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Chiral Separation of Dipeptides and Sulfoxides by CE and Investigation of the Chiral Recognition Mechanism

Dissertation

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Abbreviations

α-CD	native α-cyclodextrin
β-CD	native β-cyclodextrin
γ-CD	native γ-cyclodextrin
Asp	aspartic acid
Asn	asparagine
BGE	background electrolyte
CD(s)	cyclodextrin(s)
CE	capillary electrophoresis
CEC	capillary electrochromatography
CEKC	capillary electrokinetic chromatography
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CIL(s)	chiral ionic liquid(s)
CITP	capillary isotachophoresis
CM-α-CD	carboxymethyl-α-cyclodextrin sodium salt
CM-β-CD	carboxymethyl-β-cyclodextrin sodium salt
CM-γ-CD	carboxymethyl-γ-cyclodextrin sodium salt
CZE	capillary zone electrophoresis
DAD	diode array detector
DM-β-CD	2,6-dimethyl-β-CD
DM-β-CD50	2,6-dimethyl-β-CD with 50 % isomeric purity
DM-β-CD95	2,6-dimethyl-β-CD with 95 % isomeric purity
DS	degree of substitution
EKC	electrokinetic chromatography
EKI	electrokinetic injection
EMA	European Medicines Agency
EMO	enantiomeric migration order
EOF	electroosmotic flow

FDA	Food and Drug Administration
FS	fused-silica
GC	gas chromatography
HA-β-CD	heptakis(6-deoxy-6-amino)-β-CD heptahydrochloride
HDAS-β-CD	heptakis(2,3-di-O-acetyl-6-O-sulfo)-β-CD
HDMS-α-CD	hexakis(2,3-di-O-methyl-6-O-sulfo)-α-CD
HDMS-β-CD	heptakis(2,3-di-O-methyl-6-O-sulfo)-β-CD
HDI	hydrodynamic injection
HP-α-CD	hydroxypropyl-α-cyclodextrin
HP-β-CD	hydroxypropyl-β-cyclodextrin
HP-γ-CD	hydroxypropyl-γ-cyclodextrin
HPLC	high-performance liquid chromatography
HS-β-CD	heptakis(6-O-sulfo)-β-CD
ID	inner diameter
IL(s)	ionic liquid(s)
LIF	laser-induced fluorescence
LC	liquid chromatography
LOD	limit of detection
M-α-CD	methyl-α-cyclodextrin
M-β-CD	methyl-β-cyclodextrin
M-γ-CD	methyl-γ-cyclodextrin
MEKC	micellar electrokinetic chromatography
MEEKC	microemulsion electrokinetic chromatography
MS	mass spectrometry
NMR	nuclear magnetic resonance
Pro	proline
Rs	resolution between two signals
RSD	relative standard deviation
S-α-CD	sulfated α-cyclodextrin
S-β-CD	sulfated β-cyclodextrin

S-γ-CD	sulfated γ-cyclodextrin
SBE-β-CD	sulfobutylether-β-cyclodextrin
SDS	sodium dodecyl sulfate
SFC	supercritical fluid chromatography
Suc-β-CD	succinyl-β-cyclodextrin
Suc-γ-CD	succinyl-γ-cyclodextrin
OA-γ-CD	octakis(6-deoxy-6-amino)-γ-CD octahydrochloride
ODMS-γ-CD	octakis(2,3-di-O-methyl-6-O-sulfo)-γ-CD
T	capillary temperature
TBA	tetrabutylammonium cation
TBA-Cl	tetrabutylammonium chloride
TMA	tetramethylammonium cation
TMA-Cl	tetramethylammonium chloride
TM-β-CD	heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin
TMA-α-CD	(2-hydroxy-3-N,N,N-trimethylamino)propyl-α-CD chloride
TMA-β-CD	(2-hydroxy-3-N,N,N-trimethylamino)propyl-β-CD chloride
TMA-γ-CD	(2-hydroxy-3-N,N,N-trimethylamino)propyl-γ-CD chloride
U	applied voltage
UV	ultraviolet

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1. Introduction

1.1 Stereochemistry in pharmaceutical sciences

1.1.1 Overview on chirality

Chirality is a fundamental property present in many biological molecules, as well as in pharmaceuticals, food additives, agrochemicals and others. The term ''chiral'' was coined by Sir William Thomson (later Lord Kelvin) in 1884, when he called any geometrical figure or group of points ''chiral'', when its image in a mirror could not be brought to coincide with itself. Chirality is often illustrated with the idea of right and left-handedness. A left hand and a right hand are mirror images of each other, subsequently they are not superimposable. At the molecular level, chirality is observed in structures with the same atom bonding but a different spatial arrangement of the substituents. The chiral center is often a carbon atom having tetrahedral structure substituted with four different atoms or moieties, as shown in Figure 1 for lactic acid. But there are other examples when chirality arises from a silicon, sulfur, nitrogen or phosphorous atom. The presence of one or more chiral centers in a compound result in stereoisomers. Stereoisomers can be subdivided in two groups: enantiomers and diastereoisomers. The pairs of structures which are non-superimposable mirror images of each other are enantiomers [1, 2]. If this condition is not fulfilled, they are called diastereoisomers [3]. In contrast to the diastereomers, enantiomers possess the same physicochemical properties in achiral media. When a mixture contains equimolar amounts of the enantiomers, it is referred to as a racemate.

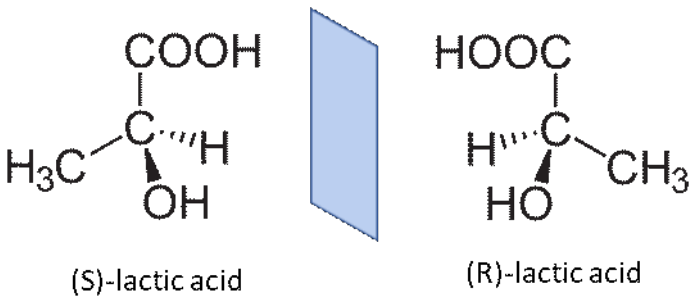

Figure 1. Enantiomers of the lactic acid.

Assignment of enantiomers is based on absolute configuration but also on the so-called optical activity. The most widely used characterization of stereoisomers is the R/S system introduced by S. Cahn, C. K. Ingold and V. Prelog, where the absolute configuration at a chiral center is designated as R (the Latin *rectus*) or S (the Latin *sinister*) to unambiguously describe a 3D structure of the molecule [4]. There are precise rules to prioritize substituents groups around a stereogenic center [1, 5]. In case of chiral drugs, containing more than one chiral center, it is important to determine the absolute configuration for each stereoisomer. Stereochemical descriptions such as D and L proposed by Emil Fischer, are used

specifically for amino acids and sugars and do not generally address other compounds. Alternatively, enantiomers can also be characterized according to the direction in which they rotate the plane polarized light. The direction is opposite for enantiomers with the same degree of the rotation angle [6, 7]. This angle is determined experimentally. The rotation of the plane of the linear polarized light can be either positive (+) or negative (-) depending on the rotation direction, whether it is clockwise (dextro (+)) or counterclockwise (levo (-)). However, there is no correlation between the optical rotation and the absolute configuration of enantiomers [1, 4]. Furthermore, optical rotation may be pH-dependent, especially in the case of acids and bases. For instance, (*S*)-(+)-lactic acid is dextrorotary in water, while its sodium salt is levorotary [1]. A racemate (±) is optically inactive, because being an equimolar mixture of both enantiomers, the net rotation of the plane of the linear polarized light is counterbalanced and consequently zero.

1.1.2 Importance of the enantiomers in pharmaceutical sciences

The importance of the stereochemistry with regard to pharmaceutical drugs has increased in the last decades. More than 50% of the 50 top-selling small molecule pharmaceuticals in current use are chiral compounds as shown in Figure 2 [8]. About 49% are marketed as single stereoisomers.

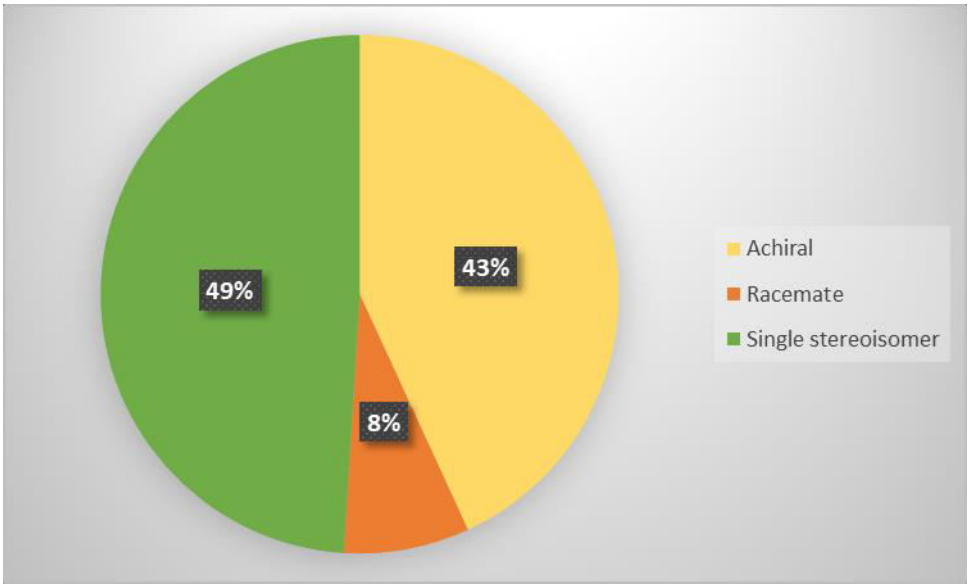

Figure 2. 50 Top selling non-peptide drugs in 2020: Percentage of achiral, racemic and single stereoisomer compounds. Adapted from reference [8].

The difference in spatial arrangement of the substituents can translate into different pharmacological activities and pharmacokinetic profiles of the enantiomers in biological media. Since the building blocks in living organisms such as enzymes or receptors are chiral, stereoselective interactions with enantiomers may result in enantiospecific binding and, consequently, to the observed pharmacological differences [7]. The "three point interaction" model by Easson and Stedman can be applied to rationalize the interaction between the active enantiomer and its binding site [9] as described in Figure 3. It is

reasonable, that a chiral drug molecule may display a different pharmacological activity than its enantiomer because the latter binds differently with biological target, especially, when the chiral center of the substance is interacting directly with the binding site. The more active enantiomer is called "eutomer", whereas the "distomer" is the stereoisomer with lower affinity or a different (toxic) pharmacological effect [10-12].

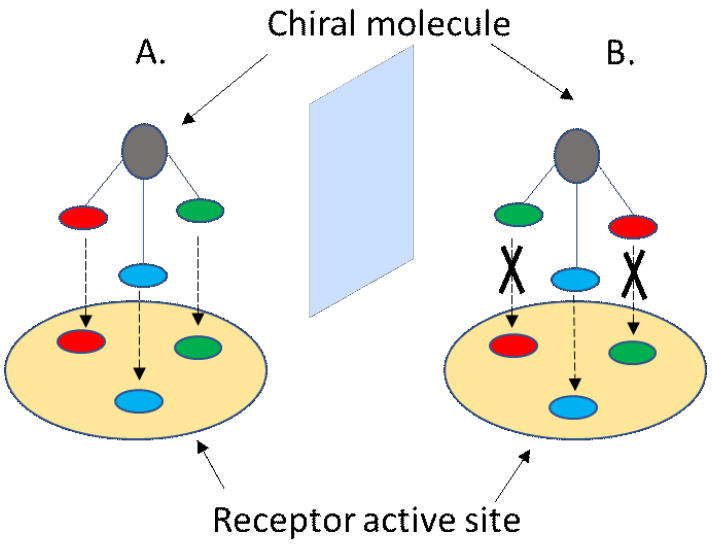

Figure 3. Interaction between the active enantiomer (A) and inactive enantiomer (B) with receptor. A. Eutomer with optimal fit to the binding site, B. Distomer with lower affinity.

Thalidomide is probably the most recognized example that illustrates the importance of the chirality for pharmaceutical drugs. A teratogenic effect of one of the enantiomers was detected when using racemic thalidomide in the 1960s as a sedative and antiemetic drug resulting in birth defects of 10 000 infants of which 40% did not survive [13, 14]. The teratogenic effect was assigned to (*S*)-(-)-thalidomide, while the (*R*)-(+)-enantiomer was considered a safe sedative drug against the sleep disorders and morning sickness. Another example is lactic acid shown in Figure 1. (*R*)-(+)-lactic acid is a very common substance in human metabolism, whereas (*S*)-(-)-lactic acid, which is produced by bacterial species can be very harmful. This is due to the fact, that overgrowth of the lactate-producing bacteria in the intestines can lead to an increase of (*S*)-(-)-lactate in plasma and cause (*S*)-(-)-lactate acidosis in patients with short-bowel syndrome. Symptoms of this acidosis reflect the neurotoxicity of (*S*)-(-)-lactate and lead to recurrent episodes of encephalopathy [15].

The thalidomide disaster resulted in the recognition that enantiomers must be treated as individual compounds because they may differ significantly in their toxicity or rate of metabolism. It also impacted the demand of a discrimination of the unwanted enantiomer for a strict control of chiral drugs. Since 1992, the regulatory authorities of the United States (US Food and Drug Administration (FDA)), Japan (Pharmaceutical and Medical Devices Agency) and Europe (European Medicines Agency (EMA)) have stated in guidelines that pharmaceutical companies must investigate the enantiomers and as a

consequence develop and market the active enantiomer instead of the racemate in case of differences in the activity [7, 16]. Thus, the number of approved racemic drugs gradually decreased over the years as illustrated in Figure 4, the approved small molecular drugs by FDA in the time range from 2007 to 2021. Nowadays, more than 80% of the approved drugs featuring stereogenic centers in their structure are single stereoisomers. For example, 22 out of 23 chiral small molecule drugs approved in 2021 were single enantiomers and only one was the racemate [7, 17, 18].

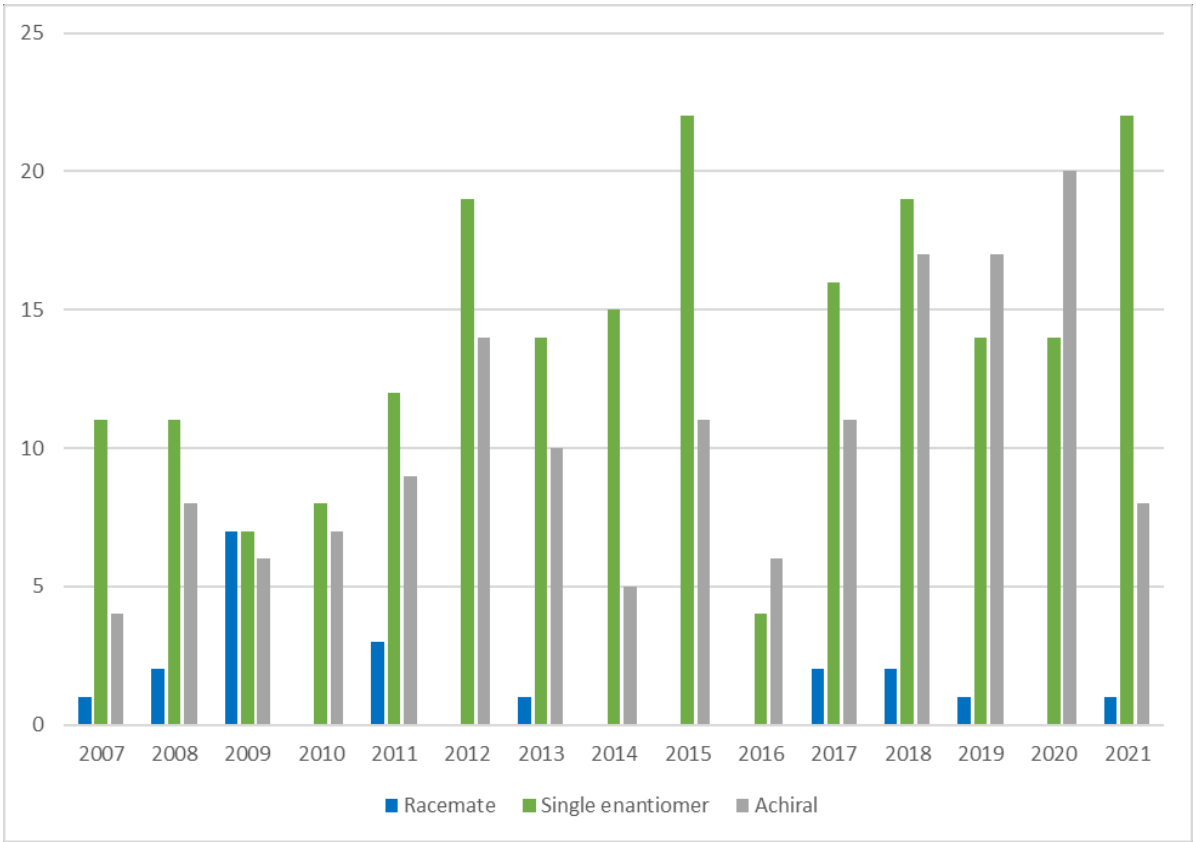

Figure 4. New small molecule drugs approved by the US FDA in the years from 2007 to 2021 according to racemate, single enantiomer and achiral. Adapted from references [7, 18].

Another fundamental change in the pharmaceutical field was the so-called "racemic switch", also known as "chiral switch". The concept was introduced in 1999 by Agranat and Caner [19]. In this marketing strategy one enantiomer of already approved racemic drugs is launched as a "new" drug [20]. Starting from ibuprofen, 14 further anti-inflammatory or other drugs were approved by the FDA between 1994 and 2011 as single enantiomer drugs of already approved racemates. The potential advantages of the chiral switch include increased therapeutic activity and selectivity, less side effects, a faster onset of the pharmacological action, reduced propensity for drug-drug interactions and a lower dose of the drug [17].

Based on the pharmacological activity of the drugs containing a chiral center, they can be categorized into three main groups:

1. ***Racemic drugs containing enantiomers with identical qualitative and quantitative properties***.

Examples are the enantiomers of promazine or fluoxetine, which have the same type and the strength of the activity.

2. ***Racemic drugs containing enantiomers with nonidentical quantitative properties***.

Examples are anti-inflammatory drugs, where the (*S*)-enantiomer typically reveals a 100 - 160 times stronger effect than the respective (*R*)-enantiomers. Similar observations were made for (*S*)-citalopram, which is a 100 times more active antidepressant than the (*R*)-enantiomer [17]. Another example is trelagliptin, whose (*R*)-enantiomer is a highly selective long-acting dipeptidyl peptidase IV inhibitor used for the treatment of the type-2 diabetes, while the (*S*)-enantiomer is essentially inactive [21].

3. ***Racemic drugs containing enantiomers with nonidentical qualitative properties***.

An example is ofloxacin, the (*S*)-enantiomer exhibits high antimicrobial activity, whereas the (*R*)-enantiomers is neurotoxic. There are other examples, such as (*S*)-penicillamine, which is antiarthritic drug, while (*R*)-penicillamine is very toxic. In the case of ketamine, the (*S*)-enantiomer is responsible for the anesthetic and analgetic effects in both animals and humans, whereas (*R*)-ketamine leads to hallucinations, agitation or restlessness [21]. Further examples of drugs with different toxicological and pharmacological effects of the enantiomers are summarized in Table 1 [17, 21]. Additional examples of drugs with different activities of the enantiomers in biological systems can be found in references [1, 16, 22].

Table 1. Examples of drugs with different physiological properties of the enantiomers. Adapted from references [17, 21].

Drugs	**(*S*)-enantiomer**	**(*R*)-enantiomer**
❖ *Ibuprofen*	Anti-inflammatory	Minimal anti-inflammatory effect
❖ *Atenolol*	β-receptor antagonist	Inactive
❖ *Methadone*	Minimal analgesic effect, can increase the risk of severe cardiac arrhythmias.	Strong analgesic
❖ *Salbutamol*	Inactive, may cause undesirable actions on lung function	Antiasthmatic, effect
❖ *Thyroxine*	Strong thyroxemic effect	Inactive
❖ *Ketoprofen*	Anti-inflammatory	Minimal anti-inflammatory effect
❖ *Bupivacaine*	Local anesthetic	The same effect, 2 times higher cardiovascular toxicity.
❖ *Omeprazole*	Acid reducer, proton pump inhibitor (PPI)	4-5 times smaller acid reducer effect.
❖ *Citalopram*	Antidepressant, selective serotonin reuptake inhibitor.	Minimal antidepressant effect
❖ *Venlafaxine*	Selective serotonin reuptake	Selective serotonin and norepinephrine reuptake.
❖ *Propranolol*	β-blocking activity	Minimal β-blocking activity, inhibits deiodinase
❖ *Modanafil*	Minimal narcolepsy effect	Strong narcolepsy effect
❖ *Barbiturates*	Sedative	Inactive
❖ *Warfarin*	Strong anticoagulant	Minimal anticoagulant effect
❖ *Verapamil*	Calcium channel antagonist. Cardiotoxicity at high concentrations.	10 - 20 times less calcium channel antagonist. Less cardiotoxicity.

1.2 Analytical techniques for enantioseparations

Since enantiomers exhibit different pharmacological effects, stereoselective analysis for the quantification of unwanted stereochemical impurities in the drug substances are crucial for drug development and regulatory assessment. Therefore, it is very important to develop efficient and sensitive methods for chiral separations. Analytical stereoselective techniques for the analysis of enantiomers are summarized in Figure 5. Among these analytical techniques, separation techniques such as chromatography or capillary electrophoresis (CE) are the most important ones for the separation of enantiomers.

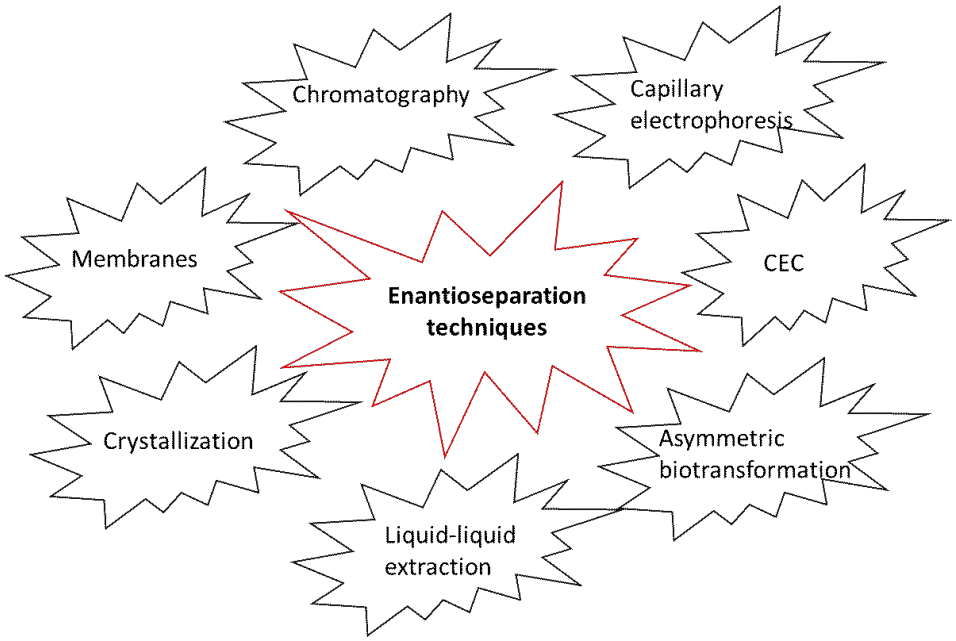

Figure 5. Stereoselective techniques for the separation of enantiomers. Adapted from reference [23].

Within the group of the chromatographic methods gas chromatography (GC), (ultra) high-performance liquid chromatography ((U) HPLC) and supercritical fluid chromatography (SFC) are equally useful for chiral drug analyses depending on the properties of the analytes. Recently nano-liquid chromatography (nano-LC) and separations using lab-on chip showed interesting results in this field [24-26]. The separations can be performed mainly in the direct or indirect mode. In indirect chiral separations, chiral derivatization reagents are used to form diastereomeric derivatives with different physical-chemical properties. Based on this difference, they are easily discriminated in achiral media [27]. In contrast, in the direct method, chiral stationary phases or chiral mobile phase additives are used. The chiral analytes undergo stereospecific interactions to form diastereomeric complexes with the chiral selector either bonded to a solid support or present in the mobile phase. This mode is more convenient and applicable for a large scale of pharmaceutical compounds. In chromatographic techniques the stationary phase is not mobile and is coated on a solid surface. However, the widely used chiral columns are very expensive.

Additionally, chiral analyses consume a relatively high amount of organic solvents and hazardous organic modifiers. On the contrary, CE is an alternative technique with low operational costs. The technique allows an easy adjustment of the concentration of the chiral selector and can be considered environmentally friendly because organic solvents are hardly used. High efficiency and flexibility are further features of this separation technique.

1.2.1 Capillary electrophoresis

First introduced to science in 1960s, CE quickly became a powerful separation tool for chiral and nonchiral compounds. The first chiral separation of enantiomers was carried out by E. Gassmann and colleagues in 1985 [28]. In CE the separation is performed by differential migration of (charged) analytes in an electrolyte solution inside a narrow capillary under the influence of an electric field. CE offers high-speed, high-resolution separations utilizing very small volumes of sample solutions. CE can be performed in several modes such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC), capillary gel electrophoresis (CGE), capillary isotachophoresis (CITP), capillary isoelectric focusing (CIEF) and capillary electrochromatography (CEC) [29]. The different modes of CE emphasize the wide spectrum of applications this technique can be used for as, for example, summarized in references [30-32].

1.2.1.1 The basic principles for electrophoretic separation

A separation in CE is based on the velocity difference of the species in an electric field. This is accomplished by two driving forces, the electrophoretic mobility (μ_{el}) of the analytes and the electroosmotic mobility of the bulk flow (μ_{EOF}).

μ_{el} is a self-electrophoretic mobility of the charged species in the electrolyte solution. For a spherical particle it is calculated according to equation (1) including analyte characteristics such as its effective charge (q) and the hydrodynamic radius (r) as well as the viscosity (η) of the electrolyte solution [33, 34]. The factor 6π refers to the spherical nature of the particle.

$$\mu_{el} = \frac{q}{6\pi\eta r} \qquad (1)$$

The ratio between the charge and the size of a particle is also referred to as charge-to-mass ratio or charge density. For a separation, the electrophoretic mobility between analytes must be different which results from differences in the charge density of the compounds. A higher charged ion with a small mass possesses a greater mobility compared to a larger ion with the same or a lower charge.

The mobility of the bulk flow, μ_{EOF}, is generated from the effect of the applied electric field on the double layer at the interface of the capillary wall and the bulk liquid phase. When an electric field is applied to

a conductive solution in a capillary with a charged inner surface, a plug-like flow of the bulk solvent is generated inside the capillary. This phenomenon is called the electroosmotic flow (EOF). For uncoated fused-silica capillaries, the inner surface of the capillary contains ionizable silanol groups. Accordingly, the strength of the EOF is dependent on the ionization of the capillary wall as a function of the pH of the bulk electrolyte solution also termed as background electrolyte (BGE). At a pH value higher than 3, the inner side of the capillary becomes negatively charged due to the increasing deprotonation of the silanol groups. At higher pH values where the silanol groups are predominantly all deprotonated, the EOF is significantly larger than at lower pH values. In addition, the negatively charged surface attracts cations from the buffer solution resulting an electrical double layer, consisting of a stagnant and a mobile layer. After applying an electrical field, the positively charged layer starts moving towards the cathode and pulls the solutes in this direction [35]. Thus, the EOF is a general mass flow affecting all particles in the solution in the same way.

The EOF can be calculated according to equation (2)

$$\mu_{EOF} = \frac{\varepsilon\zeta}{4\pi\eta} \qquad (2)$$

ε is the buffer solution dielectric constant, E is the applied electric field, ζ is the zeta potential of the solid-liquid interface (capillary wall) and η is the viscosity of the medium.

From the equation (2) can be derived, that μ_{EOF} is non-selective transport as stated above because the influencing factors are system specific and neither of them is analyte specific. The observed effective mobility, μeff, is the sum of the μ_{el} and μ_{EOF} (equation (3))

$$\mu_{eff} = \mu_{el} + \mu_{EOF} \qquad (3)$$

Thus, a separation can be enhanced or diminished depending on the relative extent of μ_{el} and μ_{EOF} [33, 36]. They can act synergistically in one direction or counteract each other. This is dependent on the properties of the analytes, as well as properties of the BGE. Both are affected by the pH of the solution. Finally, the applied voltage and the dielectric constant of the buffer solution are important factors.

1.2.1.2 CE instrumentation

One of the important features of CE is the relative simplicity of the instrument. As presented in Figure 6 the typical CE apparatus is composed of an inlet and an outlet reservoir, which are filled with the BGE, a sample vial, a high-voltage supply, electrodes, a capillary with a detection window (removed protecting coating) and a diode array detector. The separation is conducted in a capillary with an inner diameter (ID) between 10 – 100 μm and a length between 20 - 100 cm. In order to increase the stability and

flexibility of the capillaries, they are coated with polyimide. Both ends are immersed into the buffer vials, which also hold the platinum electrodes. The solution within the capillary is typically the same as in the reservoirs. The polarity of the voltage applied by the power supply can be reversed allowing the detection of the analytes at the cathodic as well as the anodic end of the capillary. A small band of the sample solution is injected at one end of the capillary from the sample vial [29, 35].

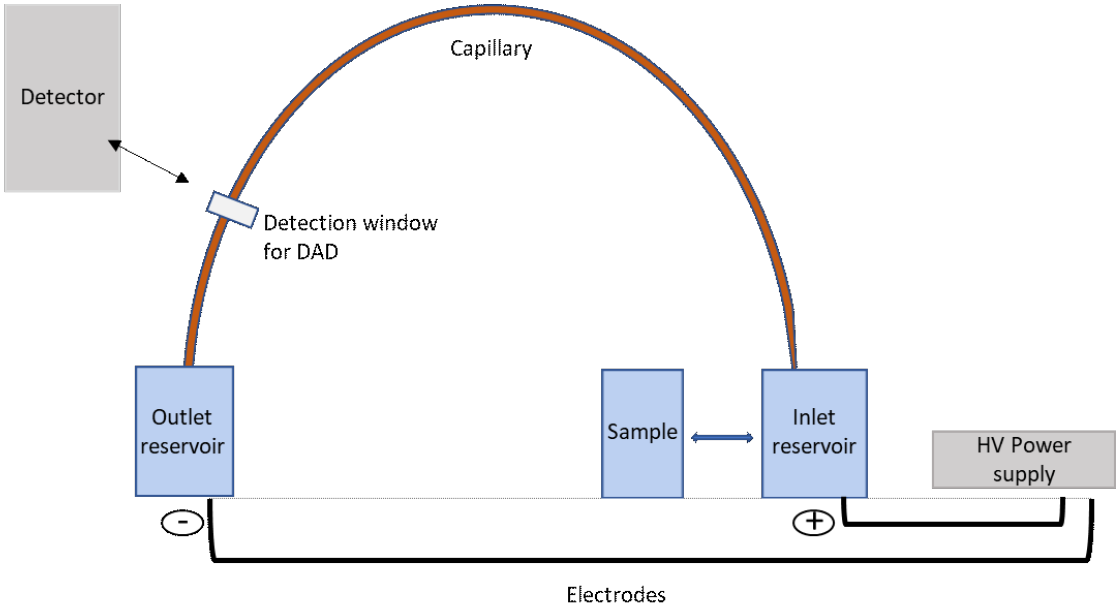

Figure 6. Basic components of CE instrumentation. During the injection, the position of the inlet reservoir is switched with the sample vial.

Sample introduction. There are two ways of sample-introduction into the capillary. These include the electrokinetic injection (EKI) and pressure-driven injection, known as hydrodynamic injection (HDI), which is a widely used method. It is performed by applying a pressure difference between the beginning and the end of the capillary. In case of EKI, the voltage is used instead of the pressure for performing the injection process [29, 35, 37].

Detection. The most widely used detection system in CE is the UV absorbance detector, which allows to perform detections at a single wavelength, as well as on multiple wavelengths using a photo diode array detector (DAD). Detection is carried out on capillary, accordingly the optical path length is very short (between 50 - 100 µm) and, subsequently, the concentration limit of detection (LOD) is relatively low. Other types of the detection systems include electrochemical detection, laser-induced fluorescence (LIF) detection or coupling CE to a mass spectrometer (CE-MS). Especially LIF and MS detection increase the selectivity in CE analysis [29, 35].

Capillary zone electrophoresis. Among the various modes of CE (see above), CZE is the most widely used technique. In this mode, the separation is based on the different velocities of the solutes due to the differences in their charge density upon application of the electric field. CZE is widely used for

analysis of pharmaceuticals as well as in the biomedical, food and environmental field as, for example, summarized in references [30, 38, 39].

Micellar electrokinetic chromatography. Introduced by S. Terabe in the 1980s [40], MEKC combines electrophoretic and chromatographic principles in the separation process. In principle, this mode is based on the distribution of the (uncharged) analytes between the aqueous phase and micelles. Because the micelles are mobile, they are also referred to as the pseudostationary phase. In addition, in case of charged compounds their mobility contributes to the overall separation process. MEKC has been applied to a variety of compounds as summarized in references [29, 35, 41].

1.3 Separation principle in chiral CE

In general, one can distinguish between direct and indirect modes in chiral separations. The direct mode is the most widely used in chiral CE separations. It requires the presence of a chiral selector, i.e., a stereochemically pure compound, which forms diastereomeric complexes with the analyte enantiomers via non-covalent interactions. Thus, direct enantioseparations in CE and in chromatographic techniques rely on the same basic principle [42]. Therefore, chiral CE separations are also referred to as capillary electrokinetic chromatography (CEKC). Nonetheless, there are significant differences between both techniques. In contrast to chromatography, in electrophoretic separations the electrophoretic mobility can be stereoselective for analytes residing in the same phase as the selector. In most of the chromatographic techniques, except for the addition of a chiral selector to the mobile phase (the so-called chiral mobile phase additive mode), the selector is fixed to a chromatographic support, and consequently, the analyte-selector complex is not mobile. In contrast, in CEKC the analyte-selector complex is mobile and can migrate to the cathode or anode depending on its charge sign [43, 44].

Assuming the formation of a complex between an analyte and a selector at a stoichiometric ratio of 1:1, the situation shown schematically in Figure 7 applies. The enantiomers (1) and (2) of a chiral analyte are complexed in a stereoselective manner by the selector. These equilibria are described by the complexation constants (complex formation constants, stability constants, binding constants) K_1 and K_2. The resulting transient diastereomeric complexes migrate according to their charge densities with the respective mobilities μ_{c1} and μ_{c2}. The non-complexed enantiomers migrate with identical mobility μ_f.

Thus, the effective mobilities of the analyte enantiomers interacting with a chiral selector are function of the mobility of the free analyte, the mobilities of the complexes and the strength of the interaction expressed as the complexation constants.

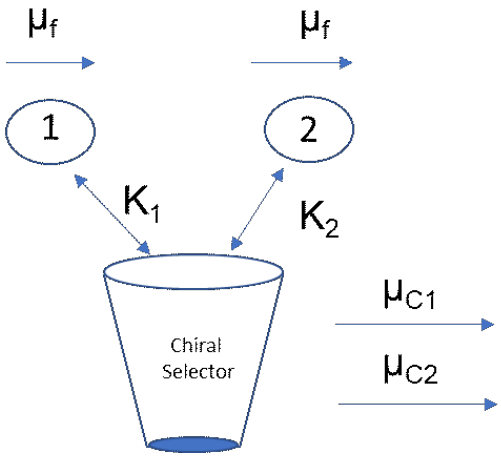

Figure 7. Schematic model of the interaction of the enantiomers (1) and (2) with the chiral selector.

The mobility difference ($\Delta\mu$) between the enantiomers can be described mathematically according to equation (4) proposed by Wren and Rowe [45, 46]:

$$\Delta\mu = \mu 1 - \mu 2 = \frac{\mu f + \mu c1 K1[C]}{1 + K1[C]} + \frac{\mu f + \mu c2 K2[C]}{1 + K2[C]} \qquad (4)$$

μ_1 and μ_2 are the effective mobilities of the first and the second migrating enantiomers, respectively. K_1 and K_2 are the complexation constants of the enantiomers (1) and (2) with the chiral selector and μ_f is the mobility of the free enantiomers, which is equal for both enantiomers. μ_{c1} and μ_{c2} are the mobilities of the analyte-selector complexes. [C] is the concentration of the chiral selector.

As can be easily derived from equation (4), a separation of the enantiomers occurs when the effective mobilities μ_1 and μ_2 differ, which can be due to differences of the affinities (complexation equilibria) of the enantiomers toward the chiral selector, i.e., $K1 \neq K2$. This is also referred to as the chromatographic enantioselective mechanism or thermodynamic mechanism of the CE separation. However, in contrast to chromatography with fixed chiral selectors, an enantioseparation is also observed in case of different mobilities of the transient diastereomeric complexes, i.e., $\mu_{C1} \neq \mu_{C2}$, which may be caused by differences in the hydrodynamic radii of the complexes due to a better fit of one of the enantiomers. This mechanism is also termed the electrophoretic enantioselective mechanism. Both mechanisms can contribute simultaneously but typically the chromatographic mechanism predominates because the hydrodynamic radii of the diastereomeric complexes do not differ to a significant extent and the effective charges of the two complexes are identical in the most cases. Nonetheless, the stereoselective electrophoretic mechanism has the following effects on CE enantioseparations:

1. The selectivity of the enantioseparation can exceed the thermodynamic selectivity of the enantiorecognition.
2. It is achievable in chiral CE to affect the enantiomer migration order (EMO) without changing the affinity pattern of the enantiomers toward the chiral selectors.
3. The most striking difference to pressure-driven chromatographic systems is the fact that, in principle, CE enantioseparations can be performed in the absence of a thermodynamic selectivity, i.e., in the case of equal complexation constants for both enantiomers. In this case the enantioseparation is solely driven by the electrophoretic mobility difference of the selector-selectand diastereomeric complexes [33, 47- 49].

1.4 Chiral selectors in CE

There is a large variety of compounds used as chiral selectors in CE enantioseparations such as cyclodextrins (CDs), linear oligosaccharides and polysaccharides, chiral surfactants, macrocyclic glycopeptide antibiotics, proteins, peptides, chiral crown ethers, aptamers or chiral ionic liquids (CILs). In addition, ligand exchange complexation and ion pair formation can be exploited for the separation of chiral molecules or pharmaceutical drugs as summarized in references [42, 44, 49, 50].

1.4.1 Cyclodextrins

Cyclodextrins and their derivatives are the most versatile chiral selectors for enantioseparations in CE. Advantages are their UV transparency, good aqueous solubility and the fact that a large variety is commercially available at relatively low prices. Furthermore, there is a wide range of derivatives.

CDs are cyclic oligosaccharides composed of D-glucopyranose units linked by α-1,4 glycosidic bonds. The most widely used CDs, α-CD, β-CD and γ-CD, differ in the number of glucose units, i.e., six D-glucosyl molecules in case of α-CD, seven molecules for β-CD and eight molecules for γ-CD. The schematic structure is shown in Figure 8, further properties have been compiled in Table 2 [51, 52].

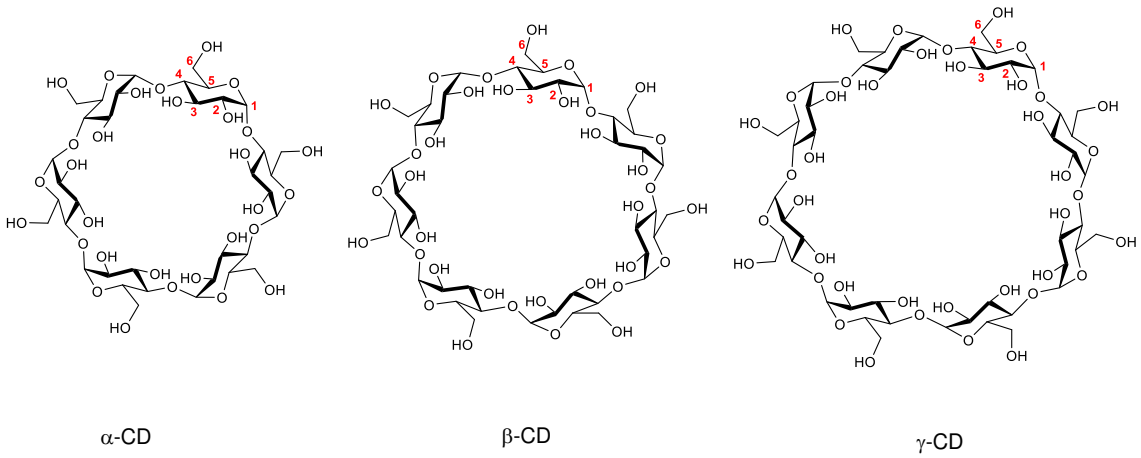

Figure 8. Schematic structures of α-CD, β-CD and γ-CD.

Table 2. Properties of native cyclodextrins [51]

	α-CD	**β-CD**	**γ-CD**
D-Glucopyranose units	6	7	8
Molecular formula (anhydrous)	$C_{36}H_{60}O_{30}$	$C_{42}H_{70}O_{35}$	$C_{48}H_{80}O_{40}$
Molecular mass (g/mol)	972.8	1135.0	1297.1
Approximate cavity diameter (Å)	4.7 - 5.3	6.0 - 6.5	7.5 - 8.3
Approximate outer diameter (Å)	14.6	15.4	17.5
Approximate height (Å)	7.9	7.9	7.9
Approximate volume of cavity (Å^3)	174	262	427
Solubility in water (g/100 mL)	14.5	1.85	23.2
Approximate pK_a (25 °C)	12.33	12.20	12.08

CDs have a circular structure in form of a truncated conical cylinder (Figure 9). As the hydroxy groups are located on the outer surface, this surface is hydrophilic and the inner cavity is hydrophobic. This results in their capability to host wide range of organic molecules. The wider, so-called secondary rim contains the secondary 2- and 3-hydroxy groups, while the narrower, primary rim is formed by the primary 6-hydroxy groups [43].

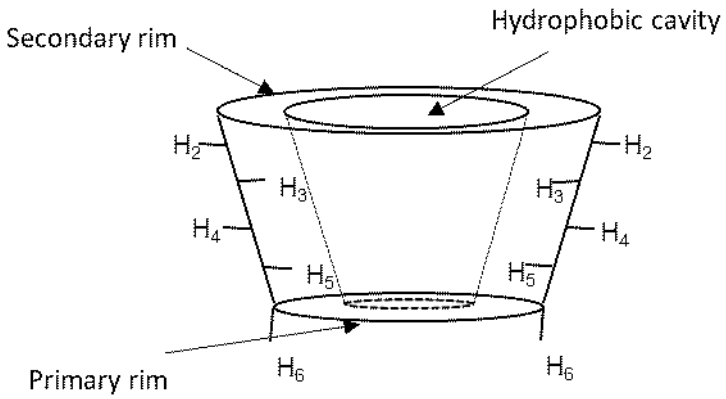

Figure 9. Schematic representation of the three-dimensional toroidal structure of CDs.

In order to modulate the physicochemical properties and molecular recognition capability, native CDs have been chemically modified. With regard to the substitution pattern, CD derivatives may be divided into "randomly substituted CDs" and "single isomer CDs". The hydroxy groups display different reactivities and selection of various regio- and site-specific chemical reactions including specific activation or protecting groups enable the synthesis of many derivatives. For example, the 2-OH group is the most acidic one, while the 6-hydroxy group is the least acidic on one hand, but the most accessible especially for spacious reagents. Substitution at the 3-OH group is the most difficult reaction. Furthermore, it is possible to selectively derivatize an individual OH group of a single glucose residue of a CD. Consequently, a large number of so-called randomly substituted and single isomer CDs have been prepared [53-55]. While the singe isomer CDs as well as the CDs bearing a substituent at a single glucose molecule are defined compounds, randomly substituted CDs are a mixture of compounds with a different degree of substitution as well positional isomers. Nonetheless, companies are able to produce such CDs in a reproducible way with a comparable composition of the batches.

Derivatization increases the size of the cavity and enables further interactions between CDs and analytes. Introducing charged substituents results in a self-mobility of the selector and often enhances the separation especially in the case of oppositely charged CDs and analytes. Moreover, the low solubility of β-CD is overcome by derivatization. Due to the formation of intramolecular hydrogen bonds between the 3-OH and the 2-OH groups of adjacent D-glucose units the structure of β-CD is rigid which is considered the reason for the poor aqueous solubility of β-CD compared to α-CD and γ-CD, see Table 2.

An important advantage of CDs over some other host molecules is their ability to form inclusion complexes [56]. Because CDs are chiral molecules, they form such complexes in a stereospecific manner. The main intermolecular forces involved in the complex formation are hydrogen bonding, hydrophobic, dipole-dipole, van-der Waals and, in case of charged CDs, electrostatic interactions. In addition to intermolecular interactions between CDs and analytes, the expulsion of so-called high-energy water from the cavity appears to play a role in inclusion complex formation [52, 57]. In the absence of a guest molecule, the cavities of the CDs are occupied by water molecules. The number depends on the size of the cavity (see Table 2). These water molecules are "unorganized" because only a limited number of hydrogen bonds are possible, less than the average of about four interactions per molecule as in bulk water. Thus, the water molecules inside the cavity cannot form a stable hydrogen-bonded network, they are "energetically frustrated". Upon inclusion of a molecule, these water molecules are expelled from the cavity into the bulk phase, where they can form more hydrogen bonds. As a result, CD-solute complexation is mainly enthalpically driven unlike the classical entropically driven complexation process based on hydrophobic interactions [52].

Examples of commercially available CD derivatives, which were used as chiral selectors in this work, have been compiled in Table 3.

Table 3. Examples of the commercially available native CDs and their derivatives. n is the number of D-glucopyranose units in the CD.

CDs	n	Substituents
Native CDs		
α-CD	6	H
β-CD	7	H
γ-CD	8	H
Neutral CDs		
Methyl-α-CD	6	CH_3, randomly substituted
Methyl-β-CD	7	CH_3, randomly substituted
Methyl-γ-CD	8	CH_3, randomly substituted
Heptakis(2,6-di-O-methyl)-β-CD	7	CH_3 in positions 2 and 6
Heptakis(2,3,6-tri-O-methyl)-β-CD	7	CH_3 in positions 2, 3 and 6
2-Hydroxypropyl-α-CD	6	CH_2-CH(OH)-CH_3, randomly substituted
2-Hydroxypropyl-β-CD	7	CH_2-CH(OH)-CH_3, randomly substituted
2- Hydroxypropyl-γ-CD	8	CH_2-CH(OH)-CH_3, randomly substituted
Negatively charged CDs		
Heptakis(6-sulfo)-β-CD	7	SO_3Na in position 6
Sulfated-α-CD	6	SO_3Na, randomly substituted
Sulfated-β-CD	7	SO_3Na, randomly substituted
Sulfated-γ-CD	8	SO_3Na, randomly substituted
Sulfobutylether-β-CD	7	CH_2-CH_2-CH_2-CH_2-SO_3Na, randomly substituted
Succinyl-β-CD	7	CO-CH_2-CH_2-COOH, randomly substituted
Succinyl-γ-CD	8	CO-CH_2-CH_2-COOH, randomly substituted
Carboxymethyl-α-CD	6	CH_2-COONa, randomly substituted
Carboxymethyl-β-CD	7	CH_2-COONa, randomly substituted
Carboxymethyl-γ-CD	8	CH_2-COONa, randomly substituted
Heptakis(2,3-di-O-acetyl-6-sulfo)-β-CD	7	CH_3CO in positions 2 and 3, SO_3Na in position 6
Hexakis(2,3-di-O-methyl-6-sulfo)-α-CD	6	CH_3 in positions 2 and 3, SO_3Na in position 6

Heptakis(2,3-di-O-methyl-6-sulfo)-β-CD	7	CH_3 in positions 2 and 3, SO_3Na in position 6
Octakis(2,3-di-O-methyl-6-sulfo)-γ-CD	8	CH_3 in positions 2 and 3, SO_3Na in position 6
Positively charged CDs		
(2-Hydroxy-3-N,N,N-trimethylammoniopropyl)-α-CD	6	CH_2-CH(OH)-CH_2-N(CH_3)$_3$Cl, randomly substituted
(2-Hydroxy-3-N,N,N-trimethylammoniopropyl)-β-CD	7	CH_2-CH(OH)-CH_2-N(CH_3)$_3$Cl, randomly substituted
(2-Hydroxy-3-N,N,N-trimethylammoniopropyl)-γ-CD	8	CH_2-CH(OH)-CH_2-N(CH_3)$_3$Cl, randomly substituted
Heptakis(6-deoxy-6-amino)-β-CD	7	NH_2 in position 6
Octakis(6-deoxy-6-amino)-γ-CD	8	NH_2 in position 6

1.4.2 Chiral ionic liquids

Ionic liquids (ILs) are organic salts with melting point below 100°C. They have negligible vapor pressure, good aqueous solubility and thermal stability. Additionally, they have relatively high conductivity and good miscibility with different solvents. Typically, ILs are composed of a large organic cation, like tetraalkylammonium, imidazolium, quinolinium, etc. and a small organic or inorganic anion, which can be halogen, amino acids, etc. [58]. CILs contain either a chiral cation or a chiral anion, or both may be chiral. The chiral anions can be amino acids, lactic acid, borates or camphorsulfonates, whereas chiral cationic parts may include amino acid esters, chiral chlorine or imidazolinium derivatives, as well as alkaloids such as ephedrine [59]. One of the advantages of CILs over achiral ILs is the fact that they can contribute to the overall enantiorecognition process. Moreover, CILs are easy to synthesize.

In CE, CILs have been used as chiral selector or as second chiral additive in combination with a primary chiral selector [58, 60]. One of the few examples of the use of a CIL as single chiral selector is the separation of the enantiomers of 1,1-binaphthyl-2,2-diylhydrogenphosphate by amino-acid derived CILs, which were composed of an amino acid ester as cation and lactic acid as anionic component [61]. However, in the vast majority of cases, the CILs are used as further chiral additives to enhance a separation based on a primary chiral selector, often a CD. The first report of the synergistic effect was published by Francois and colleagues [62]. The authors noted the improvement of the CD-mediated chiral separation of anti-inflammatory drugs upon addition of [EtChol][NTf_2] and [PhChol][NTf_2] CILs (ethyl- and phenylcholine bis(trifluoromethylsulfonyl)imide). Hadjistasi and co-workers first published the use of amino acid-based CILs as additives in combination with native β-CD [63]. Because of their amphoteric nature, amino acids can be the cationic or anionic component in CILs. Accordingly, many amino acid-derived CILs were successfully applied as co-selectors for enantioseparations of racemic

drugs [64-67].

The synergism observed between CILs and primary chiral selectors has been explained by two mechanisms. First, addition of the CIL to the BGE increases the ionic strength and, accordingly affects the EOF [68, 69]. Furthermore, the cationic component can absorb to the inner surface of the capillary reducing or even reversing the EOF. This increases the timespan, during which the analyte enantiomers can interact with the chiral selector, which results in an improvement of the enantioseparation. The second mechanism is the involvement of a CIL component in the formation of a ternary complex with the analyte and the chiral selector. Such ternary complexes have been demonstrated by molecular modeling. An example is the complex formed between the calcium antagonistic drug amlodipine, Hydroxypropyl-β-CD (HP-β-CD) and [TMLP][TF_2N] (*N*,*N*,*N*-trimethyl-L-phenylalaninol-bis(trifluoromethanesulfon)imide) [70]. Molecular modeling also revealed a complex formed from dansylated L-His, β-CD and [TMA][L-Arg] (tetramethylammonium-L-arginine) [71]. The presence of the ternary complex between FMOC-homocysteine, γ-CD and the CIL [TMA][L-Arg] was concluded from NMR studies [72].

As stated above, amino acid-based CILs have been frequently used in combination with other chiral selectors in CE [64-67]. The structures of such CILs applied in the current work are illustrated in Figure 10. The CILs are based on the amino acids Asp, Asn and Pro as anion and either the tetramethyl ammonium (TMA) cation or the tetrabutylammonium (TBA) cation.

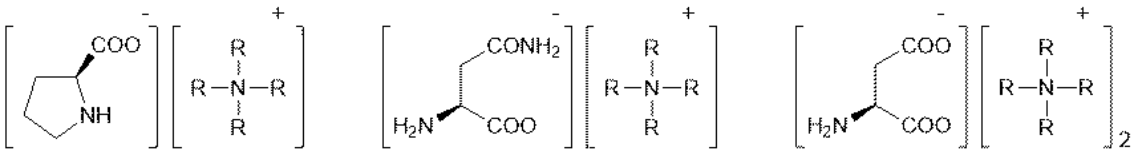

Figure 10. Amino acid-based CILs used in the current work.

1.5 CE in chiral analysis

Since its introduction in the 1980s, CE has grown into an effective separation tool in pharmaceutical [30, 73-76], biomedical [42, 49, 76-79], food and environmental analysis [49, 76, 80-84]. Especially the high selectivity, the small sample volumes required, eco-friendliness due to the reduced use of organic solvents, simplicity and accuracy increase the potential of the CE and allows to detect sensitive analytes in biological media [76-78, 85]. Additionally, as an effective method for stereoisomer separations, chiral CE is widely used for analytical enantiomer discrimination of drug substances summarized in references [30, 86-89].

1.5.1 Peptides

Peptides are highly abundant biological molecules found in all species. In humans they play an important role for controlling many (patho)physiological processes acting as hormones, neurotransmitters, enzyme substrates or inhibitors, immunomodulators, antibiotics, toxins, etc. [90-94] Examples are insulin, which regulates blood glucose level among others, or substance p, which is a neurotransmitter. Furthermore, many peptide drugs have been approved during the last years [95] and others are in clinical development [90, 96]. Finally, small peptides or peptidomimetic drugs such as the angiotensin converting enzyme inhibitors, for example, captopril or enalapril or the renin inhibitor aliskiren have been approved by the regulatory authorities [97].

Because peptides and peptidomimetic drugs are chiral compounds, effective control of their stereochemical purity is required. Due to the fact, that these compounds are hydrophilic, CE has been shown to be a powerful analytical technique and an alternative to HPLC including the separation of stereoisomers [98-100]. Furthermore, di- and tripeptides served as model compounds to study their migration behavior in enantioselective separations and to understand the effect of structural changes with regard to amino acid composition and/or their protonation state. Consequently, the enantioseparation of a set of di- and tripeptides was studied in the presence of neutral CDs [101-106], negatively charged CDs [106-112] or positively charged CDs [105]. Due to the fact that peptides are amphoteric compounds, several specific migration phenomena were observed, especially the pH-dependent reversal of the enantiomer migration order [101-106, 108, 111]. This phenomenon has also been found in case of dansylated amino acids [113], which are also amphoteric. Two mechanisms were considered as explanations for this phenomenon. The first is the change of the chiral recognition of the CE toward the peptide enantiomers when they are protonated or in the zwitterionic state [101,112]. The second mechanism is based on a complexation-induced pK_a shift resulting in the fact that the mobilities of the diastereomeric enantiomer-selector complexes becomes the driving force of the chiral separation because the mobility of the stronger complexed enantiomer exceeds the mobility of the weaker complexed one [105, 108, 112]. Such migration behavior was often observed when a dipeptide contained the amino acids Phe or Tyr but modifications of the structure of the aromatic amino acids such as the length of the side chain have not been studied to date [113].

1.5.2 Sulfoxides

Sulfoxides represent a special case of chiral compounds, because the stereogenic center is a sulfur atom. As presented in Figure 11 for 2-(benzylsulfinyl)benzamide enantiomers, the sulfur atom has a tetrahedral structure with the fourth substituent being a pair of electrons. Sulfoxides are found as natural compounds such as alliin in garlic [114]. Another example is methionine sulfoxide, the oxidation product of the amino acid methionine. It is a mixture of two diastereoisomers, methionine-(*S)*-sulfoxide and methionine-(*R)*-sulfoxide. Both free amino acid- and protein-based forms of methionine-(*S)*-sulfoxide are stereospecifically reduced by MsrA enzyme, whereas the reduction of methionine-(*R*)-sulfoxide requires additionally MsrB and fRMsr enzymes. The fRMsr enzyme is present only in unicellular organisms, thus, mammals are unable to reduce the methionine-(*R*)-sulfoxide [115]. Pharmaceutical

drugs bearing a sulfoxide moiety include sulfinpyrazone, which is a uricosuric medication used to treat gout, the nonsteroidal anti-inflammatory drug sulindac or the proton pump inhibitors such as omeprazole, pantoprazole or lansoprazole. Examples of single isomer sulfoxide drugs are the antihypertensive drug aprikalim, the proton pump inhibitor esomeprazole, the stimulant armodafinil or the immunosuppressant drug oxisurane [17, 116]. In addition, sulfoxide moieties are found in pesticides such as fipronil, propagate or methiocarb sulfoxide as well as in ligands of metal complexes used for asymmetric catalysis in synthetic chemistry [117, 118].

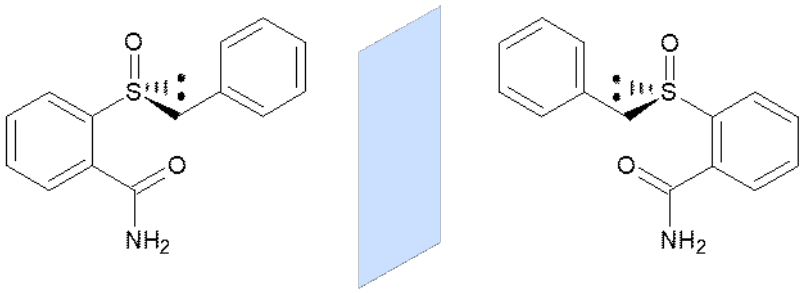

Figure 11. Enantiomers of 2-(benzylsulfinyl)benzamide.

In analytical research, sulfoxides have gained interest recently, because extremely high selectivity had been observed in the separation of the enantiomers of 2-(benzylsulfinyl)benzamide on a cellulose *tris*(3,5-dichlorophenylcarbamate) chiral stationary phase in HPLC. An enantioseparation factor (α) of 112 was reported [119]. (Benzylsulfinyl)benzamides and other sulfoxides have been studied on polysaccharide-based or glycopeptide-based chiral columns in HPLC [120-123] and SFC [124, 125], but such analytes have been scarcely studied by CE. Only two publications reported the CE enantioseparation of chiral sulfoxides or sulfinate esters and sulfoxide pesticide metabolites [126, 127].

2. Aims and Scopes

Fast equilibration, low costs, high resolution, easy replacement of the chiral selector and variation of its concentration makes CE a powerful technique for the enantioseparation of many chiral molecules with different physico-chemical characteristics. Furthermore, CE can be used to study mechanistic aspects of chiral separations.

This dissertation focuses on enantioseparations by CE in the presence of native CDs and their derivatives, either as single selectors or with combination of the CILs. The thesis is divided in two main parts.

1. Ala-Phe has been an intensively studied model peptide in the pH-dependent reversal of the enantiomer migration order [101, 104-106, 108-109, 111, 112]. Accordingly, the CD-mediated enantioseparation of a set of dipeptide analogs with structural alteration of the Phe or Ala moiety, i.e. dipeptides containing β-Ala, β-Phe, phenylglycine (Phg) or homophenylalanine (homoPhe) was investigated with the following specific aims:
   - to study the effect of the structure of the dipeptide analogs
   - to systematically investigate the effect of the type of CD (cavity size, type and pattern of the substitution) on the enantioseparations including the enantiomer migration order
   - to study the effect of the protonation state of the analytes by variation of the pH of the BGE
   - to study effect of amino-acid-derived CILs on the enantioseparation of selected peptides in the presence of neutral CDs
   - to understand the migration behavior of selected analyte-CD combinations as a function of the enantioselectivity of the CD toward the analytes (expressed as complexation constants) and the mobility of the diastereomeric complexes.

2. The second part of the work was focused on the chiral separation of the sulfoxides in the presence of CDs as chiral selectors using (benzylsulfinyl)benzamides as model compounds. Accordingly, the aims for the second part of the thesis were:
   - the effect of the structure of the (benzylsulfinyl)benzamides, i.e. the type and position of substituents
   - a systematic investigation of the enantioseparations as a function of the type of CD
   - to understand the separation mechanism as a function of analyte complexation and complex mobilities for selected analyte-CD combinations.

3. Manuscripts

3.1 Overview of manuscripts

Manuscript 1

Enantioseparation of analogs of the dipeptide alanyl-phenylalanine by capillary electrophoresis using neutral cyclodextrins as chiral selectors

M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1623, 2020, 461158. DOI: 10.1016/j.chroma.2020.461158.

Manuscript 2

Enantioseparation of alanyl-phenylalanine analogs by capillary electrophoresis using negatively charged cyclodextrins as chiral selectors

M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1632, 2020, 461585. DOI: 10.1016/j.chroma.2020.461585.

Manuscript 3

Effects of amino acid-derived chiral ionic liquids on cyclodextrin-mediated capillary electrophoresis enantioseparations of dipeptides

M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1652, 2021, 462342. DOI: 10.1016/j.chroma.2021.462342.

Manuscript 4

Enantioseparation of chiral (benzylsulfinyl)benzamide sulfoxides by capillary electrophoresis using cyclodextrins as chiral selectors

M.-L. Konjaria, R. Kakava, A. Volonterio, B. Chankvetadze, G.K.E. Scriba. Submitted to J. Chromatogr. A on 02.03.2022

Four manuscripts are described in this chapter. Enantioseparations of dipeptides and the migration order changes were investigated in the presence of neutral and charged CD derivatives at different pH values presented in manuscript 1 and manuscript 2, respectively. The synergistic effect of amino acid-based chiral ionic liquids in combination with β-CD and HP-β-CD on the separation of the peptides was investigated in manuscript 3. The last manuscript includes the investigation of the enantioseparation of chiral (benzylsulfinyl)benzamides in the presence of neutral and charged CDs. In order to rationalize the mechanisms corresponding to the different migration order of the enantiomers as function of the protonation state of peptides, and the CD cavity size or the substitution pattern and additionally, CD concentration in case of sulfoxides, binding constants and complex mobilities were determined in manuscript 1, 2 and 4.

3.2 Manuscript 1

Manuscript No. 1

Manuscript title: Enantioseparation of analogs of the dipeptide alanyl-phenylalanine by capillary electrophoresis using neutral cyclodextrins as chiral selectors

Authors: Mari-Luiza Konjaria, Gerhard K.E. Scriba

Bibliographic information: J. Chromatogr. A 2020, 1623, 461158, https://doi.org/10.1016/j.chroma.2020.461158

The candidate is

☒ First author, ☐ Co-first author, ☐ Corresponding author, ☐ Co-author.

Status: Published.

Authors' contributions (in %) to the given categories of the publication

Author	Conceptual	Data analysis	Experimental	Writing the manuscript
Mari-Luiza Konjaria	85	85	100	70
Gerhard K.E. Scriba	15	15		30
Total:	100%	100%	100%	100%

Overview:

The manuscript describes the enantioseparation of the Ala-Phe analogs by capillary electrophoresis in the presence of neutral CDs at pH values 2.5, 3.5 and 9.5. In order to rationalize the pH-dependent reversal of the enantiomer migration order upon increasing the pH from 2.5 to 3.5 the complexation constants and the apparent limiting mobilities of the diastereomeric peptide enantiomer-CD complexes were determined.

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Enantioseparation of analogs of the dipeptide alanyl-phenylalanine by capillary electrophoresis using neutral cyclodextrins as chiral selectors

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ABSTRACT

In the present study, the enantioseparation of the LL- and DD-enantiomers of the dipeptides Ala-Phe, Ala-phenylglycine (Phg), Ala-homoPhe, Ala-β-Phe, Gly-Phe and β-Ala-Phe was studied by capillary electrophoresis in the presence of native α-, β- and γ-cyclodextrin (CD) as well as their methyl and hydroxypropyl derivatives. Separations were performed under standardized conditions in fused-silica capillaries at pH 2.5, 3.5 and 9.5. All analyte enantiomers could be separated at acidic pH under at least one of the experimental conditions. β-CDs proved to be more universal chiral selectors than α- and γ-CDs. Only few alkaline conditions led to an enantioseparation. For a given dipeptide, the enantiomer migration order depended on the type of CD with regard to cavity size and degree of substitution. Little effect was found with regard to the structure of the dipeptides. pH-dependent reversal of the enantiomer migration order upon increasing the pH from 2.5 to 3.5 was observed for all dipeptides with at least one of the β-CD derivatives. In the case of β-CD, analysis of the complexation constants and the apparent limiting mobilities of the diastereomeric peptide enantiomer-CD complexes revealed, that the enantiomer migration order of Ala-Phe, Ala-homoPhe and Ala-β-Phe was determined by the stereoselective complexation by β-CD at pH 2.5. At pH 3.5 opposite chiral recognition of the enantiomers by β-CD was found for Ala-Phe and Ala-β-Phe resulting in the reversed migration order. In contrast, chiral recognition did not change in the case of Ala-homoPhe, but reversal of the enantiomer migration order was based on the apparent mobility of the diastereomeric analyte-CD complexes.

1. Introduction

Small peptides and peptidomimetics represent an important class of pharmacologically active therapeutics [1,2] as well as synthetic chiral building blocks, thus, requiring effective analytical techniques for the determination of their chiral purity. Because these analytes are often hydrophilic and ionogenic, capillary electrophoresis (CE) is a suitable technique for this purpose as summarized in several reviews [3–9]. CE offers several advantages including the thigh resolution power, the low consumption of analytes and chemicals as well as the high flexibility and ease of changing the chiral selector.

In CE, the chiral selector is added to the background electrolyte (BGE). Consequently, enantioseparations are based on two general principles [10–12]. The first one, also referred to as the enantioselective chromatographic mechanism is based on the different affinities of the chiral selector toward the enantiomers. This is reflected in the complexation constants (formation constants, binding constants) of the transient diastereomeric complexes between the selector and the enantiomers. The second one is the electrophoretic enantioselective mechanism due to differences in the limiting mobilities of the diastereomeric selector-enantiomer complexes, which may, for example, be due to differences in the hydrodynamic radii of the complexes. Both mechanisms contribute to an enantioseparation, but the chromatographic mechanism is typically predominant because differences in the hydrodynamic radii are most often negligible. However, the latter mechanism allows the enantioresolution of analytes even in the case of equal complexation constants [10,13].

Another advantage of CE is the relatively easy adjustment of the enantiomer migration order (EMO) because for the determination of the enantiomeric purity of an analyte it is desirable when the minor impurity migrates before the major compound, which may be present in 1000-fold excess, in case of peak tailing. Several techniques can be applied for reversal of the EMO as summarized in [14–16]. In the case of peptides, the pH-dependent reversal of the EMO has been observed as an additional mechanism when charged and uncharged cyclodextrins (CDs) were applied as chiral selectors [17–24]. This has been attributed to a change in

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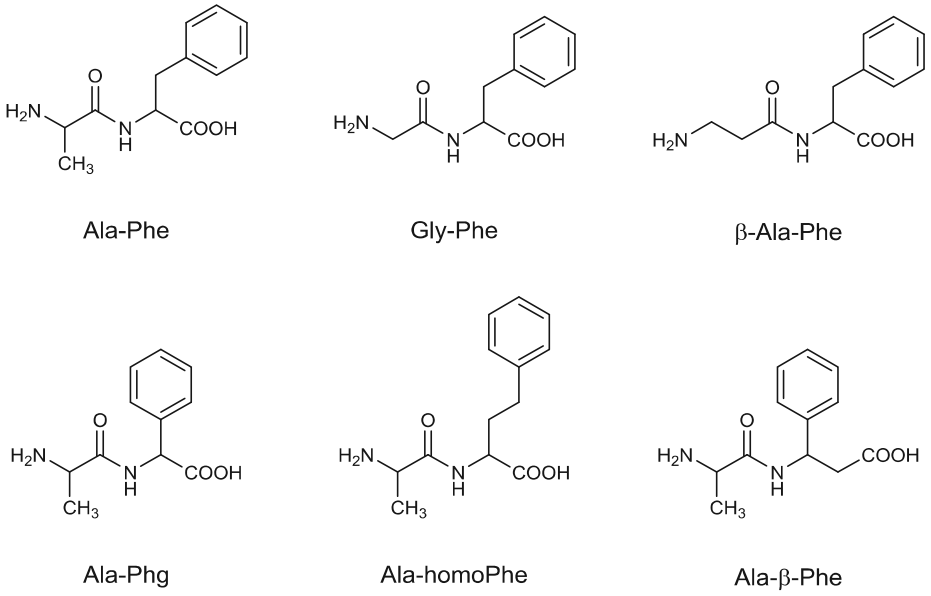

Fig. 1. Structures of the Phe dipeptides.

the chiral recognition of the selector toward the charged or uncharged peptide [20,21,23,24] as well as a shift of the protonation equilibrium of the analytes upon complexation by the CD (a so-called complexation-induced pK_a shift) [24–29].

In previous publications, the effect of the pH of the BGE on the EMO of the dipeptide Ala-Phe has been reported in the presence of various neutral CD derivatives [19,20,22–24]. It has been derived from NMR data [30–32] as well as molecular modeling [32] that complexation of Ala-Phe by CDs occurs via inclusion of the phenyl moiety into the cavity of the CDs. The aim of the present study was to investigate the effect of modifications of Phe on the CD-mediated enantioseparation in CE. Thus, length of the side chain of the amino acid was modified, i.e. exchanging Phe with phenylglycine (Phg) or homoPhe, as well as the position of the aromatic ring (β-Phe). In addition, Ala was substituted by Gly and β-Ala keeping Phe as the second amino acid. The structures of the analytes are shown in Fig. 1.

2. Material and Methods

2.1. Chemicals

β-CD was from Wacker Chemie (Munich Germany), γ-CD was from Merck (Darmstadt, Germany) and methylated γ-CD (M-γ-CD, degree of substitution (DS) ~ 13 - 16) was from the Cyclodextrin Shop (Tilburg, The Netherlands). α-CD, randomly methylated β-CD (M-β-CD, DS ~ 12), 2,6-di-*O*-methyl-β-CD 50 % purity (DM-β-CD50, DS ~ 11 - 14), 2,6-di-*O*-methyl-β-CD 95 % purity (DM-β-CD95, DS = 14), 2-hydroxypropyl-β-CD (HP-β-CD, DS ~ 4.5), 2,3,6-trimethyl-β-CD (TM-β-CD), methylated α-CD (M-α-CD, DS ~ 11), 2-hydroxypropyl-α-CD (HP-α-CD, DS ~ 4.5) and 2-hydroxypropyl-γ-CD (HP-γ-CD, DS ~ 4.5) were obtained from Cyclolab Ltd. (Budapest, Hungary). Gly-L-Phe, Gly-D-Phe, L-Ala-L-Phe and D-Ala-D-Phe were obtained from Bachen AG (Heidelberg, Germany), while β-Ala-L-Phe, β-Ala-D-Phe, L-Ala-L-β-Phe, D-Ala-D-β-Phe, L-Ala-L-Phg, D-Ala-D-Phg, L-Ala-L-homoPhe and D-Ala-D-homoPhe were prepared by standard peptide chemistry procedures reacting the respective *N*-benzyloxycarbonyl-protected amino acid *N*-hydroxysuccinimide with the second amino acid in dimethylformamide followed by hydrogenolytic deprotection in the presence of Pd/C as catalyst [33]. All other chemicals were of analytical grade. Water was purified using a TKA Genpure UV-TOC from Thermo Scientific (Waltham, USA). BGEs and sample solutions were filtered through 0.22 µm polypropylene syringe filters from BGB Analytik (Schloßböckelheim, Germany).

2.2. Capillary electrophoresis

All experiments were conducted on a Beckman P/ACE MDQ CE system (AB Sciex, Darmstadt, Germany) equipped with a UV-Vis diode array detector and controlled by 32 KARAT software for system control, data acquisition and processing. 50 µm I.D., 365 µm O.D. fused silica capillaries with a total length of 50.2 cm and an effective length of 40 cm were from BGB Analytik (Schloßböckelheim, Germany). A new capillary was rinsed at a pressure of 138 kPa (20 psi) subsequently with 0.1 M NaOH for 20 min, water for 10 min, 0.1 M NaOH for 10 min, 0.1 M phosphoric acid for 10 min and water for 10 min. Between the analyses, the capillaries were washed at a pressure of 138 kPa (20 psi) with 0.1 M phosphoric acid for 1 min and with the BGE for 3 min when performing experiments in the acidic pH range. In experiments conducted at pH 9.5 the capillary was rinsed with 0.1 M sodium hydroxide for 1 min and with the BGE for 3 min. The applied voltage was 25 kV and the capillary temperature was 20°C. UV detection was carried out at 215 nm at the cathodic end of the capillary. Dimethylsulfoxide (DMSO) was used as electroosmotic flow (EOF) marker if required.

Separations were performed in 50 mM sodium phosphate buffer or 50 mM sodium borate buffer prepared on a daily basis. Starting from 50 mM phosphoric acid and 50 mM boric acid, respectively, the pH was adjusted using 0.1 M sodium hydroxide after the addition of the CDs. Buffers containing β-CD were prepared with 2 M urea. The buffers were filtered (0.22 µm) and degassed by sonication before use. Sample solutions of the peptides (200 µg/mL prepared in purified water) were introduced at a pressure of 3.5 kPa (0.5 psi) for 5 s. The migration order was confirmed by spiking with the individual peptide enantiomers

Viscosity measurements of the buffers were performed using the CE instrument as a viscosimeter and 0.1% (m/v) riboflavin-5'-

phosphate as boundary marker according to [34]. Electrophoretic mobilities were measured in triplicate and viscosity measurements were performed four-fold.

2.3. Software

The CEVal software [35] was used for non-linear curve fitting for the determination of the complexation constants and the limiting motilities of the peptide-CD complexes.

3. Results and Discussion

The structures of Ala-Phe and the analogs are shown in Fig. 1. The modifications included shortening or elongating the amino acid side chain yielding Ala-Phg and Ala-homoPhe, respectively, reducing and increasing the distance between the phenyl ring and the chiral carbon because complexation involved inclusion of the phenyl ring into the CD cavity [23–25]. In addition, the distance between the chiral carbon and the carboxyl group was modified in Ala-β-Phe. Changes in Ala included "removal" of the methyl group to yield Gly-Phe and changing the position of the amino group to β-Ala-Phe. In case of Ala-Phe, Ala-Phg, Ala-homoPhe and Ala-β-Phe only the LL and DD stereoisomers were studied, while the LD and DL isomers were not considered.

Native α-CD, β-CD and γ-CD as well as their methyl and hydroxypropyl derivatives were studied in untreated fused-silica capillaries in the pH range 2.5 to 3.8 in 50 mM sodium phosphate electrolyte solution and at pH 9.5 using a 50 mM sodium borate buffer under standardized conditions. β-CD and its derivatives were screened at a concentration of 20 mg/mL. When an enantioseparation was not observed the experiments were repeated at CD concentrations of 10 mg/mL and 50 mg/mL. α-CD and its derivatives were tested at concentrations of 50 mg/mL, while in the case of γ-CD and derivatives concentrations of 100 mg/mL were applied. These higher concentrations were based on a previous study of the enantioresolution of di- and tripeptides by α-CD and γ-CD as well as their hydroxypropyl derivatives [36]. No further attempts for optimizing the separations were undertaken. The results of the screening are summarized in Table 1.

3.1. CD structure

In this section, only general observations based on the CD structure will be addressed. The effect of the pH of the BGE will be discussed in detail in section 3.3. Generally, β-CD derivatives provided more separations under the standardized screening conditions compared to α-CD or γ-CD derivatives and γ-CDs were more effective compared α-CDs (Table 1). The most universal selector was native β-CD. This is in accordance with a previous study using the native CDs and their hydroxypropyl derivatives for a set of di- and tripeptides with varying amino acid compositions [36]. The exception among the randomly methylated CDs is M-γ-CD, which yielded more enantioseparation than M-α-CD and M-β-CD.

In the majority of cases, the LL-configured dipeptides migrated before the respective DD stereoisomers at pH 2.5. With regard to the dipeptides containing only one chiral center, i.e. Gly-Phe and β-Ala-Phe, the migration order was D > L for most conditions in the presence of β-CD and its derivatives. In contrast it was more often L > D upon addition of α-CDs or γ-CDs, although exceptions exist. For example, the EMO of Gly-Phe at pH 2.5 is D > L in the case of HP-α-CD and HP-β-CD, while it is L > D in the presence of HP-γ-CD. Similar examples can also be found for the analytes with two chiral centers such as Ala-Phe where the EMO was DD > LL for HP-α-CD and LL > DD for HP-γ-CD, while no separation was observed at pH 2.5 in the presence of HP-β-CD. Differences of in the chiral recognition depending on the size of the CD cavity

Table 1
Enantiomer migration order of the peptides under standardized conditions in the presence of neutral CD derivatives using 20 mg/mL in the case of β-CD derivatives, 50 mg/mL α-CD derivatives and 100 mg/mL γ-CD derivatives. The faster migrating enantiomer (migration time to the detector) is listed first.

	β-CD			M-β-CD				DM-β-CD50			DM-β-CD95			TM-β-CD			HP-β-CD		
Peptide	pH=2.5	pH=3.5	pH=9.5	pH=2.5	pH=3.5	pH=9.5		pH=2.5	pH=3.5	pH=9.5	pH=2.5	pH=3.5	pH=9.5	pH=2.5	pH=3.5	pH=9.5	pH=2.5	pH=3.5	pH=9.5
Gly-Phe	ns[a)]	D>L	L>D[b)]	D>L	ns	ns		D>L	ns	ns	ns	D>L	ns	D>L	L>D	ns	D>L	D>L	ns
Ala-Phe	LL>DD	DD>LL	LL>DD[b)]	ns	ns	ns		DD>LL	ns	ns	LL>DD	DD>LL	ns	DD>LL	ns	ns	ns	DD>LL	ns
β-Ala-Phe	L>D	D>L	L>D[b)]	D>L	ns	ns		D>L	ns	ns	L>D[c)]	D>L	ns	D>L	D>L	ns	D>L	D>L	ns
Ala-Phg	LL>DD	ns	DD>LL[b)]	ns	DD>LL	LL>DD		ns	ns	ns	LL>DD	DD>LL	ns	ns	ns	ns	DD>LL	ns	ns
Ala-homoPhe	LL>DD	DD>LL	DD>LL[b)]	ns	ns	ns		ns	ns	ns	LL>DD[c)]	DD>LL	ns	LL>DD	ns	ns	ns	DD>LL	ns
Ala-β-Phe	LL>DD	DD>LL	LL>DD[b)]	LL>DD	LL>DD	ns		LL>DD	DD>LL	ns	LL>DD	ns	LL>DD	LL>DD	LL>DD	ns	LL>DD	DD>LL	ns
	α-CD			M-α-CD				HP-α-CD			γ-CD			M-γ-CD			HP-γ-CD		
Peptide	pH=2.5	pH=3.5	pH=9.5	pH=2.5	pH=3.5	pH=9.5		pH=2.5	pH=3.5	pH=9.5	pH=2.5	pH=3.5	pH=9.5	pH 2.5	pH 3.5	pH 9.5	pH=2.5	pH=3.5	pH=9.5
Gly-Phe	ns	L>D	ns	D>L	ns	ns		D>L	ns	ns	L>D	ns	ns	ns	ns	D>L	L>D	ns	ns
Ala-Phe	ns	ns	ns	DD>LL	ns	ns		DD>LL	ns	ns	LL>DD	ns	ns	LL>DD	ns	ns	LL>DD	ns	ns
β-Ala-Phe	L>D	L>D	ns	ns	ns	ns		ns	ns	ns	L>D	ns	ns	L>D	ns	ns	L>D	ns	ns
Ala-Phg	LL>DD	LL>DD	ns	ns	ns	ns		ns	ns	ns	LL>DD	ns	ns	ns	DD>LL	ns	ns	ns	ns
Ala-homoPhe	ns	ns	ns	ns	ns	ns		ns	ns	ns	LL>DD	ns	ns	DD>LL	DD>LL	ns	ns	ns	ns
Ala-β-Phe	LL>DD	ns	ns	ns	ns	ns		LL>DD	ns	ns	LL>DD	ns	ns	LL>DD	LL>DD	ns	LL>DD	ns	ns

[a)] ns, not separated under standard conditions.
[b)] concentration of β-CD 50 mg/mL.
[c)] concentration of DM-β-CD95 10 mg/mL.

have been described for small peptides [36] as well as non-peptide analytes [37–40].

Comparing the various methylated β-CD derivatives, it is interesting to note that most separations were observed for DM-β-CD95 with an isomeric purity of 95 %, which yielded comparable separations to native β-CD including the EMO in the acidic pH range. M-β-CD and DM-β-CD50 are a mixture of positional isomers as well as isomers with a varying number of substituents. These CDs were relatively poor chiral selectors for the present set of analytes. This finding was somewhat surprising as it is often observed, that randomly substituted CDs provide better enantioresolutions compared to single isomer CDs [41,42]. The substitution pattern also affected the EMO. Taking Ala-Phe at pH 2.5 as an example, the order was LL > DD in the presence of DM-β-CD95 and native β-CD, while it was opposite in the presence of DM-β-CD50 and TM-β-CD, which is completely methylated. In contrast, LL > DD was found for native β-CD as well as all methylated β-CD derivatives in the case of Ala-β-Phe at pH 2.5. Differences in the EMO depending on the substitution pattern of methylated CDs has been observed previously for dipeptides [4,19,21] as well as other analytes [15].

3.2. Peptide structure

Only few conclusions could be drawn regarding structural effects of the dipeptides. Interestingly, the enantiomers of Ala-β-Phe could be separated with all CDs at least at one of the experimental conditions except for M-α-CD (Table 1). Comparing Ala-Phe, Ala-Phg and Ala-homoPhe, little differences were found with regard to the total number of successful separations although there appeared to be a tendency, that Ala-homoPhe was entantioresolved under fewer conditions compared to the other two dipeptides. In combination with the data obtained for Ala-β-Phe it might be concluded that the distances of the phenyl ring and the carboxylic acid group to the chiral center might affect the recognition by the CDs. However, further structural analogs would be required to unequivocally prove this dependence. “Removal” of the chiral center of the *N*-terminal Ala yielding Gly-Phe and β-Ala-Phe did not affect the overall chiral recognition of these analytes by the CDs.

Interestingly, opposite EMO depending on the peptide structure was observed in some cases. For example, at pH 2.5 opposite EMO was seen for Ala-Phe and Ala-β-Phe in the presence of TM-β-CD and HP-α-CD. The same observation applied to Ala-Phg and Ala-β-Phe in the case of HP-β-CD at pH 2.5 and β-CD at pH 9.5 as well as to Ala-homoPhe and Ala-β-Phe with M-γ-CD at pH 2.5 and with β-CD at pH 9.5.

3.3. pH dependence

Generally, with the exception of β-CD, all other CDs proved to be poor chiral selectors for the analytes at pH 9.5 (Table 1). At this pH, enantioseparations were only observed for the combination of Ala-Phg and M-β-CD, Ala-β-Phe and DM-β-CD95 as well as Gly-Phe and M-γ-CD. The best separation conditions were found at pH 2.5, where at least 2-3 peptides were enantioresolved by each CD.

The pK_a values of Ala-Phe are 3.08 and 7.91 [36], while the pK_a values of the carboxyl group of dipeptides are in the range of about 2.8 to 3.6 and the pK_a values of the amino group range between 7.2 and 8.5 [43,44]. Thus, the peptides are at least partially positively charged at pH 2.5 and become increasingly uncharged in the pH range 3.5 - 3.8 (the isoelectric point is about 5.5 for most dipeptides). At pH 9.5 they are negatively charged and migrate after the EOF. Assuming complexation by the CD as the predominant separation mechanism, the stronger complexed enantiomer should migrate second in the fully protonated, positively charged stage at pH 2.5, while it migrates first when the analyte is negatively charged at pH 9.5. Therefore, identical EMO at these pH values would refer in fact to opposite chiral recognition by the CD, while opposite EMO would indicate identical chiral recognition of the enantiomers. Consequently, opposite chiral recognition can be assumed for Ala-Phe, Ala-β-Phe and β-Ala-Phe using β-CD as chiral selector as well as for Ala-β-Phe in the presence of DM-β-CD95 (Table 1). In contrast, identical recognition of the enantiomers by β-CD was found for Ala-Phg and Ala-homoPhe. Several cases of opposite recognition can also be concluded comparing the EMO of analytes at pH 3.5 to the EMO at pH 9.5 (Table 1).

Upon comparison of the EMO at pH 2.5 and at pH 3.5, reversal of the EMO was found for Ala-Phe, Ala-homoPhe, Ala-β-Phe and β-Ala-Phe in the case of β-CD as well as for Ala-Phe, Ala-Phg, Ala-homoPhe and β-Ala-Phe using DM-β-CD95 as chiral selector (Table 1). This behavior was also seen for enantioseparations of Ala-β-Phe with DM-β-CD50 and HP-β-CD as well as for Gly-Phe with TM-β-CD. As observed for enantioseparations of other dipeptides and tripeptides by neutral CD derivatives [17–24], reversal of EMO upon a change of the protonation state of the peptides appears to be a quite common phenomenon. With regard to β-CDs, this occurred for all analytes under at least one of the experimental conditions except for M-β-CD, so that conclusions about the structural requirements of the peptides cannot be drawn from the present set of analytes. α-CDs and γ-CDs were poor selectors so that the phenomenon was not observed in case of these CDs.

As outlined above, stereoselective recognition of the analyte enantiomers by the chiral selector as well as the mobilities of the diastereomeric selector-enantiomer complexes contribute to enantioseparations in CE [10,11]. Therefore, reversal of the EMO upon a change of the pH of the BGE may be based on opposite chiral recognition of the analyte as charged or uncharged species. Upon increasing the pH from 2.5 to 3.5, a larger molar fraction of the peptides exists in the zwitterionic (neutral) state so that opposite recognition of the charged and uncharged species would result in a reversal of the EMO [24,26,27]. Because the studied CDs are neutral and do not possess an electrophoretic mobility, the analysis of the peptides at pH 5.3 close to their isoelectric points is not possible. On the other hand, reversal of the EMO may be based on the fact, that the mobilities of the complexes, which might be less dominant at low pH, become increasingly dominant upon increasing the pH of the BGE, when the overall charge of the analyte decreases. This can also result in a reversed EMO [26,27]. In order to obtain information about the mechanism behind the pH-dependent reversal of the EMO, complexation constants as well as the mobilities of the diastereomeric CD-peptide complexes were subsequently determined for Ala-Phe, Ala-homoPhe and Ala-β-Phe in the case of β-CD as chiral selector. Because higher resolution was obtained at pH 3.8 compared to pH 3.5 within acceptable analysis times, these data were determined at pH 3.8. The EMO was identical at both pH values. Electropherograms of the analysis of the three peptides at pH 2.5 and 3.8 in the presence of β-CD under standardized conditions are shown in Fig. 2.

3.4. Complexation constants and complex mobility

In order to rationalize the reversal of the EMO of Ala-Phe, Ala-homoPhe and Ala-β-Phe by β-CD at the pH value 2.5 and 3.8, the complexation constants and apparent complex mobilities were determined in 50 mM sodium phosphate electrolyte containing 2 M urea and concentrations of β-CD ranging between 0 and 45 mM. The data were obtained as best fit parameters of the dependence of the effective mobility on the CD concentration at the respective pH values considering the formation of 1:1 peptide CD-complexes

Table 2
Apparent complexation constants (K) as well as actual ionic mobilities of the free analytes (μ_f) and of the analyte–β-CD complexes (μ_c) at pH 2.5 and pH 3.8. The numbers in brackets refer to the 95 % confidence intervals.

Peptide		pH 2.5			pH 3.8		
		K (M^{-1})	μ_f (10^{-9} m^2 s^{-1} V^{-1})	μ_c (10^{-9} m^2 s^{-1} V^{-1})	K (M^{-1})	μ_f (10^{-9} m^2 s^{-1} V^{-1})	μ_c (10^{-9} m^2 s^{-1} V^{-1})
Ala-Phe	LL	84	13.1	6.08	85	3.45	2.20
		(27 - 117)	(12.4 - 13.4)	(5.34 - 6.60)	(40 - 111)	(3.40 - 3.60)	(1.23 - 2.50)
	DD	119	13.1	6.25	55	3.45	2.34
		(33 - 138)	(12.4 - 13.4)	(5.64 - 6.71)	(23 - 83)	(3.40 - 3.60)	(1,.84 - 2.83)
Ala-homoPhe	LL	271	13.3	5.40	537	6.64	2.59
		(232 - 305)	(12.8 - 13.9)	(3.49 - 6.61)	(427 - 589)	(6.30 - 6.99)	(2.15 - 2.93)
	DD	395	13.3	5.66	540	6.64	2.83
		(238 - 454)	(12.8 - 13.9)	(4.70 - 6.44)	(426 - 620)	(6.30 - 6.99)	(2.38 - 3.17)
Ala-β-Phe	LL	78	15.6	6.94	156	10.3	4.78
		(39 - 87)	(15.5 - 16.2)	(4.13 - 7.24)	(60 - 276)	(9.6 - 10.9)	(3.19 - 5.50)
	DD	115	15.6	7.43	142	10.3	5.01
		(26 - 171)	(15.5 - 16.2)	(5.88 - 8.12)	(53 - 173)	(9.6 - 10.9)	(3.29 - 5.70)

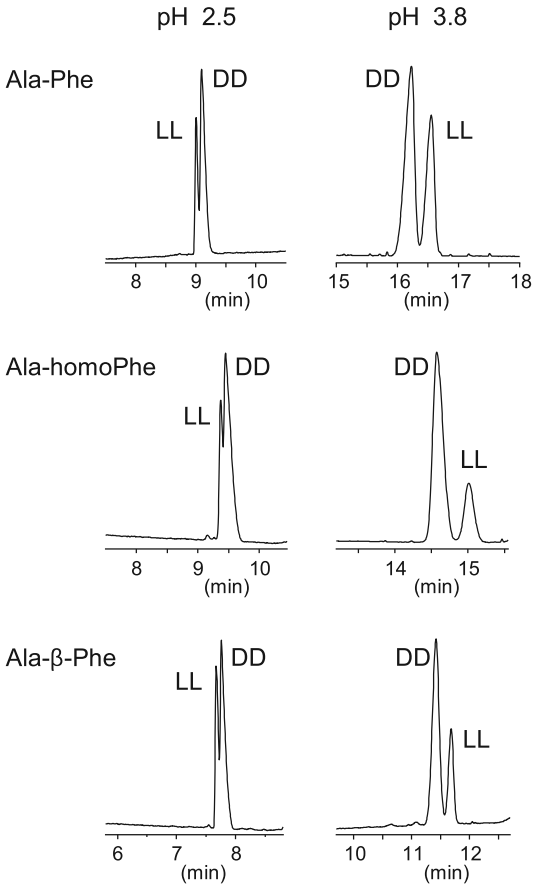

Fig. 2. Electropherograms of the enantioseparation of Ala-Phe, Ala-homoPhe and Ala-β-Phe in the presence of 20 mM β-CD as chiral selector at pH 2.5 and pH 3,8. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium phosphate solution, capillary temperature 20°C, separation voltage 25 kV, UV detection at 215 nm.

according to equation 1

$$\mu_{eff} = \frac{\mu_f + \mu_c \cdot K \cdot [CD]}{1 + K \cdot [CD]} \quad (1)$$

where μ_{eff} is the effective mobility, μ_f the mobility of the free analyte, μ_c the limiting mobility of the CD-analyte complex, K the complexation constant, and [CD] the molar concentration of the CDs. The CEVal software was used for data fitting because this software allows correction of the migration times in case of tailing peaks according to the Haarhoff-van der Linde equation [35]. The observed mobilities were corrected for the increasing viscosity upon increasing CD concentrations according to [34]. The complexation constants and the mobilities of the free analytes as well as the apparent limiting complex mobilities of the enantiomers of the three peptides are summarized in Table 2. It should be emphasized, that these data are apparent constants and mobilities rather than thermodynamic values because the ionic strength of the buffers is not known, and concentrations referred to volume (molar concentrations) and not mass of the solvent (molal concentrations). In addition, the values for the respective enantiomers are not statistically significantly different due to the limited number of experiments and the limited concentration range due to the poor solubility of β-CD. Nonetheless, they can be used to explain the observed migration behavior.

At pH 2.5, the DD-enantiomers of all three peptides displayed the higher complexation constants compared to the respective LL-isomers. In CE experiments, the EMO LL > DD was observed (Fig. 2). Thus, the EMO can be explained by the stronger complexation of the DD-stereoisomers. However, high β-CD concentrations resulted in a deterioration of the enantioresolution, which was especially evident for Ala-homoPhe (data not shown). This can be rationalized by the apparent mobilities of the diastereomeric complexes. These are also higher in the case of the DD-enantiomers so that they counteract the analyte-retarding effect of the complexation. Thus, the enantioseparation of the peptides by β-CD at pH 2.5 is governed by chiral recognition of the CD expressed as complexation constants at low CD concentrations, while at higher concentrations the contributions of the complex mobilities become increasingly effective leading to a loss in resolution.

At pH 3.8 the LL-enantiomers of Ala-Phe and Ala-β-Phe were complexed stronger than the DD-isomers, i.e. opposite to the situation at pH 2.5. Therefore, the reversal of the EMO can be explained by opposite chiral recognition of the enantiomers at both pH values. This has been reported also for other dipeptides in the presence of uncharged CD derivatives [21,24]. In contrast, the stereospecific recognition of Ala-homoPhe by β-CD did not change. The higher complexation constant was still found for the DD-enantiomer although the value was almost equal to the constant determined for the LL-isomer. Nonetheless, the EMO was DD > LL, which can be explained by the higher mobility of the complex formed between β-CD and D-Ala-D-homoPhe. This mechanism has also been found for other peptides in CD-mediated enantioseparations [21,24]. Thus, while identical EMO is observed for the three analytes, the enantioseparation mechanism at pH 3.8 differed depending on the peptide. While it is based on the strength of the complexation in case of Ala-Phe and Ala-β-Phe, it is driven by

complex mobilities in the case of Ala-homoPhe. It should be noted that reversal of the EMO as a function of the CD concentration can also be observed [14,15]. This would be the case if the enantiomers have opposite ratios of complexation constants and complex mobilities, i.e. when the weaker complex possesses the higher mobility and the stronger complex displays the lower mobility. As can be concluded from Table 1, this is not the case for the present set of analytes.

4. Conclusions

Applying standardized CE conditions, the LL- and DD-enantiomers of the six model peptides could be separated by native α-, β- γ-CD as well as their methyl and hydroxypropyl derivatives at acidic and alkaline pH of the BGE. β-CDs were more effective chiral selectors than the α- and γ-CDs. The EMO depended on the size of the cavity of the CD as well as the substitution pattern of the CD derivatives as observed for other dipeptides [4,19,20,36].

Upon increasing the pH of the BGE from 2.5 to 3.5, pH-dependent reversal of the EMO was observed for almost all β-CD derivatives, while this could not be found when using α- or γ-CDs as chiral selectors. As reported previously for neutral CD derivatives [21,24], the EMO of dipeptides in the acidic pH region is affected by the stereoselective complexation of the enantiomers by β-CD and the apparent limiting mobilities of the diastereomeric analyte-CD complexes. The predominant mechanism will determine the migration order. This can result in the reversal of the EMO upon increasing the pH of the BGE even in cases, when the chiral recognition of the enantiomers by the selector does not change.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mari-Luiza Konjaria: Data curation, Formal analysis, Writing - original draft. **Gerhard K.E. Scriba:** Conceptualization, Writing - review & editing.

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3.3 Manuscript 2

Manuscript No. 2

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Author	Conceptual	Data analysis	Experimental	Writing the manuscript
Mari-Luiza Konjaria	85	85	100	70
Gerhard K.E. Scriba	15	15		30
Total:	100%	100%	100%	100%

Overview:

The manuscript describes the enantioseparation of the Ala-Phe analogs by capillary electrophoresis in the presence of charged CDs at pH values 2.5, 3.5 and 5.3. The enantiomer migration order dependence on the CD cavity size and substituent type was studied. In order to understand mechanism of the pH-dependent reversal of the enantiomer migration order upon increasing the pH from 2.5 to 5.3, the apparent and, in case of randomly substituted CDs, averaged complexation constants and complex mobilities of the diastereomeric peptide enantiomer-CD complexes were determined.

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Enantioseparation of alanyl-phenylalanine analogs by capillary electrophoresis using negatively charged cyclodextrins as chiral selectors

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ABSTRACT

The separation of the LL- and DD-enantiomers of the dipeptides Ala-Phe, Ala-phenylglycine (Phg), Ala-homoPhe, Ala-β-Phe, Gly-Phe and β-Ala-Phe was studied by capillary electrophoresis in the presence of negatively charged α-, β- and γ-cyclodextrin (CD) derivatives. Analysis was performed under standardized conditions in fused-silica capillaries at pH 2.5, 3.5 and 5.3. All analyte enantiomers could be separated at pH 2.5 under at least one of the experimental conditions. Especially β-CD derivatives proved to be effective chiral selectors. The enantiomer migration order depended on CD cavity size and substituent type, while peptide structure had only a minor effect. Upon increasing the pH from 2.5 to 5.3, reversal of the enantiomer migration order was observed frequently. Investigation of the apparent and, in the case of randomly substituted CDs, averaged complexation constants and mobilities of the diastereomeric peptide enantiomer-CD complexes indicated that in most cases the migration order in the presence of sulfated α-, β- and γ-CD and heptakis(6-*O*-sulfo)-β-CD could be explained by the stronger binding of the second migrating analyte by the CD at pH 2.5. However, in few cases the weaker bound enantiomer migrated second, which could be attributed to the higher mobility of the respective CD complexes. At pH 5.3, similar data were obtained for sulfated β-CD and heptakis(6-*O*-sulfo)-β-CD, i.e. the strength of the complexes determined the migration order for some peptides, while the migration sequence was based on the apparent (and averaged) mobility of the diastereomeric analyte-CD complexes in other cases.

1. Introduction

Capillary electrophoresis (CE) is considered an ideal technique for the analysis of hydrophilic and ionogenic analytes, that is characterized by low consumption of materials as well as flexibility with regard to a change of the experimental conditions. For enantioseparations, the chiral selector is added to the background electrolyte (BGE). Thus, it is mobile and not fixed to a solid support as in chromatography. As a consequence, not only the different affinities of the selector toward the analyte enantiomers contribute to the enantioresolution, but also differences in the mobilities of the diastereomeric selector-enantiomer complexes [1]. The first mechanism is often referred to as the chromatographic principle and expressed in form of the complex formation constants (complexation constants, binding constants), while the second one is termed the electrophoretic mechanism and represented by the mobilities of the diastereomeric complexes. Thus, it is possible to achieve an enantioseparation selectivity higher than the thermodynamic selectivity of chiral recognition [1,2] and, moreover, enantioseparations are even possible in the case when the complexation constants of both enantiomers with the chiral selector do not differ [2,3].

Another advantage of CE is the ease of the adjustment of the enantiomer migration order (EMO). Several mechanisms can be utilized for this purpose as summarized in [4,5]. Among the many analytes involved in such studies, amino acid derivatives, dipeptides and tripeptides proved to be interesting compounds because a pH-dependent reversal of the EMO was observed using neutral cyclodextrins (CDs) [6-8] or charged CDs [9,10] as chiral selectors. In several cases, the pH-dependent reversal of the EMO was based on opposite chiral recognition by the CD when the peptide was present in the charged state or uncharged state [9,10]. In other cases, chiral recognition by the CD did not change upon increasing the pH of the BGE but the reversal of the EMO was based on the different influence of complexation constants and

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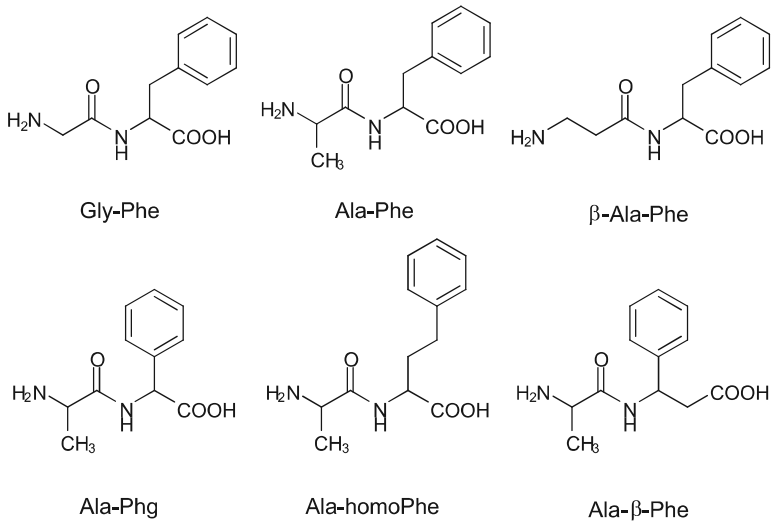

Figure 1. Structures of the Ala-Phe analogs.

complex mobilities in the studied pH range [10-13]. Thus, the EMO was determined by the binding constants at low pH, while it was affected by complex mobilities at higher pH values resulting in opposite migration order of the enantiomers.

An intensively studied model peptide in the pH-dependent reversal of the EMO has been Ala-Phe [7,9-11]. Therefore, analogs with structural modifications of the Phe and Ala moiety were prepared and the effect of these modifications on the enantioseparation by neutral CDs was investigated recently [14]. The modifications included modification of the length of the side chain of Phe, i.e. exchanging Phe with phenylglycine (Phg) or homophenylalanine (homoPhe) as well as the position of the aromatic ring yielding β-Phe. In addition, Ala was substituted by Gly and β-Ala keeping Phe as the second amino acid. The structures of the resulting dipeptides are shown in Fig. 1. It was found that the enantiomer migration order of Ala-Phe, Ala-homoPhe and Ala-β-Phe was determined by the stereoselective complexation by β-CD at pH 2.5 [14]. At pH 3.5 opposite chiral recognition of the enantiomers by β-CD was found for Ala-Phe and Ala-β-Phe resulting in a reversal of the EMO. Chiral recognition did not change in the case of Ala-homoPhe, but reversal of the EMO was based on the mobility of the complexes formed between β-CD and the analyte enantiomers.

Negatively charged CDs including randomly substituted CDs as well as single isomer CDs proved to be effective chiral selectors especially for positively charged peptide analytes [9,10,15]. An advantage of charged CDs is their electrophoretic self-mobility allowing also enantioseparations of uncharged compounds [16]. Therefore, as a continuation of previous work [14], the aim of the present study was to investigate the CE enantioseparations of Ala-Phe and the analogs by negatively charged CDs. Separations were performed at low pH of the BGE where the analytes are positively charged as well as at pH 5.3 close to the isoelectric point of the peptides so that they are essentially uncharged.

2. Material and Methods

2.1. Chemicals

Sulfobutylether-β-CD (SBE-β-CD, degree of substitution (DS) ~6.4), heptakis(6-*O*-sulfo)-β-CD (HS-β-CD) heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-CD (HDMS-β-CD), heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-CD (HDAS-β-CD), succinyl-β-CD (Suc-β-CD, DS ~3.5), hexakis(2,3-di-*O*-methyl-6-*O*-sulfo)-α-CD (HDMS-α-CD), octakis(2,3-di-*O*-methyl-6-*O*-sulfo)-γ-CD (ODMS-γ-CD) and carboxymethyl-α-CD (CM-α-CD, DS ~3.5) were from Cyclolab Ltd (Budapest, Hungary). Sulfated β-CD (S-β-CD, DS ~12-15) and sulfated α-CD (S-α-CD, DS ~8-11) were obtained from Sigma-Aldrich Chemie GmbH (Munich Germany), carboxymethyl-β-CD (CM-β-CD, DS ~3.5) was from Wacker Chemie (Munich Germany) and sulfated γ-CD (S-γ-CD, DS ~13-15) was obtained from Cyclodextrin Shop (Tilburg, The Netherlands). Gly-L-Phe, Gly-D-Phe, L-Ala-L-Phe and D-Ala-D-Phe were obtained from Bachen AG (Heidelberg, Germany), while β-Ala-L-Phe, β-Ala-D-Phe, L-Ala-L-β-Phe, D-Ala-D-β-Phe, L-Ala-L-Phg, D-Ala-D-Phg, L-Ala-L-homoPhe and D-Ala-D-homoPhe were prepared by standard peptide chemistry procedures reacting the respective *N*-benzyloxycarbonyl-protected amino acid *N*-hydroxysuccinimide with the second amino acid in dimethylformamide followed by hydrogenolytic deprotection in the presence of Pd/C as catalyst [17]. All other chemicals were of analytical grade. Water was purified using a TKA Genpure UV-TOC from Thermo Scientific (Waltham, USA). BGEs and sample solutions were filtered through 0.22 µm polypropylene syringe filters from BGB Analytik (Schloßböckelheim, Germany).

2.2. Capillary electrophoresis

All experiments were conducted on a Beckman P/ACE MDQ CE system (AB Sciex, Darmstadt, Germany) equipped with a UV-Vis diode array detector and controlled by 32 KARAT software for system control, data acquisition and processing. 50 µm ID fused silica capillaries with a total length of 50.2 cm and an effective length of 40 cm were from BGB Analytik (Schloßböckelheim, Germany). All rinsing steps were conducted at a pressure of 138 kPa (20 psi). A new capillary was treated subsequently with 0.1 M NaOH for 20 min, water for 10 min, 0.1 M NaOH for 10 min, 0.1 M phosphoric acid for 10 min and water for 10 min. Between the analyses, the capillaries were washed with 0.1 M phosphoric acid for 1 min and with the BGE for 3 min when performing experiments at pH 2.5 or 3.5. In experiments conducted at pH 5.3, the capillary was rinsed with 0.1 M acetic acid for 1 min and with the BGE for 3 min. The applied voltage was 25 kV and the capillary temperature was 20°C. UV detection performed at 215 nm at the cathodic end of the capillary in case of normal polarity and at the anodic end

when the polarity was reversed. Dimethyl sulfoxide (DMSO) was used as electroosmotic flow (EOF) marker.

Separations were performed in 50 mM sodium phosphate buffer or 50 mM sodium acetate buffer prepared on a daily basis from the respective acid and the pH was adjusted after the addition of the CDs with 0.1 M NaOH. The BGEs were filtered (0.22 µm) and degassed by sonication before use. Sample solutions of the peptides (200 µg/mL non-racemic mixture prepared in purified water) were introduced at a pressure of 3.5 kPa (0.5 psi) for 5 s. The migration order was confirmed by spiking with the individual peptide enantiomers.

Viscosity measurements of the BGEs were performed using the CE instrument as a viscosimeter and 0.1% (m/v) riboflavin-5'-phosphate as boundary marker according to [18]. Electrophoretic mobilities were measured in triplicate and viscosity measurements were performed four-fold.

2.3. Software

The CEVal software [19] was used for non-linear curve fitting for the determination of the complexation constants and the motilities of the peptide-CD complexes.

3. Results and Discussion

The structures of the analogs of Ala-Phe are summarized in Fig. 1. Structural modifications included shortening or elongating the Phe side chain yielding Ala-Phg and Ala-homoPhe, which changes the distance between the phenyl ring and the chiral carbon. It has been shown previously that complexation of Ala-Phe or Ala-Tyr by β-CD derivatives involved inclusion of the phenyl ring into the CD cavity [20-22]. In addition, the distance between the chiral carbon and the carboxyl group was increased in Ala-β-Phe. Changes of Ala included "removal" of the methyl group to yield Gly-Phe and changing the position of the amino group to β-Ala-Phe. In case of dipeptides with two chiral centers, the study was restricted to the LL and DD enantiomers.

Analyses were performed under standardized conditions in bare fused-silica capillaries at an applied voltage of 25 kV and a temperature of 20°C using 50 mM sodium phosphate in the pH range 2.5 to 3.5, and 50 mM sodium acetate at pH 5.3 as BGEs. Randomly substituted α-, β- and γ-CD derivatives containing sulfate, sulfonate or carboxy groups as well as sulfated single isomer CDs were investigated. CDs were initially screened at concentrations of 5 mg/mL and 20 mg/mL. If no resolution was observed under these conditions, higher or lower concentrations of the CDs were evaluated. In few cases, CD concentrations as low as 1 mg/mL resulted in an enantioseparation. No further attempts for method optimization were undertaken with regard to the pH and molarity of the BGE, buffer composition or applied voltage. The results are summarized in Table 1.

3.1. Effect of the CD structure

CDs containing sulfate or carboxy substituents proved to be better chiral selectors compared to the previously studied uncharged CD derivatives [14]. This can be attributed to the self-mobility of the negatively charged CDs, which is opposite to the direction of the peptides at low pH, when the analytes are positively charged. Generally, β-CD derivatives revealed higher enantioresolution ability compared to α- and γ-CD derivatives, and α-CDs were more effective than γ-CDs (Table 1). ODMS-γ-CD proved to be a rather poor selector because only two analytes could be resolved at pH 2.5 and 3.5, respectively. The single isomer HS-β-CD as well as randomly substituted CM-β-CD resolved the enantiomers of all Ala-

Table 1
Enantiomer migration order of the peptides under standardized conditions in the presence of negatively charged CDs derivatives. The EMO is listed according to detection at the cathode, the faster migrating enantiomer is listed first. Separations were performed using 2 mg/mL of SBE-β-CD and HS-β-CD, 5 mg/mL S-β-CD, S-α-CD and HDAS-β-CD, 10 mg/mL S-γ-CD and 20 mg/mL of HDMS-β-CD, CM-β-CD, CM-α-CD, Suc-β-CD, HDMS-α-CD and ODMS-γ-CD at pH 2.5 and 3.5. At pH 5.3, CD concentrations were 10 mg/mL SBE-β-CD, HDAS-β-CD and S-α-CD, 15 mg/mL HS-β-CD and S-β-CD and 20 mg/mL HDMS-β-CD, CM-β-CD, CM-α-CD, Suc-β-CD, HDMS-α-CD, S-γ-CD and ODMS-γ-CD.

Peptide	SBE-β-CD pH=2.5	pH=3.5	pH=5.3	S-β-CD pH=2.5	pH=3.5	pH=5.3	HS-β-CD pH=2.5	pH=3.5	pH=5.3	HDMS-β-CD pH=2.5	pH=3.5	pH=5.3	HDAS-β-CD pH=2.5	pH=3.5	pH=5.3	CM-β-CD pH=2.5	pH=3.5	pH=5.3
Gly-Phe	D>L	D>L	D>L	L>D	L>D	ns	L>D	L>D	D>L	D>L	D>L	L>D	D>L	D>L	ns	D>L	D>L	D>L
β-Ala-Phe	D>L	D>L	D>L	D>L	ns	D>L[b]	L>D	L>D	D>L	D>L	D>L	L>D	D>L	ns	ns	D>L	D>L	D>L
Ala-Phe	DD>LL	DD>LL	DD>LL	DD>LL	DD>LL	LL>DD	LL>DD	DD>LL	DD>LL	DD>LL	DD>LL	LL>DD	DD>LL	DD>LL	ns	LL>DD	DD>LL	DD>LL
Ala-Phg	DD>LL	DD>LL	ns	DD>LL	DD>LL	DD>LL	LL>DD	ns	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	DD>LL	DD>LL	DD>LL	DD>LL	DD>LL
Ala-homoPhe	LL>DD	LL>DD	LL>DD	DD>LL	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	DD>LL	LL>DD	LL>DD	LL>DD	LL>DD	ns	DD>LL	DD>LL	ns
Ala-β-Phe	LL>DD	DD>LL	ns	DD>LL	LL>DD	LL>DD	LL>DD	LL>DD	DD>LL	DD>LL	DD>LL	DD>LL	DD>LL	DD>LL	ns	LL>DD	LL>DD	DD>LL
	Suc-β-CD			S-α-CD			CM-α-CD			HDMS-α-CD			S-γ-CD			ODMS-γ-CD		
Peptide	pH=2.5	pH=3.5	pH=5.3	pH=2.5	pH=3.5	pH=5.3	pH=2.5	pH=3.5	pH=5.3	pH=2.5	pH=3.5	pH=5.3	pH=2.5	pH=3.5	pH=5.3	pH=2.5	pH=3.5	pH=5.3
Gly-Phe	L>D	D>L	L>D	L>D	L>D	ns	D>L	D>L	L>D	ns	D>L	ns	L>D	L>D	ns	ns	ns	ns
β-Ala-Phe	L>D	L>D	L>D	L>D	L>D	L>D	ns	ns	D>L	ns[c]	D>L	ns	L>D	L>D	ns	ns	ns	ns
Ala-Phe	LL>DD	DD>LL	DD>LL	LL>DD	ns	ns	ns	DD>LL	DD>LL	LL>DD	ns	ns	LL>DD	LL>DD	ns	ns	ns	ns
Ala-Phg	ns	ns	LL>DD	LL>DD	LL>DD	DD>LL	ns	ns	DD>LL	ns	ns	ns	LL>DD	LL>DD	ns	ns	ns	ns
Ala-homoPhe	LL>DD	LL>DD	nd	DD>LL	DD>LL	ns	DD>LL	DD>LL	DD>LL	LL>DD	DD>LL	ns	LL>DD	LL>DD	ns	LL>DD	ns	ns
Ala-β-Phe	DD>LL	LL>DD	DD>LL	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	DD>LL	LL>DD	LL>DD	ns	LL>DD	LL>DD	LL>DD	LL>DD	LL>DD	ns

ns, not separated under standard conditions; nd, not detected
[a] concentration of CD 1 mg/mL
[b] concentration of CD 30 mg/mL
[c] ns at 5 mg/mL, nd at 10 mg/mL

Phe analogs at all conditions, but HDMS-β-CD as well as S-β-CD and SBE-β-CD were also very effective chiral selectors.

As expected, opposite EMO as a function of the type of the CD was observed in several cases as also described for the Ala-Phe analogs in the presence of neutral CD derivatives [14], for other di- and tripeptides using charged or uncharged CDs [7,23] as well as non-peptide analytes [4,5]. EMO also depended on the cavity size of the CD as observed for the Ala-Phe analogs and neutral CD derivatives [14] as well as for peptide analytes [24] and non-peptide analytes [25-27]. For example, with regard to S-α-CD, S-β-CD and S-γ-CD, this was found in the case of Ala-Phe, β-Ala-Phe, Ala-Phg, Ala-homoPhe and Ala-β-Phe at pH 2.5. Further examples can be found in Table 1.

It is interesting to note that the substitution pattern also affected the migration order. Comparing randomly substituted S-β-CD with HS-β-CD, which selectively bears sulfate groups at C6 of the D-glucose moieties, opposite EMO could be observed under most of the experimental conditions. Similar results with regard to S-β-CD and HS-β-CD were obtained previously for dipeptides and tripeptides [10,15] and dependence of the migration order of dipeptide enantiomers on the isomeric purity of the CD selector has also been observed in the case of randomly methylated β-CD and 2,6-dimethyl-β-CD [23]. In randomly substituted CDs, all isomers differing in their substitution pattern and degree of substitution contribute to the enantioseparation. It has been frequently observed that randomly substituted CDs result in higher enantioselectivity compared to single isomer CDs because of this fact [28,29]. However, with the present set of analytes, HS-β-CD seems to be slightly superior compared to S-β-CD, because every analyte was enantioseparated under each tested condition. Upon methylation of the hydroxy groups in position 2 and 3 of the D-glucose units forming the wider rim of the CD torus, i.e. moving from HS-β-CD to HDMS-β-CD, the enantioresolution ability of the CDs was not significantly affected although opposite EMO was observed in some cases including Gly-Phe, β-Ala-Phe, Ala-Phe. Moreover, higher CD concentrations were required to achieve a successful enantioseparation by HDMS-β-CD. Acetylation of the 2- and 3-OH groups yielding HDAS-β-CD essentially resulted in a loss of the chiral recognition of the analyte enantiomers by the CD at pH 5.3, while comparable separations were achieved at lower pH values (Table 1).

3.2. Effect of peptide structure

Only few conclusions could be drawn regarding the effects of the structural variations because most peptides were enantioseparated by most CDs (Table 1). This may be due to the favorable counter-mobility of positively charged analytes and negatively charged CDs at low pH. The enantiomers of Ala-homoPhe and Ala-β-Phe were separated by each CD under at least one of the standardized experimental conditions applied. Often, opposite EMO was observed for the two peptides. All other Ala-Phe analogs were enantioresolved by one of the CDs except ODMS-γ-CD.

A clear tendency of the EMO regarding the "removal" of the stereogenic center of Ala or regarding the length of the side chain bearing the phenyl ring could not be observed because these variations led to a reversal of the EMO in some but not all cases. For example, comparing Ala-Phe and Gly-Phe reveals that the recognition by the CDs with regard to the stereochemistry of Phe is identical in most cases except for S-β-CD and CM-β-CD at pH 2.5 and Suc-β-CD and CM-α-CD at pH 5.3. "Shortening" the side chain of Phe, yielding Ala-Phg, resulted in opposite EMO of Ala-Phg and Ala-Phe in the presence of HDAS-β-CD or CM-β-CD at pH 2.5. In addition, more enantioseparations were observed for Ala-Phe compared to Ala-Phg. Within the pair Ala-Phe and Ala-homoPhe opposite EMO resulted for several CDs, for example for SBE-β-CD, HDMS-β-CD, HDAS-β-CD, CM-β-CD and S-α-CD at pH 2.5. Thus, elongation of the distance between the chiral carbon and the phenyl ring appeared to have a more significant effect than shortening the distance. Reversed EMO was also observed in some cases comparing Ala-Phg and Ala-β-Phe, which differ in the distance between the chiral carbon and the carboxyl group. Nonetheless, clear relationships between the chiral recognition ability of the peptides by the CDs or the EMO could be not concluded from the set of Ala-Phe analogs. This may be due to the relatively small changes in the structures of dipeptides.

3.3. Effect of electrolyte pH

The pK_a values of the carboxylic acid group of dipeptides range between 2.8 and 3.6 and the pK_a values of the amino group between 7.2 and 8.5 [30,31] so that the isoelectric point ranges between pH 5 and 6. Thus, the Ala-Phe analogs are positively charged at pH 2.5 and 3.5 and migrate toward the cathode. In contrast, the analytes are essentially present in the zwitterionic state at pH 5.3 and do not bear a significant overall charge. Thus, they do not possess a significant self-mobility and are transported by the negatively charged CDs to the anode, while the EOF directs them toward the cathode.

Overall, less enantioseparations were observed at 5.3 compared to pH 2.5 and 3.5 (Table 1). This may be attributed to a lower chiral recognition ability of the negatively charged CDs toward the uncharged analytes compared to their protonated stage due to the opposite charge of solutes and CDs. In fact, as discussed below in section 3.4, the dipeptides displayed a slight anodic self-mobility so that they appear to bear a slight negative overall charge at pH 5.3. Therefore, electrostatic repulsion may also contribute to the lower separation ability observed at this pH.

With regard to the EMO, it has to be considered that the analytes migrated before the EOF at pH 2.5 and 3.5 while they migrated after the EOF at pH 5.3. The migration order indicated in Table 1 always refers to detection at the cathode. Reversal of the EMO was observed in many cases. However, reversal of EMO between pH 2.5 (and 3.5) versus pH 5.3 does not correlate to a change of the chiral recognition due to the detection before and after the EOF, respectively. In contrast, opposite chiral recognition by the CDs may correlate to identical migration order at these pH values (see section 3.4). Most often, identical migration order of the enantiomers was noted between pH 2.5 and pH 3.5 and reversal occurred at pH 5.3. This indicates an effect of the charge of the peptide analyte with regard to the interaction with the CD [9,10]. It has been previously shown for Ala-Phe in the presence of CM-β-CD and Suc-β-CD that EMO is determined by the opposite chiral recognition of the peptide enantiomers by the CDs at pH 2.5 and 5.3, i.e. depending on the charge of the analytes, while complex mobilities determined the migration order at pH 3.5 [9]. A similar mechanism may be assumed in the other cases found for the present set of Ala-Phe analogs. For Ala-homoPhe in the presence of S-β-CD as well as Ala-β-Phe in the presence of Suc-β-CD, the EMO changed between pH 2.5 and 3.5 and again between pH 3.5 and 5.3. Fig. 2 illustrates examples, where identical EMO was observed at all pH values tested (Fig. 2A), the EMO at pH 2.5 and 3.5 were identical but different from pH 5.3 (Fig. 2B), and an example where EMO changed between pH 2.5 and 3.5 and again at pH 5.3 (Fig. 2C).

3.4. Binding constants and complex mobility

In order to rationalize the EMO of the peptides by sulfated CDs, complexation constants and complex mobilities of all six analytes were determined at pH 2.5 for S-α-CD, S-β-CD, S-γ-CD and HS-β-CD as chiral selectors. Moreover, the parameters were also deter-

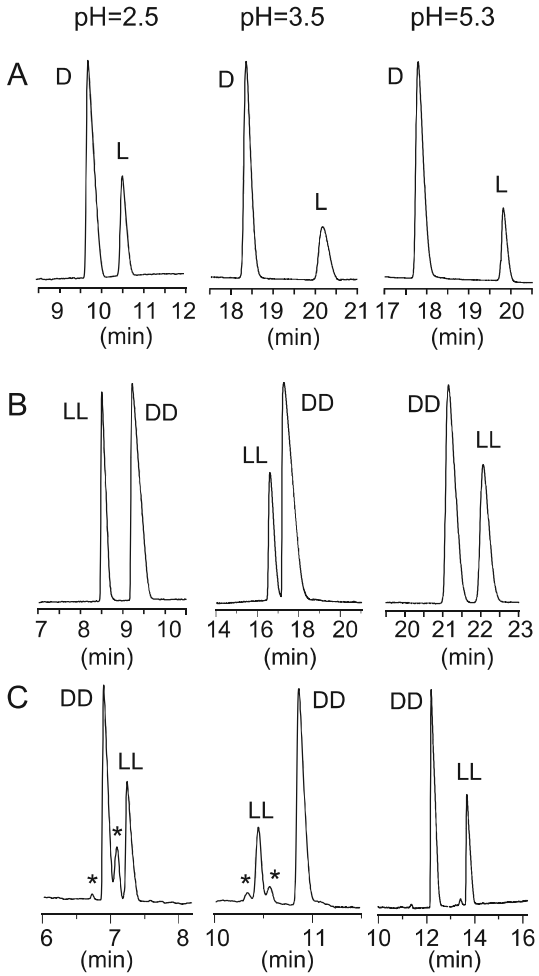

Figure 2. Examples of electropherograms illustrating the enantiomer migration order of (A) Gly-Phe in the presence of CM-β-CD, (B) Ala-Phe in the presence of HS-β-CD and (C) Ala-β-Phe in the presence of Suc-β-CD at pH 2.5, 3.5 and 5.3. Experimental conditions: 40/50.2 cm, 50µm ID fused-silica capillary, 50 mM sodium phosphate buffer at pH 2.5 and 3.5, and 50 mM sodium acetate buffer at pH 5.3; 20°C; 25 kV. For CD concentrations see header of Table 1. * synthetic impurity.

mined for β-Ala-Phe, Ala-Phe, Ala-Phg and Ala-homoPhe at pH 5.3 in the case of S-β-CD and HS-β-CD, because reversal of the EMO has been observed when increasing the pH from 2.5 to 5.3. Experiments at pH 2.5 were performed in 50 mM sodium phosphate buffer, while 50 mM sodium acetate buffer was used at pH 5.3. CD concentrations ranged between 0 and 50 mg/mL, if possible. Detection was performed at the cathode at low CD concentrations while at high concentrations reversed polarity was applied at pH 2.5. In the case of S-γ-CD, currents above 150 µA and sometimes break-down of the system were observed at concentrations above 20 mg/mL, so that experiments with this CD were limited to 20 mg/mL.

The complexation and mobility data were obtained as best fit parameters of the dependence of the effective mobility on the CD concentration at the respective pH values considering the formation of 1:1 peptide CD-complexes according to equation 1

$$\mu_{eff} = \frac{\mu_f + \mu_c \cdot K \cdot [CD]}{1 + K \cdot [CD]} \quad (1)$$

where μ_{eff} is the effective mobility, μ_f the mobility of the free analyte, μ_c the limiting mobility of the CD-analyte complex, K the complexation constant, and [CD] is the molar concentration of the CDs. For these calculations, the averaged molar mass based on the DS stated on the certificate was applied in the case of randomly substituted CDs. The CEVal software was used for data fitting because this software allows correction of the migration times in case of tailing peaks according to the Haarhoff-van der Linde equation [19]. The observed mobilities were corrected for the increasing viscosity upon increasing CD concentrations. It should be noted that the obtained data are apparent constants and mobilities rather than thermodynamic values because the ionic strength of the BGEs containing charged CDs is not known, and concentrations referred to the volume and not to the mass of the solvent. Furthermore, in the case of randomly substituted CDs, the constants are averaged over the substitution and positional isomers of the CDs. Although differences between the respective values are typically not statistically significant based on the overlapping 95 % confidence intervals, the trend of the data can still be used to rationalize the migration behavior of the analytes.

3.4.1. Electrolyte pH 2.5

Complexation constants and mobilities obtained for Ala-Phe and the analogs at pH 2.5 for S-α-CD, S-β-CD, S-γ-CD and HS-β-CD are summarized in Table 2. The data were not determined for Ala-homoPhe and S-β-CD because the peptide could only be detected at 1 mg/mL at the cathode and above 40 mg/mL at the anode (reversed polarity) so that a sufficient number of points for reliable curve fitting was not available. In the present study, lower absolute values of the binding constants and complex mobilities were derived for Ala-Phe in the presence of HS-β-CD compared to an earlier study [10]. However, the relative ratio of the values was about the same so that the same conclusions could be reached. The differences can be attributed to differences in the preparation of the BGE, different batches of the CD as well as the application of the Haarhoff-van der Linde equation in the calculations.

With a few exceptions, the D- and DD-enantiomers, respectively, are complexed stronger by the CDs compared to the corresponding L- and LL-stereoisomers. These exceptions include for S-β-CD the dipeptides Ala-Phe, Ala-Phg and Ala-β-Phe and for HS-β-CD Ala-β-Phe, where the LL-enantiomers are complexed preferably. These observations further emphasize that the dependence of the chiral recognition of stereoisomers by CDs may depend on the size of the cavity. For example, the DD-isomers of Ala-Phe, Ala-Phg and Ala-β-Phe are complexed stronger by S-α-CD and S-γ-CD but are complexed weaker than the LL-stereoisomers by S-β-CD. Moreover, S-β-CD preferentially bound the LL-enantiomers of the dipeptides containing two stereogenic centers, while the D-enantiomer was bound stronger when only a single stereogenic carbon was present in the analytes.

In most cases, the EMO could be directly related to the complexation constants at pH 2.5, i.e. the stronger complexed enantiomer migrated second (Table 2). This is in accordance with the fact that the weaker bound stereoisomer is associated with the CD to a lower extend on the timescale and, consequently, migrates faster to the cathode where detection was carried out. However, this was not true for Ala-homoPhe in the presence of S-α-CD, β-Ala-Phe in the presence of S-β-CD and Ala-β-Phe in the presence of HS-β-CD. For these peptides, the weaker bound stereoisomer migrated second. In these instances, the diastereomeric complexes of the weaker bound enantiomer exhibited the higher mobility compared to the respective stronger complexed enantiomer. Therefore, the complex of the weaker bound stereoisomer migrated faster toward the anode due to the overall negative charge. Consequently, EMO in these cases is determined by the complex mobilities rather than the complexation constants. If complexation constants and complex mobilities counteract each other, i.e. when the

Table 2
Apparent complexation constants (K) and mobilities of the free analyte (μ_f) as well as the analyte-CD complexes (μ_c) and enantiomer migration order (EMO) at pH 2.5. The EMO is listed according to detection at the cathode. The numbers in brackets refer to the 95 % confidence interval.

		S-α-CD				S-β-CD			
Peptide		K	μ_f	μ_c	EMO	K	μ_f	μ_c	EMO
		(M^{-1})	($10^{-9}m^2V^{-1}s^{-1}$)	($10^{-9}m^2V^{-1}s^{-1}$)		(M^{-1})	($10^{-9}m^2V^{-1}s^{-1}$)	($10^{-9}m^2V^{-1}s^{-1}$)	
Gly-Phe	L	93	13.1	−13.3	1	87	13.0	−10.0	1
		(61 / 132)	(12.1 / 14.1)	(−11.0 / −16.7)		(76 / 98)	(12.6 / 13.3)	(−9.1 / −10.9)	
	D	99		−13.9	2	92		−10.7	2
		(67 / 139)		(−11.6 / −16.9)		(82 / 104)		(−9.8 / −11.6)	
β-Ala-Phe	L	106	14.3	−12.3	1	65	14.2	−17.1	2
		(75 / 142)	(13.4 / 15.2)	(−10.4 / −14.8)		(60 / 70)	(13.9 / 14.4)	(−16.2 / −18.0)	
	D	113		−13.4	2	89		−10.9	1
		(82 / 148)		(−11.6 / −15.7)		(82 / 96)		(−10.2 / −11.7)	
Ala-Phe	LL	85	13.6	−13.4	1	89	13.5	−12.1	2
		(55 / 124)	(12.6 / 14.7)	(−10.6 / −17.3)		(80 / 100)	(13.2 / 13.8)	(−11.2 / −13.0)	
	DD	93		−13.3	2	84		−11.4	1
		(61 / 134)		(−10.8 / −16.9)		(75 / 94)		(−10.5 / −12.4)	
Ala-Phg	LL	93	12.4	−14.9	1	99	12.4	−7.3	2
		(51 / 148)	(11.1 / 13.7)	(−11.3 / −20.8)		(69 / 139)	(11.6 / 13.2)	(−5.4 / −9.7)	
	DD	100		−16.1	2	91		−4.7	1
		(59 / 154)		(−12.7 / −21.3)		(59 / 136)		(−2.7 / −7.4)	
Ala-homoPhe	LL	121	14.8	−13.8	2	nd	nd	nd	-
		(77 / 183)	(13.3 / 16.3)	(−10.4 / −18.3)					
	DD	125		−13.2	1	nd		nd	-
		(80 / 188)		(−9.8 / −17.4)					
Ala-β-Phe	LL	121	16.6	−12.8	1	106	16.5	−9.7	2
		(95 / 151)	(15.8 / 17.4)	(−11.3 / −14.6)		(93 / 121)	(16.1 / 16.9)	(−8.7 / −10.7)	
	DD	136		−16.7	2	99		−9.6	1
		(111 / 163)		(−15.3 / −18.3)		(86 / 113)		(−8.6 / −10.7)	

		S-γ-CD				HS-β-CD			
Peptide		K	μ_f	μ_c	EMO	K	μ_f	μ_c	EMO
		(M^{-1})	($10^{-9}m^2V^{-1}s^{-1}$)	($10^{-9}m^2V^{-1}s^{-1}$)		(M^{-1})	($10^{-9}m^2V^{-1}s^{-1}$)	($10^{-9}m^2V^{-1}s^{-1}$)	
Gly-Phe	L	182	12.7	−1.1	1	55	13.3	−11.4	1
		(93 / 335)	(11.9 / 13.6)	(−0.7 / −1.6)		(33 / 75)	(10.7 / 15.9)	(−9.5 / −13.4)	
	D	221		−0.9	2	76		−11.1	2
		(118 / 396)		(−0.6 / −1.1)		(51 / 88)		(−9.2 / −12.9)	
β-Ala-Phe	L	201	14	−0.5	1	66	14.5	−14.9	1
		(103 / 368)	(13.1 / 14.9)	(−0.2 / −0.8)		(48 / 83)	(12.2 / 16.9)	(−8.2 / −19.9)	
	D	236		−1.0	2	77		−14.5	2
		(131 / 311)		(−0.6 / −1.3)		(30 / 125)		(−8.4 / −26.3)	
Ala-Phe	LL	172	13.5	−0.1	1	40	13.9	−9.8	1
		(88 / 312)	(12.6 / 14.3)	(−0.05 / −0.2)		(25 / 55)	(10.9 / 16.9)	(−7.5 / −10.9)	
	DD	192		−0.8	2	69		−10.9	2
		(104 / 332)		(−0.4 / −1.2)		(42 / 95)		(−8.7 / −12.1)	
Ala-Phg	LL	115	12.4	−0.4	1	88	12.5	−5.8	1
		(52 / 230)	(11.9 / 12.9)	(−0.2 / −0.6)		(66 / 110)	(12.1 / 12.9)	(−3.5 / −7.9)	
	DD	196		−2.6	2	102		−3.7	2
		(123 / 320)		(−2.0 / −3.1)		(86 / 118)		(−2.1 / −5.4)	
Ala-homoPhe	LL	314	14.5	−2	1	128	14.6	−22.1	1
		(221 / 404)	(13.0 / 16.1)	(−1.5 / −2.4)		(112 / 144)	(11.9 / 17.2)	(−13.9 / −36.3)	
	DD	348		−2.4	2	160		−23.4	2
		(266 / 430)		(−1.3 / −3.9)		(140 / 182)		(−15.9 / −35.7)	
Ala-β-Phe	LL	274	16.5	−1.7	1	94	16.3	−17.2	1
		(196 / 353)	(14.9 / 17.9)	(−0.5 / −2.9)		(76 / 110)	(15.2 / 17.4)	(−14.4 / −20.1)	
	DD	284		−2.7	2	86		−18.3	2
		(206 / 362)		(−2.2 / −3.2)		(68 / 104)		(−15.1 / −21.4)	

nd, not determined

weaker bound enantiomer displays the higher mobility, the separation will deteriorate upon increasing CD concentrations as shown for Ala-Phe in the presence of S-α-CD in Fig. 3A. In fact, counteracting analyte complexation and complex mobilities may result in a reversal of the EMO upon increasing CD concentrations as, for example, discussed in detail for ketoconazole and terconazole in the presence of hydroxypropyl-β-CD [32]. However, this effect could not be observed in the present study, because of the limitations regarding the CD concentrations due to high currents above 150 µA obtained at high CD concentrations. If the stronger bound enantiomer exhibits also the higher complex mobility, separations will improve when CD concentrations are increased as illustrated for the enantioseparation of Ala-Phg by S-β-CD in Fig. 3B. Typically, the complex of the stronger bound enantiomer also exhibited the higher (anodic) mobility at pH 2.5 as also reported for other dipeptides by negatively charged CDs [10].

3.4.2. Electrolyte pH 5.3

The complexation constants and mobilities for β-Ala-Phe, Ala-Phg, Ala-Phe and Ala-homoPhe in presence of S-β-CD and HS-β-CD at pH 5.3 are summarized in Table 3. The data were not determined for β-Ala-Phe S-β-CD because only partial separation was observed even at high CD concentrations so that reliable values could not be obtained. Compared to pH 2.5, weaker complexes were observed at pH 5.3 when the analytes are in the zwitterionic state and essentially uncharged, as compared to the positively charged state at pH 2.5. This has also been described for enantioseparations of other dipeptides using negatively charged

Table 3
Apparent complexation constants (K) and mobilities of the free analyte (μ_f) as well as analyte-CD complexes s (μ_c) and enantiomer migration order (EMO) at pH 5.3. The EMO is listed according to detection at the cathode. The numbers in brackets refer to the 95 % confidence interval.

		S-β-CD				HS-β-CD			
Peptide		K	μ_f	μ_c	EMO	K	μ_f	μ_c	EMO
		(M^{-1})	($10^{-9}m^2V^{-1}s^{-1}$)	($10^{-9}m^2V^{-1}s^{-1}$)		(M^{-1})	($10^{-9}m^2V^{-1}s^{-1}$)	($10^{-9}m^2V^{-1}s^{-1}$)	
β-Ala-Phe	L	nd	nd	nd	-	35	−0.3	−19.0	2
						(28 / 43)	(-0.2 / −0.4)	(−15.6 / −24.2)	
	D	nd		nd	-	31		−20.2	1
						(25 / 39)		(−16.4 / −24.3)	
Ala-Phe	LL	23	−0.4	−16.8	2	20	−0.3	−27.5	2
		(10 / 41)	(-0.3 / −0.5)	(−12.4 / −25.8)		(14 / 26)	(−0.2 / −0.4)	(−22.6 / −35.3)	
	DD	25		−16.5	1	17		−29.4	1
		(12 / 43)		(−12.3 / −24.4)		(12 / 23)		(−23.3 / −40.1)	
Ala-Phg	LL	42	−0.3	−11.3	2	18	-0.2	−10.3	1
		(17 / 63)	(−0.2 / −0.4)	(−8.1 / −19.1)		(14 / 23)	(−0.1 / −0.3)	(−7.9 / −13.4)	
	DD	45		−10.7	1	13		−14.2	2
		(19 / 66)		(−7.8 / −18.3)		(9 / 18)		(−11.3 / −18.9)	
Ala-homoPhe	LL	35	−0.2	−18.1	2	94	−0.2	-26.3	1
		(22 / 53)	(−0.1 / −0.3)	(−14.6 / −24.1)		(59 / 139)	(−0.1 / −0.3)	(−21.5 / −34.8)	
	DD	28		−19.7	1	109		−24.9	2
		(16 / 34)		(−15.2 / −28.6)		(71 / 158)		(−20.7 / −31.7)	

nd, not determined

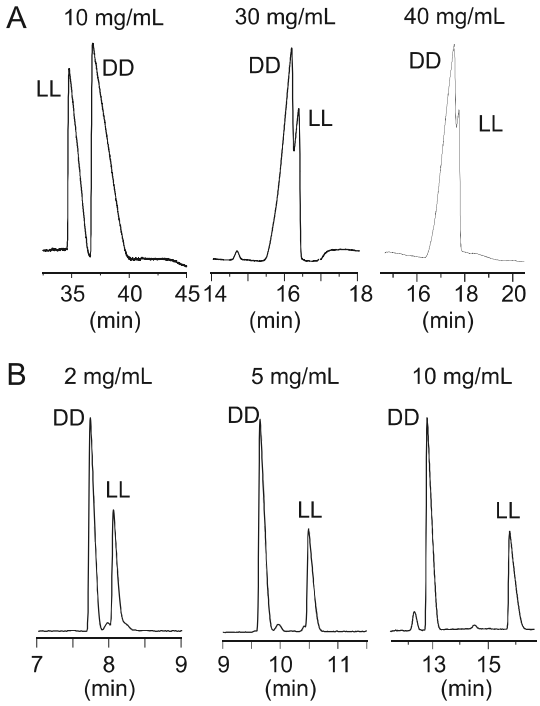

Figure 3. Dependence of the enantioseparation on the CD concentration of (A) Ala-Phe by S-α-CD and (B) Ala-Phg by S-β-CD. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium phosphate buffer, pH 2.5, 20°C, 25 kV. Separations of Ala-Phe (A) were carried out at normal polarity at 10 mg/mL (detection at cathode) and under reversed polarity at higher concentrations (detection at anode).

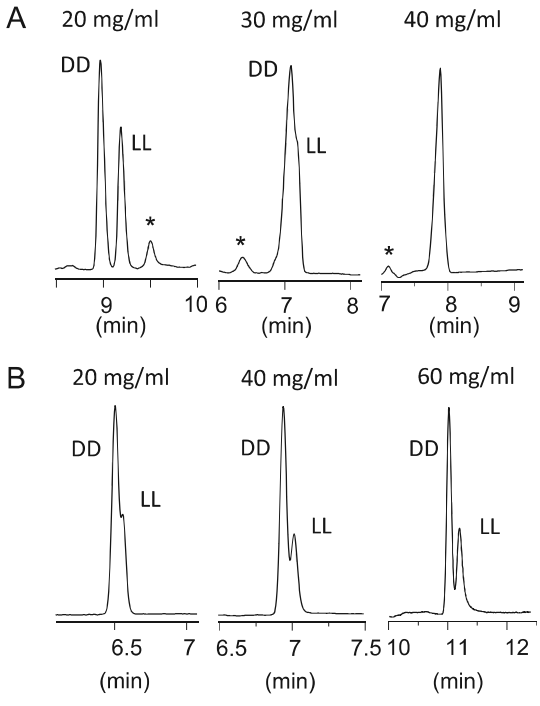

Figure 4. Dependence of the enantioseparation of (A) Ala-HomoPhe and (B) Ala-Phe on the concentration of S-β-CD. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium acetate buffer, pH 5.3, 20°C, 25 kV. * synthetic impurity.

CDs [9,10]. As can be concluded from the low anodic mobility of the free analytes (Table 3), the peptides are in fact slightly negatively charged so that electrostatic repulsion might contribute to the low binding affinity. With respect to complexation constants, chiral recognition was opposite for S-β-CD and HS-β-CD. Compared to pH 2.5, opposite chiral recognition at pH 5.3 by S-β-CD was observed for Ala-Phe and Ala-Phg and in case of HS-β-CD for β-Ala-Phe, Ala-Phe and Ala-Phg. Thus, the charge state of the dipeptides affected chiral recognition.

The weaker bound enantiomer always exhibited higher electrophoretic mobility at pH 5.3, in accordance with an earlier study on negatively charged CDs [10]. Thus, the stronger bound enantiomer should migrate first when detected at the cathode (after the EOF) because of its slower overall mobility toward the anode. This is true for Ala-Phe and Ala-Phg in the presence of S-β-CD and Ala-Phg in the presence of HS-β-CD, whereas for the other combinations the weaker bound enantiomer displaying the higher anodic complex mobility was migrating first. In these cases, the separation deteriorated when increasing the CD concentrations as illustrated for Ala-homoPhe in Fig. 4A. At low concentrations, the separation

is governed by the affinity of the CD toward the enantiomers expressed as complexation constants, while at high concentrations, when essentially all analyte molecules are bound, complex mobilities increasingly affect the overall separation. This results in a loss of enantioresolution. In contrast, when the stronger bound enantiomer migrated first even at low CD concentrations, the enantioseparation will improve at higher concentrations because of the increasing influence of the complex mobilities on the separation process. An example for this scenario is shown in Fig. 4B. Thus, as also observed at pH 2.5 the stereoselective recognition of the solutes by the CDs as well as the mobility of the diastereomeric complexes contribute to an enantioseparations and these parameters may either cooperate or counteract each other.

4. Conclusions

Under standardized CE conditions, the LL- and DD-enantiomers of the Ala-Phe and five structural analogs could be separated by negatively charged α-, β- and γ-CDs. Although the number of studied α- and γ-CD derivatives was limited, β-CD derivatives seemed to be more effective chiral selectors. The EMO depended on the size of the cavity of the CD as well as the substitution pattern of the CD derivatives as observed for other dipeptides and CDs [7,9,14,23,24].

Increasing the pH of the BGE from 2.5 to 5.3 resulted in the reversal of the EMO in many cases. However, because the peptides were detected before the EOF at pH 2.5 but after the EOF at pH 5.3, this did not correspond to a change in the chiral recognition of the peptide enantiomers by the CDs. In contrast, identical EMO at both pH values indicated changes in the recognition of the analyte enantiomers. As reported previously for other CD derivatives [8,10,11,14], the EMO of dipeptide was based on the stereoselective complexation of the enantiomers by the CDs as well as by the mobilities of the diastereomeric analyte-CD complexes and the predominant mechanism determined the migration order. This may result in a reversal of the EMO depending on the experimental conditions even in cases, when the chiral recognition of the selector toward the analyte enantiomers does not change.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mari-Luiza Konjaria: Investigation, Formal analysis, Writing - original draft. **Gerhard K.E. Scriba:** Conceptualization, Supervision, Writing - review & editing.

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3.4 Manuscript 3

Manuscript No. 3

Manuscript title: Effects of amino acid-derived chiral ionic liquids on cyclodextrin-mediated capillary enantioseparations of dipeptides

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Author	Conceptual	Data analysis	Experimental	Writing the manuscript
Mari-Luiza Konjaria	85	85	100	70
Gerhard K.E. Scriba	15	15		30
Total:	100%	100%	100%	100%

Overview:

The manuscript describes the synergistic effect of chiral ionic liquids composed of tetraalkylammonium ions and the amino acids L-Asn, L-Asp or L-Pro on the enantioseparations of dipeptides mediated by β-CD and HP-β-CD. The migration order of the enantiomers was not affected by the presence of a chiral ionic liquids in the BGE. The individual contribution of the components composing L-Asp based CILs were investigated at pH values 2.5 and 3.5 respectively. Additionally, stereochemistry of the CILs was studied upon including D-amino acid-based chiral ionic liquids combination with primary chiral selectors.

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Effects of amino acid-derived chiral ionic liquids on cyclodextrin-mediated capillary electrophoresis enantioseparations of dipeptides

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ABSTRACT

The synergistic effect of chiral ionic liquids composed of tetraalkylammonium ions and the amino acids Asn, Asp or Pro on the enantioseparations of dipeptides mediated by β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin in capillary electrophoresis was studied. Addition of a chiral ionic liquid resulted in a concentration-dependent increase in the enantioresolutions compared to the sole presence of a cyclodextrin in the background electrolyte. The extent varied with the tetraalkylammonium cation (tetramethylammonium versus tetrabutylammonium) as well as the amino acid component of the ionic liquid. The presence of a chiral ionic liquid did not counteract the pH-dependent reversal of the enantiomer migration order of the dipeptides Ala-Phe, Ala-Tyr and Phe-Phe when increasing the pH of the background electrolyte from 2.5 to 3.5. Comparing the effect of a chiral ionic liquid based on Asp with the addition of equimolar concentrations of the individual components of the ionic liquid, a diverse picture was observed. In some cases, higher resolution values were obtained with the chiral ionic liquid, while for other cases superior enantioseparations were obtained upon separate addition of the amino acid component and a tetraalkylammonium chloride. With regard to the stereochemistry of the amino acid, a superior effect was typically observed using the L-configured amino acid, but in some cases higher resolution values were found in the presence of D-Asp. The rationale for the diverse observations is not obvious and may be due to the zwitterionic nature of analytes as well as the amino acid component of the chiral ionic liquid.

1. Introduction

Ionic liquids have attracted increased attention in separation sciences in recent years [1,2]. Ionic liquids are organic salts with melting points below 100°C, often around room temperature. They have relatively high conductivity and thermal stability and exhibit low vapor pressure. The majority of applications of ionic liquids in separation sciences has been reported for CE followed by HPLC and GC [3,4]. With regard to enantioseparations, chiral ionic liquids (CILs) have been used in most cases [4–6]. In CILs, either the cation or the anion or both are chiral compounds. As chiral cations amino acid esters, alkaloids such as ephedrine, chiral choline or imidazolinium derivatives have been used, while chiral anions included amino acids, lactic acid or camphorsulfonic acid [2,6-8]. In the majority of CE applications, the synergism of CILs in combination with other selectors has been exploited. In these studies, the enantioseparation is primarily mediated by the primary chiral selector, while the CIL itself did not appear to possess significant chiral recognition toward the enantiomers. First reports of this kind date to 2007 [9] and 2009 [10], when cyclodextrins (CDs) were combined with chiral choline derivatives or a ionic liquid-type surfactant. Since then, CILs were frequently used in combination with CDs to separate many structurally diverse basic and acidic drugs [4–8] but other selectors such as vancomycin, maltodextrin, clindamycin or cyclofructans have been studied [6,8]. Only a few publications reported the use of a CIL as single chiral selector. The first application was reported in 2006 by micellar electrokinetic chromatography (MEKC) employing amino acid-based surfactants [11]. The first publication on a chiral amino acid-derived CIL as sole selector demonstrated the enantioresolution of 1,1′-binaphthyl-2,2′-diylhydrogenphosphate using amino acid ester-based CILs [12]. Other examples are the enantioseparation of basic drugs in the presence of CILs derived from lactobionic acid [13,14] or, most recently, mono- and di-tetraalkylammonium L-tartrate ionic liquids [15,16]. Functionalized ionic liquids based on

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CDs were also studied [17,18] and CILs have been used as ligand exchangers for the separation of the enantiomers of native and derivatized amino acids [4–8].

Small peptides are a special group of analytes. They are amphoteric compounds and display specific migration phenomena in CE such as the pH-dependent reversal of the enantiomer migration order [19–22]. For example, it has been shown that the LL-stereoisomer of the dipeptides Ala-Phe and Ala-Tyr migrated before the DD-isomers in the presence of β-CD as chiral selector at pH 2.5, while the opposite migration order, i.e., DD before LL, was observed when the pH was raised to pH 3.5 [19]. This behavior was rationalized based on differences of the chiral recognition of the enantiomers by the CD as a function of the charge state of the analytes or a complexation-induced pKa shift of the analytes [23]. The separation of the *all*-L and *all*-D enantiomers of di- and tripeptides has been studied in the presence of many CDs [24–28], but no reports on the application of CILs for peptide enantioseparations have been reported in this context. Therefore, the aim of the present study was the evaluation of the effect of the addition of CILs to CD-mediated enantioseparations of dipeptides in order to explore if the often-reported synergistic effect between CDs and CILs would also apply to peptide analytes. β-CD and 2-hydroxypropyl-β-CD (HP-β-CD) were selected as CDs and tetraalkylammonium-amino acids as CILs. The latter have been successfully employed in recent years in combination with CDs for the enantioseparation of structurally diverse basic drugs [29–32] as well as the amino acids Phe and Trp in an aqueous background electrolyte (BGE) [33] or dansyl-derivatized amino acids in non-aqueous CE [34].

2. Experimental

2.1. Chemicals

β-CD was from Wacker Chemie (Munich Germany) and HP-β-CD (degree of substitution ~ 4.5) from Cyclolab Ltd. (Budapest, Hungary). L-Asp, L-Asn, L-Pro, Gly-L-Phe, Gly-D-Phe, L-Ala-L-Phe, D-Ala-D-Phe, L-Ala-L-Tyr, L-Phe-L-Phe, D-Ala-D-Phe and L-Lys-L-Phe were obtained from Bachen AG (Heidelberg, Germany). D-Ala-D-Tyr and D-Lys-D-Phe were prepared by standard peptide chemistry procedures reacting the respective *N*-hydroxysuccinimide of the *N*-benzyloxycarbonyl-protected amino acid and D-Tyr or D-Phe (all from Bachem AG, Heidelberg, Germany) in dimethylformamide followed by hydrogenolytic deprotection in the presence of Pd/C as catalyst [35]. Tetramethylammonium hydroxide pentahydrate (TMA-OH), tetrabutylammonium hydroxide 30-hydrate (TBA-OH), tetramethylammonium chloride (TMA-Cl) and tetrabutylammonium chloride (TBA-Cl) were from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and obtained from commercial sources. Water was purified using a TKA Genpure UV-TOC from Thermo Scientific (Waltham, USA). BGEs and sample solutions were filtered through 0.22 µm polypropylene syringe filters from BGB Analytik (Schloßböckelheim, Germany).

2.2. Synthesis chiral ionic liquids

$[TMA]_2$[L-Asp], $[TBA]_2$[L-Asp], [TMA][L-Asn], [TBA][L-Asn], [TMA][L-Pro] and [TBA][L-Pro] as well as the corresponding D-Asp and D-Asn containing CILs were prepared according to [29,36] by mixing 1 equivalent of the amino acid and the respective amount (1 or 2 equivalents) of the tetraalkylammonium hydroxide in water. The reaction mixture was heated at 60°C for 2 h and the water was subsequently removed under reduced pressure. The residue was dissolved in ethanol when preparing TMA-containing CILs or in acetonitrile for TBA-derived CILs, filtered and dried over anhydrous sodium sulfate. The organic solvent was removed under reduced pressure and the product was dried under high vacuum overnight. The CILs were characterized by ^{1}H-NMR spectroscopy (see supplementary material).

2.3. Capillary electrophoresis

All experiments were conducted on a Beckman P/ACE MDQ CE system (AB Sciex, Darmstadt, Germany) equipped with a UV-Vis diode array detector and controlled by 32 KARAT software for system control, data acquisition and processing. 50 µm I.D., 365 µm O.D. fused-silica capillaries with a total length of 50.2 cm and an effective length of 40 cm were from BGB Analytik (Schloßböckelheim, Germany). All rinsing steps were conducted at a pressure of 138 kPa (20 psi). A new capillary was treated subsequently with 0.1 M NaOH for 20 min, water for 10 min, 0.1 M NaOH for 10 min, 0.1 M phosphoric acid for 10 min and water for 10 min. When starting experiments with a new CIL, the capillary was washed with 0.1 M NaOH for 5 min, with water for 5 min and the CIL-containing BGE for 30 min. Between the analyses, the capillary was flushed with 0.1 M NaOH for 3 min, water for 2 min and with the BGE for 8 min. The applied voltage was 25 kV and the capillary temperature was 20°C. UV detection performed at 215 nm at the cathodic end of the capillary in case of normal polarity and at the anodic end when polarity was reversed. Dimethyl sulfoxide (DMSO) was used as electroosmotic flow (EOF) marker at a concentration of 200 µg/mL. The marker was analyzed separately and injected at a pressure of 3.5 kPa (0.5 psi) for 5 s.

Separations were performed in 50 mM sodium phosphate buffer prepared on a daily basis starting from phosphoric acid. The pH was adjusted after the addition of the CDs and/or CILs or the individual components of the CILs with 0.1 M NaOH. The BGEs were filtered (0.22 µm) and degassed by sonication before use. Sample solutions of the peptides (200 µg/mL prepared in purified water) were introduced at a pressure of 3.5 kPa (0.5 psi) for 5 s. The migration order was confirmed by spiking with the individual peptide enantiomers.

3. Results and discussion

The structures of the CILs and the dipeptide analytes are shown in Fig. 1. Six chiral CILs derived from L-Asp, L-Asn and L-Pro were evaluated as chiral co-selectors in combination with β-CD and HP-β-CD for the separation of the LL- and DD-stereoisomers of the dipeptides Ala-Phe, Ala-Tyr, Phe-Phe and Lys-Phe as well as Gly-L-Phe and Gly-D-Phe. The "mixed" stereoisomers with LD or DL configuration were not available and therefore included. Ala-Phe, Ala-Tyr and Phe-Phe were selected as they exhibited pH-dependent reversal of the enantiomer migration order in the presence of β-CD [19], while Lys-Phe is a basic dipeptide and Gly-Phe a simple dipeptide with a single chiral center. Analyses were performed under standardized conditions in bare fused-silica capillaries at an applied voltage of 25 kV and a temperature of 20°C using 50 mM sodium phosphate buffer at pH 2.5 or 3.5. Under these conditions, the peptides are positively charged albeit the charge density is less at pH 3.5 due to the increased deprotonation of the carboxylic acid function at this pH. The aim of the present study was not to develop an optimized method but to explore, if the often-reported synergistic effect between CDs and amino acid-derived CILs [4-8,29-32] would also apply for dipeptide analytes. Consequently, the effect of the concentration of the CDs on the enantioseparations was not evaluated and it was fixed at 20 mM based on earlier studies [19,37] as well as the fact that 20 mM represents the limiting aqueous solubility of β-CD. Under these conditions, partial separations of dipeptide enantiomers were typically achieved in the presence of β-CD or HP-β-CD as single selectors with R_S values up to 1.2 except for Gly-Phe at pH 2.5 and Lys-Phe at pH 3.5 in

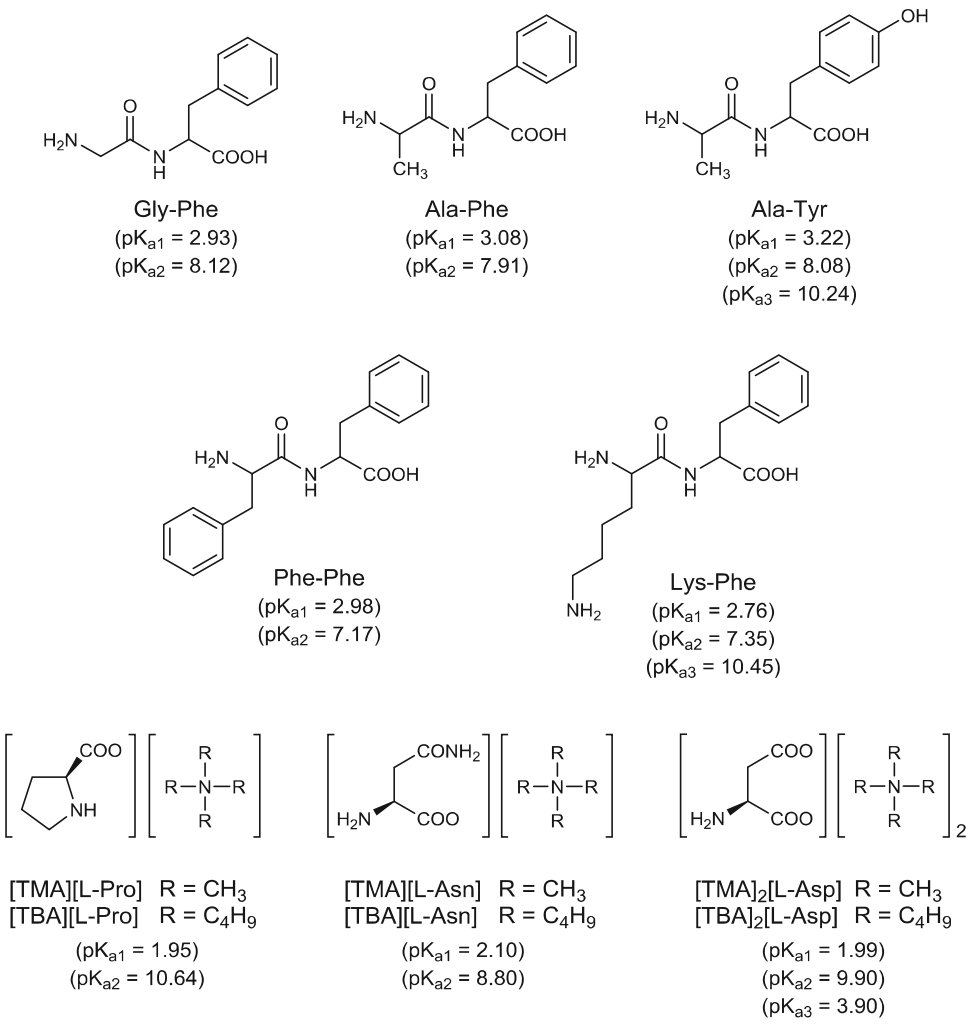

Fig. 1. Structures of dipeptide analytes and chiral ionic liquids. The pK_a values listed in brackets were taken from [40] for Gly-Phe, Ala-Phe and Phe-Phe, from [41] for Lys-Phe and from [48] for Ala-Tyr. pK_a values of the amino acids are from [46]. pK_{a1}, carboxylic acid group; pK_{a2}, α-amino group; pK_{a3}, side chain functional group, if present.

the case of β-CD (Table 1). Using HP-β-CD as selector Gly-Phe and Ala-Phe could not be enantioresolved at pH 3.5 and Ala-Phe also at pH 2.5 (Table 2). As described earlier, pH-dependent reversal of the enantiomer migration order was observed for Ala-Phe, Ala-Tyr and Phe-Phe in the presence of β-CD when increasing the pH from 2.5 to 3.5 [19,37]. This has been attributed to the increasing influence of the mobilities of the diastereomeric analyte-CD complexes over the chiral recognition of the analyte enantiomers by the CDs expressed as complexation constants [20].

3.1. Effect of the nature of the CIL

CILs were prepared from TMA-OH or TBA-OH and the respective amino acids. CILs containing two tetraalkylammonium counterions were obtained for L-Asp, while L-Asn and L-Pro-derived CILs contained a single counterion. Thus, a total of six CILs were evaluated, [TMA]$_2$[L-Asp], [TBA]$_2$[L-Asp], [TMA][L-Asn], [TBA][L-Asn], [TMA][L-Pro] and [TBA][L-Pro] (Fig. 1). The CILs were added at concentrations of 5, 15, 30 and 50 mM to the BGEs containing 20 mM of the CDs at pH 2.5. At pH 3.5 the concentration of the CILs was limited to 30 mM because analyte migration times exceeded 60 min under these conditions. The results for systems containing β-CD are summarized in Table 1, while data of HP-β-CD-mediated enantioseparations can be found in Table 2.

Generally, an enantioseparation of the peptide analytes was not observed upon use of the CILs as single additives to the background electrolyte. However, combined with β-CD or HP-β-CD, addition of the CILs resulted in significant improvements of the chiral resolution in most cases (Tables 1 and 2). R_S values as high as 4.9 were observed for the enantioseparation of Gly-Phe at pH 2.5 when using 20 mM HP-β-CD in combination with 50 mM [TBA]$_2$[L-Asp]. This refers to a 4.5-fold increase in resolution compared to the absence of the CIL. Another example is the enantioresolution of Phe-Phe, which increased from $R_S = 1.2$ in the presence of β-CD at pH 2.5 to $R_S = 4.5$ upon addition of [TMA]$_2$[L-Asp]. Except for Lys-Phe at pH 3.5 in the presence of β-CD, partial resolution was observed also in cases when no enantioseparation was observed in the system containing only the CD. Fig. 2 shows representative examples comparing the CD-mediated separations of peptide enantiomers in the absence and the presence of CILs. Addition of a CIL always led to a concomitant significant increase in migration time at both pH values studied. This can be attributed to the increasing viscosity and ionic strength of the background electrolyte upon addition of the CILs and/or the reduction of the EOF

Table 1
Enantioresolution (R_S) and migration time of the first migrating enantiomer (t1) of the dipeptides at pH 2.5 and pH 3.5 in presence of 20 mM β-CD as chiral selector and different concentration of the CILs.

		Gly-Phe				Ala-Phe				Ala-Tyr				Lys-Phe				Phe-Phe			
		pH 2.5		pH 3.5		pH 2.5		pH 3.5		pH 2.5		pH 3.5		pH 2.5		pH 3.5		pH 2.5		pH 3.5	
Selector	c (mM)	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1
β-CD	20	0.0	9.7	0.3	17.2	0.8	10.2	0.7	14.8	0.6	11.0	1.1	14.6	1.0	6.7	0	7.6	1.2	11.4	0.7	14.3
+ $[TMA]_2$[L-Asp]	5	0.0	12.6	0.7	24.1	0.8	12.7	0.6	19.8	0.6	13.6	1.3	19.3	1.1	7.5	0.0	8.9	1.3	14.3	1.0	19.1
	15	0.0	17.0	1.1	47.3	1.0	15.8	0.9	21.6	0.9	17.0	1.6	21.3	1.4	9.0	0.0	11.3	1.4	17.5	1.3	20.9
	30	0.3	24.9	nd[a]	nd	1.3	24.4	1.2	73.8	1.1	28.6	nd	nd	1.6	10.8	0.0	15.6	2.1	31.9	0.9	32.7
	50	0.4	38.7	na[b]	na	1.8	37.6	na	na	1.6	48.3	na	na	2.0	13.0	na	na	4.5	83.8	na	na
+ $[TBA]_2$[L-Asp]	5	0.0	14.7	0.4	21.7	1.2	14.6	0.9	18.4	0.8	15.7	1.4	17.8	df[c]	df	0.0	8.3	1.6	20.3	1.3	17.0
	15	0.0	17.2	0.7	31.9	1.2	16.8	0.9	26.8	1.0	18.7	1.9	26.1	1.7	9.2	sh	10.1	1.7	20.1	2.1	25.4
	30	0.0	23.1	1.5	91.8	1.7	22.7	1.3	55.9	1.2	25.5	2.2	50.4	3.1	11.2	sh	13.3	2.4	27.3	3.5	68.1
	50	0.4	29.3	na	na	2.0	28.6	na	na	1.5	32.9	na	na	3.9	13.3	na	na	3.0	36.5	na	na
+ [TMA][L-Asn]	5	0.0	15.6	0.2	18.3	0.8	15.1	0.9	14.9	0.3	16.6	1.4	16.3	0.9	8.6	0.0	7.8	0.8	17.0	1.0	14.2
	15	0.0	18.2	0.4	24.7	0.8	18.2	1.1	19.3	0.4	20.1	1.4	18.3	1.1	9.4	0.0	8.5	1.1	20.7	1.6	18.4
	30	sh[d]	22.8	1.0	33.5	1.2	21.8	1.4	28.5	0.7	24.9	1.8	29.8	1.2	9.8	0.0	10.9	1.3	25.4	1.6	29.5
	50	sh	28.4	1.0	41.2	1.2	27.3	1.4	33.0	0.8	31.4	1.7	31.4	1.4	11.3	0.0	11.4	1.8	34.1	1.9	30.5
+ [TBA][L-Asn]	5	0.0	19.3	0.6	43.4	1.5	33.1	1.3	32.2	1.0	39.5	1.7	30.7	1.1	11.0	0.0	11.1	1.8	41.6	1.7	30.0
	15	0.5	53.0	0.8	37.7	2.0	47.5	1.1	29.3	1.1	30.6	1.1	25.1	df	df	0.0	10.4	1.9	33.8	1.3	24.6
	30	0.6	72.3	0.7	36.0	2.2	46.5	1.2	23.8	1.8	60.9	1.7	23.9	2.4	15.5	0.0	9.9	3.7	85.0	1.2	21.7
	50	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na	na
+ [TMA][L-Pro]	5	0.0	13.1	1.0	18.6	0.6	12.7	1.2	18.8	0.4	14.0	1.3	17.8	0.7	7.6	0.0	8.7	0.9	14.5	1.1	18.0
	15	0.0	18.1	1.1	32.1	0.7	17.9	1.3	26.6	0.4	19.3	1.8	25.7	0.9	9.5	0.0	10.4	0.9	20.2	1.5	25.7
	30	sh	27.2	1.1	30.3	0.8	26.4	1.3	31.0	0.5	30.8	1.4	27.9	0.7	10.6	0.0	10.8	1.1	33.4	1.2	28.5
	50	sh	34.5	1.0	50.2	1.1	32.8	1.1	36.8	nd	nd	1.3	34.9	0.9	12.6	0.0	12.1	1.7	50.0	1.1	33.2
+ [TBA][L-Pro]	5	0.0	12.6	0.5	19.3	0.8	12.4	1.1	17.7	0.4	13.4	1.4	17.7	0.6	7.4	0.0	8.6	1.1	13.8	1.4	17.9
	15	0.0	16.4	1.0	28.4	1.0	16.1	1.1	23.2	0.5	17.6	1.4	22.8	df	df	0.0	9.6	1.4	18.3	1.1	22.2
	30	0.0	18.4	1.0	27.3	1.2	18.0	1.0	23.4	0.6	19.9	1.4	23.3	2.1	9.6	0.0	9.9	1.7	20.7	1.5	23.4
	50	sh	22.6	0.9	41.0	1.3	23.6	0.7	27.2	0.8	27.8	1.8	31.5	2.4	11.3	sh	11.1	1.8	30.2	1.4	29.8

[a] nd, not detectable within 60 min
[b] na, not analyzed
[c] df, deformed peak
[d] sh, shoulder.

Table 2
Enantioresolution (R_S) and migration time of the first migrating enantiomer (t1) of the dipeptides at pH 2.5 and pH 3.5 in presence of 20 mM HP-β-CD as chiral selector and different concentration of the CILs.

		Gly-Phe				Ala-Phe				Ala-Tyr				Lys-Phe				Phe-Phe			
		pH 2.5		pH 3.5		pH 2.5		pH 3.5		pH 2.5		pH 3.5		pH 2.5		pH 3.5		pH 2.5		pH 3.5	
Selector	c (mM)	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1	R_S	t1
HP-β-CD	20	1.1	9.2	0.0	20.6	0.0	9.0	0.0	16.5	0.6	9.6	0.3	15.7	sh[d]	5.8	0.9	7.5	0.6	9.9	1.1	14.6
+ $[TMA]_2$[L-Asp]	5	1.9	13.9	0.0	29.1	0.0	13.8	0.4	22.3	1.0	15.4	0.5	20.7	0.9	7.5	1.0	8.4	1.0	15.7	1.2	18.5
	15	2.4	19.2	1.7	29.1	0.0	19.4	0.6	46.8	1.3	22.0	0.7	42.5	1.0	8.8	1.1	10.3	1.2	23.8	1.6	36.1
	30	3.1	30.0	nd[a]	nd	0.7	29.3	nd	nd	1.5	36.4	nd	nd	1.1	10.9	0.9	17.0	1.6	41.4	nd	nd
	50	3.6	36.8	na[b]	na	1.0	44.1	na	na	nd	nd	na	na	1.4	12.8	na	na	nd	nd	na	na
+ $[TBA]_2$[L-Asp]	5	1.6	14.6	0.0	26.9	0.0	14.7	0.1	21.0	1.2	16.2	0.6	19.6	0.0	7.8	0.9	8.4	1.2	17.0	1.1	18.6
	15	2.1	19.0	0.2	38.3	0.7	19.2	0.4	28.9	1.6	21.8	0.6	27.8	0.6	9.3	0.8	9.8	1.6	23.6	1.4	25.8
	30	2.8	24.6	0.8	110.5	1.0	24.8	0.6	58.2	2.0	28.4	0.7	49.6	0.9	11.3	0.9	12.5	1.9	31.4	2.0	44.0
	50	4.9	53.1	na	na	1.3	59.3	na	na	3.7	66.8	na	na	1.6	15.9	1.1	15.0	nd	nd	na	na
+ [TMA][L-Asn]	5	1.7	13.8	0.4	20.3	0.6	14.1	0.7	16.8	1.3	15.3	0.4	16.7	0.9	7.5	0.8	7.7	0.9	15.8	1.3	16.4
	15	1.8	17.1	0.9	29.2	0.7	17.0	0.7	23.9	1.5	19.6	0.4	25.0	1.0	8.5	0.8	8.7	1.0	20.6	1.2	22.3
	30	2.0	22.8	1.3	22.0	0.9	22.2	0.7	23.0	1.8	25.7	0.5	21.9	1.2	9.5	0.8	8.6	1.2	27.2	1.2	21.0
	50	2.8	30.2	1.0	29.3	1.0	30.3	0.6	25.7	2.3	37.7	0.4	27.1	1.2	11.2	0.8	9.6	1.7	40.8	1.4	25.7
+ [TBA][L-Asn]	5	1.7	15.5	0.4	20.6	0.7	15.6	0.7	17.2	1.5	17.4	0.0	16.1	0.8	8.0	0.6	7.9	1.1	18.4	1.2	16.1
	15	2.0	20.9	0.4	29.4	0.9	20.6	0.6	22.0	1.8	23.7	0.0	19.7	df[c]	df	0.9	8.3	1.3	25.5	1.1	18.6
	30	2.7	27.2	0.4	28.7	1.3	26.6	0.6	22.4	2.0	31.7	0.0	20.1	0.5	11.2	1.0	8.8	1.6	35.1	1.3	20.5
	50	nd	nd	0.4	36.2	0.9	29.7	0.8	26.4	nd	nd	0.5	26.7	0.9	12.2	0.9	9.5	1.9	37.6	1.5	23.6
+ [TMA][L-Pro]	5	1.9	16.5	0.3	28.9	0.6	16.5	0.7	23.6	1.3	18.7	0.5	22.9	1.2	8.5	0.9	9.0	1.2	19.4	1.2	22.7
	15	2.2	18.2	0.4	45.9	0.5	18.1	0.5	35.2	1.6	21.1	0.8	22.6	1.2	8.6	1.0	11.0	1.5	22.3	1.2	32.8
	30	1.6	33.3	1.1	22.6	0.7	35.1	1.0	45.5	1.6	36.1	0.9	39.4	1.3	10.9	0.9	10.3	1.7	39.3	1.6	40.1
	50	nd	nd	1.5	84.9	nd	nd	1.0	43.0	nd	nd	1.3	40.3	1.6	13.6	1.0	12.2	nd	nd	1.0	25.6
+ [TBA][L-Pro]	5	1.8	13.2	0.4	24.2	0.7	13.2	0.6	17.9	1.4	14.3	0.5	20.6	0.9	7.2	0.5	8.6	1.0	14.8	1.2	19.5
	15	2.0	15.5	0.5	30.9	0.8	15.6	0.7	24.8	1.6	17.2	0.6	22.9	df	df	0.6	9.1	1.1	18.3	1.3	22.3
	30	2.3	19.7	0.5	42.0	0.7	19.7	0.6	30.5	1.6	22.2	0.5	26.7	0.3	9.5	0.8	9.7	1.3	23.8	1.4	25.0
	50	2.9	28.0	0.5	50.3	0.9	29.2	0.8	33.5	2.1	34.2	0.6	31.5	0.6	11.5	1.0	10.3	1.9	36.3	1.7	29.8

[a] nd, not detectable within 60 min
[b] na, not analyzed
[c] df, deformed peak
[d] sh, shoulder.

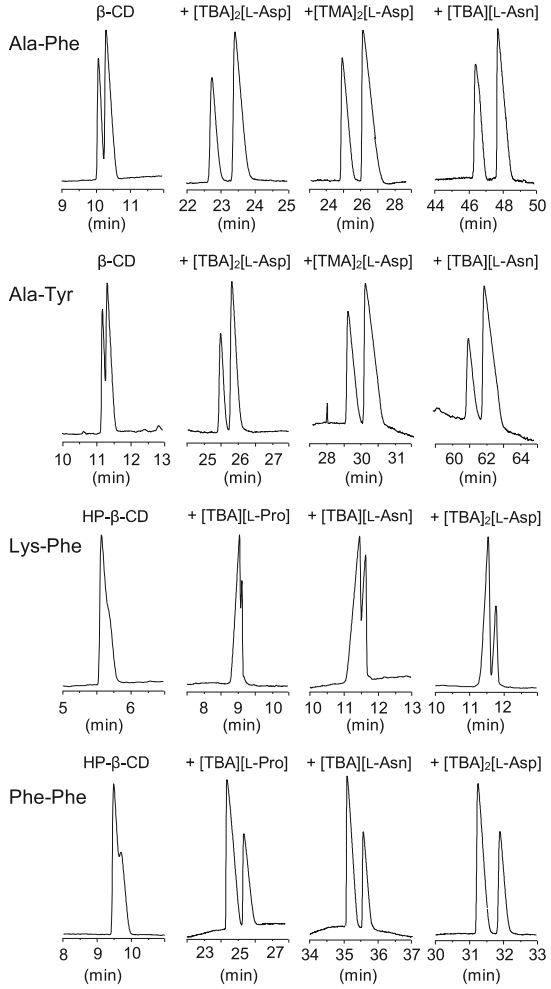

Fig. 2. Typical electropherograms showing the synergism of the addition of the CILs on the enantioseparation of Ala-Phe and Ala-Tyr by β-CD and Lys-Phe as well as Phe-Phe HP-β-CD, respectively. Experimental conditions: 40/50.2 cm, 50 µm I.D. fused-silica capillary, 50 mM sodium phosphate buffer, pH 2.5, containing 20 mM β-CD or HP-β-CD as well as 30 mM of the respective CIL, 20°C, 25 kV, 215 nm.

caused by the adsorption of the TMA and TBA cations to the capillary wall [38,39].

With regard to the type of the CIL, CILs derived from L-Asp, i.e. the CILs with two tetraalkylammonium cations $[TMA]_2$[L-Asp] and $[TBA]_2$[L-Asp], typically yielded higher R_S values than CILs based on L-Asn or L-Pro although effective enantioseparations were also observed for the latter. For example, $R_S = 3.7$ was observed for the enantioseparation of Phe-Phe with the system β-CD/[TBA][L-Asn] at pH 2.5 (Table 1) or $R_S = 2.9$ in the case of the enantioseparation of Gly-Phe using HP-β-CD/[TBA][L-Pro] at pH 2.5 (Table 2). Nonetheless, L-Pro-based CILs were overall less effective enhancing CD-mediated resolutions of the peptide enantiomers in most cases compared to L-Asp or L-Asn CILs independent of the CD applied.

Concerning the tetraalkylammonium cation type, there appears to be a clear tendency that TBA-based CILs yielded higher resolutions compared to the addition of TMA-CILs although for some analytes TMA-CILs were more effective. For some peptide enantiomers approximately equal enantioresolutions were observed independent of the type of the cation. At pH 2.5 in the presence of β-CD, TBA-based CILs yielded higher R_S values compared to TMA-CILs except for the enantioseparation of Phe-Phe where addition of 50 mM $[TMA]_2$[L-Asp] resulted in $R_S = 4.5$, while $R_S = 3.0$ was found for 50 mM $[TBA]_2$[L-Asp]. At pH 3.5, the superiority of TBA-CILs was less apparent because often comparable enantioresolutions were achieved with both types of CILs or the TMA-based CIL was more effective (Table 1). Similar conclusions can be drawn when using HP-β-CD as primary selector (Table 2). At pH 2.5 TBA-CILs were typically more effective than TMA-containing CILs, while similar efficiency of both types of CILs or a slight advantage of the TMA-based CILs was observed in several cases at pH 3.5.

3.2. Effect of CIL concentration

The concentration of additives is an important factor influencing chiral separations. In order to conclude on the effect of the CILs only, the concentration of the CDs was fixed at 20 mM, while the concentration of the CILs varied between 5 and 50 mM. Results are summarized in Table 1 (β-CD) and Table 2 (HP-β-CD). Typically, resolution of the peptide enantiomers increased with increasing concentration of CILs. In few cases, however, a decrease of the resolution was also noted. For example, in the presence of 20 mM HP-β-CD, a shoulder was observed for Lys-Phe at pH 2.5, which disappeared upon addition of 5 mM $[TBA]_2$[L-Asp]. When further increasing the concentration of the CIL, an enantioseparation was observed and $R_S = 1.6$ was obtained at 50 mM $[TBA]_2$[L-Asp]. Another example is the separation of the Ala-Tyr enantiomers at pH 2.5 with the β-CD/[TMA][L-Asn] system. The initially determined R_S value of 0.6 in the absence of the CIL dropped to 0.3 at 5 mM [TMA][L-Asn] and then slowly increased with increasing CIL concentration up to 0.8 at 50 mM [TMA][L-Asn]. Further examples can be found in Tables 1 and 2 such as the enantioseparation of Ala-Phe at pH 2.5 using β-CD and [TMA][L-Pro] or the resolution of the Phe-Phe enantiomers in the presence of β-CD and [TMA][L-Asn]. Moreover, a minor decrease of the calculated R_S values upon addition of a CIL presented in Tables 1 and 2 can be explained by small variations in the experimentally determined values required for R_S calculations. In case of some CD/CIL combinations the resolution leveled off or even decreased at high concentrations. This may be attributed to the fact that the analyte peaks also broadened because of the longer migration times in the presence of high CIL concentrations.

3.3. Effect of the pH of the background electrolyte

The pH value of background electrolyte is another important parameter for chiral separations. In the present study, only pH 2.5 and 3.5 were evaluated because pH-dependent reversal of the enantiomer migration order had been observed for Ala-Phe, Ala-Tyr and Phe-Phe using β-CD as chiral selector [19,37]. The phenomenon had not been observed in the presence of HP-β-CD [37]. Thus, no detailed pH study over a large pH range was conducted. The pK_a values of the carboxylic acid groups of the dipeptides are listed in Fig. 1 and range between 2.76 and 3.12 [40,41]. The pK_a values of the α-amino groups lie between 7.17 and 8.08 [40,41]. Thus, the peptides are positively charged at both pH values, but the charge density is higher at pH 2.5. Lys-Phe bears a second positive charge at the side chain ($pK_a = 10.45$ [41]).

Using β-CD as primary selector, a clear advantage of one pH value over the other with regard to enantioresolution could not be identified because for some analytes such as Ala-Tyr higher R_S values were observed at pH 3.5 compared to 2.5 for all CILs. In contrast, in the case of Phe-Phe resolutions at pH 2.5 exceeded those seen at pH 3.5 with some CILs, while the opposite was observed for other CILs (Table 1). The pH-dependent reversal of the enantiomer migration order was not affected in the presence of CILs. Thus, the LL-enantiomers of Ala-Phe, Ala-Tyr and Phe-Phe migrated before the DD-stereoisomers at pH 2.5 in the absence as well as

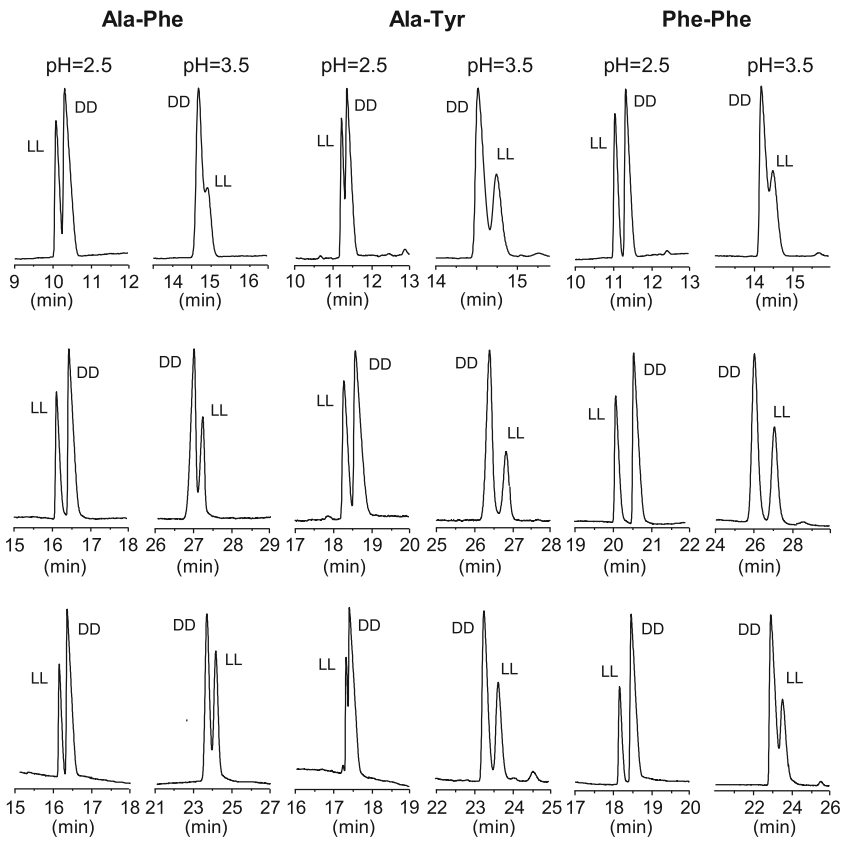

Fig. 3. Electropherograms of the enantioseparation of Ala-Phe, Ala-Tyr and Phe-Phe in the presence of (top) 20 mM β-CD and upon addition of (middle) 15 mM $[TBA]_2$[L-Asp] or (bottom) 15 mM [TBA][L-Pro] at pH 2.5 and pH 3.5. Experimental conditions: 40/50.2 cm, 50 µm I.D. fused-silica capillary, 50 mM sodium phosphate buffer, 20°C, 25 kV, 215 nm.

the presence of CILs while the migration order was always opposite at pH 3.5 under otherwise comparable conditions. Fig. 3 illustrates the addition of $[TBA]_2$[L-Asp] and [TBA][L-Pro] on the β-CD-mediated enantioseparation of the three dipeptides at pH 2.5 and 3.5. Using HP-β-CD as primary selector, R_S values were higher at pH 2.5 compared to pH 3.5 for most analytes independent of the CIL applied (Table 2). Thus, although only two CDs were studied here, it appears that the effect of the pH of the background electrolyte on chiral peptide separations depends on the CD, the CIL as well as the analyte itself.

3.4. Effect of the components of the CILs

The addition of CILs to the BGE can exert several effects [5,32]. First, the ionic strength of the BGE increases, which affects the EOF. Moreover, the cation of the CIL may be adsorbed to the capillary wall, changing the strength as well as the direction of the EOF. Both effects result in a slower migration of the analyte. Thus, the contact time between chiral selector and analyte enantiomers is increased so that the complexation equilibrium between selector and analyte can be established more often resulting in an improved enantioresolution. The effect is consequently not considered synergism between the selector and the CIL as this would also be achieved by achiral ionic liquids [6]. This has been shown for the improved separation of β-blockers by a dual CD system upon addition of glycidyltrimethylammonium chloride [38]. Formation of a ternary complex between CD, analyte and CIL, which would mean "real" synergism between a selector and a CIL, has been derived from molecular modeling studies in case of [TMA][L-Arg] [34] or an amino alcohol-derived CIL [42] but unequivocal proof of the participation of such complexes in the separation process is still lacking. Recently, the presence formation of a 1:1:1 complex formed between FMOC-homocysteine, γ-CD and the CIL (*R*)-*N,N,N*-trimethyl-2-aminobutanol-bis(trifluoromethanesulfon)imidate) could be shown by NMR spectroscopy [43].

In order to estimate the contribution of the components of the present amino acid-derived CILs to the chiral separation process, the β-CD mediated enantioseparations of Ala-Phe, Ala-Tyr, Lys-Phe and Phe-Phe in the presence of the Asp-based CILs $[TMA]_2$[L-Asp] and $[TBA]_2$[L-Asp] were compared to the separation obtained in the presence of TBA-Cl or TMA-Cl and L-Asp or mixtures of the alkylammonium chlorides and the amino acids. Concentrations of the individual components were selected so that they typically matched the concentration of 15 mM of the respective CIL, while the concentration of β-CD was 20 mM in all cases. The enantioresolution expressed as R_S values are summarized in Fig. 4 (columns a - d) at pH 2.5 (left) and pH 3.5 (right). As differences under the individual conditions do not always differ significantly the following discussion often refers to trends rather than large improvements or deteriorations of the enantioseparations.

The presence of 20 mM of the amino acids in the BGE in addition to 20 mM CD did not result in significant changes of the enantioseparation, although enhancement of the enantioseparation of some basic drugs by HP-β-CD at pH 3 and above upon addition of L-Asp or L-Glu has also been reported [44]. Along this line, addition of 30 mM TMA-Cl or TBA-Cl also affected the enantioseparation only to a minor extent (Fig. 4, columns b) compared to β-CD alone (Fig. 4, columns a). A small synergistic effect between CDs

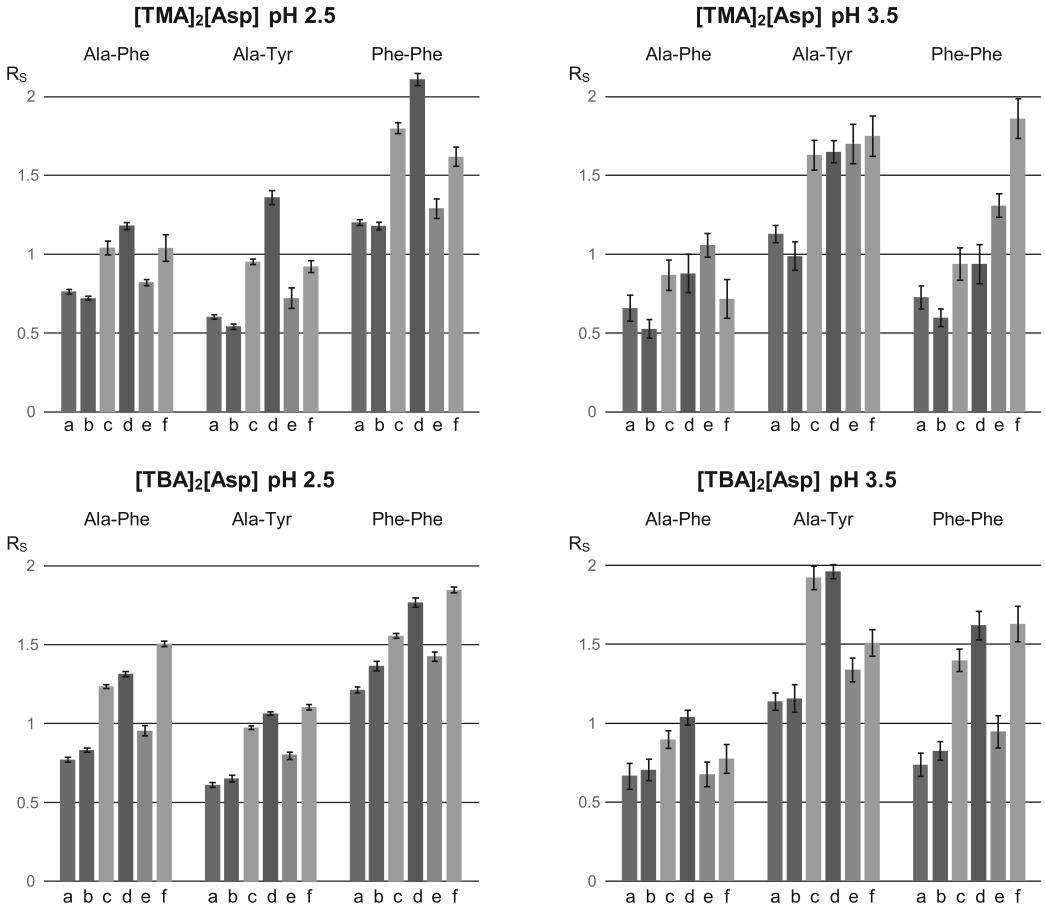

Fig. 4. Comparison of the effect of 15 mM $[TMA]_2$[L-Asp] or $[TBA]_2$[L-Asp] or equimolar concentrations of the individual components on the β-CD mediated enantioseparation (expressed as R_S values) of Ala-Phe, Ala-Tyr and Phe-Phe at pH 2.5 or pH 3.5. (a) 20 mM β-CD, (b) 20 mM β-CD + 30 mM TMA-Cl/TBA-Cl, (c) 20 mM β-CD + 15 mM L-Asp CIL, (d) 20 mM β-CD + 15 mM L-Asp + 30 mM TMA-Cl/TBA-Cl, (e) 20 mM β-CD + 15 mM D-Asp CIL, (f) 20 mM β-CD + 15 mM D-Asp + 30 mM TMA-Cl/TBA-Cl. The data are shown as mean ± SD (n = 4).

and tetraalkylammonium chlorides is agreement with other publications on the combination of tetraalkylammonium-amino acid CILs and CDs [32,45]. These studies also reported a reduced EOF in the presence of the tetraalkylammonium cation only leading to a slight improvement of the chiral separations. A substantial increase of the resolution was typically observed upon addition of either 15 mM of the CILs (Fig. 4, columns c) or the corresponding mixture of the individual components, i.e., the combination of 30 mM TBA-Cl or TMA-Cl and 15 mM L-Asp (Fig. 4, columns d). Therefore, the simultaneous presence of tetraalkylammonium cation and the amino acid is required for an increased enantioresolution so that it may be hypothesized that the CIL as entity or via the individual components participates in the chiral recognition process. At pH 2.5, the addition of the individual components resulted in somewhat higher values compared to the addition of the CILs, while at pH 3.5 essentially identical R_S values resulted for both scenarios. In contrast, Zhang et al. reported superior α-CD-mediated enantioseparation of basic drugs when the CIL [TMA][L-Arg] was added compared to the addition of equimolar concentrations of TMA-OH and L-Arg [32]. A similar observation was made by Ren et al. studying the effects of [TMA][L-Arg] on the enantioseparation of dansyl-amino acids by β-CD in non-aqueous CE [34]. The reason for this difference is not obvious but may be related to the fact that $[TBA]_2$[L-Asp] and $[TMA]_2$[L-Asp] contain two alkylammonium cations while only one is present in of the L-Arg-derived CILs studied in [32,34] as well as the different charge of the amino acid component of the CILs.

Differences in the observations at pH 2.5 (Fig. 4, left) and pH 3.5 (Fig. 4, right) may be explained by a change in the ionization status of the peptide analytes as well as the amino acids of the CILs. The reported pK_a values of the carboxylic acid groups of L-Asp are 1.99 and 3.90 [46]. Because the pI of Asp is slightly below 3, the amino acid will be slightly positively charged at pH 2.5 but partially negatively charged at pH 3.5. Therefore, it may be hypothesized that the charge state of the amino acid component of the CIL may affect the enantioseparation as well. This aspect has not been addressed in detail in studies on CILs to date. The peptide analytes are essentially positively charged at pH 2.5 and are increasingly converted to zwitterions with increasing pH [20]. Moreover, reversal of the enantiomer migration order between pH 2.5 and 3.5 is observed for the peptides in the presence of β-CD. It has been demonstrated that the β-CD mediated enantioseparation of Ala-Tyr and Phe-Phe is due to the chiral recognition of the peptide enantiomers by the CD (expressed as complexation constants) at pH 2.5, while it is increasingly affected by the mobility of the diastereomeric CD-peptide complexes at pH 3.5 [20]. Assuming a participation of the amino acid component of the CILs in the formation complex between analytes and CD, the different ionization of the amino acids as well as peptide analytes in addition to the stereochemistry of analyte and CIL resulted in a very complex sys-

tem. The effects of the individual components of this system on the enantioseparation are not obvious and difficult to rationalize. The situation is further complicated by the fact that a reversal of the enantiomer migration order is observed in the studied pH range.

Although the aim of the present study was not the development of a method for the analysis of a given dipeptide, the repeatability of the enantioseparations was estimated by analysis of the analyte migration times of the dipeptides under selected conditions, e.g., 20 mM β-CD or HP-β-CD and addition of either 15 mM $[TBA]_2$[L-Asp], $[TMA]_2$[L-Asp] or [TMA][L-Asn] as well as addition of equimolar concentrations of the individual components in some cases. The data are summarized in Tables S1 (β-CD) and S2 (HP-β-CD) (supplementary material). RSD values of migration times varied between 0.1 % and 7.5 %, with most values between 0.3 % and 2.1 %. RSD values were typically smaller at pH 2.5 compared to pH 3.5. This may be attributed to a slightly larger variation of the EOF at the higher pH value. Nonetheless, the present experimental conditions appear to result in repeatable analyte migration times.

3.5. Effect of the stereochemistry of the CILs

Zhang and colleagues noted an effect of the configuration of the amino acid component of tetraalkylammonium-amino acid CILs on the HP-β-CD-mediated enantioresolution of basic drugs [32,45]. The peak resolution decreased significantly when the system HP-β-CD/[TMA][L-Arg] was switched to the [TMA][D-Arg] CIL although R_S values were still higher upon addition of [TMA][D-Arg] compared to the sole use of HP-β-CD.

Consequently, the respective CILs containing D-Asp or addition of equimolar concentrations of D-Asp and TMA-Cl or TBA-Cl were evaluated (Fig. 4, columns e and f). Generally, a deterioration of the enantioseparation was observed when L-Asp was substituted by D-Asp. This confirms the importance of the configuration of the amino acid for the synergistic effect between CDs and CILs. However, a diverse picture is obtained when mixtures of the alkylammonium chlorides and D-Asp were employed as BGE additives. In most cases, the separate addition of the components resulted in slightly higher or about equal R_S values (Fig. 4, columns f) compared to the D-amino acid CILs (Fig. 4, columns e). In few cases, the highest resolution was even observed for the separate addition of D-Asp and TMA-Cl or TBA-Cl among the tested conditions. This is in contrast to the other systems investigated in the present study as well as reported in the literature using non-peptide analytes [32,45]. There is no apparent reason for this phenomenon, but it emphasizes the importance of the stereochemistry of buffer additives on a given enantioseparation. Moreover, enantioseparations in CE can also be based on mobility differences between the diastereomeric selector-selectand complexes [47]. Assuming a participation of the CIL amino acid in the formation of this complex, it may be speculated that the complexes formed with L- or D-amino acid possess different hydrodynamic radii and consequently different mobilities.

4. Conclusions

The aim of the study was the evaluation if the synergism of CILs and CDs reported for enantioseparations of basic and acidic analytes would also apply to dipeptides, which are amphoteric compounds. Thus, the separation of the LL- and DD-stereoisomers of model dipeptides was studied with a combination of β-CD or HP-β-CD and CILs composed of tetraalkylammonium ions and amino acids. The addition of a CIL to a CD-containing BGE at pH 2.5 and 3.5 led to a concentration-dependent increase in the enantioresolution of the dipeptide enantiomers. The increase was more pronounced compared to the addition of TMA-Cl or TBA-Cl clearly proving the synergism between CDs and CILs. The effect was most likely due to a decrease of the EOF in the presence of a CIL. Furthermore, the extent of the increase of the resolution depended on the amino acid, while the type of the tetraalkylammonium counterion had only a minor effect. The earlier reported pH-dependent reversal of the enantiomer migration order of Ala-Phe, Ala-Tyr and Phe-Phe in the presence of β-CD was also observed in the presence of CILs. Thus, the reversal based on either opposite chiral recognition of the dipeptide enantiomers by β-CD or reversal based on the increasing influence of the mobility of the CD-enantiomer complex due to a complexation-induced pK_a shift was not affected by the CILs.

When comparing the effect of the addition of Asp-based CILs versus the addition of equimolar concentrations of the individual components to the β-CD-mediated dipeptide enantioseparation at pH 2.5 and 3.5, a quite diverse picture was obtained. In some cases, the individual components yielded higher R_S values, while for other experimental conditions, CILs proved to be more effective. This may be due to the zwitterionic nature of the analytes as well as the amino acid component of the CILs. Divergent observations were also made regarding the configuration of Asp. While in most cases the L-configured amino acid was more effective compared to the D-amino acid, in a few examples D-Asp-based systems yielded the higher R_S values than the corresponding conditions employing L-Asp. Thus, further investigations including spectroscopic and/or modeling studies may be required to rationalize the observed effects with regard to addition of the individual components of the CILs as well as the stereochemistry of the amino acid involved.

Author credit statement

Mari-Luzia Konjaria: Planning and conducting research, evaluation of data, preparation of manuscript draft

Gerhard K. E. Scriba: Supervision of research, finalizing manuscript

Declaration of Competing Interest

The authors have declared no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462342.

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3.5 Manuscript 4

Manuscript No. 4

Manuscript title: Enantioseparation of chiral (benzylsulfinyl)benzamide sulfoxides by capillary electrophoresis using cyclodextrins as chiral selectors

Authors: Mari-Luiza Konjaria, Rusudan Kakava, Alessandro Volonterio, Bezhan Chankvetadze, Gerhard K.E. Scriba

The candidate is

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Status: Submitted to J. Chromatogr. A, 02.03.2022

Authors' contributions (in %) to the given categories of the publication

Author	Conceptual	Data analysis	Experimental	Writing the manuscript	Provision of material
Mari-Luiza Konjaria	70	85	90	70	30
Rusudan Kakava			10		40
Alessandro Volonterio					30
Bezhan Chankvetadze	10				
Gerhard K.E. Scriba	20	15		30	
Total:	100%	100%	100%	100%	100%

Overview:

Sulfur is the stereogenic center in case of studied sulfoxides. The enantioseparation of 16 (benzylsulfinyl)benzamides were studied by capillary electrophoresis using charged CDs as chiral selectors at pH 5.5. Additionally, some experiments in MEKC were carried out using neutral CD derivatives in the presence of sodium dodecyl sulfate at pH 9.0. The migration orders of sulfoxides were studied as function of (benzylsulfinyl)benzamide structure and CD cavity size and substitution pattern. Furthermore, the reversal of the migration order of compound 11 was noticed to be a function of the CD concentration. In order to explain mechanisms behind the CD concentration-dependent reversal of the enantiomer migration order, the complexation constants and the complex mobilities were determined for the selected set of compound-CD pairs.

Enantioseparation of chiral (benzylsulfinyl)benzamide sulfoxides by capillary electrophoresis using cyclodextrins as chiral selectors

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Abstract

Sulfur as a stereogenic center can be found in synthetic compounds and natural products. The current study evaluated the enantioseparation of 16 chiral (benzylsulfinyl)benzamides compounds by capillary electrophoresis using charged cyclodextrins (CDs) as chiral selectors in 50 mM sodium acetate buffer, pH 5.5. The sulfoxides varied in the type and position of the substituent of the benzyl moiety as well as the position and methylation of the amide group. Typically, randomly substituted CDs separated the majority of the model analytes in contrast to single isomer CDs. In case of random substitution, γ-CD derivatives displayed higher resolution ability toward the set of model compounds followed by β-CD and α-CD derivatives. Except for a few examples, the (+)-enantiomer of the analytes migrated before the (–)-isomer irrespective of the type of the CD so that the chiral recognition appeared to be also mostly independent on the structure of the sulfoxides. Evaluation of complexation constants and complex mobilities of selected CD-analyte pairs revealed that the separations were based on the stereoselective complexation by the CD expressed as complexation constants but examples for complex mobilities as the determining factor for the enantiomer migration order were also found. In case of 2-(4-bromobenzylsulfinyl)-*N*-methyl benzamide in the presence of *heptakis*(2,3-di-*O*-methyl-6-*O*-sulfo)-α-CD reversal of the enantiomer migration order as a function of the CD concentration was observed. Using neutral CD derivatives in the presence of sodium dodecyl sulfate-base micelles at pH 9.0 only few sulfoxides could be enantioseparated.

Key words: Enantiomer separation; Cyclodextrin; Sulfoxide; Enantiomer migration order; Capillary electrophoresis

1. Introduction

In most cases, the stereogenic centers of chiral molecules are carbon atoms. However, other atoms such as sulfur or phosphor in the appropriate oxidation status such as sulfonate and sulfoxide and phosphine or phosphonate, respectively, can also be stereogenic centers. Many pharmacologically active substances are chiral sulfoxides including pharmaceuticals such as proton pump inhibitors [1] or cyclooxygenase inhibitors [2] or plant secondary metabolites [3]. Furthermore, sulfoxides originate from the oxidation of sulfide groups of compounds. Because the pharmacological activity is often attributed to one of the sulfoxide enantiomers, their separation has been of great interest. This is especially true for proton pump inhibitors which have been analyzed by HPLC, sub/supercritical fluid chromatography (SFC) or capillary electrophoresis (CE) as recently summarized [1].

Furthermore, structurally simple aliphatic or aromatic sulfoxides have served as model compounds for the evaluation of various chiral separation techniques. In this context, Chankvetadze et al. noted an extremely high separation factor α larger than 110 for the enantioresolution of 2-(benzylsulfinyl)benzamide (Figure 1, compound 1) on a cellulose tris(3,5-dichlorophenylcarbamate) column using propan-2-ol as mobile phase [4]. Unmodified sulfoxides [5, 6] as well as 2-(benzylsulfinyl)benzamide derivatives [7-10] were studied on several polysaccharide-based chiral columns under various mobile phase conditions. Most recently, molecular modeling provided a rationale for the separation of 2-(benzylsulfinyl)benzamides on polysaccharide columns [11]. The enantioseparation of simple unmodified chiral aliphatic/aromatic sulfoxides as well as 2-(benzylsulfinyl)benzamide derivatives was also studied by SFC using polysaccharide-based chiral stationary phases [6, 12, 13], by HPLC with chiral columns based on teicoplanin [14-16] or cyclodextrin (CD) columns [17], respectively.

CE has been established as a suitable alternative to HPLC for liquid phase enantioseparations primarily due to the high-resolution ability and flexibility of the technique [18-20]. Consequently, CE has been used for the separation of the enantiomers of many compounds in various analytical fields including pharmaceuticals [21-24], bioanalysis [25] food [26, 27] or environmental analysis [28]. However, while the CE enantioseparations of chiral sulfoxide drugs such as proton pump inhibitors have been frequently explored [1], the resolution of the enantiomers of model sulfoxide compounds as described for HPLC above has been scarcely studied by CE. The enantioseparation of "simple" aliphatic/aromatic sulfoxides is generally challenging because these structures cannot be ionized and do not contain many groups that can interact with cyclodextrins, which are the most often applied chiral selectors in CE [29-31]. To the best of our knowledge, there is only one publication of the enantioseparation of chiral model sulfoxides and sulfinate esters by capillary electrophoresis using sulfated β-cyclodextrin (S-β-CD) and carboxymethyl-β-CD (CM-β-CD) as chiral selectors and 10 mM sodium phosphate buffer, pH 8.3 as background electrolyte [32]. Almost all analytes could be enantioresolved in the presence of S-β-CD, while CM-β-CD was less effective. Obvious structure-separation relationships could not be established but the type and position of the substituents in the aromatic ring affected analyte resolution. Because the enantioseparation of 2-(benzylsulfinyl)benzamides had not been investigated by CE previously, the aim of the present study was the evaluation of charged α-CD, β-CD and γ-CD derivatives as chiral selectors for this purpose. A set of 16 chiral (benzylsulfinyl)benzamides was used (Table 1) differing in

the position of the amide substituent and *N*-methylation as well as the type and position of the substituent in the benzylsulfinyl moiety. Moreover, the enantioseparation of the sulfoxides by neutral CDs in the micellar electrokinetic chromatography (MEKC) mode was also briefly addressed.

2. Materials and methods

2.1. Chemicals

Sulfobutylether-β-CD (SBE-β-CD, degree of substitution (DS) ~6.4) was from Cydex (San Diego, CA, USA), *heptakis*(6-*O*-sulfo)-β-CD (HS-β-CD) *heptakis*(2,3-di-*O*-methyl-6-*O*-sulfo)-β-CD (HDMS-β-CD), *heptakis*(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-CD (HDAS-β-CD), succinyl-β-CD (Suc-β-CD, DS ~3.5), *hexakis*(2,3-di-*O*-methyl-6-*O*-sulfo)-α-CD (HDMS-α-CD), *octakis*(2,3-di-*O*-methyl-6-*O*-sulfo)-γ-CD (ODMS-γ-CD) and carboxymethyl-γ-CD (CM-γ-CD, DS ~3.5), Succinyl-γ-CD (Suc-γ-CD, DS ~3.5), *heptakis*(6-deoxy-6-amino)-β-CD heptahydrochloride (HA-β-CD), *octakis*(6-deoxy-6-amino)-γ-CD octahydrochloride (OA-γ-CD), (2-hydroxy-3-*N*,*N*,*N*-trimethylamino)propyl-α-CD chloride (TMA-α-CD, DS ~2 - 4.5), (2-hydroxy-3-*N*,*N*,*N*-trimethylamino)propyl-γ-CD (TMA-γ-CD, DS ~2 - 5), α-CD, methylated β-CD (M-β-CD, DS ~12), 2,6-di-*O*-methyl-β-CD 50 % purity (DM-β-CD50, DS ~11 - 14), 2,6-di-*O*-methyl-β-CD 95 % purity (DM-β-CD95, DS 14), 2-hydroxypropyl-β-CD (HP-β-CD, DS ~4.5), 2,3,6-trimethyl-β-CD (TM-β-CD), methylated α-CD (M-α-CD, DS ~11), 2-hydroxypropyl-α-CD (HP-α-CD, DS ~4.5) and 2- hydroxypropyl-γ-CD (HP-γ-CD, DS ~4.5) were from Cyclolab Ltd (Budapest, Hungary). Sulfated β-CD (S-β-CD, DS ~12 - 15) and sulfated α-CD (S-α-CD, DS ~8 - 11) were obtained from Sigma-Aldrich Chemie GmbH (Munich Germany), carboxymethyl-β-CD (CM-β-CD, DS ~3.5), (2-hydroxy-3-*N*,*N*,*N*-trimethylamino)propyl-β-CD chloride (TMA-β-CD, DS ~5) and β-CD were from Wacker Chemie (Munich Germany) and sulfated γ-CD (S-γ-CD, DS ~13 - 15) was obtained from Cyclodextrin Shop (Tilburg, The Netherlands).

The chiral sulfoxides 2, 6, 8, 9, 12-16 (Table 1) were supplied by the group of Prof. B. Chankvetadze, Tbilisi, Georgia [33]. Sulfoxides 1, 3-5, 7,10 and 11 were synthesized using the methods as described in [34], see supplementary material for details. All other chemicals were of analytical grade. Water was purified using a TKA Genpure UV-TOC from Thermo Scientific (Waltham, USA). Background electrolyte (BGE) and sample solutions were filtered through 0.22 µm polypropylene syringe filters from BGB Analytik (Schloßböckelheim, Germany).

2.2. Capillary electrophoresis

Experiments were performed on a Beckman P/ACE MDQ CE system (AB Sciex, Darmstadt, Germany) equipped with a UV-Vis diode array detector and controlled by 32 KARAT software for system control, data acquisition and processing. 50 µm I.D., 365 µm O.D. fused-silica capillaries with a total length of 50.2 cm and an effective length of 40.0 cm were from CM Scientific (Silsden, UK). All rinsing steps were conducted at a pressure of 138 kPa (20 psi). A new capillary was treated subsequently with 0.1 M NaOH for 20 min, water for 10 min, 0.1 M NaOH for 10 min, 0.1 M phosphoric acid for 10 min and water for 10 min. Between the analyses, the capillaries were washed with 0.1 M NaOH for 2 min and with the BGE for 3 min. The applied voltage was 25 kV, and the capillary temperature was maintained 20 °C. UV

detection was performed at 220 nm at the cathodic end of the capillary in case of normal polarity and at the anodic end when polarity was reversed. Dimethyl sulfoxide (DMSO) was used as marker of the electroosmotic flow (EOF).

CE separations were performed in 50 mM sodium acetate buffer prepared on a daily basis, while a 50 mM sodium borate buffer, pH 9.0, was used for MEKC. Both electrolyte solutions contained 10 % (v/v) methanol. The pH of the BGEs was adjusted after the addition of the CDs. The buffers were filtered (0.22 µm) and degassed by sonication before use. Sample solutions of the sulfoxides (100 µg/mL prepared in water:methanol, 1:1, v/v) were introduced at a pressure of 3.5 kPa (0.5 psi) for 5 s. The migration order was confirmed by spiking with the individual sulfoxide enantiomers obtained by HPLC as described in section 2.3.

Viscosity measurements of the buffers for the determination of complexation constants were performed using the CE instrument as a viscosimeter and 0.1% (m/v) riboflavin-5'-phosphate as boundary marker according to [35]. Electrophoretic mobilities were measured in triplicate and viscosity measurements were determined four-fold.

2.3 HPLC fractionation of sulfoxide enantiomers

HPLC for obtaining the sulfoxide enantiomers was performed on a Shimadzu instrument composed of LC-10AT and LC-10AS pumps, a SPD-10A UV-VIS detector, a SIL-10A auto injector, a DGU-20A3R degassing unit, a CTO-20AC temperature controller and a SCL-10A system controller (Duisburg, Germany). The LCsolution software was used for instrument control and data acquisition. A Lux i-Cellulose 5 column (150 x 4.6 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) containing cellulose tris(3,5-dichlorophenylcarabamate) as chiral selector in combination with methanol as mobile phase was used. The flow rate was 1.0 mL/min, the temperature was set at 20 °C and detection was carried out at 254 nm. 50 µL of the solutions of the sulfoxides prepared at a concentration of 1-2 mg/mL in methanol, were injected. A minimum of 20 runs were performed for each sulfoxide and the eluate containing the respective enantiomers were pooled followed by evaporation of the solvent under reduced pressure. The compounds were obtained as amorphous off-white to yellow solids. The purity of the isolated enantiomers was estimated by HPLC analysis using the same experimental set-up. The optical rotation of the purified enantiomers was determined in ethanol using a P2000 polarimeter from Jasco (Pfungstadt, Germany).

2.4 Software

The CEVal software [36] was used for non-linear curve fitting for the determination of the complexation constants and the mobilities of the sulfoxide-CD complexes.

3. Results and discussion

The structures of the 16 chiral (benzylsulfinyl)benzamides are summarized in Table 1. They differ in the position of the amide substituent and *N*-methylation as well as the type and position of the substituent in the benzylsulfinyl moiety. The enantiomers were obtained by HPLC. In case of all sulfoxides, the first eluted enantiomer displayed dextrorotary optical rotation (data not shown). The separation of the

enantiomers of 2-(benzylsulfinyl)benzamide (compound 1) on cellulose tris(3,5-dichlorophenylcarbamate) using methanol, ethanol or propan-2-ol as mobile phases with the (+)-enantiomer eluting first has been reported [4]. Moreover, Carradori et al. showed that the enantiomers of sulfoxide 1 and the *N*-methyl derivative 2, which eluted first from a cellulose *tris*(3,5-dichlorophenylcarbamate) column using ethanol as eluent, possess the (*R*)-configuration [37]. All sulfoxides studied here also displayed the elution order (+)-enantiomer before the (–)-enantiomer on the cellulose tris(3,5-dichlorophenylcarbamate) column using ethanol as eluent (data not shown). Because the different substitution patterns of compounds 1 to 16 do not affect the absolute configuration of the enantiomers at the chiral sulfur atom (the priorities of the respective substituents according to the Cahn-Ingold-Prelog rules do not change), it is safe to assume that for all 16 sulfoxide analytes the (*R*)-configuration can be assigned to the (+)-enantiomer and the (*S*)-configuration to the levorotary enantiomer, although this has been experimentally proven only for sulfoxides 1 and 2 [37].

Because the sulfoxides cannot be ionized, charged CDs were evaluated for the separation of the enantiomers. In addition, MEKC conditions using neutral CDs as well as SDS as surfactant were briefly studied.

3.1 CE using charged CD derivatives

For the separation of neutral analytes, the carrier ability of charged CDs can be exploited [38]. Initial separations using compounds 1, 2, 6 and 8 as well as CM-β-CD or CM-γ-CD as chiral selectors were attempted at pH 2.5. However, at this pH the analytes could not be detected either at the cathode or at the anode, when reversing the polarity of the separation voltage. Consequently, the pH was increased in order to increase the magnitude of the electroosmotic flow (EOF) so that the analytes can be separated after the EOF. Using 50 mM phosphate and acetate buffers and CE concentrations of 10 mg/mL, the pH range 3.5 to 7.0 was subsequently studied. The best enantioresolution was observed 50 mM sodium acetate buffer, pH 5.5, so that this pH was selected for further studies. Addition of 10 % (v/v) methanol resulted in narrower peaks. Therefore, all screening experiments were performed in a 50 mM sodium acetate buffer, pH 5.5 containing 10 % (v/v) methanol at a separation voltage of 25 kV. Under these conditions, negatively charged CDs migrate to the anode so that uncharged analytes are detected after the EOF when detection is carried out at the cathodic end. In case of positively charged CDs, the polarity of the voltage was reversed, and the analytes were detected at the anodic end. The results are summarized for randomly substituted CDs in Table 2 and for single isomer CDs in Table 3.

3.1.1 Effect of the CD structure and substitution pattern

With the exception of CM-α-CD, randomly substituted CDs proved to be effective chiral selectors for the sulfoxides (Table 2). In the presence of S-γ-CD, CM-γ-CD, Suc-β-CD and Suc-γ-CD the enantiomers of all analytes were resolved and in the vast majority of the cases even baseline separated. Especially the γ-CD derivatives yielded high resolution with R_S values up to 20.6 (sulfoxide 7 and CM-γ-CD). β-CD derivatives were somewhat less effective closely followed by α-CD derivatives except for CM-α-CD as stated above. It may be speculated that the large cavity of the γ-CD derivatives provided a better fit for the sulfoxides as compared to the smaller cavities of β-CDs and α-CDs. The type of substituent

appeared to play a minor role. For example, S-γ-CD, CM-γ-CD and Suc-γ-CD displayed comparable efficiency resolving the enantiomers of all sulfoxide analytes, although R_S values were typically lower in case of Suc-γ-CD compared to the two other γ-CD derivatives. Furthermore, negatively charged CDs appeared to have an advantage over positively charged CDs although the number of positively charged CDs in this study was limited so that this observation may not be generalized at this point. It is interesting to note, that in most cases the (+)-enantiomers migrated first independent of the structure or charge of the CD. An exception was SBE-β-CD, where in a little over half the sulfoxides displayed the elution order (–) before (+). In case of the negatively charged CDs, separation was obtained after the EOF. Because the negatively charged CDs migrate toward the anode the weaker complexed enantiomer of the uncharged sulfoxide analytes will migrate first under normal polarity of the applied voltage and detection at the cathodic end of the capillary. The same scenario applies for positively charged CDs. In this case the polarity of the applied voltage was reversed because of the adsorption of the positively charged CDs to the capillary wall, and, consequently, a reversal of the direction of the EOF from the cathode to the anode. Thus, detection was carried out at the anodic end of the capillary. As in the presence of negatively charged CDs, the analytes migrated after the EOF while the positively charged CDs migrated toward the cathode. Thus, the weaker complexed enantiomer will also migrate first under these circumstances so that the chiral recognition did not generally change as a function of the charge of the CDs.

In few cases, the enantiomer migration order depended on the cavity size of the CDs. For example, in case of compound 7 in the presence of carboxymethylated CDs the (–)-enantiomer migrated first in the presence of CM-α-CD and CM-γ-CD, while (+)-7 migrated first when CM-β-CD was used as chiral selector (Figure 1A). The migration order was (+)-11 before (–)-11 using CM-β-CD or CM-γ-CD, while it was (–)-11 before (+)-11 in the presence of CM-α-CD. Further examples can be found in Table 2. of the enantiomer migration order on the cavity size of the CD has been observed for many non-sulfoxide enantiomers as summarized, for example in [39].

Single isomer CDs were less efficient for the enantioseparation of the sulfoxides under the standardized conditions (Table 3). Only HS-β-CD enantioseparated 15 out of the 16 sulfoxides, followed by HA-β-CD (13) and HDMS-α-CD (12). It is interesting to note that single isomer γ-CD derivatives were less effective than the corresponding β-CDs or α-CDs. In case of CDs containing a sulfate moiety in position 6 and methyl groups in positions 2 and 3, the order was HDMS-α-CD (12) > HDMS-β-CD (6) > ODMS-γ-CD (3). Derivatization of the wider secondary rim appeared to reduce the enantioseparation ability of the CDs as HS-β-CD featuring only sulfate groups at C6 separated more enantiomers compared to HDAS-β-CD or HDMS-β-CD, which contain acetyl substituents and methyl groups, respectively, in position 2 and 3 of the D-glucopyranose units of the CDs. As observed for randomly substituted CDs, in most cases the migration order of the sulfoxide enantiomers was (+) before (–) for CDs, except for HDAS-β-CD and OA-γ-CD, where the (–)-enantiomer migrated before the (+)-enantiomer in most cases. It should be noted that the polarity of the voltage was reversed in case of OA-γ-CD and detection was performed at the anodic end of the capillary. As seen in case of the randomly substituted CDs, the cavity size of the CDs affected the enantiomer migration order for some analytes. For example, the (+)-enantiomer of

compound 7 migrated first in the presence of HDMS-α-CD, while it was the (**–**)-enantiomer in the presence of HDMS-β-CD and HDMS-γ-CD (Figure 1B). Further examples can be found in Table 3. See also the discussion in section 3.1.2 below.

3.1.2 Effect of the structure of the sulfoxides

The benzamide sulfoxides differ in the position of the amide substituent and *N*-methylation as well as the type and position of the substituent in the benzylsulfinyl moiety (Table 1). Substituents in the benzylsulfinyl residue were bromine, methoxy, methyl or *tert*.-butyl groups. The parent compound 1 (2-(benzylsulfinyl)benzamide) was at least partially resolved by all but two CDs, i.e., HDMS-β-CD and HDMS-γ-CD. As stated above, in the presence of most negatively charged CDs the (+)-enantiomer of the sulfoxides migrated first. As also stated above, it is reasonable to assume from the known absolute configuration of compounds 1 and 2 [37] and the elution order of the other sulfoxide analytes from a cellulose tris(3,5-dichlorophenylcarbamate) column that the (*R*)-configuration can most likely be assigned to the (+)-enantiomer of all compounds, because none of the substitution patterns of the sulfoxides changes the absolute configuration at the chiral sulfur atom. Therefore, the chiral recognition in the vast majority of cases is independent of the structure of the sulfoxides, i.e., the type or position of the substituents. Nonetheless, a few exceptions were observed. For example, the position of the substituent in the benzyl moiety affected the migration order when HDMS-α-CD was used as chiral selector. Thus, the migration order was opposite for the compound pairs 6 and 7 as well as 10 and 11, which feature a bromine substituent in position 3 (compounds 6 and 10) or position 4 (compounds 7 and 11), respectively. Electropherograms of the enantioseparations of compounds 10 and 11 in the presence of HDMS-α-CD are shown in Figure 2A. Reversal of the enantiomer migration order was also observed for the pair of compounds 14 and 15, which bear a methyl group in position 2 or 3, respectively (Table 3). Opposite migration order was also found in case of compounds 10 and 11 in the presence of OA-γ-CD (Table 3). *N*-methylation of the benzamide group also affected the chiral recognition by CDs as the opposite migration order was observed for the compound pairs 7 and 11 in the presence of HDMS-β-CD (Figure 2B and Figure 3B) or for compounds 1 and 2, when randomly substituted SBE-β-CD was the chiral selector (Table 2). An effect of the type of substituent was found for OA-γ-CD and compounds 7 (bromine), 8 (methyl) and 9 (*tert*.-butyl), which feature the substituents in position 4 of the benzyl moiety (Table 3). Finally, the position of the benzamide group played a role for compound 1 (position 2), 2 (position 3) and 3 (position 4) in the presence of SBE-β-CD or TMA-α-CD (Table 4).

3.1.3 Determination of apparent complexation constants and complex mobilities

In order to rationalize the opposite enantiomer migration order of some analytes as a function of the position of the substituents or *N*-methylation, the complexation constants as well as the mobilities of the diastereomeric analyte-CD complexes were determined for compounds 7, 10 and 11 in the presence of HDMS-α-CD and HDMS-β-CD. The data were obtained as best fit parameters of the dependence of the effective mobility on the CD concentrations in the range of 5 to 30 mM at pH 5.5 assuming the formation of 1:1 sulfoxide CD-complexes according to equation 1

$$\mu_{eff} = \frac{\mu_f + \mu_c \cdot K \cdot [CD]}{1 + K \cdot [CD]} \quad (1)$$

where μ_{eff} is the effective mobility, μ_f the mobility of the free analyte, μ_c the limiting mobility of the CD-analyte complex, K the complexation constant, and [CD] is the molar concentration of the CDs. The software CEVal was used for data fitting because this software allows correction of the migration times in case of tailing peaks according to the Haarhoff-van der Linde equation [36]. Moreover, the observed mobilities were corrected for the increasing viscosity of the BGE upon increasing CD concentrations.

In this process it was noted that in case of compound 11 the enantiomer migration order changed as a function of the concentration of HDMS-α-CD as illustrated in Figure 3. At concentrations up to 15 mM, the (–)-enantiomer migrated before (+)-11, while at concentrations of 20 mM and above, the (+)-isomer was detected first. Comigration of the enantiomers occurred at an approximate concentration of 17.5 mM HDMS-α-CD. The dependence of the enantiomer migration order as a function of the CD concentration has been observed for several analytes as, for example, summarized in [39, 40]

The mobility and complexation data are summarized in Table 4. It should be noted that these data are apparent constants and mobilities rather than thermodynamic values because the ionic strength of the buffers is not known, and CD concentrations referred to the volume and not to the mass of the solvent. Although differences between the respective values are typically not statistically significant based on the overlapping 95 % confidence intervals, the trend of the data can still be used to rationalize the migration behavior of the analytes. As the complexes migrate toward the anode, the mobility values have a minus sign by definition. In the presence of HDMS-α-CD, the (+)-enantiomer of the three sulfoxides always displayed the higher complexation constant. Thus, the chiral recognition of the CD is identical toward the enantiomers of the three analytes and the initially assumed dependence of the chiral recognition as a function of the position of the bromo substituent is not supported by the complexation data. Nonetheless, different enantiomer migration orders were observed. In case of compound 11, opposite migration sequence of the enantiomers was noted in the initial experiments with relatively high concentrations of the CDs (Table 3 and Figure 3). However, as described above, the order was (+)-11 before (–)-11 at high concentrations, while the (+)-enantiomer migrated slower than the (–)-enantiomer at lower concentrations as observed for compound 10 over the entire concentration range. It is well known and can easily be concluded from equation (1) that complexation constants and complex mobilities may cooperate or counteract each other in a given chiral separation in CE [41]. Thus, the enantioseparation of compound 11 is dominated by the complexation constants at low concentrations (as in the case of compound 10) with the stronger bound (+)-enantiomer migrating second. In contrast, at high CD concentrations, when a larger fraction of the enantiomers is complexed, the migration order is determined by the (anodic) complex mobilities resulting in a reversal of the sequence. Similar result has been reported earlier for some basic drugs [42]. It is worth mentioning that as in [42], the mobility-dependent separation of enantiomers was characterized by a higher separation factor α compared to affinity-dependent separation (Figure 3). Comparing the mobilities of the complexes of the respective compounds, the absolute mobility in case of compound 10 is lower compared to compound 11 and the difference between the mobilities of the complexes of the (+)-enantiomer and (–)-enantiomer is much

smaller in case of compound 10, it becomes apparent, that the effect of the mobilities on the observed enantioseparation will be much smaller for sulfoxide 10 so that no dependence of the migration order on the CD concentration is observed for this analyte. Moreover, the complexation constants of the enantiomers of sulfoxide 11 are lower compared to compound 10 which also rationalizes a smaller effect of the complexation strength on the enantioseparation. In case of compound 7 a large difference between the mobilities of the enantiomer-CD complexes was found, which explains the fact that the weaker complexed (–)-enantiomer migrated second because the higher anodic mobility of this complex. HDMS-β-CD displayed opposite chiral recognition toward compounds 7 and 11 as a function of methylation of the benzamide group (Table 3). In case of sulfoxide 7 the (+)-enantiomer was bound stronger than the (–)-enantiomer, while for the *N*-methylated analog 11 the (–)-enantiomer was complexed stronger than (+)-11. Because the differences between the mobilities of the diastereomeric analyte-CD complexes were rather small, the enantioseparation of both sulfoxides is due to the opposite chiral recognition (binding strength) of the analytes by the CD as a function of the methylation of the benzamide nitrogen.

A different scenario applies comparing enantioseparations of compounds 10 and 11. The (+)-enantiomer of compound 10 with the bromo substituent in position 3 is complexed stronger than (–)-10, so that in comparison to compound 11 with the substituent in position 4 the chiral recognition of the respective enantiomers by HDMS-β-CD is opposite to each other. However, identical migration order was observed for both compounds. Thus, in case of sulfoxide 10, the observed enantiomer migration order is primarily due to the higher anodic mobility of the weaker bound (–)-enantiomer. Therefore, the overall enantioseparation is determined by the affinity of enantiomers towards the chiral selector in case of sulfoxides 7 and 11, while in the case of compound 10 it is determined by the mobilities of the transient diastereomeric associates. This illustrates one more time that both enantioselective separation mechanisms are effective in chiral CE.

3.2 MEKC using neutral CD derivatives

MEKC has the advantage that it enables to study chiral the resolution ability of neutral chiral selectors such as uncharged CDs toward uncharged analytes as the sulfoxides in the present study. Separations were carried out in fused-silica capillaries applying 50 mM sodium borate butter, pH 9.0 as electrolyte solution. Using β-CD and γ-CD as selectors at concentrations of 20 and 50 mg/mL, SDS concentrations were varied between 20 and 100 mM. Based on the higher resolution observed at 100 mM SDS this concentration was selected for further experiments. Peaks were sharper upon addition of 10 % (v/v) methanol. Subsequently, the neutral CDs were added at concentrations of 50 or 60 mg/mL, while carboxymethylated CDs were studied at 20 mg/mL. Native α-CD could not be investigated because a precipitate was formed when this CD was added to a BGE containing 100 mM SDS. The separation system of the individual CDs was not further optimized.

The results of the screening are summarized in Table S1. Only γ-CD and its derivatives HP-γ-CD and CM-γ-CD proved to be effective chiral selectors, while β-CD and its derivatives only resolved few sulfoxide enantiomers. Under the experimental conditions of the screening, HP-γ-CD and CM-γ-CD were the most universal selectors as these CDs at least partially separated most analytes. Nonetheless, the

highest resolution of R_S = 5.5 was achieved in case of compound 9 and M-β-CD. None of the analytes was enantioseparated in the presence of HP-α-CD or HP-β-CD. It is interesting to note, that in the presence of β-CD derivatives the (+)-enantiomer of the sulfoxides migrated first except for compound 16 using CM-β-CD, while the (–)-enantiomer was detected first when γ-CD or one of its derivatives was applied as chiral selector.

4. Conclusions

The chiral (benzylsulfinyl)benzamides could be effectively separated by capillary electrophoresis in a pH 5.5 background electrolyte using CDs as chiral selectors. In contrast, neutral CD derivatives under MEKC conditions resolved only few of the analytes. Interestingly, randomly substituted charged CDs were by far more efficient compared to single isomer CDs with the exception of HS-β-CD, which separated even more analytes that its randomly substituted counterpart S-β-CD. Especially in the case of random substitution, the larger cavity of γ-CD derivatives appeared to favorably accommodate the analytes because the enantiomers of all analytes were separated in the presence of S-γ-CD, CM-γ-CD and Suc-γ-CD and 14 out of 16 in the case of TMA-γ-CD. Nonetheless, except for CM-α-CD, the other α-CD derivatives also effectively resolved the analyte enantiomers. In the vast majority of cases, the (+)-enantiomers migrated before the (–)-isomers independent of the CD or the structure of the analytes. Thus, structure-separation relationships could not be concluded in the present study. Further studies should enlarge the variety of the substituents on the (benzylsulfinyl)benzamide core structure.

Analysis of the complexation constants and complex mobilities of selected analyte-CD pairs revealed that the (+)-enantiomers of compounds 7, 10 and 11 were always bound stronger by HDMS-α-CD, while the weaker (–)-enantiomer-CD complex exhibited the higher anodic mobility. The same was found for compounds 7 and 10 in the presence of HDMS-β-CD. This resulted in opposite enantiomer migration order in case of compounds 7 and 10 in case of both CDs illustrating that the effective migration order may result from either stereoselective complexation or differences of the mobility between the diastereomeric enantiomer-CD complexes. This may also lead to a reversal of the migration order of the enantiomers as a function of the CD concentration as observed for compound 11 in the presence of HDMS-α-CD. HDMS-β-CD complexed the (–)-enantiomer of sulfoxide 11 stronger resulting in the opposite migration order compared to compound 10. Summarizing, as observed for other analytes [39, 41], the enantiomer migration order may be dominated by either stereoselective complexation (expressed as complexation constants) or by the mobilities of the diastereomeric complexes. A general prediction of the mechanism responsible for analyte migration in enantioselective CE cannot be concluded from the migration order alone.

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Conflict of interest

The authors declare no conflict of interest.

5. References

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6. Figures and tables

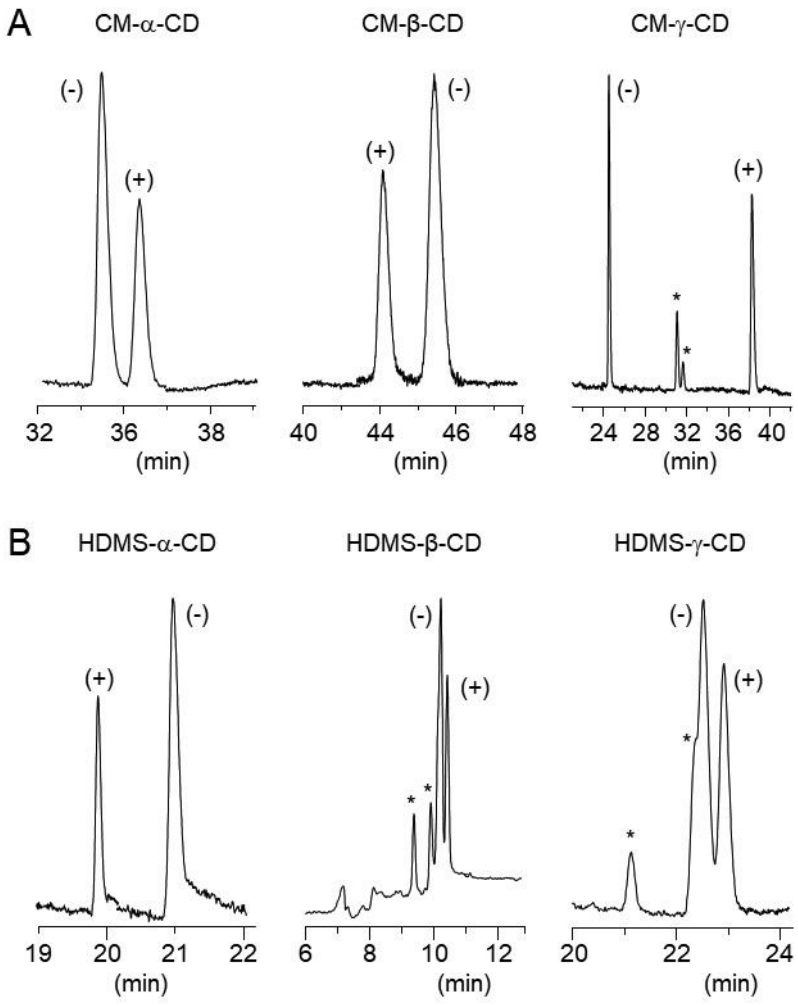


Figure 1. Electropherograms of the separation of the enantiomers of 2-(4-bromobenzylsulfinyl) benzamide (compound 7) in the presence of (A) 20 mg/mL CM-α-CD, CM-β-CD, CM-γ-CD and (B) 40 mg/mL HDMS-α-CD, HDMS-β-CD and HDMS-γ-CD. Other experimental conditions: 40/50.2 cm, 50 µm I.D. fused-silica capillary; 50 mM sodium acetate buffer, pH 5.5; 20 °C; 25 kV; detection at 220 nm. * synthetic impurity.

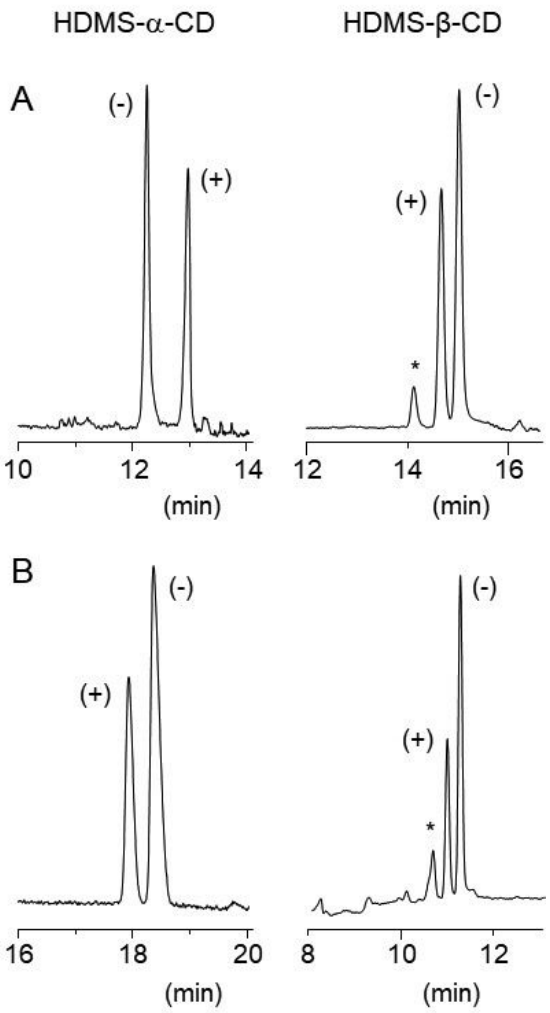


Figure 2. Electropherograms of the separation of the enantiomers of (A) 2-(3-bromobenzylsulfinyl)-*N*-methylbenzamide (compound 10) and (B) 2-(4-bromobenzylsulfinyl)-*N*-methylbenzamide (compound 11) in the presence of 40 mg/mL HDMS-α-CD and HDMS-β-CD. For other experimental conditions see Figure 2.

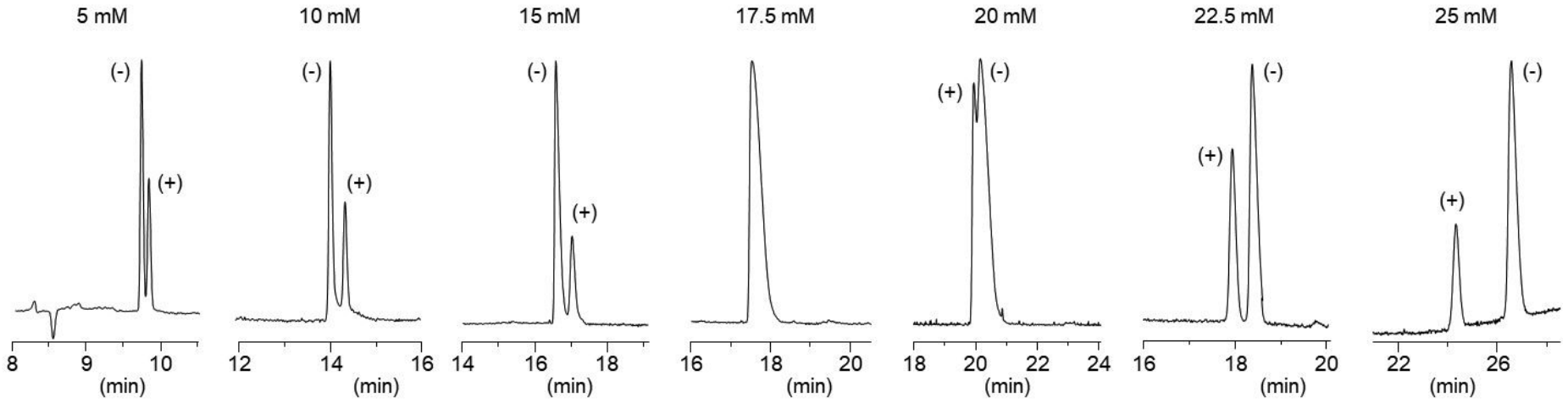
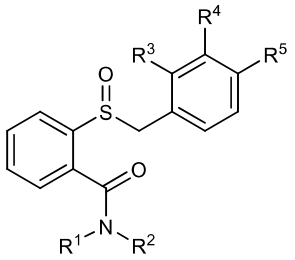
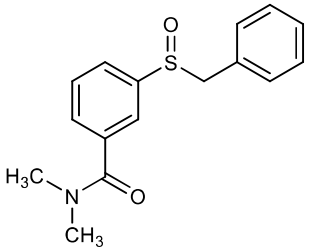


Figure 3. Electropherograms of the separation of the enantiomers of 2-(4-bromobenzylsulfinyl)-*N*-methylbenzamide (compound 11) as a function of the concentration of HDMS-α-CD in the background electrolyte. For other experimental conditions see Figure 2.

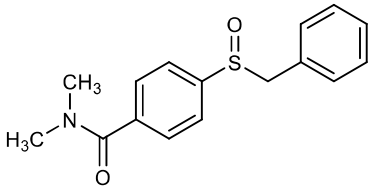
Table 1 Structures of sulfoxides



1-3, 6-16



4[a)]



5[b)]

#	IUPAC name	R^1	R^2	R^3	R^4	R^5
1	2-(benzylsulfinyl)benzamide	H	H	H	H	H
2	2-(benzylsulfinyl)-*N*-methyl benzamide	CH_3	H	H	H	H
3	2-(benzylsulfinyl)-*N*,*N*-dimethyl benzamide	CH_3	CH_3	H	H	H
6	2-(3-bromobenzylsulfinyl)benzamide	H	H	H	Br	H
7	2-(4-bromobenzylsulfinyl)benzamide	H	H	H	H	Br
8	2-(4-methylbenzylsulfinyl)benzamide	H	H	H	H	CH_3
9	2-(4-tertbutylbenzylsulfinyl)benzamide	H	H	H	H	$C(CH_3)_3$
10	2-(3-bromobenzylsulfinyl)-*N*-methyl benzamide	CH_3	H	H	Br	H
11	2-(4-bromobenzylsulfinyl)-*N*-methyl benzamide	CH_3	H	H	H	Br
12	2-(3-methoxybenzylsulfinyl)-*N*-methyl benzamide	CH_3	H	H	OCH_3	H
13	2-(3-methylbenzylsulfinyl)-*N*-methyl benzamide	CH_3	H	H	CH_3	H
14	2-(2-methylbenzylsulfinyl)-*N*,*N*-dimethyl benzamide	CH_3	CH_3	CH_3	H	H
15	2-(3-methylbenzylsulfinyl)-*N*,*N*-dimethyl benzamide	CH_3	CH_3	H	CH_3	H
16	2-(4-methylbenzylsulfinyl)-*N*,*N*-dimethyl benzamide	CH_3	CH_3	H	H	CH_3

[a)] IUPAC name: 3-(benzylsulfinyl)-*N*,*N*-dimethyl benzamide

[b)] IUPAC name: 4-(benzylsulfinyl)-*N*,*N*-dimethyl benzamide

Table 2. Enantiomeric resolution values and migration order of sulfoxides under standardized conditions in presence of randomly substituted charged CDs. The faster migrating enantiomer is indicated in brackets. CE conditions: 50 mM sodium acetate buffer, pH 5.5, 50 µm, 50.2/40 cm fused-silica capillary; 20 °C, 25 kV; detection at 220 nm at the cathodic end in the presence of negatively charged CDs and at the anodic end in the presence of cationic CDs. CD concentrations: 5 mg/mL SBE-β-CD; 10 mg/mL TMA-α-CD, TMA-β-CD, TMA-γ-CD and S-γ-CD; 20 mg/mL CM-α-CD, CM-β-CD, CM-γ-CD, S-α-CD, Suc-β-CD and Suc-γ-CD; 40 mg/mL S-β-CD.

Sulfoxide	SBE-β-CD	S-α-CD	S-β-CD	S-γ-CD	CM-α-CD	CM-β-CD	CM-γ-CD	Suc-β-CD	Suc-γ-CD	TMA-α-CD	TMA-β-CD	TMA-γ-CD
1	0.3 (-)	0.6 (+)	1.7 (+)	3.0 (+)	0.3 (-)	4.1 (+)	3.5 (+)	1.7 (+)	1.6 (+)	0.3 (-)	2.5 (+)	4.3 (+)
2	0.9 (+)	0.2 (+)	0.9 (+)	3.2 (+)	ns	4.4 (+)	3.5 (+)	5.3 (+)	2.8 (+)	0.2 (+)	7.5 (+)	7.8 (+)
3	0.2 (+)	0.9 (+)	0.2 (+)	0.8 (+)	ns	2.0 (+)	4.2 (+)	1.9 (+)	2.2 (+)	0.3 (+)	2.5 (+)	5.9 (+)
4	ns	ns	ns	1.2 (+)	ns	1.0 (+)	1.2 (+)	1.1 (+)	1.0 (+)	ns	ns	ns
5	1.1 (-)	ns	0.2 (+)	10.7 (+)	ns	1.6 (-)	1.4 (+)	1.3 (+)	1.0 (+)	ns	ns	0.2 (-)
6	2.4 (-)	2.9 (+)	1.0 (+)	16.0 (+)	2.2 (+)	0.6 (+)	4.5 (+)	1.8 (+)	1.9 (+)	3.0 (+)	2.6 (+)	14.9 (+)
7	8.9 (-)	5.4 (+)	2.1 (+)	13.3 (+)	7.6 (-)	8.9 (+)	20.6 (-)	1.4 (+)	6.5 (+)	1.1 (+)	5.4 (+)	10.7 (+)
8	1.6 (-)	ns	1.0 (+)	7.9 (+)	ns	3.2 (-)	12.3 (+)	2.8 (+)	3.2 (+)	ns	2.1 (+)	11.5 (+)
9	nd	2.4 (+)	2.4 (+)	12.8 (+)	ns	nd	9.4 (+)	5.2 (+)	1.9 (+)	2.3 (+)	ns	12.7 (+)
10	1.6 (-)	1.2 (+)	2.8 (+)	10.2 (+)	ns	3.5 (+)	1.6 (+)	2.1 (+)	2.3 (+)	3.5 (+)	5.1 (+)	11.7 (+)
11	2.4 (-)	7.5 (+)	4.9 (+)	10.4 (+)	3.5 (-)	6.1 (+)	13.1 (+)	2.4 (+)	4.3 (+)	0.8 (+)	5.8 (+)	ns
12	0.9 (-)	ns	ns	3.6 (+)	ns	ns	1.7 (+)	1.6 (+)	2.0 (+)	0.8 (+)	1.6 (+)	4.1 (+)
13	2.0 (+)	0.6 (+)	ns	8.0 (+)	ns	1.9 (-)	13.7 (+)	1.6 (+)	1.9 (+)	0.2 (+)	0.5 (+)	2.6 (+)
14	0.5 (+)	1.1 (+)	0.2 (+)	0.6 (+)	ns	3.2 (+)	7.0 (+)	2.8 (+)	3.1 (+)	0.2 (-)	1.9 (+)	5.3 (+)
15	1.0 (+)	0.7 (+)	1.3 (+)	2.0 (+)	0.6 (+)	0.9 (+)	3.2 (+)	0.8 (+)	2.0 (+)	0.9 (+)	1.7 (+)	4.4 (+)
16	0.2 (+)	1.0 (+)	ns	0.5 (+)	ns	2.0 (+)	6.8 (+)	0.9 (+)	2.1 (+)	ns	2.1 (+)	2.4 (+)

nd, not detected within 60 min; ns, not separated

Table 3. Enantiomeric resolution values and migration order of sulfoxides under standardized conditions in the presence of single isomer charged CDs. The faster migrating enantiomer is indicated in brackets. CE conditions: 50 mM sodium acetate buffer, pH 5.5, 50 μm, 50.2/40 cm fused-silica capillary; 20 °C, 25 kV; detection at 220 nm at the cathodic end in the presence of negatively charged CDs and at the anodic end in the presence of cationic CDs. CD concentrations: 20 mg/mL HS-β-CD, HA-β-CD and OA-γ-CD; 40 mg/mL HDMS-α-CD, HDMS-β-CD, ODMS-γ-CD and HDAS-β-CD.

Sulfoxide	HS-β-CD	HDAS-β-CD	HDMS-α-CD	HDMS-β-CD	ODMS-γ-CD	HA-β-CD	OA-γ-CD
1	0.8 (+)	0.8 (-)	2.2 (+)	ns	ns	2.3 (+)	0.3 (-)
2	1.9 (+)	0.2 (-)	0.9 (+)	ns	ns	3.5 (+)	ns
3	1.6 (+)	ns	ns	ns	ns	1.9 (+)	ns
4	1.5 (+)	0.3 (+)	ns	ns	ns	ns	0.5 (+)
5	ns	ns	0.5 (+)	ns	ns	0.9 (+)	0.3 (-)
6	1.8 (+)	0.4 (-)	0.7 (-)	1.0 (+)	ns	1.7 (+)	ns
7	7.3 (+)	ns	5.6 (+)	1.1 (-)	0.9 (-)	6.5 (+)	1.8 (-)
8	2.6 (+)	ns	2.2 (+)	ns	ns	3.4 (+)	0.7 (+)
9	6.0 (+)	0.5 (-)	ns	2.3 (-)	1.6 (-)	ns	1.1 (-)
10	2.4 (+)	0.7 (-)	2.2 (-)	0.8 (+)	ns	9.3 (+)	0.3 (+)
11	6.7 (+)	3.2 (-)	1.6 (+)	1.8 (+)	ns	12.0 (+)	0.7 (-)
12	0.2 (+)	ns	0.5 (+)	ns	ns	0.2 (+)	1.1 (-)
13	0.4 (+)	1.7 (-)	1.2 (+)	2.2 (+)	2.4 (+)	ns	ns
14	1.0 (+)	ns	0.2 (-)	ns	Ns	1.8 (+)	ns
15	1.1 (+)	ns	1.2 (+)	ns	ns	3.3 (+)	ns
16	1.2 (+)	ns	ns	ns	ns	1.1 (+)	ns

ns - not separated

Table 4. Apparent complexation constants (K) and mobilities of the free analyte (μ_f) as well as analyte-CD complexes (μ_c) and enantiomer migration order (EMO). The numbers in brackets refer to the 95% confidence interval. CE conditions: 50 mM sodium acetate buffer, pH 5.5, 50 µm, 50.2/40 cm fused-silica capillary; 20 °C, 25 kV; detection at 220 nm at the cathodic end.

Sulfoxide		HDMS-α-CD				HDMS-β-CD			
		K (M^{-1})	**μ_f** ($10^{-9}m^2V^{-1}s^{-1}$)	**μ_c** ($10^{-9}m^2V^{-1}s^{-1}$)	**EMO**	**K** (M^{-1})	**μ_f** ($10^{-9}m^2V^{-1}s^{-1}$)	**μ_c** ($10^{-9}m^2V^{-1}s^{-1}$)	**EMO**
7	(+)	28.6 (20.5 / 36.3)	0.004 (0.003 / 0.005)	–0.525 (–0.205 / –0.712)	1	63.6 (48.7 / 80.1)	0.005 (0.002 / 0.009)	–0.098 (–0.075 / –0.121)	2
	(-)	20.2 (15.6 / 26.2)		-0.706 (–0.301 / –1.223)	2	50.1 (40.1 / 63.5)		–0.109 (–0.088 / –0.137)	1
10	(+)	60.6 (49.0 / 72.1)	0.007 (0.003 / 0.103)	–0.082 (–0.051 / –1.202)	2	160.1 (132.8 / 180.9)	0.008 (0.005 / 0.012)	–0.046 (–0.022 / –0.069)	1
	(-)	38.9 (19.8 / 53.3)		0.101 (–0.505 / –1.435)	1	127.4 (111.3 / 142.5)		–0.054 (–0.036 / –0.078)	2
11	(+)	24.4 (15.9 / 28.7)	0.012 (0.005 / 0.015)	–0.523 (–0.252 / –0.884)	2 → 1	93.9 (78.9 / 115.6)	0.011 (0.009 / 0.018)	–0.086 (–0.062 / –0.102)	1
	(-)	19.6 (12.8 / 25.4)		–0.611 (–0.331 / –1.109)	1 → 2	113.3 (89.7 / 136.8)		–0.079 (–0.055 / –0.098)	2

4. General discussion

CE enantioseparations in the present research project focused on a systematic investigation of the enantioseparation of chiral dipeptides and sulfoxides in the presence of different CD derivatives as chiral selectors alone or a combination as additives to the BGE. The work includes three main parts. In the first part, enantioseparations of dipeptides and the migration order changes were investigated in the presence of neutral (manuscript 1) and charged (manuscript 2) CD derivatives at different pH values, while the synergistic effect of amino acid based CILs in combination with β-CD and HP-β-CD on the separation of the peptides was studied in the second part of this work (manuscript 3). The last topic includes the investigation of the enantioseparation of chiral sulfoxides in the presence of neutral and charged CDs (manuscript 4). In order to understand the mechanisms corresponding to the different migration order of the enantiomers of selected analytes, binding constants and complex mobilities were determined (manuscript 1, 2 and 4).

4.1 Separation principles

In Figure 12 six major scenarios are disclosed with regard to the experimental conditions studied in this research with respect to the peptide analytes. In the pH range from 2.5 to 3.8 peptides are positively charged and migrate toward the cathode. Neutral CDs, which do not possess any self-mobility, migrate in the same direction with the EOF (Figure 12 a). Figure 12 b represents a separation of positively charged analyte in the presence of negatively charged CDs. At the acidic pH, the analyte and the CD possess countercurrent mobilities. Under these conditions the EOF is not a dominant driving force. At lower concentrations of the CDs a detection at the cathode is possible, while at higher concentrations they can be only detected when the polarity of the applied voltage is reversed. This is due to the fact, that at higher concentrations a negatively charged CD becomes a carrier of the positively charged analyte and transports it toward to the anode. With increasing the pH value up to 5.3 or 5.5 in the presence of negatively charged CDs, where peptide analytes are neutral and migrate with the EOF, here the EOF is strong enough to detect the analytes at the cathode. Nevertheless, at relatively high CD concentrations the reversal of the polarity is needed, due to the strong anodic mobility of the negatively charged CDs (Figure 12 c). When positively charged CDs are used instead of negatively charged CDs at the same pH value, detection at the anode is carried out (Figure 12 d). In contrast to acidic pH values, at pH 9.5 peptides are negatively charged and migrate toward the anode. Because of the strong electroosmotic flow, which compensates the electrophoretic mobility of the analytes, the detection at the cathode is carried out as illustrated in Figure 12 e. Finally, when a CIL is added to the BGE at pH values 2.5 and 3.5, the anionic and cationic components of the additive dissolve in the aqueous BGE and interact with the capillary wall or with a positively charged analyte in different ways, resulting higher separation quality (Figure 12 f).

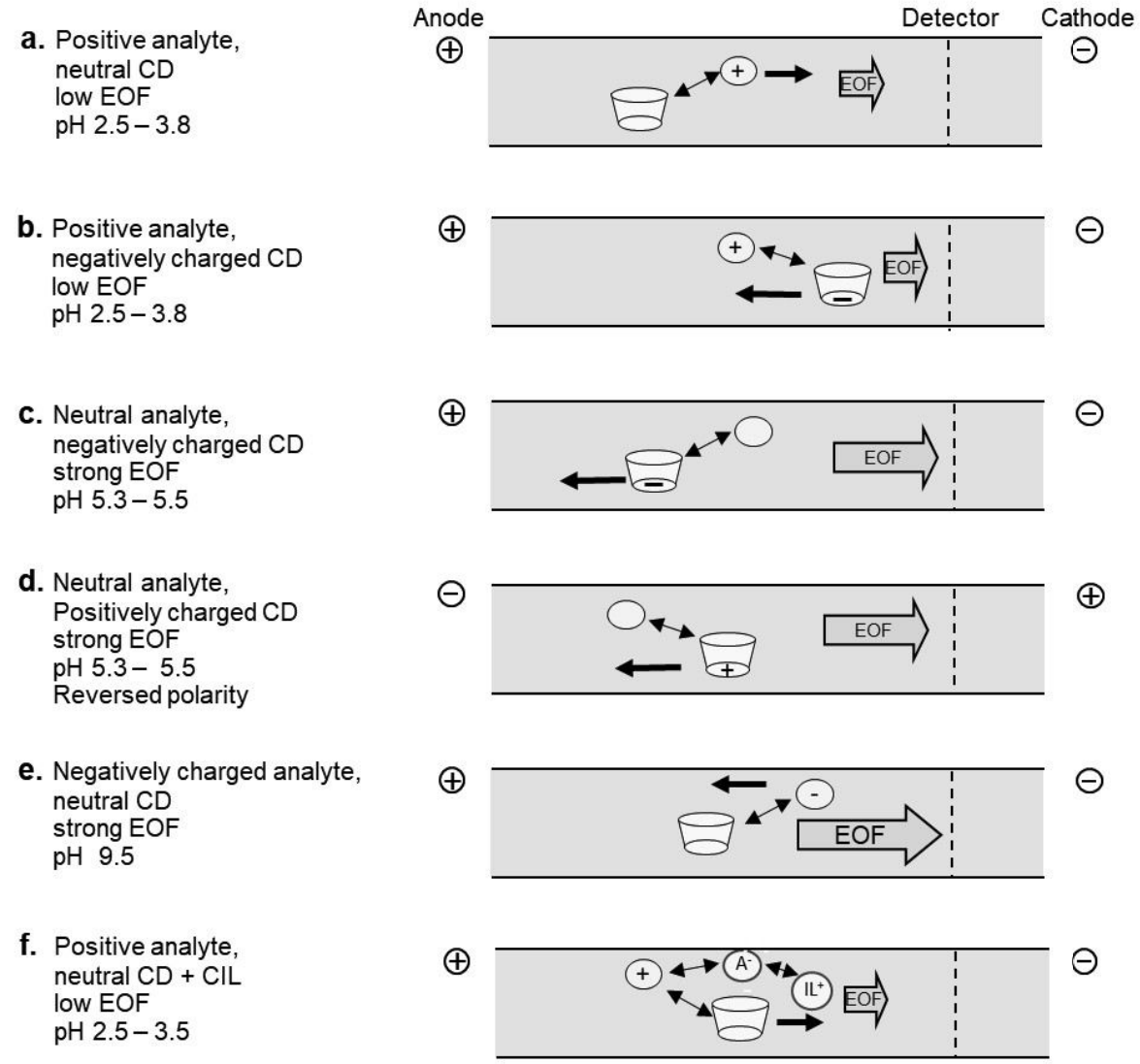


Figure 12. The schematic representation of a separation depending on a charge of the analyte, the CD and the pH value of the BGE. A basket-type structure refers to the CD, an analyte is represented with oval symbols with indication of the charge, the yellow symbols refer to the anionic and the cationic parts of the CIL.

4.2 Chiral separation of dipeptides

The dipeptide enantioseparations will be discussed with regard to peptide structure, type of the CD, the pH of the BGE and type of the CIL as an additive to the buffer solution. The separation data is summarized in manuscripts 1, 2 and 3. The structures of the studied dipeptides are illustrated in Figure 13. Only DD- and LL-enantiomers were investigated in case of the dipeptides containing two chiral centers. DL- and LD-stereoisomers were not included. Analyses were conducted at four different pH values. It must be noted that the pH plays an important role in the ionization of the functional groups of the peptides determining the net charge of the analytes. The pK_a values of the carboxyl acid group of dipeptides range between 2.8 - 3.6 and the pK_a value of the amino groups of dipeptides is between 7.2

- 8.5 [128, 129]. Accordingly, at pH 2.5, 75 – 80 % of carboxyl groups are protonated, while this fraction is between 16 – 23 % at pH 3.5. Thus, the overall net charge of the peptides is positive at pH 2.5 but the charge density is lower at pH 3.5. The peptides are neutral around pH 5.3, where they are in a zwitterionic state and are negatively charged at pH 9.5. Subsequently, neutral native α-, β-, and γ-CDs and their derivatives were used to analyze only charged peptides at pH values between 2.5 - 3.8 and 9.5, while charged CDs were also effective selectors in pH range from 2.5 to 5.3. Analyses were conducted in bare fused-silica capillaries at an applied voltage of 25 kV and the temperature was maintained 20 °C using 50 mM sodium phosphate buffer in the pH range from 2.5 to 3.8, 50 mM sodium acetate buffer for pH 5.3 and 50 mM sodium borate buffer when conducting analyses at pH 9.5. CD concentrations ranged between 1 mg/mL to 100 mg/mL depending on the type of the chiral selector. Furthermore, the neutral CDs were analyzed combination with CILs based on tetraalkylammonium ions and amino acids. The CD concentration was fixed to 20 mM and the CIL concentrations varied between 5 - 50 mM.

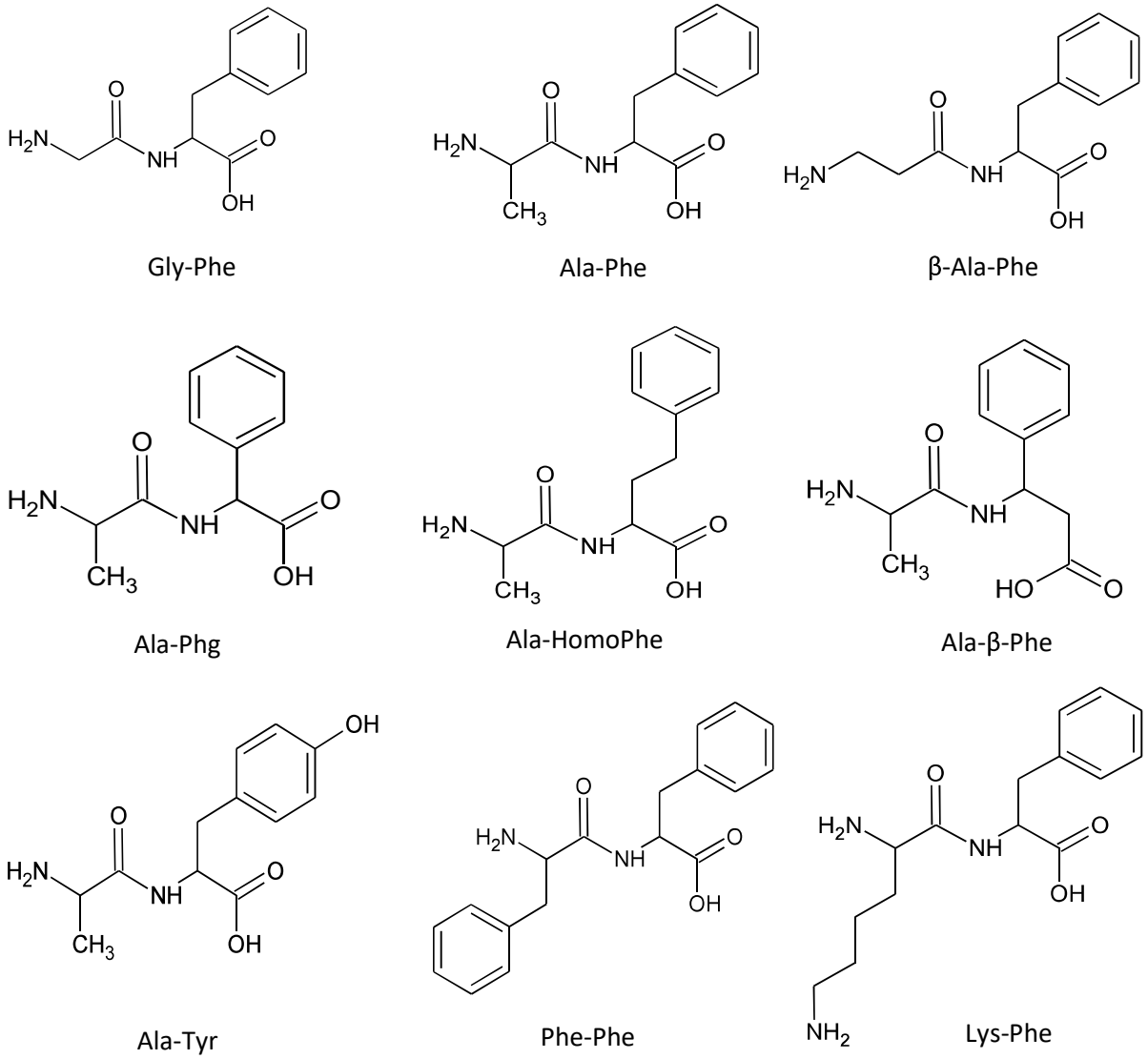


Figure 13. Structures of the dipeptides.

4.2.1 Effect of amino acid sequence on the migration order of peptide enantiomers

In this chapter the EMO of the studied peptides with respect to their structural modification will be discussed. The separation results are summarized in Table 1 in manuscript 1 and Table 1 in manuscript 2. Generally, only a few conclusions can be drawn regarding an influence of the structure of the dipeptides on the chiral separation and the EMO. Gly-Phe and β-Ala-Phe are the peptides with one chiral center. The structural difference is the insertion of a CH_2 group between the chiral center and N-terminus in β-Ala-Phe compared to Gly-Phe. According to the results, their migration order of the enantiomers was the same in the presence of neutral CDs (Table 1 of manuscript 1). On the contrary, when analysing them with negatively charged CDs, opposite EMOs were detected in several cases (Table 1 of manuscript 2). For example, in the presence of sulfated-β-CD (S-β-CD) at pH 2.5 or succinylated-β-CD (Suc-β-CD) at pH 3.5. Changing from Gly-Phe to Ala-Phe did not have any influence when analyzing them with neutral CDs. In contrast, in the presence of negatively charged CDs a few different enantiomer affinity patterns of the studied CDs toward these analytes were noted (see Table 1 in manuscript 2). Furthermore, β-Ala-Phe is a structural isomer of Ala-Phe, but this type of structural modification had only an influence in the presence of carboxymethylated-β-CD (CM-β-CD) at pH 2.5 and Suc-β-CD at pH 3.5, where the opposite EMOs of the respective dipeptides were detected (see Table 1 in manuscript 2).

One of the modifications of Ala-Phe peptides included shortening of the side chain of Phe in case of Ala-Phg or elongating the distance between the chiral center and the phenyl ring resulting in Ala-HomoPhe. These modifications yielded a reversal of the enantiomigration order in some cases, but not in all. In the presence of neutral CDs there was only one occasion of reversal of the EMO between Ala-Phe and Ala-Phg enantiomers, when analysing them with β-CD at alkaline pH value. On the contrary, when using the charged CDs as chiral selectors, there were much more cases observed. For instance, the DD-isomer of Ala-Phe migrated before LL-enantiomer in the presence of heptakis(2,3-di-O-methyl-6-O-sulfo)-β-CD (HDMS-β-CD) and heptakis(2,3-di-O-acetyl-6-O-sulfo)-β-CD (HDAS-β-CD) at pH values 3.5 and 2.5, respectively, while the first eluting enantiomer of Ala-Phg was LL under these conditions. The more significant effect on the migration order changes of the Ala-Phe analogs had the switch to Ala-HomoPhe, which revealed the opposite EMO compared to Ala-Phe in 17 studied conditions out of 35 where the separations of both peptides were achieved in the presence of neutral and negatively charged CDs. According to NMR studies on peptide-CD complexes carried out by Holzgrabe and co-workers [130], Phe-containing dipeptides penetrate the cavity at the wider rim with the phenyl ring first. Subsequently, the difference in the migration order of Ala-HomoPhe and Ala-Phe might be explained by deeper immersion of the DD-enantiomer of Ala-HomoPhe bearing longer side chain into the cavity of the studied CDs compared to the respective enantiomers of Ala-Phe or Ala-Phg at pH 2.5. This is in accordance with the observed migration orders at pH 2.5. In 11 cases out of 16 the LL-enantiomer of Ala-homoPhe was the first migrating stereoisomer when the separation of the respective dipeptide was achieved in the presence of neutral and charged CDs. In contrast, for Ala-Phe and Ala-Phg the LL-isomer was the first eluting enantiomer only in 50 % of the cases where the separation of these dipeptides was observed. Figure 14 represents a few examples of the EMO change according to structural alteration of the dipeptides where elongating as well as shortening of the side chain of Ala-Phe resulted in the opposite

migration order of the corresponding peptides in the presence of HDAS-β-CD at pH 2.5. If the migration order of Ala-Phg and Ala-HomoPhe was LL > DD, it was opposite to the migration order DD > LL observed for Ala-Phe.

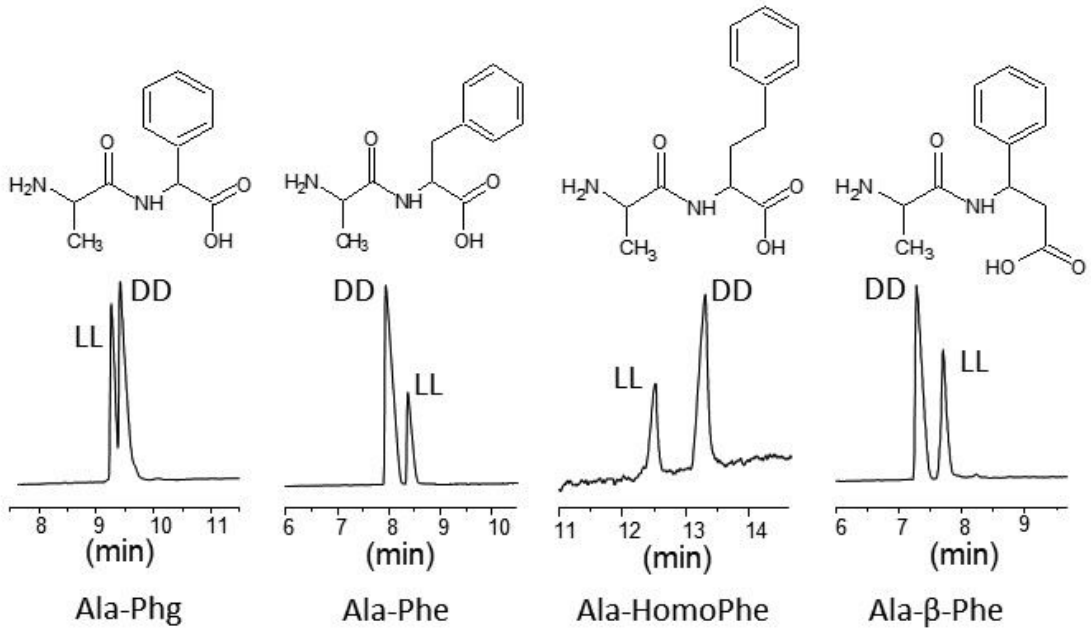


Figure 14. Enantioseparations of Ala-Phg, Ala-Phe, Ala-HomoPhe and Ala-β-Phe in the presence of 5 mg/mL HDAS-β-CD, pH 2.5. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium phosphate buffer, capillary temperature 20 °C, separation voltage 25 kV, UV detection at 215 nm.

Furthermore, when comparing Ala-Phg and Ala-β-Phe, where the structural difference is an additional CH_2 group between the chiral carbon and the carboxyl group, an opposite chiral affinity pattern of HDAS-β-CD towards these dipeptides was observed as shown in Figure 14. Further examples can be found in Table 1 of manuscript 2.

In the summary, although differences in the EMO were observed for some peptide analytes, the relatively small modifications in the structures of the Ala-Phe analogs did not allow to conclude clear relationships between the chiral recognition ability of the peptides by the CDs or the EMO.

4.2.2 Changes in peptide migration order with respect to CD type

Based on the CE measurements carried out on 6 dipeptides out of the 9 shown in Figure 13, reversal of the EMO was found as function of the substitution pattern of CDs and the cavity size of CDs. In this manner 24 neutral and charged CD derivatives were investigated. The research is presented in manuscripts 1 and 2.

Generally, negatively charged CDs revealed higher enantiorecognition ability compared to neutral CDs, especially β-CD derivatives at the acidic pH values. As shown in Figure 12 b and c, negatively charged CDs possess a self-mobility toward the anode opposite to the mobility of the positively charged peptides in the pH range from 2.5 to 3.8. Moreover, electrostatic interactions between the oppositely charged analytes and the CDs may also contribute to the formation of transient diastereomeric complexes in addition to van der Waals interactions, hydrogen bonding and dipole-dipole interactions which only contribute to complex formation in the case of neutral CDs [113]. Subsequently, the mobility difference between the enantiomers of a positively charged peptide is much higher in the presence of negatively charged CDs compared to neutral CDs, resulting a better enantioresolution.

Furthermore, introducing charged groups in a CD wider rim not only improved a separation quality but also affected the concentration of the CDs necessary for an enantioresolution. If in case of neutral CDs, a minimum concentration of 20 mg/mL was needed to obtain any kind of separation. For some negatively charged CDs even 2 mg/mL was enough to separate the enantiomers of the studied dipeptides. This can be explained with the countercurrent mobility of the negatively charged CDs and the positively charged analytes (Figure 12 b). However, at pH 3.5 higher concentrations were needed. This is due to the fact, that at this pH value a charge density of peptides is much smaller than at pH 2.5, for a more detailed discussion see section 4.2.

The migration order of the studied Ala-Phe analogs is summarized in Table 4 and 5 for selected CDs. Further examples can be found in Table 1 in manuscript 1 and Table 1 in manuscript 2. The superior effect of the charged CDs over neutral CDs can be seen in examples. For instance, Gly-Phe, which could not be separated in the presence of β-CD was successfully resolved when analysing the compound in the presence of S-β-CD or HDMS-β-CD at pH 2.5 (Table 4 and Table 5). The same applies to α-CD and γ-CD and their charged or neutral derivatives. For instance, at pH 3.5 no enantioresolution was achieved when analysing peptides with native γ-CD. In contrast, a randomly substituted sulfated-γ-CD (S-γ-CD) resolved all of the analytes at the same pH value (Table 4 and Table 5).

Table 4. Enantiomer migration order of the peptides in the presence of some neutral CDs at different pH values. The faster migrating enantiomer is listed first. CD concentrations were 20 mg/mL β-CD, M-β-CD, 50 mg/mL α-CD and M-α-CD and 100 mg/mL γ-CD and M-γ-CD at pH values 2.5, 3.5 and 9.5 respectively.

	α-CD			**β-CD**			**γ-CD**		
Peptides	**pH=2.5**	**pH=3.5**	**pH=9.5**	**pH=2.5**	**pH=3.5**	**pH=9.5**	**pH=2.5**	**pH=3.5**	**pH=9.5**
Gly-Phe	ns[a)]	L>D	ns	ns	D>L	L>D[b]	L>D	ns	ns
Ala-Phe	ns	ns	ns	LL>DD	DD>LL	LL>DD[b]	LL>DD	ns	ns
β-Ala-Phe	L>D	L>D	ns	L>D	D>L	L>D[b]	L>D	ns	ns
Ala-β-Phe	LL>DD	ns	ns	LL>DD	DD>LL	LL>DD[b]	LL>DD	ns	ns
Ala-Phg	LL>DD	LL>DD	ns	LL>DD	ns	DD>LL[b]	LL>DD	ns	ns
Ala-homoPhe	ns	ns	ns	LL>DD	DD>LL	DD>LL[b]	LL>DD	ns	ns
	M-α-CD			**M-β-CD**			**M-γ-CD**		
Gly-Phe	D>L	ns	ns	D>L	ns	ns	ns	ns	D>L
Ala-Phe	DD>LL	ns	ns	ns	ns	ns	LL>DD	ns	ns
β-Ala-Phe	ns	ns	ns	D>L	ns	ns	L>D	ns	ns
Ala-β-Phe	ns	ns	ns	LL>DD	LL>DD	ns	LL>DD	LL>DD	ns
Ala-Phg	ns	ns	ns	ns	DD>LL	LL>DD	ns	DD>LL	ns
Ala-homoPhe	ns	ns	ns	ns	ns	ns	DD>LL	DD>LL	ns

[a)] ns, not separated at standard conditions

[b)] concertation of β-CD was 50 mg/mL

Methylation of CDs increases the ability of the CD to form additional intermolecular interactions and, furthermore, affects the size and the structure of the cavity [43]. According to our results these changes had a drastic effect on an enantioresolution of the dipeptides. Randomly methylated-α-CD (M-α-CD) proved to be a rather poor chiral selector because only two peptides could be resolved at pH 2.5 and no separation was observed at other studied pH values (Table 4). The same applies for randomly methylated-β-CD (M-β-CD) and randomly methylated-γ-CD (M-γ-CD). With exception that, at pH 3.5 M-γ-CD could separate some of the dipeptides compared to the native γ-CD, which was not efficient chiral selector at this pH value. The same happened when secondary rim was completely methylated, resulting in 2,6-dimethyl-β-CD (DM-β-CD) and heptakis(2,3,6-tri-O-methyl)-β-CD (TM-β-CD). The separation ability of the respective CDs was much lower compared with native β-CD (see Table 1 in manuscript 1). In contrast, introducing charged groups on the CD rim selectively or randomly revealed much better results. For example, heptakis(6-O-sulfo)-β-CD (HS-β-CD) resolved the enantiomers of Ala-Phe analogs at all studied conditions. The same superior effect of the sulfonated and the sulfated CDs are described in the reference [111], where these types of the CDs proved to be efficient selectors for peptide enantioseparation at the acidic pH values.

Table 5. Enantiomer migration order of the peptides in the presence of some charged CDs at different pH values. The faster migrating enantiomer is listed first. CD concentrations were 5 mg/mL S-α-CD, S-β-CD, 10 mg/mL S-γ-CD, 20 mg/mL HDMS-β-CD, HDMS-α-CD and HDMS-γ-CD at pH 2.5 and 3.5, 10 mg/mL S-α-CD, 15 mg/mL S-β-CD, 20 mg/mL S-γ-CD, HDMS-β-CD, HDMS-α-CD and HDMS-γ-CD at pH 5.3.

	S-α-CD			**S-β-CD**			**S-γ-CD**		
	pH=2.5	**pH=3.5**	**pH=5.3**	**pH=2.5**	**pH=3.5**	**pH=5.3**	**pH=2.5**	**pH=3.5**	**pH=5.3**
Gly-Phe	L>D	L>D	ns	L>D	L>D	ns	L>D	L>D	ns
Ala-Phe	LL>DD	ns	ns	DD>LL	DD>LL	DD>LL	LL>DD	LL>DD	ns
β-Ala-Phe	L>D	L>D	L>D	D>L	ns	D>L	L>D	L>D	ns
Ala-β-Phe	LL>DD	LL>DD	LL>DD	DD>LL	DD>LL	DD>LL	LL>DD	LL>DD	LL>DD
Ala-Phg	LL>DD	LL>DD	DD>LL	DD>LL	DD>LL	DD>LL	LL>DD	LL>DD	ns
Ala-homoPhe	DD>LL	DD>LL	ns	nd	LL>DD	DD>LL	LL>DD	LL>DD	ns
	HDMS-α-CD			**HDMS-β-CD**			**ODMS-γ-CD**		
Gly-Phe	ns	D>L	ns	D>L	D>L	L>D	ns	ns	ns
Ala-Phe	LL>DD	ns	ns	DD>LL	DD>LL	LL>DD	ns	ns	ns
β-Ala-Phe	nd	D>L	ns	D>L	D>L	L>D	ns	ns	ns
Ala-β-Phe	LL>DD	LL>DD	ns	LL>DD	LL>DD	DD>LL	LL>DD	LL>DD	ns
Ala-Phg	ns	ns	ns	ns	LL>DD	LL>DD	ns	ns	ns
Ala-homoPhe	LL>DD	DD>LL	ns	LL>DD	LL>DD	DD>LL	LL>DD	ns	ns

[a)] ns, not separated at standard conditions.

4.2.2.1 Migration order as function of the CD substitution pattern

The enantiomigration order of the studied peptides revealed that the EMO was also dependent on the substitution pattern of the CDs. Some examples of the affinity reversal of the enantiomers of dipeptides and tripeptides in the presence of neutral and charged CDs and their derivatives as a function of a type of the substitution of CDs were reported in references [101, 113]. Similar observations were derived for the Ala-Phe analogs in the presence of uncharged and charged chiral selectors. Comparing native β-CD with randomly substituted S-β-CD opposite EMO is observed under most of the experimental conditions (Table 4 and Table 5). As presented in Figure 15 for β-CD and S-β-CD, the LL-enantiomers of the analytes migrates first in the presence of β-CD, while they are the second migrating when using S-β-CD as a chiral selector. Furthermore, randomly introducing sulfated groups to β-CD not only changed the affinity pattern of the CDs toward the respective enantiomers, but also had a positive effect on a resolution. The same applies for HS-β-CD (Figure 15), but here only Ala-homoPhe reveals opposite migration order compared with the results obtained with native β-CD. Furthermore, the migration orders observed in the presence of HS-β-CD is opposite to the EMOs obtained when using randomly substituted S-β-CD as a chiral selector for Ala-Phg and Ala-Phe (Figure 15). The isomeric purity of the

CDs was also determining factor of the migration order in case of Ala-Phe and β-Ala-Phe, when analysing them in the presence of β-CD, 2,6-DM-β-CD with an isomeric purity of 50% and 2,6-DM-β-CD with an isomeric purity of 95%. The EMO of the respective dipeptides was the same in presence of β-CD and 2,6-DM-β-CD with an isomeric purity of 95% and was opposite to the EMO observed with 50% isomerically pure 2,6-DM-β-CD (see Table 1 in manuscript 1). Similar observations were reported previously for di- and tripeptides [111, 112] once again proving the fact that not only the nature of substituent but also their location on the CD rim may determine the enantiomer affinity pattern of some dipeptides [33].

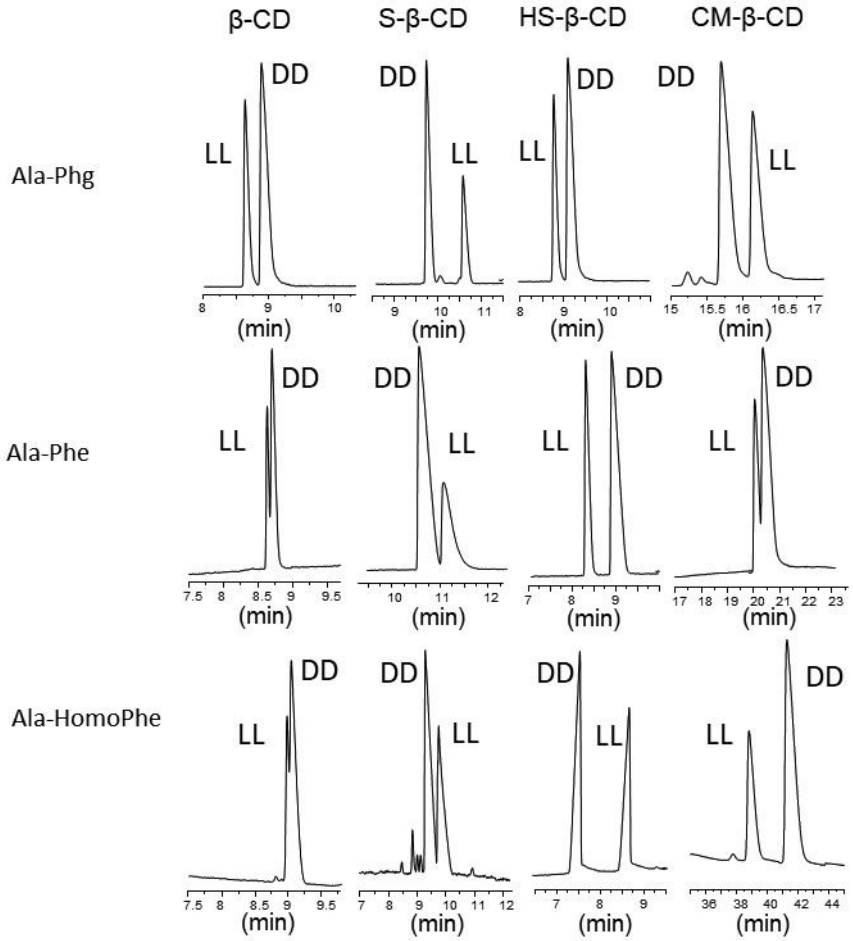


Figure 15. Electropherograms of the enantioseparation of Ala-Phg, Ala-Phe and Ala-HomoPhe, in the presence of 20 mg/mL β-CD, 5 mg/mL S-β-CD, 2 mg/mL HS-β-CD and 20 mg/mL CM-β-CD. Experimental conditions: 40/50.2 cm, 50 μm ID fused-silica capillary, 50 mM sodium phosphate buffer at pH 2.5, capillary temperature 20^0C, separation voltage 25 kV, UV detection at 215 nm.

In contrast, in case of native α-CD and γ-CD and their sulfated derivatives, the EMO of the enantiomers was identical (Table 4 and Table 5). Comparing TM-β-CD and HDMS-β-CD, which selectively bears sulfate groups at C6 of the D-glucose moieties instead of methyl groups in case of HDMS-β-CD, and TM-β-CD, which is completely methylated, opposite EMO was observed only for Gly-Phe. The

L-enantiomer migrated faster in the presence of TM-β-CD at pH 3.5, while the reversal of the migration order was observed when analysing it with HDMS-β-CD at the same pH value. Upon random carboxymethylation of the hydroxy groups of the D-glucose units, i.e., moving from β-CD to CM-β-CD, or from α-CD to carboxymethylated-α-CD (CM-α-CD), the enantioseparation ability of the CDs was significantly affected. Especially, some opposite EMOs were observed at pH 2.5 for β-CD and CM-β-CD, and at pH 2.5 and 3.5 when comparing α-CD and CM-α-CD (see Table 1 in manuscript 2). Figure 15 illustrates an improvement of the enantioseparation of the Ala-HomoPhe enantiomers when switching from native β-CD to CM-β-CD without changing the EMO. On the contrary, the opposite migration order was detected for Ala-Phg, whose respective LL-enantiomer migrated faster in the presence of β-CD, while it was migrating as the second peak with CM-β-CD.

4.2.2.2 Migration order as function of the CD cavity size

Not only the substitution pattern affected the migration order of the studied Ala-Phe analogs, but also the cavity size of the respective CDs had an important influence on the EMO. It is interesting to note, that all native α-CD, β-CD and γ-CD contain D-glucopyranose units in the same stereochemical configuration and accordingly the enantiomer affinity pattern toward these CDs should be the same, but there are many examples contradicting this hypothesis discussed in references [104, 131-135] for peptide and non-peptide analytes. The present research showed once again that the cavity size of a cyclic oligosaccharides can be a determining factor for achieving different enantiomigration order of analytes and a better enantioresolution. The chemical groups involved in non-covalent intermolecular CD-peptide interactions are the same but the distance between noncovalently interacting groups may change when increasing the cavity size in the order α-CD, β-CD and γ-CD and their derivatives [33]. In general, β-CD and its derivatives provided more separations under the standardized screening conditions compared with α-CD and γ-CD and their derivatives. In contrast to negatively charged CDs, for uncharged chiral selectors γ-CDs were more effective compared to α-CDs (Table 4 and Table 5). Nevertheless, at alkaline pH only native β-CD and its derivatives were able to separate Ala-Phe analogs (Table 4), with lower separation efficiency compared to acidic pH values. This is in accordance with the previously published data on other dipeptides in the presence of neutral β-CD derivatives at pH 9.5 [105]. This may be attributed to the strong EOF at pH 9.5, resulting in a high velocity of both analyte and CD, so that a small difference in the affinities of the enantiomers toward the CDs for the formation of the transient diastereomeric complexes were not sufficient to result in an enantioresolution [113]. In case of negatively charged CDs at pH 2.5 and 3.5, S-α-CD, S-β-CD and S-γ-CD exhibited a quite strong enantiomer resolving ability and the EMO was dependent on the cavity size of the CDs for Ala-Phe, Ala-HomoPhe, Ala-Phg, Ala-β-Phe and β-Ala-Phe as presented in Table 5. At pH 2.5 and 3.5 the enantiomer affinity pattern of S-α-CD and S-γ-CD towards the respective dipeptides was the same and was the opposite to that in the presence of S-β-CD. In contrast, for native α-CD, β-CD and γ-CD the EMO stayed unchanged, as all resolved analytes revealed LL > DD (or L > D) migration order at pH 2.5 (Table 4).

The opposite EMO as a function of a CD cavity size was observed only in several cases for the studied single isomers sulfated CDs in the pH range from 2.5 to 5.3, where the γ-CD derivative

octakis(2,3-di-O-methyl-6-O-sulfo)-γ-CD (ODMS-γ-CD) was the least effective chiral selector, compared to the other α-CD and β-CD derivatives (Table 5). Different chiral affinity pattern of HDMS-α-CD and HDMS-β-CD toward Ala-Phe at pH 2.5 and Ala-homoPhe at pH 3.5, respectively, were detected.

Taken together, introducing a charged group in a CD not only improves the enantioresolution ability of the CD, but can also significantly change the chiral recognition patterns. Charged CDs appear to be more suitable selectors for the enantioseparation of small peptides in the acidic pH range. Although the number of studied α-CD and γ-CD derivatives was limited, β-CD derivatives in general revealed higher effectiveness for the separation of this set of Ala-Phe analogs. The migration order of the enantiomers of the studied dipeptides can be opposite toward CDs having different cavity size, or different substituents on the CD rim. Moreover, not only the type of the substituents but also their location on the CD rim determined the enantiomer affinity pattern in case of some analytes.

4.2.3 Migration order as function of the pH

Beside the CD type and the analyte structure, an additional parameter the EMO is dependent on is the pH of the BGE, which determines the charge of the analytes. The individual migration orders of the studied peptides can be found in Table 4 and Table 5 for selected CDs. Further examples are summarized in Table 1 in manuscripts 1 and Table 1 in manuscript 2. Based on the previously published papers, reversal of the EMO upon a change of the pH values of the BGE appears to be a quite common phenomenon for various small peptides using neutral and charged CDs due to their amphoteric nature [101, 104, 106, 111-113]. In order to observe the migration behavior of the Ala-Phe analogs, different pH values have been extensively studied. For the selected dipeptides, the opposite migration order was observed for β-CD and DM-β-CD when changing the pH from 2.5 to 3.5 (see Table 1 in manuscript 1). In contrast, for charged CDs this phenomenon was less characteristic. This can be explained with the different protonation state of the peptides which is important for a separation in the presence of neutral CDs. Since the CD itself does not possess a self-mobility and migrates with the EOF, the apparent mobility of the enantiomer-CD transient complexes is dependent on the mobility of the analyte. On the contrary, in case of negatively charged CDs, which possess a relatively high anodic mobility, the separation mechanism toward the peptides is changed. In general, native α-CD, β-CD and γ-CD and their derivatives were able to resolve the enantiomers of the majority of the peptides at pH = 2.5 value and the resolution ability decreased with increasing the pH, with some exceptions. For instance, HS-β-CD which was equally effective for resolving this set of dipeptides at all studied pH values, or CM-α-CD which was able to resolve all the peptides only at pH 5.3 and its enantioresolving ability was much weaker at lower pH values. Further examples can be found in Table 1 in manuscript 1 and Table 1 in manuscript 2. At pH 9.5 only in case of β-CD opposite EMOs were detected, when comparing results to pH 2.5 (Table 4). This was due to the lower separation ability of neutral CDs at alkaline pH. On the contrary, some examples of the opposite migration orders were observed at pH 5.3 when comparing to the acidic pH values in the presence of charged CDs. For instance, the opposite enantiomer affinity pattern can be found for the peptides in the presence of HS-β-CD, HDMS-β-CD, CM-β-CD and CM-α-CD when increasing the pH value from 2.5 to 5.3, but exceptions exist (see Table 1 of manuscript 2). In

contrast, for negatively charged α-CD and γ-CD derivatives, the migration order of the enantiomer was not affected by pH. Only in case of Ala-HomoPhe the migration order was reversed in the presence of HDMS-α-CD when changing the pH from 2.5 to 3.5.

Figure 16 illustrates the separation examples of Ala-Phe in the presence of S-β-CD, where the identical EMO was obtained at all three pH values, and the separation of β-Ala-Phe in the presence of HS-β-CD, when the EMO at pH 2.5 and 3.5 were identical but different from pH 5.3. Interestingly, Ala-HomoPhe in the presence of S-β-CD changes the chiral recognition pattern toward the CD when increasing the pH from 2.5 to 3.5 and then from 3.5 to 5.3. The same type of interaction was observed for Gly-Phe and Ala-β-Phe in the presence of Suc-β-CD (see Table 1 in manuscript 2). This represents quite a special case from the viewpoint of enantiomer affinity pattern as a function of the pH of the BGE. In the presence of neutral CDs, the change of the chiral recognition pattern of Ala-homoPhe toward β-CD was observed, when switching the pH value from 2.5 to 3.5 and then from 3.5 to 9.5 (Table 4).

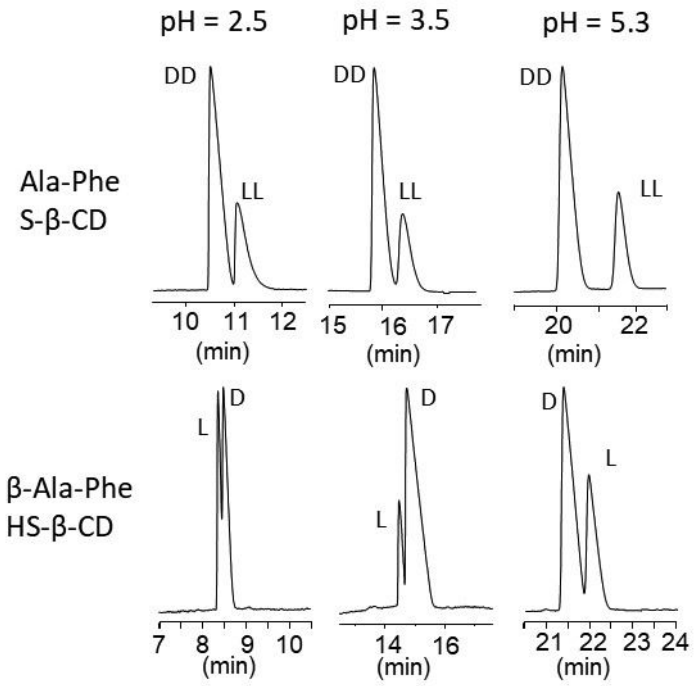


Figure 16. Dependence of the EMO at the pH values of the BGE for Ala-Phe in the presence of 5 mg/mL S-β-CD at pH 2.5, 15 mg/mL at pH 3.5 and 30 mg/mL at pH 5.3 and β-Ala-Phe in the presence of 2 mg/mL HS-β-CD at pH 2.5, 5 mg/mL at pH 3.5 and 15 mg/mL at pH 5.3 values. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium phosphate solution at pH 2.5 and 3.5 and 50 mM sodium acetate buffer at pH 5.3, capillary temperature 20 °C, separation voltage 25 kV, UV detection at 215 nm.

Taken together, enantiomers of many dipeptides showed opposite affinity to β-CD and its derivatives upon a change of a protonation state of the peptides when increasing pH from 2.5 to 3.5. α-CDs and γ-CDs were poor chiral selectors so that the phenomenon was not observed in case of these CDs. In case of charged CD, upon changing the pH from 2.5 to 5.3 opposite affinity pattern was detected in most of the cases.

4.2.4 Effect of nature and concentration of CILs as additives to the BGE

This chapter is devoted to the peptide enantioseparation as function of the CIL added to the buffer solution in combination with a primary chiral selector. The separation data is summarized in Table 1 for native β-CD and Table 2 for HP-β-CD in manuscript 3.

In the last decade increasing attention has been dedicated to the synthesis of stable CILs and their application in various separation techniques [59]. Their ability to increase the viscosity and ionic strength of the buffers results in a decrease or even a reversal of the EOF when adding them to the BGE. Due to the high molecular diversity of both ions, of which at least one is containing the chiral center, a large variety of chiral CILs can be synthesized. In the present research, amino-acid derived CILs were prepared from tetramethylammonium hydroxide (TMA-OH) or tetrabutylammonium hydroxide (TBA-OH) and the respective amino acids. CILs containing two tetraalkylammonium counterions such as $[TBA]_2$[L-Asp] and $[TMA]_2$[L-Asp] were obtained from L-Asp, while L-Asn and L-Pro-derived CILs contained a single counterion - [TBA][L-Asn], [TMA][L-Asn], [TBA][L-Pro] and [TMA][L-Pro], for the structures see Figure 10. From the peptides presented in Figure 13, Gly-D-Phe, Gly-L-Phe, and DD- and LL-enantiomers of Ala-Phe, Ala-Tyr, Phe-Phe and Lys-Phe were selected, and only β-CD and HP-β-CD were chosen as the chiral selectors. Ala-Phe, Ala-Tyr and Phe-Phe were interesting analytes according to the early observations [101], where a pH-dependent reversal of an enantiomigration order for these dipeptides was reported in the presence of β-CD. For HP-β-CD as chiral selector this phenomenon was not detected [104]. Compared to the other dipeptides studied, Lys-Phe is basic dipeptide, and it was interesting to study the behavior of this type of the analyte in the presence of the CILs. In general, an enantioresolution of the dipeptide analytes was not observed upon the use of the CILs as a single additive to the BGE. Nevertheless, in combination with the primary chiral selectors such as β-CD or HP-β-CD the CIL had a positive influence on the enantioseparation of the peptides in most of the cases (see Table 1 and Table 2 in manuscript 3). As described in Figure 12 f, CILs in the bulk solution dissociate in IL^+ cation and the A^- anions. Positively charged ions interact with the capillary wall, modifying it and as a result reducing the EOF [136-138], while anions may interact with the positively charged analytes through electrostatic interactions, causing a deceleration of the analyte enantiomers. As a result, higher resolution values are achieved. Molecular modeling studies revealed that in the CILs complexes with higher stability are formed [58]. Moreover, NMR studies on analyte/CD/CIL systems indicated the formation of 1:1:1 complex [72].

The superiority of the synergistic systems is demonstrated in Figure 17, where in the presence of 20 mM HP-β-CD the addition of 30 mM [TBA-][L-Pro], [TBA][L-Asn] and $[TBA]_2$[L-Asp] CILs resulted in an

improvement of the enantioseparations. For instance, from a partial separation of Gly-Phe in the presence of HP-β-CD alone, a baseline separation is achieved when introducing the CILs in the BGE with R_S values between 2.3 and 3.1. The same improvement was observed in the case Ala-Phe, which could not be separated by HP-β-CD, but a partial separation was obtained upon addition of the CILs. A baseline separation was observed when analysing Ala-Tyr under these conditions. Furthermore, with the addition of the CILs migration times of the respective dipeptide enantiomers increased. This can be attributed to an increased viscosity of the BGE and/or the reduced EOF as a result of the adsorption of the TMA or TBA cations on the capillary wall [68, 69].

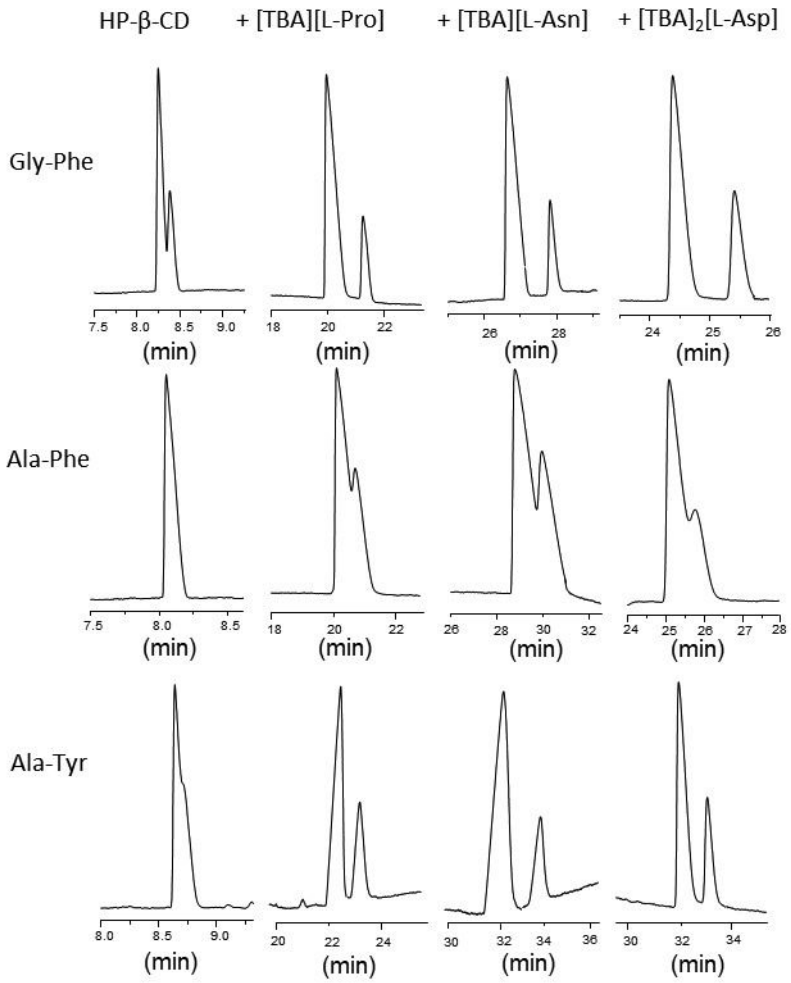


Figure 17. Electropherograms showing synergism effect after the addition of the 30 mM respective CILs with combination of 20 mM HP-β-CD on the enantioseparation of Gly-Phe, Ala-Phe and Ala-Tyr. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium phosphate buffer pH 2.5, capillary temperature 20 °C, separation voltage 25 kV, UV detection at 215 nm.

Not only the concentration of the CILs affected enantioseparations positively, but also the type of the cation and the anion of the CIL. Moreover, the stereochemistry proved to be an important parameter. Earlier investigations on the effect of the substituent groups in the CIL cation on a chiral separation indicated, that a long alkyl chain resulted in a higher resolution, which may be due a stronger

hydrophobic ability to form a more-stable layer inside the capillary [58]. The same applies for the studied tetraalkylammonium cations. The TBA-based chiral ionic liquids resulted in higher R_S values compared to the TMA-based CILs with some exceptions (see Table 1 and Table 2 in manuscript 3). Comparing the amino acid anions, L-Asp composed CILs showed a higher enantioresolution ability than L-Asn- or L-Pro-based CILs. Since the L-Asp-based CILs, such as $[TBA]_2$[L-Asp] and $[TMA]_2$[L-Asp] are containing two cations, a modification of the capillary wall was much stronger compared to the CILs were only one tetraalkylammonium cation is present, like for [TBA][L-Asn] or [TMA][L-Asn]. From this point of view, it was interesting to study the individual contribution of the components of the studied CILs to the separation process. Accordingly, the CILs $[TBA]_2$[L-Asp], $[TMA]_2$[L-Asp], [TBA][L-Asn] and [TMA][L-Asn] were chosen combination with the native β-CD and enantioseparations of Ala-Phe, Ala-Tyr and Phe-Phe were compared to the separations when using TBA-Cl or TMA-Cl as well as L-Asp or L-Asn individually or mixtures of the alkylammonium chlorides and the amino acids in combination with β-CD. Concentrations of the individual components were selected in such a way that they matched the concentration of 15 mM of the respective CIL, while the concentration of the β-CD was maintained 20 mM in all cases. Additionally, D-Asp- or D-Asn-based CILs were synthesized and included in this study, to evaluate the effect of the stereochemistry of the CIL. The enantioresolution expressed as R_S values at pH 2.5 are summarized in Figure 18.

Addition of 20 mM of the amino acid or 15 to 30 mM of TMA-Cl or TBA-Cl had only a minor effect on the separation compared to β-CD alone (Figure 18 columns a, b left and right). The resolution was improved when adding 15 mM of the CILs or the corresponding mixture of the individual components. This synergistic effect is in agreement with previously published papers [137, 139] were amino acid-based CILs were used to demonstrate the effectiveness of the CILs. In case of the L-Asn-based CILs [TBA][L-Asn] or [TMA][L-Asn], higher R_S values were observed upon addition of the CIL compared to equimolar concentrations of the individual components at pH 2.5 (Figure 18 columns c, d right). In contrast, in the case of L-Asp containing CILs, the resolution values were higher when the individual components were added to the BGE compared to the results obtained in the presence of the CILs (Figure 18 columns c, d left). This indicates that amino acid based ILs containing one or two counterions may dissociate at pH 2.5 in different ways, resulting in different composition of ions in the BGEs, which also affects the separation.

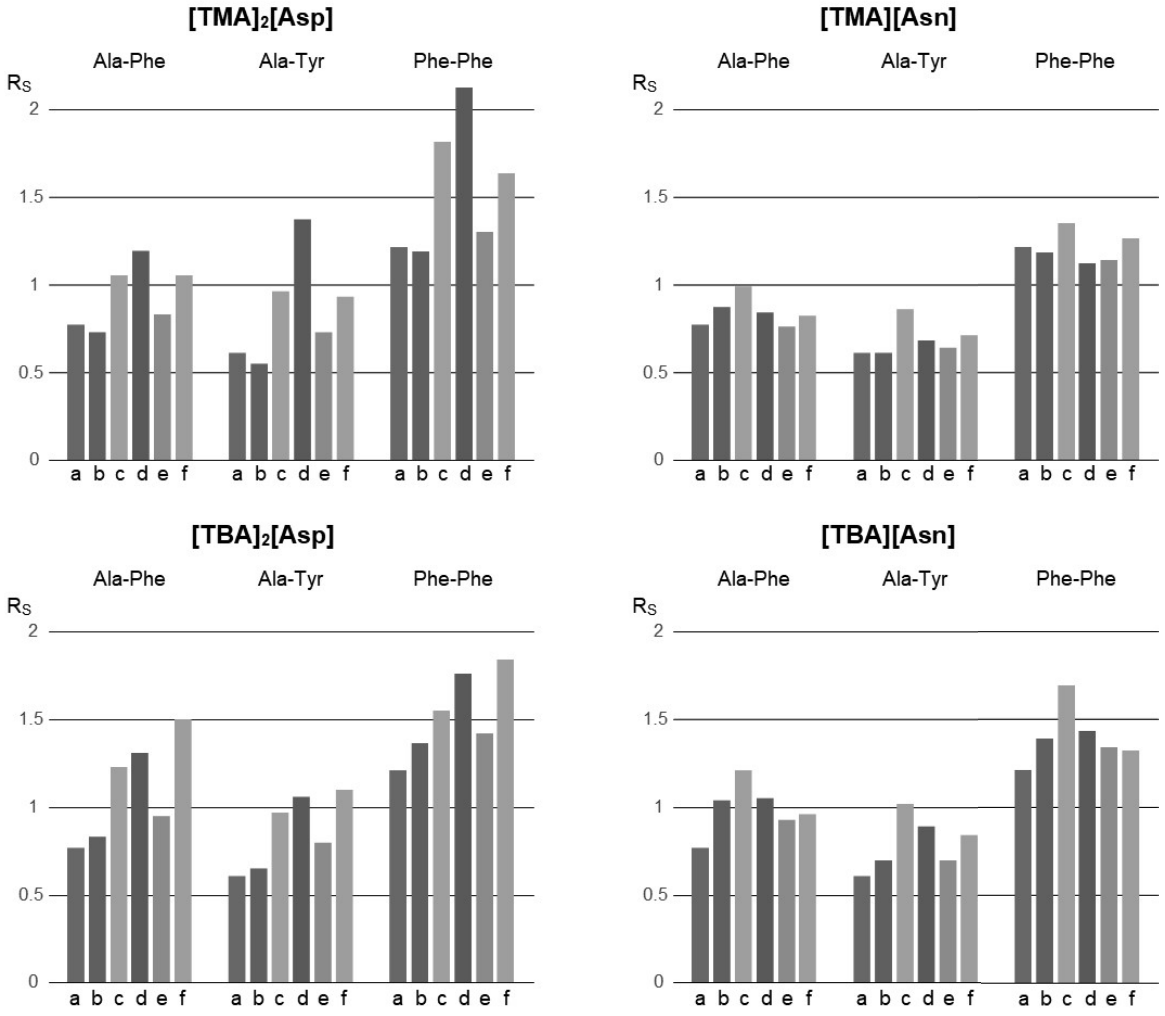


Figure 18. Comparison of the effect of 15 mM CILs or equimolar concentrations of the individual components on the β-CD mediated enantioseparation (expressed as R_S values) of Ala-Phe, Ala-Tyr and Phe-Phe at pH 2.5. In case of addition of the individual components, the concentration of TMA-Cl or TBA-Cl was 15 mM for Asn-derived CILs and 30 mM for Asp-derived CILs. (a) 20 mM β-CD, (b) 20 mM β-CD + 15/30 mM TMA-Cl/TBA-Cl, (c) 20 mM β-CD + 15 mM L-amino acid CIL, (d) 20 mM β-CD + 15 mM L-amino acid + 15/30 mM TMA-Cl/TBA-Cl, (e) 20 mM β-CD + D-amino acid CIL, (f) 20 mM β-CD + 15 mM D-amino acid + 15/30 mM TMA-Cl/TBA-Cl.

The studies conducted with D-amino acid-based CILs revealed no clear conclusions of the effect of the stereochemistry of the CILs on peptide enantioseparation. Zhang and co-workers have observed a superior effect of L-Arg-based CILs over D-Arg-derived CILs on HP-β-CD-mediated enantioseparations of basic drugs [137, 139]. This difference can be explained with the fact, that the CILs and analytes studied in the present research had a similar nature, i.e., both are amino acids or are composed of amino acids, while Zhang and co-workers studied amino acid-based CILs for the separation of basic drugs. The CILs containing either D-Asn or D-Asp were evaluated at pH 2.5 and 3.5 (Figure 18 e and f columns, left and right represents examples at pH 2.5). The tendencies described in case of the L-amino

acid-based CILs above did not change in case of D-amino acid containing CILs. The deterioration of an enantioseparation was observed when substituting L-amino acid CILs by D-amino acid CILs, with some exceptions. For instance, the highest resolution values were detected when analysing the peptides in the presence of a mixture of the individual components 30 mM TBA-Cl + 15 mM D-Asp at pH 2.5 (Figure 18 f columns, below, left), emphasizing the fact that stereochemistry of the buffer additives is crucial on a particular enantioseparation.

The pH value of the BGE is another important parameter for CIL-mediated chiral separations. Only two pH values were evaluated, i.e., 2.5 and 3.5, based on earlier studies [101, 104]. Overall, upon addition of an amino acid-based CILs as chiral additives to the BGE, the pH-dependent reversal of the enantiomer migration order was not affected. However, the effect of the pH on the separations was not conclusive. While in case of some dipeptides higher resolution values were observed at pH 2.5 with increasing CILs concentrations, in case of other dipeptides the highest R_S values were observed at pH 3.5 (see Table 1 in manuscript 3). In contrast, when the dual system consisted of HP-β-CD as chiral selector and a CIL, the synergistic effect was much stronger at pH 2.5 for most of the dipeptides than at pH 3.5 with a few exceptions (see Table 2 in manuscript 3).

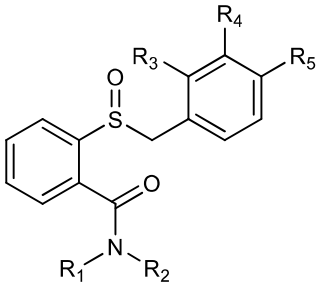
Thus, not all effects observed upon addition of a CIL could be understood partly because of the limited number of the CILs studied. Nonetheless an improvement of the enantioresolution upon addition of the CILs as BGE additives was noted. The migration order of the dipeptides was not affected by the CIL. Upon comparing the effect of the addition of the CILs versus the addition of the individual components in case of Asp and Asn-derived CILs, a general picture was hard to conclude. In some cases, the individual components yielded higher resolutions, while for other experimental conditions the CILs proved to be superior. Studies on D-Asp- and D-Asn-based CILs revealed an importance of a configuration of the buffer additives on a particular enantioseparation but clear trends could not be concluded.

4.3 Chiral separation of sulfoxides

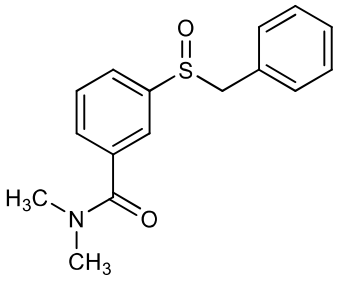
Sulfoxides are unique compounds with a sulfur atom as a chiral center. HPLC separations of sulfoxides are more frequent compared to CE, where only two papers have been published [126, 127] as discussed in chapter 1.5.2. A series of (benzylsulfinyl)benzamides and derivatives was systematically analyzed in polar organic, polar-aqueous-organic and normal phase HPLC on several polysaccharide-based chiral columns by Chankvetadze and co-workers [119-122]. In particular, a Cellulose-5 column provided the highest separation factor for 2-(benzylsulfinyl)benzamide with the impressive number α = 112 [119]. From this point of view, we were interested in examining the CDs as chiral selectors for the separation of 2-(benzylsulfinyl)benzamide and analogs in CE.

Table 6 illustrates the 16 (benzylsulfinyl)benzamides analyzed in this part of the research. As discussed for peptides, the migration order of the sulfoxides was studied with respect to structure of the analytes and the CD type. The pH was not investigated because the sulfoxides are neutral compounds. The migration orders of the individual compounds are summarized in Table 2 and Table 3 in manuscript 4.

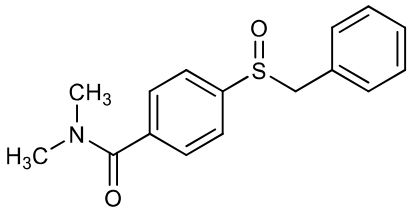
Table 6. Structures of sulfoxides



1-3, 6-16



4[a)]



5[b)]

#	IUPAC name	R_1	R_2	R_3	R_4	R_5
1	2-(benzylsulfinyl)-benzamide	H	H	H	H	H
2	2-(benzylsulfinyl)-*N*-methyl benzamide	CH_3	H	H	H	H
3	2-(benzylsulfinyl)-*N*,*N*-dimethyl benzamide	CH_3	CH_3	H	H	H
6	2-(3-bromobenzylsulfinyl)-benzamide	H	H	H	Br	H
7	2-(4-bromobenzylsulfinyl)-benzamide	H	H	H	H	Br
8	2-(4-methylbenzylsulfinyl)-benzamide	H	H	H	H	CH_3
9	2-(4-tertbutylbenzylsulfinyl)-benzamide	H	H	H	H	$C(CH_3)_3$
10	2-(3-bromobenzylsulfinyl)-*N*-methylbenzamide	CH_3	H	H	Br	H
11	2-(4-bromobenzylsulfinyl)-*N*-methylbenzamide	CH_3	H	H	H	Br
12	2-(3-methoxybenzylsulfinyl)-*N*-methyl benzamide	CH_3	H	H	OCH_3	H
13	2-(3-methylbenzylsulfinyl)-*N*-methyl benzamide	CH_3	H	H	CH_3	H
14	2-(2-methylbenzylsulfinyl)-*N*,*N*-dimethyl benzamide	CH_3	CH_3	CH_3	H	H
15	2-(3-methylbenzylsulfinyl)-*N*,*N*-dimethyl benzamide	CH_3	CH_3	H	CH_3	H
16	2-(4-methylbenzylsulfinyl)-*N*,*N*-dimethyl benzamide	CH_3	CH_3	H	H	CH_3

[a)] IUPAC name: 3-(benzylsulfinyl)-*N*,*N*-dimethyl benzamide

[b)] IUPAC name: 4-(benzylsulfinyl)-*N*,*N*-dimethyl benzamide

The chiral (benzylsulfinyl) benzamides were synthesized as described in [140]. (See page 126 in supplementary material of manuscript 4). Stereoselective HPLC was used to collect small quantities of the enantiomers of the sulfoxides for peak identification. Fractioning was carried out on an analytical cellulose tris(3,5-dichlorophenlycarbamate) column using methanol as eluent [121]. The fractions of the enantiomer peaks were collected and pooled and the optical rotation of the purified enantiomers were determined (data not shown). For all sulfoxides, the (+)-enantiomer eluted before the (**–**)-enantiomer. Subsequent CE experiments were performed with spiked samples to enable the determination of the EMO.

In order to identify suitable separation conditions for the sulfoxides, starting from pH 2.5, initial separation of compounds **1**, **2**, **6** and **8** were carried out in the presence of CM-β-CD and CM-γ-CD. The sulfoxides were not detectable at this pH either at the cathode or at the anode, when reversing the polarity of the applied voltage. Using 50 mM phosphate and acetate buffers, series of experiments were conducted in a pH range between 3.5 - 7.0 and finally the best separation conditions were selected. Acetate buffer, pH 5.5, containing 10% methanol as organic modifier was used for further studies in the presence of 19 charged CDs. The addition of the organic modifier was necessary to observe narrow peaks and to increase the solubility of the non-polar analytes in the BGE. Additionally, some experiments in the MEKC mode in the presence of neutral CDs in combination with 100 mM sodium dodecyl sulfate (SDS) as surfactant were carried out at pH 9.0 in sodium borate buffer also containing 10 % methanol.

4.3.1 Effect of structure of the sulfoxides

The (benzylsulfinyl)benzamides summarized in Table 6 differ in the position of the amide substituent and *N*-methylation, as well as the type and position of the substituent in the benzylsulfinyl moiety, which comprises a halogen atom, or a methyl group, a methoxy group or as the largest substituent a tert-butyl group. With regard to the migration order, the (+)-enantiomer migrated before the (**–**)-enantiomer in the presence of most CDs. Thus, structural modifications had only a minor effect although there were some exceptions. For instance, the type of the substituent in the benzyl ring affected the migration order when octakis(6-deoxy-6-amino)-γ-CD (OA-γ-CD) was used as chiral selector. Thus, the migration order of compound **7** featuring a bromine atom in a para position of the benzyl moiety was opposite to the EMO of compound **8** with the methyl group in the para position (see Table 3 in manuscript 4). Additionally, R_S values of compound **7** was 2 times higher than compound **8**, which is with agreement to the observation from Rodriguez and co-workers [126], where the size of the substituent and its electronegativity affected the resolution of sulfoxide analytes. Derivatives of 2-(benzylsulfinyl)benzamide with larger substituents in the benzylsulfinyl moiety showed higher resolution compared to compounds with smaller substituents in the benzyl moiety. For instance, compound **9** with the tert-butyl substituent in the para position displayed higher resolution in the presence of OA-γ-CD than compound **8** with a methyl group in para position (see Table 3 in manuscript 4). Not only the type of the substituent, but also its position in the benzyl ring was crucial in case of compounds **10** and **11**, which feature a bromine substituent in position 3 (compound **10**) or position 4 (compound **11**), respectively. The opposite migration order of the respective sulfoxides was detected in the presence of OA-γ-CD (see Table 3

manuscript 4). Moreover, Figure 19 illustrates the electropherograms of the enantioseparations of compound **10** and **11** in the presence of HDMS-α-CD. Upon changing the position of the bromine atom from meta (compound **10**) to para (compound **11**), the migration order of the respective analytes changed from (-) > (+) in case of compound **10** to (+) > (-) for compound **11**. The same scenario applied for the pairs of compounds **14** and **15,** which bear the methyl group in ortho and meta positions, respectively. This change in the analyte structure resulted in the opposite EMO when analysing these sulfoxides in the presence of HDMS-α-CD (see Table 3 in manuscript 4).

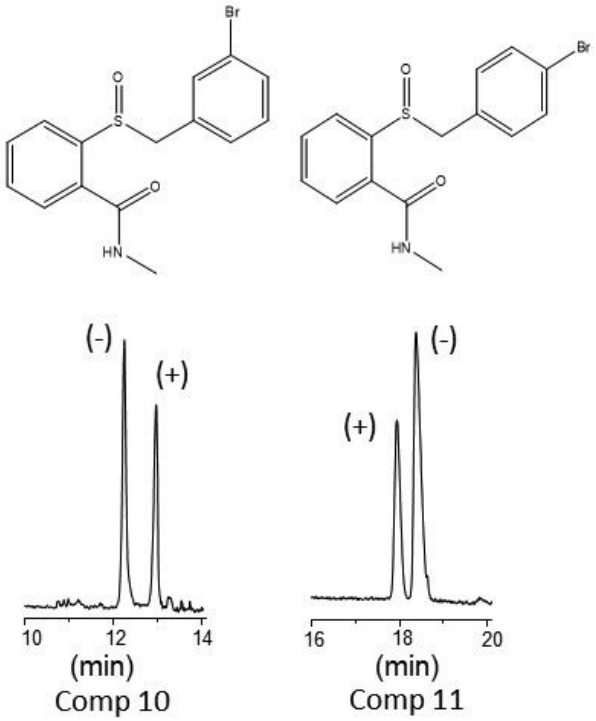


Figure 19. Enantioseparations of compound **10** (2-(3-bromobenzylsulfinly)-N-methyl benzamide) and compound **11** (2-(4-bromobenzylsulfinly)-N-methyl benzamide) in the presence of 40 mg/mL HDAS-β-CD. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium acetate buffer pH 5.5, capillary temperature 20 °C, separation voltage 25 kV, UV detection at 220 nm.

Generally, *N*-methylation of the amide groups had only a minor effect on the migration order of enantiomers of the studied 2-(benzylsulfinyl) benzamides. The opposite migration order was detected only in a few cases. For instance, compound **7** as a primary amide revealed opposite EMO compared to the *N*-methyl amide compound **11** in the presence of HDMS-β-CD (see Table 3 in manuscript 4).

Overall, the position of the benzamide group (compounds **1**, **4**, **5**), its *N*-methylation, as well as different substituents in the benzylsulfinyl moiety and their position played an important role for the affinity patterns of the studied CDs toward the sulfoxides. Nonetheless, clear structure-separation relationships could not be deduced. This may be due to the fact, that the modification on the (benzylsulfinyl)benzamide core structure did not include all possible scenarios in the present set of analytes.

4.3.2 Effect of CD structure and substitution pattern

Sulfoxides are neutral compounds and move with the bulk flow when no charged chiral selector is added to the BGE. Accordingly, scenarios c and d, illustrated in Figure 12, apply for the analyses of the sulfoxides in the presence of negatively and positively charged CDs, respectively.

Based on the CE measurements, reversal of the migration order of enantiomers of the sulfoxides was found to be a function of the cavity size and the substitution pattern as also discussed for the peptides in chapter 4.2.2. Among the randomly substituted CDs, the highest enantioresolution ability could be attributed to sulfated, carboxymethylated and succinylated γ-CDs as well as Suc-β-CD. These CDs enantioresolved all analytes. An advantage of CM-β-CD (14 separated analyte) over S-β-CD was obvious (12 separated analyte). Moreover, R_S values in the presence of CM-β-CD were much higher in most cases. This is in contrast to the results obtained in reference [126], where S-β-CD showed higher enantioresolving ability toward chiral sulfoxides and sulfinate esters compared to CM-β-CD. However, it should be mentioned that the separation conditions in reference [126] and our studies were different, and that also the structures of the analyzed sulfoxides differed between the studies.

The enantioresolving ability of the CDs was dependent on the cavity size, the individual migration orders are summarized in Table 2 and Table 3 in manuscript 4. In contrast to the peptides, where β-CD and its derivatives revealed the best separation results, for sulfoxides γ-CD derivatives showed the highest resolution abilities for the analytes, followed by β-CD derivatives while the α-CD derivatives resolved the smallest number of the compounds (see Table 2 in manuscript 4). This can be explained with the fact, that the large cavity of the γ-CD derivatives was more favorable for the sulfoxides to provide a better fit for forming the inclusion complexes. For example, in case of CM-α-CD the enantiomers of only 5 sulfoxides out of 16 were separated, while 14 were separated in the presence of CM-β-CD and all of them were resolved when CM-γ-CD was used as a chiral selector. Furthermore, the observed EMOs of the sulfoxides was a function of the cavity size in particular cases. For instance, for compound **1**, **7** and **11** the migration order in the presence of CM-α-CD were opposite to the one in case of CM-β-CD and it changed again when analysing compound **7** with CM-γ-CD, while it was the same for compounds **1** and **11** as with CM-β-CD. For sulfated and succinylated CDs the EMO was not dependent on a cavity size of the respective CDs, it was (+) > (–) in all cases. The same scenario applied for positively charged CDs, with exceptions of compounds **1** and **14**, of which respective (–)-enantiomer eluted before the (+)-isomer in the presence of (2-hydroxy-3-N,N,N-trimethylamino)propyl-α-CD (TMA-α-CD) (see Table 2 in manuscript 4).

A different behavior was observed for single isomer CDs as chiral additives to the BGE. HS-β-CD proved to be the most effective chiral selector among the studied single isomer CDs, as it could resolve 15 (benzylsulfinyl)benzamides out of 16. Examples of enantioseparations of the sulfoxides by single isomer CDs are shown in Figure 20. When introducing acetyl groups in the wider secondary rim of HS-β-CD resulting in HDAS-β-CD, the enantiomigration order was opposite compared to HS-β-CD (Figure 20 first and second column). In the presence of HDMS-β-CD, where instead of the acetyl groups methyl groups are present at C2 and C3 positions, the migration order was opposite compared to HDAS-β-CD but identical to HS-β-CD. In general, the enantiomer resolving ability of single isomer CDs was smaller

compared to the HS-β-CD. Accordingly, derivatization of the wider secondary rim of the glucose moiety had a negative effect on the separation of the chiral (benzylsulfinyl)benzamides.

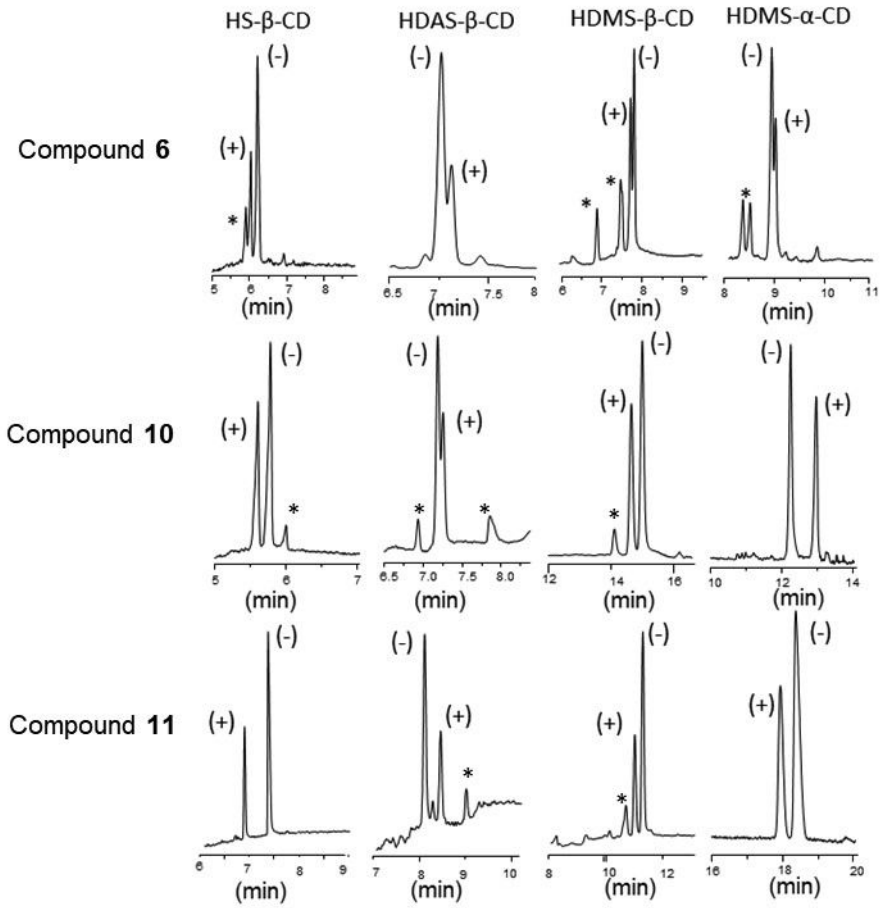


Figure 20. Enantioseparation of Compound **6**, compound **10** and compound **11** in the presence of 10 mg/mL HS-β-CD, 40 mg/mL HDAS-β-CD, HDMS-β-CD and HDMS-α-CD. Experimental conditions: 40/50.2 cm, 50 μm ID fused-silica capillary, 50 mM sodium acetate buffer pH 5.5, capillary temperature 20 °C, separation voltage 25 kV, UV detection at 220 nm. * Synthetic impurities.

In contrast to randomly substituted CDs, in case of single isomer CDs the increase of the cavity size had a drastic effect on an enantioresolving power for the analytes. The separation ability of HDMS-α-CD, HDMS-β-CD and ODMS-γ-CD decreased with increasing the cavity size. ODMS-γ-CD could resolve only three analytes out of 16, whereas the separated number of sulfoxides reached 14 in the presence of HDMS-α-CD. The same tendency was revealed when using positively charged single isomer CDs as a chiral selector. Heptakis(6-deoxy-6-amino)-β-CD (HA-β-CD) could resolve the enantiomers of 13 out of 16 sulfoxides, whereas OA-γ-CD resolved only 9. Not only the enantioseparation ability was affected by the cavity size, but also the EMO. Figure 20 represents the different EMOs of sulfoxide **6** and **10**. The migration order (+) > (-) in the presence of HDMS-β-CD changed to (-) > (+) when switching to HDMS-α-CD with smaller cavity size. In the case of compound **11**, the EMO remained the same for both CDs.

Neutral CDs were investigated for their ability to resolve the enantiomers of the sulfoxides. Because the sulfoxides are uncharged, MEKC conditions were applied using 100 mM SDS as surfactant. As in the case of charged CDs 10% methanol was added to the BGEs. The screening results are summarized in Table S1 in manuscript 4 (See page 129). 9 neutral CDs were selected, native β and γ-CD, as well as their hydroxypropyl and methyl derivatives. Native α-CD could not be investigated because a precipitate was formed when adding SDS to the BGE. Interestingly, the enantiomers of only 3 out of the 16 sulfoxides were separated by β-CD. On the other hand, in the presence of γ-CD 6 sulfoxides were enantioresolved with elution order (-) > (+), which was opposite to the EMO observed in the presence of β-CD. The best enantioresolution ability of the studied neutral CDs revealed HP-γ-CD. Cavity size of the CDs also affected the separations in the MEKC mode. The CDs with larger cavity size, separated more sulfoxides. Additionally, negatively charged CDs were also tested in the MEKC mode but their separation ability was much weaker compared to the absence of SDS in the BGE (EKC conditions). For example, CM-β-CD resolved the enantiomers of only 2 sulfoxides under MEKC conditions, while 14 were resolved under EKC conditions. Moreover, the enantioresolution ability of CM-γ-CD was deteriorated when switching from EKC to MEKC conditions (see Table 2 and Table S1 in manuscript 4). The elution order of the sulfoxides observed in EKC was opposite to the EMO obtained in MEKC when using CM-γ-CD as chiral selector. The data are not surprising, because different mechanisms apply under both modes. Distribution of the analytes and/or the analyte-CD complexes between the micellar and the aqueous phase in MEKC may affect the observed enantioseparations compared to the EKC mode.

Taken together, charged CDs revealed a superior effect over neutral CDs with regard to enantioseparations of the chiral (benzylsulfinyl)benzamides in CE. The degree of substitution played an important role in the enantioresolving ability of the studied chiral selectors, as the randomly substituted charged CDs were by far more efficient compared to the single isomer CDs. An exception was HS-β-CD, which could resolve the enantiomers of more analytes compared to its randomly substituted counterpart S-β-CD. Cavity size of the CDs appeared to be also an important parameter, especially for randomly substituted CDs, where the large cavity of the γ-CD and its derivatives provided a better fit for the sulfoxides compared to β-CDs and α-CDs. In contrast, for single isomers CDs, the lowest enantioresolving ability was observed for ODMS-γ-CD, whereas HDMS-α-CD could separate the enantiomers of a larger number of the analytes.

4.4 Mechanistic studies

CE can also be used as a convenient technique for studies of molecular recognition phenomena in non-covalent intermolecular interactions. The high peak efficiency in CE allows the observation of enantioselective effects in selector-selectand interactions that are not detectable by other techniques [141]. As discussed in the introduction, two main scenarios can correspond to different EMOs when switching the separation conditions. According to the mobility model proposed by Wren and Rowe [45], an enantioseparation is observed in the case of differences in the chiral recognition of the selector toward the enantiomers (expressed as complexation constants) or when the diastereomeric complexes differ in

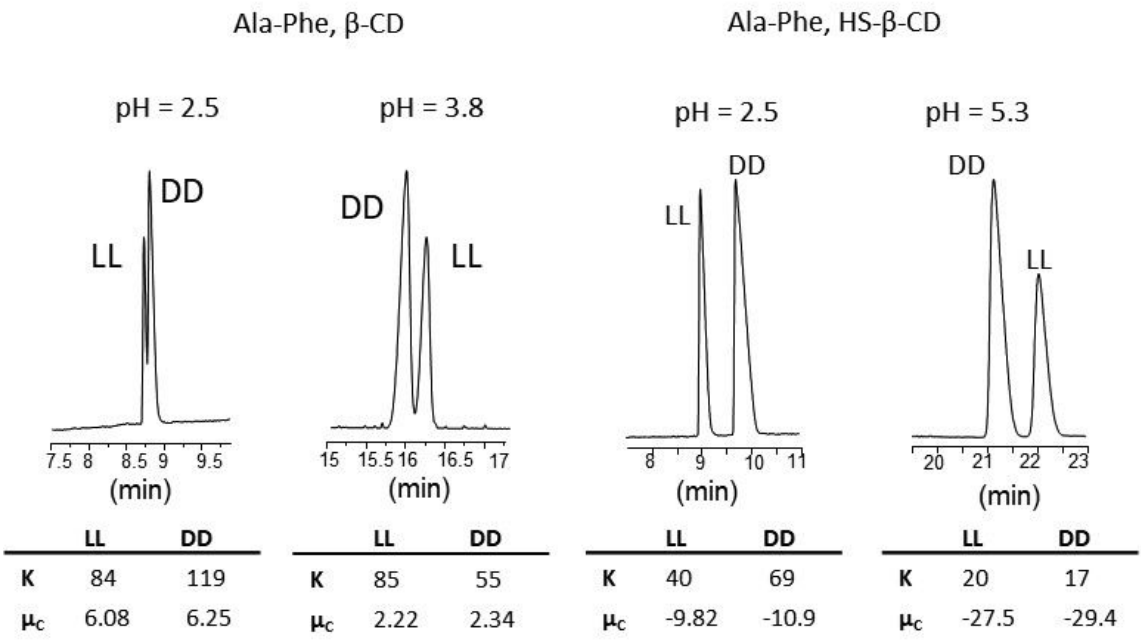
their electrophoretic mobility (see page 12). Thus, opposite EMO is observed when either the affinity of the chiral selectors toward the analyte enantiomers are different [113, 142, 143], or when the respective enantiomer-CD complexes differ in their mobilities [48, 144].

In order to rationalize the mechanism behind the reversal of the EMO of Ala-Phe analogs depending on protonation state of the peptides or the CD type and in case of the sulfoxides the opposite EMO as a function of the CD concentration and CD cavity size, the complexation constants and the apparent mobilities of the transient diastereomeric CD-analyte complexes were determined for selected dipeptides and CDs. The data were obtained as best fit parameters of the dependence of the effective mobility of the enantiomers on the CD concentration assuming the formation of 1:1 CD-analyte complexes according to equation 4 (see chapter 1.3). It must be noted that these data are apparent complexation constants and mobilities, rather than thermodynamic values, because the ionic strength of the BGEs was not determined and the concentrations refer to the volume and not to the mass of the solvent. In case of randomly substituted CDs, the constants are averaged over the degree of substitution of the CDs.

Based on the mechanistic studies carried out on the selected analytes, the data regarding the enantioseparations of dipeptides with neutral CDs at pH 2.5 and 3.5 can be found in Table 2 in manuscript 1. The results observed for dipeptides and charged CDs at pH 2.5 are summarized in Table 2 in manuscript 2 and Table 3 in manuscript 2 for pH 5.3. The data regarding the enantioseparations of sulfoxides and charged CDs are shown in Table 4 in manuscript 4. The selected examples will be classified according three main scenarios. In the first part the differences in chiral recognition of enantiomers will be discussed. The second scenario will be addressed to the enantioseparation based on the complex mobility difference of diastereomeric associates. And the final third scenario will refer to the reversal of the EMO as a function of CD concentration.

4.4.1. Chiral affinity-dependent enantiomer migration order

The first mechanism of a reversal of the EMO is based on opposite chiral recognition of the analyte enantiomers by the CD. Figure 21 illustrates the separation of Ala-Phe in the presence of β-CD and HS-β-CD at different pH values. The complex formation constants as well as the mobilities of transient diastereomeric complexes are shown below each electropherogram.



	LL	DD
K	84	119
μ_c	6.08	6.25

	LL	DD
K	85	55
μ_c	2.22	2.34

	LL	DD
K	40	69
μ_c	-9.82	-10.9

	LL	DD
K	20	17
μ_c	-27.5	-29.4

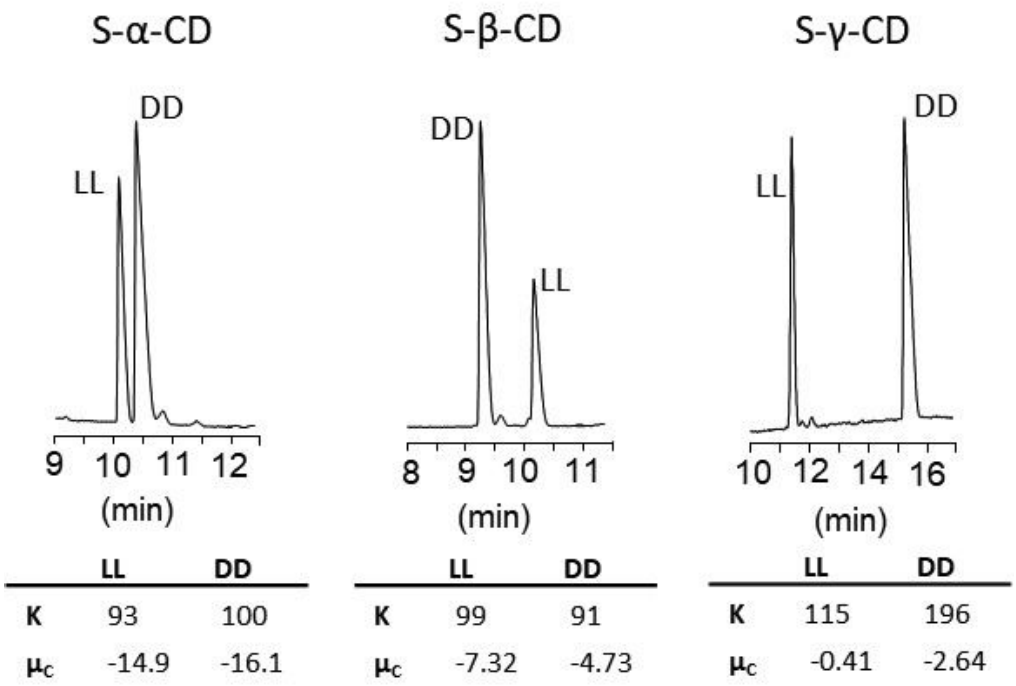
Figure 21. Enantioseparation of Ala-Phe in the presence of 20 mM β-CD as chiral selector at pH 2.5 and 3.8 and in the presence of 2 mg/mL HS-β-CD as a chiral selector at pH 2.5 and 15 mg/mL HS-β-CD at pH 5.3. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium phosphate buffer at pH 2.5 and 3.8, 50 mM sodium acetate buffer at pH 5.3, capillary temperature 20 °C, separation voltage 25 kV, UV detection at 215 nm. Corresponding apparent complexation constants (K; M^{-1}) and analyte-CD complex mobilities (μ_C; $10^{-9}m^2V^{-1}s^{-1}$) are presented in the tables below each electropherogram.

At pH 2.5, the DD-enantiomer of Ala-Phe displayed the higher complexation constant compared to the LL-enantiomer. Accordingly, the observed migration order LL > DD can be explained by the stronger complexation of the DD-enantiomer by β-CD so that the migration velocity of this enantiomer is slowed down. The same was observed for other peptides under these conditions as summarized in Table 2 in manuscript 1. This is also in accordance to previously published data [145], where the DD-enantiomers of Ala-Phe, Ala-Tyr and Asp-PheOMe revealed higher complexation constants than the LL-isomers at pH 2.5 and migrated first. Upon increasing the pH from 2.5 to pH 3.8 the migration order change of Ala-Phe was caused by the chiral affinity pattern change of β-CD toward peptides. At pH 3.8, the LL-enantiomer was complexed stronger to the CD than D-Ala-D-Phe, and, accordingly, it eluted as the second peak. Thus, the reversal of the EMO between pH 2.5 and 3.5 can be explained by opposite chiral recognition of the enantiomers by β-CD at these two pH values. This may be attributed to the different protonation state of the peptides at the studied pH values. As discussed in chapter 4.2, peptides are positively charged at pH 2.5 and become increasingly uncharged when the pH of the BGE is increased. The reversal of the EMO upon a change of the protonation state appears to be a quite a common phenomenon for peptide analytes as it has been observed in several cases [101, 106, 108, 109, 146, 147]. Li and Waldron explained this type of behavior of the peptides by the stronger association between dipeptide stereoisomers and CDs at lower pH values when the protonated carboxylic acid is more hydrophobic than the carboxylate anion. This may favor the complexation, compared to the higher

pH values, where the peptide charge density is much smaller due to increasing deprotonation of the carboxylic acid group [142].

The reversal of the EMO of Ala-Phe upon increasing the pH value from 2.5 to 5.3 in the presence of HS-β-CD is presented in Figure 21 (right). At pH 5.3, the zwitterionic peptide does not have a net charge and migrates as an uncharged compound with the EOF. Accordingly, they have the opposite mobility to the negatively charged CDs, which migrates toward the anode. Thus, as described for pH 2.5 the stronger complexed enantiomer should migrate as a second peak. Subsequently, upon increasing the pH from 2.5 to 5.3, the weaker bound LL-enantiomer of Ala-Phe at pH 2.5 was stronger bound to the CD when the pH was 5.3 resulting in opposite migration order of the enantiomers. The same applies for β-Ala-Phe in the presence of HS-β-CD when increasing the pH value from 2.5 to pH 5.3 (see Table 2 and Table 3 in manuscript 2). The smaller complexation constants obtained at pH 5.3 compared to pH 2.5 may be explained by a lower impact of electrostatic interactions between the negatively charged CDs and the neutral analyte. This has also been reported in [108, 112, 146], when analysing small peptides in the presence of S-β-CD, HS-β-CD as well as CM-β-CD.

As stated above, the chiral recognition of the peptide stereoisomers by CDs was dependent on the size of the cavity. An example is illustrated in Figure 22 for Ala-Phg at pH 2.5 when analyzing it in the presence of S-α-CD, S-β-CD and S-γ-CD with the complexation and mobility data shown below the electropherograms. The DD-enantiomer is complexed stronger by S-α-CD and S-γ-CD, while it is the weaker bound stereoisomer in case of S-β-CD. This results in the opposite EMO in the presence of S-β-CD compared to the other two CDs. In this example, the complexes of the stronger bound enantiomers also possessed the higher anodic mobility resulting in large separation factors in case of S-β-CD and S-γ-CD. Further examples include Ala-Phe and Ala-β-Phe in the presence of S-α-CD, S-β-CD and S-γ-CD. In both cases the same scenario applies as discussed for Ala-Phg above, when the DD-enantiomers of the peptides are complexed stronger to S-α-CD and S-γ-CD but are weaker bound to S-β-CD. As a result, the opposite migration order of the enantiomers was observed (see Table 2 in manuscript 2).



	LL	DD
K	93	100
μ_C	-14.9	-16.1

	LL	DD
K	99	91
μ_C	-7.32	-4.73

	LL	DD
K	115	196
μ_C	-0.41	-2.64

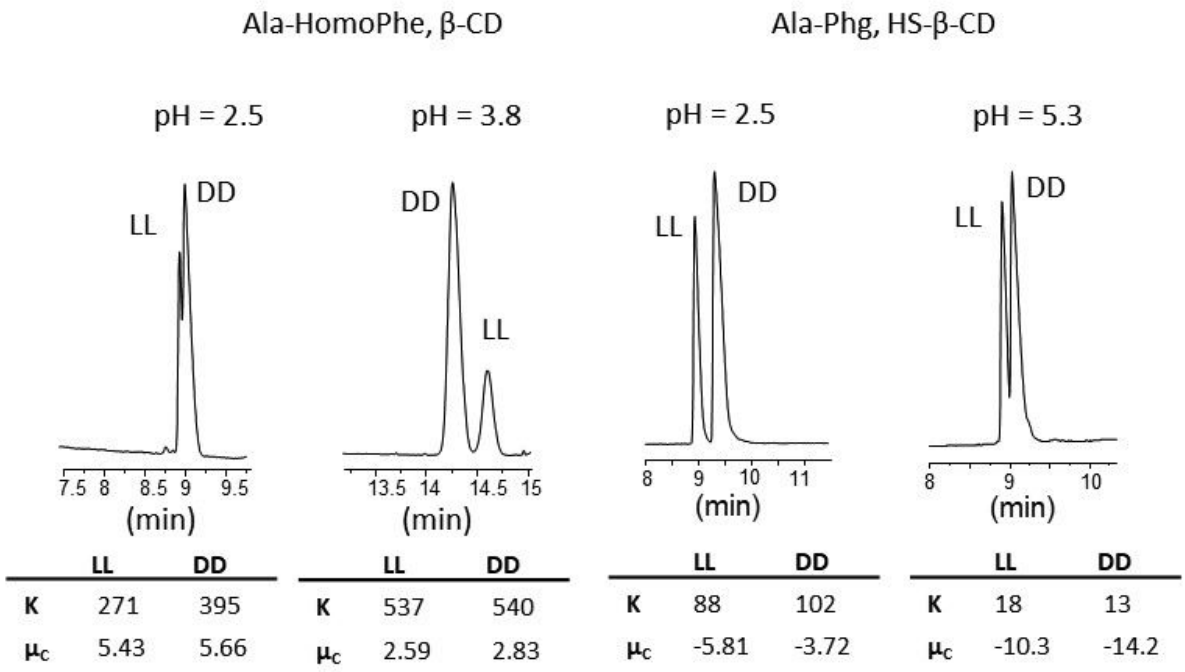
Figure 22. Enantioseparation of Ala-Phg in the presence of 5 mg/mL of S-α-CD and S-β-CD and 10 mg/mL S-γ-CD as chiral selectors at pH 2.5. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium phosphate buffer, capillary temperature 20 °C, separation voltage 25 kV, UV detection at 215 nm. Corresponding apparent complexation constants (K; M^{-1}) and analyte-CD complex mobilities (μ_C; $10^{-9}m^2V^{-1}s^{-1}$) are presented in the tables below each electropherogram.

The dependence of the EMO and, consequently, the affinity pattern of enantioresolution as a function of the size of the CD cavity was also noted in case of the sulfoxides, e.g., compound **11**. In this case, the EMO could also be rationalized based on the complexation data. In the presence of HDMS-α-CD, the stronger complexed (+)-enantiomer migrated second, while it was bound weaker than the (**–**)-enantiomer by HDMS-β-CD (see Table 4 in manuscript 4) so that revered EMO was found. Another possibility of a reversal of the migration order can be a change in the complex mobility while the chiral recognition pattern of the selector toward analytes is not altered, as discussed in more detail in the chapter below.

4.4.2. Complex mobility-dependent enantiomer migration order

In the second scenario, the enantioseparation mechanism is dominated by the complex mobilities rather than the complexation constants. Figure 23 illustrates the example of the separation of the Ala-HomoPhe enantiomers in the presence of β-CD at pH 2.5 and 3.8. The stereospecific recognition of Ala-HomoPhe toward the CD did not change with pH. The stronger complexed enantiomer is the DD-isomer at both pH values, although the value was almost equal to the complexation constant determined for the LL-enantiomer at pH 3.8. However, the EMO at pH 3.8 is opposite to the migration order observed at pH

2.5. This can be explained by the higher mobility of the complex formed between β-CD and the DD-isomer at pH 3.8. A stronger influence of the complex mobility at pH 3.8 compared to pH 2.5 was also the case reported in reference [146], where in the presence of β-CD and DM-β-CD the Ala-Tyr enantiomers did not change the affinity pattern toward these CDs when increasing the pH from 2.5 to 3.5, but opposite EMOs were observed at both pH values.



	LL	DD
K	271	395
μ_C	5.43	5.66

	LL	DD
K	537	540
μ_C	2.59	2.83

	LL	DD
K	88	102
μ_C	-5.81	-3.72

	LL	DD
K	18	13
μ_C	-10.3	-14.2

Figure 23. Enantioseparation of Ala-HomoPhe in the presence of 20 mM β-CD as chiral selector at pH 2.5 and 3.8 and Ala-Phg in the presence of 2 mg/mL HS-β-CD as a chiral selector at pH 2.5 and 15 mg/mL HS-β-CD at pH 5.3. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium phosphate buffer at pH 2.5 and 3.8, 50 mM sodium acetate buffer at pH 5.3, capillary temperature 20 °C, separation voltage 25 kV, UV detection at 215 nm. Corresponding apparent complexation constants (K; M^{-1}) and analyte-CD complex mobilities (μ_C; $10^{-9}m^2V^{-1}s^{-1}$) are presented in the tables below each electropherogram.

Comparing the enantioseparations of Ala-Phg at pH 2.5 and 5.3 identical EMO was found at both pH values (Figure 23, right). Nonetheless, the chiral recognition of the enantiomers by the CD was opposite as evidenced by the larger complexation constant of the DD-enantiomer at pH 2.5 and the stronger complexation of the LL-stereoisomer at pH 5.3. As in case of the previous example of the enantioseparation of Ala-Phe by β-CD or HS-β-CD (see chapter 4.4.1), the affinity pattern of the CD changed as a function of the charge state of the peptide. It is positively charged at pH 2.5 and essentially uncharged close to the isoelectric point around pH 5.3. The reason for the identical migration order of this peptide at both pH values is due to the fact that the enantioseparation of the charged peptide at pH 2.5 is governed by the complexation equilibrium so that the stronger complexed DD-isomer is slowed down and migrates second. In contrast, the EMO in case of the uncharged peptide analyte at pH 5.3 is caused by the mobilities of the enantiomer-CD complexes, which are directed to the anode. The mobility

is much higher in case of the weaker bound DD-enantiomer so that this enantiomer is migrating toward the anode faster than the LL-isomer. As the EOF drags the analytes toward the cathode, where the detection is carried out, the enantiomer possessing the lower (anodic) mobility is eventually detected first. Therefore, no reversal of the EMO as a function of the pH of the BGE is detected for the enantioseparation of Ala-Phg using HS-β-CD as selector. The same applies for Ala-Phe and Ala-Phg in the presence of S-β-CD, where the stronger complexed LL-enantiomers of the dipeptides at pH 2.5 are weaker bound by the S-β-CD at pH 5.3. Nevertheless, this did not result in the opposite migration order of the enantiomers. These cases can also be explained with the higher anodic mobilities of the respective weaker bound LL-enantiomers at pH 5.3, and as a result, they migrated as a second peak after the stronger bound DD-isomers (see Table 3 in manuscript 2).

Another example for the effect of the complex mobility on the EMO is the enantioseparation of β-Ala-Phe in the presence of S-α-CD, S-β-CD and S-γ-CD at pH 2.5. Using S-α-CD and S-γ-CD as chiral selectors the L-enantiomer migrated first. This was in accordance with the fact that the D-enantiomer is bound stronger by these CDs and that the mobility of the complex between the CD and this enantiomer was higher compared to the complex of the L-enantiomer. In case of S-β-CD, β-Ala-D-Phe, which is also complexed stronger than the L-enantiomer, migrated first so that opposite EMO was observed compared to the other two CDs. This could be explained by the anodic mobility of the complex of the L-enantiomer, which was significantly higher than that of the complex of the D-enantiomer. Thus, the EMO is determined by the complex mobilities in case of S-β-CD, while the complexation equilibrium (expressed as complexation constants) dominated the EMO in the presence of S-α-CD and S-γ-CD.

4.4.3. CD concentration-dependent reversal of the enantiomer migration order

As stated in chapter 1.3, the affinity pattern of a chiral selector expressed as complexation constants and the mobilities of the complexes as the second stereoselective mechanism may cooperate or counteract each other in a given enantioseparation. An example of the cooperation of the two stereoselective CE principles is the example of Ala-Phg in the presence of S-α-CD and S-γ-CD as shown in Figure 22. The separation is greatly enhanced because the complex of the stronger bound enantiomer also displayed the higher anodic mobility, so that the enantiomers are "dragged" further apart than in separations solely based on differences in the complexation constants.

In many cases of CE enantioseparations the complexation constants and complex mobilities counteract each other. An example is the resolution of the enantiomers of sulfoxide **11** in the presence of HDMS-β-CD shown in Figure 24. Upon increasing the CD concentration, an initial increase of the resolution is observed, and after reaching a maximum at about 20 mM the separation starts to deteriorate upon further increasing the concentration of HDMS-β-CD. This behavior can be rationalized as follows. The (-)-enantiomer is bound stronger by the CD, but the complex has the lower anodic mobility compared to the complex of the (+)-enantiomer. Thus, at low CD concentrations the separation is dominated by the complexation equilibrium and the weaker bound (+)-enantiomer migrates first. However, at high CD concentrations the influence of the complexation equilibrium diminishes because a large fraction of

both enantiomers is bound to the CD. Under these conditions, the effect of the complex mobilities becomes increasingly effective so that a deterioration of the enantioseparation results in case of the counteraction of the two stereoselective CE principles. Similar observations were also made in case of the peptide analytes. For example, in the case of the enantioseparation of Ala-HomoPhe by S-β-CD at pH 5.3, the chiral resolution was lost at a CD concentration of about 40 mg/mL (see Figure 4 in manuscript 2). As in the previous example, the separation behavior can be rationalized based on the complexation constants and complex mobilities(L-Ala-L-HomoPhe: K = 35 M^{-1}, μ_C = - 18.1·$10^{-9}m^2V^{-1}s^{-1}$; D-Ala-D-HomoPhe: K = 28 $M^{-1,}$ μ_C = - 19.7·$10^{-9}m^2V^{-1}s^{-1}$). The weaker bound DD-enantiomer also possesses the higher anionic complex mobility, which will lead to a deterioration of the chiral separation at high CD concentrations.

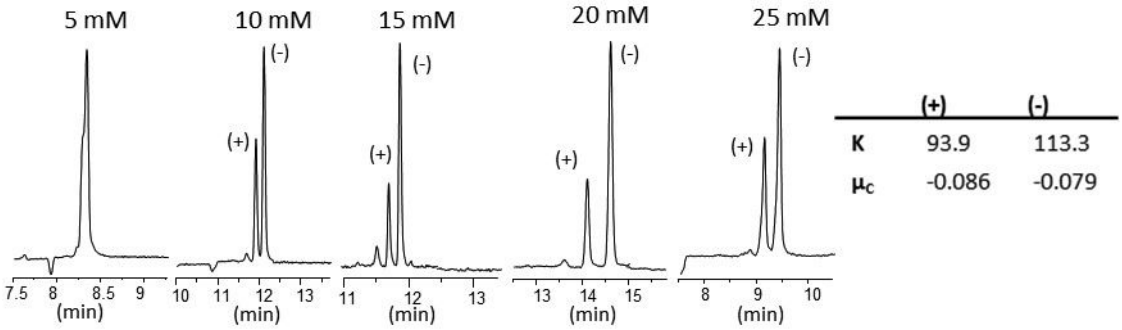


Figure 24. Enantioseparation of compound **11** in the presence of HDMS-β-CD at different CD concentrations. Experimental conditions: 40/50.2 cm, 50 µm ID fused-silica capillary, 50 mM sodium acetate buffer pH 5.5, capillary temperature 20 °C, separation voltage 25 kV, UV detection at 220 nm. Corresponding apparent complexation constants (K; M^{-1}) and analyte-CD complex mobilities (μ_C; $10^{-9}m^2V^{-1}s^{-1}$) are presented in the table.

However, in some cases the counteraction of the chiral recognition of analyte enantiomers by the selector and the mobilities of the complexes may result in a reversal of the EMO as a function of the CD concentration. This phenomenon is not very common but was observed for sulfoxide **11** in the presence of HDMS-α-CD as chiral selector at pH 5.5 as shown in Figure 25**.** At low CD concentrations up to about 15 mM the EMO was (-) > (+), while at CD concentrations above 20 mM it was reversed to (+) > (-). Comigration of the enantiomers occurred at about 17.5 mM HDMS-α-CD. Reversal of the EMO with increasing CD concentrations has been previously observed, for example, for *cis*-ketoconazole and *cis*-terconazole in the presence of HP-β-CD as chiral selector [148, 149]. Further examples have been summarized in references [48, 150].

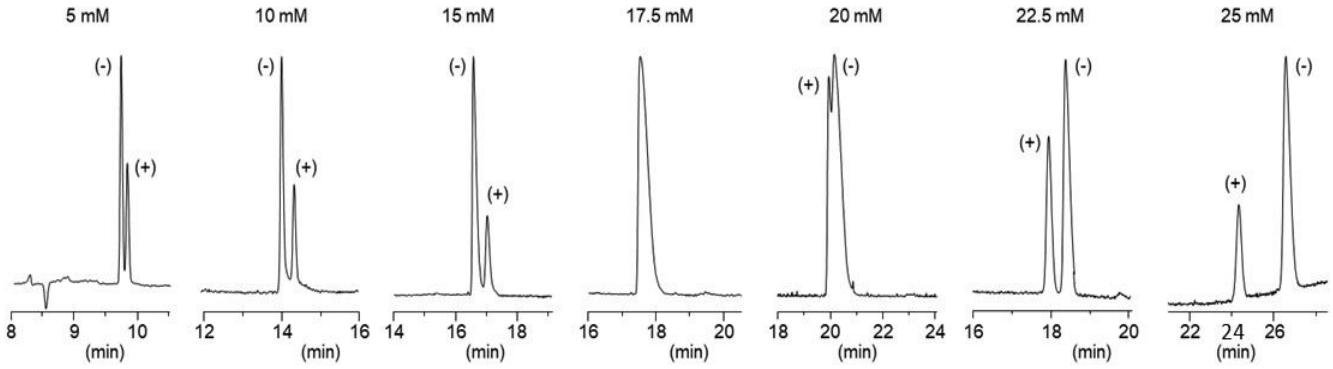


Figure 25. Electropherograms of the separation of the enantiomers of 2-(4-bromobenzylsulfinyl)-*N*-methylbenzamide (compound **11**) as a function of the concentration of HDMS-β-CD in the BGE. Other experimental conditions: 40/50.2 cm, 50 µm I.D. fused-silica capillary; 50 mM sodium acetate buffer, pH 5.5, capillary temperature 20 °C, separation voltage 25 kV, UV detection at 220 nm. (+)-enantiomer: $K = 24.4\ M^{-1}$, $\mu_C = -0.52 \cdot 10^{-9} m^2 V^{-1} s^{-1}$; (-)-enantiomer: $K = 19.6\ M^{-1}$, $\mu_C = -0.61 \cdot 10^{-9} m^2 V^{-1} s^{-1}$.

Analysis of the complexation constants and complex mobilities (listed in the legend of Figure 25) clearly indicated that the migration order of the enantiomers of sulfoxide **11** is governed by the complexation equilibrium at low CD concentrations, where the stronger bound (+)-enantiomer migrated as a second peak. On the contrary, at higher CD concentrations, when a large fraction or essentially the entire amount of the enantiomers are present in the complexed form, the weaker bound (**–**)-enantiomer which displayed the higher anodic complex mobility, migrated second. Under these conditions, the effect of the stereoselective recognition by the CD expressed as complexation constants is negligible, while the mobilities of the diastereomeric complexes determine the migration order of the enantiomers.

Summarizing, the migration behavior observed for peptide and sulfoxide analytes using CDs as chiral selectors including the migration order as well as reversal of the EMO as a function of the experimental conditions (pH or CD concentration) could be rationalized by analysis of the complex formation constants and complex mobilities. Several examples were identified where the EMO was based on either the complexation equilibrium or by the mobilities of the diastereomeric complexes. This included one example of the relatively rare case of the reversal of the EMO as a function of the CD concentration. However, as can be clearly concluded from the data, the prediction of the exact mechanism responsible for an CE enantioseparation cannot be predicted and must be derived for each case.

5. Summary

Capillary electrophoresis has proven to be a powerful technique for the separation of enantiomers as well as the investigation of the complexation between analyte and chiral selector for the understanding of mechanistic aspects of migration phenomena [33, 150, 151]. In the framework of the present thesis, the CD-mediated enantioseparation of two types of analytes with different physico-chemical properties was studied. The first group comprised primarily the dipeptide Ala-Phe and structural analogs, which are highly polar and ionizable analytes. Moreover, they are amphoteric compounds. Structural variations included replacement of Ala by β-Ala or Gly as well as the exchange of Phe with Phg, β-Phe and homoPhe. In contrast, the second type of analytes were lipophilic and non-ionizable (benzylsulfinyl)benzamides. A further special feature of these compounds refers to the fact that the stereogenic center is a sulfur atom. Special emphasis with regard to the enantioseparations was put on the EMO.

With regard to the structure of the analytes, a change of the EMO was observed only in a few cases so that general conclusion of structure-separation relationships could not be drawn from the present set of analytes. Within the group of the peptides, the length of the side chain bearing the aromatic ring, i.e., the distance between the phenyl ring and the stereogenic carbon atom seemed to play a role in the chiral recognition. Thus, opposite EMO was observed between Ala-Phe and Ala-homoPhe under about half of the studied conditions, while the EMO of Ala-Phe and Ala-Phg did not differ. The same was true for peptides with modifications of the Ala residue. The data obtained for the sulfoxides hinted at the fact that the position of a bromine substituent in the benzylsulfinyl moiety affected the chiral recognition by HDMS-α-CD or OA-γ-CD and, consequently, the EMO in the case of compounds **6** and **10**, bearing bromine in position 3, compared to compounds **7** and **11** with the bromine substituent in position 4.

As expected, the type of CD affected the enantioseparations. Generally, charged CD proved to be more efficient chiral selectors compared to uncharged CDs. This was true for both groups of analytes. In case of the protonated peptides this observation can be explained by the counter-mobility of the positively charged analytes and the negatively charged CDs as well as the fact that additional ionic interactions between CDs and analytes might contribute to the complex formation. Charged CDs were also successfully applied to enantioseparations of the uncharged sulfoxides. Because neutral CDs cannot be utilized in the EKC mode for uncharged analytes, the MEKC mode using SDS as micelle-forming agent was applied when studying these CDs. Only few enantioseparations were observed. Thus, the usability of neutral CDs for the separation of chiral (benzylsulfinyl)benzamides appeared to be limited. Furthermore, the chiral recognition ability of the CDs toward the analytes depended on the size of the CD cavity. For the dipeptides, β-CD derivatives were the most successful chiral selectors followed by γ-CD and α-CD derivatives. This can be explained by a better "fit" of the aromatic ring. In contrast, in case of the sulfoxides, γ-CDs were the most effective selectors followed by β-CD and α-CD derivatives. The larger cavity of γ-CD apparently accommodated the (benzylsulfinyl)benzamide structure best. In

addition, several examples in both groups of analytes highlighted the effect of the cavity size of the CDs on the chiral recognition of enantiomers and consequently the EMO.

Another structural feature affecting chiral separations was the substitution pattern of the CDs as evidenced by opposite EMO of dipeptide analytes. Data obtained for HDMS-β-CD and HDAS-β-CD emphasized the importance of the substitution pattern on the wider rim of the CD cavity for the chiral recognition of the peptide enantiomers. Furthermore, the isomeric purity of the investigated CDs proved to be an important parameter for the determination of the EMO of the dipeptides as observed for randomly substituted S-β-CD and HS-β-CD. In case of the sulfoxides, the separation picture is diverse. Randomly substituted charged CDs were by far more efficient compared to single isomer CDs, with the exception of HS-β-CD. For the sulfoxides, the migration order proved to be less dependent on the CD type or substitution pattern.

In case of peptides as ionizable compounds the chiral recognition by CDs may differ depending on the protonation state of the analytes, which is mediated by the pH of the BGE. Interestingly, this was observed for β-CD derivatives only when decreasing the charge of the dipeptides by increasing the pH of the BGE from 2.5 to 3.5. Using charged CDs, the dipeptides could also be studied close to their isoelectric point at pH 5.3.

Using β-CD or HP-β-CD as primary chiral selectors, addition of a CIL composed of tetraalkylammonium ions and amino acids led to an increase of the enantioresolution of the dipeptide analytes while not affecting the EMO. The synergistic effect was primarily attributed to a decrease of the EOF in the presence of the CILs. The extend of the increase in resolution was a function of the type of the amino acid, while the type of the tetraalkylammonium counterion was of minor importance. In the case of Asp-based CILs the synergism was even stronger when the individual components of the CIL were added to the BGE. With regard to the stereochemistry of the amino acid no clear picture could be obtained because in some cases the L-amino acid containing CIL displayed a more pronounced effect than the D-amino acid containing CIL, while it was vice versa in a few cases involving Asp based CILs.

In order to rationalize the reversal of the EMO of the analytes as a function of the protonation state of the analytes or the cavity size or substitution pattern of selected CDs, mechanistic studies were conducted by determining the apparent complexation constants and the mobilities of the diastereomeric CD-enantiomer complexes. The data revealed that the EMO of the dipeptides could be explained by a reversal of the chiral recognition of the enantiomers by a CD as a function of the charge state or by the fact that the complex mobilities became dominant for the migration order.

Binding constants and complex mobilities could be used to explain the CD-concentration dependence reversal of the EMO observed for compound **11** in the presence of HDMS-α-CD. At low CD concentrations the EMO was based on the stereoselective complexation of the enantiomers where the weaker complexed (**–**)-enantiomer migrated first. In contrast, at high CD concentrations when both enantiomers are essentially present in the complexed form the mobilities of the diastereomeric analyte-CD complexes became the determining factor of the migration order, so that the (+)-enantiomer migrated first under these conditions. In most cases, when the stereoselective affinity of a CD toward enantiomers

and the mobilities of the respective diastereomeric complexes counteracted each other, a deterioration of the resolution is observed at high CD concentrations.

6. Zusammenfassung

Die Kapillarelektrophorese (CE) hat sich als leistungsfähige Technik zur Trennung von Enantiomeren sowie zur Untersuchung der Komplexierung zwischen Analyten und chiralem Selektor zum Verständnis mechanistischer Aspekte von Migrationsphänomenen erwiesen [33, 150, 151]. Im Rahmen der vorliegenden Arbeit wurden Cyclodextrin (CD)-vermittelte Enantiomerentrennungen von zwei Substanzgruppen mit unterschiedlichen physikalisch-chemischen Eigenschaften untersucht. Die erste umfasste vor allem Ala-Phe und strukturelle Analoga als polare, ionisierbare sowie amphotere Substanzen. Strukturelle Variationen beinhalteten den Austausch von Ala durch β-Ala oder Gly sowie von Phe durch Phg, β-Phe und HomoPhe. Bei der zweiten Substanzgruppe handelte es sich um lipophile und nichtionisierbare (Benzylsulfinyl)benzamide. Das stereogene Zentrum ist bei diesen Verbindungen das Schwefelatom. Besonderes Augenmerk wurde bei den Trennungen auf die Migrationsreihenfolge der Enantiomere gelegt.

Nur in wenigen Fällen wurde eine Änderung der Migrationsreihenfolge der Enantiomere aufgrund unterschiedlicher Strukturen der Substanzen beobachtet, so dass aus den vorliegenden Daten keine generellen Schlussfolgerungen auf Struktur-Trenn-Beziehungen gezogen werden konnten. Innerhalb der Gruppe der Peptide schien der Abstand des Phenylrings vom Stereozentrum eine Rolle bei der chiralen Erkennung zu spielen. So wurde zwischen Ala-Phe und Ala-homoPhe bei etwa der Hälfte der Bedingungen eine umgekehrte Migrationreihenfolge der Enantiomere beobachtet, während sich die Reihenfolge der Enantiomere von Ala-Phe und Ala-Phg nicht unterschieden. Die für die Sulfoxide erhaltenen Daten deuteten darauf hin, dass die Position eines Bromsubstituenten im Benzylsulfinyl-Rest die chirale Erkennung durch z.B. durch HDMS-α-CD oder OA-γ-CD beeinflusst. Entgegengesetzte Migrationsreihenfolge wurde für die Enantiomere der Verbindungen **6** und **10** mit einem Bromsubstituenten in Position 3 im Vergleich zu Verbindungen **7** und **11** mit Bromsubstitution in Position 4 beobachtet.

Wie erwartet beeinflusste auch die Art des CDs die Trennung der Enantiomere. Ionische CDs waren in der Regel effektivere chirale Selektoren als ungeladene CD-Derivate. Dies galt für beide Substanzgruppen. Für protonierte Peptide lässt sich diese Beobachtung durch die entgegengesetzte Mobilität der positiv geladenen Analyten und der negativ geladenen CDs erklären sowie durch die Tatsache, dass zusätzliche ionische Wechselwirkungen zwischen CDs und Analyt zur Komplexbildung beitragen können. Ionische CDs wurden auch erfolgreich zur Enantiomerentrennung der ungeladenen Sulfoxide eingesetzt. Da neutrale CDs im EKC-Modus nicht für ungeladene Analyte verwendet werden können, wurde bei der Untersuchung dieser CDs der MEKC-Modus mit SDS als Mizellbildner angewendet. Dabei wurden allerdings nur wenige Enantiomerentrennungen beobachtet, so dass ungeladene CDs für die Trennung der chiralen (Benzylsulfinyl)benzamide wenig geeignet waren. Weiterhin hing die chirale Erkennung der Enantiomere durch die CDs von der Größe der Cyclodextrin-Kavität ab. Für Dipeptide waren β-CD-Derivate die erfolgreichsten chiralen Selektoren, gefolgt von γ-CD- und α-CD-Derivaten. Dies kann durch einen besseren "Fit" des aromatischen Rings erklärt

werden. Im Gegensatz dazu waren bei den Sulfoxiden γ-CDs die effektivsten Selektoren, gefolgt von β-CD und α-CD-Derivaten. Offensichtlich interagierten die (Benzylsulfinyl)benzamide besser mit der größeren Kavität der γ-CDs. Zusätzlich verdeutlichten mehrere Beispiele in beiden Substanzgruppen, dass die Größe der Kavität die chirale Erkennung und damit auch die Migrationsreihenfolge der Enantiomere bestimmt.

Ein weiteres, die chiralen Trennungen und die Migrationsreihenfolge der Peptid-Enantiomere beeinflussendes strukturelles Merkmal war das Substitutionsmuster der CDs. Dieser Effekt wurde besonders anhand der für HDMS-β-CD und HDAS-β-CD erhaltenen Daten illustriert. Zusätzlich erwies sich die Isomerenreinheit der CDs als wichtiger Parameter, was anhand von randomisiert substituiertem S-β-CD und HS-β-CD gezeigt wurde. Bei den Sulfoxiden war das Trennverhalten unterschiedlich. Randomisiert substituierte ionisierte CDs waren in der Regel effizienter im Vergleich zu den isomerenreinen CDs mit Ausnahme von HS-β-CD. Bei den Sulfoxiden war die Migrationsreihenfolge der Enantiomere meist unabhängig vom CD-Typ oder dessen Substitutionsmuster.

Im Fall der Peptide als ionisierbare Verbindungen war die chirale Erkennung durch die CDs darüber hinaus abhängig der Protonierung der Analyte, die durch den pH-Wert des BGE bestimmt wird. Interessanterweise wurde dies nur beobachtet, wenn die positive Ladungsdichte der Dipeptide durch die Erhöhung des pH-Wertes von 2.5 auf 3.5 verringert wurde. Unter Verwendung geladener CDs konnten die Dipeptide auch nahe ihrem isoelektrischen Punkt bei pH 5.3 untersucht werden.

Die Zugabe von Aminosäure-basierten chiralen ionischen Flüssigkeiten (CILs) mit Tetraalkylammonium Gegenionen erhöhte die Auflösung der Enantiomerentrennungen von Dipeptiden durch β-CD oder HP-β-CD als primäre chirale Selektoren. Die Migrationsreihenfolge der Enantiomere wurde durch den Zusatz nicht beeinflusst. Der synergistische Effekt von CD und CIL wurde in erster Linie der Abnahme des EOF in Gegenwart der CILs zugeschrieben. Variation des Aminosäure-Anions hatte eine größere Auswirkung auf die Auflösung als die Art des Gegenions. Ebenso wurde im Fall von Asp beobachtet, dass der Synergismus stärker ausgeprägt war, wenn die einzelnen Komponenten dem Elektrolyten zugesetzt wurden im Vergleich zur Verwendung des entsprechenden CILs. Hinsichtlich der Stereochemie der Aminosäure-Komponente konnte kein eindeutiges Bild gewonnen werden. In einigen Fällen wurde eine Verbesserung der Trennung durch das die L-Aminosäure enthaltende CIL erreicht, für andere Dipeptide war ein die D-Aminosäure enthaltendes CIL effektiver als das CIL der L-Aminosäure.

Um die Ursachen der Migrationsreihenfolge der Enantiomere, speziell deren Umkehrung als Funktion der Ladung der Analyte, der Größe der Kavität der CDs oder deren Substitutionsmusters zu erklären, wurden die scheinbaren Bindungskonstanten und die Mobilitäten der Analyt-CD-Komplexe bestimmt. Die Daten zeigten, dass der Änderung der Migrationsreihenfolge von Dipeptid-Enantiomeren durch eine Umkehrung der chiralen Erkennung der Enantiomere durch ein CD in Abhängigkeit von der Ladung des Analyten erklärt werden kann. Weiterhin konnte gezeigt werden, dass die Migrationsreihenfolge auch durch die Mobilität der diastereomeren Analyt-CD-Komplexe bestimmt wurde. Als besonderes Beispiel erwies sich die Enantiomerentrennung des Sulfoxids **11** in Gegenwart von HDMS-α-CD. Hierbei wurde

die Umkehr der Migrationsreihenfolge in Abhängigkeit der CD-Konzentration beobachtet. Bei niedrigen CD-Konzentrationen basierte die Migrationsreihenfolge auf der stereoselektiven Komplexierung der Enantiomere, wobei das schwächer komplexierte (–)-Enantiomer zuerst wanderte. Im Gegensatz dazu wurden bei hohen CD-Konzentrationen, wenn beide Enantiomere im Wesentlichen in komplexierter Form vorliegen, die Mobilitäten der diastereomeren Analyt-CD-Komplexe zum bestimmenden Faktor der Migrationsreihenfolge, so dass das (+)-Enantiomer unter diesen Bedingungen zuerst wanderte. Im Regelfall wurde eine Abnahme der Auflösung bei Erhöhung der CD-Konzentrationen beobachtet, wenn stereoselektive Erkennung eines CDs und die Mobilität der Komplexe einen entgegengesetzten Effekt für die Enantiomerentrennung aufweisen.

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7. Supplementary material appendices

7.1 Appendix I: Supplementary material of manuscript 3

Effects of chiral ionic liquids on cyclodextrin-mediated enantioseparations of dipeptides

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Synthesis of chiral amino acid-based ionic liquids

The CILs were synthesized as described in [1, 2] using tetramethylammonium hydroxide pentahydrate (TMA-OH, 40 % w/w aqueous solution, purity ≥ 97%) and tetrabutylammonium hydroxide 30-hydrate (TBA-OH, 98 %) from Merck (Darmstadt, Germany) and the respective amino acids, which were obtained from commercial sources.

For the preparation of L-Asn, D-Asn and L-Pro based CILs, 6.2 mmol of the amino acid were suspended in 40 mL water and 6.2 mmol of TMA-OH or TBA-OH were added. In case of L-Asp and D-Asp CILs, 6.2 mmol of TMA-OH or TBA-OH were added to a solution or suspension of 3.1 mmol of the amino acid in 40 mL water. The resulting mixtures were stirred at 60 °C for 2 hours. Subsequently, water was removed under reduced pressure at 80 °C. The residue was dissolved in 50 mL ethanol in case of TMA-containing CILs or in acetonitrile in case of TBA-amino acid CILs and filtered to remove unreacted amino acid. The filtrate was dried over anhydrous Na_2SO_4 and the organic solvent was removed under reduced pressure. The product was dried under high vacuum overnight. The CILs were obtained as with 85 to 95 % yields as pale yellow oils in most cases, except $[TMA]_2$[L-Asp], $[TMA]_2$[D-Asp] and [TMA]-[L-Asn] which were white solids. Characterization was performed by ^{1}H-NMR spectroscopy (250 MHz).

[TMA][L-Asn]: ^{1}H NMR (D_2O): δ = 2.3 (dd, J = 8.8 Hz, 14.5 Hz, 1 H, Asn CH_2), 2.58 (dd, J = 3.8 Hz. 14.5 Hz, 1 H, Asn CH_2), 3.09 (s, 12 H, CH_3), 3.50 (dd, J = 3.8 Hz, 8.7 Hz, 1 H, Asn CH).

[TMA][D-Asn]: ^{1}H NMR (D_2O): δ = 2.57 (dd, J = 8.7 Hz, 17.4 Hz, 1 H, Asn CH_2), 2.73 (dd, J = 3.8 Hz, 17.4 Hz, 1 H, Asn CH_2), 3.10 (s, 12 H, CH_3), 3.80 (dd, J = 3.8 Hz, 7.6 Hz, 1 H, Asn CH).

[TBA][L-Asn]: ^{1}H NMR (D_2O): δ = 0.86 (t, J = 7.2 Hz, 12 H, CH_3), 1.27 (m, 8 H, CH_2), 1.56 (m, 8 H, CH_2), 2.60 (dd, J = 8.7 Hz, 17.3 Hz, 1 H, Asn CH_2), 2.68 (dd, J = 3.7 Hz, 8.8 Hz, 1 H, Asn CH_2), 3.11, (m, 8 H, CH_2), 3.50 (m, 1 H, Asn CH).

[TBA][D-Asn]: ^{1}H NMR (D_2O): δ = 0.86 (t, J = 7.2 Hz, 12 H, CH_3), 1.28 (m, 8 H, CH_2), 1.57 (m, 8 H, CH_2), 2.56 (dd, J = 8.9 Hz, 17.4 Hz, 1 H, Asn CH_2), 2.73 (dd, J = 3.7 Hz, 17.4 Hz, 1 H, Asn CH_2), 3.11, (m, 8 H, CH_2), 3.80 (dd, J = 3.7 Hz, 8.9 Hz, 1 H, Asn CH).

[TMA][L-Pro]: ^{1}H NMR (D_2O): δ = 1.62 (m, 3 H), 2.0, (m, 1 H), 2.66 (m, 1 H), 2.92 (m, 1 H), 3.09, (m, 12 H, CH_3), 3.37 (m, 1 H, Pro CH).

[TBA][L-Pro]: ^{1}H NMR (D_2O): δ = 0.85 (t, J = 7.2 Hz, 12 H, CH_3), 1.26 (m, 8 H, CH_2), 1.56 (m, 8 H, CH_2), 1.65 (m, 3 H), 2.03, (m, 1 H), 2.70 (m, 1 H), 2.95 (m, 1 H), 3.10 (m, 8 H, CH_2), 3.43 (m, 1 H, Pro CH).

$[TMA]_2$[L-Asp]: ^{1}H NMR (D_2O): δ = 2.24 (t, J = 7.5 Hz, 15.2 Hz, 1 H, Asp CH_2), 2.59 (d, J = 3.3 Hz, 15.2 Hz, 1 H, Asp CH_2), 3.09 (s 24 H, TMA CH_3), 3.52 (dd, J = 3.3 Hz, 7.5 Hz, 1 H, Asp CH).

$[TMA]_2[D\text{-}Asp]$: ^1H NMR (D_2O): δ = 2.23 (dd, J = 9.9 Hz, 15.7 Hz, 1 H, Asp CH_2), 2.59 (dd, J = 3.7 Hz, 15.7 Hz, 1 H, Asp CH_2), 3.11 (s, 24 H, TMA CH_3), 3.53 (dd, J = 3.7 Hz, 9.8 Hz, 1 H, Asp CH).

$[TBA]_2[L\text{-}Asp]$: ^1H NMR (D_2O): δ = 0.86 (t, J = 7.2 Hz, 24 H, CH_3), 1.27 (m, 16 H, CH_2), 1.56 (m, 16 H, CH_2), 2.24 (dd, J = 9.8 Hz, 15.7 Hz, 1 H, Asp CH_2), 2.59 (dd, J = 3.7 Hz, 15.7 Hz, 1 H, Asp CH_2), 3.11, (m, 16 H, CH_2), 3.52 (dd, J = 3.7 Hz, 9.8 Hz, 1 H, Asp CH).

$[TBA]_2[D\text{-}Asp]$: ^1H NMR (D_2O): δ = 0.87 (J = 7.3 Hz, 24 H, CH_3), 1.27 (m, 16 H, CH_2), 1.57 (m, 16 H, CH_2), 2.26 (dd, J = 9.9 Hz, 15.7 Hz, 1 H, Asp CH_2), 2.60 (dd, J = 3.5 Hz, 15.7 Hz, 1 H, Asp CH_2), 3.11, (m, 16 H, CH_2), 3.54 (dd, J= 3.6 Hz, 9.9 Hz, 1 H, Asp CH).

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Table S1 Migration times (min) and RSD values (%) in brackets of the dipeptide enantiomers at pH 2.5 and 3.5 in the presence of 20 mM β-CD as well as 15 mM of the CILs or equimolar concentrations of the CIL components. Other experimental conditions: 40/50.2 cm, 50 µm I.D. fused-silica capillary, 50 mM sodium phosphate buffer, 20 °C, 25 kV, 215 nm.

Dipeptide		**pH**	**BGA additive**			
	L		$[TBA]_2$[L-Asp] 15 mM	30 mM TBA-Cl 15 mM L-Asp	$[TMA]_2$[L-Asp] 15 mM	30 mM TMA-Cl 15 mM L-Asp
Gly-Phe		2.5	18.43 (1.2)	20.33 (0.4)	22.52 (0.6)	22.84 (0.9)
	D		18.43 (1.2)	20.33 (0.4)	22.52 (0.6)	22.84 (0.9)
	L	3.5	31.54 (3.5)	31.62 (5.5)	35.71 (5.3)	40.84 (2.5)
	D		31.08 (3.5)	31.18 (5.5)	35.10 (5.3)	39.75 (2.5)
Ala-Phe	LL	2.5	18.81 (0.1)	19.75 (0.7)	21.64 (0.6)	21.40 (2.1)
	DD		19.25 (0.1)	20.25 (0.7)	22.27 (0.5)	21.98 (2.1)
	LL	3.5	22.99 (2.2)	24.71 (3.3)	27.10 (5.4)	35.32 (3.9)
	DD		22.75 (2.1)	24.60 (3.0)	26.71 (5.3)	34.59 (3.9)
Ala-Tyr	LL	2.5	20.77 (1.0)	21.55 (0.5)	24.38 (0.2)	24.07 (0.4)
	DD		20.99 (1.1)	21.80 (0.3)	24.60 (0.3)	24.30 (0.6)
	LL	3.5	21.74 (2.0)	22.61 (1.1)	24.55 (1.0)	31.93 (1.9)
	DD		21.28 (2.0)	22.10 (1.1)	24.11 (1.2)	31.23 (1.9)
Lys-Phe	LL	2.5	10.41 (0.3)	10.75 (0.1)	10.37 (0.3)	10.14 (0.7)
	DD		10.72 (0.3)	11.08 (0.1)	10.64 (0.3)	10.43 (0.7)
	LL	3.5	9.98 (0.9)	10.30 (2.3)	10.04 (1.3)	9.02 (1.3)
	DD		9.98 (0.9)	10.30 (2.3)	10.04 (1.3)	9.02 (1.3)
Phe-Phe	LL	2.5	17.08 (0.7)	22.52 (0.3)	24.64 (0.3)	25.33 (0.7)
	DD		17.65 (0.7)	23.32 (0.3)	25.56 (0.2)	26.43 (0.7)
	LL	3.5	21.14 (0.7)	21.54 (1.9)	23.35 (1.5)	28.66 (1.1)
	DD		20.87 (0.8)	21.06 (1.9)	22.58 (1.5)	28.28 (1.2)

Table S2 Migration times (min) and RSD values (%) in brackets of the dipeptide enantiomers at pH 2.5 and 3.5 in the presence of 20 mM HP-β-CD as well as 15 mM of the CILs or equimolar concentrations of the CIL components. Other experimental conditions: 40/50.2 cm, 50 µm I.D. fused-silica capillary, 50 mM sodium phosphate buffer, 20 °C, 25 kV, 215 nm.

Dipeptide		**pH**	**BGA additive**		
			$[TBA]_2$[L-Asp] 15 mM	$[TMA]_2$[L-Asp] 15 mM	[TMA][L-Asn] 15 mM
Gly-Phe	L	2.5	26.32 (1.3)	30.58 (2.1)	21.95 (0.8)
	D		24.85 (1.4)	28.34 (2.1)	20.59 (0.9)
	L	3.5	37.65 (2.1)	36.85 (3.5)	20.14 (3.8)
	D		37.25 (2.1)	36.16 (3.6)	19.48 (6.4)
Ala-Phe	LL	2.5	24.96 (1.0)	26.87 (1.6)	20.60 (2.1)
	DD		24.39 (1.0)	26.87 (1.6)	19.84 (2.1)
	LL	3.5	27.60 (2.0)	26.27 (7.3)	16.62 (6.3)
	DD		27.22 (2.1)	25.83 (7.5)	16.27 (6.6)
Ala-Tyr	LL	2.5	29.05 (1.0)	41.93 (1.0)	23.32 (2.0)
	DD		27.61 (1.0)	40.17 (1.0)	21.86 (2.1)
	LL	3.5	30.25 (1.8)	23.61 (4.6)	18.24 (5.7)
	DD		29.94 (1.8)	22.52 (5.5)	18.16 (3.3)
Lys-Phe	LL	2.5	11.50 (0.2)	12.49 (0.6)	9.52 (0.3)
	DD		11.43 (0.2)	11.68 (0.6)	8.92 (0.4)
	LL	3.5	9.01 (1.2)	10.46 (1.4)	8.72 (2.1)
	DD		8.96 (1.1)	10.05 (1.5)	8.26 (2.0)
Phe-Phe	LL	2.5	29.86 (0.7)	25.42 (0.9)	25.36 (2.1)
	DD		28.71 (0.7)	24.38 (0.9)	24.81 (2.1)
	LL	3.5	25.65 (1.8)	28.27 (3.6)	18.76 (2.7)
	DD		24.94 (1.8)	27.51 (3.6)	18.21 (2.2)

7.2 Appendix II: Supplementary material of manuscript 4

Enantioseparation of chiral (benzylsulfinyl)benzamide sulfoxides by capillary electrophoresis using cyclodextrins as chiral selectors

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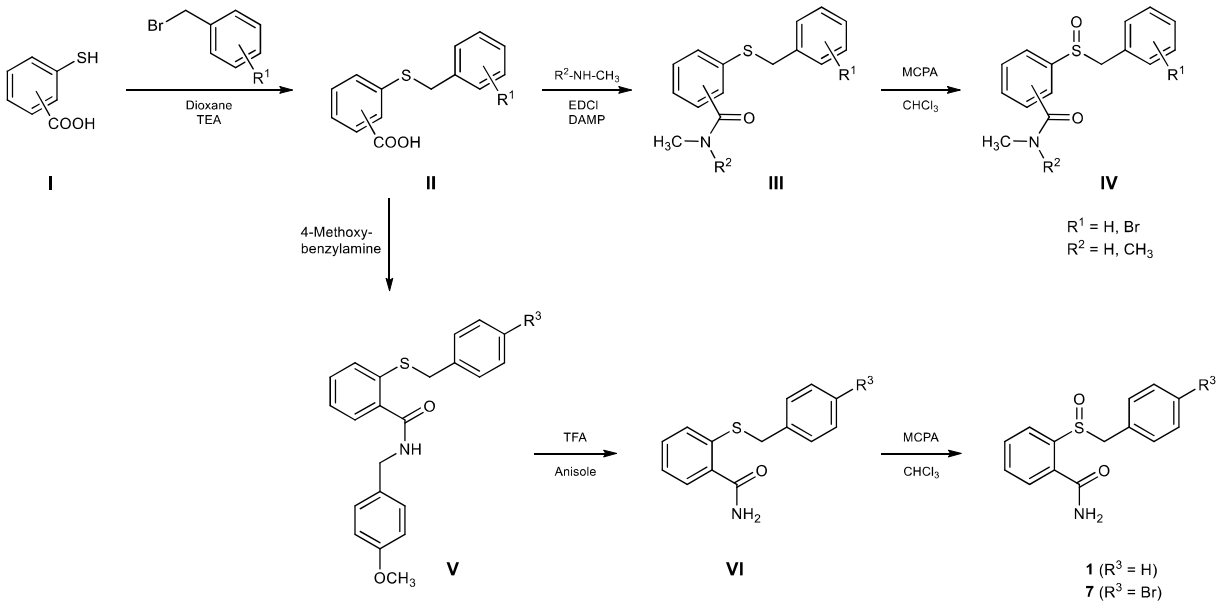
1. Synthesis of sulfoxides

All chemicals were obtained from commercial suppliers. The sulfoxides **1**, **3** - **5**, **7**, **10** and **11** (Table 1) were synthesized according to [G. Pinna et al., Tetrahedron 67 (2011) 5268-5281] as outlined in Scheme S1. The starting material was 2-, 3- or 4-mercaptobenzoic acid (**I**), which was condensed with benzylbromide or a substituted derivative in the presence of triethylamine (TEA) to yield the corresponding sulfide compound (**II**). In case of *N*-methylated target compounds, the sulfide was converted to the *N*-methyl- or *N*,*N*-dimethylbenzamides (**III**) by coupling methylamine or dimethylamine

using *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) in the presence of 4(dimethylamino)pyridine (DMAP). Oxidation of the sulfide group was mediated by 3chloroperoxybenzoic acid (MCPBA) to yield the sulfoxides (**IV**). For the synthesis of 2(benzylsulfinyl)benzamides **1** and **7**, 2-(benzylthio)benzoic acid or 2-(benzylthio)benzoic acid, respectively, was coupled (EDCI mediated) with 4-methoxybenzylamine to yield the respective *N*-4methoxybenzylamide derivative (**V**). Conversion to 2-(benzylthio)benzamide derivative **VI** was performed by heating with trifluoroacetic acid (TFA) in anisole. Oxidation with MCPBA yielded **1** or **7**, respectively.



Scheme S1 Outline of the synthesis of (benzylsulfinyl)benzamides

General procedure for sulfoxides IV

To a stirred solution of 1 eq of the mercaptobenzoic acid (**I**) and 2.1 eq. TEA in dioxane (0.1 M concentration of the acid), 1.1. eq. of benzyl bromide or a substituted derivative were added dropwise at 0 °C. After a reaction time of 10 to 60 min (monitored by TLC, silica gel 60; $CHCl_3$:MeOH 95:5, v/v), the organic solvent was removed under reduced pressure. The residue was diluted in 1 M HCl and extracted three times with ethyl acetate. The organic layers were collected, dried over anhydrous Na_2SO_4, filtered, and the organic solvent was removed under reduced pressure to yield the respective (benzylthio)benzoic acid (**II**) as an off-white solid that was utilized in the next step without further characterization.

To a solution of 1 eq. of crude **II** in CH_2Cl_2, 1 eq. EDCl and 1.1 eq. DMAP were added followed by 1.1 eq. *N*-methylamine, *N*,*N*-dimethylamine or 4-methoxybenzylamine at room temperature. The solution was stirred at room temperature for 2 h. The organic solvent was evaporated under reduced pressure, and the residue diluted with 0.1 M HCl, followed by extraction with $CHCl_3$ (three times). The organic layers were collected and extracted with saturated aqueous $NaHCO_3$ solution and dried over anhydrous Na_2SO_4. Opüon filtration, the organic solvent was removed under reduced pressure to yield the respective crude benzamides **III** or **V**.

To a solution of 1 eq. of benzamide **III** in $CHCl_3$ (0.1 M concentration) cooled to 0 °C was added dropwise 1 eq. of 75 % (w/v) solution of MCPA in $CHCl_3$. After completion of the addition, the reaction mixture was stirred at 0 °C for 30 min and quenched by addition of a saturated $NaHCO_3$ solution. The mixture was extracted three times with $CHCl_3$, the organic layers were collected and dried over anhydrous Na_2SO_4. After filtration, the organic solvent was evaporated under reduced pressure. The crude products **IV** were purified by flash chromatography (silica gel, 60, 60-200 µm; *n*-hexane:ethyl acetate 20:80, v/v). The identity of the compounds was checked by ^{1}H NMR spectroscopy and the purity was estimated by HPLC using an i-Cellulose-5 column (150 x 4.6 mm, 5 µm; Phenomenex, Torrence, CA. USA) and methanol as mobile phase, flow rate 1.0 mL/min, detection wavelength 254 nm.

2-(benzylsulfinyl)-*N*,*N*-dimethyl benzamide (**3**): White amorphous solid, HPLC purity 97.7 %. ^{1}H NMR (DMSO-d_6): δ = 2.84 (s, 3 H), 3.05 (s, 3 H), 3.99 (d, J = 12.8 Hz, 1 H), 4.4 (d, J = 12.8 Hz, 1 H), 7.09 (dd, J = 3.4 Hz, J = 9.5 Hz, 2 H), 7.33 (m, 3 H), 7.55 (m, 4 H).

3-(benzylsulfinyl)-*N*,*N*-dimethyl benzamide (**4**): Off-white amorphous solid, HPLC purity 99.1 %. ^{1}H NMR (DMSO-d_6): δ =2.76 (s, 3 H), 2.96 (s, 3 H), 4.11 (d, J = 8.1 Hz, 1 H), 4.31 (d, J = 8.1 Hz, 1 H), 7.04 (dd, J = 3.3 Hz, J = 5.9 Hz, 2 H), 7.25 (m, 3 H), 7.42 (s, 1 H), 7.51 (m, 3 H).

4-(benzylsulfinyl)-*N*,*N*-dimethyl benzamide (**5**): White amorphous solid, HPLC purity 98.2%.
^{1}H NMR (DMSO-d_6): δ =2.87 (s, 3 H), 2.99 (s, 3 H), 4.1 (d, J = 12.8 Hz, 1 H), 4.3 (d, J = 12.8 Hz, 1 H), 7.06 (dd, J = 5.4 Hz, J = 9.4 Hz, 2 H), 7.24 (m, 3 H), 7.49 (m, 4 H).

2-(3-bromobenzylsulfinyl)-*N*-methyl benzamide (**10**): White amorphous solid, HPLC purity 97.9 %.

^{1}H NMR (DMSO-d_6): δ =2.84 (d, J = 2.9 Hz, 3 H), 3.94 (d, J = 7.8 Hz, 1 H), 4.49 (d, J = 7.8 Hz, 1 H), 7.1 (d, J = 4.8 Hz, 1 H), 7.25 (m, 2 H), 7.48 (d, J = 4.5, 1 H), 7.57 (m, 3 H), 7.87 (d, J = 4.5 Hz, 1 H), 8.82 (d, J = 2.73 Hz, 1 H).

2-(4-bromobenzylsulfinyl)-*N*-methyl benzamide (**11**): Off-white amorphous solid, HPLC purity 97.5 %.
^{1}H NMR (DMSO-d_6): δ = 2.83 (d, J = 2.9 Hz, 3 H), 3.91 (d, J = 7.85 Hz, 1 H), 4.47 (d, J = 7.83 Hz, 1 H), 7.05 (d, J = 5.2 Hz, 2 H), 7.47 (d, J = 5.2 Hz, 2 H), 7.62 (m, 3 H), 7.85 (d, J = 4.5 Hz, 1 H), 8.81 (d, J = 2.7 Hz, 1 H).

General procedure for sulfoxides 1 and 7

The crude benzamides **V** obtained as described above, were dissolved in 3 mL TFA and 1 mL anisole. The mixture was heated on an oil bath at 80 °C for 4 h monitoring the reaction by TLC (silica gel, n-hexane : EtOH 50:50, v/v). After completion of the reaction, TFA was removed under reduced pressure, the residue was dissolved in CHCl3 and extracted with saturated aqueous $NaHCO_3$ solution followed by extraction with 0.1 M HCl. The organic solvent was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to yield the crude benzamides **VI**, which were oxidized using MCPBA as described for benzamides **III** above. Crude **1** and **7** were purified by flash chromatography (silica gel, 60, 60-200 µm; *n*-hexane : ethyl acetate 20:80, v/v) and analyzed by ^{1}H NMR and HPLC.

2-(benzylsulfinyl)benzamide (**1**): Off-white amorphous solid, HPLC purity 98.2 %.
^{1}H NMR (DMSO-d_6): δ = 3.81 (d, J = 7.8 Hz, 1 H), 4.5 (d, J = 7.7 Hz, 1 H), 7.17 (m, 2 H), 7.19 (m, 3 H), 7.63 (m, 4 H), 7.85 (m, 1 H), 8.34 (s. 1 H).

2-(4-bromobenzylsulfinyl)benzamide (**7**): Off-white amorphous solid, HPLC purity 98.8 %.
^{1}H NMR (DMSO-d_6): δ = 3.87 (d, J = 7.8 Hz, 1 H), 4.46 (d, J = 7.8 Hz, 1 H), 7.07 (d, J = 5.2 Hz, 2 H), 7.47 (d, J = 5.2 Hz, 2 H), 7.61 (m, 1 H), 7.68 (m, 2 H), 7.79 (s. 1 H), 7.93 (d, J = 4.6 Hz, 1 H), 8.36 (s, 1 H).

Table S1. Separation of sulfoxides with neutral CDs under standardized MEKC conditions under standardized conditions. (+) or (-) indicates the faster migrating enantiomer. CE conditions: 50 µm ID, 50.2/40 cm fused-silica capillary; 50 mM sodium borate buffer, pH 9.0, containing 100 mM SDS and 10 % (v/v) methanol; 20 °C; 25 kV; detection at 220 nm at the cathodic end. CD concentrations: 50 mg/mL HP-α-CD, β-CD, M-β-CD, DM-β-CD50, DM-β-CD95, TM-β-CD and γ-CD; 60 mg/mL HPβ-CD and HP-γ-CD; 20 mg/mL CM-β-CD and CM-γ-CD.

Sulfoxide	HP-α-CD	β-CD	M-β-CD	DM-β-CD50	DM-β-CD95	TM-β-CD	HP-β-CD	γ-CD	HP-γ-CD	CM-β-CD	CM-γ-CD
1	ns	ns	ns	ns	0.3 (+)	ns	ns	ns	1.1 (-)	ns	2.2 (-)
2	ns	ns	ns	ns	ns	1.5 (+)	ns	ns	0.8 (-)	ns	1.7 (-)
3	ns	ns	ns	0.4 (+)	ns	ns	ns	ns	0.1 (-)	ns	0.4 (-)
4	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	sh (-)
5	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.5 (-)
6	ns	ns	ns	ns	ns	ns	ns	0.7 (-)	1.8 (-)	ns	ns
7	ns	0.8 (+)	ns	ns	ns	nd	ns	0.3 (-)	1.7 (-)	ns	1.1 (-)
8	ns	sh (+)	0.9 (+)	ns	ns	ns	ns	0.3 (-)	2.1 (-)	nd	1.7 (-)
9	ns	3.2 (+)	5.5 (+)	ns	ns	ns	ns	ns	ns	nd	ns
10	ns	ns	ns	ns	ns	ns	ns	0.8 (-)	1.4 (-)	ns	ns
11	ns	ns	ns	ns	ns	nd	ns	0.5 (-)	3.2 (-)	nd	1.2 (-)
12	ns	ns	1.0 (+)	ns	ns	sh (+)	ns	ns	sh (-)	nd	sh (-)
14	ns	ns	ns	sh (+)	ns	ns	ns	sh (-)	sh (-)	ns	1.0 (-)
15	ns	ns	ns	ns	ns	ns	ns	ns	0.5 (-)	0.3 (+)	sh (-)
16	ns	ns	ns	ns	ns	ns	ns	ns	0.7 (-)	0.4 (-)	0.3 (-)

nd, not detected within 60 min; ns, not separated; sh, shoulder.

Contributions to the Manuscript Figures

Manuscript 1

Reference: M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1623, 2020, 461158

Figure 1 : Structures of the analytes were created by candidate.

Figure 2 : The experimental data for this figure were 100% acquired by the candidate, finalized by coauthor G.K.E. Scriba.

Manuscript 2

Reference: M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1632, 2020, 461585

Figure 1 : Structures of the analytes were created by candidate.

Figure 2, 3, 4 : The experimental data for these figures were 100% acquired by the candidate, finalized by coauthor G.K.E. Scriba.

Manuscript 3

Reference: M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1652, 2021, 462342.

Figure 1 : Structures of the chiral ionic liquids were created by G.K.E. Scriba, the structures of the analytes were created by candidate.

Figure 2, 3, 4 : The experimental data for these figures were 100% acquired by the candidate, finalized by coauthor G.K.E. Scriba.

Manuscript 4

Reference: M.-L. Konjaria, K. Kakava, A. Volonterio, B. Chankvetadze, G.K.E. Scriba, submitted to J. Chromatogr. A on 02.03.2022.

Figure 1, 2, 3 : The experimental data for these figures were 100% acquired by the candidate, finalized by coauthor G.K.E. Scriba.

Contributions to the Manuscripts

Manuscript 1

Enantioseparation of analogs of the dipeptide alanyl-phenylalanine by capillary electrophoresis using neutral cyclodextrins as chiral selectors

M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1623, 2020, 461158. DOI: 10.1016/j.chroma.2020.461158.

Personal Contribution (85 %) : study design and concept development, experimental work, calculation of complexation constants, data analysis and interpretation, preparation of manuscript draft.

Gerhard K.E. Scriba (15 %): conceptualization, supervision, finalizing manuscript.

Manuscript 2

Enantioseparation of alanyl-phenylalanine analogs by capillary electrophoresis using negatively charged cyclodextrins as chiral selectors

M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1632, 2020, 461585. DOI: 10.1016/j.chroma.2020.461585.

Personal Contribution (85 %) : study design and concept development, experimental work, calculation of complexation constants, data analysis and interpretation, preparation of manuscript draft.

Gerhard K.E. Scriba (15 %): conceptualization, supervision, finalizing manuscript.

Manuscript 3

Effects of amino acid-derived chiral ionic liquids on cyclodextrin-mediated capillary electrophoresis enantioseparations of dipeptides

M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1652, 2021, 462342. DOI: 10.1016/j.chroma.2021.462342**.**

Personal Contribution (85 %) : study design and concept development, experimental work, synthesis of chiral ionic liquids, data analysis and interpretation, preparation of manuscript draft.

Gerhard K.E. Scriba (15 %): conceptualization, supervision, finalizing manuscript.

Manuscript 4

Enantioseparation of chiral (benzylsulfinyl)benzamide sulfoxides by capillary electrophoresis using cyclodextrins as chiral selectors

M.-L. Konjaria, R. Kakava, A. Volonterio, B. Chankvetadze, G.K.E. Scriba. Submitted to J. Chromatogr. A on 02.03.2022

Personal Contribution (70 %): study design and concept development, experimental work, synthesis of some sulfoxides, fractioning of sulfoxide enantiomers on analytical HPLC column and determination of the optical rotation sign, calculation of the complexation constants, data analysis and interpretation, preparation of manuscript draft.

Rusudan Kakava (10 %): Synthesis of some samples, performing the method evaluation analysis.

Alessandro Voloterio (5 %): Synthesis of some samples.

Bezhan Chankvetadze (5 %): cooperation in study design and discussion.

Gerhard K.E. Scriba (10 %): conceptualization, supervision, finalizing manuscript.

List of publications and presentations

Journal Articles:

Enantioseparation of novel chiral sulfoxides on chlorinated polysaccharide stationary phases in supercritical fluid chromatography

C. West, M.-L. Konjaria, N. Shashviashvili, E. Lemasson, P. Bonnet, R. Kakava, A. Volonterio, B. Chankvetadze, J. Chromatogr. A 1499, 2017, 174-182. DOI: 10.1016/j.chroma.2017.03.089.

Enantioseparation of analogs of the dipeptide alanyl-phenylalanine by capillary electrophoresis using neutral cyclodextrins as chiral selectors

M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1623, 2020, 461158. DOI: 10.1016/j.chroma.2020.461158.

Enantioseparation of alanyl-phenylalanine analogs by capillary electrophoresis using negatively charged cyclodextrins as chiral selectors

M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1632, 2020, 461585. DOI: 10.1016/j.chroma.2020.461585.

Effects of amino acid-derived chiral ionic liquids on cyclodextrin-mediated capillary electrophoresis enantioseparations of dipeptides

M.-L. Konjaria, G.K.E. Scriba, J. Chromatogr. A 1652, 2021, 462342. DOI: 10.1016/j.chroma.2021.462342**.**

Advances of capillary electrophoresis enantioseparations in pharmaceutical analysis (2017–2020), Review

S. Krait, M.-L. Konjaria, G. K. E. Scriba, Electrophoresis, 2021, 1709-1725. DOI: 10.1002/elps.202000359.

Enantioseparation of chiral (benzylsulfinyl)benzamide sulfoxides by capillary electrophoresis using cyclodextrins as chiral selectors

M.-L. Konjaria, R. Kakava, A. Volonterio, B. Chankvetadze, G.K.E. Scriba. Submitted to J. Chromatogr. A on 02.03.2022

Oral presentations

Separation of chiral sulfoxides in high-performance liquid chromatography with chloro-substituted chiral phenylcarbamates and Abraham descriptors

M.-L. Konjaria

27/12/2016, 5th conference in Physical and analytical chemistry, Tbilisi, Georgia.

Separation of chiral sulfoxides in high-performance liquid chromatography with new chiral phenylcarbamates and Abraham descriptors

M.-L. Konjaria

06/02/2017, 76th student conference, Tbilisi, Georgia.

Separation of chiral sulfoxides with chloro- and methyl-substituted phenylcarbamates in high-performance liquid chromatography.

M.-L. Konjaria

04/01/2019, 9th Annual Symposium on Physical and Analytical Chemistry ,Tbilisi, Georgia

Poster presentations:

Separation of chiral sulfoxides in high-performance liquid chromatography with new chiral phenylcarbamates

M.-L. Konjaria, N. Shashviashvili

07/07-12/07, 2014, Georgian-German School-workshop, Tbilisi, Georgia.

Separation of chiral sulfoxides in high-performance liquid chromatography with new chiral phenylcarbamates

M.-L. Konjaria, N. Shashviashvili

15/04/2015, 3rd national conference in medical sciences, Tbilisi, Georgia.

Separation of chiral sulfoxides in high-performance liquid chromatography with new chloro-substituted chiral phenylcarbamates

M.-L. Konjaria

05/07-08/07, 2015, 26th PBA , International symposium on Pharmaceutical and Biomedical analyses, Tbilisi, Georgia.

Eigenständigkeitserklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bekannt ist, ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind.

Ich versichere, dass ich die Hilfe eines Promotionsberaters nicht in Anspruch genommen habe und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die vorliegende Dissertation wurde von mir bei keiner bisherigen Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Weiterhin versichere ich, dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Universität als Dissertation eingereicht habe.

Jena, den _____ Mari-Luiza Konjaria _____