

Chapter 1

Introduction

1.1 History and development of capillary electrophoresis

1.1.1 History

Electrophoresis is defined as the migration of charged species under the influence of an externally applied electric field. Differences in mobility of the analytes are due to their average charge, size, shape, and on the properties of the used electrolyte solution. The Russian physicist Reuss carried out the first separations based on this principle in 1809. He studied the migration of colloidal clay-particles and discovered that the liquid adjacent to the negatively charged surface of the wall migrated towards the negative electrode under influence of an externally applied electric field [1]. The theoretical aspects of this electrokinetic phenomenon that Reuss called electro-osmosis were formulated in 1897 by Kohlrausch [2]. In the late 1800's and early 1900's, electrophoretic separations in so called 'U'-shaped tubes were carried out by several researchers in their attempt to separate diphtheria toxin, globulin, and toxin/antitoxin solutions. Michealis introduced the word electrophoresis into the scientific world in 1909 during his successful attempts to determine the isoelectric points of invertase and katalase, two enzymes that were at that time not available in pure form [1].

The analytical aspects of electrophoresis were advanced by Tiselius, starting in 1925 with his PhD thesis on the development of free moving boundary electrophoresis. This resulted in the separation of complex protein mixtures based on differences in electrophoretic mobilities [3]. In 1948 Tiselius was awarded the Nobel price for Chemistry for his work on electrophoresis. In 1950 Durrum introduced paper electrophoresis, separating serum proteins, which was considered to offer several advantages over the Tiselius apparatus, soon improved by developing high voltage equipment [1]. One of the major problems concerning electrophoresis, however, was the poor resolution observed due to peak broadening caused by Joule heating and in a minor way caused by molecular diffusion, as will be explained in Chapter 2. The most common way to prevent this peak broadening was the use of stabilizers, e.g. starch gel, paper, agarose, cellulose acetate or polyacrylamide gel, as summarized by Gordon *et al.* [4]. Although the above-mentioned stabilizers minimized the problem of convection, they led to other peak broadening

phenomena, like Eddy diffusion and undesired interactions between analytes and stabilizer (if desired we call it electrochromatography). In the mid-1960's Tiselius and Hjertén developed polyacrylamide gel electrophoresis in 3-mm i.d. rotating capillaries for the separation of ribosomes and viruses [5]. In 1967 Hjertén reviewed the technique, in which stabilization was achieved by continuous rotation of the tube about its longitudinal axis and thus without the mentioned causes of peak broadening [6-8].

1.1.2 Development

Giddings deduced that in theory, when longitudinal diffusion is the only cause of peak broadening, a very high efficiency must be possible [9]. The latter was, to a reasonable extent, verified by experiments done by Everaerts *et al.* in 1970 using 200 μm i.d. Teflon® tubes [10] and by Virtanen in 1974 using thin glass tubes [11]. They discovered the stabilizing "wall effect" by using the thin tubes as a separation column in which the small diameter of the column counteracts the convective flow, leading to an increase in efficiency. The latter is a consequence of the phenomenon that the increase in efficiency is proportional to the decrease in diameter of the tube due to the increasing surface-to-volume ratio. Although the theoretical predictions made by Giddings were more or less approximated over the years [12,13], it was not until 1981 that Jorgenson and Lukacs [14-17] demonstrated an electrophoresis system using open glass capillaries of 75 μm i.d. with an on-column fluorescence detector. They applied voltages up to 30KV, which provided the predicted efficiencies (>400000 plates) illustrated by the separation of fluorescent dansylated amino acids and the separation of fluorescamine derivatized amino acids in human urine within 25 minutes. In the first review on capillary electrophoresis [18] the possible advantages of performing zone electrophoresis in open tubes of small diameter were summarized:

1. Efficient heat transfer within the electrophoresis medium is achieved, leading to minimal temperature gradients.
2. Disadvantageous effects of remaining temperature gradients are minimized by solute diffusion back and forth across the tube diameter.
3. The medium is stabilized against convective flow by the wall effect.

After these initial experiments and after the introduction of the first commercially available instruments in 1988, the potential of capillary electrophoresis as a high performance separation technique in analytical (bio)technology, bioanalysis and pharmaceutical analysis was soon acknowledged in some early papers [19-24] and emphasized by more than 3000 registered scientific publications in 2000 in contrast to 35 publications in 1990 [25]. It proved to be a powerful and useful method of analysis, especially when small amounts of sample are available or the analyte is present at very low mass levels. The mass detection limits with CE are very good, but the concentration limits are not on a level that can be compared to, for instance, HPLC. Because of the low injection volume and the small path-lengths of the UV and fluorescence detectors, the concentration sensitivity in CE was (and still is) often much lower than that of conventional detectors used in HPLC [26,27]. CE has been used in bioanalysis for the separation of the analyte under study from matrix components, metabolites and/or structurally related substances [28-34]. In pharmaceutical analysis CE has been used for the separation of inactive excipients, drug degradation products and, if appropriate, synthetic precursors or side-products [34-39]. CE has also been introduced as an analytical technique in cancer research by monitoring tumor markers by CE-MS [40,41]. In the field of forensic science, CE techniques are routinely applied, including the analysis of PCR-amplified DNA, the determination of trace amounts of explosives and their residues [42-47]. In 1996 CE was used as an analytical technique to provide scientific evidence in a case of sexual assault. DNA analysis with quantitation by CE was performed in hair samples and the results led to the conviction of the suspect [48,49].

Especially for chiral separations CE seems to be the separation technique of choice. Some major advantages of chiral separations in CE in comparison with HPLC are the low consumption of the chiral selector (reduced costs) and the high plate numbers due to a reduced peak broadening as a consequence of the absence of Eddy diffusion and mass transfer between two phases (the A- and C-term of the Van Deemter equation, respectively). Also the selectivity that is defined as the extent to which the analyte under study can be assessed in a complex mixture without interference from the other components in the mixture, obtained by a difference in mobility between the solutes under the influence of an

external electrical field, is high in CE. The latter expression was extensively discussed as one of the key parameters in electrokinetic separation sciences [28]. Therefore, because of the combination of the high plate numbers and a high selectivity, baseline separations with CE can be achieved at low(er) concentration levels of the chiral selector.

1.2 Modes of capillary electrophoresis

CZE is probably the most applied capillary electrophoretic separation technique because of its simplicity and fast separations. CZE is based on the electrophoretic properties of the analyte and the buffer solution (pH and ionic strength) only and has been applied in aqueous and non-aqueous buffer solutions with different electrolyte systems (e.g. capillary moving boundary electrophoresis, capillary isotachopheresis, and capillary isoelectric focusing). When CZE is unable to provide an acceptable selectivity towards the analytes, for instance when dealing with the separation of neutral and/or chiral compounds, modification of the separation system is necessary. These modified CE systems are in general indicated as electrokinetic chromatography (EKC) and can be subdivided, based on the nature of the used additives, into micellar electrokinetic capillary chromatography (MEKC) and capillary electro-chromatography (CEC). In MEKC, micelles are used as a *pseudo*-stationary phase in order to separate neutral and/or charged analytes. The separation principle is based on differences in mobility between the (neutral) analytes that is obtained by a difference in interaction with either the positively charged micelle (e.g. Cetyltrimethylammonium bromide, CTAB) or the negatively charged micelle (e.g. Sodium dodecylsulfate, SDS). In EKC differences in mobility are obtained by a difference in chromatographic interaction of the analytes with either a *pseudo*-stationary phase (e.g. Cyclodextrins (CDs) or free moving particles) or a stationary phase (CEC, *i.e.* CE columns fully packed with for instance octadecylsilane or columns having a thin film of stationary phase covalently bound to the inner-wall of the capillary, so called open-tubular columns).

The most interesting approaches in CE are probably the selectors that provide separations based on chiral or molecular recognition and

which are consequently able to separate enantiomers. The most widely used chiral selectors in CE are without doubt the CDs. Especially the chemically modified CDs, which are commercially available, provide a high selectivity for chiral but also achiral separations. A new promising mode seems to be the use of molecularly imprinted microspheres as (chiral) selectors in CE. These MIP particles should be able to provide a unique selectivity, based on molecular recognition. The obtained enantioselectivity is a consequence of the difference of the strength of the interaction of the chiral selector and the two enantiomers (thermodynamic enantioselectivity). The peak shape is dependent on the difference in the rate of the interaction of the chiral selector and the two enantiomers (kinetic enantioselectivity). When using a CEC system, the efficiency is to a large extent dependent on the rate of the mass transfer that occurs due to the distribution of the analytes between two phases. When a slow mass transfer occurs, it will lead to (severe) peak broadening. The latter phenomenon is described in the next section.

1.3 Peak broadening

One of the fundamental aspects in separation science is the occurrence of peak broadening, resulting in a decrease in efficiency. In 1956 Van Deemter, Zuiderweg and Klinkenberg [50] derived their famous equation ($H=Au^{1/3} + B/u + Cu$, where H is the theoretical plate height and u is the linear velocity of the mobile phase) that combines the three major sources contributing to peak broadening: 1) multiple paths (tortuosity) of an analyte through the separation bed, the so called Eddy diffusion or A-term; 2) longitudinal molecular diffusion, the B-term, and 3) slow mass transfer between the phases, the C-term. In CZE, due to the absence of both Eddy diffusion and a mass transfer, peak broadening is ideally only caused by longitudinal molecular diffusion (also called axial diffusion).

Although longitudinal diffusion in CZE will be the major contributor to peak broadening, several authors [18,51-59] have indicated additional parameters causing a decrease in efficiency:

- Temperature gradients within the capillary, caused by Joule heating, generated by the electric current through the system. Joule heating can be expressed as $W=i^2/\kappa A^2$, where i is the electric current, κ is the specific conductance, and A is the cross-sectional area of the capillary [60]. The electrophoretic mobility will increase with the temperature of the medium with approximately 2% per degree Celsius [15,56,61]. The easiest way to avoid these temperature gradients is to reduce the tube radius.
- Injection effects (column overloading). Usually, injection volumes larger than 1% of the total length of the capillary (*i.e.* 20 nL when the i.d.=50 μ m and L=1m) will lead to an increase in peak broadening.
- Peak broadening due to electromigration. If the conductivity of the sample zone is different to that of the background electrolyte, its presence in the buffer medium can change the uniformity of the field strength along the capillary. The distorted electric field leads to asymmetric and broadened zones. Furthermore, each solute band moves through the detector window with a velocity that is determined by its electrophoretic mobility and the applied voltage. The peak width per time unit is directly related to the velocity of the solutes, *i.e.* the solutes with slow velocity migrate through the detector window much slower than solutes with a high velocity, which results in an increased peak width for the former solute.
- Wall adsorption. Adsorption of solutes (especially proteins) to the wall of the capillary leads to a change of the EOF, asymmetric zone deformation, sample loss, reduction of column efficiency and irreproducible migration times.

1.4 Proposed mechanisms for chiral and molecular recognition

In general, from a geometrical point of view, a three-point interaction is mandatory for chiral and molecular recognition in host-guest chemistry (*i.e.*

to form a diastereomeric complex between the analyte and the chiral selector) as described by Easson and Stedman [62] and later acknowledged by Ogston [63] and Dalgliesh [64]. This three-point interaction model has been demonstrated on many designs, but the most elegant and informative one is probably the “left-and-right-hand” model as described in many textbooks and which was presented as a vivid model of chiral recognition by Meyer and Rais [65]. However, it can be possible that a stable enantiomer-host receptor complex occurs due to, for instance, two hydrogen-bonding interactions and one steric hindrance as is the case for some amino-acids in biological systems [66]. In this case, the three-point interaction model is not based on three attractive interactions, but on two attractive and one repulsive (non-bonding) interaction. Davankov [67,68] also mentioned that achiral components like solvent molecules and even sorbent surfaces can participate in the chiral recognition (*i.e.* the formation of the diastereomeric complex). Nevertheless, Davankov stated that the above-mentioned cases should be seen as an extension to the three-point interaction model rather than an exception of the model. Finally, Booth *et al.* [69] summarized the chiral recognition process for a ligand-receptor interaction, which consists of four steps: 1) the formation of a selectand-selector complex; 2) the positioning of selectand-selector to yield optimized interactions (conformational adjustments); 3) the formation of secondary interactions (activation of the complex), and 4) expression of the molecular fit.

For the chiral recognition by CDs, several recognition mechanisms have been proposed in the literature. A suitable model is the so-called lock-and-key mechanism or inclusion mechanism, that requires the following assumptions [70]: (a) an inclusion complex must be formed; (b) there must be a relatively tight fit between the analyte and the CD, and (c) the chiral center must be near, and must interact with the ‘mouth’ of the CD cavity. This mechanism seems to be adequate when it comes to explaining the chiral separation of, for instance, 1,1'-binaphthalene with β -CD, because in this system no hydrogen-bonding site(s) exists [66]. Furthermore, in aqueous systems intermolecular hydrogen bonds are hardly formed because of strong hydration to hydrogen bonding sites of both host and guest molecules. Using non-aqueous media in CE, strong hydrogen-bonding may contribute to the desired chiral recognition and beyond that to a possible

chiral separation. Also recognition mechanisms like point interactions through Coulomb forces, point interactions through coordinative bonds, and dipole-dipole interactions have been described in the literature [71-73]. In all cases it should be realized that when chiral/molecular recognition occurs, it does not necessarily lead to chiral discrimination (see Chapter 6).

The above-mentioned recognition mechanisms of the enantiomers and the chiral selector have been extensively studied by NMR techniques and X-ray crystallography [74]. A challenging approach is the application of molecular modeling to predict and describe the possible inclusion complexes [75-79].

1.5 Regulatory perspective to chiral separations

As has been mentioned in many textbooks, the need for chiral separations was acknowledged by Louis Pasteur who reported in 1858 the stereochemical differences between the *dextro* and the *levo* form of ammonium tartrate. He observed that the *dextro*-isomer was more readily degraded by a mold than the *levo*-isomer. The first pharmacological differences between enantiomers (adrenaline or epinephrine) were for the first time observed by Abderhalde and Müller in 1908 [69]. Nowadays, the US Food and Drug Administration (FDA), as well as regulatory authorities in Europe, China and Japan have provided guidelines indicating that preferably only the active enantiomer of a chiral drug should be brought to the market [80-84]. Exceptions are (i) that both enantiomers have a comparable pharmacological effect, which is hardly ever the case; (ii) if *in vivo* or *in vitro* enantioconversion of the eutomer occurs, or (iii) that the effect is stereospecific, *i.e.* the effect is 100% related to the eutomer and the distomer shows no side-effect. In general, however, the distomer will have a certain pharmacological activity and, moreover, it may affect the pharmacological activity of the eutomer.

A more complex situation seems to occur when the chiral compound possesses two (*e.g.* scopolamine, ephedrine, labetalol) or even more than two (*e.g.* morphine, penicillines, glucose) asymmetrical centers. A chiral compound possessing n chiral centers can exist of 2^n stereoisomers, all with their own possible pharmacological activity. Some of these stereoisomers

are geometric isomers or diastereoisomers and are supposed to be treated as separate drugs and are developed, unless *in vivo* interconversion occurs, as such. Yet, little direction is given by any regulatory authority regarding development of compounds with multiple chiral centers [83,84].

1.6 References

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