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Enantiomeric Separations using Chiral Counter-Ions

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Abstract
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This thesis describes the use of chiral counter-ions for the enantiomeric separation of amines in non-aqueous capillary electrophoresis. The investigations have been concentrated on studies of the influence, of the chiral counter-ion, the solvent, the electrolyte and the analyte, on the enantioselective separation.

Modified divalent dipeptides have been introduced in capillary electrophoresis for the separation of amino alcohols and chiral resolution of amines. Association constants for the ion-pair between dipeptide and amino alcohol could be utilized for development of separation systems with higher amino alcohol selectivity. Chiral discrimination (ion-pair formation) between the dipeptides and amines are preferably generated in non-aqueous background electrolytes (BGEs). The amount of triethylamine in the BGE determined the dipeptide charge and a divalent dipeptide promoted higher enantioselectivity than a monovalent dipeptide. An N-terminal-end blocking group and glutamic acid at the C-terminal-end of the dipeptide was important for chiral separation of the amines.

Chemometric and univariate methods have been employed for evaluation of suitable solvent compositions in the BGE. An experimental design including a single solvent as well as binary, ternary and quaternary mixtures of polar organic solvents, showed that optimal enantioresolution was obtained with an ethanol:methanol 80:20 mixture in the BGE. Furthermore, water was found to have an adverse influence on enantioselectivity and no enantioresolution was obtained with BGEs containing more than 30 % water.

An alkali metal hydroxide added to the BGE affected the chiral separation by competing ion-pair formation with the selector. The electroosmosis was reduced in order of decreasing alkali metal ion solvated radius and became anodic using K, Rb or Cs in ethanolic BGEs.

The correlation between the amino alcohol structure and the enantioselectivity was investigated using chemometrics. The obtained models showed that enantioselectivity for the amino alcohols was promoted by *e.g.* degree of substitution and substituent size on the nitrogen.

Keywords: Capillary Electrophoresis, Chiral Counter-Ion, Divalent Dipeptide, Enantioselectivity, Electroosmosis, Chemometrics, Molecular Descriptor, Structure-Enantioselectivity Relationship

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My mind rebels at stagnation. Give me problems, give me work, give me the most abstruse cryptogram, or the most intricate analysis, and I am in my own proper atmosphere.

Sir Arthur Conan Doyle

List of Papers

This thesis is based on the following papers, which are referred to in the text by the Roman numerals assigned below.

I Hedeland, Y., Haglöf, J., Beronius, P. and Pettersson, C. (2006) Effect of alkali metal hydroxides on the enantioseparation of amines using di-O-isopropylidene-keto-L-gulonic acid as the selector in NACE. *Electrophoresis*, 27(22):4469-4479.

II Haglöf, J. and Pettersson, C. (2010) Separation of amino alcohols using divalent dipeptides as counter ions in aqueous CE. *Electrophoresis*, 31(10):1706-1712.

III Haglöf, J. and Pettersson, C. (2010) Enantiomeric separation of amines using divalent dipeptides as chiral counter-ions in non-aqueous CE. *In manuscript*.

IV Haglöf, J. and Karlsson, A. (2010) Multivariate data analysis of a chiral separation system using N-derivatized dipeptides as chiral counter-ions on porous graphitic carbon stationary phase. *In manuscript*.

Table of Contents

Abbreviations

ACN	Acetonitrile
BGE	Background electrolyte
CE	Capillary electrophoresis
CV	Cross validation
CZE	Capillary zone electrophoresis
DIKGA	(–)-2,3:4,6-di-*O*-isopropylidene-2-keto-*L*-gulonic acid
$\Delta\mu$	Mobility difference
DoE	Design of experiments
EOF	Electroosmotic flow
EtOH	Ethanol
HAE	*L*-alanyl-*L*-glutamic acid
HPLC	High performance liquid chromatography
K	Association constant
μ_{eff}	Effective mobility
μ_{eo}	Electroosmotic mobility
MeOH	Methanol
μ_m	Average mobility
N	Efficiency measured as number of theoretical plates
NACE	Non-aqueous capillary electrophoresis
OPLS	Orthogonal projection to latent structures
PC	Principal component
PCA	Principal component analysis
PLS	Projection to latent structures by means of partial least squares
2-PrOH	2-propanol
Q^2	Goodness of prediction
R^2	Goodness of fit
R_s	Resolution
TEA	Triethylamine
ZAE	*N*-benzyloxycarbonyl-*L*-alanyl-*L*-glutamic acid
ZEG	*N*-benzyloxycarbonyl-*L*-glutamyl-glycine
ZEY	*N*-benzyloxycarbonyl-*L*-glutamyl-*L*-tyrosine
ZFE	*N*-benzyloxycarbonyl-*L*-phenylalanyl-*L*-glutamic acid
ZGE	*N*-benzyloxycarbonyl-glycyl-*L*-glutamic acid
ZYE	*N*-benzyloxycarbonyl-*L*-tyrosyl-*L*-glutamic acid

Introduction

The term chirality is derived from the Greek word for hand, χειρ. A molecule is said to be chiral if its mirror images are non-superimposable, *i.e.* there is a form of molecular asymmetry. Chirality was first discovered by Louis Pasteur in 1848 when investigating two different crystalline forms of tartaric acid. However, Pasteur described the molecule as 'dissymmetrical' and it was Lord Kelvin who coined the expression chiral in 1894 [1].

Where drugs are concerned, chirality is of fundamental interest because, since the environment in a biological context is chiral, the two forms of the drug, the enantiomers, may interact in different manners with *e.g.* receptors [2]. The difference between the enantiomers varies from identical pharmacological activity, like *e.g.* promethazine [3], through no or only low activity for one enantiomer, *e.g.* warfarin [4] and citalopram [5], to cases where one enantiomer is toxic, *e.g.* dopamine [6] and thalidomide [7]. There are also chiral drugs where the pharmacological activity of the enantiomers is different, *e.g.* the (*S,R*)-enantiomer of labetalol has primarily α-blocking properties, while the (*R,R*)-enantiomer is a β-blocker [8-9].

Despite early knowledge of chirality and its implications, chiral drugs were developed and marketed as racemates, *i.e.* as mixtures of equal amounts of both enantiomers, until the 1980s [10]. The main reason for this was a deficiency of suitable analytical tools for use during drug development, pharmacological and pharmacokinetic studies and quality control [8]. Chiral methods employing liquid chromatography (LC) and gas chromatography (GC) were the first to be developed [11] and, in 1992, the Food and Drug Administration (FDA) issued a statement stipulating that, only in particular cases should chiral drugs be developed as racemates [12]. Furthermore, the FDA guidelines for registration of new drugs [13] state that, for single-enantiomer drugs, the other enantiomer should be regarded as any other impurity, that the specification should include determination of enantiomeric purity and that possible racemization during storage, *i.e.* conversion between the enantiomers, should have been investigated.

Today the dominant technique for separation of chiral compounds is high performance liquid chromatography (HPLC), but capillary electrophoresis (CE) has gained increasing interest during the last decades [14], and research to obtain improved chiral separations is ongoing. Recently, Radim Vespalec wrote [15]:

> It is well-founded to expect that life-science research will ask for the solution of novel and more demanding tasks that either result from or are connected with chirality. This is the main reason why [we need] to deepen the understanding of the interactions between chiral selectors and chiral analytes and why [we should strive] to enlarge the set of accessible chiral selectors.

This thesis contains one piece of the ongoing research puzzle, namely, the development of methods for chiral separation, in addition to which, it contributes to the general body of knowledge by increasing the understanding of what factors govern interactions between chiral substances. New chiral resolving agents have been introduced in CE and the environment for the separation, *i.e.* solvents and electrolytes, has been investigated as well. In addition to this, the structure of the analyzed amines has been coupled the enantioselectivity. This is my piece, my contribution to the puzzle. Enjoy!

Chiral Separations in Capillary Electrophoresis

The pioneering work on electrophoresis in a tube was done by Tiselius during the 1930s, for which he was awarded the 1948 Nobel Prize in Chemistry [2]. During the 1950s, further research on electrophoresis in small glass tubes was done more or less simultaneously by Kolin [16] and Hjertén [17], and the first apparatus for capillary electrophoresis was described in 1967 [18]. It was not until 1981, however, that modern capillary zone electrophoresis (CZE) utilizing 75 µm internal diameter capillaries was introduced by Jorgenson and Lukacs [19-20]. CE has several advantages compared with HPLC, and not least amongst these are its simplicity, higher efficiency and the ability to separate a wide range of substances [21]. Disadvantages include lower sensitivity and worse precision [22].

Chiral separations were introduced in CE by Gassmann *et al* in 1985 [23], using a copper(II) L-histidine complex for chiral resolution of derivatized amino acids. Since then, many different groups of chiral resolving agents, selectors, have been employed: cyclodextrins [24], crown ethers [25], proteins [26], macrocyclic antibiotics [27] and low molecular weight chiral counter-ions [28-29]; the cyclodextrins being the most frequently used group of selectors [30].

Several reviews have been written on chiral separations as exemplified by these references [14, 31-34].

Non-Aqueous Solvents

The first separation method using CE with non-aqueous solvents was presented by Walbroehl and Jorgenson in 1984 [35]. However, it was not until 1996 that the first chiral separations in non-aqueous CE (NACE) were demonstrated [28-29, 36-37]. The benefits of using non-aqueous solvents for chiral analyses in CE include possible higher solubility of the selector [38] or the analyte [39], the ability of using selectors lacking chiral selectivity in an aqueous environment [40] and the potential of changing the selectivity by exchanging or mixing solvents [41]. The drawbacks of using non-aqueous solvents in CE are, *e.g.*, the difficulty of measuring the pH* (apparent pH), and the limited information available about the pKa*-values in many of the solvents used [42-44]. For a detailed discussion on the advantages and disadvantages of non-aqueous solvents in CE, see the references [41, 45].

Chiral counter-ions, or ion-pairing selectors, benefit from the use of non-aqueous solvents rather than water [46]. The level of interaction between ions in a solution depends on the dielectric constant, where a higher value demotes and a lower value promotes interaction [47]. Thus, depending on the selection of solvents, ion-pairing will be more or less pronounced. However, ion-pairing between selector and analyte might be impaired by the presence of other electrolytes that form competing ion-pairs with the selector and/or analyte ions [41].

Addition of water to the background electrolyte (BGE) generally has a negative impact on the chiral selectivity [48]. Tjørnelund and Hansen [49] showed that small amounts of water (0.5 %) might increase reproducibility, while Lodén *et al* [50] showed that the addition of higher amounts of water (up to 25 %) effectively reduced chiral selectivity.

A more comprehensive overview of chiral separations in NACE is available in these reviews [41, 46, 48, 51].

The Chiral Separation System

In capillary electrophoresis, analytes are separated according to their charge-to-volume ratio. Since enantiomers have the same charge and volume, they are inseparable in an ordinary achiral CE background electrolyte. Thus, a chiral selector is added to the background electrolyte to distinguish between the enantiomers. The selector is one of the enantiomers of a chiral compound and will, therefore, interact to a different extent with the two enantiomers, changing their apparent charge and/or volume, making enantiomeric separation possible [15].

In 1952, Dalgliesh described the mechanisms underlying chiral separation in the three-point interaction model [52]; this was later generalized by Davankov and Kurganov in 1983 [53] and Pirkle and Pochapsky in 1989 [54]. The model states that three different points of interaction are required between the chiral analyte and the chiral selector for chiral discrimination to be possible. The interactions can be either attractive or repulsive, as long as the sum of the interactions is attractive. Thus, for chiral counter-ions, the ion-ion interaction between the selector and the analytes is only one of the three points of interaction between the molecules.

In addition to the chiral selector and the solvent(s), the BGE may contain different electrolytes. These might constitute a buffer, affect the charge of the chiral selector or influence the electroosmotic flow (EOF) [41]. As mentioned above, the electrolyte components might decrease chiral selectivity by competitive non-stereoselective ion-pair formation with the chiral selector and/or analytes. A review by Fillet *et al* [55] discussed the influence of BGE electrolytes and solvents on separation selectivity in NACE.

Determination of Constants

When an analyte is introduced into a BGE containing a chiral selector, the reversible interaction between the R-enantiomer of the analyte (A) and the chiral selector (S) can be described by the following equilibrium (Eq. 1) and the corresponding equilibrium constant (Eq. 2). This is only valid for 1:1 complexes between selector and analyte, but Chankvetadze [56] concluded that this is the case for the majority of the chiral separations using chiral selectors, a statement that was further confirmed by Vespalec [57].

$$(R)A + S \rightleftharpoons (R)AS \qquad \text{(Eq. 1)}$$

$$K_{(R)AS} = \frac{[(R)AS]}{[(R)A][S]} \qquad \text{(Eq. 2)}$$

Thus, the resulting mobility for (R)A, $\mu_{(R)A,\,eff}$, is a function of the mobilities for the free analyte, μ_A, and for the complex between the analyte and the selector, μ_{AS}, as described by Eq. 3 [24]. As mentioned above, the mobilities of the free enantiomers are identical, as is also generally the case for the mobilities of the complexes between the selector and the enantiomers [15]. However, in rare cases, the mobilities for the complexes have been shown to differ [58-59].

$$\mu_{(R)A,\,eff} = \frac{\mu_A + \mu_{AS} K_{(R)AS}[C]}{1 + K_{(R)AS}[C]} \qquad \text{(Eq. 3)}$$

Wren and Rowe [60] described the resulting mobility difference, $\Delta\mu$, between the enantiomers by Eq. 4. It can be seen from this equation that there will be an optimum concentration of the chiral selector where the mobility difference is highest. This concentration is dependent on the equilibrium constants according to Eq. 5.

$$\Delta\mu = \frac{(\mu_A - \mu_{AS})(K_{(R)AS} - K_{(S)AS})[C]}{1 + (K_{(R)AS} + K_{(S)AS})[C] + K_{(R)AS} K_{(S)AS}[C]^2} \qquad \text{(Eq. 4)}$$

$$[C]_{opt} = \frac{1}{\sqrt{K_{(R)AS} K_{(S)AS}}} \qquad \text{(Eq. 5)}$$

From Eq. 3, which analogously holds for the S-enantiomer as well, it is then possible to calculate the association constants between analyte enantiomer and chiral selector, either by linearization methods or non-linear curve-fitting algorithms [61-62]. Using Eq. 5, the selector concentration that results in the highest mobility difference between the enantiomers can then be calculated. However, the optimum resolution is not necessarily found at the highest $\Delta\mu$, as discussed by Karbaum and Jira [39], since other factors con-

tribute to the resolution, see Eq. 6 below [63]. The efficiency, N, and the average mobility, μ_m also affect the resolution, μ_m being dependent on the electroosmosis and the effective mobility, too.

$$R_s = \frac{\sqrt{N}}{4} \times \frac{\Delta\mu}{\mu_m} \qquad \text{(Eq. 6)}$$

Chemometrics

Chemometrics is a generic term for mathematical tools used in chemistry. Modern analytical techniques generate very large amounts of data that cannot be analyzed by standard two-dimensional plots. The mathematical tools described here reduce the number of variables, and may thereby help to reveal information that would otherwise be hidden in the sheer volume of data [64].

Design of Experiments

The Design of Experiments (DoE) is not a chemometric tool *per se*, but rather, is a way of conducting experiments that yields as much information as possible from a limited number of experiments. Instead of studying one variable at a time, all variables are modified simultaneously according to a well defined plan. The results are then analyzed using one or more of the mathematical tools described in the subsections below [65].

There are many different designs to choose from, depending on the objective of the study – DoE can be employed for screening or optimization purposes as well as method validation [66]. Common designs include full and fractional factorial designs, Plackett-Burman designs and D-optimal designs. All of these incorporate the concepts of center points and randomization. The center points are experimental runs intentionally conducted to examine the centre of the experimental volume defined by the range of the variables. Usually at least three experiments are carried out during the course of the study. The results of these experiments help describe curvature in the experimental volume as well as determine whether there has been any variation over the time during which the study was conducted. Randomization of the experiments is conducted to eliminate possible systematic variations [67].

Principal Component Analysis

Principal component analysis (PCA) is one of the most widely used projection methods [64]. With PCA, the number of variables is reduced to a few principal components (PCs). The PCs are linear combinations of the original variables and each PC summarizes all variation in the data along one axis.

The first PC describes the direction of most variation in the data, while the second PC is orthogonal to the first and describes the direction of most variation when the first principal component is subtracted from the data. Together, the PCs describe all variation in the dataset except a small remainder, which usually consists of experimental noise [68]. The number of principal components for a dataset defines the rank of the dataset, and, while data with highly correlated variables will have few principal components, data with no correlation between the variables will have the same number of PCs as it had of original variables [69].

PCA can be used to get an overview of a large dataset as well as for the identification of important variables or groupings in the data. PCA models are usually visualized by the use of score plots, where the relations between the experimental observations are seen, and loadings plots where the relations between the variables can be observed [68].

(Orthogonal) Projections to Latent Structures

The initialism PLS stands for projections to latent structures by means of partial least squares, an expression which is, rather confusingly, sometimes shortened to both "projections to latent structures" and "partial least squares". However, PLS is an extension of PCA, where two kinds of variables are considered: ordinary variables and responses. When using PLS, the aim is to correlate the variation in the responses to the changes in the variables. This can be described as fitting two PCA models at the same time, one for the variables and the other for the responses, while simultaneously aligning them to each other [70-71].

Orthogonal PLS (OPLS) is a recent extension of PLS, where the variable data that is not correlated to the responses is excluded from the model. Thus, only data relevant for the interpretation of the responses are retained, which simplifies the model and increases its interpretability [72]. (O)PLS is commonly used when analyzing quantitative structure-activity relationships (QSAR) [70], but has also been used to describe retention relationships (QSRR) [73] and enantioselectivity relationships (QSER) [74].

(O)PLS models can be visualized in the same manner as PCA ones, with score plots for observation relations and, instead of the PCA loadings, weight plots for variable and response relations [75].

Model Significance and Validation

The significance of a model is usually estimated using R^2- and Q^2-values. R^2 describes the goodness of fit of the model, *i.e.* how well the model fits the data it is based on. Q^2 is the goodness of prediction, *i.e.* how well the model can predict responses for observations with new sets of variables. R^2- and Q^2-values close to 1.0 indicate good models, and the limits for adequate models are usually set to 0.7 for R^2 and 0.4 for Q^2 [68].

Chemometric models are often validated using either external data or cross-validation. With external data, a dataset not used in fitting the model is used for prediction. The predicted data is then compared with experimental values. This method is, however, limited to the occasions when large amounts of data are available.

Cross-validation (CV) is used when only a small amount of data is available. The data on which the model is based is divided into several subsets. The model is then fitted using all of the subsets bar one, and this exception is then used for prediction. This is then repeated for all subsets. It has been shown that, for small datasets (tens of observations), CV yields better models than one obtains by saving a small subset of the data for use in external validation [76].

Aims

The aim of this thesis was to increase the understanding of the interactions between chiral species, and therefore chiral separation. More specifically, the aims of the papers were:

- To study the influence of different alkali metal hydroxides on the enantiomeric separation of a number of test solutes (**Paper I**).
- To study the competing ion-pair formation between the chiral selector and the alkali metal ions (**Paper I**).
- To introduce a number of divalent N-derivatized dipeptides as counter-ions in aqueous CE for enhanced separation of amino alcohols (**Paper II**).
- To investigate the ion-pairing properties of the said dipeptides in an aqueous environment (**Paper II**).
- To introduce the said dipeptides as chiral counter-ions in CE (**Papers II** and **III**).
- To investigate the influence of the solvents on chiral discriminating ion-pairing (**Paper III**).
- To correlate molecular structure of some amino alcohols to the enantiomeric selectivity by means of molecular descriptors and multivariate data analysis (**Paper IV**).

Results and Discussion

This section contains the summarized results from **Papers I–IV**. For reasons of clarity, the results are not presented paper by paper, but rather grouped together by which part of the separation system, as described in subsection "The Chiral Separation System" above, they belong to. The reader is referred to the individual manuscripts for a more detailed discussion of the individual experiments and results.

Chiral Counter-Ions

Different kinds of chiral selectors have been discussed in the section "Chiral Separations in Capillary Electrophoresis" above. Chiral counter-ions are selectors that form ion-pairs with the analytes.

Eight different chiral counter-ions were utilized in the research carried out for this thesis: (–)-2,3:4,6-di-*O*-isopropylidene-2-keto-*L*-gulonic acid (DIKGA), see Figure 1, and a number of divalent dipeptides, see Table 1.

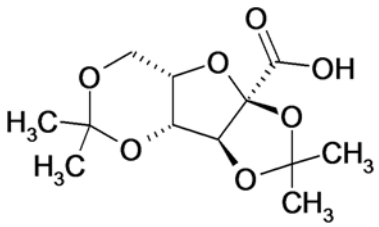


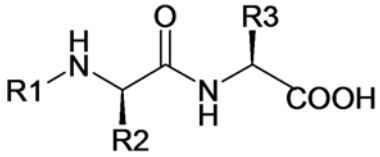
Figure 1. Structure of the chiral counter-ion DIKGA.

DIKGA is an L-ascorbic acid derivative that has been utilized as a chiral counter-ion in both HPLC and CE [40, 50, 77-78]. In **Paper I**, DIKGA was added to methanolic or ethanolic solutions containing an alkali metal hydroxide. A number of pharmacologically active amines were then enantiomerically separated using the DIKGA-containing solution as the BGE.

The divalent dipeptides consist of two amino acids, one of which is glutamic acid with a carboxylic acid in the side chain. The first three dipeptides, *N*-benzyloxycarbonyl-*L*-alanyl-*L*-glutamic acid (ZAE), *N*-benzyloxycarbonyl-glycyl-*L*-glutamic acid (ZGE) and *N*-benzyloxycarbonyl-*L*-phenylalanyl-*L*-glutamic acid (ZFE), which have been used previously as

chiral mobile phase additives in HPLC [79] were also used in **Paper IV**. In addition to these three counter-ions, *L*-alanyl-*L*-glutamic acid (HAE) was included in **Paper II**, while all seven dipeptides were used as chiral counter-ions in **Paper III**.

Table 1. Structure of the divalent dipeptides used as chiral counter-ions.



Name	R1	R2	R3
N-benzyloxycarbonyl-*L*-alanyl-*L*-glutamic acid	Z	CH_3	CH_2CH_2COOH
N-benzyloxycarbonyl-glycyl-*L*-glutamic acid	Z	H	CH_2CH_2COOH
N-benzyloxycarbonyl-*L*-phenylalanyl-*L*-glutamic acid	Z	$CH_3C_6H_5$	CH_2CH_2COOH
N-benzyloxycarbonyl-*L*-tyrosyl-*L*-glutamic acid	Z	$CH_3C_6H_4OH$	CH_2CH_2COOH
N-benzyloxycarbonyl-*L*-glutamyl-glycine	Z	CH_2CH_2COOH	H
N-benzyloxycarbonyl-*L*-glutamyl-*L*-tyrosine	Z	CH_2CH_2COOH	$CH_3C_6H_4OH$
L-alanyl-*L*-glutamic acid	H	CH_3	CH_2CH_2COOH

Z = $C(O)OCH_2C_6H_5$

In **Paper I**, the optimum concentration of the chiral selector (DIKGA) was analyzed by determining Δμ, Figure 2. For two of the analytes, pindolol and isoprenaline, the optimum total selector concentration is found at approximately 15 mM. Using a mathematical iterative process, taking into account the different ion-pairing interactions in the BGE and the autoprotolysis of the solvent, the concentration of free deprotonated selector was determined to be 0.98 mM. This corresponds well with the theoretical optimum selector concentration calculated by using Equation 5 and the ion-pairing constants determined by use of conductometry.

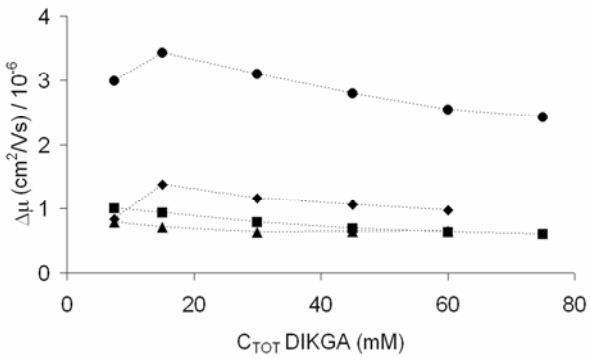


Figure 2. Selector concentration and the mobility differences (**Paper I**). BGE: (–)-DIKGA and KOH (molar ratio 5:2) in EtOH. (■) *rac*-Atenolol, (●) *rac*-isoprenaline, (♦) *rac*-pindolol and (▲) *rac*-propranolol.

In **Paper II**, the divalent dipeptides were introduced as counter-ions in aqueous CE and used to enhance the selectivity when separating different amino alcohols. It was found that the dipeptides form ion-pairs with the amino alcohols and that a high concentration of divalent dipeptide brought about a reversal in the effective mobility, as can be seen in Figure 3.

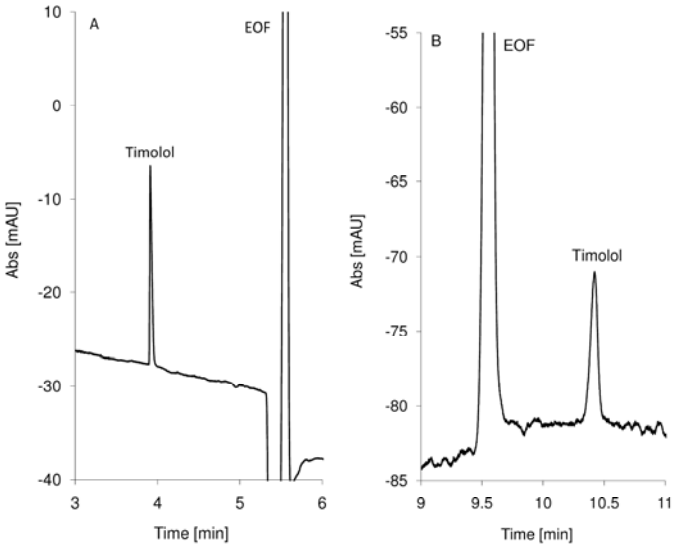


Figure 3. Electropherograms of timolol (**Paper II**). Analyte concentration = 0.5 mM. BGE: (A) 10 mM ZFE and 20 mM NaOH in H_2O. (B) 90 mM ZFE and 180 mM NaOH in H_2O.

It was observed that the dipeptides showed a stronger interaction with the amino alcohols in divalent form than in monovalent or uncharged form as is evident from Figure 4. It can also be observed that the presence of an N-terminal-end blocking group increased the interaction between the counter-ion and the amino alcohol, which might indicate that the electrostatic interaction is accompanied by a hydrophobic interaction. Diamond [80] introduced the concept of water-structure enforced ion-pairing, which might be applicable in this case. This means that the ion-pairs are held together both by attraction between the opposite charges and by a force from the water that drives the hydrophobic parts of the molecules together in order to maximize entropy.

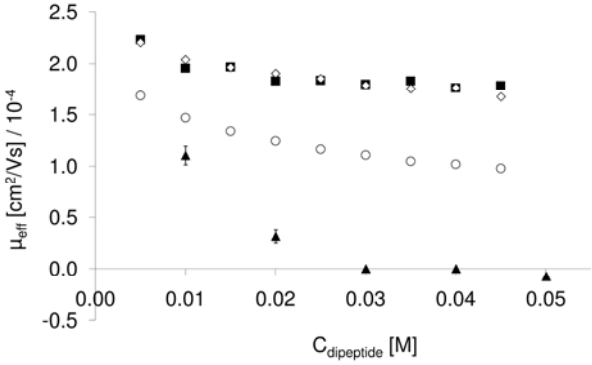


Figure 4. Influence of dipeptide concentration on the effective mobility (μ_{eff}) of timolol (**Paper II**). Analyte concentration = 0.5 mM. ■: uncharged HAE, ◊: monovalent HAE, ○: divalent HAE, ▲: divalent ZAE.

Association constants can be calculated using Equation 3 in section "Determination of Constants". The association constants between the analyzed amino alcohols and the divalent dipeptides were determined using Equation 3 and the Levenberg-Marquardt non-linear curve-fitting algorithm [81-82]. The determined constants could be used to develop separation systems with enhanced selectivity for different amino alcohols.

Since the dipeptides are chiral, they were also employed as chiral counter-ions in aqueous CE, but no enantioresolution was observed in pure aqueous BGEs. However, in methanolic BGEs with a low water content (below 25 %), separation between amino alcohol enantiomers was observed.

The chiral separation possibilities were further developed in **Paper III** using the divalent dipeptides as counter-ions with the set of divalent dipeptides being extended to include all of the counter-ions presented in Table 1. This enabled a study to be made of how the structural properties of the dipeptides affected the chiral selectivity.

No chiral selectivity was found for the dipeptide without an N-terminal-end blocking group, HAE, or for the dipeptides with the glutamic acid in other than the C-terminal position, ZEY and ZEG. It is, therefore, likely that interaction between dipeptide and amino alcohol is localized to the N-blocking group, the carboxylic acid in the side chain and/or the side chain of the second amino acid.

The use of divalent counter-ions is especially interesting in the context of the theoretical study by Wren and Rowe [60]. Besides defining many of the equations used in this thesis, they concluded that a larger difference in mobility between the free analyte and the analyte:selector complex would result in a larger mobility difference, given that no other parameters were found to have been altered. Since the dipeptides are protolytes, the effect of changes in the counter-ion charge could be studied, see Figure 5. Interestingly, the maximum mobility difference was observed at lower than double dipeptide

charge, *i.e.* around 1.5. The reason for this might be competing ion-pairing between the base, triethylamine (TEA), and the chiral counter-ion, something that was observed in **Paper I**. This decreases the concentration of the dipeptide available for interaction with the amino alcohol. There is, therefore, an optimum dipeptide:base ratio as demonstrated by Figure 5.

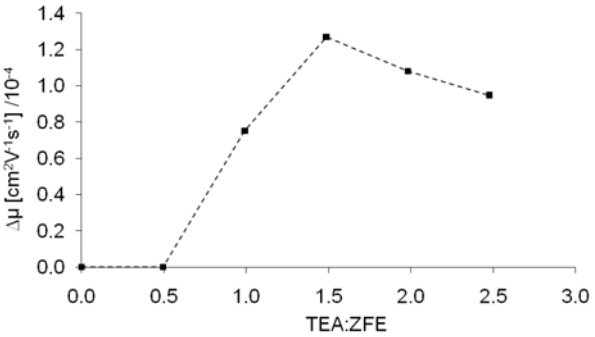


Figure 5. Mobility difference as a function of dipeptide:TEA ratio (**Paper III**). BGE: 10 mM ZFE and varying concentrations of TEA in MeOH. Analyte: 0.5 mM *rac*-timolol.

In **Paper III**, the impact of altering the concentration of the divalent dipeptides on the enantiomeric mobility difference was examined. In Figure 6, it can be seen that, while the mobility difference exhibits a maximum at around 7 mM, the resolution increases with increasing dipeptide concentration. This is attributable to the impact of the other parameters on the resolution, as is evident by examining Equation 6. In this case, the increasing resolution was mainly due to decreasing electroosmosis.

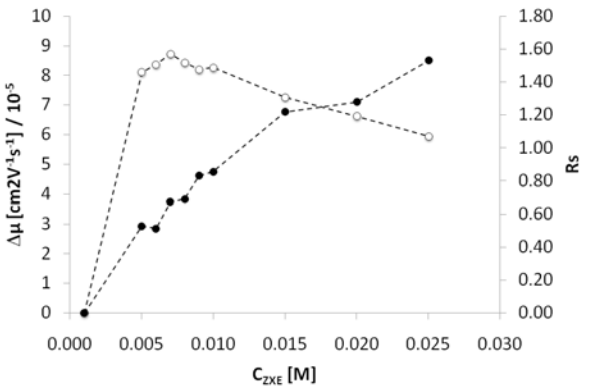


Figure 6. The mobility difference (○) and resolution (●) as a function of dipeptide concentration (**Paper III**). BGE: ZYE:TEA 1:1.5 in MeOH:EtOH 20:80. Analyte: 0.5 mM *rac*-timolol.

Electrolytes

The utilized chiral counter-ions are acidic protolytes, and the addition of a base causes them to become charged through deprotonation. In the papers constituting this thesis, all chiral counter-ions are added to the BGE along with a base. In most cases, the charge of protolytes is controlled using a suitable buffer. However, when using ion-pairing chiral selectors, ions from the buffer might form competing ion-pairs with the selector and/or the analyte. Therefore, only a base was added to the background electrolyte to deprotonate the chiral counter-ion in the investigations reported here. Thus the chiral counter-ion, being a weak protolyte, will buffer the BGE.

In **Paper I**, a number of different alkali metal hydroxides were investigated to examine their influence on the enantiomeric separation of a number of amines. NaOH was used in **Paper II**, while TEA was used in **Papers III** and **IV**.

The importance of selecting the most appropriate base to deprotonate the chiral counter-ion is evident from Figure 7. Co-migrating atenolol enantiomers were fully resolved when LiOH was exchanged for KOH.

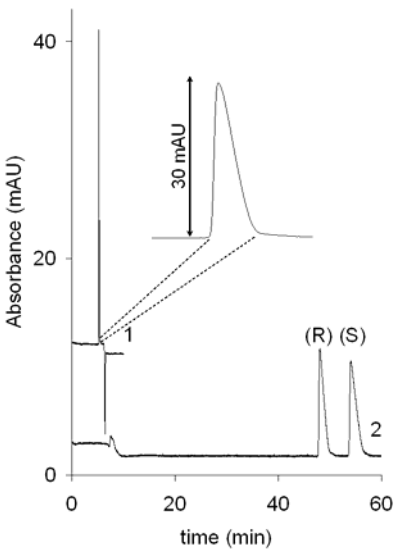


Figure 7. The influence of LiOH and KOH on the enantioseparation of atenolol. L_{det}: 8.5 cm, U: 30 kV, BGE: 50 mM (–)-DIKGA and 20 mM LiOH (1) or KOH (2) in ethanol. The positive peak in electropherogram 1 corresponds to *rac*-atenolol and the negative peak to the EOF. This figure has been reproduced from **Paper I**.

The alkali metal hydroxides were shown to influence the electroosmotic flow as well as the effective mobility of the analyzed amines and the mobility difference between the amine enantiomers. The electroosmotic flow decreased with decreasing solvated radius of the alkali metal ions, *i.e.* Li^+ >

$Na^+ > K^+ > Rb^+ > Cs^+$, as is evident from Figure 8. This trend was remarked upon by Porras *et al*, when they studied different alkali metal acetates [83]. However, as can be seen in the figure, the direction of the electroosmosis was reversed when using alkali metal ions of smaller solvated radius. Reversed electroosmosis has also been observed before, but only using hydrophobic cations in methanolic BGEs [84-85]. From simulations, Greberg and Kjellander [86] proposed that charge inversion might occur in the electric double layer in water-based monovalent electrolytes. In **Paper I**, it was proposed that the anodic electroosmosis might be attributable to attraction of an excess of the alkali metal ions with the smallest solvated radius, causing charge inversion in the diffuse layer.

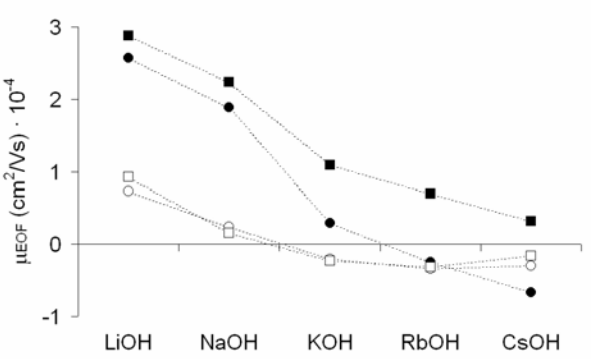


Figure 8. Influence of alkali metal hydroxide on the EOF (**Paper I**). Solute: 0.1 % mesityl oxide. x in the lower part of this legend refers to the respective alkali metal ion. (–)-DIKGA and alkali metal hydroxide in MeOH or EtOH. (■) 25 mM DIKGA and 10 mM xOH and (●) 50 mM DIKGA and 20 mM xOH in MeOH. (□) 25 mM DIKGA and 10 mM xOH and (○) 50 mM DIKGA and 20 mM xOH in EtOH.

While the effect of the alkali metal hydroxides on the effective mobility, μ_{eff}, generally showed the inverse pattern as compared with that identified for the electroosmosis, *i.e.*, increasing with decreasing solvated radius, the effect on the mobility difference was lower. Figure 9 shows how the mobility difference depends on the choice of alkali metal hydroxide in ethanolic BGEs for four amines. There is some correlation between the mobility difference and the solvated radius of the alkali metal ions, but not as obvious as for the electroosmosis or the effective mobility.

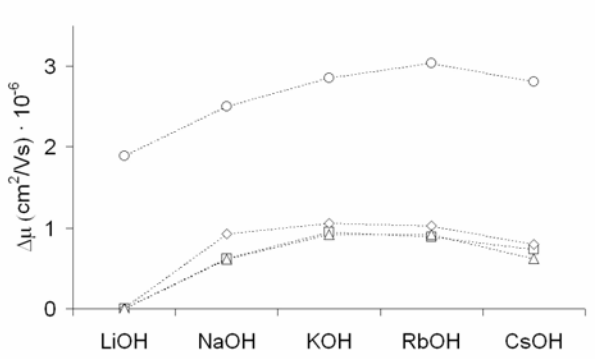


Figure 9. The influence of the alkali metal hydroxide on the mobility difference (Δμ) for the enantiomeric amines (**Paper I**). BGE: 50 mM (–)-DIKGA and 20 mM alkali metal hydroxide in EtOH. (□) *rac*-atenolol, (○) *rac*-isoprenaline, (◊) *rac*-pindolol and (Δ) *rac*-propranolol.

Ion-pairing association constants were determined for both alkali metal ion:DIKGA ion-pairs and amine:DIKGA ion-pairs by use of conductometry. It was found that the use of alkali metal ions with a higher association constant resulted in a higher enantiomeric mobility difference. This is probably owing to that a lower concentration of free DIKGA is closer to the optimum concentration described previously in Figure 2.

Solvents

In **Paper II**, water was used as the solvent, in contrast to **Paper I**, where either methanolic or ethanolic BGEs were employed. In **Paper III**, the effect of different solvents was investigated, and 2-propanol and acetonitrile were included, as well as the previously utilized methanol and ethanol. Finally, in **Paper IV**, a mixture of methanol, acetonitrile and ethyl acetate was used.

In most of the papers in this thesis, the separation is performed in non-aqueous solvents. However, since many of the solvents and electrolytes utilized are either hydrates or hygroscopic, the effect of added water on the separations has been studied. Tjørnelund and Hansen [49] investigated the effect of added water on NACE separations and found that, while only small differences in selectivity, efficiency and EOF were observed on addition of 0.5 % water to the non-aqueous BGE, reproducibility was enhanced.

In **Paper I** it was concluded that small additions of water (≤ 10 %) increased the electroosmosis and the effective mobility, while lowering the enantiomeric mobility difference. In **Papers II** and **III** it could be seen that higher amounts of water (> 25 %) removed all chiral selectivity.

In **Paper III** the solvents and their effect on the chiral separation were studied more thoroughly. A multivariate study including experimental data

like the effective mobility, electroosmosis, maximum mobility difference and optimum chiral counter ion concentration was performed. The experimental data was correlated to data obtained from the literature for the solvents, including the dielectric constant, ε^0, viscosity, η, and hydrogen donor, HBD, and acceptor, HBA, ability.

Figure 10 shows the two first components in the constructed PLS model. It can be seen that there is a strong correlation between the dielectric constant-viscosity quotient and the effective mobility and the electroosmosis, *c.f.* [87]. This is also the reason for the lack of enantioselectivity in water – the high dielectric constant decreases ion-ion interactions by shielding the ion charges [46].

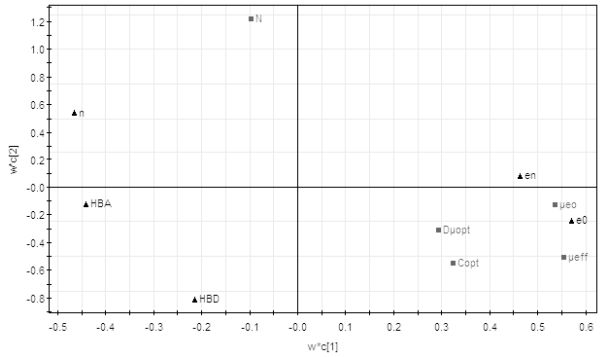


Figure 10. The first two components in the PLS model of solvent characteristics and experimental data (**Paper III**).

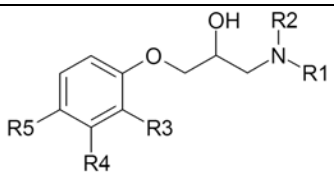
Design of Experiments was used to optimize the solvent composition in the BGE. The four solvents, MeOH, EtOH, 2-PrOH and ACN, were used in binary, ternary and quaternary mixtures together with the chiral counter-ion ZFE, with timolol as the analyte. The results showed that, compared with the methanolic BGEs, ethanol and 2-propanol increased resolution mainly by decreasing the EOF. Acetonitrile increased the enantiomeric mobility difference, but the resolution decreased owing to an increase in the electroosmosis and the effective mobility. In order to get full enantiomeric resolution ($R_s > 1.5$), while retaining as short an experimental time as possible, optimal solvent composition was determined as a 20:80 (v/v) methanol:ethanol mixture.

Analytes

In **Paper IV**, the correlation between analyte structure, laid out in Table 2, and chiral selectivity was investigated. PLS models were built from experimental data extracted from previous studies [79, 88] and molecular descrip-

tors for the molecules analyzed. It was found that models with a good fit, $R^2 = 0.866$, and predictability, $Q^2 = 0.839$, could be constructed from the assembled data. This approach has been used before, *e.g.* by Caetano and Van der Heyden [69]. However, the constructed model contained 44 molecular descriptors and, while the effect of each descriptor on the chiral selectivity could be seen, the overall picture could not be interpreted, *i.e.* the correlation between the molecular structure and chiral selectivity was obscured.

Table 2. Structure of the investigated amino alcohols



Substance	R1	R2	R3	R4	R5
1	$CH(CH_3)_2$	H	H	H	H
2 (Alprenolol)	$CH(CH_3)_2$	H	CH_2CHCH_2	H	H
3	$CH(CH_3)_2$	H	$CH_2CH_2CH_3$	H	H
4	$CH(CH_3)_2$	H	$CH_2CH_2OCH_3$	H	H
5	$CH(CH_3)_2$	H	H	$CH_2CH_2OCH_3$	H
6 (Atenolol)	$CH(CH_3)_2$	H	H	H	CH_2CONH_2
7	$CH(CH_3)_2$	H	H	H	OCH_3
8	$CH(CH_3)_2$	H	H	H	C(O)H
9 (Metoprolol)	$CH(CH_3)_2$	H	H	H	$CH_2CH_2OCH_3$
10	$CH_2CH_2CH_3$	H	H	H	$CH_2CH_2OCH_3$
11	$C(CH_3)_3$	H	H	H	$CH_2CH_2OCH_3$
12	H	H	H	H	$CH_2CH_2OCH_3$
13	$CH(CH_3)_2$	CH_3	H	H	$CH_2CH_2OCH_3$
14	$CH(CH_3)_2$	H	H	H	$CH_2CH_2OCH_3$
15	CH_3	CH_3	H	H	$CH_2CH_2OCH_3$
16	$CH(CH_3)_2$	CH_2CH_3	H	H	$CH_2CH_2OCH_3$
17	-piperidine		H	H	$CH_2CH_2OCH_3$

Therefore, another approach was chosen, namely, that of using the available information on the molecular structure to reduce the number of molecular descriptors in the model. Only descriptors relevant to any of the structural differences between the molecules analyzed were retained in the model. This resulted in a model with a goodness of fit and predictability that were almost as high as those obtained with the previous model ($R^2 = 0.831$, $Q^2 = 0.786$), but with a much enhanced interpretability.

Models were built for the three different chiral counter-ions ZAE, ZFE and ZGE, and all models contained only 2–4 molecular descriptors. Figure 11 shows the correlation between the predicted and the experimental selectivity. Only two molecules show poor predictability, one with large

nitrogen substituents (no. 13) and one with a large unbranched nitrogen substituent (no. 10).

On the whole, larger molecules showed higher enantioselectivity than smaller ones. This was especially apparent when it came to substitution on the nitrogen; a higher degree of substitution and larger substituents gave increased enantioselectivity. Not much variation could be seen in the relative position of the ring substituents, nor was it found in their size.

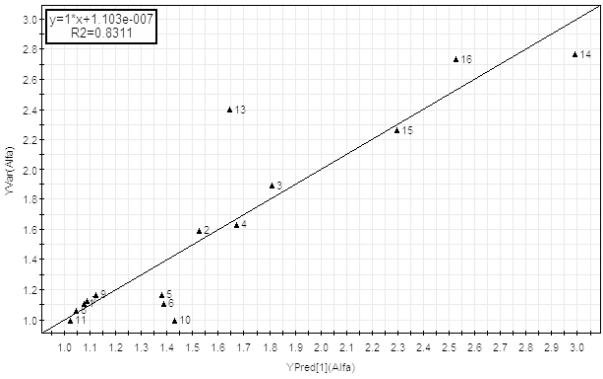


Figure 11. The final PLS model describing the relationship between α and the molecular structure, as presented by the molecular descriptors (**Paper IV**). Experimental *versus* predicted α.

Figure 12 shows the grouping of the molecules analyzed when combining two of the molecular descriptors in the model. A general correlation was revealed between the small molecules in the lower left corner and larger ones in the upper right. However, judging by the differences between the molecular descriptors, *Randic index* and *Kier Chi4p*, the model exhibits greater discrimination between the different molecules than can be attributed merely to size.

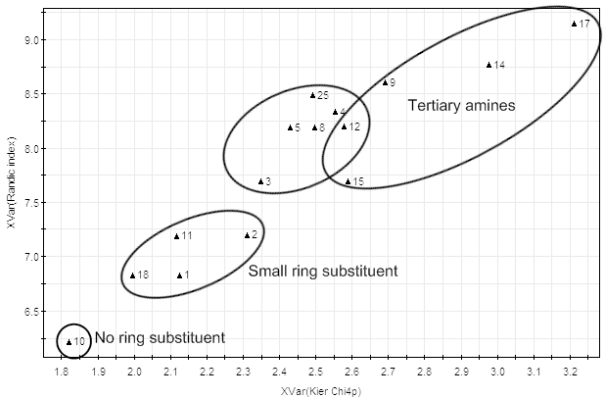


Figure 12. Final PLS model describing the relationship between α and the molecular structure as presented by the molecular descriptors (**Paper IV**). Molecule distribution as described by the molecular descriptors *Kier Chi4p* (x-axis) and the *Randic index* (y-axis).

Conclusions

In this thesis, different aspects of chiral separation systems have been investigated using chiral counter-ions in CE. The chiral counter-ion, solvents, electrolytes and the analytes have all been studied.

The divalent dipeptides ZAE, ZFE and ZGE have been introduced as counter-ions in aqueous CE. It was beneficial to use a base added to the BGE in order to control the dipeptide charge instead of a buffer, since the buffer ions impair the ion-pair formation between the analyte and counter-ion by competitive ion-pair formation with the counter-ion. Other results revealed that addition of the dipeptides to the BGE resulted in higher selectivity for the separation of different amino alcohols when used in divalent form than with the monovalent or uncharged form.

The divalent dipeptides have also been introduced as chiral counter-ions in NACE. The study included seven different dipeptides, with the intention of investigating the importance of different structural elements of the dipeptide in the chiral discriminating ion-pairing with amines. The N-terminal-end blocking benzyloxycarbonyl group was found to be important for chiral selectivity, as was the presence of glutamic acid as the C-terminal-end amino acid. As found in aqueous BGEs, divalent dipeptides produced higher selectivity ($\Delta\mu$) than monovalent or uncharged ones.

The choice of a suitable solvent composition in the BGE was found to be important for a chiral discriminating interaction between the chiral counter-ion and the analyte. The selectors used in this thesis only showed enantioselectivity in non-aqueous BGEs. No enantioselectivity was found in either aqueous BGEs, or when more than 25 % water was added to non-aqueous BGEs. The reason for this is probably the high dielectric constant of water, reducing the amount of ion-pairing in the solution. However, small variations (0.1–0.5 %) in the water content of the non-aqueous BGEs resulted in a negligible influence on the chiral separation.

Ethanol and methanol both proved to be suitable solvents for chiral separations in CE, and a mixture of 80 % EtOH and 20 % MeOH resulted in the highest resolution. Addition of acetonitrile produced higher chiral selectivity when added to methanolic or ethanolic BGEs, but the resolution was worse owing to increased electroosmosis.

Selecting the right base to transfer the chiral selector into its charged form also proved to be important. Different alkali metal hydroxides were evaluated and found to affect the majority of the separation parameters: μ_{eff}, μ_{eo},

Δμ and N. The effect was most pronounced for the EOF, where a smaller solvated radius of the alkali metal ion reduced or even reversed the electroosmosis.

Chemometrics, more specifically PCA and (O)PLS, has been shown to be a valuable tool for both optimization purposes and for investigation of structure-selectivity relationships. Models could be built from a large set of molecular descriptors, with high goodnesses of prediction and fit manifested as high R^2- and Q^2-values. However, models incorporating many descriptors lose their interpretability and also, therefore, their applicability. Models with few descriptors were demonstrated to have almost the same predictive power as models with a higher number of descriptors, with the included benefit of increased interpretability.

By correlating molecular structure, as described by the molecular descriptors, and experimentally determined enantioselectivity, it was found that molecules with a higher degree of substitution and larger substituents on the nitrogen showed higher enantioselectivity.

Future Studies

Returning to a statement made in the introduction, there is a need to increase the general understanding of chirally discriminating interactions and for new chiral selectors to be indentified. This thesis has hopefully contributed somewhat to both these areas, but many tasks remain.

In this thesis, a number of chiral counter-ions (selectors) based on amino acids were introduced in CE. Amino acids exist in a large variety, both standard, *i.e.* that can be found in eukaryotes, and non-standard. As a result of this, more research is needed to analyze potential di- and tripeptides that might be suitable for use as chiral selectors. In addition, different modifications to the dipeptides, *e.g.* the N-terminal-end blocking group used in this thesis, need investigation.

Determination of ion-pair association constants through precision conductometry made possible the determination of the pH* and the concentration of the free selector in the BGE. The powerful combination of CE and precision conductometry for the analysis can give much more information about the mechanisms governing chiral discrimination.

Chemometrics are mainly used for optimization purposes in separation science. Combining structural information and experimental data can give much information on the nature of the chirally discriminating interaction. Combination of chemometrics with NMR and/or molecular modeling might lead to new insights on the interaction between selector and analyte.

Swedish Summary / Svensk Sammanfattning

Kiralitet är en form av asymmetri på molekylär nivå och en molekyl sägs vara kiral ifall dess spegelbild inte kan roteras så att den ser exakt likadan ut som den ursprungliga molekylen. Ordet kiral härstammar för övrigt från det grekiska ordet för hand, och just händer är en ofta använd bild för att illustrera kiralitet, eftersom de ser likadana ut men är varandras spegelbilder. De två spegelbilderna av en molekyl kallas enantiomerer och har samma fysiokemiska egenskaper, t.ex. kokpunkt och viskositet, så länge de befinner sig i en omgivning som i övrigt inte är kiral.

Kiralitet är särskilt viktigt när det gäller läkemedel. I kroppen kommer läkemedelssubstansen att interagera med en miljö som i sig är kiral, eftersom kroppens enzym och receptorer är uppbyggda av aminosyrors ena enantiomer. Detta innebär att en läkmedelsmolekyls enantiomerer kan interagera på olika sätt med kroppen, och därmed ha olika verkan. Det finns exempel på allt från läkemedelssubstanser där båda enantiomererna har samma verkan, via olika verkningsgrad för enantiomererna, till fall där den ena enantiomeren är giftig. I sällsynta fall kan enantiomererna också ha helt olika verkan.

På grund av detta finns det ett behov av att kunna analysera enantiomerer; för att kunna bestämma vilken enantiomer det är, hur mycket det är av de två formerna och att helt kunna skilja dem åt. En vanlig teknik är vätskekromatografi (HPLC) där enantiomererna skiljs åt genom att de binder olika starkt till en s.k. kiral selektor som antingen sätts till den mobila fasen eller fästs på den stationära fasen.

I den här avhandlingen har en annan teknik använts, nämligen kapillärelektrofores (CE). Tekniken bygger på att molekyler vandrar med olika hastighet i ett elektriskt fält beroende på deras storlek och laddning. Enantiomerer, som har identisk storlek och laddning, skiljs åt genom tillsats av en kiral selektor till bakgrundselektrolyten (BGEn). Selektorn binder till enantiomererna och påverkar därmed deras storlek och eventuellt laddning. Eftersom enantiomererna binder olika starkt till selektorn påverkas de olika och kan separeras. I artiklarna som ingår i denna avhandling undersöks olika kirala selektorer, dessutom olika lösningsmedel, tillsatser till BGEn och enantiomerernas struktur.

I **delarbete I** undersöks olika hydroxider av alkalimetaller som tillsatser till BGEn. Den kirala selektorn som använts är en svag syra och tillsats av någon av hydroxiderna (som är starka baser) gör den negativt laddad. Detta gör

i sin tur att selektorn kan bilda neutrala jonpar med positivt laddade joner av den analyserade molekylen. I arbetet beskrivs hur hydroxiderna av olika alkalimetaller påverkar separationen av olika aminers enantiomerer. Den största effekten kunde ses på elektroosmosen, det vätskeflöde mot katoden som uppkommer i en kiselkapillär med elektrisk spänning över ändarna. En mindre solvatiserad radie (större kristallradie) hos alkalimetalljonen gav ett lägre elektroosmotiskt flöde, och vid tillräckligt liten solvatiserad radie blev flödet omvänt, dvs. mot anoden.

Valet av alkalimetall påverkade även selektiviteten mellan enantiomererna, antagligen genom att de olika alkalimetalljonerna bildar egna jonpar med selektorn och därmed konkurrerar med aminerna om de fria selektorjonerna. Olika grad av jonparbildning mellan de olika alkalimetalljonerna och selektorn gör att de påverkar jonparbildningen mellan selektorn och aminerna i olika utsträckning.

I **delarbete II** analyseras några olika dipeptider, molekyler uppbyggda av två aminosyror, för användning som motjoner i CE. Trots att det normalt sett inte bildas jonpar i vatten, visade sig dipeptiderna bilda jonpar med olika aminoalkoholer. Dipeptiderna innehåller dubbla karboxylsyregrupper, vilket gör att de kan befinna sig i både neutral, enkelt och dubbelt negativt laddad form. Enbart dipeptider i dubbelt negativt laddad form kunde påvisas bilda jonpar med aminoalkoholerna. Dipeptiderna är även modifierade med en hydrofob grupp i ena änden vilket skulle kunna göra att det omgivande vattnet hjälper till med bildandet av jonpar genom att tvinga ihop hydrofoba molekyler för att entropin ska minska.

Associationskonstanter för bildningen av jonpar mellan olika dipeptider och aminoalkoholer bestämdes och kunde användas för att utveckla bättre separationssystem för aminoalkoholerna. Slutligen användes en av dipeptiderna för att förbättra separationen av fem olika aminoalkoholer.

Delarbete III är en fortsättning av **delarbete II** och forsätter analysen av dipeptiderna, nu använda som selektorer för kiral separation av aminer. Koncentrationen av dipeptid i bakgrundselektrolyten liksom av den tillsatta basen (trietylamin, TEA) studerades för att finna optimala separationsbetingelser. Val av lösningsmedel visade sig ha stor betydelse för den kirala separationen; vatten påverkade den kirala separationen negativt, antagligen för att jonparen mellan dipeptid och amin som bildades i vatten var lösare sammanhållna än jonparen i polära organiska lösningsmedel och därmed förlorades den kirala igenkänningen. Optimal lösningsmedelssammansättning i BGEn bestämdes slutligen till en blandning av 80 % etanol och 20 % metanol.

En större uppsättning dipeptider analyserades i **delarbete III** än i **delarbete II**, vilket gjorde det möjligt att studera betydelsen hos några strukturella element i dipeptiderna. Den hydrofoba gruppen som diskuterades ovan visa-

de sig även vara nödvändig för den kirala separationen. Det var även närvaron av två karboxylsyregrupper vid den andra änden. Detta minskade antalet dipeptider som kunde användas som kirala selektorer från de sju som studerats till fyra. Dessa kunde sedan användas för kiral separation av olika aminer.

Slutligen, i **delarbete IV**, studerades hur strukturen hos analyten, i detta fall olika aminoalkoholer, påverkade den kirala selektiviteten. För detta ändamål användes kemometriska analysmetoder i kombination med molekylära deskriptorer. De senare är olika numeriska värden, bestämda antingen experimentellt eller teoretiskt, som beskriver olika aspekter av molekylen. Det kan handla om t.ex. storlek, laddning, närvaro eller frånvaro av olika funktionella grupper. Principalkomponentanalys (PCA) användes för att få en överblick av all data, medan PLS, från engelskans ”Projection to Latent Structures”, användes för att koppla samman kiral selektivitet och molekylstruktur.

Modeller med god anpassning till data liksom god predikterande förmåga kunde byggas från den insamlade datan. Eftersom modellen innehöll 44 olika deskriptorer var det dock svårt att korrelera molekylstruktur och selektivitet. Validering av modellen tydde också på att den innehöll experimentellt brus, vilket medförde risk för en överanpassad modell. Genom att använda information om vilka strukturella skillnader som fanns mellan molekylerna i modellen gick det att reducera antalet molekylära deskriptorer till ett fåtal (2–4). Dessa modeller uppvisade jämförbar anpassning till data och predikterande förmåga med den första modellen, samtidigt som tolkningen blev mycket enklare och kopplingen mellan struktur och selektivitet blev tydligare. Framförallt visade det sig att molekyler med fler substituenter på en kväveatom hade högre selektivitet än molekyler med få substituenter, liksom att större substituenter gav högre selektivitet än små.

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