A physicochemical approach of capillary electrophoresis towards method development

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Technische Universiteit Delft, op gezag van de Rector Magnificus Prof. ir. K.F. Wakker, in het openbaar te verdedigen ten overstaan van een commissie, door het College van Dekanen aangewezen, op dinsdag 17 december 1996 te 13.30 uur door

Hugo André Lisette CORSTJENS

licentiaat in de scheikundige wetenschappen Katholieke Universiteit Leuven

geboren te Maaseik (België)
Dit proefschrift is goedgekeurd door de promotor:
Prof. ir. K.CH.A.M. Luyben

Toegevoegd promotor:
Dr. ir. J. Frank

Samenstelling promotiecommissie:

Rector Magnificus (voorzitter)  Technische Universiteit Delft
Prof. ir. K.Ch.A.M. Luyben (promotor)  Technische Universiteit Delft
Dr. ir. J. Frank (toegevoegd promotor)  Technische Universiteit Eindhoven
Prof. dr. ir. C.A. Cramers  Technische Universiteit Delft
Prof. dr. G. Frens  Technische Universiteit Delft
Prof. dr. ir. G.W.K. van Dedem  Technische Universiteit Delft
Dr. T.K. Gerdings  Solvay Duphar, Weesp

Dit onderzoek is uitgevoerd bij de Vakgroep
Bioprocesstechnologie
van de Technische Universiteit Delft.
'Mijn overleden heer had het idee gehad dat de krachten hun oorsprong niet in het duister hadden maar dat ze van het licht afstammen of het licht waren, en het licht zou nog niet geschapen zijn.'

'Zou dus alles wat er is afstammen van iets wat nog niet geschapen is?'

'Het meeste licht zou naar zijn mening nog ongeschenen zijn. Maar ik geef toe dat dit gesprek, hoe aangenaam het ook is, een wending heeft genomen die mijn verstand te boven gaat.'

De dwaas (K. Ekman)
## Contents

1. **GENERAL INTRODUCTION**  
   1.1 The basic principles of capillary electrophoresis  
   1.2 Modes of capillary electrophoresis  
   
   1.2.1 Capillary zone electrophoresis  
   1.2.2 Micellar electrokinetic capillary chromatography  
   1.2.3 Capillary gel electrophoresis  
   1.2.4 Capillary isoelectric focusing  
   1.2.5 Capillary isotachophoresis  
   1.2.6 Capillary electrokinetic chromatography  
   1.3 Aim and outline of this thesis  
   1.4 References  

2. **VARIATION OF THE pH OF THE BACKGROUND ELECTROLYTE DUE TO ELECTRODE REACTIONS IN CAPILLARY ELECTROPHORESIS:**  
   **THEORETICAL APPROACH AND IN SITU MEASUREMENT**
   2.1 Introduction  
   
   2.1.1 The nature of electrode reactions  
   2.1.2 Measuring the pH  
   2.2 Materials and methods  
   
   2.2.1 Apparatus  
   2.2.2 Chemicals  
   2.2.3 Buffers and samples  
   2.3 Results and discussion  
   
   2.3.1 Theoretical approach  
   2.3.2 Experimental verification  
   2.3.3 *In situ* pH measurement  
   2.4 Conclusions  
   2.5 References
3. AN EQUATION FOR THE DESCRIPTION OF THE RESOLUTION OF CHARGED SOLUTES IN MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY 31
   3.1 Introduction 32
   3.2 Theory 33
      3.2.1 A resolution equation for charged solutes in MECC 33
      3.2.2 Evaluation of the resolution as a function of different parameters 36
         3.2.2.1 Simplification to the resolution equation for uncharged solutes 36
         3.2.2.2 The resolution as a function of $k'_i$ and $k'_j$ 37
   3.3 Experimental verification 46
   3.4 Conclusions 51
   3.5 References 51

4. A CAPILLARY ELECTROPHORETIC STUDY ON THE INFLUENCE OF CHARGE AND FRICTION ON THE MOBILITY OF SAVINASE™ AND SITE DIRECTED MUTANTS 53
   4.1 Introduction 54
   4.2 Material and methods 55
      4.2.1 Apparatus 55
      4.2.2 Chemicals 56
      4.2.3 Buffers and samples 56
      4.2.4 Calculation of the dipole moments and projected surface areas of the Savinases 58
      4.2.5 Description of the electrophoretic mobility of proteins 58
   4.3 Results 60
      4.3.1 The proteins under investigation 60
      4.3.2 Effect of the ionic strength on the migration behaviour of Savinase variants 61
      4.3.3 The migration behaviour of Savinase variants at pH 3 62
      4.3.4 The migration behaviour of Savinase variants at pH 6 64
      4.3.5 The migration behaviour of Savinase variants at pH 6 in the presence of Ca$^{2+}$ 66
   4.4 Discussion 68
      4.4.1 Effect of the ionic strength on the migration behaviour of Savinase variants 68
5. A CAPILLARY ELECTROPHORETIC STUDY OF THE INTERACTION OF METHYLAMINE DEHYDROGENASE WITH AMICYANIN

5.1 Introduction

5.1.1 Estimation of the binding constant

5.1.2 The biochemical system under investigation

5.2 Experimental

5.2.1 Apparatus

5.2.2 Chemicals and buffers

5.2.3 Simulations

5.3 Theory

5.3.1 The determination of the association constant

5.3.2 Simulation of the electrophoretic mobility using the theoretical plate model

5.3.3 The effect of the ionic strength on the association constant

5.3.4 The effect of the osmotic pressure on the association constant

5.4 Results and discussion

5.4.1 General considerations

5.4.2 The estimation of $\mu_\text{A}$, $\mu_\text{MA}$ and $K_\text{ass}$

5.4.3 The effect of the pH on the association constant

5.4.4 The effect of the ionic strength on the association constant

5.4.5 The effect of the osmotic pressure on $K_\text{ass}$

5.5 Conclusions

5.6 References

6.1 Introduction 102

6.2 Statistical approaches in the optimisation of CE 104

6.3 Optimisation procedures based on physicochemical models 107
   6.3.1 Global approaches in the optimisation of CE 107
   6.3.2 Practical approaches in the optimisation of CE 113

6.4 Conclusions 117

6.5 References 118

7. **Optimisation of Selectivity in Capillary Zone Electrophoresis and Micellar Electrokinetic Capillary Chromatography Using the Iterative Regression Strategy**

7.1 Introduction 122

7.2 Theory
   7.2.1 The general principles on which the iterative regression strategy is based 123
   7.2.2 The choice of the parameter and the accessory parameter space 124
   7.2.3 The description of the migration behaviour in capillary electrophoresis used in the iterative regression strategy 125
   7.2.4 The choice of the criterion in the iterative regression strategy 127
   7.2.5 The experimental set-up of the iterative regression optimisation strategy 128

7.3 Experimental 130
   7.3.1 Apparatus 130
   7.3.2 Chemicals 130
   7.3.3 Buffers and samples 130
   7.3.4 Optimisation 131

7.4 Results and discussion 131
   7.4.1 The choice of the solutes 131
   7.4.2 The one-parameter pH optimisation 131
   7.4.3 The simultaneous two-parameter optimisation 134
<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 Conclusions</td>
<td>138</td>
</tr>
<tr>
<td>7.6 References</td>
<td>139</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>143</td>
</tr>
<tr>
<td>SAMENVATTING</td>
<td>147</td>
</tr>
<tr>
<td>LIST OF SYMBOLS AND ABBREVIATIONS</td>
<td>151</td>
</tr>
<tr>
<td>DANKWOORD</td>
<td>155</td>
</tr>
</tbody>
</table>
Chapter 1
General introduction
1.1 The basic principles of capillary electrophoresis

Electrophoresis is defined as the movement of suspended molecules or particles in solution under the influence of an electric field. When the components of a mixture are characterised by a difference in migration speed, their separation and analysis can be in principle achieved. Although it is beyond the scope of this introduction to elaborate an historical overview of electrophoretic separations, it is noteworthy that the first electrophoretic experiments were already performed in the beginning of the nineteenth century by von Reuss [1], who observed the movement of clay particles in an electric field. An historical overview, together with many important contributions to the development of (capillary) electrophoretic analysis, both from the practical as well as the theoretical point of view, has been written by Compton and Brownlee [2].

The migration velocity $v_m$ of a charged particle in an electric field is proportional to the field strength, $E$, by its electrophoretic mobility $\mu$, as shown in equation 1.1.

$$v_m = \mu E \quad (1.1)$$

The electrophoretic mobility is the balance between the electric force that the molecule experiences and its frictional drag through the medium

$$\mu \sim \frac{\text{electric force}}{\text{frictional force}} \quad (1.2)$$

The mobility $\mu$ is affected by the experimental conditions and buffer properties. It is e.g. obvious that the net charge of weak acids and bases and thus the electric force is controlled by the pH of the background electrolyte. Less evident parameters, like the type of the buffer- and counter ion or the temperature of the background electrolyte, affect $\mu$ as well but are not always considered as such or their effect on $\mu$ is often not fully understood. Consequently, the electrophoretic mobility should not be confused with the often tabulated values for the absolute mobilities of a solute, defined at infinite dilution and complete ionisation. An improved understanding of the mobility under the actual experimental conditions is important so that well-considered adjustments of the experimental conditions enable a streamlined development of a capillary electrophoretic experiment.
In classical electrophoresis gels have been frequently used to reduce convection but the introduction of narrow capillaries, most often made of fused silica with an inner diameter ranging from 25 to 100 µm, which act themselves as anticonvective, represents one of the early stages of practical capillary electrophoresis (CE) [3-6]. Because no stabilising agents are present, 'real' free zone electrophoresis can be performed. Another major advantage of the use of narrow bore capillaries results from its very high surface area to volume ratio, favouring an efficient dissipation of the Joule heat generated during the electrophoretic process to the surrounding medium. This allows the use of field strengths up to 500 V cm⁻¹ which enables fast separations characterised by a high efficiency. Furthermore, such a capillary electrophoretic set-up is characterised by a low solvent throughput, which reduces the amount of waste and facilitates the use of complex and expensive buffer systems. In addition, the sample volume is typically in the order of a few nanoliters and this minute volume enables the analysis of solutes only available in very small amounts, provided that the concentration is sufficiently high for the detection technique employed.

A very important feature of CE is the electroosmotic flow due to the surface charge of the negatively charged silica wall under influence of the electric field. If controlled properly, the electroosmotic flow, characterised by an almost plug-flow profile, can be exploited to drag both cations and anions to the same end of the capillary, so that both type of ions can be analysed in a single run.

Although in this work several commercially available CE apparatuses were used, the basic experimental set-up is in all cases similar and relatively simple. A schematic presentation of the compartments of a CE apparatus is shown in Fig. 1.1, and consists of 2 buffer vials, at least one sample vial, a capillary from which both ends are immersed in the buffer vials and two electrodes connected to the high voltage power supply. In most cases, optical on-line detection is performed at the opposite end of the injection side, although off-line detection is possible as well.

The most obvious application area of CE is related to analytical separations. The main goal is then to achieve a desired separation followed by a qualitative and/or quantitative interpretation of the results. This may be done both in the area of research and development as well as in routine analysis. In some cases, a careful examination of the analytical data obtained from a CE experiment provides valuable information about the physicochemical properties of the solutes. Capillary electrophoresis is therefore suited to investigate properties
of the solutes directly or sideways related to the charge density and the friction of the solutes, according to Eq. 1.2.

![Diagram of capillary electrophoresis set-up]

Fig. 1.1 Schematic presentation of a capillary electrophoretic set-up

### 1.2 Modes of capillary electrophoresis

The application range of CE is considerably extended because of the various modes in which CE can be performed. Many of these modes are possible on the same CE apparatus and switching from one mode to the other generally only requires a change of the buffer system. Below, the various CE modes are described briefly.
1.2.1 Capillary zone electrophoresis

Fig. 1.2 Schematic presentation of a separation in capillary zone electrophoresis

Capillary zone electrophoresis (CZE) is the most widely used and simplest mode of CE, in which the capillary is only filled with buffer. The separation is then based on differences in the mobility values of the solutes. All neutral solutes migrate with the speed of the electroosmotic flow and will not be separated. The application range of CZE is extensive and numerous ionic species can be analysed [7].

To improve the separation, the migration behaviour of the solutes can be modified by dissolving additives in the buffer that interact in a specific manner with the solutes. Typical examples are chiral separations and the analysis of inorganic metal ions accomplished by adding the appropriate complexing agents [8, 9].

1.2.2 Micellar electrokinetic capillary chromatography

Fig. 1.3 Schematic presentation of a separation in micellar electrokinetic capillary chromatography

Micellar electrokinetic capillary chromatography (MECC) differs from CZE in that a surfactant, at a concentration higher than the critical micellar concentration, is added to the buffer. Micelles, which are usually characterised by a hydrophobic core and a charged or
polar exterior, will then be formed. Provided that the velocities of the electroosmotic flow and the pseudo-stationary micellar phase are different, the partitioning of a solute between the aqueous and the micellar phase determines its overall velocity.

Initially, this mode of CE was introduced to enable the separation of neutral solutes in an electrophoretic system and in that case the selectivity is solely due to differences in the affinity for the micellar phase [10]. It has been shown, however, that mixtures of both charged and uncharged solutes can be separated in a MECC system, hence electrophoretic and chromatographic migration mechanisms are combined [11]. Therefore, the choice of the type and concentration of the micelle is considered to be an additional tool to increase the selectivity.

1.2.3 Capillary gel electrophoresis

![Diagram](Image)

Fig. 1.4 Schematic presentation of a separation in capillary gel electrophoresis

Capillary gel electrophoresis makes use of capillaries filled with polymer networks. These gels act as a molecular sieve and because large molecules will be hindered more than small ones, they will be retarded accordingly. This mode of CE is therefore very analogous to the conventional slab gel electrophoresis. Capillary gel electrophoresis can be used for the separation of large molecules having the same charge to mass ratio but differing in size, and is therefore suited to analyse various proteins and nucleic acids [12].
1.2.4 Capillary isoelectric focusing

If the capillary is filled with an ampholyte (represented by a, b, ... in Fig 1.5), a stable pH gradient will be formed after the voltage has been turned on. Ionisable solutes will then move through the capillary and take a location according to their pI's, where they carry no charge. In this way, proteins can be analysed and separated according to their isoelectric point [13, 14].

1.2.5 Capillary isotachophoresis

Capillary isotachophoresis makes use of two buffer systems: the leading and the terminating electrolyte (represented by L and T in Fig. 1.6 respectively). The sample, which is positioned in between the leading and the terminating electrolyte, will be separated into different zones.
but all of these zones will be sandwiched between both electrolytes. The zones are ordered according to their mobility but they migrate with the same velocity. The solutes in the zones have the same concentration, determined by the concentration of the leading electrolyte. For that reason capillary isotachophoresis is often used as a preconcentration step prior to a different mode of CE [15].

1.2.6 Capillary electrokinetic chromatography

![Diagram of capillary electrokinetic chromatography](image)

*Fig. 1.7 Schematic presentation of a separation in capillary electrokinetic chromatography*

Capillary electrokinetic chromatography makes use of capillaries packed with a chromatographic packing. In a first approximation, the electroosmotic flow characterised by an almost plug-flow profile, is not affected by the packing material and can therefore be used to displace the background electrolyte. It is expected that the efficiency and the robustness of such a system is increased as compared to the pump driven high pressure columns. Only recently, this mode of CE has drawn some attention and consequently, a limited number of applications, primarily using reversed phase packings, has been presented [16,17].

1.3 Aim and outline of this thesis

Although it is generally accepted that CE has an extremely high potential in the field of analytical bioseparations, a major breakthrough at all levels of separation science still seems to be deferred. This is partly due to the omnipresence of well developed chromatographic and conventional electrophoretic applications and the limited possibilities of CE in the field
of (semi)-preparative analysis. But also the lack of more or less detailed relevant information on the electrophoretic properties of the solutes in a capillary electrophoretic set-up results in a reduced reproducibility and hampers the introduction of CE as a widespread analytical tool in various analytical environments. Consequently, due to the shortage of standard methods, the development of an application often requires a thorough knowledge of electrophoretic separations. More basic knowledge about the electrophoretic behaviour of various solutes facilitates the development of a method which fulfils all the requirements put forward by the analyst. In addition, the use of many different 'analyst-specific' procedures like the treatment of the capillaries, the purging and rinsing steps and buffer and sample preparations can be avoided.

The aim of this thesis therefore is to study several important physicochemical aspects of CE in order to exploit the full potential of the technique, both in the field of analytical separation sciences and in the estimation of physicochemical properties of biomolecules. We focused on two modes of capillary electrophoresis: CZE and MECC. The former was chosen because it is the basic and most simple form of CE, and other modes of CE profit from a better insight in the concepts of free zone electrophoresis. The latter was chosen because it has a high separation potential that can be used for many different types of samples and is characterised by a challenging number of experimental parameters.

In chapter 2 the variation of the pH of the background electrolyte in CZE due to electrode reactions is discussed and an in situ measurement approach, making use of indicator dyes, is presented. This chapter is primarily involved with the reproducibility of CE and provides a better understanding of the migration behaviour of weak acids and bases. The principles presented are applied throughout the thesis.

Chapter 3 deals with the mechanisms underlying the migration of charged solutes in MECC. The description of the migration behaviour of uncharged solutes and subsequently their resolution by means of a capacity factor has already been presented in the literature. Because MECC can also be used to separate mixtures of both charged and uncharged solutes, an extension of the classical equation for the resolution, making use of the capacity factor applicable to uncharged as well as charged solutes, is presented. This enables a better understanding of the principles and possible interactions underlying the observed migration.
Chapter 1

The next two chapters deal with the evaluation of physicochemical properties of biomolecules. In chapter 4 the observed mobility of the enzyme Savinase™ and a group of structurally closely related variants is studied at different experimental conditions such as pH, ionic strength and concentration of calcium in the background electrolyte. The observed migration behaviour is explained in terms of charge to mass ratio for all the solutes. In chapter 5 the association behaviour of two proteins amicyanin and methylamine dehydrogenase is studied at different pH, ionic strength and osmotic pressure. An association constant was estimated from the observed migration behaviour and the advantages as well as the limitations of CE in this respect are discussed.

The last two chapters are related to optimisation problems in capillary electrophoresis. In chapter 6 the most important optimisation strategies in CZE and MECC are reviewed, hence a better insight in the relevant parameters as well as optimisation approaches for both CZE and MECC is obtained. In chapter 7 an optimisation scheme applicable for both CZE and MECC experiments is presented. This optimisation approach is based on the iterative regression design that makes use of the description of the migration mechanisms presented in chapter 3. This approach enables the optimisation of the separation of many different kinds of samples.

1.4 References

Chapter 2
Variation of the pH of the background electrolyte due to electrode reactions in capillary electrophoresis:
theoretical approach and in situ measurement

Abstract

Electrode reactions during the electrophoretic process may change the pH of the buffer and subsequently the migration behaviour of solutes with resultant loss of reproducibility. A theoretical treatment of pH variations due to electrolytic processes is presented. The choice of buffer appears to have a dramatic influence on the pH variations observed, even if substantial buffer action is expected at the pH chosen. The experimental evaluation of the separation of 4-hydroxy-3-methoxycinnamic acid and 3-hydroxybenzoic acid reveals that the quality of the separation decreases continuously from a baseline separation observed in the first experiment to a comigration of the two solutes (resolution = 0) in the ninth experiment. A pH decrease of about 0.05 pH units accounts for the observed changes in the mobility.

A novel in situ pH measurement approach is presented in which the mobility, peak area, and peak height of an indicator dye are related to the pH in the capillary. This enables the identification and quantitation of pH variations during electrophoretic runs: the pH decreases at the anodic side already after the first experiment and pH variations as small as 0.02 pH units can be measured. The variations in peak height appear to be less suited. The calculated pH variations are in close agreement with the ones obtained experimentally.

Published in Electrophoresis, 17 (1996) 137.
2.1 Introduction

The pH of the background electrolyte in capillary electrophoresis (CE) affects the overall migration behaviour of solutes in different ways by (i) modifying the magnitude of the electroosmotic flow [1-3] and (ii) affecting the electrophoretic properties of weak acids and bases [4, 5] as well as (iii) the association and dissociation behaviour of solutes with various modifiers like chiral selectors, micelles and complexing agents [6-9]. Therefore, the pH of the buffer is a crucial factor in the migration behaviour of solutes in CE [7, 10-13]. An uncontrolled variation of the pH during the electrophoretic process must be avoided and stable buffer systems, which are used to maintain the pH at the desired value, are important with respect to method development, method validation, ruggedness testing and routine analysis.

In spite of the use of more or less concentrated buffers, variations in the pH of the background electrolyte are considered to be one of the main causes of poor reproducibility [14-16]. Several suggestions have been made to avoid such problems. Strege and Lagu [14] reported that the reproducibility of protein separations can be increased through buffer replenishment after each run. The same was concluded by Schwartz et al. [17] who studied both the reproducibility of the migration times as well as the peak areas of dansyl amino acids. Vinther and Søeberg [15] studied the radial pH gradient, which resulted in severe adsorption of proteins to the capillary wall and concluded that an increase of the ionic strength of the buffer resulted in a significant decrease of these effects.

2.1.1 The nature of electrode reactions

A CE apparatus can be considered an electrolytic cell in which chemical reactions take place at the electrodes driven by the electric field. Although the processes taking place at the electrodes are quite complex, three categories can be distinguished based on the rate limiting steps [18]. The first category includes processes in which the supply of reactant(s) is rate limiting, the second consists of all mechanisms in which adsorption and desorption processes are rate limiting and the third involves those reactions for which the reaction rate is limited by the electron transfer. For each category the overpotential, which is the difference between the actual potential and the equilibrium potential, is closely related to the driving force of the process.
Typical electrode reactions involve the production of $\text{H}^+$ and $\text{OH}^-$ from the electrolysis of water, which is of particular interest in CE, and shown below [19, 20]:

\[
2 \text{H}_2\text{O} + 2\text{e}^- \rightarrow 2\text{OH}^- + \text{H}_2 \quad \text{(cathode)}
\]

\[
2 \text{H}_2\text{O} \rightarrow 4\text{H}^+ + 4\text{e}^- + \text{O}_2 \quad \text{(anode)}
\]

As a result, the pH at the cathodic side tends to increase while the buffer at the anodic side tends to become more acidic. The above expressions are overall reactions [19] and no attempt is made to identify the occurrence of various electroactive intermediates, nor the appropriate chemical reactions. Strege and Lagu [14] reported a shift of 1 pH unit both at the cathodic and anodic side for a sodium phosphate buffer at a concentration ranging from 25 to 100 mM, after a voltage of 25 kV was applied for several hours. These changes were attributed to oxidation and reduction processes taking place at the electrodes. Recently Zhu et al. [21] reported that only minor pH changes in the bulk of the buffer solution were found as long as the pH of the system was close to the dissociation constant of the buffer (pH $\approx$ pK$_a$ ± 1) [22].

### 2.1.2 Measuring the pH

Usually, the pH of buffers in CE and changes therein are measured off-line in the bulk of the solution using a conventional pH electrode. However, this approach has several shortcomings. (i) The actual pH inside the capillary can be different from the pH in the bulk of the solution mainly due to a temperature difference caused by Joule heating. Several attempts have been made to measure or calculate the temperature inside the capillary and significant temperature increases as large as 50 °C were reported [16, 22, 23-26]. Further complications arise from local temperature gradients [23, 26, 28-30]. Also variations of the ionic strength close to the sample zone result in specific pH profiles [31] and subsequent aberrant migration behaviour. (ii) Because the pH is always measured at the end of the runs, no information on the dynamics of pH changes is obtained. (iii) In CE, buffer additives like organic modifiers and surfactants are used to increase the selectivity [32-34]. However, measurement of the pH in the presence of such additives is not obvious. Although several approaches to measure the pH in partially aqueous and nonaqueous systems are described and standard pH$^*$ scales are tabulated [22, 27], their practical use is often limited.
Furthermore, modifications of the pH electrode can result in unstable readings, long response time or even unreliable results.

In this chapter, approaches are presented that enable a quantitative treatment of pH changes in CE capillaries. Theoretical calculations demonstrate that substantial changes of the pH in the background electrolyte can occur and the impact on CE separations is shown. In addition, we present a novel in situ measurement approach of the pH using an indicator dye in which the aforementioned problems concerning pH measurement are avoided. The experimentally obtained pH changes are in agreement with those calculated.

2.2 Material and methods

2.2.1 Apparatus

All experiments were carried out on a BioFocus 3000 system (Bio-Rad, Hercules, CA, USA). Uncoated fused silica capillaries, 50 cm (45.4 cm effective length) × 50 μm ID or 24 cm (19.4 cm effective length) × 50 μm ID (Chrompack, Middelburg, The Netherlands) were used. The samples and the capillary were thermostated at 20 °C. The volume of each buffer in the vials was 1 ml. The samples were injected with 6.9 or 13.8 kPa s. A constant voltage of 15 or 20 kV was applied. Experiments designed to measure the pH in situ were monitored at 558 nm, which is the wavelength at which the difference in absorption coefficient between the two coloured forms of phenol red is maximal. All other runs were recorded at 215 nm. The data were collected and processed using the BioFocus 3000 data acquisition (version 4) and integration (version 3.01) software package.

2.2.2 Chemicals

2-[N-Cyclohexylamino]ethane-sulfonic acid (CHES) was from Sigma (St. Louis, MO, USA) and N,N-bis[2-hydroxyethyl]glycine (BICINE), 4-morpholinopropane-sulfonic acid (MOPS) and methanol were from Aldrich (Milwaukee, WI, USA). Phenolsulfoneftalein (phenol red) was obtained from Merck (Darmstadt, Germany). 4-Hydroxy-3-methoxycinnamic acid (4-H-3-MC) and 3-hydroxybenzoic acid (3-HB) were from Janssen (Geel, Belgium). Deionised water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).
2.2.3 Buffers and samples

The pH of the various buffer systems was adjusted with a 0.5 M sodium hydroxide solution. Prior to use, all solutions were filtered through 0.45 μm pore size filters. Phenol red was dissolved in ethanol, 10 % v/v, at a concentration of 1 mg/ml. 4-H-3-MC and 3-HB were dissolved in water at a concentration of 0.1 mM. Methanol was used as an electroosmotic flow marker.

2.3 Results and discussion

2.3.1 Theoretical approach

An oxidation-reduction process, like the dissociation of water, is generally expressed as

\[ \text{Red} = \text{Ox} + me \]  

(2.1)

where Red and Ox represent the reduced and oxidised species, respectively, and \( m \) is the number of electrons, e, involved. The current \( i \) flowing through the CE system is defined as the time derivative of the charge \( q \) that passes through the capillary:

\[ i = \frac{d}{dt} q \]  

(2.2)

Faraday's law of electrolysis states that the quantity of electricity is directly proportional to the quantity of chemical change that occurs at the electrodes and so it follows that

\[ i = m F \frac{d}{dt} n \quad \text{with} \quad q = m F n \]  

(2.3)

where \( n \) is the number of moles of the electroactive species involved and \( F \) is the Faraday constant. Note that only the faradaic current, due to the reaction, is considered here and that the capacity current - due to the charging of the double layer around the electrode - is neglected. This assumption is valid when the applied voltage remains constant during the electrophoretic process.
The number of moles involved in the electrode processes can be calculated after integration over the time segment \( t \), which is the total electrolysis time:

\[
n = \frac{1}{mF} \int_0^t i \, dt = \frac{q}{mF} = \frac{it}{mF}
\] (2.4)

If it is assumed that protons are produced in a 1 to 1 ratio with the electroactive species, then \( n \) represents the amount of protons produced and added to the buffer. For a total volume, \( V \), of the buffer this amounts to an additional concentration equal to:

\[
[H^+] = \frac{n}{V}
\] (2.5)

Buffer molecules counterbalance changes in the proton concentration and hence are used to maintain the pH at a desired value. A buffer is characterised by its dissociation constant, which limits the useful pH range where significant buffer action is recognised (= \( pK_a \pm 1.0 \)). The Henderson-Hasselbalch equation (for dilute solutions at a pH between 3.5 and 10.5) relates the pH of the system to the concentration of the basic and acidic buffer species \( c_B \) and \( c_A \), respectively, and the \( pK_a \) of the buffer:

\[
\text{pH} = pK_a + \log \frac{c_B}{c_A}
\] (2.6)

Reported \( pK_a \) values must often be corrected for the actual experimental conditions and an adjustment for the ionic strength, \( I \), can be incorporated using Davies' equation [22] applicable for zwitterionic bases:

\[
pK_a' = pK_a - (2 \, z - 1) \left( \frac{0.5 \, \sqrt{I}}{1 + \sqrt{I}} - 0.1 \, I \right)
\] (2.7)

where \( z \) is the number of charges on the buffer molecule. This correction results from the shielding of charged buffer species by oppositely charged molecules in the solution. Dissociation constants often show a pronounced dependence on the temperature [22]. In this study, the current through the system is relatively low and typically around 9 \( \mu A \), hence the actual temperature of the buffer in the capillary is assumed to be equal to the temperature.
of the cooling liquid, which is 20 °C. Because the literature $pK_a$ data are also obtained at 20 °C, no corrections are necessary.

The ratio of basic over acidic buffer species can be calculated after rearranging of the Henderson-Hasselbalch equation (Eq. 2.6).

\[
x = \frac{c_B}{c_A} = 10^{(pH - pK_a)} \tag{2.8}
\]

If only one ionisable group on the buffer molecule is involved in the observed buffer action, the total buffer concentration $c_{tot}$ is the sum of the concentration of basic and acidic species:

\[
c_{tot} = c_A + c_B \tag{2.9}
\]

Combination of Eqs. 2.8 and 2.9 allows the calculation of $c_A$ and $c_B$:

\[
c_A = \frac{c_{tot}}{1 + x} \tag{2.10}
\]

\[
c_B = \frac{c_{tot} x}{1 + x} \tag{2.11}
\]

The protons produced by the electrolytic process (see also Eq. 2.5) shift the concentration of buffer species $A$ and $B$:

\[
c'_A = c_A + [H^+]^* \tag{2.12}
\]

\[
c'_B = c_B - [H^+]^* \tag{2.13}
\]

The new pH value is then obtained from the Henderson-Hasselbalch equation:

\[
pH = pK'_a + \log \frac{c'_B}{c'_A} \tag{2.14}
\]

Here, the changes in the concentration of the buffer resulting from its electrophoretic mobility are neglected. This simplification is based on the assumption that most of the
charge is displaced by small ions with a high electrophoretic mobility like $\text{Na}^+$. Consequently, the variations in the calculated pH in this case will be hardly affected.

In Fig. 2.1, the calculated pH at the anode of a 40 mM CHES buffer as a function of the electrolysis time for different initial values of the pH is shown. The reported $pK_a$ of CHES is 9.55, but corrected for the ionic strength of the solution becomes 9.47. All the initial pH values (in the range 8.70 to 10.10) are within the buffering pH range of CHES. Figure 2.1 clearly shows that the initial value of the pH is of great importance with respect to the pH variation in time. If the chosen pH is below the dissociation constant of the buffer, the time during which the pH is more or less stable is reduced drastically. When the pH is higher than the $pK_a$ of the buffer, a substantial resistance to sudden pH changes is observed over a longer period of time although the observed pH does not remain constant in time.

![Graph showing pH vs. electrolysis time for different initial pH values](image)

**Fig. 2.1** The calculated pH of a 40 mM CHES buffer as a function of the electrolysis time for various initial values of the pH (10.10, 9.70, 9.47, 9.20 and 8.70). The current was set to 80 $\mu$A and the volume of buffer was set to 1 mL. See Section 2.3.1 for further details.
The time derivative of the pH as a function of the electrolysis time illustrates the extreme nonlinear behaviour of the system, as depicted in Fig. 2.2. The pH changes per unit time increase dramatically if the pH is well above or below the pK\textsubscript{a} of the buffer even when the pH is within the range of pK\textsubscript{a} ± 1.0. These pH changes become relatively small if the pH approaches the actual dissociation constant of the buffer. The buffer capacity is maximum if the pH equals the pK\textsubscript{a} and then the pH of the buffer is characterised by a maximum stability (dpH/dt = 0).

![Graph showing pH changes with electrolysis time](image)

*Fig. 2.2* Calculated changes in pH per unit time of a 40 mM CHES buffer as a function of the electrolysis time for various initial values of the pH (10.10, 9.70, 9.47, 9.20 and 8.70). See Section 2.3.1 for further details.

### 2.3.2 Experimental verification

Figure 2.1 clearly shows that pH changes close to 0.5 pH units are observed even if the pH is within the effective buffering range. Consequently, these changes should have a dramatic influence on the migration behaviour of weak acids and bases. Therefore, a mixture containing 4-H-3MC and 3-HB was analysed several times in a 40 mM CHES buffer at pH
10.0. The pH of the background electrolyte is chosen such that the buffer capacity is substantial but also close to the reported dissociation constant of the hydroxy group of 3-HB ($pK_a = 9.92$) [35].

The migration data are summarised in Table 2-1 and, as expected, the electrophoretic mobility of 4-H-3-MC which behaves as a strong acid at this pH is almost constant during these nine experiments. In contrast, the variation of the pH of the background electrolyte from experiment 1 to 9 influences the degree of ionisation of 3-HB and therefore its electrophoretic mobility increases. The total amount of charge that passes the capillary during these nine experiments is approximately 0.12 C. The first experiments are characterised by a baseline separation with a resolution of almost 2, but the quality of the separation, expressed as the resolution between the two solutes during consecutive experiments (see the last column in Table 2-1) decreases. In experiment 9 the difference in the electrophoretic mobility of 3-HB and 4-H-3-MC has become so small that only one peak is detected in the electropherogram.

**Table 2-1 Migration data of 4-H-3-MC and 3-HB after consecutive experiments**

<table>
<thead>
<tr>
<th>experiment</th>
<th>mobility ($10^{-9}$ m$^2$ V$^{-1}$ s$^{-1}$)</th>
<th>resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>electroosmotic flow</td>
<td>4-H-3-MC</td>
</tr>
<tr>
<td>1</td>
<td>67.19</td>
<td>-42.19</td>
</tr>
<tr>
<td>2</td>
<td>69.91</td>
<td>-44.17</td>
</tr>
<tr>
<td>3</td>
<td>69.91</td>
<td>-44.30</td>
</tr>
<tr>
<td>4</td>
<td>69.91</td>
<td>-44.17</td>
</tr>
<tr>
<td>5</td>
<td>69.91</td>
<td>-44.17</td>
</tr>
<tr>
<td>6</td>
<td>69.91</td>
<td>-44.30</td>
</tr>
<tr>
<td>7</td>
<td>69.91</td>
<td>-44.30</td>
</tr>
<tr>
<td>8</td>
<td>69.91</td>
<td>-44.30</td>
</tr>
<tr>
<td>9</td>
<td>69.91</td>
<td>-44.30</td>
</tr>
</tbody>
</table>

a) The total capillary length was 24 cm, and the applied voltage was 15 kV, the current observed was around 80 $\mu$A, and the analysis time of one experiment was 3 min.

22
The variation in the pH necessary to explain the observed changes in the electrophoretic mobility of 3-HB can be estimated based on the charge model presented by Sillero and Ribeiro [36], resulting in Eq. 2.15:

$$\mu_{ep} \sim \frac{q}{f} \sim \frac{1}{f} \frac{10^{(pH - pK_a)}}{1 + 10^{(pH - pK_a)}}$$

(2.15)

where $q$ is the charge on the molecule and $f$ is the friction coefficient. Application of Eq. 2.15 shows that the observed mobility changes can be brought about by a small decrease of the pH from 10.0 to 9.96. This value is close to the final pH value, 9.94, calculated using the theoretical approach described in section 2.3.1.

Although (small) pH changes affect the electroosmotic flow as well, a masked dependence of the electroosmotic flow on the number of experiments performed is not observed (see also Table 2-I). However, the electroosmotic flow is not only affected by changes in pH but also by other variables such as the ionic strength [37, 38]; and these are not considered here.

### 2.3.3 In situ pH measurement

The migration behaviour of 3-HB is affected by the pH of the background electrolyte (see above). Therefore, changes in the migration behaviour of such a solute can be exploited to probe the pH of the buffer inside the capillary. Based on this principle, we developed an in situ measurement of the pH using indicator dyes that are commonly used in titrations of weak acids and bases. Indicator dyes have physicochemical properties which make them more suited as pH indicators in CE as compared to weak acids such as 3-HB. (i) These molecules show weak acid-base properties so that a change in the mobility is expected if the pH, when chosen close to the pK$_a$ of the dye, changes. (ii) The dyes were selected for the visible end point detection of titrations, based on the change of colour upon (de)protonation of the molecule. The accompanying large spectral changes (reflected in the peak area and height) can be exploited to monitor the pH in a capillary when the detection wavelength is properly chosen. (iii) A large number of such dyes has been described in the literature [39], covering a pH range from 0 to 14.

A MOPS-BICINE buffer was chosen to investigate the influence of electrode reactions on the pH and to test the suitability of indicator dyes in the study and quantification of a pH
drift. The pK_a's of MOPS and BICINE are 7.20 and 8.35, respectively, so that adequate buffer capacity is expected between 6.5 and 9.0. Phenol red is an acid-base indicator with a pH transition interval between pH 6.4 (yellow) and pH 8.2 (red) due to an ionisable hydroxyl group. The indicator dye was injected in the MOPS-BICINE buffer at various pH values ranging from 6.2 to 9.76. As illustrated in Fig. 2.3 the electrophoretic mobility and peak area and height are strongly dependent on the pH of the buffer and sigmoidal behaviour as a function of the pH is recognised in all cases. The dotted lines are fitted curves based on the charge model presented by Sillero and Ribeiro [36] (see Eq. 2.15). The friction coefficient and the dissociation constant of the dye are the fitted parameters, obtained from a non-linear least-square fit. The electrophoretic mobility of phenol red at low pH values (pH ~6) is not zero but close to $-20 \times 10^{-9}$ m² V⁻¹ s¹ because of a negatively charged SO₃⁻ group.

The fitted pK_a value of phenol red equals 7.90, 7.84 and 7.49 for the mobility, peak area and peak height curve, respectively. They agree fairly well with the literature value of 7.92 [39]. The fitted dissociation constant based on the peak height shows less agreement but it is known that peak height is less suitable for precise quantitative measurements, mainly due to changes in peak shapes, often observed in CE [40-44]. The reproducibility of the fitting of the aforementioned parameters was good because the day-to-day deviations were smaller than 4 % in all cases.

When phenol red is injected several times in a MOPS-BICINE buffer at pH 8.04, the observed mobility, peak height and area change significantly from one experiment to the other as shown in Fig. 2.4. Although at this pH the buffer capacity is expected to be substantial, all three parameters show the same behaviour and decrease almost linearly during 10 consecutive experiments. The total amount of charge which has then passed the capillary is about 0.076 C. The experimentally observed changes in mobility, peak area and height can be related to pH values using the equations that were used to fit the curves in Fig. 2.3. These pH values of the background electrolyte as a function of the number of experiments and thus electrolysis time are shown in Fig. 2.5. The pH values calculated from the theory in section 2.3.1, extended for two buffer systems, are plotted as a dotted line in Fig. 2.5. The drift in pH, whether determined experimentally from the behaviour of phenol red using different criteria or calculated, is remarkably similar in all cases, and pH variations as small as 0.02 pH units can easily be measured. The pH values after the first experiment are 8.07, 7.97 and
Fig. 2.3 The change of (A) the electrophoretic mobility (■), (B) the peak area (*) and (C) the peak height (▲) of phenol red injected in a 15 mM MOPS - 15 mM BICINE buffer with pH values 6.20, 6.50, 6.90, 7.16, 7.33, 7.50, 7.71, 7.96, 8.08, 8.24, 8.41, 8.75 and 9.76. The total capillary length was 50 cm and the applied voltage was 20 kV. The dotted lines represent the fitted curve in each case. See Section 2.3.2 for further details.
Fig. 2.4 (A) The electrophoretic mobility (■), (B) the peak area (⋆) and (C) the peak height (▲) of phenol red, injected in a 15 mM MOPS - 15 mM BICINE buffer with pH 8.04 as a function of the number of experiments performed. The current observed was 9 µA and the volume of the buffer was 1 mL. See Section 2.3.3 for further details.
8.02, based on experimental changes of the mobility and peak area and theoretical calculation, respectively, and are close to the initial value of 8.04. The final pH after ten experiments is about 0.5 pH units lower. The pH value obtained after the first experiment, using the change in the peak height of phenol red, is 8.16 and deviates significantly from the expected value. However, this is not surprising because the fitted curve shown in Fig. 2.3 already shows some deviations compared to the other data. Nevertheless, although the peak height-derived pH values are systematically higher than the other pH data, they display the same trend compared to the other methods. Changes in pH of the background electrolyte are already observed after the first experiment. This illustrates that even if the buffer is well chosen with respect to pH and pKₐ value, significant pH changes can occur that may have a detrimental effect on the migration behaviour of the solutes.

Fig. 2.5 The calculated pH of a 15 mM MOPS - 15 mM BICINE buffer, based on the observed changes in electrophoretic mobility (■), peak area (★) and peak height (▲) of phenol red during 10 consecutive experiments. The dashed line represents the pH calculated based on the theoretical approach. See Section 2.3.3 for further details.
2.4 Conclusions

As mentioned earlier, the chemical processes taking place at the electrodes in CE can be complex and are in most cases still unidentified. Nevertheless, based on simple theoretical principles of electrolysis, a quantitative treatment of pH variations, resulting from these processes, is possible. The calculated pH variations can be significant, even if the buffer capacity of the background electrolyte is substantial, and it is shown that these pH changes may have a strong influence on the quality of a CE separation. Indicator dyes commonly used in the titration of weak acids and bases appear to be useful indicators in the in situ pH measurement presented here. The calculated pH variations and the experimentally obtained ones are in close agreement. In spite of the complexity of electrode reactions, the detrimental pH variations observed should be minimised to improve the reproducibility in CE. Careful measurement of the variation of the pH as presented above should be considered as a valuable asset, even if much attention has been paid to the selection of an appropriate buffer system. Based on the in situ measured pH variations, appropriate measures like replenishment of the buffer at the cathodic and anodic side must be considered.

2.5 References


29


Chapter 3
An equation for the description of the resolution of charged solutes in micellar electrokinetic capillary chromatography

Abstract

The migration behaviour of charged solutes in micellar electrokinetic capillary chromatography is affected by both the electrophoretic properties of the solute and its interaction with the micelles. Both must therefore be considered to obtain an adequate description of the migration behaviour and thus resolution. A novel equation is presented, in which the resolution is expressed as a function of the micellar interaction (capacity factor for charged solutes) and the electrophoretic mobility (free zone migration time) of the charged solute. Simulations reveal the dependence of the resolution of two closely migrating solutes on both distinct interactions, which is clearly different as compared to the separation of neutral solutes and general guidelines with respect to better separation are deduced. Experimental data illustrate that this equation can be used to gain more insight in the corresponding migration mechanisms. Furthermore, using this knowledge, it is possible to adjust the experimental parameters to achieve better resolution.

J. Chromatogr., in press.
3.1 Introduction

Micellar electrokinetic capillary chromatography (MECC) is a special mode of capillary electrophoresis (CE) initially developed to separate neutral solutes [1-4]. The electroosmotic and the micellar migration velocities are driven by a potential difference across the capillary. The migration behaviour of a neutral solute results from its partitioning between the aqueous and the micellar phases. Selectivity between solutes then originates from differences in their interaction with the micelles. Such a separation mechanism is of course analogous to that observed in conventional chromatography and therefore, a capacity factor \( k \) was introduced to describe the interaction between the solute and the micellar phase [1]

\[
k = \frac{t_m - t_{eo}}{t_{eo} \left(1 - \frac{t_m}{t_{mc}}\right)}
\]  

(3.1)

where \( t_m \), \( t_{eo} \) and \( t_{mc} \) are the migration times of the solute, the electroosmotic flow and the micellar phase respectively.

In practice, many samples also contain charged solutes and it has been shown that MECC is suitable to separate such mixtures as well [4-7], so that the number of relevant parameters which influence the migration is extended.

The migration behaviour of charged solutes is related to both the electrophoretic properties of the solute and its interaction with the micelle. The velocity in the aqueous phase is defined by the charge density of the solute but when the solute is associated with the micellar phase, its velocity is mainly determined by the micellar mobility. Hydrophobic forces play a major role when the solute interacts with the core of the surfactant aggregate and electrostatic attraction (or repulsion) plays a dominant role if prominent charged sites are present on both the solute and the micelle [8].

Otsuka et al. introduced a modified capacity factor to describe the micellar interaction of a charged solute [9]
where \( t_{m0} \) is the migration time of the solute in the absence of micelles. Note that \( k \) is used in the case of uncharged solutes, while \( k' \) pertains to charged solutes. The capacity factor as defined in Eq. 3.2 is analogous to the capacity factor for neutral solutes and is only a measure for the interaction between the solute and the micelle. However, the overall migration velocity is not only determined by the micellar interaction, quantitatively expressed as \( k' \), but also by the charge density of the solute and this results in an electrophoretic mobility in the aqueous phase different from the electroosmotic mobility. As such, translation of capacity factor values to the observed migration behaviour and subsequent resolution of two (closely migrating) solutes is not obvious.

Here, we present an equation describing the resolution as a function of capacity factors for charged solutes. The information comprised in \( k' \) values is translated into easily interpretable information coupled to the electropherogram of interest. By means of simulations the influence of different parameters on the resolution is studied and using experimental data, it is shown that this equation is useful for a better understanding of the migration principles and in the search for good separation conditions.

### 3.2 Theory

#### 3.2.1 A resolution equation for charged solutes in MECC

The mole fraction \( X \) of a solute in the aqueous phase is defined and related to the capacity factor \( k' \) as

\[
X = \frac{n_{aq}}{n_{aq} + n_{mc}} = \frac{1}{1 + k'} \quad \text{with} \quad k' = \frac{n_{mc}}{n_{aq}}
\]  

(3.3)
where $n_{aq}$ and $n_{mc}$ are the number of moles in the aqueous and micellar phase respectively. The overall velocity of a solute $v_m$ can then be expressed as a weighted sum of the velocity of the solutes in the absence of micelles $v_0$ and the velocity of the micellar phase $v_{mc}$

$$v_m = X v_0 + (1 - X) v_{mc}$$  \hspace{1cm} (3.4)

It follows that

$$X = \frac{v_m - v_{mc}}{v_0 - v_{mc}} = \frac{t_{mc} - t_m}{t_m - t_{m0}} \frac{t_{m0}}{t_{mc} - t_{m0}} \quad \text{with} \quad \nu = \frac{l}{t}$$  \hspace{1cm} (3.5)

where $l$ is the migration distance. Combination of Eqs. 3.3 and 3.5 results in

$$k' = \frac{1}{X} - 1 = \frac{t_m - t_{m0}}{t_{m0} (1 - \frac{t_m}{t_{mc}})}$$  \hspace{1cm} (3.6)

so that the migration time of a solute in a micellar system can be expressed as a function of the capacity factor, the migration time in the absence of micelles and the micellar migration time (see also Ref. 1, 9 and 10):

$$t_m = \frac{t_{m0} (1 + k')}{{1 + \frac{t_{m0}}{t_{mc}} k'}}$$  \hspace{1cm} (3.7)

The resolution $R_s$ between two solutes $i$ and $j$ is defined as

$$R_s = \frac{t_{mj} - t_{mi}}{4 \sigma_j} = \frac{\sqrt{N}}{4} \left(1 - \frac{t_{mi}}{t_{mj}}\right)$$  \hspace{1cm} (3.8)

where $\sigma$ is the standard deviation of the peak and $N$ the efficiency $(t_{mj}/\sigma_j)^2$. Substitution of Eq. 3.7 in Eq. 3.8 results in the resolution equation applicable to samples containing charged solutes:
\[ R_s = \frac{\sqrt{N}}{4} \frac{t_{m0j} (1 + k'_j) - t_{m0k} (1 + k'_k) + \frac{t_{m0k}}{t_{mc}} \frac{t_{m0j}}{t_{mc}} (k'_j - k'_k)}{t_{m0j} (1 + k'_j) (1 + \frac{t_{m0i}}{t_{mc}} k'_j)} \] (3.9)

This equation relates the resolution to the capacity factors and \( t_{m0} \) values which are solute specific parameters. Here it is assumed that the electrophoretic mobility of the solute is constant and not affected by the addition of surfactant. All solute specific changes in the migration behaviour like interactions with the micellar phase or with the monomers surfactant molecules and shifts of the dissociation constants of weak acids and bases due to the presence of the surfactant molecules, are lumped in one capacity factor.

To calculate the capacity factor according to Eq. 3.6, data obtained from two experiments need to be combined: a free zone experiment to measure \( t_{m0} \) and a micellar experiment to measure \( t_m \) and \( t_{mc} \). The relevance of such a capacity factor is, however, dependent on the validity of the following three assumptions:

(i) The electroosmotic flow should not change significantly when going from a free zone to a micellar system. When significant changes occur, corrections must be included to adjust the \( t_{m0} \) values according to the electroosmotic mobility measured in the micellar system. In this way, the \( k' \) values are unaffected by changes in the observed electroosmotic flow.

From simple electrophoretic principles it follows that the observed apparent mobility of a solute in a free zone system \( (\mu_{app \ ZE}) \) equals the sum of the electroosmotic \( (\mu_{eo \ ZE}) \) and the electrophoretic mobility of the solute \( (\mu_{ep}) \):

\[ \mu_{app \ ZE} = \mu_{eo \ ZE} + \mu_{ep} \] (3.10)

If the electroosmotic flow in the micellar experiment \( (\mu_{eo \ MECC}) \) is different from that in the free zone system, the apparent mobility \( (\mu_{app}) \) corresponding to the desired \( t_{m0} \) value can easily be calculated using the electroosmotic mobility measured in the micellar system.

\[ \mu_{app} = \mu_{eo \ MECC} + \mu_{ep} \] (3.11)
Combination of Eqs. 3.10 and 3.11 results in

\[ \mu_{\text{app}} = \mu_{e0 \text{ MIECC}} + \mu_{\text{app CZE}} - \mu_{e0 \text{ CZE}} \]  \hspace{1cm} (3.12)

so that \( t_{m0} \) values, necessary to calculate capacity factors, can always be calculated.

(ii) The kinetics of mass transfer between the aqueous and the micellar phase should be fast so that a solute is moving either in the aqueous phase or in the micellar phase and the intermediate state does not significantly contribute to the migration behaviour.

(iii) The micellar mobility should be unaffected by the incorporation of a solute molecule. The validity of this assumption is related to the type of surfactant and solute and the actual micellar mobility depends on the net charge and the mass of both species. Sodium dodecyl sulphate (SDS) which is used throughout the experimental part of this study has a molecular weight of 288, carries one negative charge and has an aggregation number of 62 while the test solutes have molecular weights less than 200 and carrying at most one charge. It is therefore unlikely that both the charge or the size of SDS micelles are noticeably affected by the incorporation of one solute molecule. Similarly, the same assumption is made if the micellar mobility is measured using a hydrophobic marker like Sudan III which is assumed to be totally solubilised in the micellar phase.

3.2.2 Evaluation of the resolution as a function of different parameters

The examples chosen here assume that the electroosmotic and the solute migration time are smaller than the micellar migration time. The interaction with the micelles always results in a slower migration velocity as compared to the free zone system. These assumptions are in accordance with the experimental conditions most frequently found in the literature. The equations presented, however, are not restricted in any way and are applicable for all possible values of \( t_{e0} \), \( t_{m} \) and \( t_{me} \).

3.2.2.1 Simplification to the resolution equation for uncharged solutes

The resolution equation as expressed in Eq. 3.9 is applicable to samples containing both charged and uncharged solutes. In case of uncharged solutes, \( t_{m0} \) and \( t_{m0} \) equal \( t_{e0} \) and Eq.
3.9 reduces to Eq. 3.13 which is the well-known resolution equation for uncharged solutes in MECC derived by Terabe et al. [10].

\[
R_s = \frac{\sqrt{N}}{4} \frac{(k_j - k_i) + \frac{t_{eo}}{t_{mc}} (k_i - k_j)}{(1 + k_j) (1 + \frac{t_{eo}}{t_{mc}} k_i)}
\]

\[
\sqrt{N} \frac{\alpha - 1}{\alpha} \frac{k_j}{1 + k_j} \frac{1 - \frac{t_{eo}}{t_{mc}}}{1 + \frac{t_{eo}}{t_{mc}} k_i} \quad t_{m0i} = t_{m0j} = t_{eo} \quad (3.13)
\]

where $\alpha$ is the selectivity defined as the ratio of the two capacity factors. Assuming a constant value for $N$, the separation between two solutes is only dependent on the differences in the micellar interaction and thus their capacity factor values. This is clearly different when compared to the equation for charged solutes (Eq. 3.9) where both the micellar interaction as well as the electrophoretic properties of the solutes must be taken into account.

3.2.2.2 The resolution as a function of $k'_i$ and $k'_j$

In the resolution equation for uncharged solutes (Eq. 3.13), the capacity factors are the only solute specific parameters and maximisation of the resolution can be performed by adjusting the capacity factors. This was illustrated by Terabe et al. [10] who evaluated the effect of $k$ on the resolution. Assuming both the efficiency and the selectivity of neutral solutes being independent of the capacity factor, Eq. 3.13 was simplified to yield the $f(k)$ function as shown in Eq. 3.14.

\[
f'(k) = \frac{k_j}{1 + k_j} \frac{1 - \frac{t_{eo}}{t_{mc}}}{1 + \frac{t_{eo}}{k_i}} \approx \frac{k}{1 + k} \frac{1 - \frac{t_{eo}}{t_{mc}}}{1 + \frac{t_{eo}}{k}} \quad k_i \approx k_j \approx k \quad (3.14)
\]

The $k$ value which corresponds to the maximum of this function depends on the ratio of $t_{eo}/t_{mc}$. It was concluded in this study [10] that intermediate values of $k$ (2 to 5) would result
in optimal separation conditions. This is in agreement with the results obtained from other studies in which the resolution of neutral solutes in MECC was investigated [11, 12].

If the assumption $k_i \approx k_j$ is omitted, both capacity factors must be considered to evaluate the resolution. In this study, the complete resolution equation for uncharged solutes (Eq. 3.13) is used to evaluate $R_s$ as a function of $k_i$ and $k_j$, and only $N$ is assumed to be independent of the capacity factor. This results in the iso-resolution plot in Fig. 3.1 which is characterised by a high degree of symmetry, the diagonal from low to high $k$ values being the symmetry axis. On this diagonal, $k_i$ and $k_j$ are equal and consequently the resolution is always zero.

![Fig. 3.1 Iso-resolution plot as a function of $k_i$ and $k_j$ (logarithmic scale) for two uncharged solutes (Eq. 3.13). The value of $N$ is set to 10000, $t_{co}$ and $t_{mc}$ are set to 3.1 and 10 respectively.](image)

At low $k$ values, the term $k_j/(1+k_j)$ will be small and dominates the $R_s$ value. At high $k$ values, both solutes will migrate close to the $t_{mc}$ and the separation will be poor due to the
last term in Eq. 3.13. At intermediate values of \( k \), only relatively small differences between \( k_i \) and \( k_j \) are required to obtain a significant increase of the resolution and this range of \( k \) values should be preferred, in agreement with the aforementioned studies. Furthermore, maximisation of the difference between \( k_i \) and \( k_j \) always results in an increase in the observed resolution.

If charged solutes are present, the micellar interaction can be quantified using the appropriate capacity factor for charged solutes but then the resolution equation for charged solutes (Eq. 3.9) must be used to evaluate the quality of the separation and to estimate \( k' \) values resulting in adequate resolution. Three situations are distinguished:

**case 1**: \( t_{m0i} = t_{m0j} = t_{m0} \)

The electrophoretic properties of both solutes are equal and simplification of Eq. 3.9 to an equation analogous to the one for uncharged solutes results in:

\[
R_s = \frac{\sqrt{N}}{4} \frac{\alpha - 1}{\alpha} \frac{k'_i}{1 + k'_j} \frac{1 - \frac{t_{m0}}{t_{mc}}}{t_{mc} - t_{mc} - \frac{t_{m0}}{k'_i}}
\]

\( t_{m0i} = t_{m0j} = t_{m0} \) (3.15)

An analogous equation describing the resolution for this particular case has already been presented [13].

It is clear that the iso-resolution surface as a function of the capacity factors is similar to that for uncharged solutes as illustrated in Fig. 3.2. However, the absolute values of the resolutions are smaller in the case of \( t_{co} < t_{m0} \) and larger in the case of \( t_{co} > t_{m0} \). This can be explained by a decrease or increase of the separation window (due to the last term on the right-hand side in Eq. 3.15).

**case 2**: \( t_{m0i} < t_{m0j} \)

The electrophoretic mobility of a solute is defined by its net charge and the frictional drag, which can be expressed as molecular mass or volume [14-17]. If two solutes differ in charge and/or frictional drag, their electrophoretic mobilities are different which can be measured
as variations in the migration times in a free zone system. The observed difference is not only determined by these physicochemical properties but also by the experimental settings such as the polarity of the voltage, pH, electroosmotic mobility and the presence of coatings to reduce or inverse the electroosmotic flow. As a result, many different combinations of these parameters can be classified as belonging to case 2. Calculation of the iso-resolution plot requires values for \( t_{m0} \) and \( t_{n0} \) but is not dependent on these experimental conditions. Such a plot which refers to case 2 is shown in Fig. 3.3.

**Fig. 3.2** Iso-resolution plot as a function of \( k'_i \) and \( k'_j \) (logarithmic scale) for two equally charged (cationic) solutes (Eq. 3.15). The value of \( N \) is set to 10000, both \( t_{m0} \) and \( t_{n0} \) are set to 2.34 and \( t_{eo} \) is set to 10. See also case 1 in the text. The dotted line represents the trajectory of the resolution of the pair 3,4-dihydroxybenzylamine (solute i) - dopamine (solute j). The arrow indicates the direction of increasing concentration of SDS.
At low capacity factor values, separation is achieved based on the individual differences in charge density hence the separation principle simplifies to a free zone mechanism and Eq. 3.9 reduces to

$$R_s = \frac{\sqrt{N}}{4} \frac{t_{m0j} - t_{m0i}}{t_{m0j}} \quad k_i' \approx k_j' \approx k' < 1 \quad (3.16)$$

This region, denoted A in Fig. 3.3, can be found at the lower left corner of this figure.

If the solutes interact very strongly with the micellar phase, the migration times of both solutes will be close to $t_{mc}$ so that both $k_i'$ and $k_j'$ are large and almost equal and Eq. 3.17 shows that the resolution then becomes very low.

$$R_s = \frac{\sqrt{N}}{4} \frac{t_{m0j} k_j' - t_{m0i} k_i' + \frac{t_{m0i} t_{m0j}}{t_{mc}} (k_i' - k_j')}{t_{mc}} \approx \frac{t_{m0j} t_{m0i}}{t_{mc}} k_i' k_j'$$

$$\frac{\sqrt{N}}{4} \frac{t_{m0j} - t_{m0i}}{t_{m0j}} \approx 0 \quad k_i' \approx k_j' \approx k' > 1 \quad (3.17)$$

This region, denoted B in Fig. 3.3 can be found at the upper right corner of this figure.

At intermediate values of $k'$, the migration velocity of the solute must be seen as a weighted sum of the micellar and the electrophoretic velocity (see also Eq. 3.4 with $X$ being the weight factor). Consequently, the resolution may either increase or decrease at increasing concentration of the micellar phase and thus capacity factor values, depending on the relative magnitudes of the electrophoretic mobilities and capacity factor values. Therefore, no simplification of Eq. 3.9 is possible.

If solute j interacts more strongly with the micellar phase than i ($\log k_j' > \log k_i'$), the weight factor $X$ in Eq. 3.4 is smaller for solute j than for i. From this micellar interaction it follows that solute j will be more retarded in the capillary than i. Since in the free zone system, the migration time of solute j is already larger than i, the difference in the migration times and thus resolution will further increase at higher concentrations of the micellar phase (or
increasing $k'$ values.). This region of increasing resolutions (compared to a free zone system) is denoted C in Fig. 3.3.

Fig. 3.3 Iso-resolution plot as a function of $R_i$ and $R'_j$ (logarithmic scale) for two differently charged solutes in which $t_{150} < t_{100}$ (Eq. 3.9). The value of $N$ is set to 10000, $t_{150}$ and $t_{100}$ are set to 3.56 and 5.53 respectively and $t_m$ is set to 10. See also case 2 in the text. The dotted line represents the trajectory of the resolution of the pair 2,3-dichlorophenol (solute i) - 3-methylbenzoic acid (solute j). The arrow indicates the direction of increasing concentration of SDS.

If solute i interacts more strongly with the micellar phase ($\log k'_j < \log k'_i$), this solute is more retarded by the micelles than solute j. Now the weight factor $X$ in Eq. 3.4 is larger for j than for i. Solute i, which is the fastest migrating solute in the free zone system, will be, in the presence of micelles, more retarded than j so that the difference in the migration times will decrease at higher concentrations of the micellar phase (or increasing $k'$ values). This region of decreasing resolution (compared to a free zone system) is denoted D in Fig. 3.3.
It is obvious that if \( k'_i \) further increases the migration time difference will further decrease until both solutes comigrate. This can be seen in Fig. 3.3 as the line where \( R_s \) equals zero. Here, the electrophoretic mobilities and the capacity factors of both solutes are such that the overall migration velocities of both solutes are the same. If the resolution in Eq. 3.9 is set to zero, the equation can be solved for \( k'_j \) which is then expressed as a function of \( t_{m0i}, t_{m0j} \) and \( k'_i \):

\[
    k'_j = \frac{\frac{t_{m0i} - t_{m0j} + t_{m0i} k'_i (1 - \frac{t_{m0j}}{t_{mc}})}{t_{m0j} (1 - \frac{t_{m0j}}{t_{mc}})}}{t_{m0j} (1 - \frac{t_{m0j}}{t_{mc}})}
\]  

(3.18)

This equation can always be solved for any value of \( t_{m0} \) and \( k'_i \) but only the solutions with a \( k'_j \) value higher than or equal to zero are meaningful.

If this difference in \( k' \) values increases even more (\( \log k'_j < \log k'_i \)), a peak order reversal occurs and the migration time difference increases again. This region of increasing resolution is denoted \( E \) in Fig. 3.3. Here the migration mechanism of solute \( i \) can be primarily attributed to the micellar interaction while for solute \( j \) both the electrophoretic mobility as well as the micellar interaction contributes to the migration velocity.

In table 3-1 a brief overview with a qualitative analysis of the resolution is given for the different situations described above.

The resolution is not necessarily zero if \( k'_i \) equals \( k'_j \) as in case 1 and Eq. 3.9 simplifies to

\[
    R_s = \frac{\sqrt{N}}{4} \frac{(t_{m0j} - t_{m0i})}{t_{m0j} (1 + \frac{t_{m0i}}{t_{mc}} k')}
\]

\[
    k'_i = k'_j = k'
\]

(3.19)

From this equation it follows that \( R_s \) will reach a maximum for \( k' = 0 \). If the micellar interaction increases equally for both solutes (\( k'_i = k'_j \)), the differences in electrophoretic properties will become less significant with respect to the overall migration behaviour and therefore the resolution will decrease.
Furthermore, maximisation of the difference between $k'_i$ and $k'_j$ does not necessarily result in an increase in the resolution as is the case for uncharged or equally charged solutes. Again, both electrophoretic behaviour and micellar interaction must be considered to describe the migration.

**Table 3-I** Evaluation of the resolution as a function of the relative magnitude of the capacity factors (see case 2 in the text and $t_{ma} < t_{me}$)

<table>
<thead>
<tr>
<th>Region</th>
<th>$R_s$ Fig. 3.3</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k'_i = k'_j = k' = 0$</td>
<td>A +</td>
<td>Separation is based on a free zone mechanism and defined by the difference between $t_{ma}$ and $t_{moj}$ (see also Eq. 3.16).</td>
</tr>
<tr>
<td>$k'_i$ and $k'_j &gt; 0$</td>
<td>B 0</td>
<td>The interaction between the solutes and the micelle is extremely high, hence the overall migration velocity of both solutes equals the migration velocity of the micellar phase.</td>
</tr>
<tr>
<td>$k'_j &gt; k'_i$</td>
<td>C ++</td>
<td>Solute j, which is the slowest migrating solute in a free zone experiment, is even slower in a micellar system.</td>
</tr>
<tr>
<td>$k'_j &lt; k'_i$</td>
<td>D -</td>
<td>Migration of solute j is mainly determined by electrophoretic properties and migration of solute i is mainly determined by micellar interaction: these two migration mechanisms partly compensate each other, resulting in a decrease in $R_s$.</td>
</tr>
</tbody>
</table>

$$k'_i = \frac{t_{moj} - t_{mi} - t_{mi} k' (1 - \frac{t_{ma}}{t_{me}})}{t_{moj} (1 - \frac{t_{ma}}{t_{me}})}$$

/ 0 The micellar interaction of solute i results in an increase in the migration time but this is counterbalanced by the electrophoretic properties of solute j. This equation (Eq. 3.18) can be derived from Eq. 3.9.

$\leq k'_i$ | E + | After comigration, a change in the peak order occurs followed by an increase of the resolution. |

44
case 3: $t_{m0i} > t_{m0j}$

The iso-resolution plot shown in Fig. 3.4 is similar to that of case 2. However, due to the reversal of the relative magnitude of the $t_{m0}$ values, the narrow region of low resolutions is now found at relatively high values of $k'_j$ and relatively low values of $k'_i$. Although the experimental set-up can be quite different from that in case 2, both cases can be treated in an analogous way and all the different regions that can be distinguished in Fig. 3.3 can also be identified in Fig. 3.4.

![Iso-resolution plot](image)

**Fig. 3.4** Iso-resolution plot as a function of $k'_i$ and $k'_j$ (logarithmic scale) for two differently charged solutes in which $t_{m0i} > t_{m0j}$ (Eq. 3.9). The value of $N$ is set to 10000, $t_{m0i}$ and $t_{m0j}$ are set to 5.53 and 2.34 respectively and $t_{mi}$ is set to 10. See also case 3 in the text. The dotted line represents the trajectory of the resolution of the pair 3-methylbenzoic acid (solute i) - 3,4-dihydroxybenzylamine (solute j). The arrow indicates the direction of increasing concentration of SDS.
In all the situations described above, the resolution becomes worse when both $k'$, and $k'_j$ increase to relatively high values. High capacity factors should therefore always be avoided, not only because of an increase in migration time but also because of the loss of separation.

Differently charged solutes can be separated at low capacity factor values, a free zone experiment should then be preferred in stead of a micellar system. However, if the electrophoretic differences are very small or if mixtures of more than two solutes, charged and/or uncharged, must be separated, the use of a micellar system is a viable alternative.

### 3.3 Experimental verification

The approach described in the theoretical part provides general guidelines concerning the (desired) migration behaviour of solutes. However, there are only roughly defined relations between a desired capacity factor and the experimental settings in CE [8, 11, 18-23] to actually obtain this capacity factor. Nevertheless, the equations describing the micellar interaction and the resolution for charged solutes in MECC are helpful in understanding migration and separation principles and to evaluate changes in various experimental parameters.

To illustrate this, migration data of five solutes at different concentrations of SDS were analysed using the equations presented above. These experiments were performed on a home-built system. An uncoated fused silica capillary, 58 cm (44.5 cm effective length) x 53 μm ID (Polymer Technologies, Phoenix, AZ, USA), thermostated at 40 °C was used. All runs were performed in a phosphate buffer at pH 7 and an ionic strength of 0.05 M. The samples were injected hydrodynamically raising the sample vial 5 cm for 20 seconds. A constant voltage of 18 kV was applied and the runs were monitored at 210 nm.

The observed migration times together with $t_{co}$ and $t_{mc}$, are shown in Fig. 3.5. 4-Methylcatechol is not charged at the pH chosen and migrates at the speed of the electroosmotic flow in the free zone system. The two cationic solutes, 3,4-dihydroxybenzylamine and dopamine, migrate faster than the electroosmotic flow and are characterised by the same electrophoretic mobility. 2,3-Dichlorophenol and 3-methylbenzoic acid are negatively charged and migrate significantly slower than the electroosmotic flow. Furthermore, the migration time difference at zero SDS concentration
reveals that 3-methylbenzoic acid has a higher electrophoretic mobility than 2,3-dichlorophenol.

It can be seen that several changes of the order of migration occur at increasing concentrations of surfactant. For example, changes of the relative peak positions are recognised for the peak pairs 2,3-dichlorophenol - 3-methylbenzoic acid and 3,4-dihydroxybenzylamine - 4-methylcatechol, close to 20 mM and 8 mM SDS respectively. Even more dramatic is the migration behaviour of dopamine, which is the fastest migrating solute in the free zone system, but the slowest migrating solute at high concentrations of SDS.

![Graph](image)

Fig. 3.5 Migration times of the test solutes, the electroosmotic flow and the pseudo-stationary micellar phase as a function of the concentration of SDS. All experiments were performed in an uncoated fused silica capillary (ID: 53 μm, total length: 58 cm, length to detector window: 44.5 cm) using a phosphate buffer at pH 7.0 and variable concentrations of SDS. Solutes: 3-methylbenzoic acid (\(\ast\)), 2,3-dichlorophenol (\(\circ\)), 4-methylcatechol (\(\ast\)), 3,4-dihydroxybenzylamine (\(\triangle\)), dopamine (\(\bullet\)), \(\xi_0\) (\(\boldsymbol{\bullet}\)) and \(\xi_{nc}\) (\(\circ\)). These data were kindly provided by J. K. Strasters.
From these migration data, capacity factors for all solutes can be calculated as shown in Fig. 3.6. Corrections for the small changes in the electroosmotic flow at higher SDS concentrations are introduced as described in section 3.2.1. To calculate the capacity factor for the charged solutes (both anionic and cationic), Eq. 3.6 should be used.

From the migration times in the free zone system it follows that 3-methylbenzoic acid has a high negative charge density. Due to electrostatic repulsion with the SDS micelles, there is hardly any interaction between this solute and the surfactant aggregate so that the capacity factor is close to zero. The migration behaviour of 3-methylbenzoic acid is solely determined by the electrophoretic properties of this solute (apart from changes in the electroosmotic flow). Consequently, the migration time of this solute is fairly constant.

![Graph showing capacity factor vs. SDS concentration](image)

**Fig. 3.6** Capacity factors of the test solutes as a function of the concentration of SDS. The capacity factor values are calculated according to the equation for charged solutes (Eq. 3.6). Dotted lines represent the least square fits for the $k^*$ values. All experiments were performed in an uncoated fused silica capillary (ID: 53 μm, total length: 58 cm, length to detector window: 44.5 cm) using a phosphate buffer at pH 7.0 and variable concentrations of SDS. Solutes: 3-methylbenzoic acid (†), 2,3-dichlorophenol (†), 4-methylcatechol (♦), 3,4-dihydroxybenzylamine (☻) and dopamine (■). These data were kindly provided by J. K. Strasters.
4-Methylcatechol which is uncharged at pH 7.0 is retarded at higher concentrations of SDS and this can be attributed to the interaction with the micellar phase. Either Eq. 3.6 for charged solutes or Eq. 3.1 for uncharged solutes can be used to calculate the value of the capacity factor.

3,4-Dihydroxybenzylamine, dopamine and 2,3-dichlorophenol carry significant charges but show interaction with the micelles as well. Therefore, both the electrophoretic mobility of the solutes as well as the interaction with the micellar phase must be considered to evaluate the migration mechanism of these solutes. The capacity factors of 3,4-dihydroxybenzylamine and dopamine are larger compared to the other solutes. This is probably due to the electrostatic attraction between the positively charged solutes and the oppositely charged micelle.

The linear relationship between the capacity factors and the surfactant concentration is satisfactory ($r^2$ is typically higher than 0.98) except for 3-methylbenzoic acid but this may be related to the calculation of small capacity factors. The concentration of surfactant at a $k'$ value of zero results from extrapolation of the regression lines in Fig. 3.6 and equals the critical micellar concentration (CMC) of SDS. Here, typical values around 4 mM are found which are smaller than the theoretical value of 8 mM. However, it is known that the actual CMC is affected by the buffer properties like the ionic strength and the temperature. The migration data of 3,4-dihydroxybenzylamine do not seem to be suitable for the determination of the CMC (CMC = ±2.5 mM) but the exact reason is not clear.

The resolution according to Eq. 3.9 was calculated for 3,4-dihydroxybenzylamine - dopamine. The migration properties of this pair of solutes is discussed in section 3.2.2.2 under case 1. The calculated resolution is shown in Fig. 3.2 as the dotted trajectory. Since both solutes carry the same charge, they cannot be separated in a free zone system. The adequate separation at 10 mM SDS is mainly caused by the interaction of 3,4-dihydroxybenzylamine with the micelles. The trajectory in Fig. 3.2 reveals that the quality of the separation decreases when the surfactant concentration increases above 10 mM. This loss of resolution results from a dramatic increase of the micellar interaction of dopamine. Close to 15 mM SDS the solutes comigrate, as they do in the free zone system. Apparently, both solutes are slowed down to the same extent by the micellar interaction resulting in equal capacity factors. Close to 15 mM SDS a peak crossing occurs and 3,4-dihydroxybenzylamine now migrates faster than dopamine. Increasing the concentration
of SDS from 20 to 50 mM does not result in an improvement of the resolution. As mentioned earlier, high capacity factor values should not be preferred because excessive micellar interaction does not improve the quality of the separation and, in addition, the overall migration times increase. It is obvious that in this case the separation is solely based on the differences in the micellar interaction. Consequently, a larger difference in the respective $k'$ values results in an increase of the resolution which also results from Fig. 3.2.

The separation of 2,3-dichlorophenol and 3-methylbenzoic acid is an example of case 2, as discussed in section 3.2.2.2. Again, the trajectory is shown as a dotted line in Fig. 3.3. Both solutes are negatively charged but have a different electrophoretic mobility. As mentioned earlier, 3-methylbenzoic acid shows no interaction with the SDS micelles hence $k'$ of 3-methylbenzoic acid is close to zero (log $k'$ between -2.5 and -0.9). The separation at low SDS concentrations is solely due to the differences in the electrophoretic properties. At increasing concentrations of SDS, the migration time difference and thus resolution decreases which can be explained by an increase of the interaction between 2,3-dichlorophenol and the micelles (see also Figs. 3.5 and 3.6). Finally, the two peaks overlap at 20 mM SDS, as is depicted both in Figs. 3.3 and 3.5. In contrast, the capacity factor values, shown in Fig. 3.6, are continuously diverging as a function of the concentration of SDS hence a comigration of 2,3-dichlorophenol and 3-methylbenzoic acid is not supported by the data. This illustrates that an increase of the resolution for charged solutes cannot always be obtained increasing the difference in capacity factor values as is the case for uncharged or equally charged solutes. At higher concentrations of surfactant the capacity factor of 2,3-dichlorophenol further increases, resulting in a peak crossing and an increase of the resolution at the expense of the analysis time. This is also revealed in Fig. 3.3 where the dotted line descends to lower resolution values at low capacity factor values, crosses the valley ($R_1 = 0$) and then ascends at the other side towards increasing resolutions at high capacity factor values.

The dotted line in Fig. 3.4 refers to the separation of two oppositely charged solutes: 3-methylbenzoic acid and 3,4-dihydroxybenzylamine. Because of the large differences in the charge density of both solutes, the separation is quite good in the free zone system and at low SDS concentrations. Increasing the SDS concentration up to 30 mM reduces the resolution resulting in a comigration of both solutes. Again, this can be explained by a dramatic increase of the micellar interaction of 3,4-dihydroxybenzylamine. At even higher concentrations of SDS the peak order reverses and the resolution increases. It is obvious that for an adequate separation of these two solutes the addition of SDS is not required.
3.4 Conclusions

The examples discussed here clearly illustrate that by using the appropriate capacity factor and resolution equations, it is possible to gain more insight in the migration behaviour of charged and neutral solutes in MECC, in particular related to their interactions with the micellar phase. Evidently, the migration behaviour of every (relevant) pair of solutes can be evaluated in this way. Careful examination of the data enables the adjustment of the appropriate parameters, in this case the concentration of SDS, to optimise the separation between various peak pairs. Obviously, for optimisation purposes, smaller sets of experiments should be used to predict the resolution of these peak pairs. Extension to other parameters, like the pH of the buffer, should also be possible.

Acknowledgement

The authors would like to thank Joost Strasters who kindly provided the experimental data, used in this work.

3.5 References

Chapter 4
A capillary electrophoretic study on the influence of charge and friction on the mobility of Savinase™ and site directed mutants

Abstract

Capillary zone electrophoresis is used to study the electrophoretic properties of the enzyme Savinase™ and variants thereof, differing in the number of ionisable residues but for every variant, an equal number of basic and acidic amino acids was introduced. The mobilities of the solutes are studied under different experimental conditions like the ionic strength, the pH and the addition of Ca^{2+} to the background electrolyte.

At pH 3 the acidic residues are protonated, hence the migration order is related to the number of basic residues introduced. Furthermore, these data reveal a proportionality between the theoretical net charges based on the amino acid composition and the actual charges deduced from the experiments. Calcium ions shield negative charges on Savinase and consequently, the migration order measured at pH 6 in the presence of Ca^{2+} is the same as in the low pH buffer. A parameter which accounts for the partial shielding of the charges by Ca^{2+} is estimated by means of a modified mobility equation. If no calcium ions are added to the buffer at pH 6, the observed migration order is less easily understood. It is shown that changes in the friction, expressed as the molecular mass or the radius of the proteins, cannot be responsible for the migration order. Assumed differences in the net effective charge typically smaller than 0.01, however, result in a good agreement between the measured and the calculated mobilities.

This research was performed in close collaboration with Dr. M.R. Egmond and Dr. J.C.J. Peelen, Unilever Research Laboratory, Vlaardingen

To be submitted for publication
4.1 Introduction

Capillary zone electrophoresis (CZE) is characterised by a high separation power and therefore has been shown to be an excellent method for the separation and analysis of structurally closely related proteins. Furthermore, the absence of stabilising agents and (pseudo-) stationary phases in CZE probably entails only minor disturbances of the three dimensional structure of the proteins, hence their native state is preserved. However, it is not always easy to link the experimentally observed mobility to structural properties of proteins, even though several equations are available, expressing the mobility as a function of physicochemical properties. This is mainly due to an inaccurate estimate of the charge density under the experimental conditions and the lack of appropriate parameters to quantify the friction. Furthermore, many of these equations are build up of semi-empirical parameters which hampers a broad application.

Several studies have been reported on the physicochemical properties of native proteins investigated by means of capillary electrophoresis (CE). Yao et al. [1] introduced a simple method for the determination of the isoelectric point of proteins, searching for the pH of the buffer at which the electrophoretic mobility of the protein equals zero. Gao et al. [2] described a method to estimate the effective charge of a protein in solution at any given pH by means of electrophoretic data of appropriate 'standards' and relating these data to the observed mobility of the protein.

An effective search for minor differences in structure and/or composition of proteins was presented by Wiktorowicz et al. [3] who compared the electrophoretic behaviour of native and three site directed mutants of RNase T1. Although two of the variants were supposed to have an equal charge to mass ratio, baseline separation was observed. Apparently, the way in which the charged residues of the variants are sensed by the electric field is different and seems to be related to the ionic environments of the mutated amino acid residues. The effect of charge heterogeneity on the electrophoretic behaviour of various recombinant-DNA derived proteins like human growth hormone, a T4 receptor protein and tissue plasminogen activator was demonstrated by Wu et al. [4]. Each type of protein was investigated separately and the electropherograms obtained at different pH's showed a partial separation in some cases. This was explained by small differences in charge and molecular weight of the solutes. Temperature induced transformations of BR96 antibody resulted in 4 isoforms which could be distinguished by capillary zone electrophoresis, micellar electrokinetic capillary
chromatography and circular dichroism [5]. Below 70 °C, these transformations were reversible and attributed to the formation of conformers. Twelve S. nuclease variants, obtained by site directed mutagenesis, were subjected to a CZE study at different pH values by Kálmán et al. [6, 7]. The authors suggested the occurrence of three pH dependent conformational states that are characterised by different radii. Furthermore, they tried to estimate both the charge differences between the wild type and the mutants and the actual charge of the individual solutes. Besides, several pairs of solutes, differing solely one amino acid residue, were characterised by mobility differences of about 2 to 3 × 10^{-10} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}, which was attributed to radii differences smaller than 0.5 Å.

Savinase™, a strongly basic protease and a series of variants differing in the total number of acidic and basic amino acid residues obtained by genetic engineering [8], were analysed using gel electrophoresis and ion-exchange chromatography. The data obtained were used to investigate the effect of additional charged amino acid residues on the surface potential and charge distribution. In the presence of several cations, in particular calcium, a clear relationship was observed between the total number of positively charged residues added and the electrophoretic and ion-exchange behaviour at pH 6.4. This was attributed to non-specific screening of the negative charges on the enzyme. In the same study, calculation of the overall electrostatic potential of the variants, characterised by an increased number of ionisable amino acids but an equal number of basic and acidic residues, indicated that in the absence of cations, the solutes appeared to become slightly more negative with increasing numbers of charges added.

That study [8] prompted us to analyse the electrophoretic behaviour of the Savinases in a free zone system at different concentrations of buffer and calcium ions and at several pH values. By means of a mobility equation designed for proteins [9], the experimentally observed migration behaviour was related to the physicochemical properties of the proteins.

4.2 Material and methods

4.2.1 Apparatus

The experiments were carried out on a 3DHPC system (Hewlett-Packard, Avondale, USA), using coated eCap Neutral Capillaries, 40 or 38.5 cm (31.5 and 30 cm effective length) x 50 μm ID (Beckman, Fullerton, CA, USA). Due to the deactivation of the silica wall, the
electroosmotic flow was strongly reduced. The samples and the capillary were thermostated at 20 °C. The samples were injected with a pressure of 2 or 6 kPars. A constant voltage between 12.5 and 20 kV was applied and the actual voltage was determined by the electric field and current limit with respect to the coated capillary as prescribed by the supplier of the capillary. All the runs were recorded at 214 nm and the data were collected and processed using the 3DHPCE acquisition and integration software package.

4.2.2 Chemicals

Citrate and 4-morpholineethanesulfonic acid (MES) buffers (Beckman, Fullerton, CA, USA) as prescribed by the supplier of the coated capillaries were used throughout this study. Calciumchloride was from BDH (Poole, UK), phenylmethylsulfonyl fluoride was from Boehringer (Mannheim, Germany) and 2-propanol was from Fluka (Buchs, Switzerland). Deionised water was prepared using a Milli-Q system (Millipore, Milford, MA, USA).

4.2.3 Buffers and samples

Stock solutions of the buffers were diluted in water or in a 12 mM CaCl₂ stock solution to obtain the desired concentration of buffer and CaCl₂. Because calcium ions showed to be the most effective in shielding negative charges on Savinase [8], this cation was chosen in this study. In Table 4-I, some properties of the buffers are summarised.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>CaCl₂ concentration (mM)</th>
<th>pH</th>
<th>current (µA)</th>
<th>[field strength] (V/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mM citrate - 15 mM MES</td>
<td>0</td>
<td>6</td>
<td>8.8</td>
<td>[500]</td>
</tr>
<tr>
<td>5 mM citrate - 5 mM MES</td>
<td>0</td>
<td>6</td>
<td>7.5</td>
<td>[493.5]</td>
</tr>
<tr>
<td>10 mM citrate</td>
<td>0</td>
<td>3</td>
<td>10.7</td>
<td>[325]</td>
</tr>
<tr>
<td>5 mM citrate - 5 mM MES</td>
<td>3</td>
<td>6</td>
<td>8.8</td>
<td>[500]</td>
</tr>
</tbody>
</table>
Wild type Savinase and several variants were obtained by genetic engineering [8] and were provided in a lyophilised form or in a 1:2 glycerol/water stock solution. Prior to injection, the samples were diluted to 0.5 - 1 mg/ml. To prevent autolysis, 10 µl of a solution of 10 mg phenylmethylsulfonyl fluoride in 1 ml 2-propanol was added to the protein sample.

Savinase belongs to the class of subtilisins and is used as an additive for washing powder. It contains 269 amino acid residues and the amino acid sequence of the wild type Savinase and its 3-dimensional structure have been reported [10]. The enzyme has a molecular mass of about 27 kD and its isoelectric point, based on the amino acid composition, is close to 10, although isoelectric focusing experiments reveal a pI value of 11.15 [11]. In Table 4-II, some properties of Savinase and variants thereof are summarised.

<table>
<thead>
<tr>
<th>Label</th>
<th>changes introduced into Savinase sequence</th>
<th>ΔM [% difference]</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type /</td>
<td></td>
<td>0 [0%]</td>
</tr>
<tr>
<td>US 205</td>
<td>S156E, A158R, A172D, N173K (=a)</td>
<td>185 [0.69%]</td>
</tr>
<tr>
<td>US 211</td>
<td>T38R, H120D, N140D, S141R (=b) + a</td>
<td>288 [1.08%]</td>
</tr>
<tr>
<td>US 216</td>
<td>Q12R, P14D, T22R, N43R, Q59E, N76D, A98R, S99D (=c) + a + b</td>
<td>546 [2.05%]</td>
</tr>
<tr>
<td>US 219</td>
<td>T213K, N248D, T255E, S256K, S259D, A272R + a + b</td>
<td>756 [2.84%]</td>
</tr>
</tbody>
</table>

The number of substitutions increases from 0 to 22 and the systematic replacement of uncharged relatively light amino acid residues by charged heavier ones results in a relatively small increase (≤ 3%) in the molecular mass. Because clustering of charged groups with the same sign was avoided, conformational changes are unlikely, although small perturbations cannot be excluded. Furthermore, all mutations were at least 10 Å from the active site. This results in enzymatic and stability properties comparable to that of the wild type Savinase. For a detailed description of these data, the reader is referred to Ref. [8].
4.2.4 Calculation of the dipole moments and projected surface areas of the Savinases

The dipole moments of the Savinases are calculated using an in-house written application which calculates the dipole vectors, from the centre of mass as origin, by multiplying the partial charge of an atom (in one electron units) by its distance (in Angstroms) from the origin in the x, y and z directions. This is repeated for every atom of a variant and summed for the x, y and z directions. The length of this vector represents the size of the dipole moment. The partial charges are calculated at pH 6 by Insight II (MSI, San Diego USA) on a Silicon Graphics workstation.

Using Insight II the Savinases are positioned in such a way that the dipole vector is perpendicular to the computer screen. The proteins are rendered using van der Waals radii. After the rendering the number of pixels used for the rendering are counted by an in-house written application. By counting the pixels of a sphere of known radius the number of pixels are related to square Angstroms. In this way the areas of the frontal surface projections normal to the dipole vectors of the Savinase variants are calculated.

4.2.5 Description of the electrophoretic mobility of proteins

The electrophoretic mobility \( \mu \) of a solute is affected by the charge density and the friction, and the following equation based on the Debye-Hückel-Henry theory, is suggested for proteins [9, 12]:

\[
\mu = \frac{ze \phi(\kappa r)}{6\pi \eta r (1+\kappa r)}
\]  
(4.1)

where \( z \) is the theoretical protein valence, \( e \) is the electronic charge, \( r \) is the hydrodynamic radius, \( \eta \) is the viscosity of the buffer and \( \phi(\kappa r) \) is Henry's function, which varies in a sigmoidal fashion from 1 (for \( \kappa r \) equal to 0) to 1.5 (for \( \kappa r \) being \( \infty \)). The inverse of \( \kappa \) is the Debye length and equal to the thickness of the ionic double layer surrounding the protein. The Debye parameter \( \kappa \) can be expressed as \( 2^{1/2}Ne_0^{1/2}(e_0\varepsilon'RT)^{1/2} \) where \( N \) is Avogadro's number, \( e_0 \) is the dielectric constant of the buffer, \( \varepsilon' \) is the permittivity of free space, \( R \) is the gas constant and \( T \) is the absolute temperature. A more convenient equation is obtained
when the radius of the protein is substituted by its molecular mass as shown in Eq. 4.2 [9, 13]

\[ r = \left( \frac{3MV}{4\pi N} \right)^{1/3} \frac{f}{f_0} \]  \hspace{1cm} (4.2)

which results in Eq. 4.3:

\[ \mu = \frac{z_4 e \varphi(\kappa)}{6\pi \eta \frac{f}{f_0} M^{1/3} + \frac{6\pi \eta \sqrt{2} N e}{(e_0 e' RT)^{1/2}} \frac{(\frac{f}{f_0})^2}{(4\pi N^{2/3}) I^{1/2} M^{2/3}}} \] \hspace{1cm} (4.3)

where \( z_4 \) is the actual protein valence, \( f/f_0 \) is the frictional ratio, which is an indirect measure for the protein asymmetry and ranges from 1 for a perfect globular to more than 3 for a cylindrically shaped protein, \( V \) is the protein partial specific volume and \( M \) is its molecular mass. The actual protein valence \( z_4 \) is affected by charged species in solution but is proportional to the theoretical valence \( (z = F z_4) \). The proportionality factor \( F \) is pH independent but may be affected by buffer properties like ionic strength and the type of buffer. Both Eqs. 4.1 and 4.3 enable the description of the mobility of the proteins as a function of the properties of the solutes and the experimental set-up.

Several parameters of Eqs. 4.1 and 4.3 are not known accurately or may be affected by the experimental conditions and must therefore be estimated. The solutes investigated here are globular proteins, hence the frictional ratio is set to 1 throughout this study. The partial specific volumes \( V \) of the proteins are calculated from the amino acid composition in combination with tabulated values for the specific volumes of the individual amino acid residues [14] and ranges from 0.709 x 10\(^{-3}\) m\(^3\) kg\(^{-1}\) for wild type Savinase to 0.711 x 10\(^{-3}\) m\(^3\) kg\(^{-1}\) for variant US 219. These values are relatively close to 0.75 x 10\(^{-3}\) m\(^3\) kg\(^{-1}\), a value often used for proteins [14]. Furthermore, the variation in the calculated partial specific volumes is extremely small (\( \leq 0.3\% \)). Tabulated values for \( \kappa \) [12] and calculated values for \( r \) according to Eq. 4.2 enable an estimation of \( \varphi \kappa \) which is in the range of 0.8 to 1.4 so that Henry's function is assumed to be 1 in all cases.
According to the denominator in Eq. 4.3, the friction varies in a continuous way from $M^{1/3}$ to $M^{2/3}$, depending on the size of the protein and the ionic strength of the buffer. In a first approximation, the ratio of both terms varies from 0.7 to 1.3, depending on the buffer used. Consequently, none of these terms may be neglected under the experimental conditions used here.

4.3 Results

4.3.1 The proteins under investigation

Proteins can be considered complex molecules composed of both positively and negatively charged entities. If these charged sites are distributed in a non-uniform way over the molecule, a permanent dipole moment is present. The dipole properties together with the cross-sectional surface areas for the Savinases are calculated as explained in section 4.2.4 and summarised in Table 4-III.

<table>
<thead>
<tr>
<th>Table 4-III Properties of the Savinase variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Label</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>wild type</td>
</tr>
<tr>
<td>US 205</td>
</tr>
<tr>
<td>US 211</td>
</tr>
<tr>
<td>US 216</td>
</tr>
<tr>
<td>US 219</td>
</tr>
</tbody>
</table>

These data reveal an increase of the surface areas at increasing number of substitutions and the radii of the solutes are in the range of 20.73 to 21.87 Å. Comparison of the dipole vectors of the proteins reveals that the z-coordinates are similar for all solutes. The dipole vectors of wild type Savinase and US 205 point to the third quadrant in the (x,y) plane, while the dipole vectors of all other solutes point to the fourth quadrant.
Due to the high isoelectric point of Savinase, an eCap neutral capillary with a deactivated silica wall had to be used to suppress interactions with the capillary. Figure 4.1 shows an overlay of some typical electropherograms obtained in the 5 mM citrate - 5 mM MES buffer at pH 6.0 and even though some peak tailing is observed, adsorption appears to be satisfactorily suppressed. In this system most of the mutant protein preparations migrate as a single peak, in some cases a small impurity migrating at about the same position as wild type Savinase is observed.

![Graph showing electropherograms of Savinase and variants](image)

**Fig. 4.1** Overlaid electropherograms of Savinase and some of its variants analysed in a 5 mM citrate - 5 mM MES buffer at pH 6.

### 4.3.2 Effect of the ionic strength on the migration behaviour of Savinase variants

The migration data of wild type Savinase and its variants obtained at different experimental conditions are shown in Fig. 4.2. The mobilities are expressed as \((\mu_{\text{variants}} - \mu_{\text{wild type}})\) and are thus relative to the mobility of wild type Savinase.

The ionic strength values of the 5 and 15 mM citrate - MES buffers at pH 6 are calculated to be 13 and 39 mM respectively and an increase of \(I\) results in a decrease of the apparent
mobility of the wild type enzyme from 12.20 \times 10^{-9} to 8.11 \times 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}. Furthermore, Fig. 4.2 reveals that the relative mobilities of all proteins become smaller at higher ionic strength but the migration order of the Savinases is preserved and not affected by the increase of the ionic strength over the range studied here.

Fig. 4.2 The mobility of the variants, compared to the mobility of the wild type enzyme, measured in different buffer systems (■: 15 mM citrate - 15 mM MES at pH 6; □: 5 mM citrate - 5 mM MES at pH 6; △: 10 mM citrate at pH 3 and ○: 5 mM citrate - 5 mM MES, 3 mM Ca^{2+} at pH 6).

4.3.3 The migration behaviour of Savinase variants at pH 3

The experiments performed in the 10 mM citrate buffer at pH 3.0 reveal a straightforward proportionality between the measured mobility and the number of substituted amino acids, as shown in Fig. 4.2. At this pH, the introduced acidic residues will for the greater part be protonated and carry no charge. Consequently, the net charge of the variants becomes more positive according to the number of basic residues introduced. The differences in the charge between the variants and wild type Savinase can be calculated from pK_a values of the relevant amino acid residues [15] and the Henderson-Hasselbalch equation, and the data in Table 4-IV show that large differences in the protein valence up to 10 are expected ($\Delta z_{\text{pH}}$).
Capillary electrophoresis of Savinase

Table 4-IV The estimated valence differences between the wild type of Savinase and variants thereof

<table>
<thead>
<tr>
<th>Label</th>
<th>( \Delta z_{pH3} )</th>
<th>( \Delta z_{pH6} )</th>
<th>( \Delta z_{pH6+Ca} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>/</td>
<td>0.009</td>
<td>/</td>
</tr>
<tr>
<td>US 205</td>
<td>1.86</td>
<td>-0.006</td>
<td>0.025</td>
</tr>
<tr>
<td>US 211</td>
<td>3.66</td>
<td>0.011</td>
<td>0.099</td>
</tr>
<tr>
<td>US 216</td>
<td>7.32</td>
<td>-0.011</td>
<td>0.397</td>
</tr>
<tr>
<td>US 219</td>
<td>10.07</td>
<td>-0.004</td>
<td>0.751</td>
</tr>
</tbody>
</table>

The experimentally observed mobilities obtained in the low pH buffer can be evaluated by means of Eq. 4.3 which results in a good linear fit \( (r^2 = 0.95) \) as shown in Fig. 4.3.

![Graph](image-url)

**Fig. 4.3** The calculated mobility, according to Eq. 4.3, as a function of the experimentally observed mobility for Savinase and variants thereof. The experiments were performed in a 10 mM citrate buffer at pH 3. The solid line represents the linear least-square fit.
4.3.4 The migration behaviour of Savinase variants at pH 6

It is surprising that the electropherograms in Fig. 4.1 show an almost baseline separation of all the Savinase variants, since at neutral pH (=6) both basic and acidic residues added are fully ionised. Even more remarkable is the migration order which is no longer related to the number of substitutions.

Evaluation of these data according to Eq. 4.3 and shown in Fig. 4.4, reveals that a pronounced scattering of the data results in a bad linear relation ($r^2 = 0.66$ and 0.034 for the 5 and 15 mM buffer systems respectively). Apparently, the observed mobilities at pH 6 are not described satisfactorily by this equation. The friction may, however, be affected by a favoured spatial orientation in an electric field due to the dipolar properties of the proteins, provided that the proteins are not perfectly globular. Therefore, it was tried to obtain a better description of the experimental data by means of a radius which corresponds to the calculated frontal surface area (see also Table 4-III) in combination with a favoured spatial orientation instead of the molecular mass (see also Eq. 4.1).

![Diagram](image.png)

**Fig. 4.4** The calculated mobility, according to Eq. 4.3, as a function of the experimentally observed mobility for Savinase and variants thereof. The experiments were performed in a 5 mM citrate - 5 mM MES buffer at pH 6 (●) and in a 15 mM citrate - 15 mM MES buffer at pH 6 (★). The dotted and the solid line represent the linear least-square fits for the experiments performed in the 5 and 15 mM buffer respectively.
The mean component of a dipole in the direction of an externally applied electric field \( \bar{u} \) can be calculated [16] using the Boltzmann distribution

\[
\bar{u} = -\frac{u}{a} + u \frac{e^{a} + e^{-a}}{[e^{a} - e^{-a}]} \quad \text{where} \quad a = \frac{uE}{k_{B}T}
\]

(4.4)

where \( u \) is the dipole moment, \( E \) is the electric field strength and \( k_{B} \) is the Boltzmann constant. The ratio \( \bar{u}/u \) is a measure for the alignment of a dipole in an electric field and ranges between 0 if there is no specific orientation and 1 if the dipole is perfectly aligned in the field.

The friction can now be expressed as a weighted sum of the hydrodynamic radius \( r \), calculated from the surface area of the standard planes and \( \bar{r} \), which is the average hydrodynamic radius of the protein when there is no alignment in the electric field and the protein rotates freely according to Brownian movement. This results in Eq. 4.5 in which \( \bar{u}/u \) is the weighting factor. An estimate for \( \bar{r} \) is calculated over 5 random orientations at pH 6 and equals 21.43 Å.

\[
\text{friction} \sim \frac{\bar{u}}{u}r + (1 - \frac{\bar{u}}{u}) \bar{r}
\]

(4.5)

The parameters necessary to calculate \( \bar{u}/u \) can be found in Tables 4-I and 4-III. However, both the dipole moment as well as the electric field strength may be affected by the experimental conditions and are therefore not exactly known. The former may be estimated to be too low since the induction of a dipole moment in an externally applied field is not included here. The real value of \( E \) may be higher than the values shown in Table 4-I as a result of stacking effects in the close vicinity of the sample plug. Consequently, \( \bar{u}/u \) is estimated to be between 3.5 x 10^{-3} and 0.20. (\( E \) values of 500 and 10000 V cm\(^{-1} \) and dipole moments of 250 and 750 debye were used; the latter value is deduced from literature data for a series of proteins [16].) If it is assumed that the deviations of the parameters used in Eq. 4.4 are similar for all variants, mutual comparison of the \( \bar{u}/u \) values is possible, although the calculated \( \bar{u}/u \) value for each individual solute may be inaccurate. The friction according to Eq. 4.5 is shown for all solutes in Fig. 4.5 and reveals an increase of the friction as the number of substitutions increases. However, these deviations are typically smaller than 0.5 %.
Next to variations in the friction, small differences in the overall charge may play a part in the observed mobility as well. By means of Eq. 4.3, these charge variations are estimated in such a way that the theoretically expected linear behaviour between the calculated and measured mobilities is obtained. These charge differences are summarised in Table 4-IV ($\Delta z_{\text{pK16}}$) and are typically in the order of 0.01 and result in an almost perfect linear fit (data not shown).

4.3.5 The migration behaviour of Savinase variants at pH 6 in the presence of Ca$^{2+}$

Addition of 3 mM CaCl$_2$ to the citrate - MES buffer at pH 6.0 appears to have a dramatic effect on the observed migration order, which becomes the same as in the low pH buffer (Fig. 4.2). This results from shielding of the negative charges on the Savinases by Ca$^{2+}$. Consequently, the mechanism underlying the migration behaviour in this buffer system is analogous to the protonation of acidic groups taking place in the citrate buffer at pH 3.
Calculation of the mobilities according to Eq. 4.3 requires an estimate of the actual changes in the net charge as well as in the friction caused by the Ca\(^{2+}\) addition. To calculate the valence differences (\(\Delta z_a^{pH6-Ca}\)), it is assumed that there is no selectivity in the shielding by Ca\(^{2+}\) with respect to the type of acidic residue added. Consequently, it is assumed that the efficiency of shielding is proportional to the number of substituted negatively charged residues. For the sake of simplicity, the stoichiometry of the association has not been taken into account. Furthermore, the actual valence difference ranges between \(\Delta z_a^{pH6}\) which is close to zero according to the data in Table 4-IV and \(\Delta z_a^{pH3}\) which is assumed to be the highest attainable valence difference as a result of complete shielding of all the acidic residues added. From the above it follows that the valence difference can be estimated according to Eq. 4.6

\[
\Delta z_a^{pH6-Ca} = \frac{\Delta z^{pH6}}{F} + \frac{\Delta z^{pH3}}{F} \frac{n}{2m}
\]  

(4.6)

where \(n\) is the number of substitutions and \(m\) accounts for the efficiency of the shielding of the negative charges by Ca\(^{2+}\). If \(m\) equals the total number of negatively charged residues introduced, a perfect shielding of all charges comparable with protonation at low pH is then obtained. A higher value of \(m\) indicates that the negative charges are only partly shielded. The proportionality factor \(F\) is included as well to convert the theoretical protein valence to the actual one.

The actual molecular mass of Savinase in the presence of calcium ions \((M_{Ca})\) is estimated according to Eq. 4.7, which has a form analogous to Eq. 4.6

\[
M_{Ca} = M + \frac{M_{ion} n}{2m}
\]  

(4.7)

where \(M\) and \(M_{ion}\) are the molecular masses of the Savinase variant and the shielding ion in solution respectively.

Equations 4.6 and 4.7 can be substituted into Eq. 4.3 so that the mobility is expressed as a function of the modified charge and friction. The linear least square fit of the experimental data to this modified mobility equation results in a value for \(m\) of 13.8 \((r^2 = 0.986)\). From
this it follows that the changes in the molecular mass are smaller than 0.12 %, hence no effect of changes in \( M_{\text{Ca}} \) on the mobility are expected and the actual charge differences calculated according to Eq. 4.6 \( (\Delta z^a_{\text{pH+Ca}}) \) are summarised in Table 4-IV.

4.4 Discussion

4.4.1 Effect of the ionic strength on the migration behaviour of Savinase variants

Figure 4.2 reveals that an increase of the ionic strength from 5 to 15 mM citrate - MES does not affect the migration order. This suggests that interactions between buffer molecules and one or more Savinase variants do not occur to a degree that compromises a comparison of the electrophoretic behaviour of Savinase at different experimental conditions. The absolute mobilities as well as the mobility differences, however, are reduced at higher ionic strength, which is probably caused by shielding of charged sites on the Savinases by ions in solution. A theoretical decrease of 1.35 of the mobility of the wild type Savinase is predicted by Eq. 4.3, which is in fair agreement with the measured decrease of 1.5 fold.

This suggests that small differences in the electrophoretic properties should be preferably studied in a buffer of low ionic strength, although a 3 fold decrease of the efficiency compared to the high ionic strength buffer (data not shown) must also be taken into account. Furthermore, in spite of the reduction in the measured mobilities and the scatter of the experimental data, the ratio of the slopes of the linear least square fits in Fig. 4.4 is close to 1 (0.056/0.071≈0.8), so it seems that the physicochemical properties on which the mobility differences are based, are affected to the same extent by the range of ionic strength studied here.

4.4.2 The migration behaviour of Savinase variants at pH 3

The migration order at pH 3 is related to the calculated valence differences at low pH in a straightforward manner, based on the amino acid composition and a good linear fit in Fig. 4.3 is obtained. Changes in the friction, due to the small increase of the molecular mass or conformational changes at acidic pH values, may affect the electrophoretic mobility as well.
The latter is not included here but these differences are probably much smaller compared to the changes in the charge and therefore not of decisive importance for the calculation of the mobility.

Figure 4.3 and the data in Table 4-IV reveal that a two fold increase of the net charge does not result in a two fold increase of the mobility. The proportionality factor $F$ introduced in section 4.2.5 accounts for this reduction of the effect of an additional charge on the observed mobility. Previously, Compton [17] obtained a value for $F$ by taking the ratio of the calculated and experimental mobility at one pH value. In the present study, $F$ is obtained from the slope of the least square linear fit in Fig. 4.3 and equals 10.7. This indicates that only ten percent of the added charge is recovered as an increase of the observed mobility. In the study cited above, $F$ values ranging from 2.8 to 8.4 were calculated for several proteins and this indicates that $F$ strongly depends on the type of protein and probably to the type and concentration of the buffer as well.

4.4.3 The migration behaviour of Savinase variants at pH 6 in the presence of Ca$^{2+}$

The gel electrophoretic and ion-chromatographic experiments performed by Egmond et al. [8] suggest that shielding of negative charges on Savinase by Ca$^{2+}$ affects the overall charge of the proteins. The basic residues are not affected by the presence of cations so that the mutated proteins become increasingly positive according to the number of substitutions. This is confirmed by CE experiments in the buffer at pH 6 in the presence of Ca$^{2+}$ presented here. In our study, however, only 3 mM CaCl$_2$ is added to the background electrolyte, which is at least 3 times lower than the concentration used by Egmond et al. [8]. This illustrates the sensitivity of CE and its suitability to study small differences in structural properties of proteins.

From the above it follows that the mechanism underlying the mobility differences in the pH 6 buffer in the presence of Ca$^{2+}$ and in the low pH buffer is analogous. This is confirmed by a fairly constant value of 1.93 for the ratio of the mobilities measured in both buffer systems for each variant (the relative standard deviation is 2.3%). This mobility ratio of about two indicates that shielding of the negative charges by Ca$^{2+}$ is less efficient compared to protonation at low pH. This is also expressed in the fitted value for $m$ of 13.8. The data in Table 4-IV, however, indicate that the ratio of the actual charges in both buffer systems
changes from 7.0 for US 205 to 1.3 for US 219, hence a constant value of about 1.9 is not obtained. This suggests that the changes in the actual charge due to the shielding of Ca\(^{2+}\) are underestimated at a low number of substitutions and overestimated if more than 16 charged residues are incorporated. This non-linearity was also observed by Grossman et al. [18], who related the mobility of a series of peptides to the logarithm of \((z + 1)\).

Although our experiments indicate that the actual charge of the proteins is clearly affected by the pH and the addition of Ca\(^{2+}\), the estimation of reliable values for \(\Delta z\) is not straightforward. In addition, it should not be overlooked that both the charge as well as the friction may change simultaneously, which further hampers an accurate calculation of the mobility under different experimental conditions for the series of proteins analysed here. Nevertheless, for both buffer systems discussed above, it is likely that the observed mobility differences are mainly due to the changes in the net charge while the small changes in the friction, expressed as molecular weight, are overruled by the charge differences.

4.4.4 The migration behaviour of Savinase variants at pH 6 in the absence of Ca\(^{2+}\)

The migration order is strikingly different in the buffers at pH 6 in the absence of Ca\(^{2+}\). It follows from Table 4-II that histidine at position 120 is substituted by aspartic acid in US 211, US 216 and US 219. Even though the pK\(_a\) of this histidine residue is not exactly known, this may explain why these solutes are characterised by a smaller mobility as compared to wild type Savinase and US 205. It does, however, not explain the exact peak order for the individual solutes.

The differences in the calculated mobilities in Fig. 4.4 are solely brought about by the calculated differences in the molecular mass. The linear fit is, however, bad and the observed mobility differences are much larger than would be expected based on the increase of \(M\). It is suggested that the molecular mass does not adequately accounts for the small differences in the friction. The introduction of the radii and the dipole properties of the proteins in the electric field is used here in an attempt to refine the calculation of the mobility.

It has been shown in the literature that a favoured spatial orientation of a dipolar molecule in an electric field affects the friction in case of non-globular solutes. Rowe et al. [19] analysed a series of monosubstituted alkyl pyridines and concluded that size and shape
effects affect the observed mobilities. A theoretical investigation on the effect of particle
orientation in an electric field was performed by Han and Yang [20]. Although no
experimental work was included, the authors concluded that both the permanent dipole
moment and the induced dipole moment contribute to the positioning of the solute in the
capillary, affecting the mobility.

The radii, necessary to calculate the friction according to Eq. 4.5, are deduced from the
surface areas shown in Table 4-III. These data indicate that the Savinases are not perfectly
globular shaped and that the dipole vectors of the Savinases point to different directions. The
increase of the radii as the number of substitutions increases seems to be in agreement with
the aforementioned increase of the molecular weight as shown in Table 4-II, although the
increases in the surface areas are much more pronounced. Although inaccurate values for \( E \)
and \( u \) may affect the absolute values of the friction calculated according to Eq. 4.5, the
relative values for the friction and the order thereof can be evaluated. The friction data
shown in Fig. 4.5 cannot be brought in line with the experimentally observed mobility data
and do not improve the agreement between the measured and calculated mobilities.

It is obvious that the friction calculated here contains only limited information on the
3-dimensional shape of the Savinases. Therefore, it is possible that this aberrant calculated
mobility is caused by the lack of relevant spatial information on each variant, which is
obviously not available. From this, it follows that a satisfying relationship between the
friction (expressed as molecular mass or radius) and the observed mobility, for the system
studied here, is not obtained yet.

It is remarkable that Kálmán et al. [6, 7] attributed small differences in the mobilities of S.
nuclease mutants in the order of 2 to 3 \( \times 10^{-10} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1} \) to differences in the hydrodynamic
radius as small as \( \pm 0.4 \text{ Å} \) (1.6% of the protein radius) as a result of the substitution of only
one amino acid (< 0.1 % of the protein molecular mass). In our study, however, differences
in the radii as large as 1.1 Å cannot be used to explain the experimentally observed
migration behaviour. Therefore, we believe that the data in the former study should be
interpreted with care. In addition, the radii of the S. nuclease mutants are not unambiguously
defined and actually no confirmation of an actual increase of the radius is given. Therefore,
if mobility differences are observed for proteins that differ in one or only several amino acid
residues, both the effect on the friction and the charge must be considered simultaneously,
even if only uncharged residues are substituted.
Consequently, we investigated the effect of small changes in the overall charge on the mobility. A change in the net charge of the solutes may be caused by a shift of the dissociation constant of one or more of the 7 (or 6) histidine residues, as already mentioned by Egmond et al. [8] or by differences in the local ionic environment of the introduced charged residues and the way these sites are exposed to the aqueous phase [3]. Although it is not possible to find an unambiguous support for changes in the net charge from our experiments, the data in Table 4-IV reveal that small variations in the actual charge (the theoretical valence may be about one order of magnitude higher) cause significant changes in the calculated mobility, which results in a dramatic increase of the linearity of the fit (data not shown) according to Eq. 4.3.

4.4.5 Remaining considerations

The calculations performed by Egmond et al. indicated a small decrease of the electrostatic potential at increasing number of charged residues [8], which would result in a proportionality of the mobility obtained from the CE measurements and the number of substitutions. Although a relation between these calculations and our experiments may not be straightforward, the structural decrease of the electrostatic potential is not supported by the migration data obtained at pH 6 and many aspects of the migration behaviour of these Savinase variants at this pH remain unexplained.

4.5 Conclusions

This study illustrates that the high separation power of CE enables the examination of changes in the physicochemical properties of proteins and thus their mobilities resulting from subtle differences in the amino acid composition. In addition, the set of Savinase mutants studied here is very well suited to study these small differences. The high separation selectivity of CZE is preeminently illustrated by the baseline separation for all solutes obtained in the citrate - MES buffer at pH 6.0, where only minor differences in the physicochemical properties of the solutes are expected. Equations expressing the mobility of a solute as a function of its physicochemical properties are presented in the literature. Despite some inaccuracies in the estimation of several parameters, these equations have been shown to be useful to study the electrophoretic behaviour and structural properties of proteins.
The addition of calcium ions to the buffer at pH 6 and the use of a pH 3 buffer results in a migration order which is primarily explained by a change in the overall charge. The data obtained at pH 3 allow the estimation of the proportionality factor between the theoretical and actual protein valence. The experiments performed in the presence of Ca$^{2+}$ enable the estimation of the efficiency of the shielding of negative charges on Savinase by Ca$^{2+}$. Although changes in the friction may occur as well, the agreement between the experimental and calculated mobility data reveal that charge differences can be considered much more important than changes in friction.

The migration order obtained in the buffer at pH 6 cannot be explained easily. Our attempts to describe the changes in the friction by means of the molecular mass and the radius of the proteins perpendicular to the dipole vector are not successful. However, the analysis of these data is complicated because of the mutual effects in both charge and friction, upon substitution of one or more amino acid residues, and both must be considered. The data presented here reveal that the introduction of small changes in the actual charges of the individual mutants enable a better description of the experimental mobility data. In analogy with the data obtained in the buffer at pH 3 and in the buffer at pH 6 in the presence of calcium ions, it is concluded that the differences in charge are probably more important than variations in friction.

4.6 References


Chapter 5
A capillary electrophoretic study of the interaction of methylamine dehydrogenase with amicyanin

Abstract

Capillary zone electrophoresis is used to study the binding behaviour of amicyanin and methylamine dehydrogenase (MADH), both from Thiobacillus versutus. A systematic approach is presented to estimate the apparent association constant in which the concentration of amicyanin is included as an additional fit parameter in the handling of the experimental mobility data, next to \( K_{\text{ass}} \) and the mobilities of the complex and the free solute. Only in case of relatively high affinity (\( K_{\text{ass}} \geq 1 \times 10^6 \text{ M}^{-1} \)) and thus at high concentration ratio of amicyanin to MADH, the amicyanin concentration affects the value for the fitted association constant. For the system studied here, association constants up to \( 2 \times 10^6 \text{ M}^{-1} \) can be determined. Simulations, based on a theoretical plate model and the evaluation of the peak shape of amicyanin confirm these results.

An increase of the ionic strength of the background electrolyte results in a relatively small increase of the association constant of the MADH-amicyanin complex, although a biphasic dependence of \( K_{\text{ass}} \) on the ionic strength cannot be excluded. An increase of the pH (from 7.4 to 8.9) and the osmotic pressure (up to 65 bars) results in a significant increase of \( K_{\text{ass}} \). The former may be caused by a (de)protonation step of one or both proteins. The latter indicates that the interaction primarily involves hydrophobic forces and the formation of the complex is accompanied by the removal of about 40 water molecules at the interfacial surfaces. These results are in accordance with recently published literature data.

To be submitted for publication
5.1 Introduction

5.1.1 Estimation of the binding constant

The formation of molecular complexes, defined as the association of two or more interacting molecules, is widespread in nature [1-3] and plays a vital role in many biochemical processes. The stability of such complexes is affected by the structural and/or electronic features of the binding sites and the study of the extent of binding contributes towards an understanding of intermolecular forces. This knowledge can be exploited in various fields e.g. the study on enzymatic catalysis [4] and the optimisation of therapeutic drug dosage [5-7]. Accordingly, the association behaviour of molecules and the different parameters affecting the binding have been subjected to various theoretical and experimental investigations [8-10].

Any physicochemical property of the free and/or complexed molecules that is altered upon complex formation may serve as a basis for measuring the binding constant(s). Consequently, many different analytical techniques like spectroscopy, potentiometry, liquid-liquid partitioning, chromatography and dialysis are used in this field [8]. Recently, affinity capillary electrophoresis (ACE), a special mode of capillary electrophoresis (CE), has appeared to be suitable in the study of the binding behaviour of molecules like the association of enantiomeric drug substances and cyclodextrins [11-13] or metal ions and various ligands [14], while biochemical systems like antibody-antigen [15], lectin-sugar [16] and protein-ligand [17] have been studied as well.

Because CE belongs to the homogeneous separation systems that can be performed in the absence of stabilising agents, the suitability of this technique in the study of complex formation is further enhanced. Many complications related to the phase dependent behaviour of solutes that often appear in two-phase systems like chromatography and liquid-liquid partitioning are absent. Furthermore, CE is considered to be an equilibrium technique because the measurement itself does not perturb the actual formation of the complex. Consequently, the binding constant derived from the CE measurements resembles the 'real' binding constant without a major disturbance provoked by the measurement. Here, it is assumed that the applied potential difference does not affect the association. Lastly, the extent of complex formation is directly measured as a shift in the observed electrophoretic
mobility so that the use of an external indicator is not necessary which reduces the complexity of the experimental set-up.

5.1.2 The biochemical system under investigation

Methylamine dehydrogenase (MADH) occurs in a number of methylotrophic bacteria [18] and catalyses the oxidation of primary amines. The native enzyme contains four subunits arranged in an $\alpha_2\beta_2$ configuration, has a molecular weight of about 125000 and an isoelectric point close to 3.9. The physiological electron acceptor for MADH is amicyanin, a copper-containing monomeric protein with a molecular mass of 14000 and an isoelectric point of 4.8.

The complex formation of amicyanin and MADH has been the subject of several studies. Absorption spectroscopy and ultrafiltration revealed that the interaction is mainly driven by electrostatic attraction although it was suggested that hydrophobic interactions contribute as well [19]. This predominance of electrostatic interaction was also put forward by Backes et al. [20], who performed a resonance Raman spectroscopy study. In the former study, the stoichiometry of the complex was determined to be 2 amicyanin:1 MADH, which is in accordance with the $\alpha_2\beta_2$ configuration of the enzyme. In the same study, gel filtration chromatography indicated that a complex with an association constant smaller than $1 \times 10^5$ M$^{-1}$ is formed. An association constant of $2.2 \times 10^5$ M$^{-1}$ was derived from ultrafiltration measurements performed by Davidson et al. [21]. Furthermore, addition of 200 mM NaCl strongly reduced the complex formation, again indicating the importance of electrostatic interactions. These results are in contradiction with crystallographic studies that reveal a major contribution of hydrophobic forces [22-24] and this dominance of hydrophobic forces was also inferred from chemical cross-linking experiments [25].

A strong pH dependence of the complex formation was found by Gorren and Duine [26]. A rise of the pH from 7 to 10 resulted in a 10 fold increase of the association constant from $1 \times 10^5$ to $1 \times 10^6$ M$^{-1}$. The observed increase of the oxidation rate of MADH by amicyanin at increasing concentrations of cations like Na$^+$, K$^+$ or Cs$^+$ was attributed to the electron transfer itself and not to an increase of the stability of the complex [27].

In this chapter, CE is used to study the interaction of MADH and amicyanin, both from *Thiobacillus versutus*. Some features and limitations of CE in the estimation of binding
constants and the accessory data handling are discussed. The effect of pH, ionic strength and osmotic pressure on the association behaviour is studied and some suggestions with respect to the interaction mechanism are put forward.

5.2 Experimental

5.2.1 Apparatus

All experiments were carried out on a BioFocus 3000 system (Bio-Rad, Hercules, CA, USA) with an uncoated fused silica capillary, 58 cm (53.4 cm effective length) x 75 μm ID (Chrompack, Middelburg, The Netherlands). The samples and the capillary were thermostated at 20 °C. The samples were injected with 6.9 kPa s and a constant voltage of 10 kV was applied. The experiments were monitored at 200 nm and the data were collected and processed using the BioFocus 3000 data acquisition (version 4) and integration (version 3.01) software package.

5.2.2 Chemicals and buffers

di-Sodium hydrogen phosphate was from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate, sodium pyrophosphate and sodium chloride were from J. T. Baker (Deventer, The Netherlands). di-Sodium pyrophosphate and glycerol (99.5 % pure) were from Brunschwig (Amsterdam, The Netherlands) and mesityl oxide was from Fluka (Buchs, Switzerland). Deionised water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

MADH from *Thiobacillus versutus* was purified as described previously [28, 29]. Amicyanin was a kind gift from Dr. S de Vries. Stock solutions of both proteins were prepared in a 4 mM phosphate buffer, pH 7.16 and the concentrations of MADH and amicyanin were determined spectrophotometrically to be 30.6 and 35 μM, using an $A_{280}^{\text{mg/ml}}$ of 1.14 and 1.13 respectively [29, 30]. The purity of both proteins was checked using ion-chromatography (HiTrap Q, Pharmacia, Uppsala, Sweden) and capillary zone electrophoresis and no cross contamination could be detected.
Stock solutions of 12 and 16 mM phosphate buffer at pH 7.4, 12 and 16 mM pyrophosphate buffer at pH 8.9, 0.33 M potassium chloride and a 68 % glycerol solution in water were prepared. Appropriate amounts of these stock solutions were diluted in water to obtain the desired concentrations of buffer, MADH and glycerol as well as the desired ionic strength. Corrections for the volume changes associated with the addition of glycerol were incorporated. The changes of pH associated with the addition of KCl and glycerol were assumed to be negligible. The former assumption is based on the relatively small changes of the ionic strength introduced here. The latter assumption is confirmed by literature data [31] where only an increase of 0.15 pH units is observed for a 50 % glycerol in 10 mM phosphate buffer, compared to the pure phosphate buffer at pH 7, whereas the maximum concentration of glycerol in this study is only 20 %. The amicyanin stock solution was adjusted to a final concentration of 2 μM prior to injection.

5.2.3 Simulations

The simulation software was programmed in Igor Pro (version 3) and ran on a Power Macintosh 6100/60.

5.3 Theory

5.3.1 The determination of the association constant

If a molecule $A$ forms a complex with a molecule $M$, CE can be used to estimate the association constant $K_{\text{ass}}$, provided that the difference in the electrophoretic mobility of the free and the complexed form of $A$ is sufficiently large

$$M + A \rightarrow MA \quad \text{with} \quad K_{\text{ass}} = \frac{[MA]}{[M]_{\text{free}} [A]_{\text{free}}} \quad (5.1)$$

where $[MA]$, $[M]_{\text{free}}$ and $[A]_{\text{free}}$ are the concentration of the complex, free $M$ and free $A$ respectively. If it assumed that the kinetics of the formation and dissociation of the complex are fast, the total concentration of each solute, indicated by the subscript 0, can be expressed as:
\[ [A]_0 = [A]_{\text{free}} + [MA] \quad (5.2) \]

\[ [M]_0 = [M]_{\text{free}} + [MA] \quad (5.3) \]

The observed electrophoretic mobility of solute \( A \) (= \( \mu_{cp} \)) is a weighted sum of the electrophoretic mobility of the complexed and the free form of \( A \)

\[ \mu_{cp} = X_A \mu_A + X_{MA} \mu_{MA} ; \quad X_A + X_{MA} = 1 \quad (5.4) \]

where \( \mu_A, \mu_{MA}, X_A \) and \( X_{MA} \) are the electrophoretic mobilities and the fractions of the free and complexed form of \( A \) respectively. These fractions are defined as \( X_A = [A]_{\text{free}}/[A]_0 \) and \( X_{MA} = [MA]/[A]_0 \). Rewriting of Eq. 5.4, using Eqs. 5.1 to 5.3 enables the expression of the mobility of \( A \) as a function of the mobilities of the free and complexed form of \( A \) and the association constant:

\[ \mu_{cp} = \frac{\mu_A + K_{ass} [M]_{\text{free}} \mu_{MA}}{1 + K_{ass} [M]_{\text{free}}} \quad (5.5) \]

Equation 5.5, or a simplified form in which \([M]_{\text{free}}\) is set to \([M]_0\) (which can only be done if \([M]_{\text{free}} \gg [MA]\)), can be used to determine the association constant [12, 13].

Deranleau [32, 33] showed that the relative error in the determination of the association constant is minimal in the 20 to 80 % saturation range of \( A \) (0.2 \( \leq X_A \leq 0.8 \)) and actually no information on the association behaviour is obtained in the extreme cases where \( X_A \) equals 0 or 1. To satisfy the above condition, the concentration range of \( M \) in the background electrolyte should be chosen according to the actual magnitude of the association constant. In case of very high affinities, the maximum concentration of \( M \) should be very low while the detection limit of \( A \) sets the minimum concentration of \( A \). In this particular situation, the solutes \( A \) and \( M \) may be present in the same concentration range so that the condition \([M]_{\text{free}} \gg [MA]\) no longer holds. In that case the following alternative approach can be used.
The limiting mobility values of solute \( A \) (\( \mu_A \) and \( \mu_{MA} \)) equal the mobility of amicyanin in the absence of \( M \) and that in a large excess of \( M \) (\( \approx 10 \, K_{as}^{-1} \)) respectively, and both values can therefore be obtained experimentally. Once \( \mu_A \) and \( \mu_{MA} \) are known, Eq. 5.4 allows the calculation of the fractions \( X_A \) and \( X_{MA} \) from the observed mobility of \( A \) at any concentration of \( M \). It is then possible to calculate \( K_{as} [M]_{\text{free}} \) according to

\[
\frac{X_{MA}}{X_A} = \frac{[MA]_{\text{free}}}{[A]_0} \frac{[A]_0}{[A]_{\text{free}}} = K_{as} \frac{[M]_{\text{free}}}{[A]_{\text{free}}}
\]  

(5.6)

A theoretical value for \( [M]_{\text{free}} \) can be calculated from the total concentrations of \( A \) and \( M \) by solving Eq. 5.7

\[
K_{as} = \frac{[MA]}{([M]_0 - [MA]) ([A]_0 - [MA])}
\]  

(5.7)

This leads to the expression of the free concentration of \( M \) as shown below

\[
[M]_{\text{free}} = [M]_0 - [MA] =
\]

\[
[M]_0 - \frac{1}{2} \frac{K_{as} ([A]_0 + [M]_0) - \sqrt{(1 + K_{as} ([A]_0 + [M]_0)^2 - 4 \, K_{as}^2 [M]_0 [A]_0)}}{K_{as}}
\]  

(5.8)

A first estimate of \( K_{as} \) then enables the calculation of \( [M]_{\text{free}} \). A plot of \( K_{as} [M]_{\text{free}} \) obtained from the experimental data (Eq. 5.6) as a function of \( [M]_{\text{free}} \) (Eq. 5.8) should result in a straight line with a slope of \( K_{as} \). The value for \( K_{as} \) from this plot obtained can be used to recalculate the free concentration of \( M \) according to Eq. 5.8 and this is repeated until the changes in the association constant are less than a preset value.

If it is assumed that the values for \( K_{as} [M]_{\text{free}} \) according to Eq. 5.6 are accurate, deviations from linearity can only be due to errors in the constants in Eq. 5.8. In this study, we focused on the effect of the concentration of \( A \) so that the number of parameters is four (\( \mu_A, \mu_{MA}, K_{as} \) and \( [A]_0 \)). Note that the approach presented by Wren and Rowe [12], based on Eq. 5.5, is a three parameter fit (\( \mu_A, \mu_{MA} \) and \( K_{as} \)).
In this study, amicyanin is injected in a buffer containing various concentrations of MADH, representing solute $A$ and $M$ respectively in the above theoretical section. The observed mobility difference between the free and complexed form of amicyanin results from the high charge density of MADH and thus a reduced overall migration speed of the complex. Furthermore, due to the $\alpha_2\beta_2$ configuration of MADH, two distinct binding sites for amicyanin are recognised which have been shown to be identical and independent [21]. Accordingly, the MADH concentration present in the background electrolyte is multiplied by two to obtain the value for $[M]_o$. Furthermore, it is assumed that the contribution of amicyanin to the electrophoretic mobility of the complex is small so that no differentiation is necessary between the single and double associated MADH.

5.3.2 Simulation of the electrophoretic mobility using the theoretical plate model

To evaluate the experimental results, simulations based on a theoretical plate model were performed [34]. A recent example of this approach on the study of drug - protein interaction by means of high-performance chromatographic frontal analysis [35] has been presented. This approach enables a quantitative survey of the experiments and the estimation of the association constant in particular.

The capillary is divided into a series of perfectly mixed compartments or theoretical plates. A schematic presentation of two consecutive compartments $h$ and $h+1$ at time $t$ and $t+1$ are shown in Fig. 5.1. During one unit time, all free amicyanin is transferred to the next plate so that the concentration of free amicyanin in plate $h$ at time $t$ is exactly the same as in plate $h+1$ at time $t+1$. Due to the mobility difference between complexed and free amicyanin, the concentration of bound amicyanin in plate $h+1$ at time $t+1$ depends on the amount of bound amicyanin which is transferred from plate $h$ at time $t$ and the amount of complex which remains behind from plate $h+1$ at time $t$. If it is assumed that the mobility of MADH is not affected by the complex formation ($\mu_M = \mu_{mm}$) the total concentration of MADH will be constant in all compartments. The mass balances for free and bound amicyanin can be found in Fig. 5.1.

After the transfer, an immediate equilibrium between MADH and amicyanin is assumed and the concentrations of free and bound amicyanin are recalculated according to Eq. 5.8. Electropherograms can be generated by repeating these steps for the desired number of time steps.
5.3.3 The effect of the ionic strength on the association constant

As mentioned in section 5.1.2 the ionic strength affects the complex formation of amicyanin and MADH. An empirical extension of the extended form of the Debye-Hückel equation [36] enables the evaluation of the association constant as a function of the ionic strength $I$

\[
\log K_{\text{ass}} = \log K_{\text{ass}(0)} - z_A z_M \left( \frac{I^{1/2}}{1 + I^{1/2}} - 0.2 I \right)
\]  

(5.9)

where $K_{\text{ass}(0)}$ is the association constant at zero ionic strength and $z_A$ and $z_M$ are the number of charged sites on both molecules involved in the complex formation. This equation is valid at relatively low values of the ionic strength. A plot of $\log K_{\text{ass}}$ as a function of $(I^{1/2}/(1+I^{1/2}) - 0.2 I)$ results in a straight line from which $K_{\text{ass}(0)}$ and $z_A z_M$ can be estimated from the intercept and the slope respectively. The slope of the linear fit is a measure for the dependence of the complex formation on the ionic strength. Accordingly, $z_A z_M$ is a measure for the ionic interaction upon complex formation.
5.3.4 The effect of the osmotic pressure on the association constant

When complex formation is accompanied by a volume change, it can be expected that an increase in hydrostatic pressure will cause a shift in the equilibrium composition. This was experimentally verified for the porphyrin cytochrome c - cytochrome c oxidase complex by Kornblatt et al. [37], who observed a three-fold increase of the apparent association constant as a result of an increase of the hydrostatic pressure to 2.5 kbars.

The change in the dissociation constant with hydrostatic pressure is given by Eq. 5.10 [38]

\[
\frac{\delta (\ln K_{\text{dis}})}{\delta P} = \frac{\Delta V + \beta V_p P}{RT} \approx \frac{\Delta V}{RT}
\]  

(5.10)

where \(K_{\text{dis}}\) is the dissociation constant of the complex \((K_{\text{dis}} = 1/K_{\text{ass}})\), \(R\) is the gas constant, \(T\) is the absolute temperature, \(P\) is the applied hydrostatic pressure, \(\Delta V\) is the molar volume change, \(\beta\) is the compressibility of the system and \(V_p\) is the volume of the molecules involved in the complex formation. If the compressibility of the system is very small, the simplification in Eq. 5.10 is allowed and then a plot of \(\ln K_{\text{dis}}\) as a function of the applied pressure should result in a straight line from which \(\Delta V\) can be estimated from the slope. The reaction volume thus obtained reflects changes in conformation and solvation and both a positive or a negative \(\Delta V\) may be obtained.

Similarly, a shift in the equilibrium may be observed when an osmotic pressure is imposed upon the system. The addition of an osmolyte like glycerol lowers the water activity, increases the osmotic pressure and provides a way to appraise the importance of water molecules close to the surface in the complex formation. Consequently, comparable experimental results are expected if an osmotic instead of a hydrostatic pressure is imposed upon the system. Although this may not be identical from a physicochemical point of view, the equations presented above enable the evaluation of changes in the complex formation caused by an external pressure. In addition, Garner and Rau [39] showed by means of gel electrophoresis for the DNA - gal repressor system, that only in case of specific interaction between both molecules, the removal of water from the interfacial surfaces upon complex formation was observed.
Conversion of the glycerol concentration into the osmotic pressure $\pi$ is done according to Barrow [40] and results in

$$\pi = \frac{R T}{V_{\text{aq}}} \ln \frac{n_{\text{aq}} + n_{\text{glyc}}}{n_{\text{aq}}}$$ (5.11)

where $V_{\text{aq}}$ is the molar volume of water and $n_{\text{water}}$ and $n_{\text{glycerol}}$ are the moles of water and glycerol in solution respectively. The latter data are from ref. [41].

5.4 Results and discussion

5.4.1 General considerations

The determination of the electrophoretic mobility of amicyanin necessary to estimate the association constant requires an accurate electroosmotic flow measurement. A negative baseline disturbance, always present in the electropherograms, appears to be a reliable marker for the electroosmotic mobility at different concentrations of MADH.

For comparison, the migration behaviour of mesityl oxide which is often used as an external electroosmotic flow marker, was analysed at different concentrations of MADH in the background electrolyte ranging from 0 to 8 μM. The electroosmotic mobility increased almost 30% at increasing concentrations of MADH but the variation in the experimentally obtained electroosmotic mobility at a fixed concentration of MADH, using both mesityl oxide and the negative baseline disturbance was typically smaller than 2% (data not shown). Consequently, to minimise the effect of the composition of the sample plug on the amicyanin-MADH association, no external marker was added to the sample and the baseline disturbance was used as a measure for the electroosmotic flow throughout this study.

A representative set of electropherograms is depicted in Fig. 5.2. In Fig 5.3, the modeled migration behaviour of amicyanin obtained under the same conditions as the electropherograms in Fig. 5.2 is shown.
Chapter 5

Fig. 5.2 Representative set of electropherograms of amicyanin, injected in a 9.9 mM phosphate buffer at pH 7.4. At each peak, the variable concentration of MADH in the background electrolyte, expressed in μM, is included.

The experimental migration time of amicyanin as well as the electroosmotic migration time increases with increasing concentrations of MADH (see also Fig. 5.2). If the latter results from changes in the viscosity of the background electrolyte, corrections must be incorporated, as pointed out by Penn et al. [13]. Therefore, the current was measured at different concentrations of MADH which enables the calculation of a relative viscosity value as shown below

\[
\frac{i}{i_0} = \frac{\eta_0}{\eta}
\]

(5.12)

where \(i_0\), \(i\), \(\eta_0\) and \(\eta\) are the current and the viscosity in the absence and presence of MADH respectively. Throughout this study, no significant decrease of the current within one set of experiments at different MADH concentrations was observed (\% RSD ≤ 3 \%). This result is not surprising because the maximum concentration of MADH (22 μM) is relatively low.
Consequently, the increase in the viscosity is negligible and no corrections were incorporated.

![Graph](image)

**Fig. 5.3** Representative set of modeled electropherograms of amicyanin. The concentration of MADH is (for each peak from left to right) 1.36, 4.06, 6.76, 10.8, 16.22, 21.62 and 150 μM respectively.

The system peaks in the vicinity of the electroosmotic flow peak are reproducible within one set of experiments. Both the area and height of these system peaks increase at higher MADH concentrations in the background electrolyte, probably due to a rise of the background absorbance of the buffer and/or an increase of the disturbance of the equilibrium inside the capillary due to the injection of the sample at higher concentrations of MADH. It is assumed that these system peaks do not affect the migration behaviour of amicyanin hence they are not considered here.

### 5.4.2 The estimation of $\mu_A$, $\mu_{MA}$ and $K_{ass}$

In Table 5-I the values for $\mu_A$, $\mu_{MA}$ and $K_{ass}$, obtained from both the experimental and the simulated data are summarised for all the different buffer systems used. The mobility values $\mu_A$ and $\mu_{MA}$ are estimated from the raw experimental migration data, e.g. by fitting these data to the simplified form of Eq. 5.5. The association constant is deduced from the linear
regression as explained in section 5.3.1. The best estimate for \( \mu_A, \mu_{MA} \) and \( K_{ass} \), necessary to start the modelling procedure, is directly obtained from the experimental data.

Because the experimentally determined association constant is used as an input parameter in the plate height model to simulate the mobility behaviour of amicyanin at different concentrations of MADH in the background electrolyte, it follows that the newly obtained \( K_{ass} \) derived from these simulated mobility data, must be equal to the experimental input value. Deviations between the \( K_{ass} \) values obtained from the experiments and the simulations indicate an imperfection of the estimation of the association constant and suggest that these estimates of \( K_{ass} \) may be unreliable. In all cases the \( r^2 \) values are included as a measure for the linearity of the fit.

It follows from Table 5-I that the absolute values of \( \mu_A \) and \( \mu_{MA} \) decrease at increasing values of the ionic strength and osmotic pressure. The former probably results from shielding of charges on the proteins by oppositely charged species in solution while the latter is caused by an increase of the viscosity or a decrease of the dielectric constant of the medium at increasing concentration of glycerol. This results in a significant decrease of the observed mobility differences between \( \mu_{MA} \) and \( \mu_A \), although they are still large enough to differentiate between the free and complexed form of amicyanin.

In the phosphate buffer system, the association constant ranges from \( 6 \times 10^4 \) M\(^{-1} \) to \( 5 \times 10^5 \) M\(^{-1} \) depending on the ionic strength and the glycerol concentration. Due to differences in the analytical techniques, experimental conditions and the source of the proteins, comparison with other studies is not obvious. However, the \( K_{ass} \) values found in this study are in the same order of magnitude as the value obtained by Davidson et al. \( (K_{ass} = 2 \times 10^5 \) M\(^{-1} \)) [21] and Gorren and Duine [26] \( (K_{ass} = 1 \times 10^5 \) M\(^{-1} \)). The actual concentration used in the fitting procedure (see also Eq. 5.8) does not affect the value of the association constant nor the \( r^2 \) value of the fit. As expected, handling of the experimental and modelled data results in the same association constants. The conformity of these results indicates that the assumed migration mechanism in the model is correct.
The interaction of MADM with amicyanin

Table 5-I: The fitted parameters with the standard error for all buffer systems: $\mu_A$ and $\mu_{MA}$ are obtained from a non-linear fit of the mobility data to Eq. 5.5 and $K_{ma}$ is obtained from the linear regression of a plot of $K_{ma}$ vs MADHfree (Eq. 5.6) as a function of [MADH]free (Eq. 5.8). Stars indicate that no converging results are obtained.

<table>
<thead>
<tr>
<th>Buffer System</th>
<th>$\mu_A$ (10$^{-9}$ m$^2$ V$^{-1}$ s$^{-1}$)</th>
<th>$\mu_{MA}$ (10$^{-9}$ m$^2$ V$^{-1}$ s$^{-1}$)</th>
<th>$K_{ma}$ (10$^3$ M$^{-1}$)</th>
<th>$[A]_0$ (μM)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.9 mM phosphate buffer, pH 7.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1=26 mM, 0% glycerol</td>
<td>exp</td>
<td>-13.85 ± 0.27</td>
<td>-24.76 ± 1.93</td>
<td>62 ± 2.3</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>63 ± 0.1</td>
<td>/</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>1=35 mM, 0% glycerol</td>
<td>exp</td>
<td>-12.25 ± 1.9</td>
<td>-21.85 ± 4.76</td>
<td>110 ± 10.5</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>112 ± 0.7</td>
<td>/</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>1=55 mM, 0% glycerol</td>
<td>exp</td>
<td>-10.65 ± 0.61</td>
<td>-16.32 ± 3.60</td>
<td>113 ± 14</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>112 ± 0.1</td>
<td>/</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>1=26 mM, 10% glycerol</td>
<td>exp</td>
<td>-9.91 ± 0.66</td>
<td>-16.19 ± 2.38</td>
<td>187 ± 23</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>185 ± 0.3</td>
<td>/</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>1=26 mM, 20% glycerol</td>
<td>exp</td>
<td>-7.01 ± 0.38</td>
<td>-10.26 ± 0.55</td>
<td>479 ± 60</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>508 ± 8</td>
<td>/</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>9.9 mM pyrophosphate buffer, pH 8.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1=100 mM, 0% glycerol</td>
<td>exp</td>
<td>-16.95 ± 0.06</td>
<td>-17.63 ± 0.06</td>
<td>2300 ± 1300</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>exp</td>
<td>1817 ± 850</td>
<td>0.1</td>
<td>0.997</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>2500 ± 300</td>
<td>2</td>
<td>0.950</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>1600 ± 13</td>
<td>0.1</td>
<td>0.998</td>
<td></td>
</tr>
<tr>
<td>1=130 mM, 0% glycerol</td>
<td>exp</td>
<td>-13.89 ± 0.05</td>
<td>-14.72 ± 0.05</td>
<td>3350 ± 525</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>exp</td>
<td>1985 ± 180</td>
<td>0.1</td>
<td>0.967</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>3700 ± 450</td>
<td>2</td>
<td>0.932</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>1800 ± 32</td>
<td>0.1</td>
<td>0.998</td>
<td></td>
</tr>
<tr>
<td>1=100 mM, 20% glycerol</td>
<td>exp</td>
<td>-9.55 ± 0.03</td>
<td>-10.06 ± 0.03</td>
<td>*****</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>exp</td>
<td>14700 ± 2000</td>
<td>0.1</td>
<td>0.944</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>*****</td>
<td>2</td>
<td>****</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sim</td>
<td>4900 ± 370</td>
<td>0.1</td>
<td>0.973</td>
<td></td>
</tr>
</tbody>
</table>

a) data deduced from experiments, b) data deduced from simulations
The data obtained in the pyrophosphate buffer systems show some remarkable differences compared to those from the phosphate systems. If the amicyanin concentration is assumed to be 2 μM, the linearity of the fit for both the experimental and the simulated data is not good. The relatively low $r^2$ value is not caused by an extensive scatter of the data but due to a clear convex curvature of the data points. However, if the total concentration of amicyanin is included as the fourth parameter in the fitting procedure, the linearity of the plot is improved dramatically and smaller values for $K_{ass}$ are found. This is illustrated in Fig. 5.4 where the $r^2$ value and the association constant for the pyrophosphate buffer without added salt or glycerol are shown for different values of the amicyanin concentration. It is clear that both $K_{ass}$ and $r^2$ asymptotically reach constant values and below a critical amicyanin concentration, the changes in the quality of the linear fit or the estimated association constant are no longer significant.

![Graph](image)

**Fig 5.4** The estimated association constant (■) on the left y-axis and the $r^2$ value of the corresponding least-square linear fit (□) on the right y-axis as a function of the concentration of amicyanin on a log-scale. Amicyanin is injected in a 9.9 mM pyrophosphate buffer at pH 8.9.

Although the migration model used here adequately describes most of the experiments, there seems to be a limitation at high values of $K_{ass}$ as illustrated by the pyrophosphate buffer system containing 20 % of glycerol. Although the raw data do not show any clear deviation
from those of the other measurements, it was impossible to find a converging solution when the concentration of amicyanin was set to 2 μM. Moreover, when the concentration of amicyanin was included in the fitting procedure, the linear fit of the data points is not satisfactory and a clear curvature remains as is shown in Fig. 5.5. Furthermore, a large difference between the association constants obtained from the experiments and from the simulations occurs. This difference, together with the non-linearity of the data points indicate that in these cases the evaluation of the association constant may not be reliable.

As mentioned in section 5.3.1, in case of high affinity, the maximum concentration of MADH in the background electrolyte is limited hence an unfavourable high concentration ratio of amicyanin to MADH occurs and the actual concentration of amicyanin which determines the observed mobility is clearly lower compared to the injected concentration. It is obvious that in a (gaussian) peak the concentration of amicyanin decreases from a maximum value at the apex to zero at both flanks of the peak. Consequently, the ratio of MADH to amicyanin, and thus $X_{MA}$ and $X_A$ vary over the peak and the overall electrophoretic mobility of amicyanin is not constant but changes according to Eq. 5.4. It is
suggested that the fitted concentration of amicyanin corresponds to a concentration ratio of amicyanin to MADH so that the fractions $X_A$ and $X_{MA}$ are such that the calculated mobilities correspond to the measured ones, hence the experimental results can be fitted quite well.

If the association constant increases to very high values as in the pyrophosphate - glycerol buffer, the experimental data cannot be fitted well, probably due to the fact that the differences in the concentrations of MADH and amicyanin are too large. It is suggested here that in case of very high affinity, the use of a single (average) amicyanin concentration is no longer adequate to describe a set of experiments obtained at different concentrations of MADH. The $r^2$ values and the association constants obtained from the experiments and the simulations, summarised in Table 5-I, indicate that reliable results for $K_{ass}$ can be obtained up to about $2 \times 10^6$ M$^{-1}$.

An additional confirmation of the above presented migration principle can be derived from the behaviour of the amicyanin peak shape. The convey of amicyanin molecules from the bulk to the front of the peak and the following inevitable reduction of the migration velocity results in a relatively sharp front of the peak. However, at the backside of the peak, no such regulating mechanism occurs and amicyanin molecules which stay behind due to complex formation, will move into a buffer zone containing even more free MADH and will migrate even slower. Consequently, significant peak tailing, proportional to the actual mobility difference between the free and complexed form of amicyanin, should occur. The symmetry factor $\alpha$ as defined in Eq. 5.13 is used as a measure for the symmetry of the peak:

$$\alpha = \frac{(t_{end} - t_m) - (t_m - t_{start})}{t_m}$$  \hspace{1cm} (5.13)

where $t_{start}$, $t_{end}$ and $t_m$ are the times of the peak start, end and apex respectively. The value of $\alpha$ becomes zero for a perfectly symmetrical peak, a positive $\alpha$-value indicates tailing of the peak while a fronting peak is characterised by a negative $\alpha$-value.

In Table 5-II, the mean $\alpha$-values for each set of experiments are shown. Note that the experimentally observed tailing of the amicyanin peak is not affected by the actual concentration of MADH in the background electrolyte, although a more pronounced spreading of the data is observed in the pyrophosphate systems, as reflected in the standard
deviation. There is a strong correlation between the mobility difference and $\alpha$, characterised by a correlation coefficient of -0.980, which confirms the mechanism proposed above.

**Table 5-II** The mobility difference between the complexed and free form of amicyanin and the corresponding average symmetry factor with its standard deviation of the amicyanin peak for all different buffer systems.

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>$\mu_{MA} - \mu_A$ ($10^{-9}$ m$^2$ V$^{-1}$ s$^{-1}$)</th>
<th>$\alpha$ (standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>9.9 mM phosphate buffer, pH 7.4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I = 26$ mM, 0% glycerol</td>
<td>-10.9</td>
<td>0.036 (0.008)</td>
</tr>
<tr>
<td>$I = 35$ mM, 0% glycerol</td>
<td>-9.6</td>
<td>0.029 (0.014)</td>
</tr>
<tr>
<td>$I = 55$ mM, 0% glycerol</td>
<td>-5.67</td>
<td>0.015 (0.012)</td>
</tr>
<tr>
<td>$I = 26$ mM, 10% glycerol</td>
<td>-6.28</td>
<td>0.015 (0.009)</td>
</tr>
<tr>
<td>$I = 26$ mM, 20% glycerol</td>
<td>-3.25</td>
<td>0.009 (0.011)</td>
</tr>
<tr>
<td><strong>9.9 mM pyrophosphate buffer, pH 8.9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I = 100$ mM, 0% glycerol</td>
<td>-0.68</td>
<td>0.001 (0.009)</td>
</tr>
<tr>
<td>$I = 130$ mM, 0% glycerol</td>
<td>-0.83</td>
<td>-0.001 (0.006)</td>
</tr>
<tr>
<td>$I = 100$ mM, 20% glycerol</td>
<td>-0.51</td>
<td>-0.005 (0.013)</td>
</tr>
</tbody>
</table>

**5.4.3 The effect of the pH on the association constant**

The association constants in Table 5-I reveal that an increase of the pH from 7.4 to 8.9 results in a 14 fold increase of the association constant from $1.1 \times 10^4$ to $1.6 \times 10^6$ M$^{-1}$. This is a slightly higher value then the 10 fold increase reported by Gorren and Duine [26]. Interpretation of these data should however be done by keeping in mind that both a buffer change from phosphate to pyrophosphate, as well as an increase of the ionic strength from 55 to 100 mM is implicitly introduced.

Nevertheless, our CE experiments support a substantial increase in $K_{obs}$ with increasing pH. The exact reason is not clear, although the (de)protonation of one or two ionisable groups
was suggested by Gorren and Duine [26]. However, if there is a significant change in the overall charge of the proteins, this should be reflected in the values for $\mu_A$ and $\mu_{MA}$. From Table 5-I, it follows that the electrophoretic mobility of amicyanin in the free form becomes more negative at higher pH values, which can be explained by deprotonation. Note that the simultaneous increase of the ionic strength probably reduces the effect of an additional negative charge on the solute due to shielding so that the observed mobility difference reduces as well. The pH driven changes of the electrophoretic mobility of the complex are more difficult to evaluate, mainly due to the ionic strength effects in the phosphate buffer. Nevertheless, the electrophoretic mobility seems to be hardly affected or tends to become less negative in the pyrophosphate buffer compared to the phosphate buffer having an ionic strength of 55 and 35 mM respectively. Based on these electrophoretic data it is improbable, although not impossible, that deprotonation of MADH occurs over the observed pH range. Furthermore, these mobility data confirm the assumption made in section 5.3.1, that the contribution of amicyanin to the electrophoretic mobility of the complex is relatively small.

5.4.4 The effect of the ionic strength on the association constant

An increase of the ionic strength from 26 to 35 mM in the phosphate buffer results in a two fold increase of the association constant. If the ionic strength is raised to 55 mM, $K_{ass}$ does not further increase. This seems to be in contrast with literature data where a decrease of the MADH-amicyanin complex stability at increasing ionic strength is observed. However, it should be noted that the ionic strength range studied here is much smaller than the ranges typically studied in the literature.

An increase of the association constant at increasing but relatively low ionic strength can be explained by a decrease of the electrostatic repulsion between MADH and amicyanin, caused by shielding. However, the association constant between 35 and 55 mM is relatively constant and the behaviour at higher ionic strength values is difficult to predict. A system for which an increase of the association constant is followed by a decrease at increasing ionic strength is for instance the cytochrome $c_L$ - methanol dehydrogenase system [42]. An inverse relation is however found for ferredoxin-ferredoxin:NADP$^+$ reductase [43]. The former was explained by conformation and/or hydration changes at low salt concentration and a decrease of electrostatic attraction at higher ionic strength. The latter was explained by the combination of ionic interaction at low ionic strength and hydrophobic interaction at higher ionic strength on the complex formation. Both suggestions proposed above, may be applicable to the
MADH-amicyanin complex formation. It is however also possible that the accessibility of the negative charges, involved with the electrostatic repulsion, for ions in solution is larger compared to the accessibility of oppositely charged groups, concerned with the attractive forces, which also results in a biphasic dependence of $K_{as}$ on the ionic strength.

Unfortunately, due to the relatively high current passing through the capillary at an ionic strength of 55 mM ($i = 46 \, \mu A$), it is expected that at higher values of the ionic strength, the Joule heat is no longer efficiently dissipated which affects the association and migration behaviour. Therefore, buffers with an ionic strength above 55 mM were not included in this study.

Complex formation of the analogous aromatic amine dehydrogenase (AADH) - azurin system is also favoured by an increase of the ionic strength [44] and the charge interaction term in a phosphate buffer at pH 7.5 is -2.5. The association constant at zero ionic strength and the $z_Nz_M$-term for the MADH-amicyanin system, estimated according to Eq. 5.9 are summarised in Table 5-III. Due to the limited number of experimental data points and the large error in the fitting procedure, these data must be interpreted with care. Nevertheless, the discrepancy of the charge interaction term for both systems, together with a reduced cross-reactivity [44], illustrates that in spite of the analogy, some major differences in the complex formation must be present. Our data confirm the hypothesis postulated in ref. 44 that a possible cause of this difference may be attributed to the ability of the MADH-amicyanin system to form one or two salt bridges. The AADH-azurin system lacks the potential to form salt bridges and consequently the complex formation shows a different and reduced ionic strength dependence.

<table>
<thead>
<tr>
<th>Table 5-III</th>
<th>The values for the association constant at zero ionic strength and the charge interaction term determined in the phosphate and pyrophosphate buffer.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{as}$ ($L$)</td>
<td>$z_Nz_M$</td>
</tr>
<tr>
<td>$(10^3 , \text{M}^{-1})$</td>
<td></td>
</tr>
<tr>
<td>phosphate buffer</td>
<td>14.4</td>
</tr>
<tr>
<td>pyrophosphate buffer</td>
<td>643</td>
</tr>
</tbody>
</table>
From Table 5-III it follows that the ionic strength dependence is reduced by a factor of two, while the \( K_{\text{ass}} \) value is increased 45 fold, changing from the phosphate to the pyrophosphate buffer. The latter is mainly due to the increase of the pH. The former may be related to the difference in the ionic strength range studied in the two buffer systems. Other possible causes can be related to a deprotonation of one or both solutes at the high pH as already mentioned in section 5.4.3. Furthermore, (specific) interaction between the solutes and the buffer molecules, although not probable, cannot be excluded. Based on the experimental results presented here, however, no unambiguous choice between the various options is possible yet.

### 5.4.5 The effect of the osmotic pressure on \( K_{\text{ass}} \)

The data in Table 5-I reveal a dramatic increase of the association constant at higher osmotic pressure values, characterised by a linear increase of \( \ln K_{\text{ass}} \) as a function of the osmotic pressure.

A linear least-square fit of \( \ln K_{\text{ass}} \) as a function of the osmotic pressure for the data obtained in the phosphate buffer results in a straight line with a \( r^2 \) value of 0.98. The \( \Delta V \) values, calculated from the slopes are -768 and -790 ml/mole for the phosphate and the pyrophosphate buffer respectively. Slightly different results may be obtained if the compressibility of the system \( \beta \) in Eq. 5.10 is not neglected. However, the \( r^2 \) value indicates that this contribution will be very small and that the simplification in Eq. 5.10 is allowed for the system investigated here. The almost identical slopes indicate that the effect of the osmotic pressure on the association behaviour is hardly affected by the pH and ionic strength variations when changing from the phosphate to the pyrophosphate buffer.

The values for \( \Delta V \) are relatively high compared to literature data: e.g. the cytochrome b5 -cytochrome c and the cytochrome c oxidase - cytochrome c systems are characterised by a \( \Delta V \) of -50 and 200 ml/mole respectively. Because the formation of the complex is promoted by the addition of glycerol and the changes in \( K_{\text{ass}} \) values are much more pronounced compared to the ionic strength changes, it is concluded that the interaction of MADH and amicyanin is primarily driven by hydrophobic forces.

It is obvious that the value of \( \Delta V \) obtained here is much too large to be caused only by structural changes of specific residues on both proteins. If it is assumed that no specific
interaction with the glycerol molecules appear and the volume changes are solely attributed to the displacement of water molecules, the number of water molecules involved is close to 40. The crystal structure reveals that approximately 715 and 770 Å² of accessible surface area is lost upon complex formation [24] for the amicyanin and MADH molecule respectively. The number of water molecules in contact with a residue with a surface area of about 750 Å² is then estimated to be 60 (the volume of one water molecule is set to be 30 Å³), which is relatively close to the number of water molecules estimated here. Besides, conformational changes of both proteins may affect the number of water molecules involved but is of course not included here. These data are in accordance with the statement of Garner and Rau [39] that only in case of specific interaction upon complex formation, water molecules will be removed from the interfacial surfaces.

5.5 Conclusions

It is shown that capillary zone electrophoresis can be successfully used to study the complex formation of amicyanin and MADH. The experimental set-up is relatively simple, only small amounts of buffer and sample are required and the choice of the experimental parameters is quite flexible. The possible range of the ionic strength is, however, severely limited by an unacceptable increase of the current, while the glycerol concentration is somewhat limited by an increase of the migration times. The approach presented here to determine $K_{ass}$ values includes the concentration of amicyanin as a fit parameter, which enables a reliable estimation of binding constants up to $2 \times 10^6$ M⁻¹ for the MADH-amicyanin system, even if the ratio of the concentration of MADH to amicyanin is then relatively small. An improvement of the fit showed, may result from the incorporation of the time dependent concentration profiles of both amicyanin and MADH over the capillary, which seems at present too demanding in terms of computation time.

It is concluded that both electrostatic and hydrophobic interactions play a role in the complex formation of amicyanin and MADH. Since no stationary phase or stabilising medium is present, it is expected that CZE does not favour one of both interactions and therefore, it follows that the hydrophobic interaction is predominant. CE confirms the increase of $K_{ass}$ at increasing pH. Although the mobility of free amicyanin becomes more negative in the buffer at higher pH values, the suggestion that the enhanced complex formation is related to a (de)protonation of the proteins cannot be unmistakably verified.
5.6 References


[18] V.L. Davidson in V.L. Davidson (Editor), *Principles and applications of quinoproteins*, Marcel Dekker, New Yor, 1993, pp 73-95.

The interaction of MADH with amicyanin


Chapter 6
Optimisation of Selectivity in Capillary Electrophoresis with Emphasis on Micellar Electrokinetic Capillary Chromatography: a review

Abstract

Separations in capillary electrophoresis and especially in micellar electrokinetic capillary chromatography are characterised by a large number of parameters and therefore difficult to optimise. This paper reviews recent approaches suitable for optimisation of selectivity in capillary electrophoresis. Typical features of optimisation strategies applicable to capillary electrophoresis and micellar electrokinetic capillary chromatography in particular are discussed. A distinction is made between statistical approaches using fitting procedures of polynomial equations, and practical optimisation schemes based on physicochemical models describing the migration behaviour. Besides speeding up the search in finding satisfactory separation conditions, additional knowledge may be obtained about the migration and separation mechanism(s) when a systematic approach is applied. However, due to the complexity and the number of available optimisation schemes, these approaches should not be used as black-box systems. The analyst has a crucial role in optimising a separation.

6.1 Introduction

Capillary electrophoresis (CE) is a rapidly expanding analytical technique that can be used to separate many different compounds. The separation of biological molecules such as peptides, proteins [1-6] and nucleic acids [7, 8] as well as inorganic ions [9, 10] and pollutants [11, 12] has been reported in the literature. In spite of the fact that CE is characterised by a high efficiency, the desired separation is often only obtained after considerable experimentation. Although the adjustment of system parameters like sample characteristics to introduce stacking [13, 14], the use of electrokinetic injection with its discriminating capabilities [15, 16] or the selection of an appropriate detection wavelength for detection or spectral recognition [17, 18], should not be overlooked, system optimisation will not be dealt with. The purpose of the present review is to give an overview of the guidelines and strategies that are currently available to achieve an adequate selectivity with the minimum number of experiments.

Selectivity in capillary zone electrophoresis (CZE) is strongly influenced by the pH of the buffer. In addition, the type and concentration of buffer and the presence of an organic modifier can affect both selectivity and efficiency. In micellar electrokinetic capillary chromatography (MECC), a special mode of CE [19], two additional parameters have a remarkable influence on the separation i.e. the type and concentration of surfactant. As a result numerous factors will affect MECC experiments.

The temperature affects different physicochemical parameters like viscosity, pKₐ and pH values, absolute mobilities and the critical micellar concentration (CMC) of various surfactants and thus the separation. As a result, an efficient temperature control is inevitable in method development and although temperature changes can be used to improve the selectivity [20, 21], we will focus on methods in which the temperature is assumed to be constant.

Clearly, an appropriate optimisation strategy should be used to find good separation conditions in the shortest time and/or after only a few experiments. Basically, all optimisation strategies consist of three distinct steps: the choice of the appropriate parameter(s) and the parameter space, a model or algorithm to describe the migration behaviour and a criterion to evaluate the resulting electropherogram. For an overview the reader is referred to Ref. [22].

102
The choice of the parameter(s) to be optimised is mainly influenced by the analytical technique itself and therefore, this choice often seems quite obvious. In contrast, the choice of the limits of each parameter which usually defines the parameter space is more difficult to rationalise. Although generally the absolute minimum and maximum value of a parameter is physically defined, these limits seldom will be the actual limits used in the optimisation procedure. For example, optimisation of the pH as the parameter having much influence on selectivity in CZE is a rather obvious choice. However, the actual pH range under investigation is difficult to define without relevant knowledge about the sample to separate. In this respect dissociation constants as well as pH stability data are essential in CZE. Unfortunately, in many cases relevant data concerning the sample under investigation are not available so that the experience of the analyst becomes important.

After the choice of the appropriate parameters and their limiting values, experiments must be performed to explore the migration of the solutes as a function of the parameter(s). A description of the behaviour of the analytical system in the entire parameter space is then generally obtained by interpolation using an algorithm that relates the migration of the solutes to the parameter(s). Differences can be found among the various models used and the theoretical basis underlying the model can be quite different. Approaches based on physicochemical properties like dissociation constants, mobility data and diffusion coefficients of the solutes and buffer properties like ionic strength and pH as well as models based on strictly mathematical equations, treated in a statistical way, are reported. It is clear that there is a correlation between the accuracy of the model and the time and effort it takes to satisfactorily predict the migration behaviour of different solutes and hence to find good separation conditions.

The final step in the optimisation strategy is the evaluation of the migration behaviour of the solutes predicted in the parameter space in terms of the quality of the separation. The goal of an optimisation may vary considerably from one case to another e.g. the separation of two enantiomers requires a different criterion than a peptide map in which many different unknown solutes must be detected. Therefore, this goal must be translated into appropriate objective mathematical functions defining the criterion. The criterion relates the quality of an observed electropherogram to a desired one and this choice is critical and affects both the optimisation procedure to follow and the results obtained. Furthermore, it is not necessary to search for the global optimum in the parameter space but it satisfies to find experimental conditions resulting in sufficient separation. Many different criteria are proposed in the
literature and some of them which are used in the optimisation of chromatographic experiments are also useful in CE and can be found in Ref. [22]. Recently, Hayashi et al. [23] studied the precision and throughput in MECC and concluded that these statistical parameters are suitable as criteria in MECC. The criterion should always be carefully evaluated in relation to the ultimate goal of the analyst.

Although for high-performance liquid chromatography (HPLC) several optimisation schemes have been published [22], these approaches often require substantial modification before they can be used to optimise a CE separation. Recently, some books on capillary electrophoresis have paid some attention to method development and optimisation strategies in CE [24-26]. In the first part of this review the possibility to apply statistical approaches is discussed while in the second part some feasible optimisation schemes are commented.

6.2 Statistical approaches in the optimisation of CE

The optimisation of CE and especially MECC experiments is complex due to the number of parameters affecting the separation. Further complications can arise from the mutual interaction of the parameters. Examples illustrating this phenomenon have been reported both for micellar liquid chromatography [27] and MECC [24]. This explains why the development of physicochemical models describing the separation mechanisms is not an easy task. Often the behaviour of the system is approximated by simple mathematical equations for which only a minimum amount of knowledge is required.

An example of such an approach is the use of a simplex algorithm. The principle of a simplex method is covered extensively by the literature [22, 28]. In general, the simultaneous optimisation of \( n \) parameters results from a fitting procedure of the response (or criterion) \( y \) with a first-order model to the parameters \( x \) as shown in Eq. 6.1.

\[
y = b_0 + b_1 x_1 + b_2 x_2 + ... + b_n x_n
\]

The optimum is approached in a sequential way constructing geometrical figures (called simplex) in the parameter space using previous experimental results. These sequences are repeated until the separation is satisfactory or until no further improvement is observed.
The advantage of this approach is that it is applicable to any type and number of parameters and that knowledge about the separation mechanism is not required to calculate the response or define the parameter settings for the next measurement. However, an important drawback of the simplex method is the large number of experiments that is generally needed to reach the optimum. This is clearly illustrated by Castagnola et al. [29] who optimised pH, concentration of organic modifier and concentration of surfactant in the separation of derivatised amino acids. Although the variable-sized weighted simplex optimisation design was used to speed up the procedure, still 10 to 15 steps were required to reach separation conditions that are satisfactory in terms of the mean resolution of all the relevant peak pairs. In addition, the choice of the starting conditions is very critical since different starting conditions can lead to different solutions. Finally, it should be noted that by applying simplex methods much information is lost since only the information of the last n+1 experiments is retained. An adequate description of the response surface is not obtained in this way and this is a serious disadvantage when the response surface is complex.

Another multi-parameter optimisation procedure, called the overlapping resolution mapping scheme (ORM), was introduced for CE by Li and co-workers [30-35]. After defining the parameters and the accompanying parameter space, the initial experiments are performed, and the response (resolution $R_s$) of each peak pair is used to determine the coefficients of a polynomial equation that not only accounts for the effect of each parameter but also includes mathematical interaction effects between the parameters, expressed as $x_i x_j$-terms in Eq. 6.2:

$$R_s = b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 + b_{123} x_1 x_2 x_3$$ (6.2)

Once the coefficients of the polynomial equations are known, the resolution for each peak pair over the whole parameter space can be predicted and visualised as a resolution map. The optimum separation conditions can then be deduced from an overlay of all the resolution maps. Applications of this method include the separation of solutes such as sulphonamides [30, 31], flavonoids [31, 32], derivatised amino acids [33, 34], drug substances [34] and porphyrins [35].

It should be realised that in this strategy peaks are not identified, hence the actual migration behaviour of the solutes is not followed. Significant errors may result from changes in the relative peak positions. This problem was discerned by Glaich et al. [36] who initially developed the ORM approach for HPLC. Due to such effects, the response surface may be
complex and discontinuous and overlapping of the resolution maps can then be expected to yield unreliable results. For this reason peak tracking should be considered as a valuable asset and this task is much facilitated by using advanced detection techniques such as diode-array spectrophotometry or mass spectrometry.

A third type of experimental design that has been shown to be useful for optimisation purposes is the Placket-Burman statistical design which is a fractional factorial design that can be used if the number of parameters is one less than a multiple of four. Dummies should be added to meet the required number of parameters. A dummy can be used to estimate the variability of the system and the significance of the effects found for the true physical parameters. Statistical treatment of the data can often be used for the screening of many parameters and the models used to describe the results of the experiments are typically first-order in each parameter. The most important parameters found with this screening procedure can then be studied in a full multi-level factorial design.

Vindevogel and Sandra [37] used this approach to obtain a satisfactory separation of a mixture of testosterone esters. Seven parameters are evaluated by means of eight initial experiments in which the effect of pH and the concentrations of buffer, acetonitrile, sodium dodecyl sulphate (SDS) and sodium heptyl sulphate on the analysis time, the noise, the efficiency and the resolution are studied. Interpretation of the results should, however, be done very careful since the observed changes in migration behaviour may be due to multiple interactions.

An important advantage of these factorial-design type of procedures is that they are applicable under many different experimental circumstances and that there is no restriction concerning the type of solutes and parameters in the optimisation. However, since in this way no general rules are obtained concerning migration mechanisms, the results are restricted to the separation under investigation. A change in the separation conditions requires that the whole procedure has to be followed over again.
6.3 Optimisation procedures based on physicochemical models

When relevant knowledge of the mechanism of a given type of separation is available, optimisation protocols can be developed that make use of these separation principles, expressed by an appropriate algorithm.

In section 6.3.1 approaches in which fundamental equations describing the migration behaviour and the resolution will be treated. These general equations are based on a theoretical description of the separation process. The parameters describing the migration are then evaluated and adjusted to reach a maximum value of the resolution. In such a way, global guidelines, pointing to the desired migration behaviour, can be formulated. However, the translation of these guidelines into practical separation conditions is often not obvious and therefore the practical applicability is limited.

In section 6.3.2 specific physicochemical models are shown describing the migration behaviour of particular solutes as a function of one or more parameters. The experimental separation conditions can then be adjusted in such a way that the criterion reaches satisfying values. Clearly, these procedures are suitable to solve practical optimisation problems.

6.3.1 Global approaches in the optimisation of CE

In MECC uncharged solutes are separated according to differences in micellar solubility. In analogy with HPLC, the equations describing the capacity factor $k$ and the resolution $R$, in MECC are based on a classical chromatographic description as shown in Eqs. 6.3 and 6.4 [38].

$$k = \frac{t_m - t_{co}}{t_{co} \left(1 - \frac{t_m}{t_{inc}}\right)}$$

(6.3)
\[ R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_j}{1 + k_j} \cdot \frac{1 - \frac{t_{eo}}{t_{mc}}}{1 + \left( \frac{t_{eo}}{t_{mc}} \right) k_i} \quad (6.4) \]

Here, \( t_m \) is the migration time of the solute, \( t_{mc} \) is the migration time of a solute totally solubilised in the micelles (e.g. Sudan III), \( t_{eo} \) is the migration time of an uncharged solute that has no interaction at all with the micelles (e.g. methanol), \( \alpha \) is the selectivity and defined as the ratio of two capacity factors, \( N \) is the number of theoretical plates and subscripts i and j denote two (closely migrating) compounds. The definition of the capacity factor \( k \) is clearly analogous to the conventional chromatographic definition of the capacity factor as expressed in Eq. 6.5:

\[ k_{\text{chrom}} = \frac{t_m - t_0}{t_0} \quad (6.5) \]

where \( t_0 \) is the retention time of a non-retained solute and the additional term in the denominator in Eq. 6.3 accounts for the size of the migration window in MECC.

It is obvious that this limited migration range, expressed as the ratio of \( t_{mc} \) over \( t_{eo} \), is important with respect to the peak capacity and separation capabilities of a micellar system. This is illustrated in Fig. 6.1 showing three simulated electropherograms of two solutes having identical \( \alpha \)-values but different values of \( k \). The separation is superior at intermediate values of \( k \) (electropherogram B in Fig. 6.1). Low capacity factors result in relatively small micellar interactions and a lack of selectivity and high capacity factor values result in longer migration times as well as in bad separations because all the solutes are migrating close to \( t_{mc} \).

Extension of the migration window can be achieved by altering the electroosmotic and/or the micellar electrophoretic mobility [39, 40]. Recently, Ahuja et al.[41] demonstrated the use of a mixed pseudo-stationary micellar phase of SDS and Brij 35. The electroosmotic and micellar electrophoretic mobilities are matched by adjusting the ratio of the concentration of Brij 35 and SDS so that the micellar mobility equals the electroosmotic mobility but has the opposite sign. This results in a real stationary micellar phase and an infinite migration
Fig. 6.1 Simulated electropherograms illustrating the drawbacks of a limited elution range, typical for MECC. Both the migration window \((t_m/t_{eq} = 2)\) and the selectivity \((\alpha = 2)\) are held constant. (A) \(k_i = 0.1; k_j = 0.2\). (B) \(k_i = 1; k_j = 2\). (C) \(k_i = 10; k_j = 20\).
range is obtained, even at relatively high pH values close to 7 where electroosmotic velocities are significant.

The prediction of conditions for optimal separation of neutrals in MECC formulated by Foley [42] is based on the assumption that selectivity is mainly determined by the partitioning of the neutral solutes between the water and the micellar phase. Accordingly, the concentration of surfactant is the most important parameter to be optimised in that case.

The surfactant concentration \([M]\) is related to the capacity factor \(k\) as shown in Eq. 6.6:

\[
[M] = \frac{k + V \cdot CMC \cdot (k + P_{wm})}{V \cdot (k + P_{wm})} \approx \frac{k}{P_{wm} \cdot V} + CMC, \quad P_{wm} > k \tag{6.6}
\]

where \(P_{wm}\) is the partition coefficient of a given solute for the water and the micellar phase, \(V\) is the partial molar volume of the surfactant and CMC is the critical micellar concentration. Assuming that both \(N\) and \(\alpha\) are independent of \(k\), the optimum capacity factor \(k_{opt(R)}\) is derived from Eq. 6.4:

\[
k_{opt(R)} = \sqrt{\frac{t_{mc}}{t_{co}}} \tag{6.7}
\]

Substitution of \(k_{opt(R)}\) in Eqs. 6.6 and 6.4 allows the calculation of the optimal surfactant concentration (equation not shown) and the corresponding best resolution:

\[
R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{\sqrt{\frac{t_{mc}}{t_{co}} - \sqrt{\frac{t_{mc}}{t_{co}}}}}{2 + \sqrt{\frac{t_{mc}}{t_{co}} + \sqrt{\frac{t_{mc}}{t_{co}}}}} \tag{6.8}
\]

By adjusting the concentration of surfactant it is possible to optimise a separation, independent of the hydrophobicity of the solutes. The same can be done to optimise the resolution per unit time (equations not shown). It is obvious that both approaches do not predict the same optimal parameter settings.

The authors conclude that there is an optimal region for the capacity factor. In general, intermediate values of \(k\) are preferable. Low values of \(k\) will lead to a short analysis time.
but will suffer from bad resolution, except for samples that are very easy to separate. High
capacity factors result in excessive retention and a loss in resolution since all the solutes
migrate close to the $t_{mc}$. This phenomenon is not known in conventional column
chromatography.

Ghowsi et al. [43] have rewritten the equations based on chromatographic principles using
an electrophoretic approach in MECC for neutral solutes. The resolution is not only
expressed as a function of the capacity factor but also as a function of $\mu_{ep}^*$ which is the
average effective electrophoretic mobility of a neutral solute. $N$ is dependent on the capacity
factor and thus on the effective migration time of each solute separately and in addition the
efficiency is characterised by a Van Deemter-like behaviour of plate number versus voltage.

Using these equations Ghowsi et al. [43] performed a theoretical optimisation for three
modes of MECC differing in the net migration velocity of the micelles. Although the
approach and the accompanying equations are quite different compared to the optimisation
procedure of Foley [42], the results are similar: when the micelles move to the positive
electrode, resolution is maximal if the neutral solute is carried by the micelles to the same
extent as the solute is carried by the electroosmotic flow in the opposite direction.
Obviously, the analysis time will then be infinite. When the micelles are stationary or
moving to the negative electrode, the optimum capacity factor can be calculated by deriving
the appropriate equations and it can be shown that maximum resolution will be reached for
$k$ values close to 5. In a more qualitative way Terabe et al. [38] came to a similar conclusion
starting with equations derived for conventional chromatography.

Based on these global guidelines together with many experimental observations, Terabe [44]
has formulated an introductory guide for optimisation in MECC which is summarised by the
flow chart in Fig. 6.2. Here, theoretical knowledge is translated in experimental CE
conditions. The first experiment is performed with standard MECC conditions and based on
this result, the analyst is advised to change the experimental settings concerning the type and
concentration of surfactant or other buffer additives so that optimal capacity factor values
are approached. The applicability is demonstrated by Bevan et al. [45] who optimised the
resolution for mixtures of synthetic oligonucleotides. Although small deviations from theory
were observed, high concentrations of urea at various SDS-concentrations, organic modifiers
and the use of bile salts and cationic micelles could be used to control the capacity factors
and increase the chances to find good separation conditions.
Fig. 6.2 Introductory guide to the method development of a MECC experiment formulated by Terabe [44].
6.3.2 Practical approaches in the optimisation of CE

It is obvious that for charged solutes the pH will be the first parameter of choice. Kennedler and Friedl [46] have derived a relation between the resolution of monovalent ions in CZE and the pH of the buffer. Both selectivity and efficiency depend on the charge number and thus for weak electrolytes on the pH as expressed by Eq. 6.9 for two solutes i and j:

\[
R_{ij} = \left( \frac{\mu_{act,i}}{\mu_{act,j}} - 1 \right) + \left( \frac{\mu_{act,i}}{\mu_{act,j}} \Delta_j - \Delta_i \right) \frac{e U}{32 k_B T} (1 - \Delta_j)^{3/2} + \frac{\mu_{act,i}}{\mu_{act,j}} (1 - \Delta_j)^{3/2}
\]

(6.9)

where \( \mu_{act} \) is the actual mobility, \( \Delta_i = 10^{pK_a - \text{pH}} \), \( \Delta_j = 10^{pK_a - \text{pH}} \), \( e \) is the electric charge, \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature and \( U \) is the applied voltage. This equation was also extended to multivalent ions [47]. The resolution as a function of pH of the buffer and applied voltage was calculated for different solutes and optimal settings can easily be deduced. As expected, the predicted resolution is a complex function of the pH in the case of multivalent ions.

Use of these relations requires accurate knowledge about the acid-base properties of the solutes and in the study cited above, the relevant \( pK_a \) values were taken from the literature. In addition, the mobilities of the solutes have to be known very precisely and since not much literature data is available, these values should be determined experimentally by measuring the migration time of a solute as a function of the pH. Obviously, this requires a large number of experiments.

It is recognised by the authors that the model proposed is limited by the lack of consistent data on the analyte properties [48]. Nevertheless, both for mono- and multivalent ions an optimal pH was predicted, resulting in a baseline separation of the entire series of substituted benzoic acids and phenols investigated. It was found that a change of the pH as small as 0.04 may have a dramatic influence on the resolution and in spite of uncertainties in both the mobilities and the dissociation constants, this effect is predicted by this approach.

Jacquier et al. [49] also optimise the pH for the separation of monovalent ions. With only one experiment when the \( pK_a \) value is known and two experiments when the dissociation
constant is unknown, the migration time of a solute over the pH range can be predicted. In addition, an estimate of the molecular diffusion coefficient and hence the peak width is obtained. The electroosmotic flow is modelled in a very simple way using the dissociation constant of the silanol groups.

Although in this approach the number of experiments is very limited and also some uncertainty remains concerning the electroosmotic mobility and the dissociation constants, the predicted behaviour of the solutes is in reasonable agreement with the experimental results. Using this strategy, the separation of three different solute mixtures: three chlorophenol geometric isomers, three nitrophenol geometric isomers and three chloroaniline geometric isomers was rapidly achieved.

The mobility of several monovalent substituted phenols is predicted by Smith and Khaledi [50] modelling the electrophoretic mobility $\mu_{ep}$ as a function of the pH of the buffer and the acid dissociation constant $K_a$:

$$\mu_{ep} = \mu_{A^-} \cdot \frac{K_a}{[H^+] (1 + \frac{K_a}{[H^+]})}$$ (6.10)

where $\mu_{A^-}$ is the electrophoretic mobility of the anionic form of the acid. The parameters $\mu_{A^-}$ and $K_a$ can be determined by fitting Eq. 6.10 to the measured $\mu_{ep}$ at various pH values.

Although this approach is analogous to that of Friedl and Kenndler [47], there are also some important differences. The $pK_a$ values obtained by fitting Eq. 6.10 to the experimental data are apparent dissociation constants depending on the actual CE conditions and they are not necessarily close to literature data. (Titration data illustrate that for amino acids and small peptides apparent dissociation constants may differ significantly [51], illustrating the effect of the surfactant on the dissociation behaviour of these solutes.) As few as four measurements may be sufficient to obtain a reliable fit. Here, the choice of the pH range to be scanned is important and is more easy when physicochemical data of the solutes are available. Limitations on the actual prediction of migration times is discerned so that the migration order prediction for closely migrating peaks may fail, especially for solutes having almost identical $pK_a$ or mobility values.
The well-known observed linear relation between the capacity factor and the concentration of surfactant in MECC was used by Pyell and Bütehorn [52] to increase resolution. Two experiments at different SDS concentrations enable the calculation of $k$ and thus migration times of all the solutes at different concentrations of SDS. This one-parameter optimisation procedure resulted in baseline separation of a mixture of seven methyl nitroanilines. Furthermore, the optimisation of the concentration of modifiers like urea and glucose was performed in a similar way, using appropriate logarithmic relations between the migration and the concentration of the modifier.

The description of the behaviour of ionisable solutes in a micellar system is complicated due to the combination of the electrophoretic and chromatographic migration mechanisms. Khaledi and co-workers extended the procedure described earlier to the separation of both negatively [53-55] and positively [55, 56] charged solutes in a micellar system. Here, the two important parameters are pH and the micellar concentration. Assuming that the net migration of an ionisable solute is the weighted average of the migration parameter of the solute in the associated (a) or non-associated (b) forms both in the aqueous and micellar phase, the net mobility of an ionisable solute can be expressed as:

$$
\mu_{ep} = X_{aq,A} \cdot \mu_{aq,A} + X_{aq,B} \cdot \mu_{aq,B} + (X_{mc,A} + X_{mc,B}) \cdot \mu_{mc}
$$  \hspace{1cm} (6.11)

where $\mu_{aq}$ is the mobility of the solute in the aqueous phase in the associated and non-associated forms, respectively (subscripts a and b), $\mu_{mc}$ is the mobility of the micelle and the $X$ values are the mole fractions of the solute in the micellar and aqueous phase (subscripts mc and aq) in the associated and non-associated forms. Ion-pair formation between the charged solute and the oppositely charged surfactant constitutes an additional mechanism affecting migration, which is also considered. Note that this ion-pair complex and the uncharged solute molecules in the aqueous phase are assumed to migrate with the electroosmotic velocity and that $\mu_{eo}$ is not included in Eq. 6.11 since it is not important in the estimation of the electrophoretic mobility. Rewriting Eq. 6.11 results in a general expression which relates the mobility of a solute to all possible equilibrium constants, acid-base dissociation constants, pH and mobilities of the micellar phase and the solute under investigation (equation not shown). Four situations can be distinguished: an acid or basic solute that migrates in a micellar system containing positively or negatively charged micelles. For each case, the general mobility expression is then rewritten and simplified e.g. ion-pair formation is only considered for oppositely charged solute and surfactant molecules.
As an example, the relevant interactions between a weak base and a negatively charged surfactant are schematically illustrated in Fig. 6.3. Consequently, the relevant apparent parameters are estimated using five experiments at different pH and SDS concentration settings. Subsequently, the mobility of each solute can be predicted over the entire pH and SDS range. The equations were experimentally verified and the results are briefly summarised in Table 6-I. Note that fine-tuning of the separation of the aromatic amines was achieved by adding SDS and acetonitrile and this is done independent of the proposed optimisation strategy and this means that insight in the separation mechanisms is required.

Fig. 6.3 Relevant equilibria of a cationic solute (BH⁺ and B) in a micellar system containing negatively charged micelles. From Ref. [55].
Table 6-I Predicting capabilities of migration times of charged solutes in MECC using the phenomenological approach developed by the group of Khaledi.

<table>
<thead>
<tr>
<th>solute - buffer system</th>
<th>predicting capabilities</th>
<th>remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>acidic solutes - anionic micelle</td>
<td>++</td>
<td>/</td>
</tr>
</tbody>
</table>
| basic solutes - anionic micelle | + | * ion-pair formation is assumed to be very important and free BH⁻ is not present  
  * the separation is further improved increasing the SDS-concentration (improve peak shape) and add 10% acetonitrile (extend the migration window) |

6.4 Conclusions

The use of a systematic optimisation strategy in the development of CE applications is highly recommended and should be preferred to trial and error. In this way satisfactory separation conditions are established in the shortest time and/or with only a few experiments. Furthermore, detailed information on the separation mechanisms may be obtained. This will be of great use in the development of different applications by the analyst and will facilitate the improvement of existing optimisation schemes and the introduction of new and better strategies in method development.

At present there is a wide choice of optimisation procedures. For some of these strategies only a minimal knowledge of separation principles is needed, while other approaches, based on the assumption of a particular separation mechanism require more specific information about the solutes to separate. The choice of a particular strategy depends on the goal of the analyst and the feasibility of such an approach.

An important aspect of optimisation is the proper choice of the parameter(s) and the parameter space. In some cases the significance of this aspect seems to be underestimated but, unfortunately, general rules do not exist. The choice of the type of parameter may seem obvious in most cases but the choice of the parameter space is more complicated, more difficult to justify and often influenced by the analyst’s experience. It should be realised that
badly chosen start conditions may drastically reduce the chances to find useful separation conditions. Due to the high complexity and the linking of the parameters, a multi-parameter approach in which several parameters are optimised simultaneously in combination with a peak identification procedure should be favoured.

More knowledge of CE separations makes predictions of the migration behaviour more accurate but a good balance should be found between the required knowledge of the analytical system and the effort required to obtain it. In some cases this knowledge is already included in the optimisation approach by the designer so it does not need to be provided by the analyst. However, the lack of physicochemical data often hampers such optimisation protocols.

None of the strategies discussed here can be used as a black-box or a stand-alone system. The analyst himself is an important factor and by choosing the appropriate optimisation strategy he has a major influence on the outcome of the optimisation procedure. Adequate interpretation of the measurements can be of decisive importance. Therefore it remains crucial that the strategy or the algorithm as well as the separation technique is known in detail by the analyst.

6.5 References

Chapter 7

Optimisation of selectivity in capillary zone electrophoresis and micellar electrokinetic capillary chromatography using the iterative regression strategy

Abstract

An iterative regression design has been used in the optimisation of selectivity in capillary zone electrophoresis and micellar electrokinetic capillary chromatography. This strategy, initially developed for HPLC, is modified in respect of the choice of the parameter(s) and the parameter space and the description of the migration behaviour of (charged) solutes in capillary electrophoresis. The six test solutes could not be fully resolved by adjusting the pH in a free zone system but the simultaneous two-parameter optimisation of the pH and the concentration of surfactant resulted in baseline separation of all the solutes. Further improvement may be obtained when distorted peak shapes are also considered at the level of the parameter choice and the criterion.

7.1 Introduction

The utility of capillary electrophoresis (CE) has been demonstrated in many different areas of separation science and its significance is still increasing [1-10]. In addition, special modes of CE such as micellar electrokinetic capillary chromatography (MECC) and capillary gel electrophoresis have considerably broadened the application range of CE [11-13].

Selectivity and efficiency in capillary zone electrophoresis (CZE) are mainly determined by the properties of the buffer (e.g. pH, concentration and type) and the counter ion [14-17]. Organic modifiers and temperature also affect the separation [18-21] and in MECC the type and concentration of surfactant are two additional parameters which must be considered when searching for good separation conditions [22-24].

An important advantage of method development for CE compared to HPLC is the flexibility to use buffers with different properties so that, in general, long equilibration or regeneration times are not necessary. Nevertheless, because of the numerous factors affecting CZE and especially MECC, the use of a structured optimisation approach is recommended, in particular when mutual interactions of the parameters further increase the complexity [25].

Several studies on method development and optimisation strategies are presented in the literature [14, 25-36] and for an overview, the reader is referred to Ref. [37]. Here we present a multi-parameter optimisation scheme based on the iterative regression strategy. This approach is characterised by a wide applicability and can be used in the optimisation of the separation of both charged and uncharged solutes. The optimisation of different modes of CE can easily be implemented although we will focus on the optimisation of CZE and MECC. The applicability of this approach is illustrated with the separation of a mixture of six anionic solutes (five substituted benzoic acids and one substituted cinnamic acid). Studies on the migration behaviour of analogous compounds, containing one or more phenolic hydroxyl groups, have been reported in the literature [14, 35, 38-43].
7.2 Theory

7.2.1 The general principles on which the iterative regression strategy is based

The iterative regression strategy developed by Drouen et al. [44] was initially developed for HPLC. The general principles with reference to a two-parameter optimisation are illustrated in Fig. 7.1.

After defining the appropriate parameters and parameter space, initial experiments are performed (A, B, and C in Fig. 7.1). For each solute in a mixture a linear relationship is assumed between its migration and the parameters examined, resulting in a plane describing the migration behaviour of the solute in the parameter space which is defined by the data obtained in the experiments A, B and C. A first approximation of the best separation conditions is then obtained from a plot of a function which relates the quality of the separation (the so-called criterion) to the assumed migration behaviour of the solutes.

![Fig. 7.1 Schematic representation of the iterative regression optimisation approach for simultaneous two-parameter optimisation.](image)

123
In practice more or less significant deviations from linearity are observed so that the predicted optimum does not necessarily coincide with the real optimum. Additional measurements are then performed in an iterative manner and this further segmentation of the parameter space improves the prediction of the migration behaviour of the solutes. The process is repeated until a satisfactory separation is obtained or no further improvement occurs. The calculation and interpolation of the migration data require that in each experiment all peaks are identified. It should, furthermore, be stressed here that parameter optimisation can only be performed within the chosen parameter space.

These basic principles, originating from the HPLC optimisation, are preserved in CE method development. Because of inherent differences between HPLC and MECC, however, the strategy is modified to fit some special requirements.

7.2.2 The choice of the parameter and the accessory parameter space

It has been shown that pH, buffer concentration, type of buffer, temperature, applied voltage, and the addition of complexing agents and modifiers can be subjected to parameter optimisation in CE [17, 20, 27-29, 32-36, 45-49]. Furthermore, selectivity in a MECC experiment can be manipulated with a surfactant which is characterised by its net charge, critical micellar concentration (CMC), aggregation number, hydrophobic character and concentration.

This large number of significant parameters is in contrast with reversed phase (RP) HPLC experiments where elution behaviour is primarily influenced by the type and concentration of the organic modifier in the mobile phase, although sometimes other and less obvious parameters, e.g. the steepness of the gradient, are also used to increase selectivity [50].

In this study the pH of the buffer and the concentration of surfactant were considered to have a major influence on the separation and were therefore chosen as parameters. The iterative regression approach is, however, applicable to every parameter that can be changed continuously over the parameter space. Parameters that have a discrete scale (e.g. the type of the buffer ion) are more difficult to adjust using this strategy.

The minimum and maximum value of each parameter limits the parameter space. The selection of these extremes is crucial to the chance of finding useful separation conditions.
The proper choice is determined by the physicochemical properties of the system, i.e. acid dissociation constants, pH stability data of the solutes, and CMC values of different surfactants. Unfortunately, such data are not always available and the experience of the analyst then becomes an important factor.

The minimum number of initial experiments which must be performed equals the number of parameters plus one, resulting in appropriate geometrical figures: a line is composed of two experiments in a one-dimensional parameter space, a triangle of three experiments in a two-dimensional parameter space, and a tetrahedron of four experiments in a three-dimensional parameter space. In practice different experimental arrangements are used so that the minimum and maximum parameter values are easy to define and larger areas in the parameter space are covered. For one parameter only two initial experiments are needed, this increases to five initial experiments for two parameters and the formation of four triangles forming a square, whereas the simultaneous optimisation of three parameters requires no fewer than fifteen experiments resulting in twenty-four tetrahedrons in a cube. In practice, however, we believe that the majority of separation problems can be solved by the simultaneous optimisation of two parameters only.

7.2.3 The description of the migration behaviour in capillary electrophoresis used in the iterative regression strategy

In RPHPLC migration is adequately described using the logarithm of the capacity factor $k_{\text{chron}}$. Both experimental and theoretical considerations support a linear relationship between $\ln k_{\text{chron}}$ and the organic modifier-water ratio of the mobile phase [51].

In CZE, both migration time $t_m$, directly available from the experiment, and the mobility defined in Eq. 7.1 can be used as a measure of migration

$$\mu_{\text{app}} = \frac{l}{t_m E} = \frac{IL}{t_m U} = \mu_{\infty} + \mu_{\text{ep}}$$

(7.1)

where $\mu_{\text{app}}$ is the apparent mobility, $\mu_{\infty}$ is the electroosmotic mobility, $\mu_{\text{ep}}$ is the electrophoretic mobility, $l$ is the effective capillary length from inlet to detection window, $L$ is the total capillary length, $E$ is the electric field strength, and $U$ is the applied voltage.
In general it can be expected that both migration time and mobility will behave in a nonlinear manner as a function of the parameter to be optimised and in particular the pH of the buffer. Equation 7.1 implies, furthermore, that when the mobility is assumed to be a linear function of the experimental parameters, the predicted migration time will be a nonlinear (convex) function of these parameters, which does not seem to have any practical relevance. A linear relationship between migration time and the experimental parameters is, therefore, assumed, even if a few more experiments are sometimes required for adequate prediction of the migration behaviour of solutes in CZE.

In MECC interaction of the solutes with micelles is exploited to increase selectivity. Hence, the migration mechanism for charged solutes contains both an electrophoretic component, based on the charge density of the solute in the aqueous phase, and a chromatographic component, due to the partitioning between the aqueous and the micellar phase. This micellar interaction can be described by a capacity factor $k'$ based on chromatographic principles:

$$k' = \frac{(t_m - t_0)}{t_0 (1 - \frac{t_m}{t_{nc}})}$$  \hspace{1cm} (7.2)

where $t_m$ is the migration time in the micellar experiment, $t_{nc}$ is the migration time of the micellar phase, and $t_0$ is the migration time of a solute in the absence of micelles.

For a neutral solute, $t_0$ will be equal to the migration time $t_{eo}$ because of the electroosmotic flow. Equation 7.2 then reduces to Eq. 7.3 which is the well-known expression for the capacity factor $k$ for neutral solutes in MECC.

$$k = \frac{(t_m - t_{eo})}{t_{eo} (1 - \frac{t_m}{t_{nc}})}$$  \hspace{1cm} (7.3)

The calculation of $k'$ according to Eq. 7.2 can be performed only by combining data from two experiments - a free zone experiment to determine $t_0$ and a micellar experiment to determine $t_m$ and $t_{nc}$.
The use of the capacity factor (Eq. 7.2) instead of migration time, mobility, and capacity factor for neutral solutes (Eq. 7.3) has three advantages for the optimisation approach. Firstly, the capacity factor is not restricted to neutral solutes but can be also used for charged solutes. Secondly, as a consequence of the combination of experimental data in the absence and presence of micelles, free zone migration and migration due to micellar interaction are separated so that a better understanding of the migration mechanisms is obtained. Thirdly, it has been shown experimentally that the capacity factor (for both charged and uncharged solutes) is linearly dependent on the concentration of surfactant [34, 52].

7.2.4 The choice of the criterion in the iterative regression strategy

The criterion is often based on the resolution $R_s$ which can be calculated from the migration times of the solutes and the efficiency as shown in Eq. 7.4:

$$R_s = \frac{\sqrt{N} \ (t_j - \bar{t})}{2 \ (t_j + \bar{t})}$$  \hspace{1cm} (7.4)

The influence of the observed peak shapes on the resolution can be substantial, especially for chiral separations or when indirect UV-detection is used; several studies have been performed on the influence of various factors, e.g. the buffer type and mobility, the ionic strength of both sample and buffer, and the addition of organic modifiers and urea on peak shapes [49, 53-55]. Here, we focus on the optimisation of the individual migration times; the parameters chosen primarily affect the migration velocity of the solutes and to a much lesser extent the peak shapes, so we assume that $N$ is constant.

To evaluate the quality of an electropherogram, resolution values for all the (relevant) peak pairs are considered. The normalised resolution product $r$ is, therefore, calculated according to Eq. 7.5.

$$r = \prod_{i=1}^{n-1} \frac{R_{k,i+1}}{1 - \sum_{i=1}^{n-1} R_{k,i+1}} = \prod_{i=1}^{n-1} \frac{R_{k,i+1}}{R_s}$$  \hspace{1cm} (7.5)
where $n$ is the number of peaks, $R_{i,i+1}$ is the resolution between peak $i$ and $i+1$ and $\overline{R_s}$ the mean resolution; $r = 0$ when two peaks overlap and $r = 1$ when all resolutions are evenly spread in the electropherogram. If baseline separation of all the relevant solutes is required, then it follows from Eq. 7.5 that if one or more $R_s$ values are significantly higher than the resolution corresponding to baseline separation, the overall criterion value $r$ will decrease. An upper limit value for the $R_s$ is therefore introduced to avoid this decrease in $r$. This upper limit should be slightly higher than that for baseline separation.

7.2.5 The experimental set-up of the iterative regression optimisation strategy

The optimisation protocol presented here is summarised in the flow chart in Fig. 7.2. The effect of pH is evaluated in a free zone system where information on the electrophoretic behaviour of the different individual solutes is collected and a one-parameter optimisation is performed.

If the observed separation is not satisfactory, even after the addition of data obtained at other pH-parameter settings, a two-parameter optimisation can be performed. The pH is once more chosen as a parameter and the minimum and maximum pH values must be the same as in the free zone system. In addition, a second parameter is selected to perform the multi-parameter optimisation. In this study, the concentration of the surfactant SDS was chosen.

If the separation is still inadequate, the optimisation procedure has to be extended to different parameter settings: (i) the same parameters with an extended parameter space can be chosen; (ii) one parameter can be replaced by another; (iii) a third parameter can be added resulting in a three-parameter optimisation. If the criterion value is sufficiently high, small changes in the experimental conditions may be introduced to fit some special needs of the analyst. In this way less important criteria, such as the total analysis time, can be optimised.

The decision whether to refine the calculated response surface by adding more experiments or extend the number of parameters or parameter space is taken by the analyst and this requires an insight in the separation mechanism as well as in the algorithm.
Fig. 7.2 Flow chart illustrating the iterative regression optimisation scheme in MECC.
Chapter 7

7.3 Experimental

7.3.1 Apparatus

All experiments were performed on a BioFocus 3000 system (Bio-Rad, Hercules, CA, USA). An uncoated fused silica capillary 25 cm (21.4 cm effective length) x 50 μm ID (Chrompack, Middelburg, The Netherlands) was used throughout the study. The samples and the capillary were thermostated at 25 °C. The samples were injected with 6.9 kPa-sec. A constant voltage of 10 kV was applied and the highest current observed did not exceed 22 μA, which is low enough to avoid problems associated with inefficient heat dissipation. Runs were recorded using the fast scanning detector mode of the BioFocus 3000 between 200 nm and 360 nm. The data were collected and processed using the BioFocus 3000 data acquisition and integration software package.

7.3.2 Chemicals

2-[N-Cyclohexylamino]ethane sulfonic acid (CHES) and sodium dodecyl sulphate (SDS) were from Sigma (St. Louis, MO, USA). 2,4-Dihydroxybenzoic acid, 2-hydroxybenzoic acid, methanol and Sudan III were from Aldrich (Milwaukee, WI, USA). Benzoic acid was obtained from Merck (Darmstadt, Germany). 3-Hydroxybenzoic acid, 4-hydroxybenzoic acid and 4-hydroxy-3-methoxycinnamic acid were from Janssen (Geel, Belgium). Deionised water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

7.3.3 Buffers and samples

The pH of the 30 mM CHES buffers was adjusted with a sodium hydroxide solution (ca 0.5 M). The buffers used in the MECC experiments were prepared by dissolving SDS in CHES buffer adjusted to the appropriate pH. The samples were dissolved in water to a concentration of 0.17 mM. Prior to use, all solutions were filtered through 0.45 μm pore size filters. The experimental migration data were obtained by analysing mixtures of the solutes so that changes in the micro-environment and mutual interference of the ions in the sample plug are taken into account. A saturated solution of Sudan III in methanol was centrifuged and diluted 1 to 5 in buffer. Methanol was used as an electroosmotic flow marker and Sudan III was used as a marker for the micellar phase.
7.3.4 Optimisation

The optimisation software was written in Turbo Pascal 6 (Borland International, CA, USA) and ran on a PC.

7.4 Results and discussion

7.4.1 The choice of the solutes

Six acidic solutes were chosen to demonstrate the applicability of the iterative regression design for the optimisation of CE. Relevant physicochemical properties of these acids are shown in Table 7-I. Furthermore, the UV spectra of the solutes are significantly different, so that peak tracking was greatly facilitated when the fast scanning detection mode was used.

<table>
<thead>
<tr>
<th>Solute name</th>
<th>Molecular weight</th>
<th>pK_{a1}</th>
<th>pK_{a2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoic acid (B)</td>
<td>122.12</td>
<td>4.19</td>
<td>-</td>
</tr>
<tr>
<td>2-Hydroxybenzoic acid (2-HB)</td>
<td>138.12</td>
<td>2.97</td>
<td>13.40</td>
</tr>
<tr>
<td>3-Hydroxybenzoic acid (3-HB)</td>
<td>138.12</td>
<td>4.06</td>
<td>9.92</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid (4-HB)</td>
<td>138.12</td>
<td>4.48</td>
<td>9.32</td>
</tr>
<tr>
<td>2,4-Dihydroxybenzoic acid (2,4-HB)</td>
<td>154.12</td>
<td>3.30</td>
<td>-</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxycinnamic acid (4-H-3-MC)</td>
<td>194.19</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

7.4.2 The one-parameter pH optimisation

The data in Table 7-I can be used to determine both the parameter and the parameter space. From the pK_{a} data, changes in the electrophoretic mobility may be expected for some of these weak acids at pH values higher than 8. Therefore, the pH is optimised between 8.73 and 9.73. The lowest limit is set by the buffer capacity of CHES (pK_{a} = 9.55). The upper
limit is determined both by the $pK_a$ values of the solutes and the increase in current arising as a result of the addition of NaOH to adjust the pH. Moreover, as explained in section 7.2.3, a relatively small pH range under investigation probably increases the accuracy and reliability of the prediction of the migration behaviour.

The results of the one-parameter optimisation, using experimental data at pH 8.73, 9.23 and 9.73, are shown in Fig. 7.3. The plate number is set to 150000. Using this value of $N$, reasonable agreement is found between the calculated and the measured peak widths. The resolution threshold is set to 2.0, which is slightly higher than that of baseline separation.

![Graph](image)

**Fig. 7.3** Optimisation results for six test solutes for the one-parameter pH optimisation. In the upper frame the linear interpolation of the migration times of the individual solutes is shown using experimental data at pH 8.73, 9.23 and 9.73. In the lower frame, the corresponding criterion value $r$ is depicted. Abbreviations as given in Table 7-1.

Figure 7.3 shows that even within a relatively small parameter range of one pH unit, the assumed linear relationship between migration time and pH is not always observed, as is evidenced by non-linearity in some of the migration lines; the (expected) sigmoidal migration behaviour as a function of pH can be partly recognised. Comparison of the migration data
and the physicochemical properties in Table 7-1 shows that the experimental behaviour of some of these solutes deviates from the expected behaviour, e.g. the deviation from linearity is largest for 2-HB but the electrophoretic properties of this solute should be more or less unaffected by changes in pH from 8 to 10, whereas for 3-HB, which has a pKₐ of 9.92, the behaviour is strikingly linear. Differences between the expected and observed migration behaviour may be due to discrepancies between the literature pKₐ values and the actual data under CE conditions. The overall migration behaviour is, furthermore, influenced both by changes in the electroosmotic flow and by changes in the electrophoretic properties of the solutes.

The criterion value r at the upper half of the pH range between 9.23 and 9.73 is zero, due to the co-migration of 3-HB and 4-H-3-MC. An optimum is found at pH 8.88 and the corresponding value of r is 0.66. The calculated and experimentally measured electropherograms at this optimal pH are similar, as can be seen in Fig. 7.4, although the observed separation between 2-HB and 4-HB is not as good as that calculated. It is clear that the separation is still unsatisfactory especially for the fast migrating solutes.

![Fig. 7.4 Predicted and experimental electropherogram of six test solutes at the optimum parameter settings (pH of the buffer is 8.88) obtained from the one-parameter pH optimisation. Abbreviations as given in Table 7-1.](image-url)
The difference in pH between these experiments is small (0.25 or 0.5 pH units), so it is assumed that only minor improvements may be obtained by further segmentation of the pH-parameter space and the achievement of baseline separation of all the solutes in this way is unlikely.

### 7.4.3 The simultaneous two-parameter optimisation

As a second parameter, the concentration of SDS was evaluated between 10 and 50 mM. The lowest concentration of SDS used is determined by its CMC (≤ 8 mM). The highest concentration of SDS is limited by the increase in current at increasing SDS concentrations.

The parameter space for the two-parameter optimisation is shown in Fig. 7.5 where the filled circles represent the five initial experiments, hence the parameter space is divided into four triangles A, B, C and D. The measured migration times at the different parameter settings are summarised in Table 7-II.

![Diagram](image)

**Fig. 7.5** Schematic representation of the experimental set-up for the simultaneous two-parameter optimisation. The pH is varied from 8.73 to 9.73 and the concentration of SDS ranges from 10 to 50 mM. The filled circles represent the five initial experiments and the open circles represent two additional measurements.
At pH 9.73, 2,4-HB migrates slower than the micellar phase at any given concentration of SDS. This results in a negative capacity factor $k'$ and this cannot be explained in terms of micellar interaction. At high pH values 2,4-HB carries three negative charges and it is assumed that the interaction between SDS micelles and 2,4-HB is negligible because of the electrostatic repulsion of two species both carrying a high negative charge. The migration behaviour is, therefore, solely determined by the electrophoretic behaviour of 2,4-HB. As a result of this behaviour simplification is allowed and a linear interpolation of migration times, in stead of capacity factors, is preferred. If in such cases $k'$ is used for the interpolation, the calculated migration times will, moreover, be unreliable at certain values of $k'$, depending on the ratio $t_0/t_{mc}$ (Eq. not shown).

**Table 7-II** The migration times of the solutes and $t_{co}$ and $t_{mc}$, at all the measured parameter settings.

<table>
<thead>
<tr>
<th>Buffer composition</th>
<th>SDS (mM)</th>
<th>10</th>
<th>10</th>
<th>50</th>
<th>50</th>
<th>30</th>
<th>30</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.73</td>
<td>9.73</td>
<td>9.73</td>
<td>8.73</td>
<td>9.23</td>
<td>9.73</td>
<td>9.23</td>
<td></td>
</tr>
<tr>
<td>Solute</td>
<td>Migration times (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1.53</td>
<td>1.95</td>
<td>2.32</td>
<td>1.91</td>
<td>1.87</td>
<td>2.14</td>
<td>2.17</td>
<td></td>
</tr>
<tr>
<td>2-HB</td>
<td>1.59</td>
<td>2.04</td>
<td>2.44</td>
<td>1.99</td>
<td>1.95</td>
<td>2.24</td>
<td>2.35</td>
<td></td>
</tr>
<tr>
<td>3-HB</td>
<td>1.49</td>
<td>2.14</td>
<td>2.90</td>
<td>1.88</td>
<td>1.91</td>
<td>2.61</td>
<td>2.40</td>
<td></td>
</tr>
<tr>
<td>4-HB</td>
<td>1.53</td>
<td>2.62</td>
<td>3.37</td>
<td>2.05</td>
<td>2.33</td>
<td>2.96</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>2,4-HB</td>
<td>1.75</td>
<td>2.92</td>
<td>3.84</td>
<td>2.3</td>
<td>2.63</td>
<td>3.33</td>
<td>3.20</td>
<td></td>
</tr>
<tr>
<td>4-H-3-MC</td>
<td>1.44</td>
<td>2.14</td>
<td>2.78</td>
<td>1.75</td>
<td>1.91</td>
<td>2.37</td>
<td>2.44</td>
<td></td>
</tr>
<tr>
<td>$t_{co}$</td>
<td>1.18</td>
<td>1.23</td>
<td>1.34</td>
<td>1.21</td>
<td>1.20</td>
<td>1.16</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td>$t_{mc}$</td>
<td>1.83</td>
<td>2.65</td>
<td>3.38</td>
<td>2.77</td>
<td>2.70</td>
<td>2.93</td>
<td>3.01</td>
<td></td>
</tr>
</tbody>
</table>

In Fig. 7.6 the criterion $r$ is shown as a function of pH and SDS concentration. Five initial experiments are used to calculate this iso-criterion plot. It clearly shows that the response surface is quite complex and characterised by several local optima. Peak overlap, e.g. at low pH and low concentrations of SDS, results in a criterion value of zero. The $r$ value is low
at pH values close to 9 and more or less independent of the SDS concentration. This is mainly a result of poor separation of B and 4-H-3-MC and, to a lesser extent, to the low resolution between 3-HB and 4-H-3-MC at this pH. The overall optimum is situated at high pH values and at relatively high concentrations of SDS. The iso-criterion plot indicates a broad region in the parameter space where the separation is satisfactory.

Two experiments, at pH 9.73 with 30 mM SDS and at pH 9.23 with 50 mM SDS, shown as open circles in Fig. 7.5, were added to segment the parameter space further, thus forming smaller triangles (B.1, B.2, C.1 and C.2). These experiments are situated in the parameter space so that it is likely that additional information on the optimum parameter settings can be obtained. The recalculated response surface is shown in Fig. 7.7. As expected, half of the iso-criterion surface has not changed (triangles A and D in Fig. 7.5) but in the other half small changes are visible. This indicates that the linear model is in reasonable agreement with the real migration behaviour.

![Diagram](image)

**Fig. 7.6** The iso-criterion plot, obtained from the simultaneous two-parameter optimisation using five initial experiments.

**Fig. 7.7** The iso-criterion plot, obtained from the simultaneous two-parameter optimisation using seven experiments.
The region characterised by high values of $r$ is not shifted dramatically, although more pronounced dependence on pH can be observed. The region of good separation is now narrower than in Fig. 7.6. The predicted optimum is found at pH 9.63 with 38 mM SDS and the corresponding criterion value equals 0.99, close to the maximum value of 1.

The electropherogram measured at the optimum parameter settings, together with the predicted electropherogram, are shown in Fig. 7.8. Again, predicted and experimental results are quite similar and all the solutes are now baseline separated.

Fig. 7.8 Predicted and experimental electropherogram of six test solutes at the optimum parameter settings (pH of the buffer is 9.63 and the concentration of SDS is 38 mM) obtained from the simultaneous two-parameter optimisation. Abbreviations as given in Table 7-I.
As mentioned before extrapolation to other parameter values outside the parameter space is not allowed. However, closer inspection of the predicted migration lines, which diverge at higher pH values (data not shown), and the response surface indicates that an extension of the parameter space in this direction should result in better separation as a result of further improvement of the separation between 3-HB and 4-H-3-MC. In less than four minutes total analysis time the separation is, nevertheless, adequate so that the effect of an increase of the pH in this micellar system was not further investigated.

7.5 Conclusions

It is shown here that the iterative regression optimisation design can be successfully used for method development in MECC by performing a one-parameter pH optimisation in the free zone mode, followed by a simultaneous two-parameter optimisation in a micellar system. This approach avoids unnecessary complex experiments in a micellar system if the separation can be performed in a relatively simple free zone system. Especially when little is known about the sample, this approach can be very helpful and time-saving. Extension of this method to different parameters and thus other modes of capillary electrophoresis should be relatively easy.

Since experimental data are used, ideal behaviour of the solutes is not expected and physicochemical properties of the solutes do not need to be known to start the optimisation procedure, although physicochemical data are helpful in defining the parameter space. The optimisation strategy is applicable to both charged and uncharged solutes and a number of different parameters can be subjected to optimisation. Identification of all the solutes in the mixture is, however, required and although peak tracking in CE combined with the conventional UV-detection systems may still give some problems, the introduction of advanced detection methods, such as rapid scanning UV-detection, diode array spectrophotometry and mass spectrometry, will greatly simplify peak identification and subsequent optimisation.

The applicability of the iterative regression strategy is illustrated by the optimisation of the separation of six anionic solutes. Changes in the pH and in the concentration of SDS are evaluated simultaneously and the corresponding response surface is complex but the optimum
parameter settings can easily be deduced. These settings result in baseline separation of all the solutes.

Peak shapes in CE may be distorted and adversely affect the quality of the electropherogram and in some cases, this effect should be accounted for in the criterion as well as in the choice of the parameter.

7.6 References


Summary

Analytical chemistry has often been considered as a branch of chemistry in which (new) developments are directed by other scientific disciplines. The rise of the biotechnology and related fields, characterised by the development and production of high quality products available in often low concentrations and/or in complex matrices, has favoured new developments in analytical chemistry. Moreover, faster analyses are required to increase the efficiency at which the (production) processes are controlled while higher demands concerning the accuracy, the precision, the robustness and the sensitivity of the analytical methods arise. As a consequence of these factors, the development of capillary electrophoresis (CE) has been accelerated considerably.

Advantages of CE are among others its speed of analysis, its extremely high resolving power, the small sample volumes and the numerous modes in which CE can be performed. Improvements, however, related to the limited detection sensitivity, the restricted possibilities in the field of upscaling and the overall reproducibility of CE experiments should be aimed for. Future instrumental developments will be of decisive importance with respect to the first two limitations. An improved reproducibility and accordingly an increase of the number of routine applications, demands among others, an extension of the knowledge on the electrophoretic behaviour of solutes in a CE system. The aim of this thesis is therefore to investigate various physicochemical aspects of CE so that the migration behaviour of the solutes can be better understood.

The migration behaviour in capillary zone electrophoresis is mainly determined by the charge of the solutes. As a consequence, the pH of the background electrolyte is an important parameter with respect to the electrophoretic properties of weak acids and bases. In chapter
2 calculations based on the principles of electrolysis illustrate that pH variations of the buffer, due to electrode reactions, may occur. Experimental data demonstrate that these pH changes may affect the mobilities and thus the separation of the solutes in a dramatic way. A method based on the analysis of the peak area, the peak height and the electrophoretic mobility of an injected indicator dye enables the in situ determination of pH variations of the buffer as small as 0.02 pH units. This pH measurement is therefore extremely useful to estimate the (buffer) stability in a CE system so that, if necessary, corrective measures can be taken.

Micellar electrokinetic capillary chromatography is a special mode of CE in which an increase of the selectivity results from the partitioning of a solute between the aqueous and pseudo stationary micellar phase. In chapter 3 a new resolution equation is presented in which the electrophoretic behaviour of ionisable solutes as well as the effect of the partitioning between the aqueous and micellar phase are taken into account separately. By means of simulations, a better insight in the relevant migration mechanisms is obtained. This is illustrated by the experimental analysis of a mixture of charged and neutral solutes at increasing concentrations of surfactant.

In chapter 4 free zone experiments are used to study the mobility of the enzyme Savinase™ and variants thereof, and the observed differences are explained by their physicochemical properties. At pH 3 the observed migration order is related to the increase of the number of positively charged residues but the effective charge appears to be a factor 11 lower than the theoretical charge. The same migration order is seen at pH 6 in the presence of Ca²⁺, which is explained by shielding of the negative charges on Savinase by calcium ions. A modified mobility equation enables the quantitation of this shielding. The migration behaviour at pH 6 in the absence of Ca²⁺ ions is probably determined by subtle variations in the charge as well as in the friction of the proteins. The effect of the friction, expressed as the molecular mass or the radius of the protein, does not result in a satisfactory description of the observed migration order. In contrast, by introducing small variations of the actual net charge it is possible to obtain an excellent correlation between the calculated mobility and the experimentally observed migration.

The use of CE in the study and the quantitation of the association of methylamine dehydrogenase (MADH) and amicyanin is described in chapter 5. A systematic approach in which the amicyanin concentration is introduced as an additional fit parameter allows the
determination of association constants up to $2 \times 10^6 \text{ M}^{-1}$. The fitted and injected amicyanin concentrations become different if the concentration ratio of MADH to amicyanin is small. Simulations based on a plate model and the analysis of the peak shape of amicyanin confirm the hypothesis with respect to the migration mechanism. Experiments performed at various ionic strength and osmotic pressure suggest that the interaction between MADH and amicyanin is mainly attributed to hydrophobic forces. The number of water molecules removed from the interfacial surface area upon complex formation is estimated to be 40. An increase of the association constant at increasing pH may result from the deprotonation of one or more residues.

Method development in CE has often been considered as relatively simple, mainly because of the fact that long equilibration times upon a change of buffer can often be avoided. In chapter 6 an overview is presented of the most important optimisation strategies applicable to free zone electrophoresis and micellar electrokinetic capillary chromatography. Statistical approaches often require only a minimum amount of knowledge about the system to separate and are therefore characterised by a broad applicability. More efficient strategies make often use of relevant physicochemical knowledge, if available. It is therefore important not to consider these strategies as black box mechanisms. In this way, optimisation strategies can be used as a guideline to determine optimal experimental conditions in a fast and convenient way.

In chapter 7 an optimisation strategy based on the iterative regression strategy is presented. A one parameter (pH) optimisation in a free zone system is, if necessary, followed by a two parameter (pH and concentration of surfactant) optimisation in a MECC set-up. The prediction of the migration over the parameter space makes use of a linear interpolation. This strategy is applicable to a large number of solutes although peak tracking of the relevant solutes should always be performed. Because experimentally determined migration parameters like the migration time, the mobility or the capacity factor are used, no ideal behaviour of the solutes is supposed. Moreover, many different parameters can be subjected to parameter optimisation. The applicability of the strategy is demonstrated by the optimisation of the separation of a mixture of 6 substituted benzoic acids in a micellar system, resulting in a baseline separation for all components.
De analytische chemie wordt vaak beschouwd als een tak van de scheikunde waarbij de (nieuwe) ontwikkelingen worden gestuurd door andere wetenschappelijke vakgebieden, zowel binnen als buiten de scheikunde. De sterke opkomst van de biotechnologie en aanverwante disciplines, gekarakteriseerd door de ontwikkeling en produktie van hoogwaardige produkten in vaak geringe concentraties en/of in complexe matrizes, heeft de analytische scheikunde nieuwe impulsen gegeven. Bovendien zijn steeds snellere analyses vereist zodat een (produktie)proces efficiënter kan worden gestuurd terwijl strengere eisen aan de accuraatheid, de precisie, de robuustheid en de gevoeligheid van de analytische methoden worden gesteld. Mede onder invloed van deze factoren is de ontwikkeling van de capillaire elektroforese (CE) in een stroomversnelling geraakt.

Potentiële voordelen van CE zijn de snelheid van analyse, het extreem hoge scheidend vermogen, de kleine monstervolumina en de ruime keuze aan varianten waarin CE bedreven kan worden. Capillaire elektroforese is voor verbeteringen vatbaar op het vlak van de detectiegevoeligheid, de opschalingsmogelijkheden en de reproduceerbaarheid. Met betrekking tot de eerste twee beperkingen zullen de toekomstige ontwikkelingen van en aanpassingen aan de apparatuur van doorslaggevend belang zijn. Een toename in de reproduceerbaarheid en daarmee samenhangend de introductie van betrouwbare routine-applicaties vereist onder meer een uitbreiding van de kennis over het elektroforetische gedrag van componenten in een CE systeem. Het doel van dit proefschrift bestaat erin een aantal fysisch-chemische aspecten van CE te onderzoeken zodat het migratiegedrag beter begrepen kan worden.

Het migratiegedrag in capillaire zone elektroforese wordt in belangrijke mate bepaald door de op de component aanwezige lading. Bijgevolg is de pH van de buffer een belangrijke
parameter met betrekking tot de elektroforetische eigenschappen van zwakke zuren en basen. In hoofdstuk 2 wordt aan de hand van berekeningen gebaseerd op de principes van elektrolyse, geïllustreerd dat pH verschuivingen van de buffer ten gevolge van elektrodereacties op kunnen treden. Experimenten tonen aan dat deze pH verschuivingen de mobiliteiten van de componenten en dus ook hun scheiding dramatisch kunnen bevorderen. Een methode gebaseerd op de analyse van het piekoppervlak, de piekhoogte en de elektroforetische mobiliteit van geïnjecteerde kleurindicatoren maakt het mogelijk kleine pH verschuivingen van de buffer van ongeveer 0,02 pH eenheden in situ te bepalen. Deze pH meting is bijgevolg uitermate geschikt om de (buffer)stabiliteit van het CE systeem in te schatten zodat, indien nodig, maatregelen getroffen kunnen worden.

Micellaire elektrokinetische capillaire chromatografie is een speciale vorm van CE, waarbij een toename van de selectiviteit wordt nagestreefd via de verdeling van de component over de wateige en de pseudo-stationaire micellaire fase. In hoofdstuk 3 wordt een nieuwe resolutievergelijking gepresenteerd waarbij zowel het elektroforetische gedrag van de ioniseerbare componenten als ook het effect van hun verdeling over de wateige en micellaire fase afzonderlijk in rekening worden gebracht. Door middel van simulaties wordt een beter inzicht verkregen in de relevante migratiemechanismen. Dit wordt geïllustreerd aan de hand van de experimentele analyse van een mengsel van zowel geladen als neutrale componenten bij toenemende concentraties aan oppervlakte actieve stof.

In hoofdstuk 4 wordt aan de hand van vrije zone experimenten de mobiliteit van het enzym Savinase™ en enkele varianten bestudeerd en verklaard door verschillen in fysisch-chemische parameters. Bij pH 3 zijn alle geïntroduceerde zure aminozuren geprotoniseerd en de waargenomen migratievolgorde wordt gerelateerd aan de toename in het aantal positief geladen residuen. De effectieve lading op het eiwit blijkt echter een factor 11 lager te liggen dan de theoretische lading. Een zelfde migratievolgorde wordt waargenomen bij pH 6 in aanwezigheid van Ca²⁺. Dit wordt verklaard door een afscherming van negatieve ladingen op Savinase door de metaalionen. Een gemodificeerde mobiliteitsvergelijking laat toe de efficiëntie van deze afscherming te kwantificeren. Het migratiegedrag bij pH 6 in afwezigheid van Ca²⁺ wordt waarschijnlijk bepaald door subtiele variaties in zowel de lading als in de frictie van Savinase en zijn varianten. De invloed van de frictie, uitgedrukt als het molecuulgewicht of de straal van het eiwit, leidt niet tot een bevruggende beschrijving van het waargenomen migratiegedrag. Het in rekening brengen van kleine variaties in de
effectieve lading leidt tot een berekende mobiliteit die wel in overeenstemming kan worden gebracht met de experimentele migratie.

Een studie naar het gebruik van CE voor de kwantificering van de associatie van methyamine dehydrogenase (MAH) en amicyanine wordt gepresenteerd in hoofdstuk 5. Een systematische aanpak, waarbij de amicyanineconcentratie wordt geïntroduceerd als een bijkomende te fitten parameter, maakt het mogelijk associatieconstanten tot $2 \times 10^6$ M$^{-1}$ te bepalen. De gefitte en geïnjecteerde amicyanineconcentraties wijken enkel van elkaar af indien de concentratieverhouding MMDH tot amicyanine klein is. Simulaties gebaseerd op een plaat-model en de analyse van de piekvorm van amicyanine bevestigen de hypothese met betrekking tot het migratiemecanisme. Experimenten uitgevoerd bij verschillende ionsterkten en osmotische drukken geven aan dat de interactie tussen MMDH en amicyanine vooral gebaseerd is op hydrofobe krachten en in mindere mate bepaald wordt door ionogene interactie. Het aantal watermoleculen dat bij de complexvorming in de onmiddellijke nabijheid van het contactoppervlak tussen beide eiwitten verdreven wordt, is geschat op 40. Een toename van de associatieconstante bij stijgende pH kan het gevolg zijn van deprotonering van één of meerdere residuen.

Methode-ontwikkeling in CE wordt vaak als relatief eenvoudig bestempeld, mede doordat het veranderen van de buffer meestal niet gepaard gaat met lange equilibratietijden. In hoofdstuk 6 wordt een overzicht gepresenteerd van de belangrijkste optimiseringsstrategieën, toepasbaar in vrije zone elektroforese en micellaire elektrokinetische capillaire chromatografie. Statistische methoden vereisen meestal slechts een minimale kennis over het te scheiden systeem en/of de analysetechniek en worden daarom gekenmerkt door een brede toepasbaarheid. Efficiëntere strategieën maken veelal gebruik van fysisch-chemische kennis van zowel de te scheiden componenten als de analysetechniek, indien beschikbaar. Het is echter van belang dat deze strategieën niet als 'black-box' mechanismen worden gebruikt. Op deze manier kunnen optimiseringsstrategieën als leidraad dienen om snel en efficiënt de optimale experimentele condities te bepalen.

In hoofdstuk 7 wordt een optimiseringsstrategie, gebaseerd op de iteratieve regressiestrategie, gepresenteerd. Een één parameter (pH)-optimalisering in een vrije zone systeem wordt, indien nodig, gevolgd door een twee parameter (pH en de concentratie aan oppervlakte actieve stof) optimalisering in een MECC opzet. Het voorspellen van het
migratiegedrag over de ganse parameterruimte is terug te voeren tot lineaire interpolaties. Deze strategie kan worden toegepast op een breed scala aan monsters, waarbij het wel noodzakelijk is de relevante componenten te herkennen in de diverse elektroferogrammen, verkregen onder variabele experimentele condities. Het gebruik van experimenteel verkregen migratieparameters (tijd, mobiliteit of capaciteitsfactor) leidt er toe dat geen ideaal gedrag van de componenten wordt verondersteld. Bovendien wordt deze strategie gekenmerkt door een grote vrijheid in de keuze van de te optimaliseren parameters. De toepasbaarheid van deze strategie wordt geïllustreerd aan de hand van de optimalisering van de scheiding van een mengsel van 6 gesubstitueerde benzoëzuren in een micellair systeem, resulterend in een basislijnscheiding van alle componenten.
## Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$b$</td>
<td>parameter to be fitted</td>
<td>[-]</td>
</tr>
<tr>
<td>$c$</td>
<td>concentration</td>
<td>[mole m$^3$]</td>
</tr>
<tr>
<td>$e$</td>
<td>electronic charge</td>
<td>[C]</td>
</tr>
<tr>
<td>$e_0$</td>
<td>dielectric constant</td>
<td>[-]</td>
</tr>
<tr>
<td>$e'$</td>
<td>permittivity of free space</td>
<td>[C$^2$ J$^{-1}$ m$^{-3}$]</td>
</tr>
<tr>
<td>$E$</td>
<td>electric field strength</td>
<td>[V cm$^{-1}$]</td>
</tr>
<tr>
<td>$f/f_0$</td>
<td>frictional ratio</td>
<td>[-]</td>
</tr>
<tr>
<td>$F$</td>
<td>Faraday constant</td>
<td>[C mole$^{-1}$]</td>
</tr>
<tr>
<td>$i$</td>
<td>proportionality factor actual to theoretical charge</td>
<td>[-]</td>
</tr>
<tr>
<td>$I$</td>
<td>ionic strength</td>
<td>[mole m$^3$]</td>
</tr>
<tr>
<td>$k$</td>
<td>capacity factor for neutral solutes</td>
<td>[-]</td>
</tr>
<tr>
<td>$k'$</td>
<td>capacity factor for charged solutes</td>
<td>[-]</td>
</tr>
<tr>
<td>$k_B$</td>
<td>Boltzman constant</td>
<td>[J K$^{-1}$]</td>
</tr>
<tr>
<td>$k_{chrom}$</td>
<td>capacity factor in conventional chromatography</td>
<td>[-]</td>
</tr>
<tr>
<td>$K_{ass}$</td>
<td>association constant</td>
<td>[mole$^{-1}$ m$^3$]</td>
</tr>
<tr>
<td>$K_{diss}$</td>
<td>dissociation constant</td>
<td>[mole m$^3$]</td>
</tr>
<tr>
<td>$l$</td>
<td>effective capillary length</td>
<td>[m]</td>
</tr>
<tr>
<td>$L$</td>
<td>total capillary length</td>
<td>[m]</td>
</tr>
<tr>
<td>$m$</td>
<td>shielding factor</td>
<td>[-]</td>
</tr>
<tr>
<td>$M$</td>
<td>number of electrons</td>
<td>[-]</td>
</tr>
<tr>
<td>$M$</td>
<td>molecular mass</td>
<td>[kg mole$^{-1}$]</td>
</tr>
<tr>
<td>$n$</td>
<td>number of substitutions, peaks, moles or parameters</td>
<td>[-]</td>
</tr>
<tr>
<td>$N$</td>
<td>Avogadro's number</td>
<td>[mole$^{-1}$]</td>
</tr>
<tr>
<td>$P$</td>
<td>theoretical plate number</td>
<td>[-]</td>
</tr>
<tr>
<td>$P_{nw}$</td>
<td>hydrostatic pressure</td>
<td>[Pa]</td>
</tr>
<tr>
<td>$pI$</td>
<td>partition coefficient</td>
<td>[-]</td>
</tr>
<tr>
<td>$q$</td>
<td>isoelectric point</td>
<td>[C]</td>
</tr>
<tr>
<td>$R$</td>
<td>gas constant</td>
<td>[J mole$^{-1}$ K$^{-1}$]</td>
</tr>
<tr>
<td>$R_s$</td>
<td>resolution</td>
<td>[-]</td>
</tr>
</tbody>
</table>
List of symbols and abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r$</td>
<td>normalised resolution product</td>
<td>[-]</td>
</tr>
<tr>
<td></td>
<td>hydrodynamic radius</td>
<td>[m]</td>
</tr>
<tr>
<td>$t$</td>
<td>correlation coefficient</td>
<td>[-]</td>
</tr>
<tr>
<td>$t_0$</td>
<td>time</td>
<td>[s]</td>
</tr>
<tr>
<td>$t_m$</td>
<td>migration time in the absence of micelles</td>
<td>[s]</td>
</tr>
<tr>
<td>$t_{m0}$</td>
<td>retention time of unretained solute in chromatography</td>
<td>[s]</td>
</tr>
<tr>
<td>$t_{eo}$</td>
<td>electroosmotic migration time</td>
<td>[s]</td>
</tr>
<tr>
<td>$t_m0$</td>
<td>migration time of a solute</td>
<td>[s]</td>
</tr>
<tr>
<td>$t_{mc}$</td>
<td>expected migration time in the absence of micelles</td>
<td>[s]</td>
</tr>
<tr>
<td>$T$</td>
<td>migration time of the micellar phase</td>
<td>[s]</td>
</tr>
<tr>
<td>$U$</td>
<td>absolute temperature</td>
<td>[K]</td>
</tr>
<tr>
<td>$u$</td>
<td>dipole moment</td>
<td>[debye]</td>
</tr>
<tr>
<td>$V$</td>
<td>voltage</td>
<td>[V]</td>
</tr>
<tr>
<td>$v_0$</td>
<td>expected velocity in the absence of micelles</td>
<td>[m s$^{-1}$]</td>
</tr>
<tr>
<td>$v_m$</td>
<td>velocity</td>
<td>[m s$^{-1}$]</td>
</tr>
<tr>
<td>$v_{mc}$</td>
<td>velocity of the micellar phase</td>
<td>[m s$^{-1}$]</td>
</tr>
<tr>
<td>$V$</td>
<td>volume</td>
<td>[m$^3$]</td>
</tr>
<tr>
<td></td>
<td>partial molar volume</td>
<td>[m$^3$ mole$^{-1}$]</td>
</tr>
<tr>
<td></td>
<td>partial specific volume</td>
<td>[m$^3$ kg$^{-1}$]</td>
</tr>
<tr>
<td>$x$</td>
<td>concentration ratio</td>
<td>[-]</td>
</tr>
<tr>
<td></td>
<td>parameter to be optimised</td>
<td>[-]</td>
</tr>
<tr>
<td>$X$</td>
<td>mole fraction</td>
<td>[-]</td>
</tr>
<tr>
<td>$y$</td>
<td>response, criterion</td>
<td>[-]</td>
</tr>
<tr>
<td>$z$</td>
<td>theoretical chargenumber</td>
<td>[-]</td>
</tr>
<tr>
<td>$z_a$</td>
<td>actual chargenumber</td>
<td>[-]</td>
</tr>
</tbody>
</table>

Greek

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>selectivity</td>
<td>[-]</td>
</tr>
<tr>
<td></td>
<td>symmetry factor</td>
<td>[-]</td>
</tr>
<tr>
<td>$\beta$</td>
<td>compressibility</td>
<td>[Pa$^{-1}$]</td>
</tr>
<tr>
<td>$\eta$</td>
<td>viscosity</td>
<td>[N s m$^{-2}$]</td>
</tr>
<tr>
<td>$\kappa$</td>
<td>Debye parameter</td>
<td>[m$^{-1}$]</td>
</tr>
<tr>
<td>$\mu_{eo}$</td>
<td>electroosmotic mobility</td>
<td>[m$^2$ V$^{-1}$ s$^{-1}$]</td>
</tr>
</tbody>
</table>

152
List of symbols and abbreviations

$\mu_{ep}$  electrophoretic mobility  [$m^2 V^{-1} s^{-1}$]
$\mu_{nc}$  micellar mobility  [$m^2 V^{-1} s^{-1}$]
$\pi$  osmotic pressure  [Pa]
$\sigma$  standard deviation  [s]
$\phi(\kappa)$  Henry's function  [-]

Subscripts

A, B  solutes A and B
i, j  solutes i and j
aq  aqueous phase
mc  micellar phase
glyc  glycerol

Abbreviations

AADH  aromatic amine dehydrogenase
ACE  affinity capillary electrophoresis
B  benzoic acid
BICINE  N,N-bis[2-hydroxyethyl]glycine
CE  capillary electrophoresis
CHES  2-[N-cyclohexylamino]ethane-sulfonic acid
CMC  critical micellar concentration
CZE  capillary zone electrophoresis
2-HB  2-hydroxybenzoic acid
2,4-HB  2,4-dihydroxybenzoic acid
3-HB  3-hydroxybenzoic acid
4-HB  4-hydroxybenzoic acid
4-H-3-MC  4-hydroxy-3-methoxycinnamic acid
HPLC  high performance liquid chromatography
ID  inner diameter
MADH  methyamine dehydrogenase
MECC  micellar electrokinetic capillary chromatography

153
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES</td>
<td>4-morpholineethanesulfonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>4-morpholinopropane-sulfonic acid</td>
</tr>
<tr>
<td>ORM</td>
<td>overlapping resolution mapping scheme</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reversed phase high performance liquid chromatography</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
</tbody>
</table>
Dankwoord

Uiteraard hebben vele mensen hun bijdrage geleverd bij het tot stand komen van dit werk en op deze plaats zou ik allen willen bedanken die een steentje hebben bijgedragen.

Karel Luyben die als promotor mij de mogelijkheid heeft geboden dit analytisch gericht onderzoek te kunnen starten. Hans Frank die als co-promotor steeds in staat was nieuwe invalshoeken te ontdekken, waarbij zijn enthousiaste bijdrage zowel stimulerend als richtinggevend is geweest bij het uitvoeren van dit onderzoek.

Hugo Billiet die steeds paraat stond met allerhande praktische kennis over analysetechnieken en optimaliseringsstrategieën. Gert van der Steen die een deel van het praktische werk heeft uitgevoerd, waarbij vooral tijdens het laatste jaar zijn inbreng steeds belangrijker is geworden. André Oord voor het schrijven van het optimaliseringsprogramma.

Alle mensen van de systeemgroep, bio-instrumentatie, elektronische en technische dienst, waarbij Joop Langeveld en Peter Vetter een extra woord van dank verdienen doordat zij er (bijna) in geslaagd zijn de temperatuur in het capillair efficiënt te controleren.

Istvan Valko, Ann Tibbet, Radim Vespalec, Michel Schrapenzeel, Tom Vandemoortele en Koen Hellem die de afgelopen jaren in meer of mindere mate samen met mij deze techniek hebben mogen ontdekken.

Pim Muyselaar en Henk Claessens van de TU Eindhoven en Maarten Egmond en Sjaak Peelen van Unilever voor de boeiende discussies buiten het laboratorium.

Alle mensen van de bio-analytische groep die samen met mij de stap richting Kluysterlaboratorium hebben gezet.

Mijn ouders die mij steeds de mogelijkheid hebben geboden te studeren.

Ann voor zowel de mentale duwtjes in de rug als de broodnodige afleiding.
Stellingen behorende bij het proefschrift

A physicochemical approach of capillary electrophoresis:
towards method development

1. Doordat capillaire elektroforese wordt gekenmerkt door een extreem hoog schattend vermogen, is naast het opzetten en uitvoeren van analytische scheidingen, het bestuderen van het (elektroforetisch) gedrag van moleculen een belangrijk toepassingsgebied. In dit laatste geval zal de interpretatie van de gegeenerteerde data van doorlaggevend belang zijn met betrekking tot de juistheid en bruikbaarheid van de verkregen resultaten.
   dit proefschrift

2. De vaststelling dat, nadat een computergestuurd multiparameter optimaliserings-programma de scheiding van 18 aromatische amines heeft geoptimiseerd, de nauwelijks beargumenteerde toevoeging van 10% acetonitril aan de buffer de kwaliteit van deze scheiding nog dramatisch verbetert, is een geruststellend gegeven met het oog op toekomstige onderzoeksmogelijkheden op het gebied van de methodeontwikkeling in capillaire elektroforese.

3. De klassieke resolutievergelijking die door Otsuka et al. wordt gebruikt in de studie naar het gedrag van geladen componenten in micellaire elektrokinetische capillaire chromatografie is in deze vorm niet toepasbaar.
   dit proefschrift

4. De twee-marker techniek voor de bepaling van migratie-indices van geladen componenten in vrije zone elektroforese, zoals toegepast door Liang et al., maakt in feite gebruik van een ingewikkelde vorm van de gewone mobiliteitsvergelijking en draagt derhalve niet bij tot een betere beschrijving van het migratiegedrag.

5. De aannemer dat het partieel specifiek volume van de micellaire fase en dus ook dat van het gevormde complex tussen de micel en verschillende nucleosiden constant is na toevoegen van glucose is in tegenspraak met het voorgestelde interactiemechanisme dat uitgaat van het verdrijven van solventmoleculen tijdens complexvorming.
6. De veronderstelling dat vrije zone capillaire elektroforese wordt bedreven in afwezigheid van een stationaire fase gaat meestal voorbij aan het belang van de capillairwand.


8. De efficiëntie waarmee een analyse kan worden opgezet is evenredig met de kennis inzake de identiteit en (relatieve) hoeveelheden van de aanwezige componenten.

9. In de drukbevolkte hoogtechnologische informatiemaatschappij wordt het steeds moeilijker de bomen nog in het bos te zien.

10. Het feit dat de muziek van de Beatles door velen als tijdloos wordt omschreven getuigt van een slecht ontwikkeld tijdsbesef.

11. Het doorsturen van reclameboodschappen via RDS autoradiosystemen zou uit veiligheidsoverwegingen verboden moeten worden.

12. De typische stijl van de impressionisten is mede bepaald door één of meerdere oogkwalen waaraan de meeste kunstenaars die tot deze stroming behoorden leden. Richard Kendall

13. Het onvoorstelbare is onuitvoerbaar.

Hugo Corstjens
Delft, 17 december 1996