tool for analytical-scale enantioseparations in the pharmaceutical, food, and chemical industries as well as in clinical and environmental laboratories.

CE is possibly the most widely applied technique for enantiomeric separations for reasons concerning its high efficiency, versatility, and feasibility for incorporating a large number of chiral selectors that greatly facilitate method development, speediness, more environmentally friendly and cost-saving technique (a low consumption of chiral selector and reagents) for many applications with respect to HPLC techniques. The high peak efficiency that can be obtained in
CE (between 100,000 and 200,000 plates in a typical capillary) is one of the major advantages. Chiral recognition is mostly based on subtle energy differences between the temporary diastereomeric associates. For this reason, the separation factor between the enantiomers is rather small, and while a factor in the range 1.01–1.02 may appear sufficient for baseline resolution in CE, for a common HPLC column with acceptable efficiency, the separation factor must exceed 1.10 in order to observe baseline-resolved peaks. Therefore, weak enantioselective interactions that are not detectable in HPLC may appear visible in CE. Moreover, the success rate might be higher in CE because it enables to optimize the chiral selector concentration, which is impossible in chromatographic techniques where chiral stationary phases (CSPs) are usually employed, and also due to the fact that various chiral selectors can be combined in CE whereas this is a technically difficult and not very flexible approach in chromatographic techniques. Another interesting point is the fact that a chiral method can be developed faster in CE compared to chromatographic techniques, where this is labor-intensive and requires a long time. This can be explained by the fact that chiral selectors in CE can be screened in a short time due to their resolving ability toward a given chiral analyte and in various concentrations.

One of the most important topics in the field of chiral separations is the order of migration or elution of the enantiomers, because the possibility to reverse that order is very useful to determine the optical purity of chiral compounds (it is always desirable that the enantiomeric impurity migrates before the major compound). In chromatographic techniques, the reversal of the enantiomer elution order can only be achieved by changing the affinity pattern of the enantiomers toward the chiral selector, that is, changing the chiral selector, the composition of the mobile phase, or the separation temperature [16,17]. However, in CE it is possible to reverse the enantiomer migration order (EMO) in the same way as in chromatographic techniques, but also relatively easy by the variation of the concentration of the chiral selector or a manipulation of the electrophoretic mobility terms, effects that are not possible in chromatographic techniques as described above. Thus, in CE the possibilities to reverse the EMO increase without changing the affinity pattern of the enantiomers toward the chiral selector, manipulating electrophoretic principles that affect the effective mobilities of the enantiomers, that is, the direction and magnitude of the mobilities of the EOF, of the free enantiomers, of the chiral selector, and/or of the transient diastereomeric selector–enantiomer complexes [18,19].

In spite of these advantages, CE is also not free of drawbacks, which include a low concentration sensitivity with optical detection, problems with the migration time and peak height/area repeatability, and its coupling with MS, which have been quite successfully solved in the last few years. Currently, the idea about the lower concentration sensitivity of CE techniques is still widespread. However, this should not always apply because there are many different approaches commonly used to increase the sensitivity of chiral CE, as illustrated in Chapter 2 as well as in some interesting reviews [20,21]. Furthermore, it must also be noted that in nonbioanalytical applications of enantioselective CE, commonly the issue
is not the absolute concentration sensitivity of the method, but the “relative concentration sensitivity,” that is, the possibility to detect the lowest possible concentration of the enantiomeric impurity in the presence of a large excess of the major enantiomer. Thus, in this particular case, the separation factor becomes a more critical issue rather than the overall concentration sensitivity of the method. Therefore, as described above, CE is more powerful for the adjustment of the separation factor as compared to chromatographic techniques, and offers more opportunities for a reversal of the EMO, this being a significant advantage for the determination enantiomeric impurities at a very low level.

In summary, chiral CE has been established in the last two decades as one of the most flexible and powerful techniques for analytical-scale enantioseparations, and its use has shown impressive possibilities as can be deduced from the large number of reviews published on this topic in the last 10 years [22–25].

16.1.4
Fundamentals of Chiral CE

This section deals with the basic theory of chiral separations in CE. As mentioned in Chapter 1, in electrophoresis the mobility of an analyte is determined by the mobility of the EOF ($\mu_{EOF}$) as a general mass flow, and the electrophoretic mobility of a particle ($\mu_{ep}$), as a function of effective charge ($q$), and size of the analyte represented by the radius ($r$), for a spherical particle according to:

$$\mu_{ep} = \frac{q}{6\pi\eta r}$$

where $\eta$ is the viscosity of the background electrolyte. This means that $\mu_{ep}$ is an analyte-specific property, modified by properties of the medium. In the case of acidic or basic analytes, the charge is a function of the pH of the electrolyte solution. In addition, generally, the mobility of an analyte is the sum of the electrophoretic mobilities of all different species in which the analyte may exist weighted by the mole fraction ($\phi$) of the respective species:

$$\mu_{ep} = \sum_n \phi^n \cdot \mu^n_{ep}$$

where $n$ is the number of the species present under the experimental conditions.

Therefore, the effective mobility of an analyte ($\mu_{eff}$) is the sum of two electrophoretic forces, $\mu_{EOF}$ and $\mu_{ep}$, according to:

$$\mu_{eff} = \mu_{EOF} + \mu_{ep}$$

Now, in accordance with the above, $\mu_{ep}$ can be a selective transport being different for two charged analytes while $\mu_{EOF}$ is a nonselective transport, that is, it is entirely system specific and not analyte specific. Thus, a separation of two analytes, A and B, in CE is observed when the effective mobilities of the analytes differ, that is, when the electrophoretic mobilities of both are different:

$$\Delta \mu = \mu^A_{eff} - \mu^B_{eff} = \mu^A_{ep} - \mu^B_{ep}$$
As mentioned in Section 16.1.2, the electrophoretic mobilities for the enantiomers of a chiral compound are identical, and therefore their separation is possible only based on the formation of transient diastereomeric complexes with a chiral selector, according to the chromatographic mechanism. Thus, assuming 1:1 complexation between the enantiomers (R or S) and the chiral selector (CS), and assuming that for a given chiral separation the enantiomers exist only in a complexed and noncomplexed form, a simple model was proposed for the calculation of the effective electrophoretic mobility difference between a pair of enantiomers by Wren and Rowe [26] as shown below:

\[
\Delta \mu = \mu_{\text{eff}}^R - \mu_{\text{eff}}^S = \frac{\mu_{\text{free}} + \mu^{(R-CS)}K_R[CS]}{1 + K_R[CS]} - \frac{\mu_{\text{free}} + \mu^{(S-CS)}K_S[CS]}{1 + K_S[CS]}
\]

where \( \mu_{\text{free}} \) is the mobility of the free enantiomers (both equal), \( \mu^{(R-CS)} \) and \( \mu^{(S-CS)} \) are the mobility of the enantiomer-selector complexes, \( K_R \) and \( K_S \) the complexation constants of the enantiomers, and \([CS]\) the concentration of the chiral selector. Thus, an enantioseparation in CE is a function of the mobility of the free enantiomers, the mobilities of the enantiomer-selector complexes, the enantiomer-selector complexation constants, and the concentration of the chiral selector. Since the enantiomers and the chiral selector can be neutral, anionic, cationic, or zwitterionic, their charges determine the mechanism and the migration in CE. Thus, the nature of the chiral selector contributes not only to the separation selectivity but also, in the case of charged selectors, to the direction and magnitude of their electrophoretic mobility.

A chiral separation by CE is based on two mechanisms, one of them is considered chromatographic and the other one electrophoretic. The chromatographic enantioselective mechanism results from the different affinities of the enantiomers toward the chiral selector reflected in the different complexation constants (\( K_R \neq K_S \)). The electrophoretic enantioselective mechanism is due to differences in the mobilities of the enantiomer-selector complexes (\( \mu^{(R-CS)} \neq \mu^{(S-CS)} \)), caused, for example, by differences in the hydrodynamic radii of the complexes due to a “better fit” of one of the enantiomers. Both mechanisms can contribute simultaneously, or can even be giving one of the following two scenarios: (a) the mobilities of the two enantiomer-selector complexes are equal (\( \mu^{(R-CS)} = \mu^{(S-CS)} = \mu^{\text{complex}} \)); or (b) the complexation constants are equal (\( K_R = K_S = K \)); this scenario is not possible in the case of immobilized chiral selectors such as in chromatography. In such cases, the above equation can be rewritten as [27]:

\[
\begin{align*}
(a) \quad \Delta \mu &= \frac{[CS](\mu_{\text{free}} - \mu^{\text{complex}})(K_R - K_S)}{1 + [CS](K_R + K_S) + K_RK_S[CS]^2} \\
(b) \quad \Delta \mu &= \frac{K[CS](\mu^{R-CS} - \mu^{S-CS})}{1 + K[CS]}
\end{align*}
\]

Many mathematical models have been proposed to describe the influence of the main parameters of a chiral separation by CE such as the concentration of the chiral selector, pH, EOF, nature of the background electrolyte coion, and
organic solvent additives. Most models assume a 1:1 complexation between the enantiomers and the chiral reagent although equations describing multiple complexation equilibria have been developed [28–30]. However, given the basic character of this section, we have only considered the model of Wren and Rowe [26], which has frequently been applied to investigate migration phenomena. This simple model has the disadvantage that it does not account for the protonation equilibrium of the analyte in its free and complexed form. Thus, analyte protonation equilibrium was included by Vigh and coworkers in a series of articles [31,32]. On the other hand, Dubsky et al. developed a theoretical model based on that proposed by Wren and Rowe to rationalize the often observed superior enantioselectivity of randomly substituted chiral selectors compared to single isomer selectors [33].

16.1.5 Chiral Recognition Mechanisms Studies

Currently, it is not possible to predict a priori the result of a chiral-recognition process in CE. Therefore, spectroscopic techniques (mainly NMR but also UV, fluorescence, IR, and circular dichroism techniques) and molecular modeling can be used as complementary and supporting tools to chiral CE measurements [34,35]. Thus, these techniques can be used to find the optimal conditions for an enantiomeric separation by CE or to explain different effects observed, for instance, the opposite EMO when different chiral selectors are employed.

NMR has contributed to the investigation of the chiral recognition mechanism based on the variation of the structure of the selectands or the selectors in order to establish “structure–separation” relationships. Some research groups have frequently employed NMR techniques to obtain supporting and complementary data to chiral CE experiments [13,14,36–40]. Compared to other spectrometric techniques, NMR provides separate resonance signals for noncovalent diastereomeric complexes between the selector and both enantiomers. Thus, NMR allows the use of racemic samples or nonracemic mixtures of enantiomers for the stereoselective determination of the stoichiometry of the resulting complexes and the equilibrium binding constants of the selectand–selector complexes. Moreover, by means of nuclear overhauser effect and rotating frame overhauser enhancement techniques information about the spatial proximity of atoms or substituents, and the intermolecular interactions, revealing the structure/geometry of diastereomeric complexes can be obtained.

Molecular modeling techniques are powerful tools to obtain information regarding three-dimensional structures of selector–selectand complexes and their interactions. They have been employed to understand in advance whether chiral discrimination could be achieved with a given chiral selector and predict the result of the enantioseparation. Their combination with CE provides a better understanding of the chiral recognition mechanisms [41]. Also, molecular modeling was used to understand the structures of analyte–selector complexes and to explain migration phenomena observed in CE [42]. In order to improve
the knowledge of the mechanisms of enantiomer recognition pattern in CE, NMR experiments and molecular modeling have been performed in integrated studies [43–45].

Finally, chemoinformatic approaches to connect available experimental data obtained with separation techniques and several mathematical algorithms describing properties of chiral molecules have also been applied to the field of enantioselective molecular recognition [46]. These strategies enable to obtain reliable prediction systems, deduce chiral recognition mechanisms, generate insight for the conception of new chiral selectors, and corroborate and assist experimental techniques such as chiral CE.

16.2
CE Modes for Chiral Analysis

As described in the introduction, the present chapter is focused on chiral CE analyses that have been performed by direct separation methods. As has been described, the enantioseparation by CE is achieved by the combination of electrophoretic and chromatographic principles, these being the two main modes used in chiral CE, Electrokinetic Chromatography (EKC) and Capillary Electrochromatography (CEC), the two approaches primarily chosen. In the former mode, the chiral selector acts as a chiral pseudostationary phase in the BGE whereas in the latter the selector is immobilized inside the capillary forming a true stationary phase (like in HPLC). In the following sections, a brief description of these two modes along with the possibility of using nonaqueous media in the BGE (nonaqueous capillary electrophoresis (NACE)), with emphasis on the selection of the chiral selector is provided.

16.2.1
Electrokinetic Chromatography

EKC is the most widely used mode in chiral CE. This mode was defined by Terabe in 1989 as “an analytical separation method which utilizes the experimental technique of CZE in combination with the principle of chromatography” [47], so that its separation principle relies on the different partition of enantiomers between the bulk solution and the chiral pseudo-phase (chiral selector). Different research works published in the literature attribute to CZE the chiral separations of charged analytes with neutral chiral selectors whereas just the separation of neutral analytes with charged selectors is attributed to EKC. Nevertheless, bearing in mind that from a mechanistic point of view there is no difference if an analyte or a chiral selector is charged or vice versa [9,48], that is, if a neutral chiral selector can resolve the enantiomers of a charged chiral analyte then the enantioseparation of the neutral chiral analyte should also be possible with the charged chiral selector [9]. Therefore, in this chapter all chiral
separations carried out by using a chiral selector in solution will be included within the EKC mode regardless of the selector charge. Different separation schemes can be applied depending on the analyte and selector charge. Table 16.1 shows the most frequent approaches employed. Using neutral selectors only charged analytes can be separated unless a second migration principle is introduced (for instance, a charged pseudo-phase capable of interacting with a neutral analyte such as a charged micelle in MEKC). Charged selectors have been found to be particularly useful when they possess a charge opposite to that of the analyte. The charge not only serves to improve enantioresolution because of the large mobility difference between the analyte and the selector, but also increases the strength of the analyte–selector interactions (via electrostatic forces) and it allows the use of low concentrations of the chiral selector. However, at acidic pH and normal polarity, when the concentration of the negatively charged selector is high or the binding analyte-selector is strong, a basic or neutral analyte could not reach the detector (set at the cathode) because the selector transports it toward the anode. To avoid that, the polarity is reversed and the detection is carried out at the anode. At alkaline pH and normal polarity, charged selectors can be used to separate neutral and basic compounds, since at these pH values, basic analytes are uncharged and they are therefore transported by the EOF to the detector, as they are neutral compounds. Finally, analogous scenarios can be described for positively charged selectors.

Table 16.1  Separation schemes in EKC.

<table>
<thead>
<tr>
<th>Selector</th>
<th>Analyte</th>
<th>pH&lt;sup&gt;b)&lt;/sup&gt;</th>
<th>Polarity&lt;sup&gt;b)&lt;/sup&gt;</th>
<th>Analyte</th>
<th>EOF&lt;sup&gt;c)&lt;/sup&gt;</th>
<th>Mobility</th>
<th>Selector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral</td>
<td>Basic</td>
<td>Low</td>
<td>Normal</td>
<td>Translated by EOF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid</td>
<td>Basic</td>
<td>High</td>
<td>Normal</td>
<td>→→ → ←</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anionic</td>
<td>Basic</td>
<td>Low</td>
<td>Normal</td>
<td>→→ ←</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basic</td>
<td>Low</td>
<td>Reversed</td>
<td>Normal</td>
<td>← ← → ←</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral</td>
<td>Low</td>
<td>Reversed</td>
<td>Translated by EOF</td>
<td>← ←</td>
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</tr>
<tr>
<td>Basic&lt;sup&gt;d)&lt;/sup&gt;</td>
<td>High</td>
<td>Normal</td>
<td>Translated by EOF</td>
<td>← ←</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral</td>
<td>Low</td>
<td>Normal</td>
<td>Translated by EOF</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cationic</td>
<td>Neutral</td>
<td>Low</td>
<td>Normal</td>
<td>← ←</td>
<td></td>
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</tr>
<tr>
<td>Acid</td>
<td>Low/high</td>
<td>Normal</td>
<td>→ ←</td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Low, acid pH. High, alkaline pH.
b) Normal polarity: inlet (anode); detector and outlet (cathode). Reversed polarity: inlet (cathode); detector and outlet (anode).
c) At pH < 2.5, EOF is suppressed.
d) Basic analytes are uncharged at alkaline pH so that they are transported by the EOF as neutral compounds.
Only two kinds of chiral selectors, ligand-exchange (LE) compounds and cyclodextrins (CDs) were used for CE enantioseparations before 1990. Today there are different chiral selectors available for enantiomeric separation by EKC, such as monomeric and polymeric surfactants, antibiotics, chiral crown ether, linear mono-, oligo-, and polysaccharides, peptides, and proteins [49–54] but CDs are without doubt the most widely employed due to their excellent properties related to availability, diversity, universality, price, and safety.

CDs are well-known host molecules with large ring-like structures composed of α-(1,4)-linked D-(+)-glucopyranose units whose shape is a truncated cone with a relatively hydrophobic inside and a hydrophilic outside owing to the presence of hydroxyl groups. The inclusion of the enantiomers into the CD cavity and the establishment of secondary interactions (hydrogen bonds and dipole–dipole interactions) with the hydroxyl groups on the CD rim is the most widely accepted mechanism for the enantioresolution of a chiral analyte with CDs. However, it is important to point out that the complete inclusion is not always a major requirement since a partial inclusion or external interaction could be sufficient. Usually, the chiral recognition ability of native CDs is improved by the modification (chemical reactions) of the hydroxyl groups of the CD rim with different functional groups such as methyl, sulfate, acetyl and propyl, among other. Even though a big number of new CD derivatives (neutral, positively, and negatively charged) are synthesized every year, hydroxypropyl-, sulfated-, or native β-CDs are the most frequently used chiral selectors as can be observed in Figure 16.2 which shows the number of applications of different CDs collected in

![Figure 16.2](image)

*Figure 16.2* Most employed cyclodextrins in chiral CE analysis. (Data obtained from the number of applications reported for each CD in two recent reviews [23,55].)
two recent review articles [23,55]. The synthesis of all these CD derivatives can give rise to single-isomer or randomly substituted derivatives. The first ones are synthesized to yield only a single molecular species whereas the latter potentially possess several molecular structures with different degrees of substitution (an average degree of substitution is then usually given). Generally, the use of single-isomer CD derivatives is preferred since randomly substituted CDs are not well suited for obtaining reproducible results (differences in the degree of substitution and in the position of the substituents imply a high variability in the selectivity obtained from different commercial suppliers and even from batch to batch for the same supplier), to develop validated analytical methods or for mechanistic studies.

Along with CDs, surfactants and antibiotics are also very frequently employed as selectors in EKC. The main advantage of the use of surfactants as chiral selectors is their capability to simultaneously separate anionic, cationic, and neutral species, as described in Chapter 4 which deals with this CE mode called Micellar Electrokinetic Chromatography (MEKC). Among the great variety of natural and synthetic monomeric surfactants (including digitonin, saponin, bile salts, and sugar- and amino based surfactants), bile salts are the most popular. Nevertheless, polymeric surfactants (also called micellar polymers) have received great attention in the last few years because they are very compatible chiral selectors for MS detection [54]. Properties such as zero critical micelle concentration, high stability (which prevents dissociation of monomers during electrospray process), and less ion suppression (due to the low surface activity) among others, make micellar polymers a simple and interesting alternative to more conventional micelles for chiral MEKC–MS [56,57].

Regarding antibiotics, their potential to act as chiral selectors results from the high number of stereogenic centers and functional groups which are able to establish multiple interactions (electrostatic, inclusion, hydrogen bonding, etc.) with chiral analytes. Glycopeptides, polypeptides, ansamycins, aminoglycosides, macrolides, and lincosamides are the six groups of antibiotics that have been so far employed as chiral selectors [25,58]. Among them, the macrocyclic glycopeptides, which include vancomycin, ristocetin A, eremomycin, avoparcin, teicoplanin, and balhimycin, have demonstrated powerful enantioselectivity toward numerous compounds (mainly those containing free carboxylic groups) although they have as drawbacks their low solubility in water, the adsorption on the capillary wall which results in poor separation efficiency, and the strong UV absorption that causes a decreased detection sensitivity. Strategies such as optimizing the capillary washing procedures, working at a pH close to the isoelectric point of the antibiotics, adding organic solvents to the buffer, using coated capillaries, or performing the separation in counter current migration technique (CMT), can be adopted to avoid/minimize adsorption and absorption phenomena [59]. CMT is carried out by using a buffer pH in which the selector and the analyte possess opposite charges so that they migrate in the inverse direction (counter current). This methodology can be performed in the partial filling mode where only part of the capillary is filled with the chiral selector leaving the detector
path free of antibiotics. Nowadays, a newest group of antibiotics with weak UV absorption, such as macrolides (erythromycin, azithromycin, clarithromycin and boromycin) or lincosamides (clindamycin phosphate), are being currently employed as chiral selectors.

Other chiral selectors were also employed to a lesser extent, such as chiral crown ethers, linear mono-, oligo-, and polysaccharides, proteins, or LE compounds [50,52,54,60,61]. Likewise, it should be mentioned that in certain cases in which a baseline enantioseparation cannot be achieved by using only a chiral selector, the combination of different selectors (dual systems) may result in an improvement in the resolution and peak efficiency. Although some research works have employed systems based on the combination of polysaccharides or polymeric surfactants [62,63] the most frequently used dual selector systems include different CDs. Although the use of dual systems based on the combination of two charged CDs (two anionic CDs or one negatively and other positively charged) has been reported in the literature [64], the two main types of dual system incorporate two neutral CDs derivatives [65,66] or one neutral and one charged CD where the neutral CD provides the chiral recognition and the charged one insures the migration [67]. An overview of mathematical models describing by means of formulae the electromigration behavior of analytes under interaction with two or more chiral selectors has recently been published [68].

Finally, a current trend in CE is the use of chiral ionic liquids as selectors [69]. They not only were used to establish synergic systems with other selectors such as CDs, antibiotics, or polysaccharides [70–72] but also were employed as the sole chiral selector in a few applications [73,74]. However, although ionic liquids have received significant attention, their contributions in the chiral CE field are still scarce so that probably in the near future new works on this subject will be carried out.

16.2.2
Capillary Electrochromatography

CEC combines the high-efficiency features of CE (movement of analytes by electrical forces) with the greater selectivity characteristics of HPLC. In this separation technique, the movement of the mobile phase through a capillary that is filled, packed, or coated with a stationary phase is achieved by an electroosmotic flow (which may be assisted by pressure). Compounds are separated by the combined action of partitioning between the static stationary phase and mobile phase and, if they are charged, by the different electrophoretic mobilities. Although CEC has also become an important mode of CE in chiral separations, compared with EKC it is still not a mature separation technique mainly due to the limited commercial availability of CEC columns or the relatively complicated homemade fabrication of the CSPs.

Three different capillary column types have been employed to carry out enantioseparations by CEC: open tubular columns (OTCs), packed columns (PCs), and monolithic columns [75–78].
OTCs are based on the use of an open capillary whose inner wall is coated or bonded with CSPs. Among the high variety of immobilization and surface modification chemistries developed for preparing OTCs, the adsorptive coating of chiral selectors onto the surface of fused silica capillaries is the simplest procedure [79]. Cyclodextrins, celluloses, proteins, molecularly imprinted polymer (MIPs), and polymeric surfactants have been used as coating materials for CSPs. Usually, chemically bonded phases are preferred to physically adsorbed phases for their better stability and longer lifetimes. However, physical coating is easier to fabricate, less expensive, and in some instances these OTCs show stability and separation performance as good as those of covalent coatings [77].

Although CSPs used in LC could be developed as CEC packing stationary phases, nowadays they are not commercially available, which is the main drawback of PCs. However, they are the most frequently used columns in chiral CEC. In the relatively complicated homemade preparation of PCs, the packed bed must be stabilized with retaining frits on both capillary ends to keep the packing in the capillary during the analysis. The most serious problem derived from the use of PCs is the bubble formation that occurs often at the end-frit that may result in current instabilities and even current breakdown. Different types of CSPs such as CDs, macrocyclic antibiotics, proteins, LE compounds, and polysaccharides have usually been employed for chiral CEC with PCs. In particular, the use of polysaccharides (they have shown to be high enantioselective towards basic, acid and neutral compounds) offers the advantage of availability and the easy derivatization of the hydroxyl group. Thus, the introduction of electron-donating (methyl) and electron-withdrawing (chlorine) groups improves the enantiomer recognition. Usually, the most widely used polysaccharides are cellulose tris (3-chloro-4-methylphenylcarbamate), cellulose tris(4-chloro-3-methylphenylcarbamate), and amylose tris(5-chloro-2-methylphenylcarbamate) [80–82].

Monolithic columns are prepared by in situ polymerization or solidification of monolithic stationary phases with homogeneous and porous structures. Among the advantages of monolithic columns are a high phase ratio, large column capacity, simple preparation process, and wide choice of polymer monomers [78]. In general, these columns may be divided into (i) monolithic silica-based columns (prepared via in situ poly-condensation of alkoxy silane and where the chiral selector can be immobilized by physical adsorption, packing, or on-column derivatization), (ii) polymer monolithic columns (prepared by in situ polymerization and chiral selectors are often added in copolymerization so as to prepare columns with chiral selectivity); (iii) particle-fixed monolithic columns (prepared by immobilizing the traditional stationary phase particles on the surface of the capillary using sol–gel techniques, sintering methods or polymerization of organic polymers); and (iv) MIP columns (in which one enantiomer of the analyzed chiral compound is used as the imprinted molecule, also known as the template molecule, to prepare MIPs that would show selective chiral recognition toward the original molecule). In the last years, molecular imprinting technique has
experienced a high development. Recent improvements in the preparation of MIPs and their latest applications in CEC have recently been reviewed [83]. Even if most applications of MIPs are in the chiral analysis of small compounds, advances in the analysis of larger molecules are expected in the near future. In the last few years, inorganic–organic hybrid monoliths are becoming an attractive alternative to pure silica-based monoliths since they possess the advantages of easy fabrication, more pH stability, and less shrinkage. The development and immobilization of chiral selectors on hybrid monolithic matrices is a new interesting research area in chromatographic separation science [84,85].

Despite the great evolution of CEC as a separation technique for chiral separations during the last few years, its full potential has not yet been achieved. Even though current efforts are focused on the search of new CSPs mainly monolithic columns and gold nanoparticles, further developments in the stationary phase technology are required to broaden the application range of this separation technique.

16.2.3 Nonaqueous Capillary Electrophoresis

Most applications of chiral CE were usually carried out in aqueous medium. However, when enantiomers, chiral selectors, or electrolytes are insoluble or partially soluble in water, NACE (both in EKC and in CEC modes) gains a special relevance. From a practical point of view, the use of nonaqueous solvents offers certain advantages compared with aqueous media; for instance, their low conductivities and resultant low currents enable the use of higher field strengths without excessive Joule heating (providing shorter run times and enhanced efficiencies), a decrease in wall adsorption effects and, easier online coupling to MS detection. Also, their lower dielectric constants favor ion-pairing effects leading to modifications in selectivity. Moreover, the nature and properties of the organic solvent have a strong influence on the separation selectivity, efficiency, and resolution since it not only increases the solubility of compounds, but also reduces the EOF and improves separations by selectively changing the acid–basic properties of the analytes often enabling the separation of solutes with similar electrophoretic mobilities in aqueous medium [86,87]. In spite of NACE being frequently used for its improved selectivity (for instance, a better separation selectivity of polypeptides is achieved using nonaqueous than aqueous media) [88], it does not automatically induce better selectivity than aqueous CE.

One of the most attractive features of NACE is the large choice of organic solvents available, in both pure or mixture forms. Although there are no criteria for the selection of suitable solvents except the trial-and-error method, it is essential that they remain physically and chemically stable, should have good solubility of analytes, selectors, and electrolytes, and should be available at an acceptable quality and at a reasonable price. In addition, for rapid resolutions, their relative permittivity should be maximized, whereas their viscosities should
be minimized. Physicochemical properties (including relative permittivity, viscosity, UV cutoff, boiling point, surface tension, and autoprotolysis constant) of the most commonly used organic solvents in NACE can be found in a recent review article [24]. Due to its favorable properties, such as dielectric constant, viscosity, and a useful UV range for detection, methanol is the most commonly used organic solvent in NACE. As in aqueous CE, the presence of electrolytes is needed to perform efficient and reproducible separations. To ensure full protonation of basic compounds, electrolytes composed of formic or acetic acid (usually between 0.1 and 2 M) with a low concentration of ammonium formate or acetate (25–20 mM) are generally used. For the analysis of acid compounds the electrolytic composition is free of acids.

Even though the mechanism of chiral separations in NACE is complementary to aqueous CE, the stabilities of the diastereomers formed in aqueous and nonaqueous media are different which involves a little difference in the enantiomeric resolution. In principle, organic solvents with lower dielectric constants than water constitute a more favorable environment for chiral discrimination due to their ability to promote intermolecular interactions [45]. In fact, a chiral separation not feasible in aqueous media can be sometimes favored in NACE by different factors. For instance, the use of organic solvents decreases the interactions of enantiomers with the chiral selector. This fact results in different configuration structures of the diastereomers from those formed in an aqueous medium so that the change in structures can be responsible for differential NACE migrations which result in chiral resolution (and sometimes in a change in the EMO). The opposite affinity patterns of propranolol enantiomers toward different CDs in aqueous and nonaqueous buffers is a clear example of the differences that can exist between the molecular mechanisms of the separation of enantiomers in aqueous CE and NACE [89,90]. Inclusion and/or external complexes types are formed between CDs and propranolol depending not only on the chemistry and structure of the chiral selector but also on the medium in which the complexes are formed. The different structures of diastereomers gave rise to a different enantioseparation and even to the reversal of the EMO when heptakis(2,3-dimethyl-6-sulfo)-β-CD was employed as the chiral selector.

The chiral selectors most commonly employed in the NACE-EKC mode include neutral and charged cyclodextrins as well as ion-pair selectors [24], but it is also possible to use other selectors, mainly antibiotics [91].

Although it is possible to find in the literature research works and reviews (or subsection in reviews) focused on chiral separations by NACE [24,49,92,93] not much work has been developed in this field in recent years. This fact is clearly shown in Figure 16.3 which provides information on the number of research works recently published (2012–2014) dealing with chiral separations by CE. As can be observed, EKC is undoubtedly the most extensively used mode for the enantioseparation of standards and real samples in different fields whereas CEC and NACE have been used to a lesser extent. For instance, although the potential of different stationary phases employed in CEC has been demonstrated to carry out the enantioseparation of standard samples, this CE mode still lacks the
robustness required for practical applications. Therefore, although CE is considered as a mature and relevant technique in the field of chiral separations, not all the CE modes have reached nowadays their full potential.

16.3 Chiral Separations by CE-MS

UV is widely known as the most employed detection system in CE due to its low cost, simplicity, and versatility. Nevertheless, when a high selectivity and sensitivity is required, UV is not the preferred detection system. Laser Induced Fluorescence (LIF) is commonly used as the detection system in CE due to its excellent sensitivity. However, just a reduced number of molecules possess fluorescence moieties giving rise to the need of molecule-labeling as an essential step prior CE analysis. By adding a derivatization step, sample treatment becomes more tedious, leading in most cases to derivatization problems when complex matrices are analyzed. Moreover, since derivatization reactions are generally not specific, by-products can be generated.

MS can be a useful detector in chiral CE as it does not require a derivatization step. Moreover, it is more sensitive than UV detection, and also offers the possibility to unambiguously identify an analyte by its mass to charge ratio (m/z) which is translated into an excellent selectivity as discussed in Chapter 2. In addition, when MS/MS experiments are performed, structural information can also be obtained from the fragmentation mass spectrum. As has been highlighted in

![Figure 16.3](image.png) Number of research papers published from 2012 to 2014 dealing with "chiral capillary electrophoresis" obtained from Web of Science (Thomson Reuters) database using the keywords "capillary electrophoresis" and "chiral" or "enantio*" (using * as a wildcard in the search query).
different reviews [56,94,95] and book chapters regarding chiral CE–MS [96], the promising MS advantages along with the outstanding chiral resolution power of CE seem to be the perfect combination to achieve sensitive enantioseparations.

However, CE–MS hyphenation is not always an easy task. The presence of nonvolatile chiral selectors may cause ion suppression and contamination of the ionization source and optics leading to a sensitivity decrease. Regarding this issue, the most suitable CE mode to be coupled with MS detection is CEC as there is no contamination caused by the entry of the chiral selector into the MS system since it is attached to the CSP of the capillary column. Nevertheless, in EKC–MS, contamination problems might arise as a consequence of the free chiral selector present in the separation electrolyte. Two approaches are mainly being used to avoid the entry of the MS incompatible chiral selector: CMT and partial filling technique (PFT) [56]. As previously mentioned, in the former, a charged chiral selector migrates away from the detection system in opposite direction to the analytes. In the latter, the CE capillary is first flushed with the BGE without the chiral selector and, before the sample is injected, a band of BGE containing the chiral selector is introduced into the capillary. Although PFT was mainly designed to avoid strong UV-absorbing chiral selectors reaching the detection window [97], it has extensively been used in MS detection. In a very recent article, it has been proved that combination of CMT and PFT offers good results [98]. Without a doubt, PFT is the strategy most commonly employed in the EKC–MS hyphenation as can be observed in the numerous works reported in the literature. Nevertheless, the trend of enhancing this coupling should be oriented toward the development of strategies that allow the entry of chiral selectors in MS system. So far, just a few of these strategies have been reported which include, mainly, the use of low-concentrated chiral selectors [99,100], and the use of micelle polymers. As mentioned in Section 16.2, micelle polymers are an excellent alternative to the MS-incompatible micelles widely used in MEKC, as they have been proven to not only be suitable to enter the MS system but also to offer many advantages such as low background noise, less ion suppression, and more stable electrospray [56,57]. Some interesting strategies with promising results were published more than 10 years ago but these trends have not been further exploited. These include the work reported by Moini et al. [101] where amino acids–crown ether complexes exhibited a higher sensitivity than the amino acids on their own, and the work of Iwata et al. [102] where authors employed a chiral selector (highly sulfated γ-CD) that did not ionize in the working ESI mode and whose complex with the analyte broke down in the ionization source.

To conclude, in spite of its numerous advantages, chiral CE–MS applicability is extremely limited due to the incompatibility of the chiral selectors frequently employed in EKC. Instead of working on strategies in favor of avoiding the entry of the chiral selector in the MS system, future trends must be focused on the development of MS-compatible chiral selectors or new interfaces and systems compatible with those available nowadays.
Applications of Chiral Separations by CE

To achieve a successful chiral separation by CE, the optimization of different experimental parameters is critical. These parameters include nature, concentration, and pH of the BGE, concentration and type of chiral selector, temperature, and voltage, among others. The most important step to achieve a successful chiral separation is to choose the most suitable chiral selector. However, few predictions can be made and frequently, the identification of suitable selectors for a specific pair of enantiomers requires considerable experimentation. Therefore, although trial-and-error approaches are often employed in the selection of the most appropriate conditions, the use of experimental design methodologies is becoming more frequent. Experimental design is aimed at the reduction of the number of experiments and, unlike the trial-and-error approach, it also considers the dependence among the optimized parameters. By using these strategies, two steps are distinguished in the method optimization: a screening and an optimization phase. In the former, screening designs are applied to investigate all factors that might affect the separation (usually the screening is focused on the selection of the most suitable chiral selector) while in the latter the most important factors are optimized using response surface designs to identify the optimal conditions based on the selected response, for instance, migration time or enantioresolution [103–105]. Concerning the screening phase, several designs are employed based on the number of variables. If a maximum of four variables are optimized, a full factorial design is appropriate, whereas if more than four variables are studied a fractional design is applicable. For a larger amount of variables the Plackett–Burman design (PBD) is the method of choice [48,104]. Examples of response surface designs are the three-level full factorial design, Box–Behnken, and central composite designs (CCD), the latter being the most employed. A recent work describes the optimization of a chiral EKC methodology to enantioseparate a diaryl-pyrazole sulfonamide derivative by means of an experimental design [106]. In the screening phase, authors selected three different factors affecting the enantioseparation (concentration of the two CDs employed, amino-β-CD and β-CD, and the percentage of methanol present in the BGE), while in the optimization phase, the CCD was selected to determine the optimal conditions based only on the obtained Rs. By means of the obtained second-order polynomial model authors found that the concentration of both CDs exerted a strong influence on the Rs while the methanol percentage did not affect the Rs.

The above-mentioned strategies are useful not only to optimize the separation of racemic mixtures but also to determine enantiomeric impurities. In this case, a sufficient enantioseparation (at least 3.0) is recommended due to the different concentration between the main enantiomer and the enantiomeric impurity. Moreover, it is also advisable that the enantiomeric impurity is the first-migrating peak since when achieving low levels of quantification the main enantiomer is usually overloaded, thus it might hide the enantiomeric impurity.
Nowadays, a large number of works have been published dealing with the use of CE as a powerful technique for the enantioseparation of compounds of interest in different research fields such as pharmaceutical, bioanalysis, food and environmental analysis, among others. In the following sections, a brief overview of the most recent and relevant applications of chiral CE in real samples is presented.

16.4.1 Pharmaceutical Analysis

The commercialization of drugs as single enantiomers is strongly demanded by the pharmaceutical industry as discussed in Section 16.1.1. As can be observed in several reviews found in the literature [23–25,107], a large number of CE methodologies dealing with the enantioseparation of chiral drugs have been published in recent years. In fact, a great variety of drugs can be analyzed in various matrices ranging from raw materials until the obtaining of final pharmaceutical products. Drugs are not generally complex matrices so the use of cleanup techniques is not usually required. Table 16.2 groups some of the most recent and relevant works dealing with pharmaceutical analysis by chiral CE. Generally, the goal of most of the published works is the analysis of drugs marketed as single enantiomers (for which the detection of very low levels of enantiomeric impurities is required) following the requirements claimed by the ICH guidelines [120]. Thus, Deng et al. [108] developed a method to enantioseparate the antidepressant drug escitalopram whose $R$-enantiomer counteracts the activity of the $S$-enantiomer. Authors employed this method to analyze raw material and tablets, finding 0.05% of the $R$-enantiomer content. Despite the fact that the impurity was the second-migrating enantiomer, the high resolution obtained enabled to overload the main compound without interfering the impurity signal (see Figure 16.4).

When an enantiomer of an active principle has undesirable properties the control of its optical purity is of vital importance. This is the case for the drug penicillamine whose $D$-enantiomer is active whereas the $L$-enantiomer is highly toxic even in small amounts. Song et al. [109] developed a method to separate penicillamine enantiomers that also included a one-pot labeling technique to add a charged group and a chromophore to this drug since it has poor UV-Vis absorbance. Authors found undetectable amounts of $L$-enantiomer in commercial tablets (LODs in the $\mu$g/ml range). As can be observed, developing sensitive methods is a demand in the pharmaceutical analysis since it helps in detecting lower amounts of the enantiomeric impurity. Sueyoshi et al. [110] developed a method including a preconcentration step based on transient trapping obtaining LODs in the ppb range for the enantiomers of the drug chlorpheniramine. This methodology was sensitive enough to detect 43 ppb of the enantiomeric impurity in the presence of 5 ppm of the active principle in a commercial pharmaceutical formulation.

Although the most employed detection system in the pharmaceutical analysis is UV, MS has been demonstrated to be a powerful tool to develop high
Table 16.2  Some of the most recent and relevant applications of chiral CE in pharmaceutical analysis.

<table>
<thead>
<tr>
<th>Application</th>
<th>Analyte</th>
<th>Sample</th>
<th>CE mode and detection system</th>
<th>Chiral selector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysis of drugs as single active enantiomers</td>
<td>Escitalopram</td>
<td>Raw materials and tablets</td>
<td>EKC-UV</td>
<td>Sulfated-β-CD</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td>Penicillamine</td>
<td>Tablets</td>
<td>EKC-UV</td>
<td>β-CD</td>
<td>[109]</td>
</tr>
<tr>
<td></td>
<td>Chlorpheniramine</td>
<td>Solution</td>
<td>EKC-UV</td>
<td>Sulfated-β-CD</td>
<td>[110]</td>
</tr>
<tr>
<td></td>
<td>Duloxetine</td>
<td>Capsules</td>
<td>EKC-MS²</td>
<td>2-hydroxypropyl-β-CD</td>
<td>[111]</td>
</tr>
<tr>
<td></td>
<td>Zopiclone</td>
<td>Tablets</td>
<td>EKC-UV</td>
<td>β-CD and 1-ethyl-3-methylimidazolium-1-lactate</td>
<td>[112]</td>
</tr>
<tr>
<td></td>
<td>Tolderodine and methoxytolterodine</td>
<td>Pills</td>
<td>EKC-UV</td>
<td>Phosphonated-γ-CD</td>
<td>[113]</td>
</tr>
<tr>
<td></td>
<td>Esomeprazole</td>
<td>Raw material and capsules</td>
<td>EKC-UV</td>
<td>2-hydroxypropyl-β-CD</td>
<td>[114]</td>
</tr>
<tr>
<td></td>
<td>Naproxen</td>
<td>Raw material</td>
<td>EKC-UV</td>
<td>Vancomycin and L-valine tert-butyl ester bis(trifluoromethane) sulfonamide</td>
<td>[71]</td>
</tr>
<tr>
<td></td>
<td>Replaginide</td>
<td>Raw material and tablets</td>
<td>EKC-UV</td>
<td>2,6-di-O-methyl-β-CD</td>
<td>[115]</td>
</tr>
<tr>
<td></td>
<td>Levornidazole</td>
<td>Raw material and injection solutions</td>
<td>EKC-UV</td>
<td>Sulfated-α-CD</td>
<td>[116]</td>
</tr>
<tr>
<td>Analysis of racemic drugs</td>
<td>Tioconazole and isoconazole</td>
<td>Creams</td>
<td>MEKC-UV</td>
<td>2-Hydroxypropyl-β-CD and 2,6-dimethyl-β-CD</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>Zopiclone</td>
<td>Tablets</td>
<td>EKC-UV</td>
<td>Carboxymethyl-β-CD</td>
<td>[117]</td>
</tr>
<tr>
<td></td>
<td>Fluoxetine</td>
<td>Tablets and pills</td>
<td>EKC-UV</td>
<td>Sulfated-β-CD</td>
<td>[118]</td>
</tr>
<tr>
<td></td>
<td>Meptazinol and three intermediates</td>
<td>Tablets</td>
<td>EKC-UV</td>
<td>Carboxymethyl-β-CD</td>
<td>[119]</td>
</tr>
</tbody>
</table>
sensitivity and selective chiral methodologies. The use of an EKC-MS² strategy for the enantioseparation of duloxetine enabled to obtain a LOD of 20 ppb for the enantiomeric impurity (tenfold lower than with UV detection), and authors found undetectable amounts (lower than 0.02%) of the enantiomeric impurity in the analyzed pharmaceutical formulations [111]. In spite of the massive effort in commercializing pure enantiomer drugs, nowadays, several drugs are still being commercialized as a racemic mixture. This might be due to the fact that both enantiomers are active, or because one of them is inactive and a pure enantiomeric synthetic path is not possible, and in these cases the quantification of both enantiomers is required. An interesting work was focused on the development of a MEKC-UV method to simultaneously quantify antifungal drugs tioconazole and isoconazole commercialized as racemic mixtures in cream formulations [66].

Figure 16.4  Electropherogram corresponding to the analysis of 500 μg/ml of an escitalopram tablet and the determination of its enantiomeric impurity (R-enantiomer) by EKC-UV. (a) Full size. (b) Enlarged view. CE conditions: BGE: 25 mM phosphate buffer (pH 7.0) containing 1.6% (w/v) sulfated-β-CD; voltage: -20 kV; temperature: 25 °C; short-end injection: 5 s at 0.5 psi. (Reproduced with permission from Ref. [108].)
As above mentioned, the stereochemistry of biologically active molecules acquires great importance as a result of the inherent enantioselectivity of biological processes. Generally, biological matrices are complex, the compounds of interest are present in low concentrations (pg or ng/ml) and these analytes often coexist with metabolites with similar physicochemical properties which makes the separation more difficult. However, the high separation efficiency of CE combined with the possibility of performing sample cleanup processes or using online preconcentration techniques makes it a relevant tool for the determination of enantiomeric compounds in this kind of matrices. Likewise, it should be highlighted that CE is an excellent option to analyze biological samples such as tissues or single cells due to the low consumption of samples required in CE analysis. Table 16.3 shows some of the most recent and relevant applications of chiral CE in bioanalysis. Many works were focused on the development and optimization of chiral methodologies enabling the enantioseparation of different drugs to study their enantioselective metabolism both in humans (in vivo studies) and in cells or biological molecules studied outside their normal biological context (in vitro studies). The determination of amino acid enantiomers was also an important point since they are considered as potential disease biomarkers being generally involved in neuroscience studies.

The in vivo study of the enantioselective metabolism of a racemic drug required the analysis of biological fluids of patients after the administration of the drug. Warfarin and metabolites, verapamil and norverapamil, methylphenidate, or propranolol were some of the drugs investigated. As an example, Tabani et al. [121] found lower concentrations of the more active enantiomer S-propranolol than its R-enantiomer in a patient’s urine after administration of a racemic tablet of propranolol. It is also worth highlighting the study of in vivo racemization of some drugs since, under certain circumstances, the active enantiomer can be converted into the racemic mixture [131].

In vitro studies on the enantioselective metabolism can be performed in different matrices, for example in cells [124] or in enzymes [125,126]. An interesting approach, EKC–MS enabled to study the metabolism of DOPA in human neuronal cells finding that these cells metabolized L-DOPA effectively leaving the D-enantiomer intact [124]. Asensi-Bernardi et al. [125] proved that the in-line combination of electrophoretically mediated microanalysis with PFT employing sulfated-β-CD could be used to study the metabolism of verapamil by CYP3A4.

Determination of amino acids in biological samples is extremely important since they can act as biomarkers of pathologies and they give information about how different biological processes take place. As an example, using a MEKC-UV methodology with an LE compound (Cu(II)-L-proline) as the chiral selector it was possible to find differences in the content of some amino acids (tyrosine, phenylalanine, and tryptophan) between diabetic and healthy patients [127].
Table 16.3  Some of the most recent and relevant applications of chiral CE in bioanalysis.

<table>
<thead>
<tr>
<th>Application</th>
<th>Analyte</th>
<th>Sample</th>
<th>CE mode and detection system</th>
<th>Chiral selector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo studies of enantioselective metabolism of drugs</strong></td>
<td>Propranolol</td>
<td>Urine and plasma</td>
<td>EKC-UV</td>
<td>2-Hydroxypropyl-β-CD</td>
<td>[121]</td>
</tr>
<tr>
<td></td>
<td>Warfarin and metabolites</td>
<td>Plasma</td>
<td>MEKC-MS</td>
<td>Polysodium N-undecenoyl-1, l-leucylvalinate</td>
<td>[57]</td>
</tr>
<tr>
<td></td>
<td>Verapamil and norverapamil</td>
<td>Serum</td>
<td>EKC-UV</td>
<td>2,3,6-tri-O-methyl-β-CD</td>
<td>[122]</td>
</tr>
<tr>
<td></td>
<td>Methylphenidate</td>
<td>Oral fluids</td>
<td>EKC-UV</td>
<td>2-Hydroxypropyl-β-CD</td>
<td>[123]</td>
</tr>
<tr>
<td></td>
<td>DOPA</td>
<td>PC-12 nerve cells</td>
<td>EKC-MS&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Sulfated-β-CD</td>
<td>[124]</td>
</tr>
<tr>
<td></td>
<td>Verapamil</td>
<td>Cytochrome P3A4</td>
<td>EKC-UV</td>
<td>Sulfated-β-CD</td>
<td>[125]</td>
</tr>
<tr>
<td><strong>In vitro studies of enantioselective metabolism of drugs</strong></td>
<td>Fluoxetine</td>
<td>Cytochrome 2D6</td>
<td>EKC-UV</td>
<td>Sulfated-β-CD</td>
<td>[126]</td>
</tr>
<tr>
<td><strong>Determination of amino acids as disease biomarkers</strong></td>
<td>Tyrosine, phenylalanine, and tryptophan</td>
<td>Urine</td>
<td>MEKC-UV</td>
<td>Cu(II)-l-proline</td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td>8 amino acids</td>
<td>Cerebrospinal fluid</td>
<td>MEKC-LIF</td>
<td>β-CD</td>
<td>[128]</td>
</tr>
<tr>
<td></td>
<td>10 amino acids</td>
<td>Plasma</td>
<td>EKC-LIF</td>
<td>β-CD</td>
<td>[129]</td>
</tr>
<tr>
<td></td>
<td>Aspartate and glutamate</td>
<td>Brain of chickens</td>
<td>EKC-LIF</td>
<td>2,6-Di-O-methyl-β-CD and 6-monodeoxy-6-mono (3-hydroxy)-propylamino-β-CD</td>
<td>[130]</td>
</tr>
</tbody>
</table>
However, authors state that they presented just a preliminary study as they need a larger group of samples to confirm it.

It is important to note that several D-amino acids such as D-aspartate and D-serine are known to be involved in neurological processes. Samakashvili et al. investigated the D- and L-amino acids content in cerebrospinal fluid samples of patients with different Alzheimer’s disease stages by MEKC using LIF detection after derivatization with FITC [128]. Eight FITC amino acids were baseline enantioseparated and authors found differences in some of the L enantiomers, but no significant differences for D-serine. Lorenzo et al. [129] developed a methodology to enantioseparate 10 amino acids and then studied their level in plasma from patients with bipolar disorder. Authors found levels of these amino acids in concordance to the published results for bipolar patients. On the other hand, the analysis of aspartate and glutamate enantiomers in chicken's brain tissues revealed that the D-aspartate content was 1–2% of the total aspartate content (see Figure 16.5) [130].

16.4.3
Food and Beverage Analysis

Chirality of food constituents provides information about food origin and age [22]. Moreover, the determination of the enantiomers of a certain compound may also be considered as an indicative of food quality and safety. Table 16.4

![Figure 16.5](image)

**Figure 16.5** Electropherogram showing the analysis of chicken brain tissue sample by EKC-LIF. Peak identification: internal standard (1), D-aspartate (2), L-aspartate (3), and L-glutamate (4). CE conditions: BGE: 100 mM borate buffer (pH 8.0) containing 5 mM 6-monodeoxy-6-mono(3-hydroxy)-propylamino-β-CD and 8 mM (2,6-di-O-methyl)-β-CD; voltage: –24 kV; temperature: 25 °C; polyacrylamide-coated fused silica: 60 cm × 75 μm I.D. (Reproduced with permission from Ref. [130].)
<table>
<thead>
<tr>
<th>Application</th>
<th>Analyte</th>
<th>Sample</th>
<th>CE mode and detection system</th>
<th>Chiral selector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection of fraudulent manufacturing, storage or processing</td>
<td>Catechins including gallocatechin</td>
<td>Green tea</td>
<td>MEKC-UV</td>
<td>2-Hydroxypropyl-β-CD</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>Lactic acid</td>
<td>Milk and yogurt</td>
<td>EKC-C&lt;sup&gt;4&lt;/sup&gt;D</td>
<td>Vancomycin</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td>Tartaric acid</td>
<td>Wine and grape samples</td>
<td>EKC-C&lt;sup&gt;6&lt;/sup&gt;D</td>
<td>Cu(II)-trans-4-hydroxy-L-proline</td>
<td>[134]</td>
</tr>
<tr>
<td>Detection of adulterations</td>
<td>9 Amino acids</td>
<td>Fruit juices</td>
<td>MEKC-LIF</td>
<td>β-CD</td>
<td>[135]</td>
</tr>
<tr>
<td></td>
<td>Carnitine</td>
<td>Foodstuffs</td>
<td>EKC-MS&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Succinyl-γ-CD</td>
<td>[99]</td>
</tr>
<tr>
<td></td>
<td>Isocitric and citric acids</td>
<td>Fruit juices</td>
<td>EKC-UV</td>
<td>d-Quinic acid</td>
<td>[136]</td>
</tr>
<tr>
<td></td>
<td>Lipoic acid</td>
<td>Dietary supplements</td>
<td>EKC-UV</td>
<td>2,3,6-Tri-O-methyl-β-CD</td>
<td>[137]</td>
</tr>
<tr>
<td></td>
<td>Malic, tartaric, and isocitric acids</td>
<td>Fruit juices</td>
<td>EKC-UV</td>
<td>d-Quinic acid</td>
<td>[138]</td>
</tr>
<tr>
<td>Effects of processing</td>
<td>Tryptophan, phenylalanine, and glutamate</td>
<td>Beers</td>
<td>MEKC-UV</td>
<td>β-CD</td>
<td>[139]</td>
</tr>
<tr>
<td></td>
<td>Ornithine and 18 protein amino acids</td>
<td>Wine and beer</td>
<td>EKC-UV</td>
<td>Sulfated-β-CD and acetylated-γ-CD</td>
<td>[140]</td>
</tr>
<tr>
<td>Characterization of transgenic crops</td>
<td>Arginine, asparagine, alanine, glutamate, and aspartate</td>
<td>Transgenic and wild yeasts</td>
<td>MEKC-LIF</td>
<td>β-CD</td>
<td>[141]</td>
</tr>
<tr>
<td></td>
<td>Arginine, asparagine, alanine, glutamate, and aspartate</td>
<td>Transgenic and wild soybean</td>
<td>EKC-MS</td>
<td>3-Monodeoxy-3-monoamino-β-CD</td>
<td>[142]</td>
</tr>
</tbody>
</table>
includes some of the most recent and relevant articles regarding chiral analysis in food or beverage samples sorted by their particular application such as detection of adulterations, detection of fraudulent manufacturing, storage or processing, investigation of the effects of processing, and characterization of transgenic crops. As an example, the development of a chiral MEKC-UV method to enantioseparate the antioxidant gallocatechin in green tea samples (see Figure 16.6) revealed that its \((-\text{enantiomer})\) can be considered a marker of long-term storage [132]. Authors concluded that epimerization did not significantly occur during aging in the studied samples.

The presence of \(d\)-amino acids in food matrices might involve a fraudulent action such as an adulteration or a racemization reaction as a consequence of fermentation processes or abrasive conditions employed during food manufacture [143]. As recent examples, \(L\)-asparagine was proposed to be a marker to detect adulterations of pomegranate juices with apple juices by MEKC-LIF [135] and a recent work based on an in-capillary preconcentration technique (sweeping and stacking) with MEKC-UV method enabled to find \(D,\text{L}\)-tryptophan, \(D,\text{L}\)-phenylalanine and \(D,\text{L}\)-glutamate in various types of beers [139] demonstrating the potential of this method to carry out analysis of beverages. Furthermore, an EKC-UV method using a dual CD system sulfated-\(\beta\)-CD and acetylated-\(\gamma\)-CD was first developed for the enantiomeric separation of 18 protein amino acids and ornithine [140]. A previous derivatization step with 6-aminoquinolyl-\(N\)-hydroxysuccinimidyl carbamate (AQC) was achieved to enable the sensitive UV

Figure 16.6  Electropherogram showing the analysis of an infusion from (a) “not aged” and (b) “aged” green tea samples by MEKC-UV. Peak identification: \((-\text{epicatechin})\) (EC), \((-\text{epicatechin gallate})\) (ECG), \((-\text{epigallocatechin})\) (EGC), caffeinem (CF), \((-\text{epigallocatechin gallate})\) (EGCG), \((+\text{-catechin})\) \((+\text{-C})\), \((+\text{-gallocatechin})\) \((+\text{-GC})\), \((+\text{-gallocatechin gallate})\) \((+\text{-GC})\). CE conditions: BGE: 25 mM borate-phosphate buffer (pH 2.5) containing 90 mM SDS and 25 mM \((2\text{-hydroxypropyl})\)-\(\beta\)-CD; voltage: 15 kV; temperature: 25 °C; fused silica capillary: 30 cm × 50 \(\mu\)m I.D. (Reproduced with permission from Ref. [132].)
detection of amino acids as well as to make possible their interaction with the CDs. Later, a new fast in-capillary derivatization methodology allowing the full automatization of the derivatization and separation steps was undertaken [144]. Both methods were applied to the analysis of fermented foods to investigate the extent of the presence of ornithine enantiomers. Finally, by studying the $\text{D/L}$-amino acids profile it is possible to identify a transgenic foodstuff from the wild one. Thus, Giuffrida et al. using a MEKC-LIF method found differences in the release pattern of some $\text{D}$-amino acids between transgenic and wild yeast strains [141] and also differences in the amino acid levels of transgenic soybean were found compared to the wild foodstuff [142] by means of an EKC–MS methodology.

The importance of nonprotein amino acids relies on the fact that they can be used as markers for adulterations and also play an important role in the quality control of food. Carnitine is a nonprotein amino acid significant for its beneficial nutritional and pharmacological properties. However, it has been proven that the $\text{D}$-enantiomer causes several health issues, thus, its use as food component is forbidden by legislation. Sánchez-Hernández et al. [99] achieved the unequivocal identification and quantification of carnitine enantiomers by EKC–MS² in 22 different foodstuffs including drinks, biscuits, capsules, and tablets. $\text{D}$-carnitine was found in levels under 3% for most of the analyzed samples and for one of them even the illegal use of a racemic mixture was demonstrated.

The enantioseparation of organic acids present in foodstuff may also serve as indicative of adulterations or fraudulent actions. Pormsila et al. [133] developed a methodology enabling the enantioseparation of $\alpha$-hydroxy- and $\alpha$-amino acids and it was applied to the analysis of lactic acid in fresh milk and yogurt and determining their levels some days after they were opened. It was observed that $\text{L}$-lactic acid was present in fermented products (i.e., in fresh yogurt and milk days after it was opened) and $\text{D}$-lactic acid was found in a yogurt sample as contamination with bacteria took place. The isocitric and citric acid ratio is interesting to be studied as it might be an indicator of the authentication of fruit juices. Analysis of fruit juices reported by Kodama et al. [136] could be considered for authenticating juices as this ratio was in the established limits. Adulteration of dietary supplements with a synthetic mixture of lipoic acid can be identified as only the $\text{R}$-enantiomer is naturally occurring as an antioxidant while the $\text{S}$-enantiomer can only be present when an adulteration has been made. Six out of nine analyzed dietary supplements contained the racemic mixture this being a clear example of adulteration [137].

16.4.4 Environmental and Agrochemical Analysis

The importance of chirality in environmental analysis is mainly related to the different activity of the two enantiomers of a racemic pollutant. It may lead to variations in microbial degradation rates, metabolic pathways, biological uptake, and toxicity which means that one of the two enantiomers might be more
persistent in the environment than the other [145]. In the case of agrochemical products, the use of pure enantiomers in their formulation enables the use of a minor amount of the agrochemical decreasing environmental contamination when only one of the enantiomers has the desired activity. Table 16.5 groups some of the most recent and relevant articles concerning the analysis of environmental samples and agrochemical formulations.

To analyze environmental samples where pollutants are in trace or even ultra-trace levels, it is imperative to use the methodologies with an adequate sensitivity which involves, in most cases, the use of different offline sample treatments or in-capillary preconcentration techniques prior to chiral CE analysis. Petr et al. [146] developed a MEKC-UV approach combined with a novel in-capillary preconcentration strategy allowing the sensitive determination of ketoprofen enantiomers at nanomolar level in spiked waste water. This novel strategy is based on the electrokinetic accumulation of ketoprofen enantiomers on the pH boundary followed by enantioselective mobilization by a SDS mixture, sulfated-\(\beta\)-CD, and trimethyl-\(\beta\)-CD. Another sensitive method is the determination of the enantiomers of fenoprofen by means of large-volume sample stacking with EOF as the pump plus anion-selective exhaustive injection [147]. This in-capillary preconcentration technique allowed obtaining LODs of 0.38 ng/ml for fenoprofen enantiomers, but due to the high salt concentration of the analyzed river water, unfortunately the LOD increased more than 100 times.

Chiral analysis of commercial agrochemical products is required to control the enantiomeric purity of commercial formulations labeled as pure enantiomers and to avoid the noncontrolled manufacturing of these products resulting in concentrations of these compounds differing from the labeled content. As examples, some methods were developed by MEKC [148] and EKC [149] with UV detection in order to control the enantiomeric composition of different commercial agrochemical formulations. Also, a CEC-UV methodology using a novel cellulose-based CSP was developed enabling the enantioseparation of nine pesticides. The method was applied to the quantification of metalaxyl in a commercial fungicide product and its concentration was found to differ from the labeled one [80].

Chiral CE methodologies also give information regarding enantioselective degradation of an agrochemical in different environmental samples. Jarman et al. [150] developed two MEKC and EKC methods, both with UV detection to study the enantioselective transformation of some pesticides when they were added to aerobic slurries. Among the tested pesticides, authors observed an enantioselective transformation of metalaxyl, ruelene, and dichlorprop in some of the studied samples and then calculated their half-lives. For instance, in one of the studied slurries, the half-life of the active \(R\)-enantiomer was about four times lower than the more persistent \(S\)-enantiomer. On the other hand, Chu et al. [151] studied the enantioselectivity of the degradation of the fungicide imazalil in soil. Among all the degradation processes assayed, the UV irradiation offered the highest degradation rate of imazalil. However, none of the degradation processes tested showed enantioselective degradation in the studied samples.
Table 16.5  Some of the most recent and relevant applications of chiral CE in environmental and agrochemical analysis.

<table>
<thead>
<tr>
<th>Application</th>
<th>Analyte</th>
<th>Sample</th>
<th>CE mode and detection system</th>
<th>Chiral selector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental analysis</td>
<td>Ketoprofen</td>
<td>Spiked waste water</td>
<td>MEKC-UV</td>
<td>Sulfated-β-CD and 2,3,6-tri-O-methyl-β-CD</td>
<td>[146]</td>
</tr>
<tr>
<td></td>
<td>Fenoprofen</td>
<td>River water</td>
<td>EKC-UV</td>
<td>Vancomycin</td>
<td>[147]</td>
</tr>
<tr>
<td></td>
<td>cis-bifenthrin</td>
<td>Polyvalent commercial insecticide formulation</td>
<td>MEKC-UV</td>
<td>Sodium cholate and 2,3,6-tri-O-methyl-β-CD</td>
<td>[148]</td>
</tr>
<tr>
<td></td>
<td>Metalaxyl and benalaxyl</td>
<td>Commercial agrochemical formulations</td>
<td>EKC-UV</td>
<td>Succinyl-γ-CD (for metalaxyl) and succinyl-β-CD (for benalaxyl)</td>
<td>[149]</td>
</tr>
<tr>
<td></td>
<td>9 Pesticides</td>
<td>Commercial metalaxyl products, spiked soil and tap water</td>
<td>CEC-UV</td>
<td>Cellulose tris(4-chloro-3-methylphenylcarbamate)</td>
<td>[80]</td>
</tr>
<tr>
<td></td>
<td>5 Pesticides</td>
<td>Spiked aerobic slurries</td>
<td>EKC-UV (imazaquin and dichlorprop) and MEKC-UV (metalaxyl, fonofos, and ruelene)</td>
<td>γ-CD (metalaxyl and fonofos), 2,6-di-O-methyl-β-CD (imazaquin), 2-hydroxypropyl-β-CD (ruelene), 2,3,6-tri-O-methyl-β-CD (dichlorprop)</td>
<td>[150]</td>
</tr>
<tr>
<td></td>
<td>Imazalil</td>
<td>Soil</td>
<td>EKC-UV</td>
<td>β-CD</td>
<td>[151]</td>
</tr>
<tr>
<td></td>
<td>14 amino acids</td>
<td>Fertilizers</td>
<td>EKC-MS²</td>
<td>β- or γ-CD</td>
<td>[100]</td>
</tr>
</tbody>
</table>
The racemization of amino acids in hydrolyzed protein fertilizers was performed by Sánchez-Hernández et al. [100] by means of an EKC–MS² methodology. High racemization was observed for fertilizers which underwent chemical hydrolysis while low D-amino acid content was found in those which experienced enzymatic hydrolysis as can be seen in Figure 16.7.

16.4.5

Other Areas of Application

Although it is evident that chiral CE is mainly employed in pharmaceutical, clinical, food, and environmental fields, this technique offers numerous possibilities in other areas. For example, an interesting EKC-UV method was applied in the analysis of the doping chiral drug amphetamine in urine, obtaining LODs far below the legal limits thanks to the coupling to a single drop microextraction technique [152]. Also, the development of an EKC-UV method in combination with a dispersive liquid–liquid microextraction was applied to the determination of multiple chiral drugs of abuse in forensic samples such as banknotes, silver paper, and kraft paper [153].

Chiral analysis of plant extracts was also possible by means of CE methodologies. The determination of toxic gossypol enantiomers in cottonseeds and flower petals [154] and of the stereoisomers of the plant hormone jasmonic acid in wounded tobacco leaves [155] was carried out, finding endogenous (−)-jasmonic acid and (+)-epi-jasmonic acid at different concentrations.

Some of the chiral compounds employed in the cosmetic industry are considered as emergent contaminants so their optical purity must be carefully controlled. Chiral polycyclic musks were analyzed in perfumes and personal care products by MEKC-UV using sodium dodecyl sulfate as the surfactant and a dual CD system composed of HP-γ-CD and γ-CD as the chiral selector [156].

Research works focused on the use of LE compounds as chiral selectors (e.g., amino acid ionic liquids and Zn(II)-l-alanine) have been described in the literature to determine the kinetics of enzymes. Basically, the separation principle is based on the interchange between the enantiomers and LE in the coordination sphere of a bivalent metal ion. These methodologies have shown a high power in the enantioresolution of dansyl-D/L-amino acids and they have been mainly applied to evaluate the kinetic constants of different enantioselective enzymes (tyrosinase, D-amino acid oxidase, and l-amino acid oxidase) by monitoring the concentration change of substrates (namely, tyrosine or methionine) [157–159].

Finally, EKC has demonstrated to be a very useful tool to determine the enantiomeric purity of products obtained by catalytic asymmetric reactions [160–162].

16.5

Concluding Remarks and Future Trends

CE is nowadays a powerful alternative to chromatographic techniques such as GC or HPLC to carry out chiral separations. The high efficiency, simplicity, and
Figure 16.7 Extracted ion electropherograms of the CE–MS² transition and their respective spectra of (a) fluorescein isothiocyanate (FITC)-glutamate of a fertilizer sample after enzymatic hydrolysis, (b) FITC-alanine of a fertilizer sample after chemical hydrolysis and (c) FITC₂-ornithine of a fertilizer sample after chemical hydrolysis. CE conditions: BGE: 5 mM γ-CD (a and b), 5 mM β-CD (c) in 50 mM ammonium carbonate buffer (pH 10.0); voltage: 20 kV; temperature: 25 °C; hydrodynamic injection: 15 s at 50 mbar. (Reproduced with permission from Ref. [100].)
low consumption of chiral selectors, reagents, and samples confer CE with interesting features in chiral analysis. Indeed, CE methodologies possess attractive capabilities being considered excellent tools to enantioseparate a wide range of analytes in different research fields. Although CE has successfully been applied in bioanalysis and in the analysis of environmental samples, pharmaceutical and food analysis are without doubt the research areas in which chiral CE has mainly been employed. In the case of pharmaceutical analysis, the application of chiral CE methodologies enabled the determination of the enantiomeric purity of chiral drugs as well as the quantitation of the majority enantiomer and the enantiomeric impurities if present. In the case of food analysis, the detection of some enantiomers can reveal food adulterations, fraudulent actions, or the effects of some food processing or aging.

EKC, CEC, and NACE are the CE modes employed to achieve chiral separations, the first one being the favorite. Despite the fact that many chiral selectors are available to carry out enantioseparations by CE, the search for new chiral selectors continues. However, until now, there is no new class of chiral selectors competing with CDs from the viewpoint of availability, diversity, universality, price, and safety. Thus, it seems that CDs will remain (at least for the near future) the strongly dominating class of chiral selectors in CE.

Choosing the suitable chiral selector for the separation of a specific pair of enantiomers is usually based on “trial-and-error” approaches which require considerable experimentation. An enhancement of our still limited knowledge of the molecular principles/mechanisms governing chiral recognition represents therefore a major challenge for the future to choose and create suitable chiral selectors for given enantiomers. The implementation of novel strategies allowing a synergistic interaction between experimental data and molecular modeling may lead to further progress in this field.

Usually, a high sensitivity is required to carry out a chiral analysis so that the excellent potential demonstrated by CE is related with the development of pre-concentration strategies and the use of highly sensitive detectors. The MS detector has been one of the most employed alternative detection systems for developing chiral separations by CE. Despite CE–MS coupling providing additional selectivity and structural information, this hyphenation must be carefully performed due to the frequent incompatibility between the chiral selector and the MS detector which might reduce its applicability. Both the advances regarding ESI interfaces and the development of chiral selectors compatible with MS can contribute to increase the number of applications of CE–MS for chiral separations.

Even though CE offers at present a well-established group of analytical techniques for chiral separations, the full potential application of the developed methodologies has not yet been achieved so that it is easy to conclude that the future of enantioseparations by CE seems to be very promising.
Acknowledgments

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