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„Enantioselective Separation of Fluorescence Tagged Amino Acids using Chiral Anion Exchangers”

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TABLE OF CONTENTS

ABBREVIATIONS

Ac	Acetyl or acetyl protection group
AcOH	Acetic acid
ACQ	6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate
AIBN	2,2'-Azobis-(2-methyl-proponitril)
AMQ	6-Aminoquinoline
AX	Anion exchange
B	Benzyl protection group
Boc	*tert*-Butyl oxalyl protection group
CE	Capillary electrophoresis
CIP	Cahn Ingold and Prelog
ClAc	Chloro acetyl protection group
CSP	Chiral stationary phase
CMP	Chiral mobile phase
CX	Cation exchange
DAD	Diode array detector
DMF	Dimethylformamide
DNB	3,5-Dinitro-benzyl carbonyl
DNS	5-Dimethylamino-naphtyl-sulfon protection group
DNPyr	2,4-Dinitropyridin
DNZ	3,5-Dinitro-1-methyl-benzyloxalyl
F	Formaldehyde protection group
FITC	Fluoresceine isothiocyanate
FLD	Fluorescence detector
FMOC	Fluorenyloxalyl protection group
HILIC	Hydrophilic interaction liquid chromatography
HCl	Hydrogenchloride
LIF	Laser-induced-fluorescence
M	Molar
mm	Milli meters
MeOH	Methanol
mM	Milli mol per liter
NH_4AcO	Ammonia acetate
nm	Nano meters
o.n.	Over night
OPA	ortho-Phtalaldehyde
pH_a	Apparent pH
SP	Stationary phase
SO	Chiral selector
t_0	void time
(v/v)	Volume per volume
µm	Micro meters
WAX	Weak anion-exchanger
UV	Ultra violet
VIS	Visible
Z	Methylphenyloxalyl protection group
ZX	Zwitterionic exchange

Abbreviations of analytes

AABA	Alpha aminobutyric acid
ACHSA	2-Amino-cyclohexyl carboxyl acid
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Cys-S-acetamide	Cysteine acetamide
Cys-S-AcOH-	Cysteine acetic acid
DNB-AA	3,5-Dinitrophenyl amino acid
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
HCys-acid	Homocysteic acid
His	Histidine
HPro	Hydroxyproline
HSer	Homoserine
Ile	Isoleucine
Isoser	Isoserine
Leu	Leucine
Lys	Lysine
Met	Methionine
Nipe	Nipecotinic acid
NVal	Norvaline
Phe	Phenylalanine
β-Phe	beta-Phenylalanine
Pipe	Pipecolinic acid
Pro	Proline
Trp	Tryptophane
p-X-DL-Phe	para-Halide-DL-phenylalanine
p-β-X-DL-Phe	para-β-Halide-DL-phenylalanine
Val	Valine

1 AIM OF THE STUDY

The aim of this study should be the investigation of different types of fluorescence labels for amino acid derivatization and the enantioselective separation of these derivatives using a variety of weak anion-exchange type chiral stationary phases. Two fluorescence tags were applied for N-terminal protection of amino acids, 6-aminoquinolyl-succinimid carbamate (AQC) and fluoresceine-isothiocyanate (FITC). The first label was a very common fluorescent tag for quantitative amino acids analysis and the other was frequently used as a fluorescent label for biochemical assays and labeling of cells, cell compartments, enzymes and amino acid residues. The comparison of separation performance of these two fluorescence tags should be conducted. AQC was a rather expensive derivatization reagent, of which the chemistry was well known and the derivatization procedure was fully optimized and easy to handle. Fluoresceine isothiocyanate was cheaper compared to AQC. A successful application of FITC for amino acid enantioseparations should demonstrate the equality of these two tags. In turn of enantioseparation, AQC was often used for amino acid analysis, whereas FITC is a less common tag for this propose. Therefore the enantioseparation of amino acids, derivatized with FITC, should be investigated. The fluorescence-active molecule fluoresceine was much bigger than the derivatized amino acids. Therefore it was expected that enantioseparations would not be as straight forward as with smaller N-terminal tags like DNB,- DNZ,- N-Acetyl,- Z,- etc. Fluoresceine would highly interact with quinine-based selectors in a non-enantioselective manner. Hence, an enantioseparation of amino acids carrying N-terminal the fluoresceine label would be an evidence for the high selectivity provide from a quinine-based chiral selector.

The range of amino acids included all proteinogenic amino acids, as well as amino phosphonic acids, -sulfone and -sulfonic acids and some common amino acid analogues of phenylalanine, since they were used as pharmaceutical drugs. Furthermore, some special separation problems should be investigated, such as the separation of stereoisomers of isoleucine, 4-hydroxyproline and threonine.

Some dipeptides had been chosen as model substances to proof the applicability of these AQC-tagged dipeptides for the enantioselective separation on anion-exchange type quinine-based CSP. Glycoylphenylalanine (GlyPhe) and glycoylproline (GlyPro) and their reversed counterparts, PheGly and ProGly were derivatized with AQC and their enantioseparation should be investigated.

Moreover, AQC should be applied for the separation of alanine peptides and alanine-glycine peptides, as well as for phenylalanine peptides. The separation of alanine enantiomers of all-D/L-peptides up to the hexamer, of stereoisomers of tri- and tetrapeptides and of glycine-alanine di- and tripeptides were investigated. The separation of phenylalanine peptides, all-D/all-L peptides up to the pentamer and the stereoisomers of tri- and tetrapeptides, which had a pronounced hydrophobic character, were also studied.

Furthermore, the synthesis and application of new quinine-based chiral stationary phases, which exhibited a zwitterionic character should be conducted. Previous studies had introduced a second ion-exchange group into the structure of the quinine selector. The combination of a strong anion- with a

cation-exchange group, a sulfonic acid with and a quaternary amine, in one chiral selector, the quinine molecule, was up to date not yet investigated. The application of these sulfobetaine-type columns should be examined in course of enantioseparation of acidic and zwitterionic compounds. Further, because this type of selector showed a lack in enantioselective discrimination ability, their application as HILIC columns should be explored. A set of typical HILIC compounds, acids, base, zwitterions, nucleosides, nucleotides and alkaloids were applied to investigate the separation performance of the sulfobetaine-type quinine-based columns. The retention mode should be investigated and their HILIC-character was compared with a *tert*-butyl carbamate modified quinine-based column (QN/QD-AX) and a commercially available zwitterionic HILIC column.

2 INTRODUCTION

2.1 Chirality

Chirality (handedness) is a phenomenon occurring in the organic as in the anorganic world. The greek word "*cheiro*" means "hand" and is an acronym for the fact that a chiral molecule is not superimposable with its mirror image. They are called enantiomers or optical antipodes. Enantiomers have identical constitutions attached to a central C-atom, the "chiral center", but differ in the spatial arrangement of their binding atoms towards each other. Such an object cannot be brought in relation with one of the symmetry elements of the second class, such as mirror planes, center of symmetry or rotary-reflection axis. A pair of enantiomers has the same physico-chemical properties, if they are exposed to an achiral environment. They have the same density, melting- and boiling points. The optical activity is the only physical property in which enantiomers differ to each other. One enantiomer will rotate the plane of polarized light towards the right direction (+) and the other will turn it, with the same amount, to the left (-). Note that their chemical properties in a biological system may be diverse as one counterpart may be a useful drug while the other is a harmful poison, such as α-phtalimidoglutarimid, also known as contergan®. Optical rotation activity is a substance specific property and is a direct method which can be used to distinguish to antipodes. Diastereoisomers, on the other hand, are stereoisomers, which are not mirror images of one another. Diastomers have different physico-chemical properties. A racemate is a 50/50 mixture of each enantiomer and therefore optically inactive. The mixing ratio of two enantiomers can be described by the enantiomeric excess (ee) (Formula 1) [1].

$$ee = \frac{(S)-(R)}{(S)+(R)} * 100 \quad (S)>(R)$$

Formula 1: Enantiomeric excess

2.1.1 Chirality elements

Different kinds of chiral elements can be found in nature. The most abundant and obvious one is related to a single chiral atom. The tetrahedonal carbon atom is predestinated for inducing chirality. Once four different ligands are connected to the same carbon atom, this carbon atom becomes a "chiral center" and chiral nature or asymmetry is induced (Figure 1).

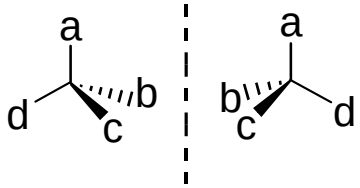


Figure 1: Chiral tetrahedonal atom and its two possible antipodes

Other atoms, which can function as chiral centers are Si, Ge, N, P, As, Sb and S. They also lead to a chiral molecule with a pyramidal structure. Furthermore, consider that nitrogen with its three different connecting atoms can only possess chiral nature, when its free pair of electrons is regarded as a forth participant and if additional rigidity is induced by denied degrees of conformational movement in such an arrangement.

When considering a molecule as chiral, it is also important to take the flexibility of this molecule into account. The stability of a chiral compound depends for example on the energy barrier that prevents the inter-conversion of the two enantiomeric forms and predefines the lifetime of one enantiomeric form. Other chiral elements are for example a chiral allene. The molecular rigidity of allene is here the most important factor for chirality. Examples are two allene-metal-ion complexes or metallocenes. If the interconversion by rotation is restricted, the so called atropisomers become chiral too. The chirality of helicenes is based on a steric crowing of condensed aromates. These chiral elements mentioned above possess one of the elements, a chiral center, a chiral axis, a chiral plane or a chiral helicity (Figure 2).

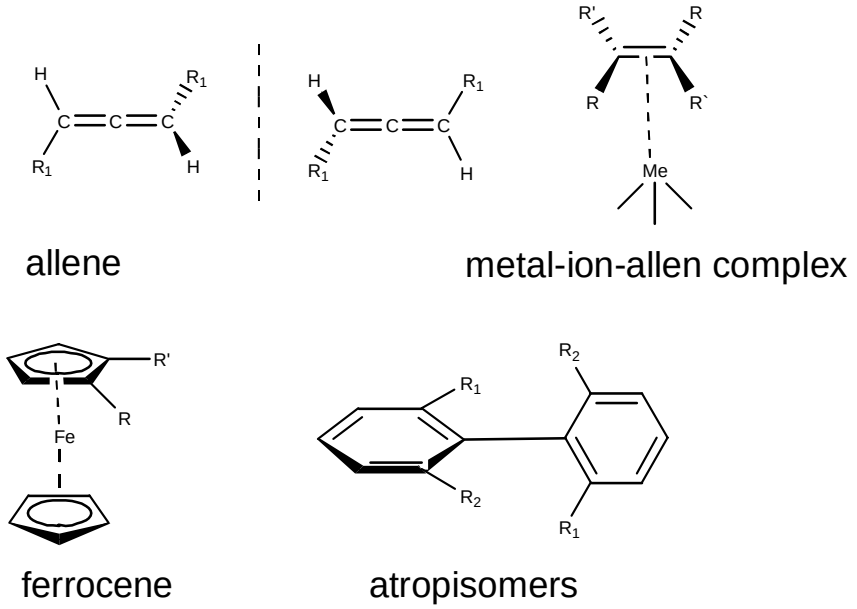


Figure 2: Chirality elements

2.1.2 Nomenclature for chirality

The accepted nomenclature for the exact and unique description of a chiral center, or more general for chirality itself, was introduced in 1966 by Cahn-Ingold and Prelog (CIP rules). Further details can be found in the original article [2]. In the first step four ligands attached to a chiral center are numbered according to the CIP-rules. Checking the direction in which these ligands are numbered leads to a clockwise or anti-clockwise arrangement. In the former case the chiral center is designated as (R) for *rectus* (*right*) or as (S) for *sinister* (*left*). For amino acids and lower carbohydrates the Fischer convention (D - *dexter* and L - *laevus*) is also common. Note that *dexter* means right side and *laevus*

stands for left side. Find below an example for the Fischer projections using glyceralaldehyde as a reference compound (Figure 3).

Figure 3: Reference compound for the "Fischer Projection" D/L-glyceraldehyde

Natural amino acids have all (S)-conformation, except glycine, which is non chiral and cysteine which has to be nominated as (R), according to the priority rule of CIP nomenclature. Regarding the Fischer projection, these natural amino acids have the L-descriptor. The majority of mono carbohydrates are right handed, according to the Fischer convention (D).

2.1.3 Diversity of chirality

Amino acids, which are the building blocks of peptides and proteins, give biomolecules such as enzymes the first instance of chirality. The different substructures, namely the primary, secondary, tertiary and quaternary structures of a protein, facilitate the 3D conformation of enzymes. This multi-layer design of larger proteins leads to the unique ability of enzyme to molecularly recognize target analytes and to catalyze certain chemical reactions. A simplified model for enzyme-educt interaction is the “lock-key” model where an educt molecule fits into a specific binding pocket of the enzyme, which is thereby induced to catalyze a transformation reaction with this analyte.
The term “chiral pool” is related to a range of naturally available small chiral substances, which exist mostly in high enantioselective purity and are available in large quantities at low cost [1].

Widening the horizon to biomolecules, like enzymes, polysaccharides and deoxyribonucleic acids, it may be obvious that these classes of substances are the building material for every biological matrix. DNA possesses a chiral nature, due to its helicity. Enzymes, the biocatalysts, are built of chiral amino acids. In addition, the functionality of enzymes is based on their substructures. Besides enzymes are often build up as complexes, containing a number of subunits and cofactors. Frequently metal ions can be incorporated. Polysaccharides can exist as a complex mixture of different polysaccharides or may even form aggregates with other biological compounds such as proteins. The diversity of polysaccharides is much greater than that of proteins or DNA. Cellulose is the most abundant biological substance in the world. It is composed of β-glucopyranoside units, which are 1,4-glycosidically bonded to each other. Cellulose exhibits a superstructure, where hydrogen bonds, helicity and to a certain degree also branching is involved in structure formation. It is well known that cellulose molecules have

chiral cavities which are based on this superstructure.

The reason why an amino acid is almost exclusively produced in its L-form by each living system is still a non-understood phenomenon. In biological tissues of higher animals, D-amino acids are normally only present in trace amounts. Lately, higher levels of D-amino acids have been recognized in special tissues and body fluids, for example in kidney and urine, of mammals and humans. They are expected to be potential drug candidates and biomarkers [3]. For example, D-Ser and D-Asp are known to have physiological relevance for humans. D-Ser is a neuro-medulator of N-methyl-D-aspartate, a subtype of the glutamate receptor in the brain. D-Asp is a hormonal regulator, found in the secretion of various glands. Several serious diseases also brought into relation with D-amino acids, namely Alzheimer, schizophrenia or renal disorders [4]. It is reported that D-Pro will be excreted in larger amounts into rat urine, showing nephrotoxic and neurotoxic properties in rat and chicks trials [5]. The exact and accurate analysis of trace amounts of amino acids taken from body fluids still poses an analytical challenge.

2.2 Impact of chirality on biological systems

One has to recognize that the whole life is based on chirality, each biocatalyzed reaction in this world is stereochemically determined. Chirality is a fundamental aspect of the living world and it provides a huge diversity for biological systems. In a more dedicated field of application, regarding the interaction of a drug with a biological system, the drug induces firstly a cascade of reactions which will then lead to a physiological response. Since each reaction is stereoselective, the two enantiomers of a drug compound cannot provide the same response in biological matrices. Which enantiomer exhibits the desired positive effect, depends on the chemical complementary between the bioactive compound and the biological interaction site [1]. An example can be given by the different taste of D- and L- amino acids. While the D-enantiomers of asparagine, histidine, isoleucine, leucine, tryptophane and tyrosine have a sweet taste, the L-enantiomers are tasteless or bitter. Also the antipodes of terpenes, such as carvone and limonene, exhibit different odors. (R)-(–)-carvone smells like spearmint, whereas (S)-(+)-Carvone is associated with the smell of caraway. (R)-(+)-Limonen and (S)-(–)-limonen smells like orange and lemon, respectively (Figure 4) [6], [7].

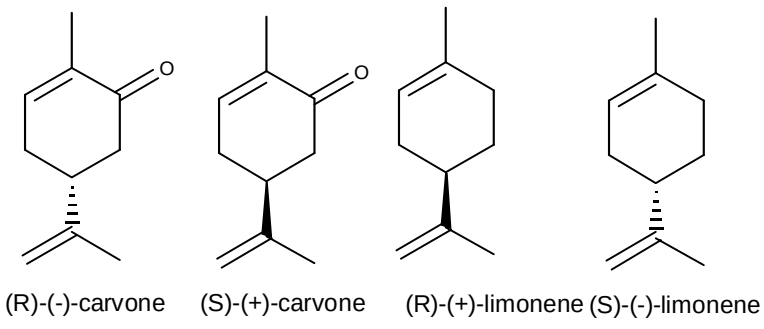


Figure 4: Pairs of enantiomers of carvone and limonene

2.2.1 The impact of chirality on pharmaceutical drug development and design

Comparing affinities, the more affine enantiomer is called eutomer and the other one distomer. The eudismic ration expresses their pharmacological activity or the degree of complementarity with the molecule interaction site. The ratio increases with the activity of the eutomer, a relationship known as the "Pfeiffers rule" [8], [9].

It was the contergan® disaster in the early sixties of the past century, which has depicted the necessity for "enantiopolishing" of bioactive drugs. The drug contergan®, also known as thalidomide, has been applied as a racemate. While the eutomer, (R)-(+)-thalidomide, has a sedative effect although the racemate has been prescribed as sleep inducing drug to pregnant women. The other enantiomer, the distomer (–)-thalidomide, has exhibited a teratogenic behavior. As a result, the application of the racemate has provided serious malformation occurring by many newborns. This tragic case has also highlighted the need for detailed pharmacological and pharmacokinetical studies. Since thalidomide undergoes a natural *in vivo* racemization, even the production and intake of the pure (R)-(+)-enantiomer may inevitably have lead to the same crucial effect in pregnant women [7], [6].

Drug regulatory authorities have issued many new guidelines and edicts, concerning the enantiomeric purity of chiral drugs and the characterization of the pharmacological profile of such a drug. Special attention is given to the so-called "*isomeric ballast*". Two enantiomers have to be regarded as different compounds and one has to consider that the enantiomers can have very different pharmacological and toxicological profiles. Nowadays racemates will seldom be approved, simply because their enantiomers are unstable *in vitro* or undergo racemization *in vivo*, have similar pharmacokinetic, pharmacodynamic and toxicological properties or because their separation is technically not feasible [7], [6].
Nonetheless, the pharmaceutical industry still puts apparent effort into the development of enantiomerically pure chiral pharmaceuticals, produced either by an asymmetric synthesis or post-purified via enantioselective clean-up steps. Rational and target oriented drug design as well as the investigation of stable formulations for potential new drugs are important attempts for research and drug development [1].

2.3 Systematic approach to enantiomerically pure substances

There are two approaches to get an enantiomerically pure antipode, the chiral approach and the racemic approach. The first is using the stereoselective (asymmetrical) synthesis, which in the best case yield an enantiomer with high enantiomeric excess (ee).

The racemic approach is less time and cost intensive, but has to furnish the separation of stereoisomers, diastereomers and mostly also enantiomers in order to obtain the pure antipodes of interest in acceptable yield. An enantioselective step has the benefit that both enantiomers are separated in high purity, which

may be favorable in terms of drug research and development, concerning structure optimization. The separation part of the racemic approach can be split into separation techniques like enantiospecific crystallization and enantioselective chromatographic separation techniques using resins or membranes as chromatographic media. In general, liquid chromatography using a chiral stationary phase (CSP) is the most frequently used tool for obtaining pure enantiomers as illustrated below. It is also regarded as the technique of choice for the determination of enantiomeric excess of organic compounds after enantiopolishing from their racemic mixture (Figure 5).

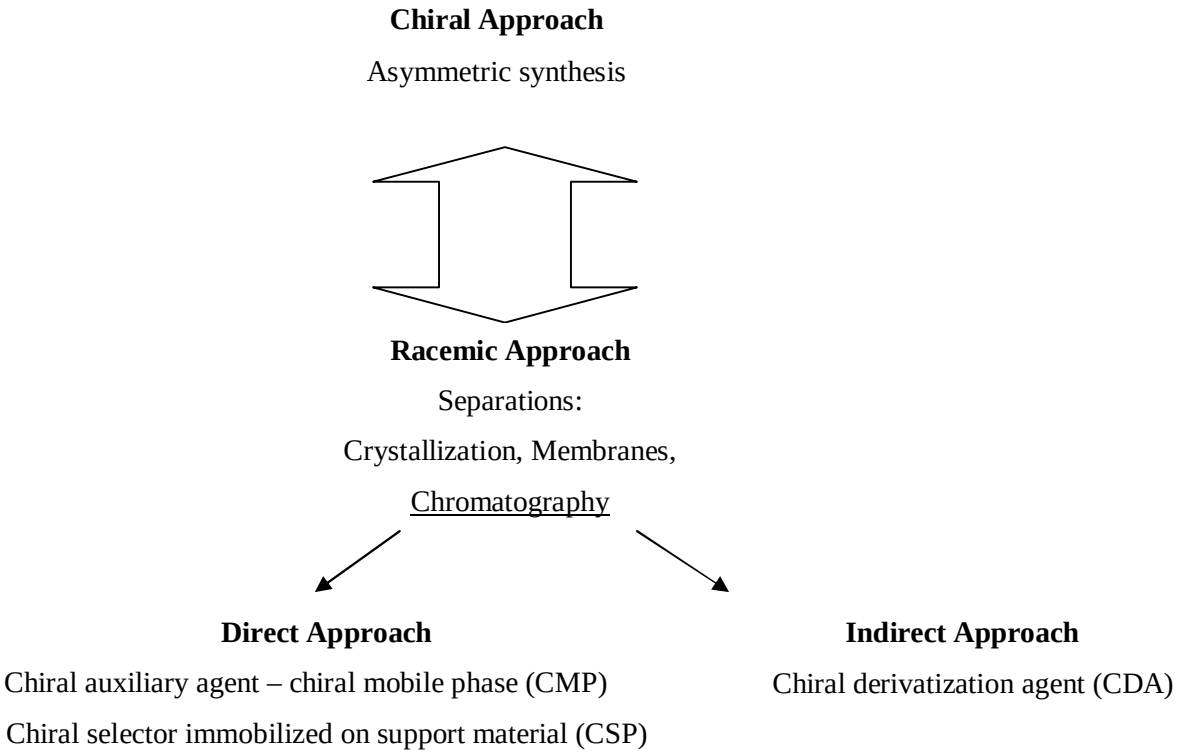


Figure 5: Illustration of the different approaches for getting an enantiomerically pure substance

Optical resolution via chromatographic separation techniques is again subdivided into the indirect and the direct approach. The indirect pathway normally involves the derivatization of a racemate, or a mixture of enantiomers, employing a suitable enantiomerically pure derivatization reagent, producing thereby diastereomers. Note that diastereomers do not coincide in their configurational state and exhibit different conformational arrangements. These substances have different physico-chemical properties and can be separated using an ordinary separation systems, like a common liquid chromatography system using a reversed phase column. Attention must be paid to chiral derivatization agent (CDA), of which the highest possible ee shall to be used. Otherwise the derivatization reaction itself may lead into the formation of a mixture of diastereomers and one or more pairs of enantiomers. Another problem can be the kinetic enantiomer resolution, since the formation of diastereomers will have different rates of reaction. Excess of derivatization reagent are applied and completion of the reaction has to be

monitored. The diastereomers should not have an equal detection response, if for example UV detection is used. This method is less prone to errors compared to the direct separation approach.

The direct approach fulfills an enantioseparation using either a chiral auxiliary reagent, which is added to the mobile phase, or a chiral stationary phase (CSP), for the separation of the two antipodes. Both methods have in common that a transient complex of chiral selectors (SO) and chiral analytes (SA) are formed. A difference in stability and persistence of the SO-(S)-SA and SO-(R)-SA complexes lead to diverging retention properties during the chromatographic separation process.
The indirect approach uses a chiral mobile phase (CMP) and a non-chiral chromatographic system. The latter is a simplification of the instrumentally requirement. The SOs can be divided into ligand-exchange metal-ion complexes, ion pairs and inclusion complexes, such as cyclodextrines, proteins, macrocyclic molecules, amino acids and crown-ether. Solubility in the mobile phase and availability of bigger amounts of these chiral auxiliary compounds are prerequisites for their routine application. The addition of a chiral auxiliary to the mobile phase is not applied routinely and is therefore of minor interest (Figure 6).

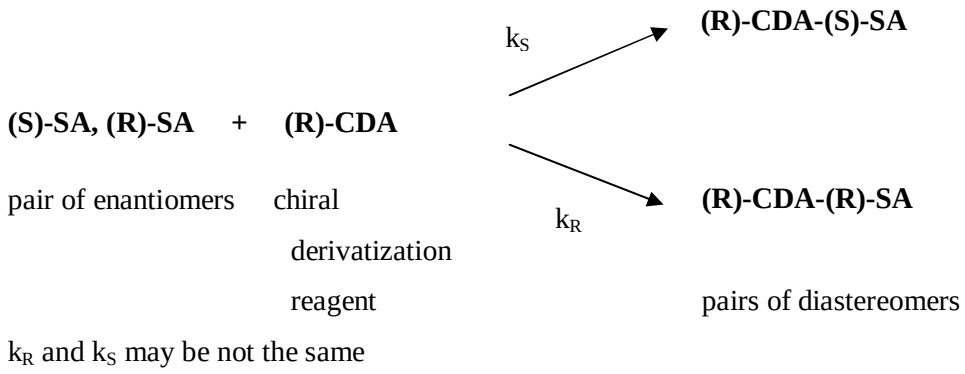


Figure 6: The indirect approach of an enantioselective chromatography

Novel, tailor-made CSP are developed and examined extensively by many researchers all over the world. A series of different kinds of CSPs are nowadays available for enantioseparations in analytical scale as in preparative scale. Chiral selectors are either coated or more frequently covalently bonded onto a solid support material, mostly silica gel. From the macroscopic viewpoint, enantiodiscrimination is based on symmetry factors, namely that the steric fit of one enantiomer is higher than that of its mirror image, but also thermodynamic interaction properties come into account. Explanations for the chiral discrimination process are often based on empirical considerations, combining thermodynamic parameters and structural elements, the binding sites of SA and SO.

An overview of the different types of CSPs, their applications, advantages and drawbacks is provided in reference [9]. Shortly, the different types of CSPs will belong to one of following categories: a) A SO-

SA-complex is formed mostly by attractive interactions such as hydrogen bonding, π-π-interactions, dipole stacking, but also by hydrophobic interactions b) While primarily attractive interactions form the complex, inclusion complexes may also play an important role. c) The SO offers chiral cavities, where the solute can enter and an inclusion complex is formed. d) The analyte takes part in a diastereomeric metal complex formation, which resembles a chiral ligand-exchange mechanism. e) Alternatively, proteins or macrocyclic molecules build the chiral selector with hydrophobic and attractive interaction as driving forces. Enantioseparation using CSPs exhibits some important advantages, namely method robustness, the chiral columns are reusable and a high performance chromatographic system is standard equipment in any analytical laboratory. Importantly, the antipodes can be won both as purified substances. The upscale to preparative approaches, using chiral stationary phases, hits more the point, because of the possibility of the use of bulk mobile phases and automatization [6], [7], [9].

2.4 Enantioseparation using liquid chromatography

In the first instance, enantioseparation by liquid chromatography (LC) is related to molecular recognition, which describes an interaction of two or more species of chemical molecules, whereas chiral recognition highlights some special aspects of the more general molecular recognition. Both imply the formation of a transient complex in a mixture due to a selective interaction. During an enantioseparation via a liquid chromatographic process, one enantiomer shows different interaction with the stationary phase than its counterpart, which means that the interaction pattern of the pair of enantiomers can be different or an enantiomer can be completely discriminated from a selective interaction. An interaction can be attributed to structural elements of both, the chiral selector (SO) and the analytes (SA). The structural elements have normally some relationship in their chemical behavior. Mostly, the principle of structural complementary is applicable. In a LC process for enantioseparation, the selector and the enantiomer forms a diastereomeric non-covalent complex located at the interface of mobile and stationary phase. A chromatogram is an illustration of a time-averaged view of these events, which reflects the change of free energy according to an SO-SA complex and the free enantiomer.
The difference in free energy of the diastereomeric adsorbate complex is indirectly a result of structural relationships, dynamics and solvation. To control the extent of these differences is the practical issue of a successful enantioseparation [10].

2.4.1 The three point interaction model

The three-point-interaction model describes the mechanism of chiral recognition [11]. The thesis postulates that at least three simultaneous interactions between the chiral selector and at least one of the enantiomers are necessary for a chiral recognition. Furthermore, one of these interactions has to be stereochemically determined, which means that the replacement of one enantiomer with another has to alter or diminish the interaction between selector and analyte. The three point interaction model is often illustrated as a tetrahedron model, where three interaction sides form a plane. Dependant of chirality of

the analytes and the chiral selector the interaction will takes place. Mostly, these interactions have an attractive nature, but this is not a requisite, repulsive steric interactions take also part in the chiral recognition process.

It ought to be mentioned that considering the conformational and configurational rigidity of chiral analytes and chiral selectors, a chiral species with rigid conformational degrees of freedom appears with an arrangement of its interaction sights being strongly dependent on its configuration. This complies more satisfyingly with the geometrical requirements for the three point interaction model compared to a species with many of possible conformational states [10].

The three point interaction is a good matching model for chiral recognition but the latter cannot be reduced to the three point attachment model [11], [12].

Another way of chiral discrimination can be achieved by the use of a chiral matrix or with stationary phases containing chiral cavities. In the case of chiral matrixes, the enantiomers are in a chiral "environment" and distinction will arise from chirality itself. A chiral cavity provides different accessibilities of the enantiomer pairs for the cavity, which may be comparable to a kind of size exclusion mechanism [9].

2.4.2 Thermodynamic aspects of enantioseparations

The parameters, which describe the varied behavior of a pair of enantiomers, during enantioseparation, are the capacity and the selectivity factor. Both are thermodynamically determined and enable the quantification of the difference in free energy of the diastereomerical SO-SA complexes. In principle, the capacity factor describes the retention for an analyte in a defined set-up (Formula 2).

$$k_{1,L} = \frac{t_{R,L} - t_{Ro}}{t_{Ro}}$$

Formula 2: Retention factor exemplified for the L-enantiomer

Retention factor includes the thermodynamic contribution of enthalpic and entropic forces (Formula 3).

$$k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + ln\theta$$

Formula 3: Retention factor in thermodynamic increments expressed

The selectivity factor for enantioseparation is described as the ratio of the retention factor of the later eluting enantiomer to its earlier eluted counterpart (Formula 4).

$$\alpha = \frac{k_L}{k_D}$$

Formula 4: Selectivity coefficient

The equation can be also written as (Formula 5):

$$\alpha = \frac{e^{\frac{-\Delta G_D^m}{RT}}}{e^{\frac{-\Delta G_L^m}{RT}}}$$

Formula 5: Selectivity coefficient expressed as the ratio of the e-function of enantiospecific free energy ΔG

Whereas $-\Delta G_D^m$ is the molar free energy of adsorption. The essential difference of free energy due to the formation of diastereomeric adsorbates can be expressed by the below stated equation (Formula 6):

$$\Delta(\Delta G)^m = -RT\, ln\, \alpha_{DL} = \frac{e^{\frac{-\Delta G_D^m}{RT}}}{e^{\frac{-\Delta G_L^m}{RT}}}$$

Formula 6: Difference of Gibbs free energy of the two enantiomers, expressed in term of the selectivity coefficient

The combination of Formula 3 and Formula 6 provides the following expression (Formula 7):

$$ln\, \alpha = -\frac{\Delta\Delta H}{RT} + \frac{\Delta\Delta S}{R}$$

Formula 7: Gibbs-Helmholtz equation

ΔΔH and ΔΔS shows the difference of enthalpy and entropy in course of chromatographic separation. This relationship is visualized by the van't Hoff plot (lnα *vs.* 1/T), with which an enthalpic or entropic control of enantioseparation can be distinguished. ΔΔH/R is the slop of the linear graph and ΔΔS/R is the intercept on the y-axis. The model makes the presumption that the retention of analytes is mainly driven by enantiospecific interactions, otherwise one has to deal with additional non-enantiospecific retention increments (Formula 8) [13], [10].

$$k_{app,R} = k_{ns} + k_{se,R} \quad \text{and} \quad \alpha_{app} = \frac{k_{ns}+k_{se,R}}{k_{ns}+k_{se,S}}$$

Formula 8: Retention factor and selectivity coefficient expressed as the sum of stereoselective (se) and non-stereoselective (ns) contributions

Investigations have shown that the entropy contribution to the chiral enantioseparation is frequently negligible. The entropic contribution is related to solvation effects and reordering of SO-SA complex [9].

It should be taken into account that high absorption energies (ΔG) can lead to higher retention times and as a consequence of the chromatographic process to stronger broadening of peak shapes. An operator has to maximize the difference in free energy $\Delta(\Delta G)$, while keeping (ΔG) at an acceptable level in order to maintain good chromatographic efficiencies. For an estimation of the performance of an enantioseparation, note that a chromatographic system with good efficiency, which furnishes tight

peaks, can afford small values of enantioselectivity $\Delta(\Delta G)$ in order to achieve acceptable performances [10].

2.5 Fluorescence labeling

2.5.1 General considerations

The labeling or derivatization of an analyte provides essential advantages, if the substance of interest lacks a suitable chromophore or fluorophore for a spectroscopical detection. Such a label or tag will also provide a much lower limit of detection for the labeled species compared to its native form. Furthermore, labeling may also change the chemical behavior of the molecule. A changed strategy of chromatographic separation or detection may even be beneficial. For example amino acid analysis (AAA), can be done using an ion-exchange chromatography, because amino acids have zwitterionic character. Some amino acids have a lack of chromophore and cannot be detected with a common UV/VIS detector. After a derivatization step, which incorporates a spectroscopically detectable tag, into the analyte molecule, more common reversed phase or electrophoretical separation techniques are applicable and most of the frequently used detectors can be applied [14]. The derivatization increases sensitivity and selectivity, which is especially interesting and even necessary for quantitative analysis of analytes from biological tissues. If the tagging reaction is highly selective, than only a group of substances or only a certain type of functional entities will be derivatized and can be addressed to a special separation or detection strategy. Generally, there are two approaches for derivatization. "*Pre-column derivatization*", describes a derivatization step, followed by an optional purification step and a chromatographic separation with detection. A "*post-column derivatization*", however means that after a chromatographic separation has taken place, the actual derivatization step is conducted in e.g. a sample loop, followed by the immediate detection of the derivatives. The advantage of the pre-column derivatization compared to the post-column derivatization is that the procedure is more flexible, concerning reaction time, heating steps and a chromatogram will not have a continuously higher baseline and band broadening. Also, dilution effects will not disrupt the chromatogram. On the other hand, the final procedure for a pre-column derivatization has to assure the quantitative derivatization of both, the standards and analytes, derived from real samples. It should be kept in mind that the derivatization reaction performed for a range of analytes will be highly competitive and differing reaction rates can be expected. Furthermore, reagent related peaks can interrupt the separation performance of analyte molecules [15].

The fundamental aspects of fluorescence spectroscopy and their detection ability will not be discussed. For further reading, the following references may be of interest [16], [17].

2.5.2 Common fluorescence labels for amino acid derivatization

Chemical structures of some common fluorescence labels are shown in Figure 7. Most tagging-reagents are reactive to amines, primary and ideally also to secondary. Ortho-phtalaldehyde (OPA) is one of the most common fluorescence reagents. It reacts only with primary amines in the presence of a thiol species, like mercaptoethanol and produces an isoindole derivative. The derivative can be detected with an excitation (ex) wavelength of 340 nm and an emission (em) wavelength of 440 nm. The reagent is used in excess, but since ortho-phtalaldehyde itself is not fluorescence active, it will not interfere in the separation of the isoindole derivative. OPA reacts at ambient temperatures and alkaline conditions with primary amines in several minutes, which facilitates the derivatization. Limit of quantification of OPA derivatives of <1 pmol are reported in literature [18].

Another frequently applied tag is 9-fluorenylmethylchorformate (FMOC-Cl). It is also commonly used in peptide synthesis as a fluorescence active protection group. The chloroformates react in alkaline buffered solution with primary as well as with secondary amines. The reaction mechanism, a S_n2 based Schotten-Baumann reaction, is well explored. It leads to a carbamate derivative. Several side products are known, which are formed in turn of the derivatization. A symmetric carbonate is formed and FMOC reacts also with water, which produces FMOC-OH. All by-products have to be separated from the analyte derivative peaks. A clean-up step, employing an extraction with apolar solvent can remove most of the excess reagent. FMOC methods are often applied as fully automatized HPLC amino acid analysis. The excitation wavelength is 260 nm and emission wavelength 315 nm, the fluorescence quantum yield is 0,5. Lysine, tyrosine, histidine and cystine and tryptophane will be double derivatized and these derivatives have increased lipophilicity. In a reversed phase chromatography system the hydrophobic derivatives will have extended retention times. Because of an internal quenching effect, the limit of detection for the double derivatized analytes is normally higher. Lowest reported LOD is 50 fmol for amino acid derivatives [19].

Also, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) is a common fluorescence tag, which is reactive to primary and secondary amino acid. NBD-F reacts within minutes in an alkaline media. A secondary amine is formed. The reaction conditions are different compared to the other here described procedures [20]. The excess of derivative resembles also a drawback, because a possible coelution of the regent peak hinders the separation of a whole series of analytes. Excitation wavelength is 470 nm and emission wavelength is 530 nm. The reported LOD is below 100 fmol. Recently NBD-F is often used for 2D-AA-analysis in a reversed phase system, coupled with an enantioseparation column in the second dimension for the determination of the amount of D-amino acids in biological fluids [4], [5],[3].

Dansylchlorid (DNS), 5-(dimethylamino) naphthalin-1-sulfonylchloride, is also often used. The so called “dansylation” is frequently conducted as a pre-column derivatization in capillary electrophoresis.

It exhibits a supreme UV absorption, but strong quenching effects arise in aqueous solutions. Therefore it is used as a model compound and much less in actual analytical applications, because of unfavorable by-products and quenching effects [15].

A broad range of other fluorescence active labels can be found in literature, all with their applications, all descriptions include their field of applications as well as their advantages and drawbacks. For instance, naphyltene-2,3-dicarboxaldehyde (NDA) has the same reaction mechanism as OPA, but produces more stable derivatives [15]. Fluorescamine is a non-fluorescent label, which reacts with primary and secondary amines to yield highly fluorescent derivatives. It is used for the labeling of proteins and amino acids prior to quantitative analysis as well as for the chiral separation of single amino acids [21].

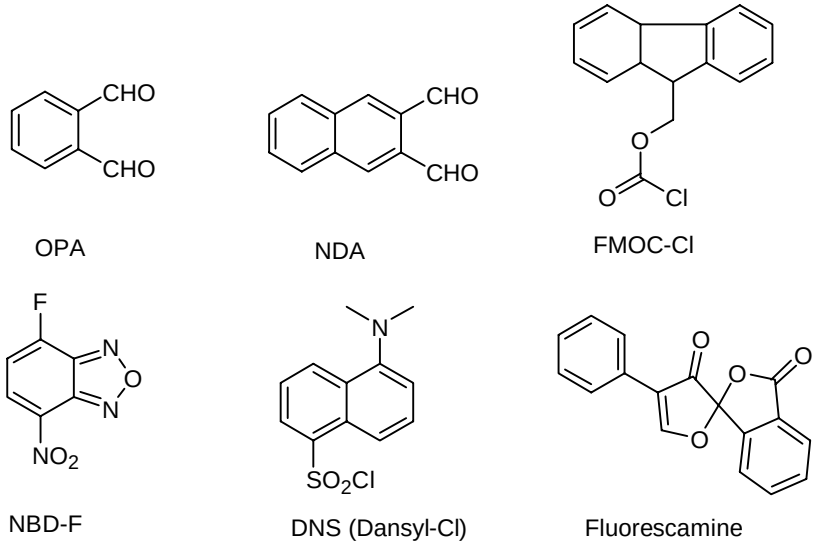


Figure 7: Common fluorescence labels

2.6 AccQ – Fluorescence Tag

The AQC tag (AQC 6-Aminoquinolyl-N-Hydroxysuccinimidyl Carbamate) is a fluorescence active, and amine reactive urea derivative that was introduced by Cohen et al. [22]. The active N-hydroxysuccinimide ester is very reactive with nucleophiles like primary and secondary amines under alkaline conditions and the reaction led within several seconds to mixed urea derivatives (Figure 8).

The excess amount of AQC is quickly hydrolyzed to 6-aminoquinoline (AMQ), the N-hydroxy succinimide and carbon dioxide in the aqueous buffer solution with pH 8 following [23]. Within several days the symmetric bis-quinolyl urea will be generated, because AMQ is a weak nucleophile and can react with still existing AQC. This symmetric urea is not soluble in the derivatization mixture and

precipitates. Due to te derivatization, the amino acid derivatives show a blue shift in the emissions spectra. Typically, the excitation wavelength applied is 250 nm and the emission wavelength of the derivative is about 395 nm. In comparison the 6-aminoquinoline is also excited at 250 nm, but its emission maxima is shifted to 520 nm. UV detection is also applicable with excitation coefficients typically exceeding 22,000 [24]. Dependencies of fluorescence intensities towards water content and pH in solution are yet known [22].

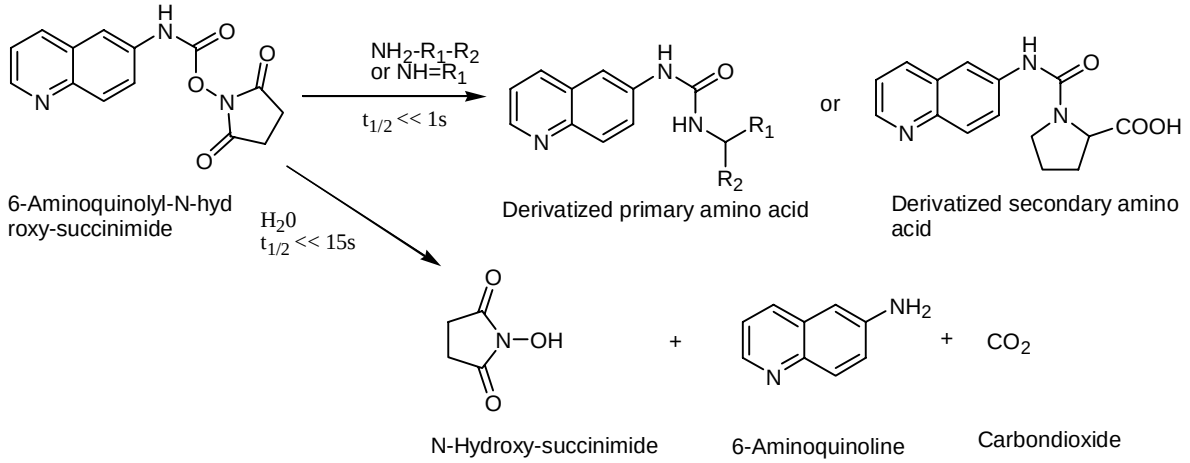


Figure 8: Reaction pathway of 6-Aminoquinolyl-N-Hydroxysuccinimidyl Carbamate

The urea derivatives are stable, for at least one week. The major application field is amino acid analysis (AAA) [25], [26], [27]. Also for the analysis of biogenic amines in blood, body fluids and food are procedures reported in literature [28], [29]. However, standard operating procedures (SOPs) for AAA are well established but several uncertainties can be found in literature. Due to an internal fluorescence quenching effect induced by the indole ring of tryptophane and the AQC quinoline group, of the AccQ-tag, this amino acid can barely detected via fluorescence detection, UV detection can be successfully applied [30], [24]. Besides lysine also other diamino species like ornithine or thiol-containing amino acids such as cystine will be double derivatized. The phenolic hydroxyl group of tyrosine forms an unstable and transient intermediate, which is thermally destroyed within ten minutes at 55°C, in the course of the derivatization procedure. Under alkaline conditions, cysteine will be oxidized to the dimer species. Derivatization of free cysteine or cysteamine forms also the bis-derivative via a thioamide linkage [31]. Alcohols react very slowly with AQC, as has been proven by analyzing a solution of AQC in methanol, but there is yet no evidence that the hydroxyl groups of serine or threonine are also derivatized. Chromatographic conditions for the separation of all twenty proteinogenic amino acids are well established [28], [25] and mostly contain only slight modifications from the original procedure described by Cohen et al. [22]. Using mild acidic conditions, between pH 4,95 and 5,75 for the mobile phase, the excess of derivatization reagent AMQ elutes prior to most of the analyzed amino acids, except of acidic compounds such as cysteic acid. The tuning of the mobile phase pH, allows the

modification of elution strength and elution order. This affects, especially the polar amino acids, in a strong manner. Employing a high performance liquid chromatographic system with a quaternary pump combined aqueous eluents with different pH values, allows a supreme resolution optimization for all twenty amino acids within one chromatographic run [25]. Generally, the pH dependencies of the AQC-amino acid derivatives are a drawback, leading to a reduced stability against mobile phase uncertainties of the method.

2.7 FITC – Fluoresceine isothiocyanate

The common fluorophore fluoresceine can be linked via a thiourea linkage to nucleophiles such as primary amines (Figure 9). Fluoresceine is mostly used in biochemistry for labeling of cells, cell active substances like anti bodies, proteins and intra cellular compartments. In the field of amino acid analysis, fluoresceine isothiocyanate is only used in a few cases. It was determined that the derivatization efficiency reaches a maximum, when the reaction time is set to twenty hours or longer in a mixture of 20 mM borate buffer pH 9,8 with 10 % acetonitrile [32]. Applications using fluorescence emission are conducted by laser-induced-fluorescence (LIF) at an excitation maximum of about 395 nm and an emission maximum near 520 nm. Separation of 21 derivatized amino acids during one run employing capillary electrophoresis (CE) with different types of back ground buffers has been performed [33]. Enantioseparations of twenty racemic amino acids were successfully applied using a mixture of β-cyclodextrine and sodium taurochlorate as chiral selectors [34].

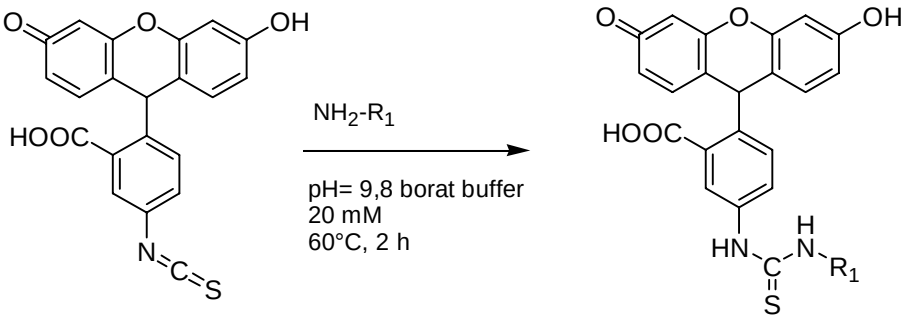


Figure 9: Reaction pathway of fluoresceine isothiocyanate with primary amines

2.8 Strategies for the analysis of cysteine

Cysteine is one of the twenty proteinogenic amino acids. In biological matrices this amino acid is mostly present as the dimer cystine. For quantitative analysis of cysteine different approaches can be found in the literature. The analysis and quantification of native cysteine is mostly not practicable, because of the formation of the dimer cystine, especially on air and in neutral to alkaline conditions.

Therefore a number of derivatization procedures for the reactive thiol group were introduced. In many AAA, using the before mentioned AQC-label, this amino acid is analyzed as the cystine species [28]. This approach is based on the assumption that just one species of cysteine, the dimer, is present in the sample. A proposal, which will not always hit the goal [24]. The derivatization with halide alkyls, such as iodo acetamide or iodo acetic acid, is often recommended. Samples, containing a biological matrix, have to be reduced with 2-mercaptoethanol [35] or tris-(2-carboxyethyl)-phosphine (TCEP) [36] [37] or other phosphines [38] to get the monomer cysteine in first instance. After reduction of dithiols, derivatization with a number of reagents is investigated in the literature. Iodo acetate and iodo acetamide are the most frequently used derivatization reagents [35], both lead to an acid stable derivative and therefore are suitable for an acid hydrolysis in the case of an AAA of a biological matrix. 4-vinylpyridine is another good known derivatization reagents [28, 39] [40]. Another approach is the derivatization of cysteine with 3,3´-dithiopropionic acid, where a mixed disulfide results due to the reaction. Frequently, cysteine and methionine are oxidized during a performic acid hydrolysis to cysteic acid and methionsulfone, respectively and quantification is conducted afterwards [41, 42] (Figure 10).

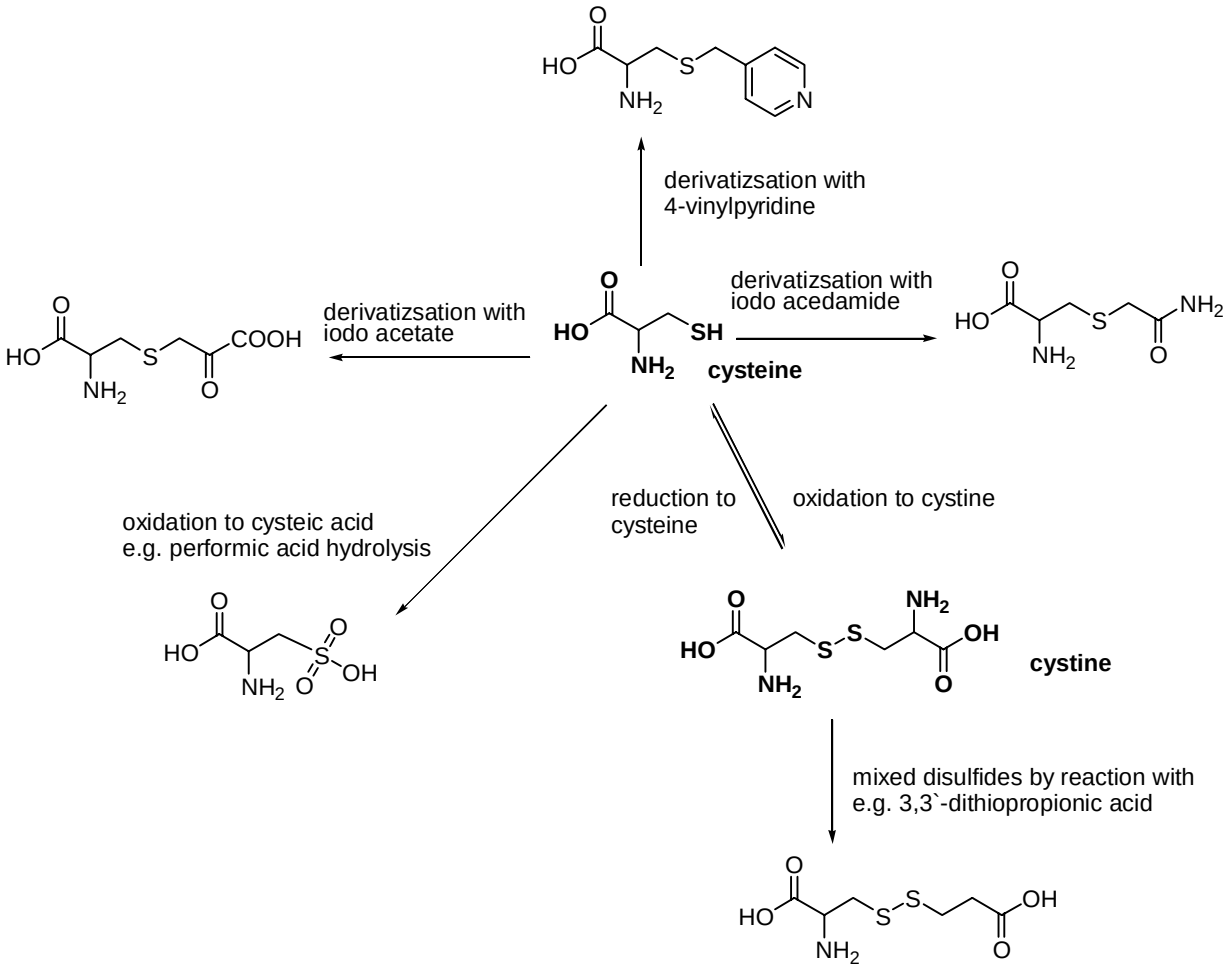


Figure 10: Derivatization strategies for the analysis of cysteine

However, the determination of cysteine is still an unsolved problem for quantification and for enantioselective analysis.

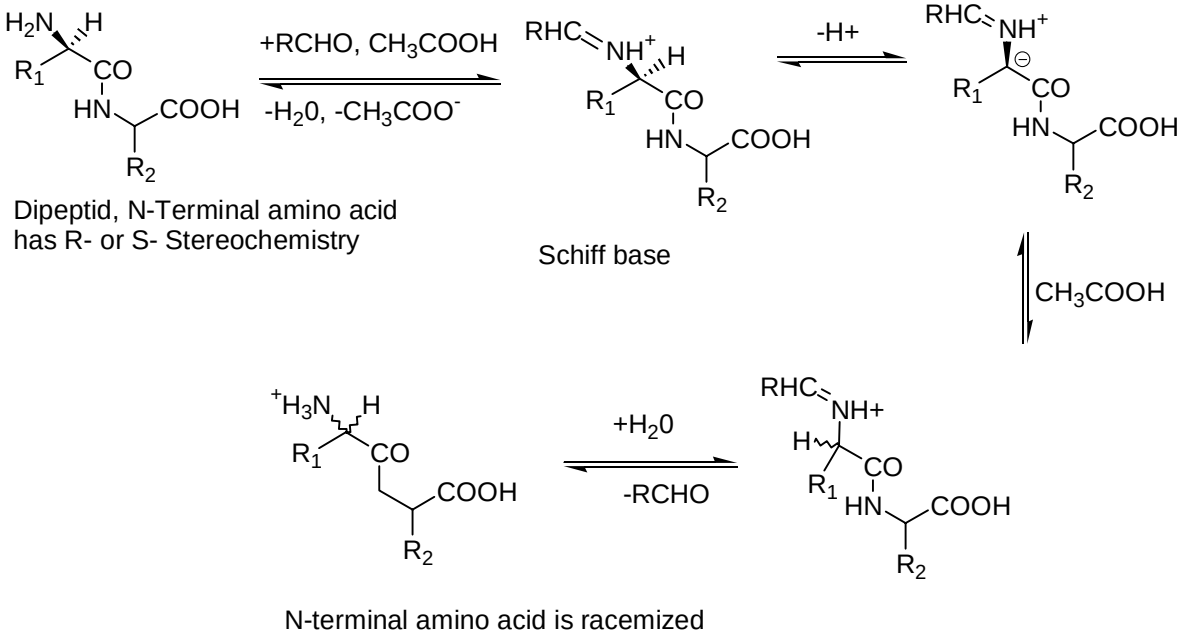
2.9 Investigation of the chiral separation of peptides and peptide stereoisomers

The enantioseparation of peptides via liquid chromatography is not as straight forward as it is regarding to amino acids. Especially the growing number of stereoisomers due to the growing number of amino acids in a peptide chain renders them often to be hardly separable. Dipeptides containing glycine, especially if glycine is N-terminal located, are often badly enantioseparated. The task of peptide stereoisomers separation is often present in peptide synthesis.
An investigation of retention mechanism and chiral discrimination process of a quinine-based chiral selector has also been investigated [43], [44] and [45]. For all-D/L-alanine peptides, up to the hexamer, the chiral discrimination is found to decrease with growing chain length. The overall retention is also decreased with the addition of each amino acid. A glycine effect was found. An achiral bridge at the C-terminus affects the chiral recognition process more strongly than an achiral bridge at the N-terminus of di- or tripeptides. A glycine in the peptide chain back bone gives the molecule more conformational degrees of freedom and more SO-SA complexes can be formed. This variety in complex formation leads to a reduced or diminished enantiodiscrimination [46].

2.9.1 Amino acid racemization

A racemization of an amino acid can be performed in an acidic milieu and on addition of an aldehyde species, according to the protocols of [47], [48]. These references showed excellent yields up to >90 % in case of an amino acid, but no investigation were found due to di- or oligopeptides. The reaction mechanism was proposed in the article from Yamada et al. [47] (Scheme 1).

A N-terminal located acidic hydrogen atom can be racemized during the formation of a Schiff base. Further articles like [49] have given evidence that during an acidic treatment under influence of high temperature over a longer period of time, peptides are able to form diketopiperazine-like intermediates and hence the inverted peptides and/or an inversion of the stereochemistry may appear. If the formation of a diketopiperazine species is involved during the racemization, then the inverted dipeptide with randomized stereochemistry had to be expected (Figure 11).



Scheme 1: N-terminal amino acids with R- or S- stereochemistry. Racemization of the N-terminal amino acid during the formation of a Schiff base

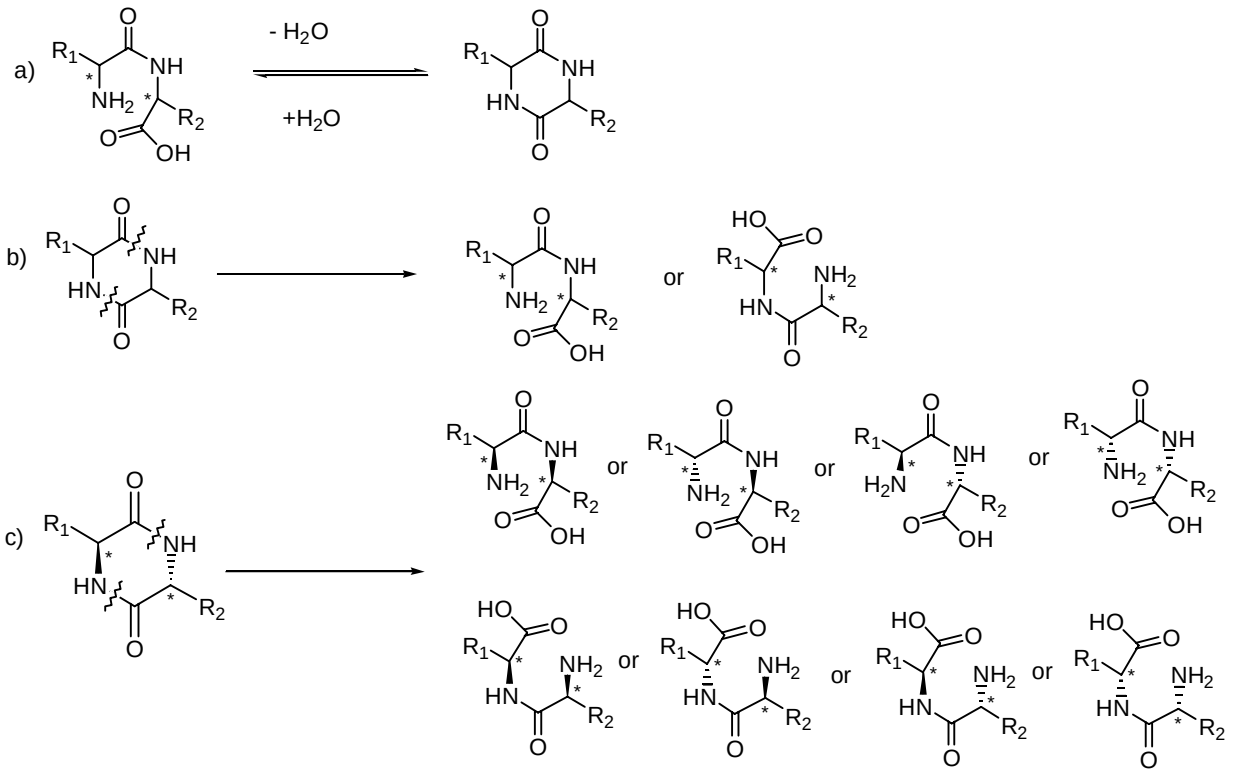


Figure 11: Possible drawbacks of a racemization of dipeptides: a) formation of a diketopiperazine b) ring opening of the amide bonds of a diketopiperazine leads to the original dipeptide or to the inverted dipeptide c) ring opening of the diketopiperazine and randomization of the stereochemistry of the two chiral carbon atoms leads to eight stereoisomers in case of a dipeptide with two chiral centers.

2.10 Quinine-based chiral stationary phase

Quinine was first time available as a pure substance in 1820. It was retrieved from the bark of the medicinal plant, cinchona. Quinine, cinchona and quinidine were known for their fever reducing properties. In fact, the chiral nature of the alkaloid quinine was first recognized by Louis Pasteur, who postulated that quinine and quinidine were left- and right handed compounds. Today they are known as stereoisomers. Pasteur had used the chiral nature of quinine for the racemization of tartaric acid and he enabled thereby the stereoselective crystallization of racemic tartaric acid with quinine, the first enantioseparation ever mentioned in human history. He also made one crucial and fascination postulation, namely that the living world itself and all biomolecules had to be asymmetric in their nature. Pasteur was with his great mind, ahead of his time [50].

2.10.1 The quinine molecule as chiral selector

More recently, the chiral nature of quinine was used for enantioseparation purposes. The separation of racemic arylalkylcarbinole and binaphtole derivatives were achieved with quinine-based CSP [51] using the normal phase mode of aqueous-free apolar mobile phases. The immobilization of the chiral selector via the radical starter mediated addition of the olefinic double bond on a mercaptopropyl support was of great importance, because the hydroxyl group on C9 position was free for interaction with the analytes anitpodes and free for further chemical modifications. For the chiral recognition the stereogenic centers at position C8/9 were known to be of highest importance. The hydroxyl group at C9, in the neighborhood of these chiral stereocenters, turned soon into the focus of interest for simple chemical modification and its influence on chiral recognition. The introduction of an acetyl group indicated a stronger reduction in enantioseparation compared to the native quinine [52]. The authors proposed a model for the chiral recognition mechanism of quinine-based CSPs, where enantiodiscrimination would progress via the formation of a diastereomeric transition state of SO and SA molecule. They emphasized that a single attractive interaction, namely a hydrogen bonding of the hydroxyl group on the selector and hydrogen bond acceptor site on the analyte, combined with a differing steric fit of the two antipodes, would ensure an enantioselective separation.

Lindner et al. had introduced a *tert*-butyl carbamate group (Figure 12), located at the C9 position of quinine, which enabled the separation of racemic acidic compounds in normal phase mode but also in hydro-organic or polar-organic mobile phase mode [53]. This new type of CSP is classified as weak anion-exchanger type chiral stationary phase (WAX-CSP).

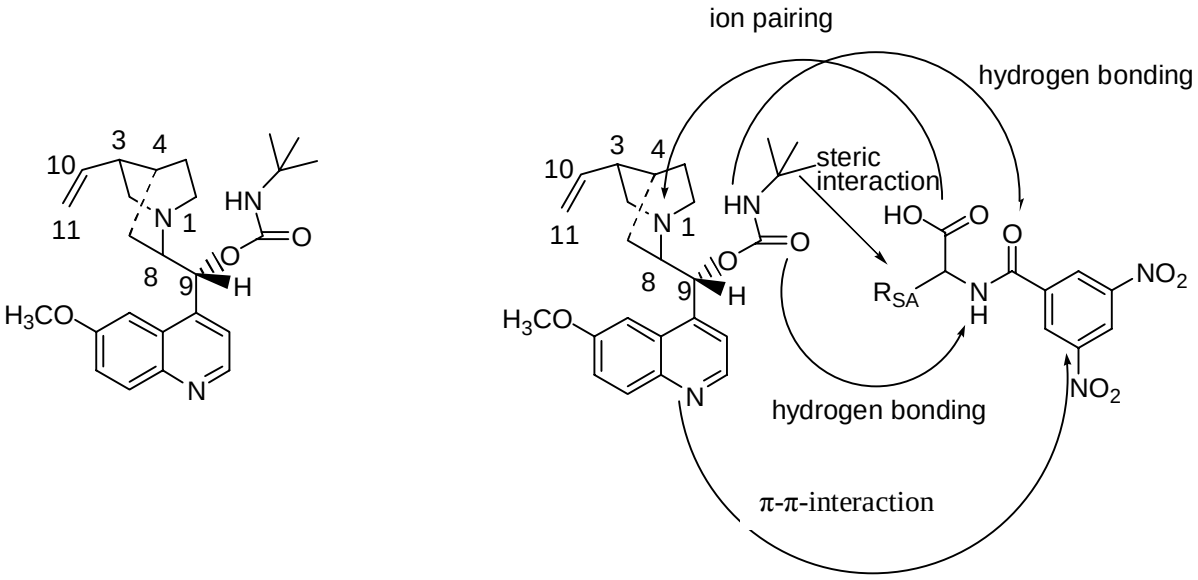


Figure 12: *tert*-Butyl-carbamate-quinine selector (1S, 3R, 4S, 8S, 9R) (left) and possible non covalent interactions between the chiral selector and a 3,5-Dinitrobenzyl-derivative (right) [53].

The tertiary amine of the quinuclidine group is protonated at pH 4-7 and therefore available for long range coulomb interactions with deprotonated acidic analytes. The retention is predominantly controlled over an ion-pairing process. The ion-pairing reinforce the formation of a specific association of SA and SO. The chiral recognition mechanism has been the topic of detailed studies [54]. The binding pocket is restricted of the quinoline, the carbamate and the quinuclidine group which span the stereogenic centers of C8/9 of the quinine molecule. If the acid functionality of a SA is blocked, e.g. because of acetylation, the chiral recognition is diminished. In order to observe chiral separation on quinine-based CSPs, the SA should possess acid groups like carboxylic, sulfonic, phosphoric and phosphonic acid. The acidic group has not to be attached directly to the stereogenic center, but better enantioseparation are achieved if the stereogenic center is located in alpha, beta or gamma-position to the acidic functional group. For SAs, having more than one acidic group, reduced enantioselectivity is often observed. The presence of two equally strong interaction sites, such as π-π- or acidic interaction, may lead to a competitive effect, which disturbs a directed orientation of the SA into the binding pocket of the SO. SO-SA complexes with varied stability are formed and the chiral discrimination mechanism will be reduced because of that [53].

Quinine and quinidine are so called “pseudo-enantiomers” because of inverted stereochemistry according to the stereocenters of C8 and C9, but in fact they are diastereomers. The chiral discrimination process has been studied in detail for N-(3,5-dinitrophenyl-)-leucine as SA and the *tert*-butyl-carbamoyl-quinine as SO. The presence of a π-π-interaction site is not a condition of chiral discrimination, but it stabilizes the transient state complex. It seems that a π-acidic aromatic moiety, like DNB- interacts much stronger with the corresponding binding site on the SO, the π-basic quinolyl ring. For an optimal fit of the π-π-interaction site, the type of linkage e.g. carbamoyle or aroyle, its flexibility and the relative distance to the stereogenic center is of great importance. Bulky side chains of amino

acids increase the chiral discrimination, whereas a modification at or near the stereogenic center with bulky substituents leads to its reduction. Generally, higher lipophilicity and steric bulkiness of the amino acid side chain leads not only to a higher retention of the later eluting antipode, but also to increased enantioselectivity. A space filling substituent at amino acid side chain stabilizes also the SO-SA complex. The X-ray structure of a most favorable SO-SA complex and the importance of the orientation of the stereocenters concerning the C8/9 position, have been investigated by Maier et al. [54].

2.10.2 Liquid chromatography using a chiral anion-exchange type quinine-based column

A typical ion-exchange behavior of the quinine-based columns is observed. The influence of mobile phase parameters on elution time and chiral recognition is well known. Elution of carboxylic acid is controlled by adjusting the pH value of the mobile phase, according to the WAX type of quinine-based CSPs. Strong dependencies of retention and enantioselectivity will arise if the pH value is altered from acidic to basic. The best results are obtainable in a pH range of 5-6, more generally pH should be adjusted near the pKa of the analyte for partially ionization. Parameters, like the pKa of the analyte, counterion and the selector (quinuclidine group), pH of the mobile phase accounts for overall retention. A hydrophobic retention increment, which does not take part in the chiral discrimination process, exhibits stronger contribution when the pH is decreased. The buffer type and concentration used is also of high importance. The buffer concentration affect the enantioselectivity in a minor extend, but the retention is decreased in the following order, citrate, phosphate, acetate and formate buffers.

For the enantioseparation of amino acids or in general, zwitterionic SAs on quinine-based CSPs, N-terminal protection for the amine group is a necessity. In summary, for primary amino acids the DNB, B, Ac, Clac, F, Fmoc, Z, Boc, DNS, DNP, DNPyr, DBD and NBD protection groups are suitable to receive an enantiodiscrimination[53] [4]. Secondary amino acids, like proline, are only separated as DNP, DNPyr, and DBD derivatives [53]. The influence of the N-protection group has been explored by systematic alteration of type of protection group and of the type of the linkage. The N-protection group is also an important interaction site for chiral discrimination. The relative orientation, and therefore the fit of the SO-SA complex, is influenced by its functionality, shape and conformational arrangement of functional groups. A hydrogen bond interaction between the carbamate moiety of the SO and the corresponding functional group on the analyte, based on the linkage type, is of predominant importance. The variation of linkage enables the study of different chiral recognition mechanism and their driving forces. Furthermore, the elution order can be changed on a quinine-based CSP by changing the protection group. Other manipulations at the amine side, for example N-methylation, lead mostly to a complete loss of enantiorecognition and enantioseparation.

2.11 Quinine-based zwitterionic-exchanger chiral stationary phases

Standard quinine-based CSPs, CSP 1 and 2 (Table 2 and Figure 12), exhibit a weak anion-exchange (WAX) behavior. Thus the applicability of this type of CSP is limited to chiral acids. To expand the range of applicable chiral analytes on the quinine-based CSP, modification of this low-molecular mass selector (SO) are done to introduce a second ion-exchange motif. By combining a strong cation-exchanger site with the WAX moiety of quinine, a zwitterionic species is generated. The application scope of these zwitterionic ion-exchanger-type CSPs has been reported by Hoffmann et al. [55], [56] and [57]. Typically the spectrum of these type of CSPs can be expanded to α- and β- amino acids and to peptides. In principle, such a zwitterionic-type CSP can perform an anion-exchange-mode (AX), a cation-exchange-mode (CX) as well as a zwitterionic-exchange-mode (ZX) separation. Investigations by Hoffmann et al. give evidence for synergistic effects between the two oppositely charged groups during the molecular recognition process, especially for zwitterionic analytes [55]. In both cases, the CX and AX mode, one site is responsible for the ion-exchange mechanism, whereas the other existing ionic site can act as an intramolecular-counterion (IMCI), and therefore takes part in the ion-exchange equilibrium and is also responsible for analyte retardation. In ZX the oppositely charged site are not only involved in a simultaneous ion-pairing process, but can also act as an IMCI [57]. Several different CX sites have been introduced into a quinine-based CSP and have been thoroughly investigated (Figure 13).

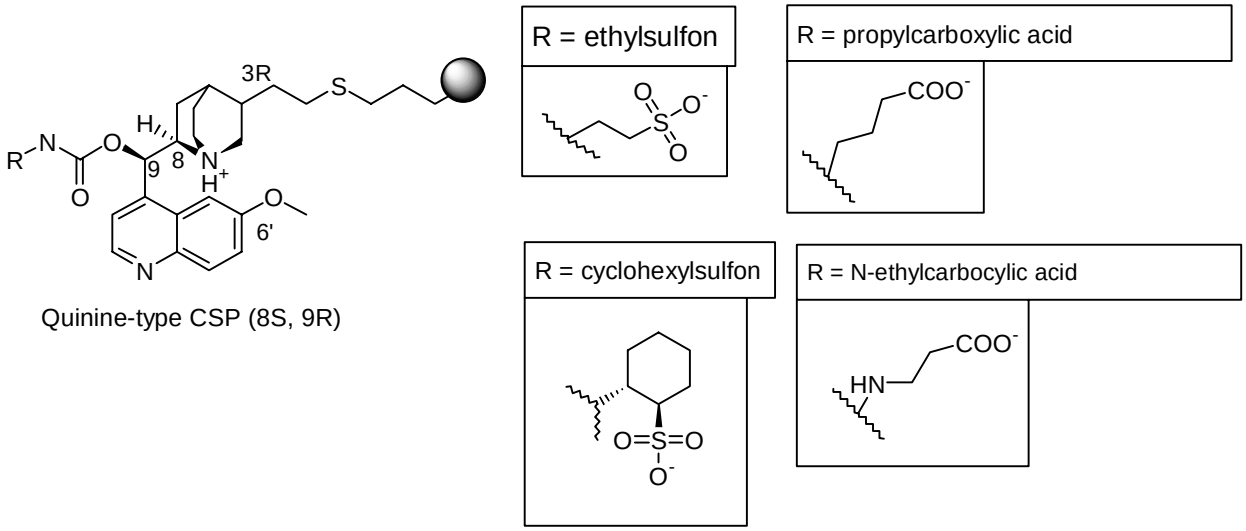


Figure 13: Quinine-based zwitterionic-type CSP

Due to the closeness of molecule position C9 of the quinine structure to the two stereogenic center and the anion exchange side, modifications are often implemented on this position. Further modifications have also been constructed on the 6'- and the 3R-position.

2.12 HILIC – Hydrophilic interaction liquid chromatography

The hydrophilic interaction liquid chromatography (HILIC) separation mode means the use of a hydro-organic mobile phase and a very polar stationary phase. The latter one can be bare silica or silica based separation materials, modified for example with amines, amides, zwitterionic or polyoles e.g. saccharide groups. HILIC is assigned to aqueous normal phase liquid chromatography (NPLC). It is a complementary separation technique to the NP mode, which uses silica and water free highly apolar mobile phases and HILIC is often orthogonal to the reversed phase liquid chromatography (RPLC). It is applied for the separation of highly polar or charged analytes, such as nucleosides, nucleotides, amino acids, peptides, proteins, oligosaccharides, carbohydrates, pharmaceutical drugs and neurotransmitters. RPLC, the most frequently applied separation mode, lacks for sufficient retention of highly polar analytes, whereas HILIC exhibits its benefits in the separation of polar compounds [58].

In HILIC, the mobile phase consists of an organic solvent, mostly acetonitrile (MeCN) that is mixed with pure water or an aqueous buffer. As a rule of thumb, retention increases with decreasing content of aqueous additive and with increasing polarity of the (hydrophilic) stationary phases. The retention mechanism in HILIC is more complex than in RP mode. Hydrogen bonding and dipole-dipole interactions may be responsible for the retention of polar compounds, but a partitioning between an aqueous rich layer on the silica surface and the organic rich mobile phase may also be involved [59].
Mobile phase normally consists of acetonitrile where water or buffer up to 5-40 % is added. Mostly volatile buffers such as ammonium formate or ammonium acetate are used. In an aqueous-organic mobile phase, which has to contain at least 0,5-1 % water, a water-rich adsorbed diffuse multi layer will be formed on the polar surface of the stationary phase. A liquid-liquid partition takes place between the water rich layer and the organic-modifier rich bulk mobile phase. The elution strength of the mobile phase increases with the polarity of the bulk organic solvent, for example in the order of methanol>ethanol>isopropanol>acetonitrile. A proton-donor/acceptor interaction also contributes to the retention behavior. The buffer type and composition influences the retention and selectivity in HILIC separations, especially when charged analytes and charged moieties on the stationary phase are involved [60].

There is a big variation of available HILIC stationary phases regarding their chemical structure compared to the common n-alkyl chain based RP columns. Widely accepted HILIC columns have an amine,- amide or a diol functional groups bound to a silica support and are mostly used for the separation of carbohydrates, proteins and oligosaccharides. Newer HILIC columns are assembled with a polymeric structure of poly(succinimide) derivative or with a sulfoalkylbetaine functional group, immobilized on silica gel [58]. Other available separation materials consist of cyanoalkyl, polyethylene glyco-, cyclodextrine, pentafluorophenylpropyl, polyvinylalcohol or polypeptides modified silica support. Generally these bonded stationary phases show higher retention for acidic compounds, which

results from a combination of a partition and sometimes an ion-exchange mechanism if a basic moiety is involved [61].

Tailing of peaks are more common in HILIC than in RP separation. Another advantage of HILIC is the compatibility with common coupling techniques like mass spectrometry employing soft ionization, like electro spray ionization (ESI) or charged aerosol detection (CAD). The combination HILIC-ESI-MS has a promising future in the analytical chemistry field [61].
A HILIC retention increment for the *tert*-butyl carbamate modified quinine-based CSPs was proposed in literature [62],[63].

2.12.1 Zwitterionic-HILIC stationary phases

Chromatographic stationary phases with zwitterionic functional groups are introduced for ion-exchange purposes. In HILIC, a zwitterionic type stationary phase (SP) allows the simultaneous separation of anionic and cationic compounds in a single analytical run. The first zwitterionic HILIC stationary phases were of the sulfoalkylbetaine type. These sulfobetaines adsorb strongly water on its surface and hydrogen-bonding and dipole-dipole interactions are of primary importance, the electrostatic interactions affect the separation to a smaller extent [61]. Typical analytes for zwitterionic HILIC phases are glucosinolate, aminoglucosides, peptides and glycopeptides, as well as purines, pyrimidine bases and nucleosides.
Mostly, this column type consists of a sulfobetaine or a phosphorylcholine species which contains a quaternary amine and a negatively charged sulfonic or phosphonic group (Figure 14). Ion-exchange interactions are believed to play a marginal role in the performance of zwitterionic HILIC columns. Amine based columns exhibit stronger ion-exchange characteristics. The two oppositely charged groups are believed to shield each other off from their environment, which is called intra molecular counter ion effect (IMCI). It is known that sulfobetaine adsorbs water strongly on its surface. Partitioning of the analyte between the aqueous layer and the bulk phase will account for retention, but electrostatic interactions will affect the separation of analytes, which carry either a positive or a negative net charge [61].

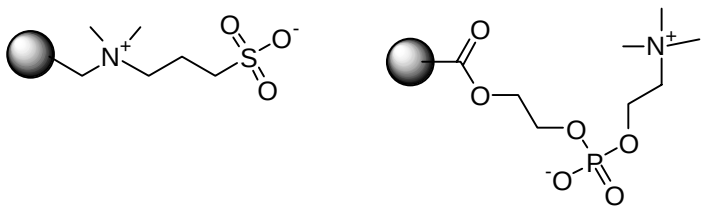


Figure 14: ZIC-HILIC stationary phase SeQuant® and KS-polyMPC [60]

2.12.2 Mixed RP/HILIC mechanism on polar columns in an aqueous-organic mobile phase

In both modes, RP and HILIC, a polar interaction with the silica support or a lipophilic interaction with hydrophobic structure elements, respectively, will contribute to the entire retention behavior. Depending on the percentage of organic solvent applied, a mixed retention mechanism will occur during a HILIC separation. This mixed mode mechanism is controlled by RP interactions at highly aqueous mobile phase compositions and become more HILIC type with growing content of organic solvent. In such a case, a plot with logarithmic retention factor log(k) *versus* φ of water content will show a characteristic U-shape. The minimum of retention states a transition state of the RP or HILIC mechanism and can be illustrated with such a plot [64], [60]. The mixed mode interaction offers the possibility for separation with better efficiency and improved selectivity. However, the latter also induces a challenge, because peak broadening appears quiet often under HILIC conditions and the prediction of elution order is not so straight forward with mixed mode HILIC separations compared to reversed phase chromatography [65].

3 EXPERIMENTAL

3.1 Chemicals

The source of used chemicals is given in this chapter. The chemical purity is provided in the brackets, where unknown means that the information of the purity was not available, due to the missing information of the provider or of an in-house production.

The AccQ® Tag Ultra Derivatization Kit was purchased from Waters Corporation (Milford, USA), using 6-aminoquinoline succinimide carbamate (AQC) as fluorescence label.

The following chemicals were purchased from Sigma (Vienna, Austria):
Acetic acid (≥99,8 %), ammonium acetate (≥98 %), ammonium formate (≥98 %), formic acid (≥97 %), ammonium hydroxid solution 25 % (w/w) 1,3-propansulton solid (98 %), iodoacetamide (98 %), iodoacetate (98 %), glycine (99 %), DL-Ala (99 %) L-Ala (99 %), DL-Ile (98 %), L-Ile (99 %), DL/L-Leu (99), DL/L-Tyr (99 %), DL/L-Val (98,5 %), DL-Thr (98 %), L-Thr (99,5 %), DL-Trp (99 %), L-Trp (99,5 %), L-Phe (99 %), p-F-DL-Phe (98 %), DL-cysteine (98 %), L-cysteine (99 %), DL-cystine (99 %), DL/L-His (98 %), DL-Lys (unknown), L-Lys (98 %), DL/L-Met (99 %), L-Arg (99,5 %), alpha amino butyric acid (99 %), DL-allo-Ile (99 %), DL-pipecolinic acid (97 %.), L-pipecolinic acid (98 %), L-Gln (99,5), D-Gln (98 %), DL/L-nipecotinic acid (98 %), cis-aminocyclohexylcarboxylic acid (98 %), 4-Cl-DL-Phe (98 %), 2-F-DL-Phe (98 %), DL/L-Asp (99 %), DL-Asn (98 %), L-Asn (99 %), DL/L-Ser (98 %), DL-allo-Thr (99 %), L-allo-Thr (90 %) trans-D/L-4-hydroxyproline (98/99 %), cis-D/L-hydroxyproline (unknown), beta-DL/L-Phe (99 %), DL-isoser (98), DL-homoser (99 %), DL/L-3,4-Dihydroxyphenylalanine (99 %), N-methyl-DL-Leu (98 %), N-methyl-DL-Val (99 %), DL-dichlorprop (95 %), 2-amino-cyclohexylsulfonic acid (unknown), DL/L-methioninsulfone (unknown), DL-phoshporleucin (96 %), triethylamine (98 %), (-)-adenosine (99 %), adenine (99 %), 2´-desoxyadenosine (99 %), cytosine (99 %), R-(-)-phenylephrine (99 %), 4-OH-phenyl-acetic acid (98 %), nicotinic acid (99 %), alpha methyl tryptophane (98 %), (-)-thiamine (99 %), DL-flurbiprofene (98 %), ibuprofene (99 %) and theobromine (99 %) theophylline (99 %).

DL-homocysteic acid (unknown purity) was purchased from Vespa Biochemicals (unknown supplier).

Amino acids and peptides purchased from Bachem (Bubendorf, Switzerland):
D-Pro (99 %), D-Phe (99 %), DL-Pro (98 %), DL-Arg (99 %), D-allo-Ile (95 %), DL/L-Gly-Pro (99 %), DL/L-GlyGlyPro (96 %), Gly-DL-Phe (97 %), Gly-D-Phe (98 %), DL/L-Phe (97 %), LLL-Phe3 (unknown), DD/LL/LD/DL Phe2 (unknown), all-D/L-Phe4/5/6/7/8 (unknown), DL-Pro-Gly (99 %), DD/LL-Ala2 (99 %), L-Ala-Gly-Gly (91,9 %), DL-Ala-Gly-Gly (95 %), Gly-D-Ala (unknown), DL-Ala-DL-Ala (unknown), All-D/L-Ala5/6 (98 %).

Peptides purchased from AnaSpec Inc. (Fremont, USA):
All possible stereoisomers of PhePhePhe tripeptides (85 %), all-D/L-alanine hepta- and octapeptides (85 %).

Amino acids and peptides purchased from Pi-Chem (Graz, Austria):
All possible combinations of stereoisomers of D/L-phenylalanine tetrapeptides (90 %) and all possible stereoisomers of alanine tripeptides and tetrapeptides (90 %)

Amino acids purchased from Loba Chem. (Fischamed, Austria):
DL-p-Br-Phe (unknown), DL-cystine (95 %).

Ortho-phosphoric acid 85 % (wt), was purchased from Fisher Scientific (Loughborough, UK).

Methanol (HPLC grade), acetonitrile (HPLC grade), dichloromethane (technical) and methanol (technical) were from VWR (Vienna, Austria) purchased.

2-Butanone (98 %) was from ABCR (Karlsruhe, Germany) purchased.

Salicylaldehyde 98 %, TLC Silica gel 60 F_{254} and TLC Silica gel amino modified 60 $F_{254,}$ mercapto propyl modified silica gel 60, particle size 5µm and porosity 120 Å were from Merck (Darmstadt, Germany).

Chemicals from other unknown sources:
DL-Ala2, Gly-DL-Ala, LL-Ala2, Gly-L-Ala, Gly-DL/L-Ala-Gly, Gly-DL-Ala-Gly, Gly-L-Ala-Gly (in-house production), TCEP (in-house production).

High recovery vials, 1,5 ml crimp top, 12x32 mm were purchased from Sigma-Aldrich, Vienna, Austria.
Thermo magnetical stirrer was purchased from IKA GmbH (Darmstadt, Germany).
Thermomixer compact was purchased from Eppendorf (Hamburg, Germany).
Satorius micro balance M3P was purchased from Satorius (Göttingen, Germany).
Thermo incubator MP Series was purchased from Binder (Tuttlingen, Germany).
Micro balance Mettler AB 104 was purchased from Mettler Toledo (Vienna, Austria).
Mercapto propyl modified silica gel, 5 µm particle size and porosity 120 Å, was produced in-house. The coverage of thiol groups is about 650 µmol per gram silica gel [54] [66].

Thin layer chromatography was performed on silica gel pressing plates 60 F_{254} from Merk (Darmstadt, Germany).

Table 1 summarizes analytical HPLC columns which were used in this study.

Table 1: Denomination of applied analytical columns in contrast to this study

Short name	Full name, column dimensions, particle and pore size
RP C18	Phenomenox Gemini C18 150 x 3,0 mm, 3µm
zwitterionic-HILIC	Macherey-Nagel, Nucleodur® 100-3 HILIC, 150 x 4,6 mm, 5µm

3.2 Analytes

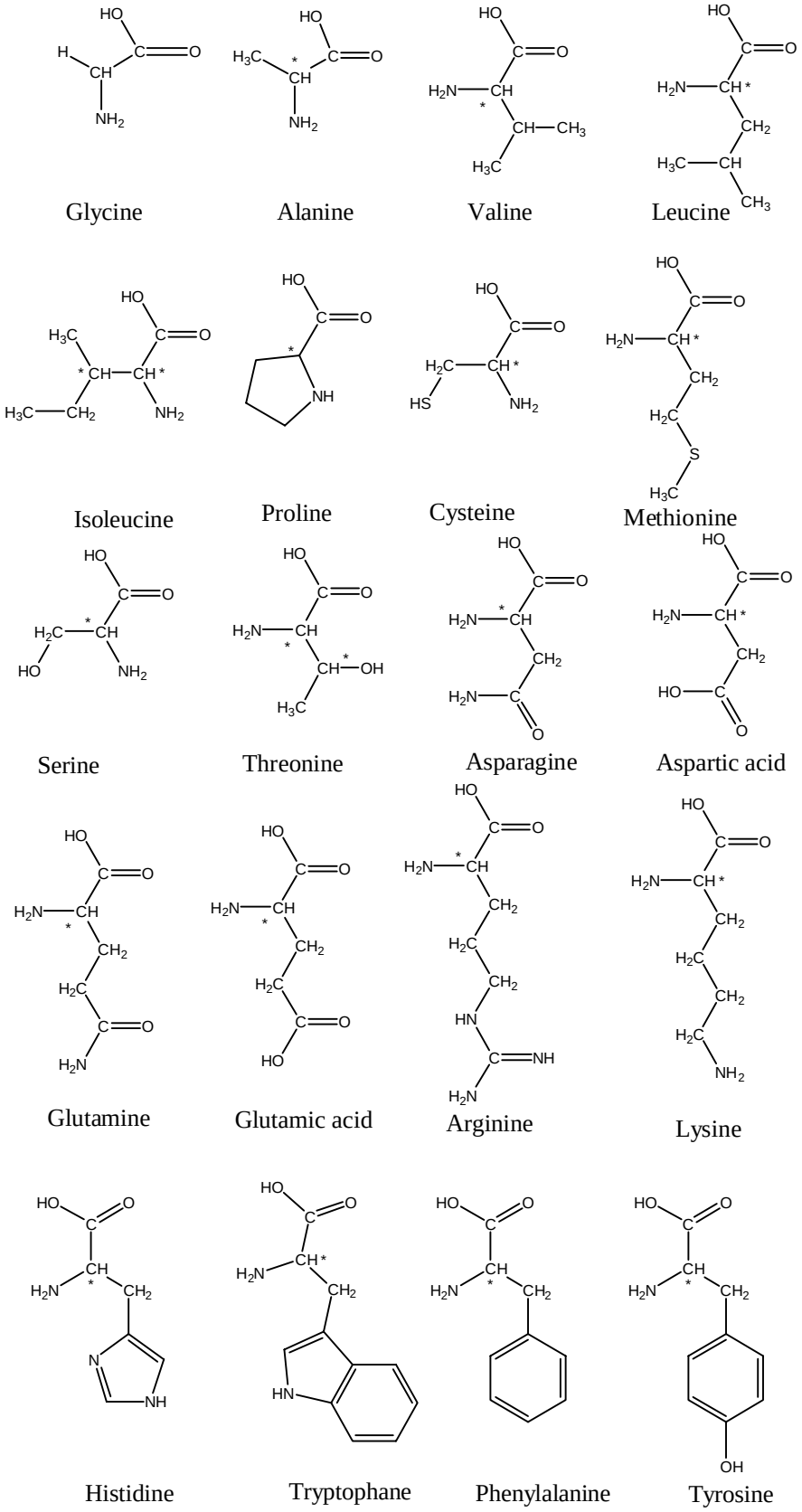
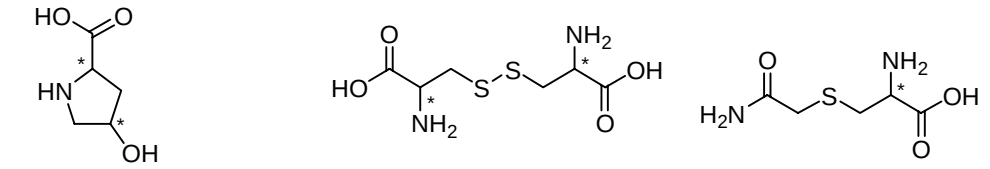
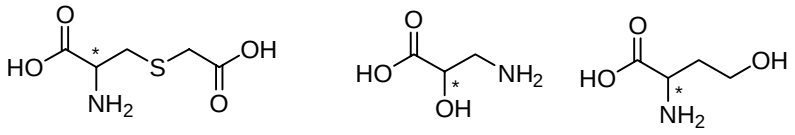


Figure 15: Structures of native proteinogenic amino acids used in this study

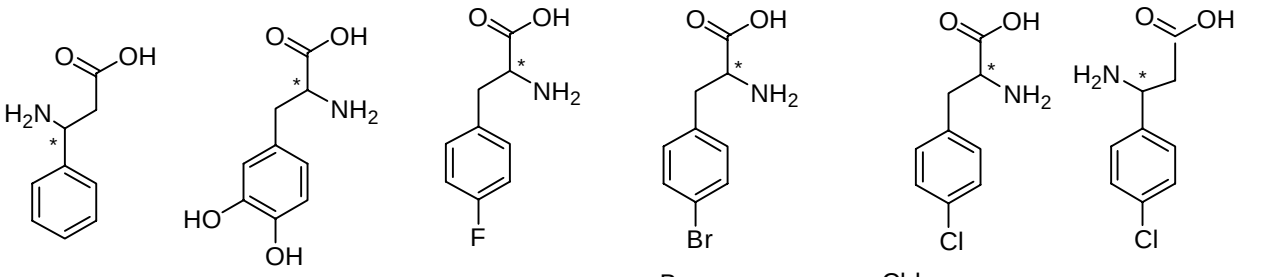


cis/trans-4-Hydroxyproline Cystine Cysteine-S-acetamide



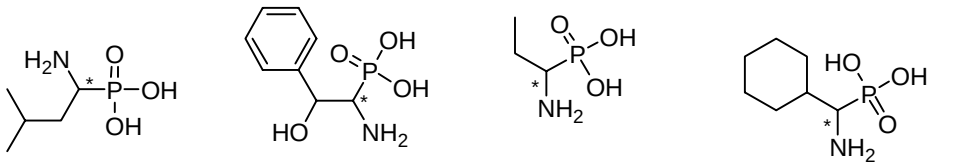
Cysteine-S-acetic acid Isoserine Homoserine

Figure 16: Stereoisomers of threonine and cysteine/cystine derivatization products

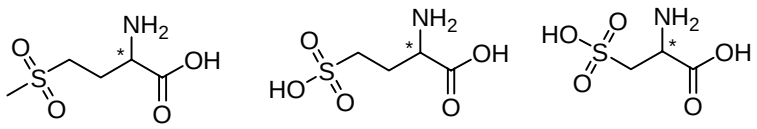


ß-Phenylalanine 3,4-Dihydroxy phenylalanine (DOPA) p-Fluoro-phenylalanine p-Bromo-phenylalanine p-Chloro-phenylalanine p-Chloro-ß-phenylalanine

Figure 17: Phenylalanine analogues

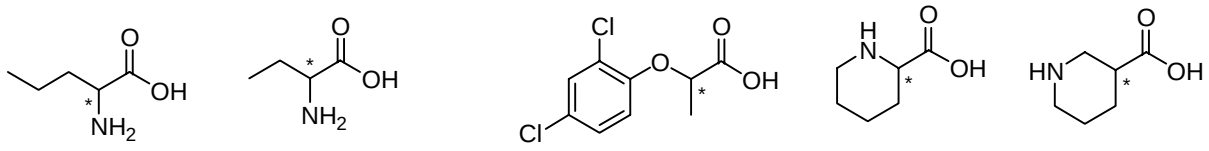


Phospholeucine alpha-Amino-3-OH-3-phenyl-phosphonic acid alpha-Amino-propyl-phosphinic acid alpha-Amino-2-cyclo hexyl-phosphonic acid



Methioninsulfone Homocysteic acid Cysteic acid

Figure 18: Amino phosphonic and sulfone acids



Norvaline alpha-Aminobutyric acid Dichlorprop Pipecolinic acid Nipecotinic acid

Figure 19: Test substances

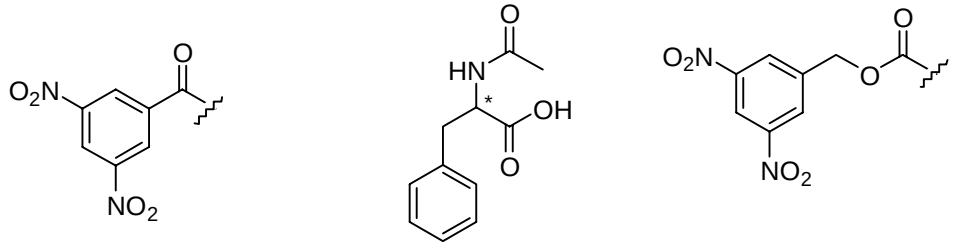


Figure 20: N-Terminal protection groups which were used in this study.

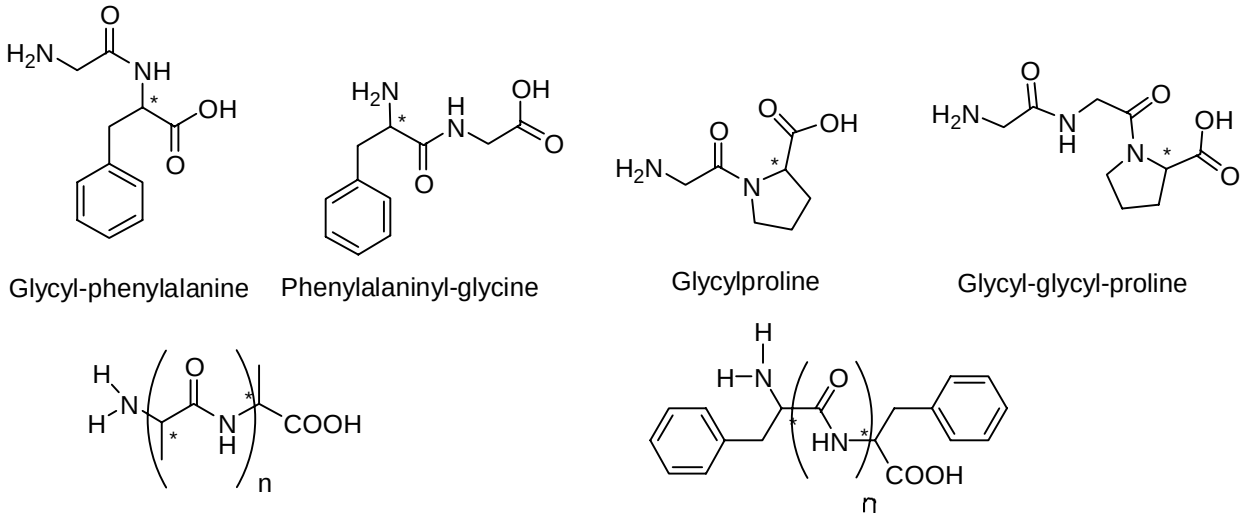


Figure 21: Dipeptides and oligo- peptides which were used in this study

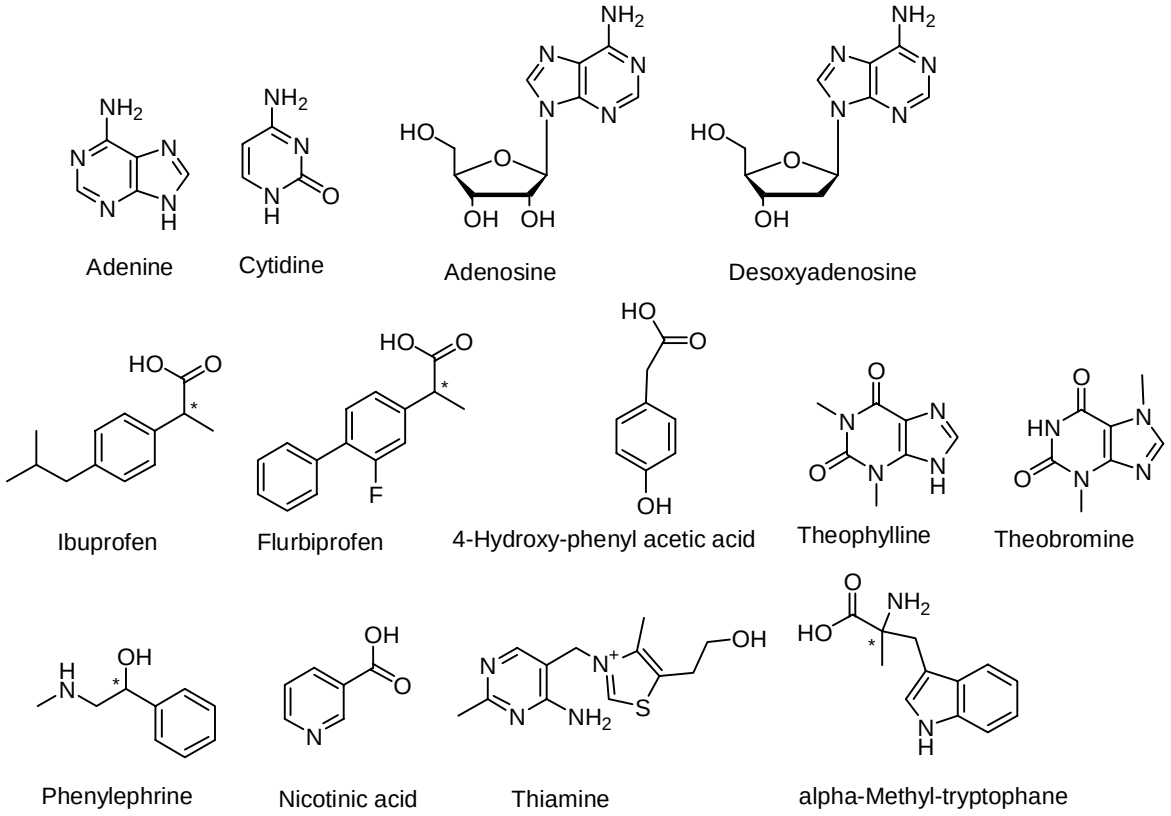
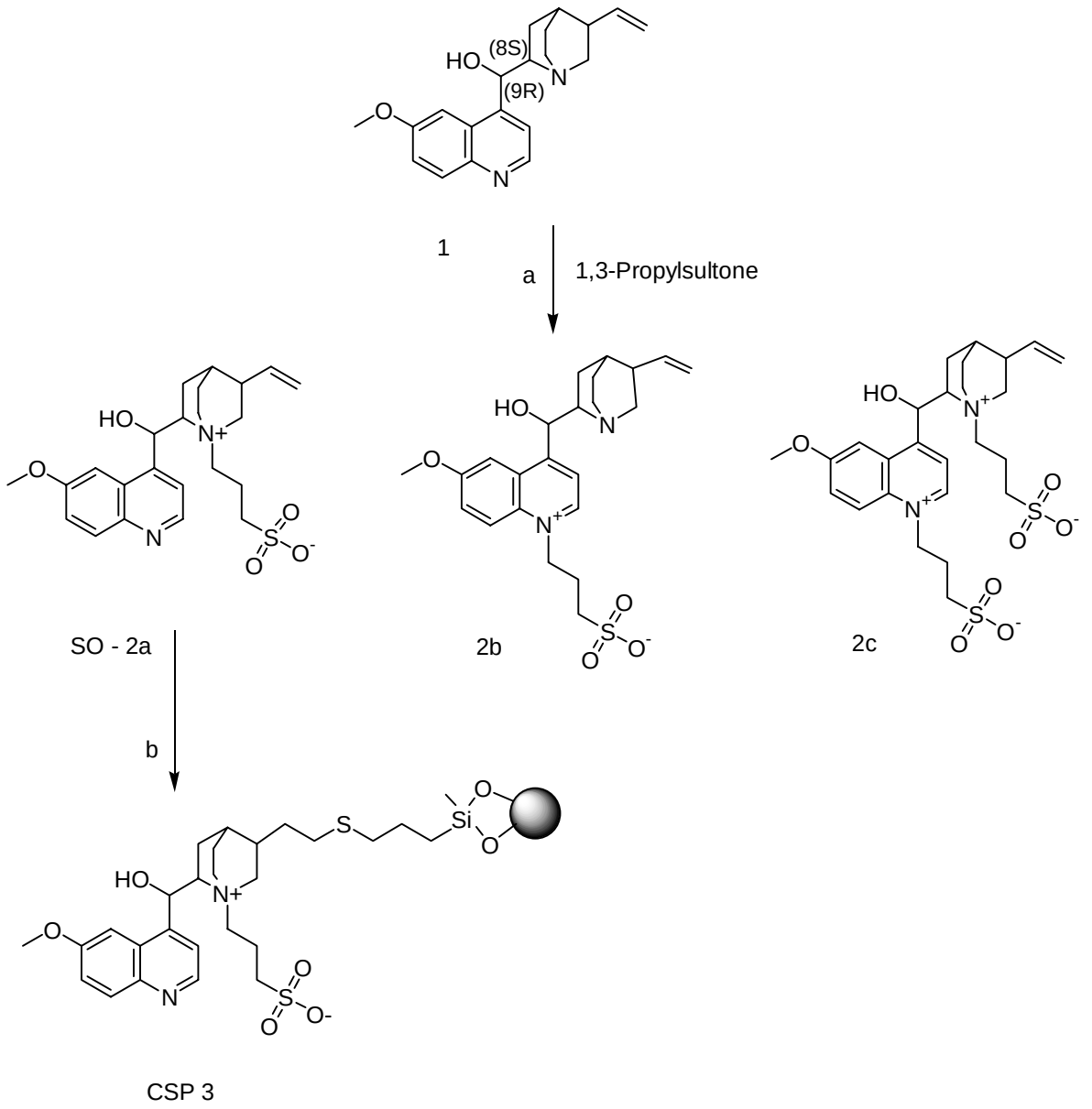


Figure 22: Set of representative HILIC compounds

3.3 Synthesis part

3.3.1 Synthesis of a quinine-based sulfobetaine modified chiral selector



Scheme 2: Reaction pathway for the synthesis of CSP 3; conditions: a) 1,3- propylsultone in 2-butanone, reflux 8 h, recrystallisation using 2-butanone and methanol provides SO 2a, side products are 2b and 2c, b) MeOH/2-butanone 1:1 reflux 8 h, radical starter AIBN

Scheme 2 shows the synthesis of selector **2** and the immobilized selector on mercapto propyl modified silica gel. 7,13 g (21 mmol) of commercially available free base of quinine **1** was dissolved in 80 ml 2-butanone and 2,1-fold mole excess of 1,3-propylsultone (44 mmol) was added. The mixture was refluxed under nitrogen over night, providing a white to yellow solid. The mixture was allowed to cool down to room temperature. After TLC check using 60 TLC plates and in dichloromethane/methanol 3:1 with 1-3 % (v/v) acetic acid as mobile phase, three different species were identified as side products

from the alkylation reaction, of which one was produced in bigger amounts compared to the others. The flask was stored in the freezer over night during which further precipitation occurred. The solid was filtered off, using a filter frit with porosity 4 and was washed three times with 30 ml of 2-butanone. The educt was almost completely washed away, since the product was soluble in 2-butanone. A crystallization in 2-butanone gave a precipitate, containing three produced quinine derivatives. The three products were supposed to be the two possible mono-alkylated and the di-alkylated quinine species. A recrystallization was carried out. Selector **2** was isolated during recrystallization with methanol and 2-butanone. Thereby the solid was dissolved in a small amount of boiling methanol and 2-butanone was added dropwise. As soon as precipitation occurred, the addition of 2-butanone was stopped. The solution was allowed to cool to room temperature and it was allowed to age over night. The solid was filtered off and yielded selector **2** in acceptable purity, with a yield of 2,3 g (23 %). Other substances, which occurred, were thougth to be the quinine derivative, alkylated on the quinoline ring and the quinine derivative alkylated on both nitrogen atoms. Due to great differences in the nature of these amphoteric substances, recrystallization could be applied to separate these side products from selector **2**.

Elementary analysis: C= 10,84 %, H= 1,90 %, N= 0,85 %, S= 2,44 % (%w, averaged)

^{1}H-NMR [MeOD]: δ= 8,73 (d, 1H), 7,94 (d, 1H), 7,80 (d, 1H), 7,44 (d, 1H), 7,3 (d,1H), 6,4 (s, 1H), 5,7 (m, 1H), 5,15 (d, 1H), 5,00 (d, 1H), 4,37 (m, 1H), 4,05 (s, 3H), 4,01 (m, 1H), 3,9 (m, 1H), 3,76 (m, 2H), 3,53 (m, 2H), 3,05 (m, 2H), 2,86 (s, 1H), 2,46 (m, 2H), 2,27 (m, 1H), 2,19 (m, 1H), 2,09 (m, 1H), 2,01 (m, 1H), 1,36 (m, 1H)

^{13}C-NMR [MeOD]: δ= 160 (C_{ar}H), 148 (C_{ar}H), 146 (C_{ar}H), 144 (C_{ar}H), 138 (CH), 131 (C_{ar}H), 127 (C_{ar}H), 123 (C_{ar}H), 121 (C_{ar}H), 117 (=CH2), 101 (C_{ar}H), 68 (CH), 66 (CH2O), 62 (CH_2), 61 (CH_2), 57 (OCH_3), 55 (CH_2), 49 ($CH_2SO_3^-$), 39 (CH), 27,7 (CH), 26 (CH_2), 22 (CH_2), 20 (CH_2)

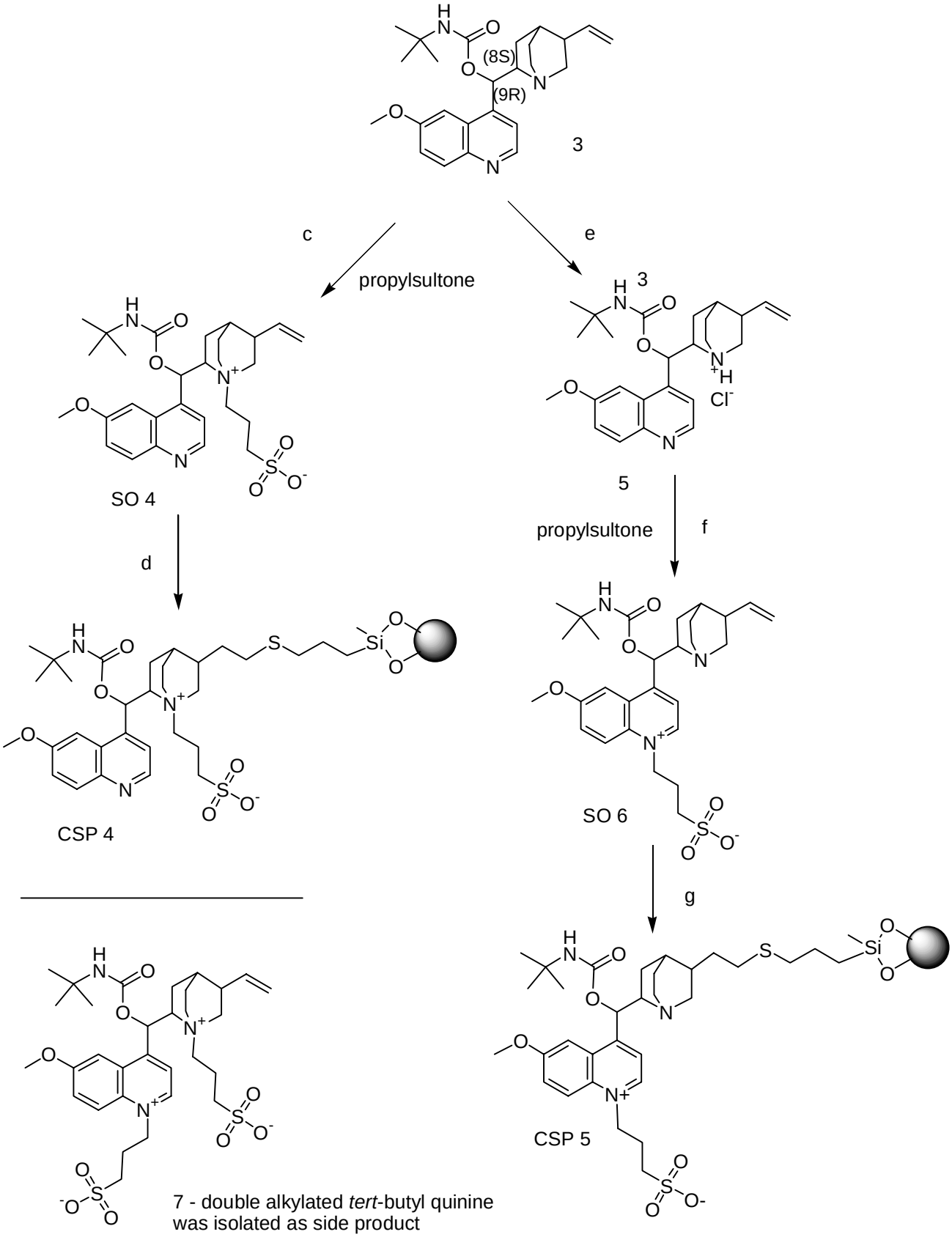
3.3.1.1 Immobilization of selector 2 on mercapto propyl modified silica gel – chiral stationary phase 3 (CSP 3)

The general immobilization procedure was described in [67] and is well established. For the immobilization onto mercapto modified silica gel, a radical mediated addition reaction was used, where the vinyl group of the quinine moiety reacts with a surface bound thiol group to give the corresponding thio-ether linkage. To a slurry of 3,49 g of silanol-endcapped, mercapto propyl modified silica gel, selector **2** (1,7 mmol), dissolved in 50 ml of a mixture of H_2O/water 1:1, was added. Because of the "problematic" zwitterionic nature of **2**, a loading of selector on the silica gel of 500 µmol/g silica gel was aspired. First the selector was coated on the silica gel. The coating was done for a better distribution and accessibility of the zwitterionic selector two the pores of the mercapto propyl modified silica gel. A methanol/water mixture was added and the liquid was ultra sonicated. Then the solvent was

evaporated under reduced pressure and dried in the vacuum oven at 60°C. Immobilization was carried out in a methanol/2-butanone mixture (40 ml) and radical starter 2,2'-Azobis-(2-methyl-proponitril) (AIBN) (70 mg) was added. The mixture was stirred with a mechanical stirrer and was allowed to reflux under nitrogen over night. Then the silica gel was filtered off, using a filter frit with a porosity of 4, washed with water, methanol and dichloromethane several times and dried under vacuum at 60°C.

Elementary analysis indicated an immobilization of 318 µmol/g selector per gram silica gel. The immobilization yield, concerning the coverage of available thiol groups on the mercapto propyl modified silica gel was 49 %.

3.3.2 Synthesis of a *tert*-butyl-quinine-based sulfobetaine modified chiral selector



Scheme 3: Synthesis pathway of CSP 4 and CSP 5, c) *tert*-butyl-carbamoylated-quinine and propylsulton in 2-butanone, reflux over night, d) MeOH/2-butanone, AIBN reflux over night, e) methanolic hydrochloric acid in methanol f) *tert*-butyl-carbamoylated-quinine and propylsultone in chloroform, reflux o.n., g) MeOH/2-butanone and AIBN reflux o.n.

Scheme 3 shows the synthesis of CSP 4. Quinine was already modified at the hydroxyl group in position C9 following the procedure from reference [54]. Because of the more apolar character of *tert*-butyl-carbamoylated quinine (tBuCQN) compared to quinine, 10,5g (24,8 mmol) of **3** were easily dissolved in 80 ml 2-butanone and afterwards 2,1 mole equivalents of propylsultone (50 mmol) were added. The mixture was refluxed over night. Precipitation occurred again in context of the alkylation. The mixture was allowed to cool down to room temperature and the solid was filtered off using a glass filter frit with porosity 4. The solid was washed with 2-butanone at 5°C. After TLC check using dichloromethane/methanol 3:1 with 1-3 % (v/v) acetic acid, it could be observed that three different sulfobetaine species were generated **4, 6, 7**, as it was expected for the alkylation reaction. In comparison to the equivalent reaction of native quinine, the yield of the products was quite different. It was proposed that the more apolar tBuCQN was easier dissolved in 2-butanone and because of that zwitterionic species did not precipitate to the same extend as observed for quinine during the alkylation reaction. Furthermore, the combination of an excess of alkylation reagent and better soluble intermediates, the double alkylated species was preferentially formed.

Recrystallization was performed as earlier described for selector **2**, but without yielding selector **4** in acceptable purity, since co-precipitation of the other alkylated products occurred. A column separation was performed, using silica gel, to yield the product of interest in acceptable purity. The zwitterionic character of alkylated quinine was inhibited by the addition of acetic acid to the mobile phase. The elution strength was increased with increasing amounts of acid added. A step gradient was applied beginning with the addition of 1 % (v/v) acetic acid and was raised to 5 % (v/v) in the mobile phase. The solid was dissolved in small volumes of dichloromethane/methanol mixture 20:1 and was applied onto the silica gel bed (about 200 g). A step gradient was necessary for the generation of an efficient zone separation of the three containing substances. Elution and proper separation of three different alkylated species was done with mixtures of dichloromethane/methanol (20:1 to 5:1) with addition of acetic acid as mentioned before. Selector **4** was identified as the second of four eluting quinine-based substances (Yield: 3,6 g or 26,7 %).

Another fraction, the third of four, was also collected and structure analysis revealed that this compound conformed to the structure of selector **6 (**Error! Reference source not found.**)** (Yield: 1,22 g, 9 %). It was found that during the drying at 60°C in the vacuum, a chemical alteration occurred with this compound. Selector **6** was found to be thermo labile.

Elementary analysis: C= 12,54 %, H= 2,04 %, N= 0,89 %, S= 2,73 % (%w, averaged)

^{1}H-NMR [D2O]: δ= 8,80 (d, 1H), 8,32 (s, 1H), 8,07 (d, 1H), 7,87 (d, 1H), 7,65 (d, 1H), 7,3 (d, 1H), 6,95 (s, 1H), 5,58 (m, 1H) 5,0 (d, 1H), 4,97 (d, 1H), 4,01 (s, 3H), 3,82 (m 5H), 3,62 (m, 1H); 3,38 (m, 1H), 3,04 (m, 2H), 2,87 (m, 1H), 2,34 (m, 2H), 2,23 (m, 2H), 2,10 (m, 1H), 2,01 (m, 1H), 1,63 (m, 1H), 1,2 (s, 9H)

^{13}C-NMR [D2O]: δ= 160 (C_{ar}H), 153 (COON), 146 (C_{ar}H), 146 (C_{ar}H), 143 (Car), 138 (C_{ar}), 137 (-

CH=), 127 ($C_{ar}H$), 126 (C_{ar}), 125 ($C_{ar}H$), 123 ($C_{ar}H$), 120 ($C_{ar}H$), 117 (=CH_2), 102 ($C_{ar}H$), 68 (CH), 66 (CHO), 61 (CH_2), 60 (CH_2), 57 (OCH_3), 53 (CH_2N), 51 (C), 47 (CH_2SO_3), 37 (CH), 28 (CH_3), 26 (CH), 25 (CH_2), 22 (CH_2), 19 (CH_2).

3.3.2.1 Immobilization of selector 4 on mercapto propyl modified silica gel – chiral stationary phase 4 (CSP 4)

Immobilization procedure for selector **4** was the same as for CSP 3 (chapter 3.3.1.1). Elementary analysis indicated selector coverage of 202 µmol/g selector per gram of modified silica gel or an immobilization yield of 32 %.

3.3.3 Synthesis of a *tert*-butyl-quinine-based sulfobetaine modified chiral selector – another approach for the synthesis of selector 6

An alternative approach for the synthesis of selector **6** was investigated, because this compound was produced only in minor amount compared to selector **4** and the double alkylated quinine derivative (Scheme 3). The mono hydrochloric salt of *tert*-butyl carbamoyl quinine **5** was produced by adding 1,1 mole equivalents of methanolic hydrochloric acid to 8,35 g (19,4 mmol) of compound **3** in water free methanol. The solvent was evaporated and the mono-chloric salt **5** was obtained. Then 1,1 mole equivalents of propylsultone was used for the alkylation reaction, which was repeated several times using different solvents, e.g. dichloromethane, chloroform and methanol. In chloroform a mono-alkylated species was generated in larger quantity compared to the corresponding reaction using 2-butanone. For clean-up, column chromatography with silica gel was performed. The reaction mixture was evaporated and dissolved in a small volume of a mixture of methanol/water 1:1, before it was coated onto silica gel. The flash column was filled with 120 g pure silica gel and the selector, coated onto silica gel, was added on the top. Elution was performed with mixtures of dichloromethane/methanol from 20:1 to 5:1 with varying amounts of acetic acid ranging from 0,1 to 3 % (v/v). Fractions of selector **6** were collected as the third of four separated substances. The solvent was evaporated, but acetic acid remained in high amount, because of its higher vapor pressure compared to organic solvents. The sticky solution was dried under vacuum. After some days white crystals were generated. Due to the risk of hydrolysis of the carbamate linkage at these solution in pure acetic acid, a precipitation by addition of 2-butanone was done. The precipitate **6** was filtered off, was washed with 2-butanone and dried under vacuum (Yield 1,75 g, 16,7 %).

Elementary analysis: C= 9,35 %, H= 1,71 %, N= 0,79 %, S= 2,10 % (%w, averaged)

^{1}H-NMR [D2O]: δ= 9,11 (d, 1H), 8,48 (d, 1H), 8,08 (d, 1H), 7,94 (d(1H), 7,70 (d, 1H), 6,76 (s, 1H), 5,72 (m, 1H), 5,12 (m (1H), 5,04 (d, 1H), 4,16 (t, 1H) 4,07 (s, 3H), 3,81 (m, 1H), 3,78 (m, 1H), 3,64 (m,

2H), 3,50 (m, 1H), 3,35 (m, 1H), 2,95 (m, 2H), 2,81 (s, 1H), 2,47 (m, 2H), 2,21 (m, 1H) 2,03 (m, 1H), 1,93 (m, 3H). 1,20 (s, 9H).

^{13}C-NMR [D2O]: δ= 161 (C_{ar}), 152 (C_{ar}H), 144 (C_{ar}), 146 (C_{ar}), 138 (-CH=), 137 (C_{ar}H), 134 (C_{ar}), 136 (C_{ar}H), 123 (C_{ar}H), 119 (C_{ar}H), 116 (=CH2), 103(C_{ar}H), 70 (CH), 66 (CHOR), 64 (CH_2), 60 (CH_2), 60 (CH_2), 57 (OCH_3), 57 (CH_2), 47 (CH_2SO_3), 36 (CH), 28 (CH_3), 27 (CH_2), 25 (CH_2), 24 (CH_2).

3.3.3.1 Immobilization of selector 6 on mercapto propyl modified silica gel – chiral stationary phase 5 (CSP 5)

The first immobilization approach was carried out as described for CSP 3 or 4. Mercapto propyl modified silica gel (3 g) was taken into a round beaker and 1,5 mmol of selector **6** was added. The selector was dissolved with methanol and the solution was ultra sonicated. The solvent was evaporated under reduced pressure and the coated silica gel was dried. The immobilization was performed in a mixture of methanol/2-butanone 1:1. Radical starter AIBN was added (70 mg) and the solution was stirred with a mechanical stirrer under reflux over night. The silica gel was filtered off and washed several times with methanol, dichloromethane and 2-butanone. Elementary analysis showed unsatisfactory loading of selector **6** onto the silica gel where the selector loading was 67 µmol/g selector per gram silica gel. It was assumed that the difficult zwitterionic character of the selector had caused a worse behavior and disabled a good immobilization.

In a second approach the selector **6** was added in a higher excess into the immobilization reaction (3 mmol) and 3,4 g of mercapto modified silica gel was used. Radical starter was also added in a higher amount (350 mg). The reaction slurry was mixed in 120 ml of MeOH/2-butanone 1:1 with a mechanical stirrer and it was allowed to reflux for ten hours under nitrogen atmosphere. After that the selector **6** modified silica gel was filtrated off and washed according to the procedure described for CSP 4. Elementary analysis was done, giving a calculated selector coverage of 188 µmol/g selector per gram of silica gel and an immobilization yield of 29 %.

Table 2 gives an overview of CSPs used for enantioseparation analysis during this master thesis.

Table 2: Denomination of investigated CSP columns together with their dimension and selector coverage

CSP code	CSP short name	CSP full name	column dimensions, particle and pore size	Selector coverage [µmol SO/g Silica gel]
CSP 1	QN-AX	*tert*-butyl-carbamoyl-quinine-based chiral stationary phase	150 x 4 ID 5 µm, 120 Å	380
CSP 2	QD-AX	*tert*-butyl-carbamoyl-quinidine-based chiral stationary phase	150 x 4 ID 5 µm, 120 Å	380
CSP 3	QN-SB-V2	N-1-propylsulfonic acid modified quinine-based chiral stationary phase	150 x 4 ID 5 µm, 120 Å	318
CSP 4	tBuCQN-SB-V2	N-1-propylsulfonic acid, *tert*-butyl-carbamoyl modified quinine-based chiral stationary phase	150 x 4 ID 5 µm, 120 Å	202
CSP 5	tBuCQN-SB-V3	N-1'-propylsulfonic acid, *tert*-butyl-carbamoyl modified quinine-based chiral stationary phase	150 x 4 ID 5 µm, 120 Å	188
CSP 6	lcQN-AX	Low coverage *tert*-butyl-carbamoyl-quinine-based chiral stationary phase	150 x 4 ID 5 µm, 120 Å	142

3.3.4 Racemization of dipeptides

3.3.4.1 Pretest with phenylalanine

The procedure for the racemization of amino acids via the formation of a Schiff-base was tested by racemization of L-phenylalanine [48]. An aliquot of 6,1 mmol of L-Phe and 615 mmol (100 fold excess) of acetic acid, HPLC grade, were taken into a round bottom flask. It was necessary to heat the mixture up to 40°C to dissolve phenylalanine in acetic acid. Then 20 % mole equivalents of salicylaldehyde were added and the mixture was refluxed for two hours. The acetic acid was then evaporated under reduced pressure, providing a yellow to orange sticky solid. About 40 ml water was added and the mixture was heated under stirring to 40°C to dissolve the solid. A spatula tip of charcoal was added and the slurry was stirred at room temperature. The charcoal and all other insoluble contents were filtered off, using a folded filter and the filtrate was evaporated under reduced pressure. 30 ml of methanol was added and the white crystalline precipitate was separated using a glass filter frit (porosity 4) and was dried under vacuum (yield 900 mg or 90 %).

The racemization efficiency was investigated using enantioseparation chromatography. The solid was derivatized with AQC and injected into a HPLC run using CSP 2 and method 1. As a result, a pair of enantiomeric peaks, with equal peak area, was obtained.

3.3.4.2 Racemization of dipeptides

Reaction mechanism was considered to be exemplified in Scheme 1.

For racemization of L-Phe-Gly, 5,3 mmol of dipeptides was added to fifteen-fold molar excess of acetic acid (76 mmol). The peptide was easily dissolved in the organic acid and 0 % mole equivalents of salicylaldehyde (2,1 mmol) was added. The mixture was refluxed for two hours before it was evaporated under reduced pressure to dryness. After addition of 40 ml water and a spatula tip of charcoal, the liquid was stirred for 30 min at room temperature. Then the adsorbent was filtered off and the filtrate was evaporated under reduced pressure. By adding methanol, precipitation occurred and the solid was isolated with a glass filter frit (porosity 4). The racemization yielded 467 mg or 39 % of a white solid. The same reaction was carried out for Gly-D-Phe using 2,6 mmol of the peptide (Yield 370 mg or 77 %). It was a contrasting test, because the N-terminally located glycine might not be racemized if the racemization was conducted using a Schiff base intermediate (Scheme 1). If just this mechanism was involved in the racemization, the Gly-D-Phe should not been altered. Otherwise, by the formation of a diketopiperazine, an inverse dipeptide and/or a changed stereochemistry should be observed.

3.4 Derivatization procedures

3.4.1 Derivatization procedure for amino acids using AQC

For derivatization of amino acids common protocols as described in reference [68] were conducted.
AQC was purchased from Waters Corporation, Millford, USA. The kit contained a screw vial with solid AQC, water free acetonitrile and vial of 20 mM borate buffer pH 8,8. Three milligrams of AQC tag were dissolved in one milliliter of acetonitrile. The solution had to be heated to 55 °C for ten minutes to complete the solubilisation process. The solution was protected from light and was stored tightly wrapped with parafilm® in a desiccator. The solution could be used for more than one week. A partial hydrolysis of the tag was recognized, but due to an excess of used derivatization reagent to analyte, an influence on derivatization efficiency could not be observed.
Amino acid standard solutions were prepared with a concentration of 2,5 mM in 0,1 M HCl. Further dilutions were done in bidistilled water. For a typical derivatization 80 µl of borate buffer was added to HPLC vial with 100 µl glass insert or a high recovery vial. From the diluted amino acid solution ten micro liters were added and the solution was immediately mixed. Then ten micro liters of derivatization reagent were added and the solution was mixed immediately. Afterwards the vial was heated at 55°C for ten minutes prior to analysis. The formation of the symmetric bis-urea derivative had led to a precipitation in the derivatization solution after several days. This solid was removed by centrifugation of the mixture and liquid phase was used.

3.4.2 Derivatization procedure for amino acids using FITC

Several protocols for the derivatization of free amino acids with fluoresceine-isothiocyanate were taken into account [33], [32] [69]. The last mentioned protocol [69] yielded a good derivatization of amino acids, concerning the reaction time and temperature. Therefore the derivatization was carried out in an alkaline milieu, a 20 mM borate puffer pH 9,8, adjusted with 10 % (w/v) NaOH. 100 µl of the borate buffer was added in a light protected reaction tube. From amino acid standard solutions, of about 50 mM, an aliquot was added. FITC was prepared as a 10 mM solution in dry dimethylformamide (DMF) and 10 µl were added to the reaction tube. The amino acid was added in a slightly excess to FITC for each derivatization reaction. The solution was mixed and stored in a thermoshaker at 60°C in the dark for two hours. It was investigated that a pH >9 was necessary to obtain proper derivatization yields as well as a temperature of 60°C during a reaction time of about 2 hours. Other protocols, which had performed the derivatization in the dark over night, had been found to be faulty. The AA-FITC derivatives could be stored in the freezer for at least one weak, if only a qualitative analysis was to be conducted. Absorption maximum was found at 235 nm, other absorption maxima were 250 nm and 460 nm. Fluorescence detection was done with an excitation wavelength of 460 nm and an emission wavelength of 520 nm.

3.4.3 Reduction of cystine and derivatization with iodo acetamide, iodo acetate and 4-vinylpyridine

A cystine solution with a concentration of 16 mM was prepared in 0,1 M HCl. Iodo acetamide and iodo acetate solutions were freshly prepared in acetonitrile with a concentration of 50 mM. The reduction of cystine was carried out by the addition of a reduction agent. A TCEP solution with 50 mM in water was prepared freshly before every use. The reduction was done with 50,- 100 and 200 fold molar excess of TCEP relative to disulfides in an aqueous media. The reaction was allowed to proceed in the dark at room temperature for two hours. Then the derivatization with the iodo species and afterwards the derivatization with AQC were conducted in the same solution.

To an aliquote of diluted amino acid solution with TCEP, a 10-fold molar excess of the iodo species was added and the reaction was allowed to proceed in the dark for two hours. The AQC derivatization was done according to the above mentioned protocol (capture 3.4.1). The cysteine derivatives were stable for at least one week in the freezer. The 4-vinylderivative was produced in-house.

3.5 Analysis protocols

3.5.1.1 General conditions and aspects for the enantioselective separations

Standard parameters for the enantioseparation were a flow rate of 1 ml/min and a column temperature of 25°C. Isocratic elution mode was performed. For each column the void volume was determined by an injection of 10 % (v/v) acetone in mobile phase and detection at 280 nm. A sequence of analytical runs

included one or more blank runs followed by a series of analyte injections, a blank run and/or a column washing run. A reagent blank was always included after each longer sequence in order to identify reagent related peaks in the chromatogram. Samples were kept in the autosampler plate, which was temperature controlled to 5 °C or were stored in the freezer.

Mobile phases were degassed in a sonification bath prior use. Identification of enantiomer peaks were achieved by injection of a racemic mixture and a single injection of one of the enantiomers. Furthermore, the peak areas of the racemic mixture had to be equal. With the application of quinine instead of quinidine-type CSPs, the elution order of most of the used analytes were reversed. This provided another hint to the enantiomeric character of an observed peak pair.

Newly synthesized columns were washed with 1 % (v/v) acetic acid in MeOH, until the baseline of both detectors, DAD and FLD was constant. The columns were stored in methanol.

3.5.2 Methods

3.5.2.1 Method 1

Standard method 1 was used for chiral separations, mainly for AQC-derivatives. *tert*-Butyl carbamate modified quinine/quinidine based chiral stationary phases were used. The column temperature was controlled to 25 °C. The mobile phase was MeOH/AcOH/NH_4AcO 98/2/0,5 % (v/v/w). The flow rate was 1 ml/min. Detection was carried out with a diode array detector at an absorption wavelength of 248 nm and with a fluorescence detector at an excitation wavelength of 248 nm and an emission wavelength of 395 nm.

3.5.2.2 Method 2

Method 2 was used for enantioseparations, mainly for the separation of alanine peptides and stereoisomers. *tert*-Butyl carbamate modified quinine/quinidine based chiral stationary phases were used. The column was tempered to 25 °C. The mobile phase was MeOH/H_2O 80/20 20 mM NH_4AcO and pH_a 6,0 adjusted with acetic acid. The flow rate was 1 ml/min, if not. Detection was carried out with a diode array detector at an absorption wavelength of 248 nm and with a fluorescence detector at an excitation wavelength of 248 nm and an emission wavelength of 395 nm.

3.5.2.3 Method 3

Method 3 was set up as method 2, besides the mobile phase. MeOH/H_2O 80/20 200 mM NH_4AcO and pH_a 6,0 adjusted with acetic acid, was used. Method 3 was used for the enantioseparations of phenylalanine peptides, therefore the flow rate of 0,5 ml/min was applied

3.5.2.4 Method 4

Method 4 used a reversed phase system for the separation of AQC tagged amino acids. An Agilent HPLC 1200 Series, with quaternary pump, degasser, column oven, diode array detector and a

fluorescent detector was applied. Phenomenox Gemini 150x3,00 mm 3µ 110 Å RP C18 column was used. Mobile phase and elution gradient were based on reference [28], but were modified. Eluent A was an aqueous buffer, 100 mM NaAcO, 0,1 % (v/v) triethylamine and pH 5,0, adjusted with acetic acid. Eluent B was a mixture of acetonitrile/water 60/40. The gradient was [t (min)/%A] 0/100, 0,5/98, 18/93, 19/90, 29,5/67, 38/67, 39/0, 43/0, 43,5/100 and 50/100 all linear segments. The flow rate was set to 0,8 ml/min and the column temperature was 40°C. Detection was carried out with a diode array detector at an absorption wavelength of 248 nm and with a fluorescence detector at an excitation wavelength of 248 nm and an emission wavelength of 395 nm.

3.5.2.5 Method 5

Method 5 was equal to method 4, besides the type of buffer. For the investigations using a mass analyzer with electrospray ionisation, a volatile buffer was used. Eluent A^{MS} was an aqueous buffer, 100 mM NH_4AcO, 0,1 % (v/v) triethylamine and pH 5,0, adjusted with acetic acid.

3.5.2.6 Method 6

Method 6 was used for the enantioseparations of fluoresceine-labeled amino acids. *tert*-Butyl carbamate modified quinine/quinidine based chiral stationary phases were used. The mobile phase was a mixture of MeOH/ACN 80/20, 150 mM FA, 75 mM NH_4AcO. Flow rate of 1 ml/min was applied and the column temperature was 25 °C. Detection was carried out using a diode array detector at the absorption maximum of 235 nm.

3.5.2.7 Method 7

Method 5 was used for separations in the HILIC mode. The mobile phase was a mixture of ACN/H_2O 90/10 with 10 mM NH_4AcO, buffer pH 5,0 or 8,0 was adjusted with acetic acid or ammonia hydroxide. Column temperature was maintained at 25 °C and a flow rate of 1 ml/min was applied. Different columns were applied using method 5 e.g. CSP 2, CSP 3, CSP 4, CSP 5 and Nucleodur® 100-3. The detection was carried out using a diode array detector. The highest absorption maximum of an individual analyte of one of following wavelength, 215, 248, 280 and 360 nm was taken into account.

3.5.3 Screening for chiral enantiodiscrimination of the synthesized sulfobetaine-type quinine and *tert*-butyl carbamate modified quinine-based chiral stationary phases

The study of the chiral recognition and enantiomer discrimination ability of the synthesized sulfobetaine-type quinine-based selectors was conducted as follows: First the standard mobile phase, MeOH/AcOH/NH_4AcO 98/2/0,5 % (v/v/w) was used for the separation of a small set of typical test substances, DNB-DL-Leu, DL-Ac-Phe and DL-Z-Phe. AQC-derivatives of representative amino acids were also tested. Then the mobile phase was altered and the test set was extended. The mobile phases of choice were MeOH/AcOH/NH_4AcO 99/1/0,25 % (v/v/w), 20 mM AcOH in MeOH, 20 mM TEA in MeOH, 50 mM Na_2HPO_4 buffer, where pH 2,0 was adjusted with H_3PO_4 and the mobile phase was a

mixture of MeOH/buffer 50/50. A hydro-organic mobile phase with MeOH/H_2O 80/20 20 mM NH_4AcO pH_a 6,0, adjusted with acetic acid, was also tested.

3.5.4 Application of the synthesized sulfobetaine-type quinine and *tert*-butyl-carbamate-quinine-based stationary phases in the hydrophilic interaction liquid chromatography mode

Each of the three columns, CSP 3, CSP 4 and CSP 5 were used for the separation of a set of typical HILIC compounds (Figure 22) using a mobile phase, containing ACN/buffer 90/10, where the buffer contained 100 mM NH_4AcO. The pH was adjusted with acetic acid to 5,0 and 8,0. For another study the apparent pH value in the hydro-organic phase was adjusted to 5,0. The samples were dissolved and diluted with a mixture of ACN/H_2O 90/10.

For the investigation of the retention mechanism exhibited by the synthesized HILIC columns, some representative analytes were tested using CSP 3. A series of test analytes were injected and different mobile phase compositions were used for elution. Mobile phase A was a mixture of ACN/aqueous buffer 5/95, containing 10 mM NH_4AcO and mobile phase B was 95/5 ACN/ aqueous buffer containing 10 mM NH_4AcO. The pH of the buffer was adjusted to 5,0 with acetic acid. The thereby obtained data were processed in a plot of log(k) *vs.* φ H_2O. A trendline was fitted to the distribution of data points. Note, that an U-shape of the trendline would imply a mixed retention mechanism.

3.6 Instrumentation and methodologies

3.6.1 Instrumentation

Chromatographic analyses were mainly done with an Agilent 1200 Series HPLC system (Agilent Technologies Waldbronn, Germany). The HPLC was equipped with a quaternary pump, as well as with an autosampler, degasser and a column oven. Detection of fluorescence active compounds was accomplished with a fluorescence detector (Type G131GA). Non-fluorescence active analytes were detected using a DAD (Type G1321A).
Data analysis was carried out with Chemstation software for LC 3D system, from Agilent Technologies.

Mass spectrometric analyses were performed on a quadrupole-Qtrap mass spectrometer, MDS Sciex 4000 QTrap, from Applied Biosystems (Foster City, USA) connected with an Agilent 1200 Series HPLC or an Ion-trap MS 1100 Series LC/MSD Trap SL from Agilent (Santa Clara, USA) connected with a HPLC 1100 Series from Agilent. Mass spectrometric analyses were performed in direct infusion, or after chromatographic separation in scan- or product-ion mode. After identification of the molecular ion mass, fragmentation analysis was performed. The analyses were also done in scan mode using positive or negative ionization. Also MS^n investigations were done. The AQC-amino acid derivatives

were in some cases identified with mass spectrometric analysis, using a RP phase method 5.

Elementary composition of the potentially interesting stationary phases were determined with a CHNS-O element analyzer (Carlo Erba) and the selector coverage on silica gel was calculated from mass percent of nitrogen detected (w-% N).

NMR spectra, ^{1}H- and ^{13}C-NMR, were recorded at room temperature with a Bruker DEX400 and DRX600 spectrometer. The spectra were recorded in different totally deuterated solvents, mostly CCl_3D or CD_3OD and for the zwitterionic sulfobetaine-quinine derivatives D_2O and CD_3COOD were used. The solvent signals were also used as reference signal. NMR data analysis was done with SpinWork® software (version 3).

IR spectra were measured with a Bruker Tensor 27, Diamond ATR FTIR. Spectras were normalized and data analysis were done with OPUS® 2,4 software.

3.7 Chromatographic parameters

3.7.1 Retention factor k

The retention factor k is a standardized value, containing the retention time t_{R1} and the void time t_{Ro} of the chromatographic system (Formula 9).

$$k = \frac{t_{R1} - t_{R0}}{t_{R0}}$$

Formula 9: Retention factor

3.7.2 Selectivity α_{ij}

Selectivity is described as the quotient of the retention factors of two peaks, related to each other. It describes the separation performance and selectivity of two analyte peaks for one chromatographic system. In the concept of enantioseparation, enantioselectivity is defined by Formula 10.

$$\alpha_{RS} = \frac{k_S}{k_R}$$

Formula 10: Enantioselectivity coefficient

Enantioselectivity is a parameter for the difference in selectivity expressed for a pair of enantiomers retained in the same chromatographic run. Alpha values above 1,0, means that one enantiomer is stronger retained than the other. High values indicate a different affinity of the selectand for the chiral selector and provide baseline separation.

3.7.3 Resolution R_S

The resolution is one of the most significant parameter describing the separation performance and efficiency of two peaks. A resolution above 1,5 indicates a baseline separation. The Rs-value depends on the plate number, the retention factor and the selectivity. Many different formulas are known. The best known one is presented in Formula 11:

$$R_S = \frac{\sqrt{N}}{4} * \frac{k}{k+1} * \frac{\alpha - 1}{\alpha}$$

Formula 11: Chromatographic resolution

It shall be mentioned that resolution values presented in this work are calculated values from the Agilent Chemstation software. In preparative chromatography of enantiomers, high resolution is desirable, since it increase the loadability and therefore the productivity of a column.

3.7.4 Plate number N

The plate numbers for a single analyte peak describes the efficiency of a chromatographic column in a quantitative way. It describes the number of equilibration zones of the analyte between the mobile and the stationary phase. In context of chiral separation it also describes the quality of the column packing and the selector coverage. Different formulas are known from literature. A general one is presented in Formula 12. There t_{R1} means the retention time of the peak and w_{FWHM} is the peak width at half maximum. Note, that all plate numbers in this work are calculated by the Agilent Chemstation Software.

$$N = 5{,}54 * \sqrt{\frac{t_{R1}}{w_{FWHM}}}$$

Formula 12: Plate number

3.7.5 Selectivity coefficients s^2

The influence of a chromatographic parameter on the separation selectivity, e.g. pH, can be demonstrated with a selectivity plot. The natural logarithm of the retention factors, obtained from a chromatogram of two different applied parameters, has to be calculated. A linear plot of the ln(k) values is fitted to a linear relationship and the square of the correlation coefficient R^2 can be calculated. The selectivity coefficient can be found by the use of Formula 13.

$$s^2 = 1 - R^2$$

Formula 13: Selectivity coefficient

A s^2 value of zero indicates that the two applied parameters (mobile phase, columns, or different

packing) do not exhibit any selectivity difference. A s^2 value of one means the two compared parameters are totally uncorrelated or orthogonal and an alteration of one of both leads to a change in selectivity.

4 RESULTS AND DISCUSSION

4.1 General aspects concerning the analytes and labels

6-Aminoquinoline succinimide carbamate (AQC) is a fluorescence tag often used for the sensitive determination of amino acids. Primarily the tag was introduced as an amino acid label for quantitative approaches in natural biological matrices. Especially for the determination of trace amounts of amino acids, in presence of bulk quantities of matrix or the other antipode, fluorescence detection had shown to be most suitable for detection and quantification [70], [71]. Furthermore, the possibility of a N-terminal derivatization of amino acids via an urea linkage was not investigated, concerning the enantioselective separations of N-derivatized analytes using a *tert*-butyl carbamate modified quinine-based chiral stationary phase. It should be conducted how far this new linkage group would influence the enantioseparation. Most important was the enantioseparation of proteinogenic amino acids, followed by a number of non-proteinogenic amino acids, with commercial, health or pharmaceutical relevance. Because of good enantioseparation results for amino acids N-derivatized with AQC, a number of dipeptides and mixtures of stereoisomers were investigated according to their separation abilities on quinine-based CSPs. A series of alanine as well as phenylalanine peptides up to the hexamer was also derivatized and chromatographed. The approach should be further investigated and it was thought to yield a deeper insight into the chiral recognition process. A comparison with later done experiments with peptides should be also conducted. Fluoresceine isothiocyante (FITC) was linked with amino acids via a thiourea bridge. FITC, a relatively large molecule compared to the rather small proteinogenic amino acids, should be successfully applied using a WAX-type quinine-based chiral stationary phase. Enantioseparations of amino acids labeled with fluorescein were already investigated [72]. Therefore the behavior of this structural linkage-analogue, compared to the urea bridge of AQC-derivatives, was studied.

A set of HILIC suitable compounds, highly polar and/or charged substances, were chosen for the investigation of the performance of the generated sulfobetaine-type CSPs regarding their applicability in the HILIC mode.

4.2 Investigation of AQC-tagged proteinogenic amino acids using reversed phase HPLC

4.2.1 Separation of a mix of AQC-tagged proteinogenic amino acids via reversed phase HPLC

The derivatization procedure for AQC derivatization was conducted according to literature [28]. Concerning the choice of HPLC vials for the derivatization, it was found that the recommended high recovery vials, had no significant advantages compared to ordinary scrimp HPLC vials with 150 µl glass inserts. The reproducibility as the peak areas were found to be equal for both types of HPLC vials.

A reversed phase method was conducted for following reasons. First, the derivatization had to be monitored, concerning the optimization of the derivatization procedure and for investigation of unexpected occurring species in term of the derivatization. Secondly, a mixture of proteinogenic amino acids were derivatized and separated in one chromatographic run. Method 4 was used for the investigations. Some unexpected reagent related peaks were found in each chromatogram. The retention time dependency on buffer pH for 6-aminoquinoline and all AQC amino acids derivatives was observed [73].

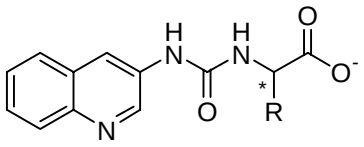


Figure 23: Scheme of an AQC-amino acid derivative

An exact adjustment of pH value was necessary to keep the variation of the retention time as low as possible. A pH >5,0 leads to an increase of retention and to a peak overlapping with acidic amino acids. The NH_3-related peak elutes under the mentioned conditions in chapter 3.5.2.4 at about 22,8 min, which means an overlapping with the His-AQC derivative. A striking problem was the overlapping of reagent peaks with analyte derivatives (Figure 24), eluting at 33,1 min, 34,1 min, 40,78 min and 40,88 min. As it could be seen in Table 14, the system peaks will overlap with Lys, Ile and Leu. Another fact was the different sensitivity of the fluorescence detection. As described in [22], fluorescence quenching had increased with increasing water content of the mobile phase. Derivatized amino acids with two chromophores could underlie a quenching effect, namely cystine, Phe and Trp. Phe as Lys were less influenced from this effect, Trp was not detectable by fluorescence detection but exhibited strong UV absorption and could be analyzed via UV detection. For cystine it was found that the LL/DD-cystine exhibited less fluorescence quenching than the DL-meso-cystine, which may originate from the difference in conformation of these molecule variants. The three stereoisomers were partially separated

in two peaks and a mono-substituted species was found rarely in small quantities in the chromatogram. The species could be identified with mass spectrometry investigations. The parameters were the same as for the dipeptide racemization analysis (3.3.4 Racemization of dipeptides). A chromatogram illustrating the separation of nineteen amino acids, without Trp is shown Figure 24. Ser and Gln coeluted as well as Thr and Arg. His was not present in the mixture.

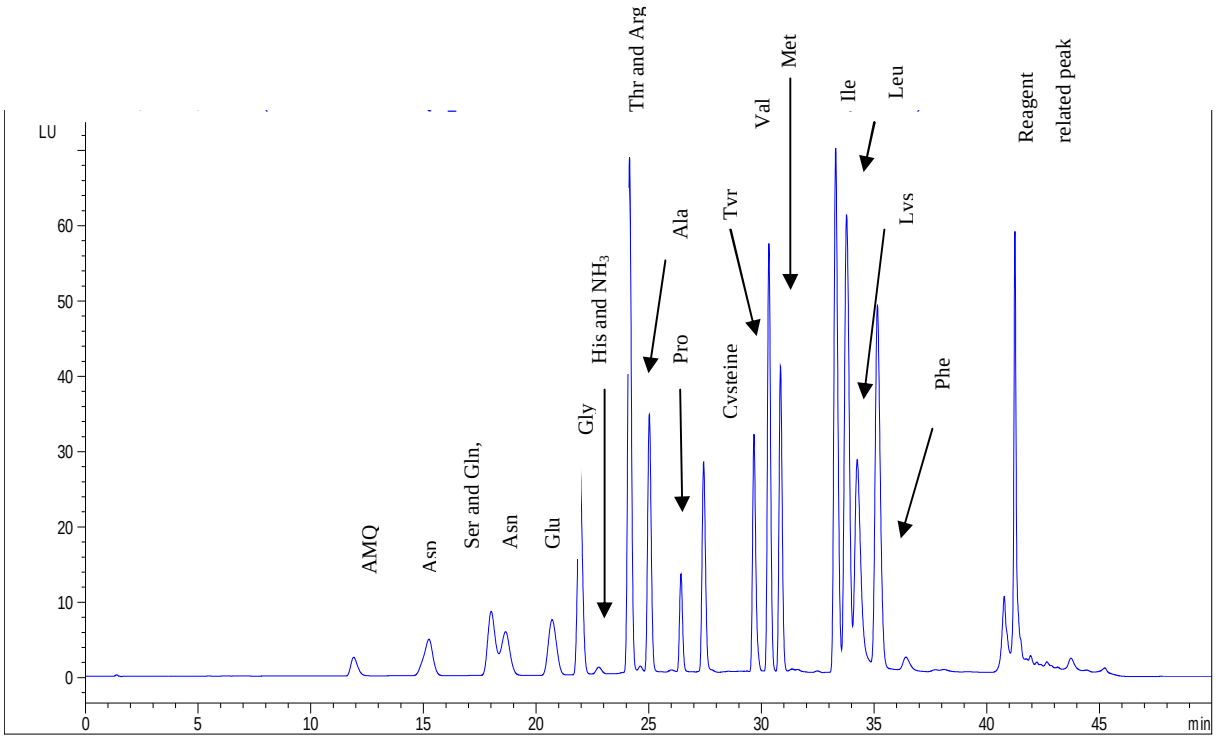


Figure 24: Chromatogram of a separation of nineteen ACQ-tagged amino acids. Tryptophan was not included because of a fluorescence quenching effect. Detection was carried out with an FLD excitation wavelength of 248 nm and an emission wavelength of 395 nm. The separation conditions, column and gradient were used as described in method 4.

4.2.2 Cysteine/Cystine derivatization

The cysteine/cystine problem was an issue of this master thesis. A reduction of cystine with tris-(2-carboxyethyl)-phosphine (TCEP) and a derivatization with iodo acetate and iodo acetamide was investigated prior derivatization with the AQC label. The reduction and derivatization of cysteine/cystine was performed according to the protocol from reference [74]. The retention behavior was investigated on both, a reversed phase column, to find a suitable Cys-derivative for later acidic hydrolysis of biological matrixes and for an enantioseparation of DL-Cys-derivatives with a quinine-based chiral stationary phase.

Surprisingly, the reduction of a cystine standard with TCEP and derivatization with AQC had led to a new peak in the chromatogram (Figure 26). The new peak had a different emission maximum than

AMQ (Figure 27). The emission maxima were at 395 nm and 520 nm, respectively. Mass spectrometric investigations revealed a molecular mass of 187 g/mol, using method 5. For that reason the new appearing peak was related to a carbamic acid derivative of AMQ (Figure 25), which was proposed to be an unstable intermediate during an acid or base catalyzed degradation of 6-aminoquinolyl-succinimidyl carbamate in aqueous solutions, as referred in literature [23]. It was supposed that in presence of TCEP the carbamic acid was more stable and could be detected in the time frame of derivatization and subsequent RP analysis. This finding stood in contrast to reports in literature [75], where the authors proposed a TCEP-AQC educt, which should be responsible for the new appearing peak.

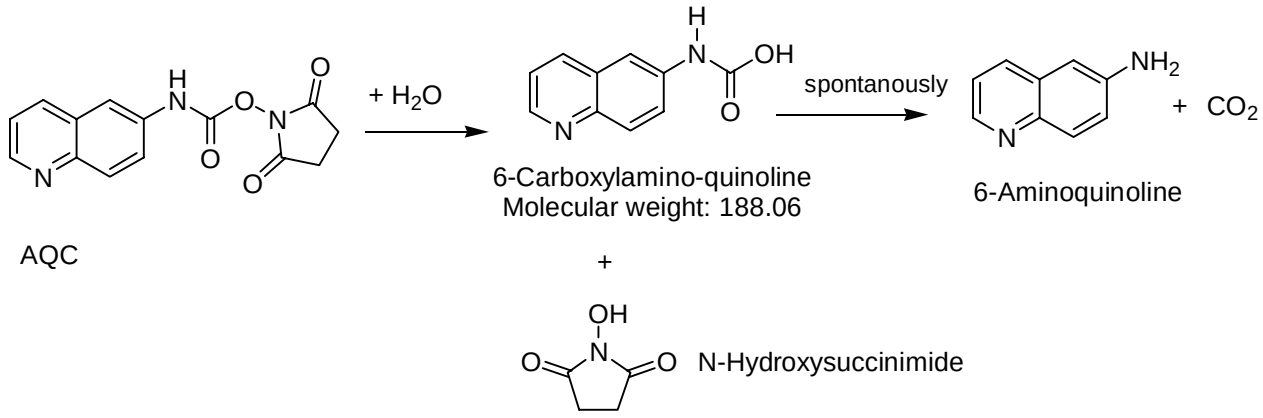


Figure 25: Hydrolysis of AQC in buffered aqueous solutions leading to the unstable carbamic acid derivative according to reference [23]

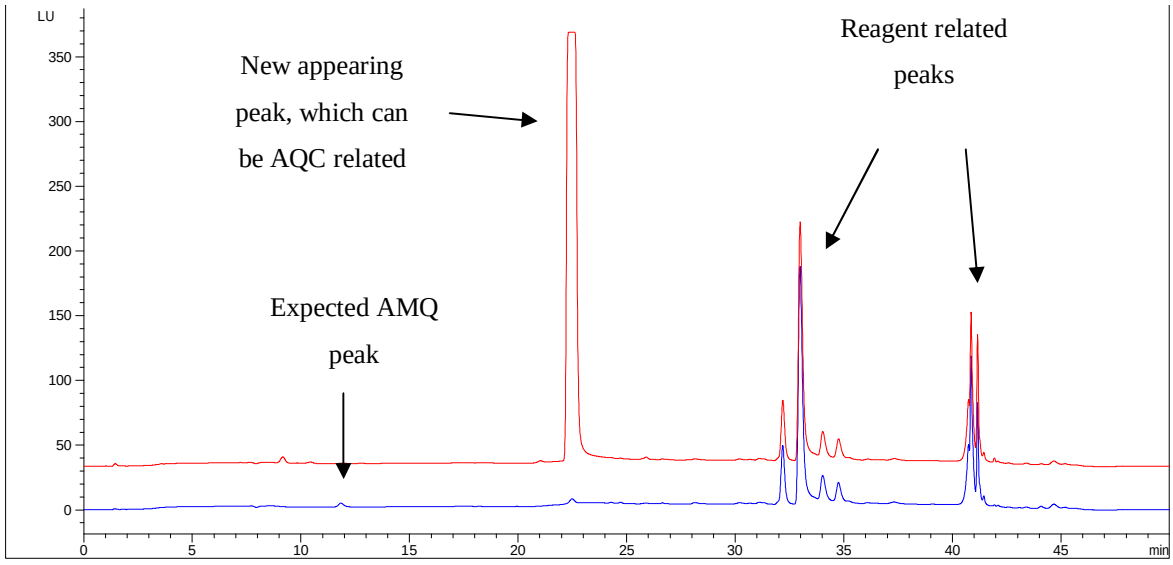


Figure 26: Overlay of chromatograms from reagent blanks. One just contained AQC (blue) and the other 10 nmol AQC and 100 nmol TCEP (red). AMQ exhibites different emission spectrum to AQC and its peak is not shown here. The investigation was carried out using method 4.

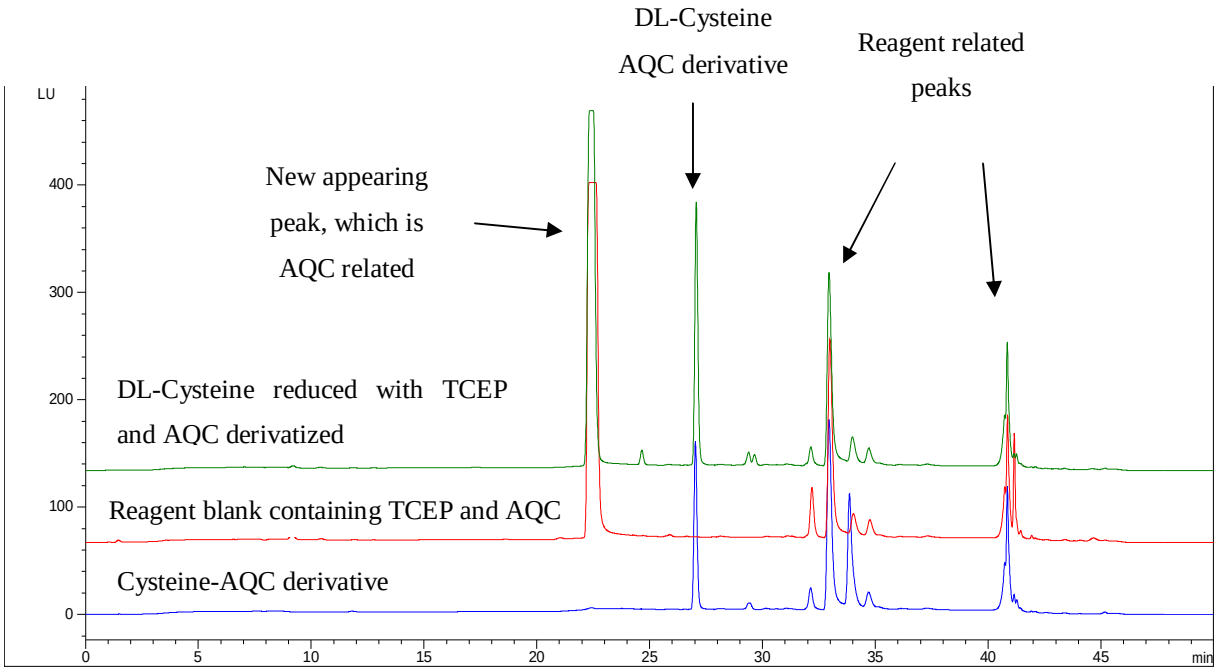


Figure 27: Overlay of the reagent blank with TCEP and AQC, L-cysteine with AQC derivatized and DL-cystine reduced with 100 molar excess of TCEP and derivatized with AQC. The investigation was carried out using a RP system and method 4.

The retention behaviour of cysteine-S-acetate and cysteine-S-acetamide were investigated during the reversed phase run. The additional acid functional group of cysteine-S-acetic acid had led to a strongly reduced retention, leading to retention times before AMQ. In such an aqueous media the quenching was remarkably strong. A lower sensitivity for this compound could be disadvantageous for further investigations. The acetamide derivative had a retention time of 24,6 min and coeluted with other amino acids such as Thr and Arg, which resembles an undesirable retention behaviour for the cysteine-acetamide derivative. The cysteine-vinylpyridine derivative had a retention time of 31,5 min, which would be preferable, because it would elute in a retention window between the AQC derivatives of Met and Ile. Stability problems occurred in term of the vinylpyridine derivatization and this mode of derivatization had to be dismissed.

On a quinine-based CSP the retardation of the cysteine-S-acetate was very strong, using CSP 1 enantioselectivity was 1,55 and Rs was 5,03, but the retention factor for the first and the second eluting enantiomer were 33,14 and 51,33, respectively. When CSP 6 was applied, a low-coverage-QN-AX column with a selector coverage of 142 µmol/gram silica gel, enantioselectivity was 1,6 and Rs was 4,08 and additionally the retention factors were acceptable, 6,54 and 10,58, respectively for the first and the second eluting antipode. Cys-S-acetate had higher retention than homocysteic acid, which had as second acid moiety a sulfonic acid. In comparison of these two bi-acidic AQC derivatives, it was expected, that the stronger the acid, the stronger the retention on an anion-exchange type CSP. The pKa value of cysteine is 1,92 and of the sulfonic acid functionality it is1,3. The acetamide moiety has an pKa

value in the range of 3,12 to 4,76, according to the pka values of iodo acetate and acetic acid, respectively. It could be stated, that the expected behaviour was not observed instead, the bi-carboxylic acid derivative Cys-S-acetate had a stronger retention than homocysteic acid.

The elution of cysteine-S-acetamide was achieved in an acceptable time scale with k_2 of 3,96. It was separated with an alpha >1,5 and a resolution between 4 and 5 using either CSP 1 or CSP 2 (Figure 28).

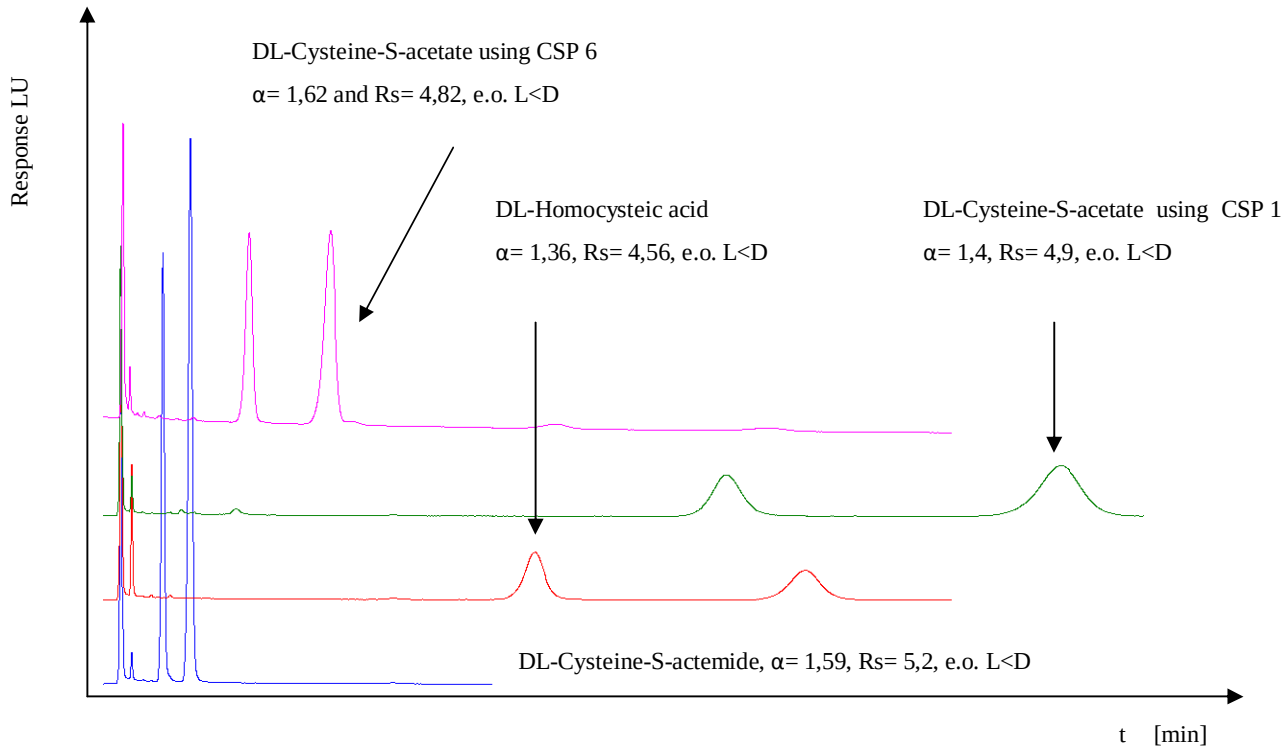


Figure 28: Chromatograms of the enantioseparations of cysteine derivatives. Cys-acetamide matched all requirements of good enantioselectivity and resolution within a short time. The cys-acetate derivatives had an expanded retention on less strong retained than the cys-S-acetate species. Method 1 was applied.

4.3 Racemization of dipeptides

A racemization of an amino acid could be conducted via the formation of a Schiff base and the racemization of an acidic proton located at the N-terminal end [47]. The racemization was illustrated in Scheme 1.

The procedure was established by the racemization of phenylalanine and then the racemization of GlyPhe and PheGly was carried out. The products were derivatized with AQC and the racemization progress was controlled via liquid chromatography runs on CSP 1 using method 2. For the racemates of PheGly, a peak pair of enantiomers was found and the contrasting test with Gly-D-Phe yielded one peak, namely the unchanged educt compound. The retention times of DL-PheGly were different, compared to the peak pair of Gly-DL-Phe (Figure 29).

A diketopiperazine would not show retention on an anion-exchange type CSP and furthermore the AQC derivatization was not suitable for an amide bond. For that reason, the racemized DL-PheGly and the

Gly-D-Phe were analyzed using a reversed phase system, where the diketopiperazine was expected to show retention, whereas a zwitterionic dipeptide would be eluted very quickly. The analysis was performed using a RP column (Table 1). Isocratic elution with mobile phase ACN/10 mM NH_4AcO 20/80 aqueous buffer, where the buffer pH 5,0 was adjusted with acetic acid was conducted. Detection was carried out at 248, 280 and 360 nm with a diode array detector. The chromatograms did not show any peaks, which could be related to diketopiperazine species.

The DL-PheGly racemate and the L-PheGly enantiomer were also analyzed with mass spectrometry. Both were derivatized with AQC and were also injected in their native form into a HPLC-RP C18-system using a modified gradient of literature [28] (method 6). The mass analyzer was a MDS Sciex 4000 QTrap, from Applied Biosystems, parameters were: scan range 180-550 m/z, ESI negative mode, maximal accumulation time of 300 ms, nebulizer 60 psi, dry gas 11 ml/min, dry temperature 350°C, with an auto MS^2 mode for ion trap fragmentation. Signals with mass 391 m/z and 221 m/z were found for the AQC-derivative and the native dipeptide, respectively. The mass peak for a diketopiperazine, 204 m/z, was not observed.

The racemization of the N-terminal chiral amino acid was tested and the inversion of the dipeptide was not observed. The stereochemistry was solely changed for N-terminal located stereocenters containing an acidic proton and a C-terminal located stereocenter was not influenced by the racemization procedure. A diketopiperazine species was not observed, neither in an enantioselective nor in a reversed phase analysis using both an UV and a mass spectrometric detection. These investigations provided evidence, that the N-terminal racemization of dipeptides followed the reaction mechanism as illustrated in Scheme 1 [47] and did not lead to a formation of diketopiperazines (Figure 11), with an eventual inversion of the dipeptide or an inversion of the stereochemistry.

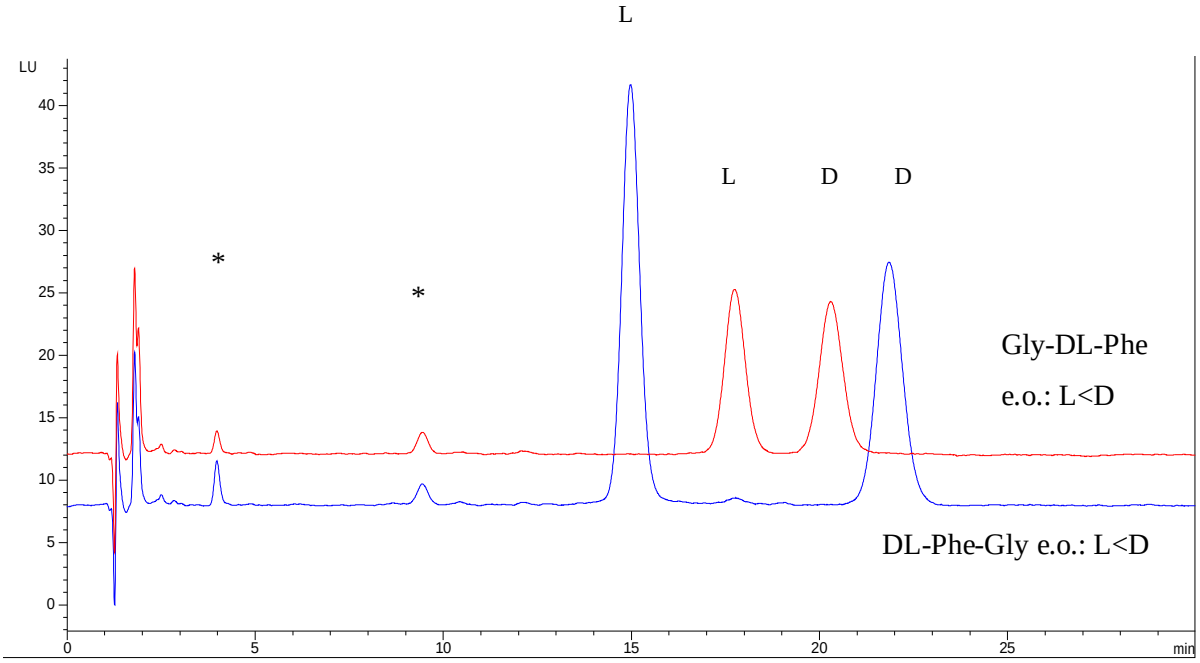


Figure 29: Overlay of chromatograms of AQC derivatives of Gly-DL-Phe and DL-Phe-Gly dipeptides. A QD-AX column was used with a hydro-organic mobile phase. The investigation was carried out using method 2.* unknown AQC reagent related peaks

FTIR investigation was conducted. It was thought that the amide bond of dipeptide would be different to an amide bond of a diketopiperazine in their IR spectrum. Absorption band for an amide bond in solid state, for N-mono-substituted amides, was expected at 1690-1640 and 1550-1510 cm^{-1}. Exaclty the same absorption bands at 1659 cm^{-1} and at 1515 cm^{-1} were found, in both, the racemized DL-PheGly and the untreated Gly-DL-Phe samples. A diketopiperazine formation would cause altered absorption bands. The finding of equal absorption bands indicated the presence of the same amide bond species in both mentioned samples and was a further confirmation of a successful racemization [16].

4.4 Enantioseparations of fluorescence tagged amino acid-derivatives using *tert*-butyl carbamate modified quinine/quinidine alkaloid-type chiral stationary phases

In this chapter, the evaluated data for all separations regarding N-terminal labeled amino acids are presented. The chromatographic parameters, such as retention factor k, enantioselectivity α_{RS} and resolution Rs will be discussed. Mainly, the separation of AQC-tagged amino acids and peptides were presented. Further, another fluorescence-label for N-terminal derivatization of amino acids, namely fluoresceine isothiocyanate (FITC), was used for the derivatization of proteinogenic amino acids. The two fluorescence tags were compared, according to their enantioselectivity and separation performance.

4.4.1 General considerations

Enantioseparation of racemic mixtures of amino acids, derivatized with AQC were performed via quinine/quinidine alkaloid-type chiral stationary phase (CSP 1 and 2). Identification of the (S/R)-enantiomers was done by comparison of the peak areas of a racemate, which should be equal. Furthermore, an injection of a pure enantiomer accomplished the identification of the single enantiomer by comparing retention times. The combination of a quinine and quinidine based CSP reversed the enantiomer elution order and furnished another identification possibility for the two enantiomer peaks.
In many cases it was found that a mobile phase, consisting of methanol with 2 % (v/v) AcOH and 0,5 % (w/v) NH_4AcO, provided good enantioseparations for the large set of analytes with different chemical properties. For this reason it was chosen as standard mobile phase for investigations using quinine-based CSPs in the polar-organic phase mode (method 1). Further standard conditions were a flow rate of 1 ml/min and column temperature was 25 °C. Detection of analytes were performed with diode array detector (DAD) at the absorption maxima of the AQC-amino acid derivatives of 248 nm and with fluorescence detector (FLD) at an excitation maximum at 248 nm and an emission maximum of 395 nm. It was recognized that a reduction in flow rate had led to a better resolution of the enantiomers or stereoisomers, but provided stronger peak broadening. Synthesis and preparation of the *tert*-butyl-carbamoyl modified quinine and quinidine based CSP's were performed according to in-house protocol [54]. Further column descriptions were provided in Table 2.
Generally, due to the weak anion-exchanger character of CSP 1 and 2, analytes with an additional acid functional group showed higher retention times, whereas basic or neutral molecules were less retained. According to that, amino acids with a second acid functional group in the side chain will undergo much stronger retention compared to its mono acid counterpart.
Derivatization was done according to the procedure described in chapter 3.4.1.

4.4.2 Evaluation of the separation performance of AQC-tagged amino acids on *tert*-butyl carbamate quinine/quinidine-based chiral stationary phases

4.4.2.1 Proteinogenic amino acids

The separation data of proteinogenic amino acids were summarized in Table 10, Table 11 and Table 12. The separations were done under the same conditions on CSP 1 and CSP 2 using method 1. The enantioselectivity values from the two columns were presented in Figure 30. Comparison of the alpha values from the separation of AQC tagged proteinogenic amino acids was performed for QN-AX and QD-AX CSPs. Alpha values, higher than 1,5, were aspired to yield good chromatographic performance for the enantioseparation on both columns. It was found that above the critical alpha value of 1,5, the QD-AX column exhibited better alpha values, compared to QN-AX. Aspartic acid and glutamic acid showed unsatisfactory separations on both columns. Aspartic acid was partially separated only by QD-AX with no separation observed on QN-AX. The elution order of aspartic acid was different than to all

other proteinogenic amino acids, expect proline. Using a QN-AX column the L-enantiomer of aspartic acid eluted firstly. Other γ-carboxylic acid amino acids, like homocysteic acid exhibited also a reversed elution order. Cysteic acid, which had a beta carboxylic acid moiety and glutamic acid, with their acid functionality on the δ-position, did not exhibit such an effect concerning a changed elution order. It is known, that a second acid functionality led to a reduced enantiodiscrimination due to more SA-SO complexes could be formed. Concerning aspartic acid, two nearly equal carboxylic acid moieties will interact with the selector and the overall enantiodiscrimination was diminished. Homocysteic acid had one sulfonic acid moiety and therefore the SO-SA complexes differed in their nature. Enantioselectivity was 1,6 and resolution 5,06 for homocysteic acid. A second acid functionality at the γ-position led to a diminished enantiodiscrimination, but the effect depended on the acidity of the additional acid moiety.

For some amino acids, such as threonine, asparagine and tryptophane, the QN-AX exhibited higher alpha values than QD-AX.

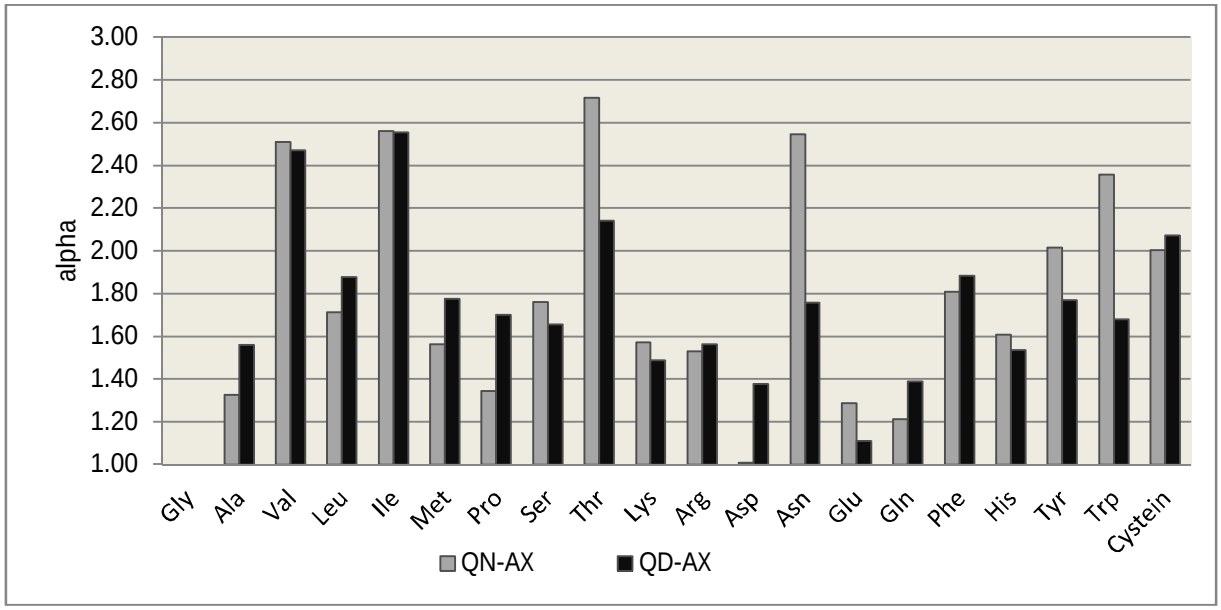


Figure 30: Alpha values of the separation of proteinogenic amino acids derivatized with AQC

Figure 31 showed the resolution values of the separation of proteinogenic amino acid derivatized with AQC. Predominately, the QD-AX CSP exhibited better resolution values and higher plate numbers. Exceptions were threonine, asparagine, tyrosine and tryptophane. However, for threonine, asparagine, tyrosine and tryptophane, the resolution was significantly higher on the QN-AX than on the QD-AX column.

The measurements with the QD-AX CSP was done on an Agilent 1100 Series HPLC with a multi wavelength detector and the QN-AX separations on an Agilent 1200 Series HPLC with DAD and FLD. Table 3 illustrated the investigation, concerning the significant different resolution values and plate numbers, which were obtained for the separation on QD/QN-AX. Three analytes were injected on an

Agilent 1200 Series HPLC using the same chromatographic parameters, except the columns. Once the QD-AX was used and afterwards the QN-AX. Generally, QN-AX exhibited a lower performance than QD-AX. The application of two different HPLC systems did not affect the resolution or the plate numbers. The difference had to be based on the applied columns. The procedure for selector synthesis, immobilization and column packing were well established [66], [51]. The column packing process might be less prone for errors.

Table 3: Investigation of resolution and plate numbers for QN- and QD-AX column. Three amino acids were derivatized with AQC and injected on an Agilent 1200 Series HPLC. The mobile phase was MeOH/AcOH/NH_4AcO 98/2/0,5 % (v/v/w).

	DL-Ser		DL-Pro		DL-Ile	
	QN-AX	**QD-AX**	**QN-AX**	**QD-AX**	**QN-AX**	**QD-AX**
Rs	5.73	6.41	2.77	6.69	7.88	9.95
N_1	2639	4039	2802	4217	2409	3496
N_2	2910	4372	2960	4316	2924	4296

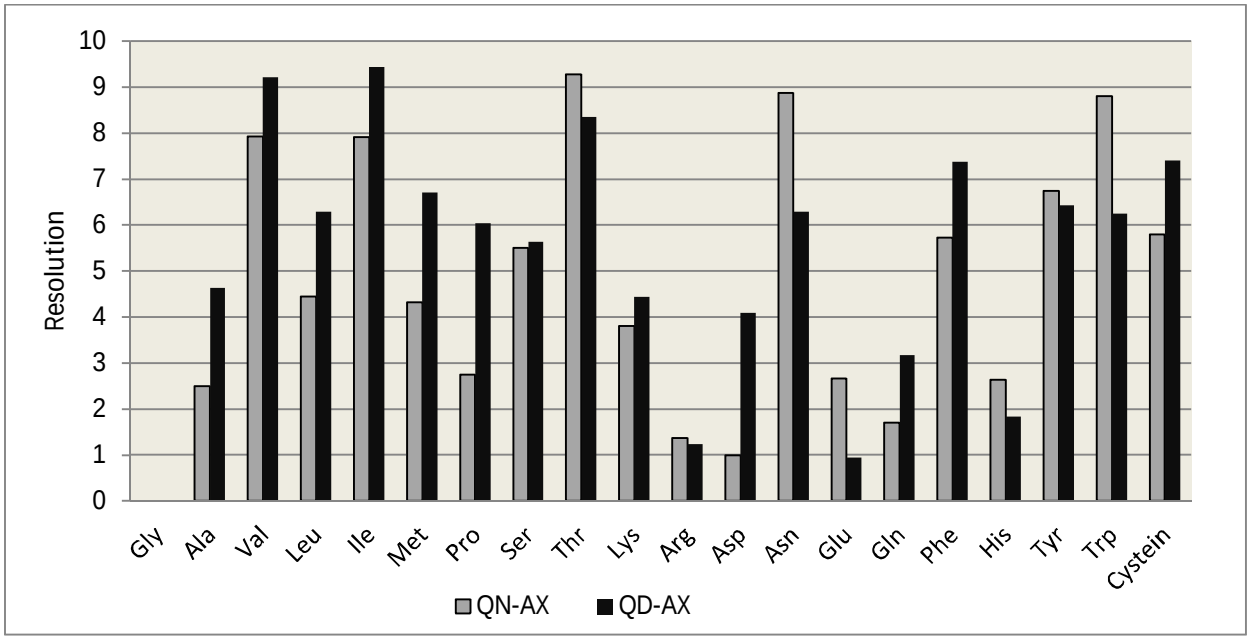


Figure 31: Resolution of the separation of proteinogenic amino acids derivatized with AQC

4.4.2.2 Comparison of polar-organic and hydro-organic mobile phase for the separation of AQC-amino acid derivatives

A comparison of separation performance for two common mobile phase compositions, namely polar-organic and the hydro-organic was established for QN-AX CSP. The result for the enantioseparation of a set of proteinogenic amino acids, derivatized with AQC and separated with a hydro-organic mobile phase were summarized in Table 13 and the result for polar-organic mode were listed in Table 10.

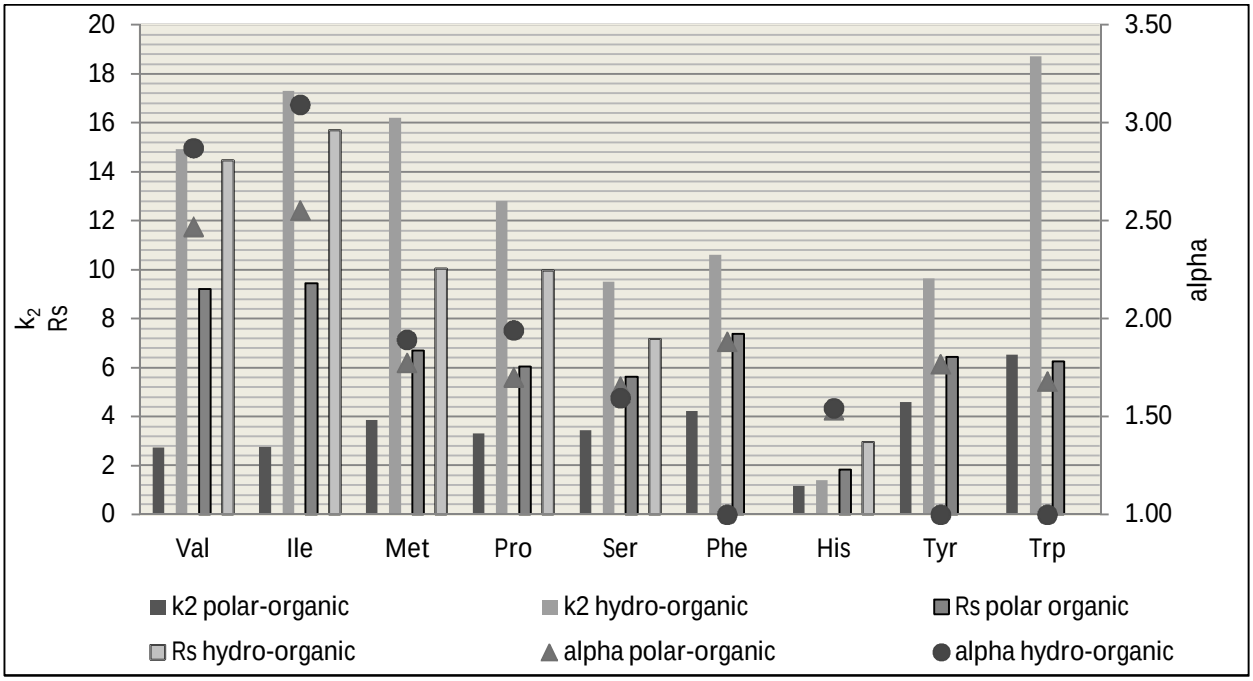


Figure 32: Comparison of chromatographic parameters of AQC-amino acid derivatives with two different mobile phase compositions. The hydro-organic mobile phase, MeOH/H_2O 80/20 containing 20 mM NH_4AcO and pH_a was set to 6,0, was applied.

As shown in Figure 32, the k_2, alpha and resolution values were plotted. Obviously, the retention of the derivatives was much shorter when the polar-organic phase mode was applied, because different buffer types and buffer salt concentrations were used. The polar organic buffer consisted of 350 mM acetic acid and 65 mM of the corresponding base (method 1), whereas the hydro-organic mobile phase used 20 mM NH_4AcO with pH_a 6,00, adjusted with acetic acid (method 2). The enantioselectivity was approximately same for both mobile phase compositions, with moderately better alpha values for the hydro-organic mode. However, Phe, Tyr and Trp were not enantioseparated with the hydro-organic mobile phase. The resolution was stronger influenced by the hydro-organic mobile phase and was in all observed cases better than observed for the polar-organic mode. For example, Pro had an alpha of 1,35, if polar-organic mobile phase was used, but with hydro-organic mobile phase alpha was raised up to 1,94. On the other side, the retention was increased with the hydro-organic mobile phase (Figure 33). This negative effect was illustrated in Figure 34 for the valine derivative. The separation performance of valine enantiomers was sufficient in polar organic mode, allowing enantioseparations within ten minutes. In comparison, the hydro-organic mobile phase exhibited rather same enantioselectivity and an unnecessarily high resolution. The run time was 23 minutes, compared with seven minutes for the polar organic mode. It was found that in general an optimized mobile phase depended on the side chain of amino acids, which account mainly for the chromatographic behavior of their AQC derivatives. Further, the hydro-organic mobile phase could be applied using a higher buffer concentration to yield shorter retention times.

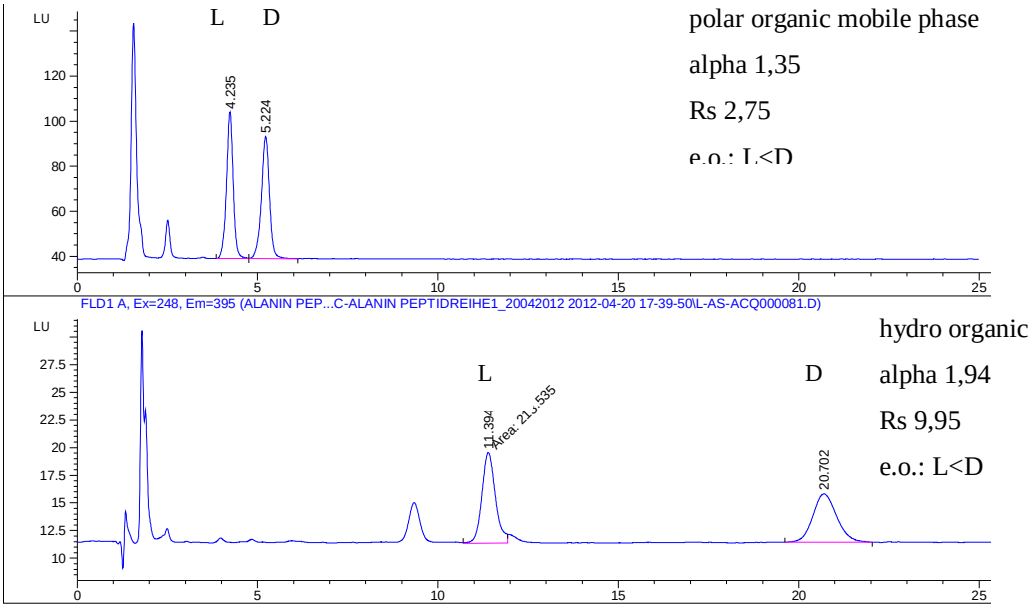


Figure 33: Comparison of chromatograms of AQC-Pro derivatives. First chromatogram shows the separation using method 1. The second chromatogram shows the separation applying method 2.

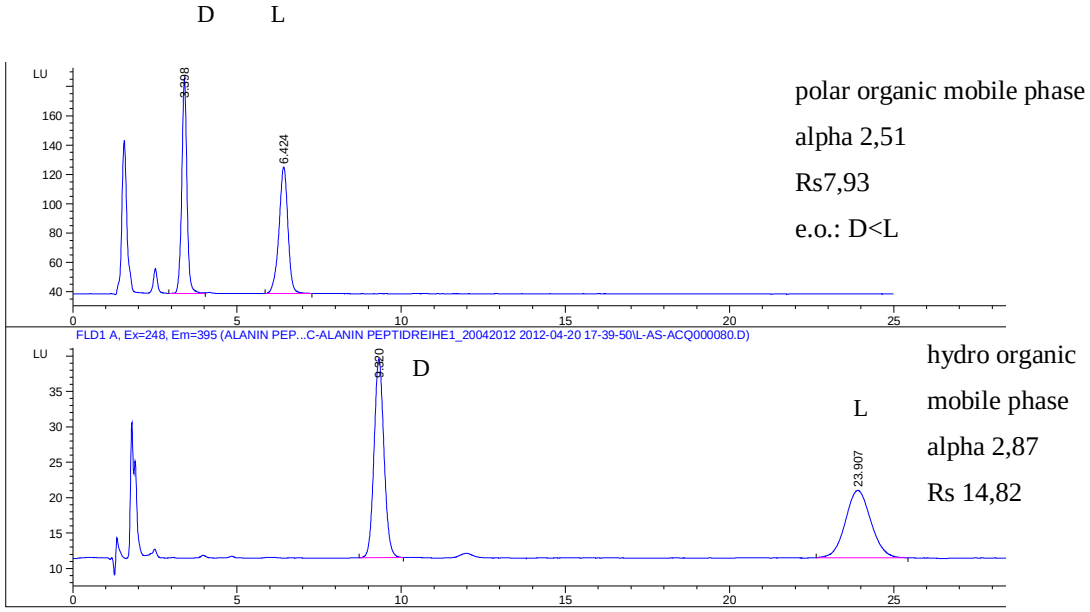


Figure 34: Comparison of chromatograms of AQC-Val derivatives. First chromatogram shows the separation using method 1. The second chromatogram shows the separation applying method 2.

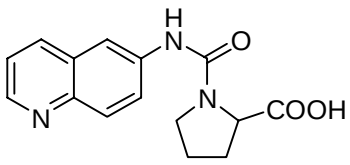
4.4.3 Separation of AQC derivatives for non-proteinogenic amino acids

It was decided to investigate the separation performance of an extended set of amino acids, derivatized with the AQC-tag.

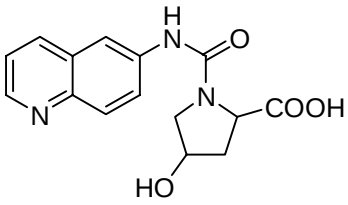
4.4.3.1 Stereoisomers of 4-Hydroxyproline

4-Hydroxyproline could exist as four stereoisomers. The most common stereoisomer in biological tissues is the 2S, 4R-trans-4-L-hydroxyproline. Hydroxyproline is often found in collagen, as an important structure element. This amino acid is generated during a posttranslational modification of the genetically coded proline.

In comparison, the two columns (CSP 1 and CSP 2) exhibited the same low performance for the hydroxyproline derivatives, when standard conditions were applied (Table 4). It was found that the separation of all four stereoisomers was much better if the flow rate was reduced from 1 ml/min, which is a standard value, to 0,4 ml/min. The retention factor and enantioselectivity values were not influenced by reducing the flow rate, but the resolution was increased (Figure 36). It should be mentioned that the elution order of proline and hydroxyproline was changed. From the enantioseparation of AQC-tagged proteinogenic amino acids on QD-AX it was known that the L-enantiomers eluted first, except for proline and aspartic acid. The latter was only partially separated and exhibited extraordinary long retention times. D-Proline was the first eluting enantiomer, when QD-AX column was used. For this secondary amino acid, it was thought that the construction of the urea derivative had an influence on the interaction of the selector with the amino acid derivative. For 4-hydroxyproline it was found that the elution order was reversed as well.



AQC-proline derivative



AQC-4-hydroxyproline derivative

Figure 35: Structure of secondary amino acids, proline and 4-hydroxyproline, derivatized with AQC

Figure 35 shows the structure of the AQC derivatives of secondary amino acids. As illustrated the urea derivative led to a tertiary amine functionality. In contrast to primary amino acids, the AQC derivatives of secondary amino acids might not interact with the selector using a hydrogen bonding of the carbamate and the alpha amine moieties of the amino acid. The urea linkage bridge exhibited a second amine, which might be capable for hydrogen bonding. Especially for secondary amino acids, the characteristic of the urea linkage bridge come into account for the formation of the SO-SA complex. A hydrogen bonding was a requirement of the multiple point interaction of a chiral molecule with the

quinine selector [53]. It was thought that the different participating moieties of the AQC derivatives of secondary or primary amino acid accounted for the reversed elution order.

Table 4: Separation data for four stereoisomers of hydroxyproline, derivatized with AQC-tag and separated on QN/QD-AX column applying method 1.

Analytes		to	t_1	t_2	k_1	k_2	alpha	Rs	e.o.
trans-4-D or trans-4-L-Hydroxyproline (2R, 4S and 2S, 4R)	QN-AX	1.40	4.03	4.59	1.88	2.28	1.21	0.94	L
cis-4-D or cis-4-L-Hydroxyproline (2R, 4R allo and 2S, 4S)	QN-AX	1.40	4.45	5.10	2.18	2.64	1.21	1.10	L
trans-4-D or trans-4-L-Hydroxyproline (2R, 4S und 2S, 4R)	QD-AX	1.50	4.37	5.78	1.91	2.85	1.49	2.34	D
cis-4-D or cis-4-L-Hydroxyproline (2R, 4R allo and 2S, 4S)	QD-AX	1.50	5.20	6.30	2.47	3.20	1.30	1.59	D

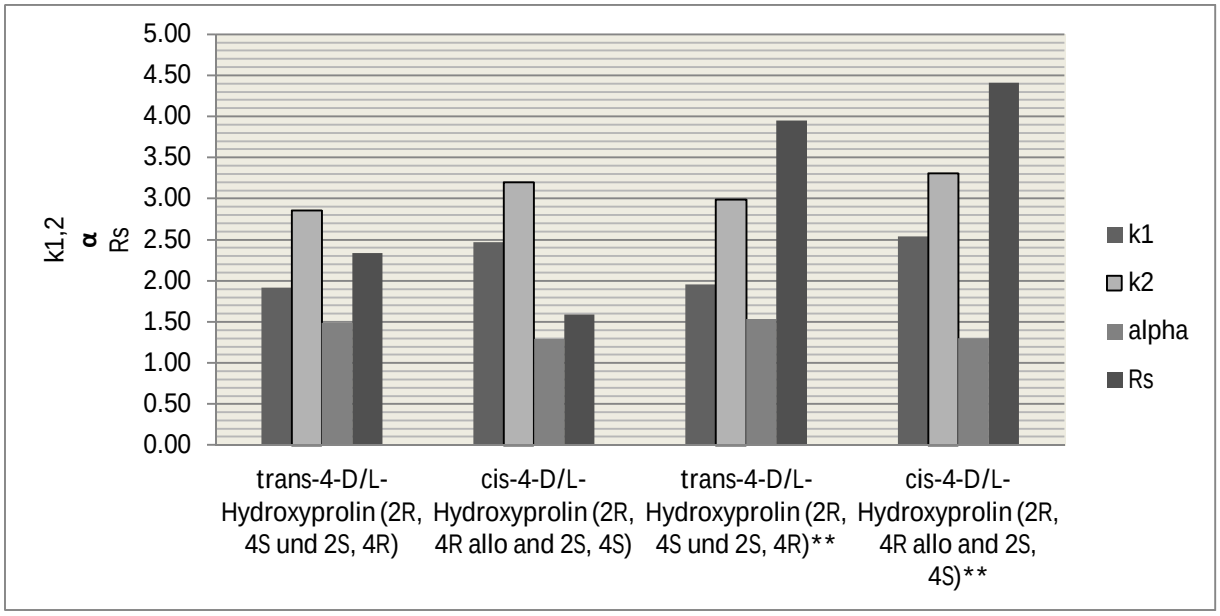


Figure 36: Comparison of separation performance of two pairs of 4-hydroxyproline enantiomers on QN/QD-AX applying two different flow rates, standard mobile phase condition with flow rate 1 ml/min and reduced flow rate 0,4 ml/min**

All four stereoisomers were well separated on both columns, CSP 1 and CSP 2, by applying the standard polar-organic, as well as the hydro-organic mobile phase composition (Table 17). In direct comparison, the latter provided slightly better performance according to enantioselectivity and resolution. Two hydro-organic mobile phases were applied and varied in their buffer concentration. The chromatographic parameters were the same, except that for one method a flow rate of 0,5 ml/min were

used. Better enantioselectivity and resolution was obtained for the hydro-organic mobile phase with higher buffer concentration of 200 mM NH_4AcO. When applying the flow rate of 0,5 ml/min, the best separation performance was achieved for the four stereoisomers. It could be stated that for the separation of 4-hydroxyproline stereoisomers the optimal mobile phase composition was $MeOH/H_2O$ 80/20, 200 mM NH_4AcO pH_a 6,0 and a flow rate of 0,5 ml/min.

4.4.3.2 Stereoisomers of Threonine

For the amino acid threonine, in total eight stereoisomers can be found, D/L-threonine, D/L-allo-threonine, DL-isoserine and D/L-homoserine. Threonine contains two stereogenic carbon atoms, L-Thr has 2S, 3R, D-Thr 2R, 3S, and the D/L-allo-Thr species have 2S, 3S, and 2R, 3R configuration, respectively. Homoserine can often be found in drugs as a mimetic compound to threonine. It is also produced during the enzymatic digestion of L-methionine. Homoserine has also been used for several syntheses of amino acid related compounds. Isoserine has been added to the set, because it can be used for the same purposes as homoserine. Isoserine is a precursor compound in organic synthesis, known as a bioactive substance important for the proliferation of yeast and plants [6].

The results for the separation of each of the single racemic mixture could be seen in Table 5. Obviously, under standard conditions, all eight stereoisomers were not separated neatly. The L-enantiomers of threonine and homoserine were expected to coelute and L-isoserine eluted very close to them. The resolution was better for the QD-AX column. It should be mentioned, that the four stereoisomers of DL-threonine and DL-allo-threonine were separated using method 1 (Figure 38). The elution order was L-Thr<L-allo-Thr<D-allo-Thr<D-Thr and the enantioselectivity values were1,21; 1,78 and 1,27, respectively to the $2^{nd}/1^{st}$, $3^{rd}/2^{nd}$ and $4^{th}/3^{rd}$ pair of stereoisomers. Resolution values were 1,34; 4,13 and 2,52 for the same order as mentioned before.

Isoserine had the highest retention factors in this series of related compounds (Table 5). This amino acid had a α-hydroxy functionality, which means that a –I-effect might acidify the carboxylic acid moiety. The stronger the acid functionality is, the stronger the ion-exchange interaction and therefore the stronger the SO-SA complex. The retention was generally higher but also the enantiodiscrimination for the two enantiomers was therefore raised. The structure of isoserine is illustrated in Figure 16 and provided an explanation for the better enantioseparation performance of this amino acid compared to the other structure analogues.

Isoserine was excluded for the investigation of the separation of threonine analogues, because only the racemate was available and therefore the peak identification was impossible in a mixture of eight compounds. For this reason a mixture of all six remaining enantiomers was separated with a slower flow rate of 0,4 ml/min. Chromatographic parameters were listed in Table 6. Again, the L-enantiomers of homoserine and threonine coeluted. All other enantiomers were separated with an alpha >1,5 and resolution in the range of 3,94 and 8,98. Figure 37 showed a chromatogram of this separation study. Under the applied conditions, the L-antipodes of homoserine and threonine coeluted.

Table 5: Stereoisomers of threonine and their separations parameters of each single racemic mixture using method 1 for both QN/QD-AX.

	QN-AX						QD-AX					
Analytes	**t_1**	**t_2**	**k_1**	**k_2**	**alpha**	**Rs**	**t_1**	**t_2**	**k_1**	**k_2**	**alpha**	**Rs**
DL-Thr	3.95	8.19	1.82	4.85	2.66	8.88	3.99	7.26	1.66	3.84	2.31	8.89
DL-allo-Thr	4.40	6.74	2.14	3.81	1.78	5.33	4.30	6.64	1.87	3.43	1.84	6.58
DL-Isoser	8.17	15.10	4.84	9.79	2.02	5.82	3.99	7.26	1.66	3.84	2.31	8.89
DL-Homoser	4.00	5.52	2.14	2.94	1.37	3.94	4.30	6.64	1.87	3.43	1.84	6.58

Table 6: Chromatographic parameters for a mixture of homoserine, threonine and allo-threonine are presented. QD-AX column applied under method 1.

Analyte	**to**	**k_{1-3}**	**k_{4-6}**	**$\alpha_{3/1,2}$ $\alpha_{4/3}$**	**$\alpha_{5/4}$ $\alpha_{6/5}$**	**Rs $_{1,2/3}$ Rs $_{3/4}$**	**Rs $_{4/5}$ Rs $_{5/6}$**	**N $_{1-3}$**	**N $_{4-5}$**	**e.o.**
DL-Homoser	3.54	1.81	2.85	1.13	1.30	1.61	4.24	5990	7801	L-Hser=L-Thr<L-allo-Thr<D-Hser<D-allo-Thr<D-Thr
DL-Thr	3.54	1.81	4.09	1.39	1.11	5.20	1.81	\	7855	
DL-allo-Thr	3.54	2.04	3.70	\	\	\	\	6820	7612	

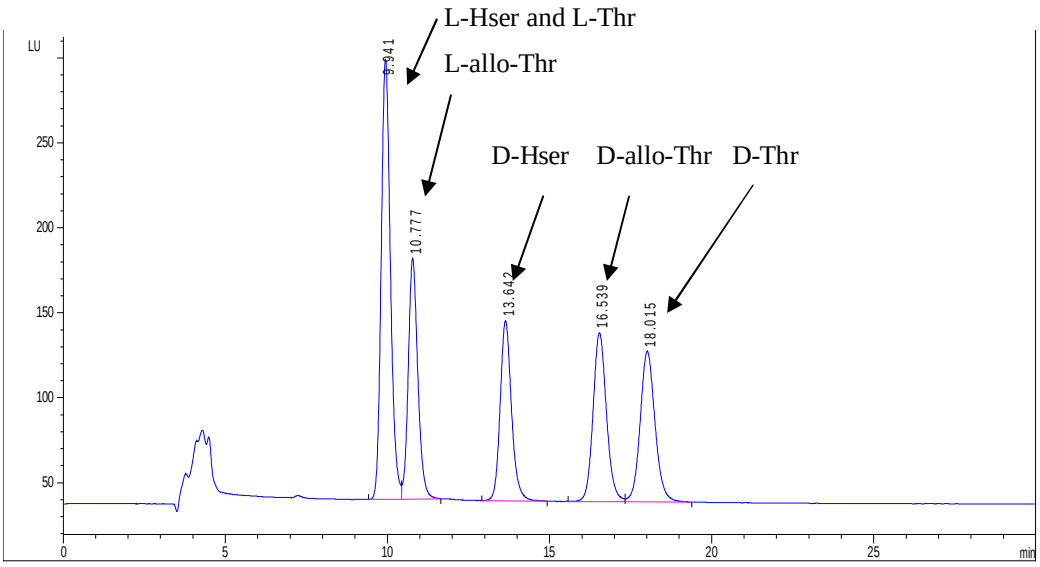


Figure 37: Chromatogram of an injection of a mixture of all six enantiomers of threonine analogues using a QD-AX column and method 2.

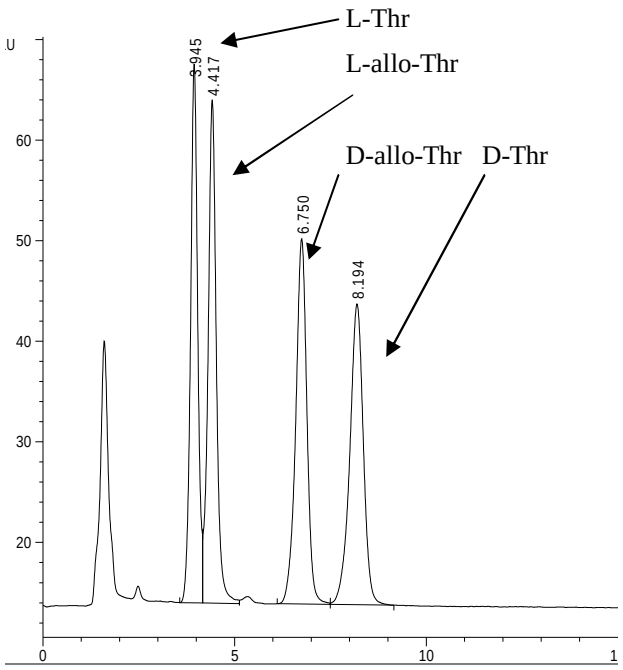


Figure 38: Separation of a mix of DL-threonine and DL-allo-threonine using a QN-AX column and method 1.

4.4.3.3 Phenylalanine/Proline-Glycine-Dipeptides

The enantioseparation and detection of chiral peptides were known as a challenging field in enantioseparation science. Several small peptides exhibited biological activity and especially in synthesis small peptides were common precursor compounds. On this account, the separation of dipeptides with a suitable sensitive detection was useful in this field of chemistry. Glycine moieties were often introduced during a derivatization as linkage groups [76-78]. Dipeptides, containing one glycine moiety, were optically active. It was known that the introduction of a glycine often hindered an otherwise easily achievable enantioseparation [43, 45].

For the investigation of the separation performance of specific dipeptides, derivatized with AQC, a study was conducted using CSP 1 and 2 and two different mobile phases were tested. The Gly-Phe species was always worse separated than the Phe-Gly species and QD-AX exhibited better separation performance than QN-AX. Resolution was increased by decreasing the flow rate. For example Gly-DL-Pro had a Rs of 0,74, 1,07 and 2,05 at a flow rate of 1,0; 0,4 and 0,25 ml/min, respectively (Table 7). It should be mentioned that the achieved separation performance was not supreme (method 1), but beat most of the published data, mentioned in the references above. Especially, the Gly-Pro species was often excluded of such investigations, because bad separation was expected. The present study used another mobile phase composition in hydro-organic mode with a different acid to base ratio (method 2). This mobile phase composition was known to yield good separation, especially for di- and higher peptides [43, 45]. The relatively low resolution of Gly-DL-Pro (Table 7) was possibly caused by the formation of rotamers. Rotamers were converted into each other by simple rotation of C-C bonds. It was known that glycine containing peptides were predestinated for rotames, because the peptide back bone

became more volatile. Rotamers also produced broader peaks in a chromatogram, because many different conformational intermediate states were possible. The formation of rotamers would provide an explanation of the reduced enantioselectivity and decreased resolution of glycine containing dipeptides.

As listed in Table 8, in most cases the resolution was increased with hydro-organic (method 2 or method 3), compared to the polar-organic mobile phase using method 1. The alpha values were worse for DL-Phe-Gly, but became better for Gly-DL-Phe. For Gly-DL-Pro, the hydro-organic mobile phase seemed to be the better choice, according to the mobile phase. Comparing the data of Gly-Gly-DL-Pro and Gly-DL-Pro, it was obvious that the introduction of another glycine moiety strongly hindered an enantioseparation. A different picture was observed when comparing the Ala-Gly peptides. Gly-DL-Ala and DL-Ala-Gly had rather the same k-values, but quiet different alpha and resolution values. Another glycine moiety on the N-terminal or the C-terminal end, respectively, resulted in reduced separation of the enantiomers (Table 8).

Table 7: Chromatographic parameters for the separation of AQC derivatized Phe/Pro-Glycine dipeptides using CSP 1 and 2 and method 1.

QN-AX polar-organic mode					**QD-AX polar-organic mode**				
Analyte	**k_1**	**k_2**	**alpha**	**Rs**	**Analyte**	**k_1**	**k_2**	**alpha**	**Rs**
Gly-DL-Phe	2.91	3.44	1.18	1.60	**Gly-DL-Phe**	1.36	2.06	1.51	2.26
Gly-DL-Pro	2.17	2.68	1.23	0.74	**Gly-DL-Pro****	2.54	3.62	1.42	2.05
Gly-DL-Pro *	2.24	2.64	1.18	1.07	**DL-Phe-Gly**	2.19	3.35	1.53	4.44
DL-Phe-Gly	1.95	3.18	1.63	4.26					

*flow rate 0.4 ml/min

**flow rate 0.25 ml/min

polar-organic mobile phase: MeOH/AcOH/NH_4AcO 98/2/0,5 % (v/v/w)

Table 8: Separation parameters of special dipeptides, derivatized with AQC and separated on a QD-AX column using method 2.

Analytes	**k_1**	**k_2**	**alpha**	**Rs**
Gly-DL-Phe	8.99	13.56	1.51	6.26
DL-Phe-Gly	10.83	12.53	1.16	2.32
Gly-DL-Pro	6.66	8.63	1.30	1.17
Gly-Gly-DL-Pro*	5.60	5.70	1.02	0.32
Gly-DL-Ala	6.31	7.07	1.12	1.68
DL-Ala-Gly	5.71	7.55	1.32	4.11
DL-Ala-Gly-Gly	4.67	4.67	1.00	\
Gly-Gly-DL-Ala	4.29	4.73	1.10	1.12

* flow rate 0.4 ml/min

hydro organic mobile phase: MeOH/H_2O 80/20, 20 mM NH_4AcO adjusted to pH_a 6.00 with acetic acid

4.4.3.4 Stereoisomers of Isoleucine

Isoleucine contained two stereogenic carbon atoms and therefore four stereoisomers. An enantioseparation was easy to perform for each of the racemic mixtures, D/L-Ile and the D/L-allo-Ile. A mixture of all four stereoisomers could not be separated when using method 1 or method 2. It should be mentioned that the QN-AX phase was able to separate at least the L-enantiomers, whereas with QD-AX, both, the D- and L-enantiomers coeluted (Figure 39). The separation data were summarized in Table 15 and Table 16.

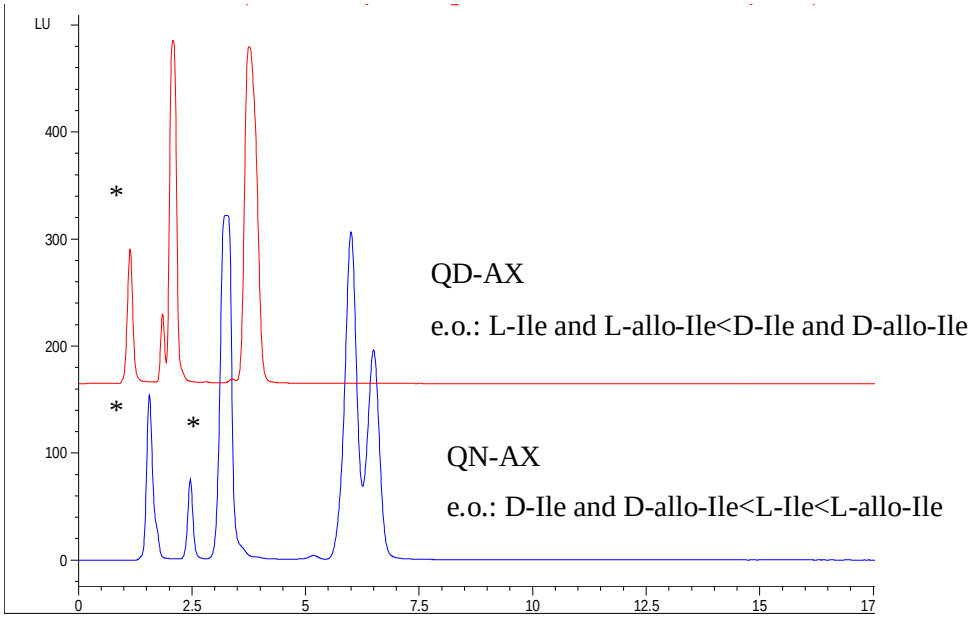


Figure 39: Chromatogram of four stereoisomers of isoleucine, separated on QN-AX (blue) and QD-AX (red) using method 1. * reagent related peak

For further investigation, the other type of mobile phase, the hydro-organic ones, with varied buffer concentration were tested (Table 17). Summarizing, a separation protocol for all four stereoisomers of isoleucine could not be established using *tert*-butyl carbamate modified quinine/quinidine chiral stationary phases. Three types of mobile phase and various chromatographic parameters were tested. The enantiodiscrimination of the first two eluting enantiomers was too small for proper peak separation. The later eluting enantiomers were partially separated on QN-AX column and were not separated on QD-AX column.

4.4.3.5 Other investigated enantioseparations of AQC-tagged amino acids and general aspects of the investigation

The separation parameters for all investigated amino acids, except the proteinogenic ones, were collected in Table 16 and Table 17. Concerning the elution order, normally, the D-enantiomer eluted first when a *tert*-butyl carbamate modified quinine-based CSP was applied. For secondary amino acids,

such as proline, 4-hydroxyproline but also for beta-phenylalanine, the AQC derivatization led to a reversed elution order. Amino acids containing a γ-carboxylic acid moiety, exhibited also a reversed elution order.

Due to the weak anion-exchanger type of QN/QD-AX columns, amino acids with a second acid moiety were much stronger retained than the mono acidic amino acids. Some bi-acidic analytes were tested, for example cysteic acid, methioninsulfone, some phosphor amino acids, such as phospholeucine and cysteine-S-acetic acid. As a rule of thumb, the higher the acidity of the second acid moiety the stronger might be the overall retention of both enantiomers. The enantiodiscrimination was also enhanced, due to a stronger SO-SA complex formation, expect of aspartic acid. A good enantioseparation of acids could often be achieved, when a so called “low coverage” column (CSP 6) with less immobilized selector, was applied. The separations on a low coverage column provided no significant loss in enantioselectivity or resolution, but were established in shorter run times.

The amino acid cysteine often exists as the dimer, cystine, especially in biological context. Cystine was double derivatized with AQC and because of strong π-π-interactions with the selector, the elution time was also high. Cystine provided three stereoisomers with DD/LL-cystine as enantiomers and the DL-cystine as a meso-species. The ratios of the peaks were expected to be 1:2:1.

No quenching effect [30] was observed in a polar-organic mobile phase, but was observed when a reversed phase run in hydro/acetonitrile was performed. Two common derivatization strategies for the thiol group of cysteine, with iodo acetamide and iodo acetic acid were conducted and their enantioseparation performance was investigated. It was observed that the acetamide modification offered a much better chromatographic behavior on a WAX-type CSP than the acetic acid derivative, because the latter was extraordinarily strong retained. Nevertheless, both analytes were separated with comparable performance.

Phenylalanine analogues, such as 3,4-dihydroxy-DL-phenylalanine and several bromo,- chloro- or fluoro-derivatives were successfully derivatized with AQC. The chromatographic parameters were illustrated in Figure 40. It was found that the two beta-Phe species had the highest enantioselectivity and the best resolution in this series of analytes. Especially, beta-DL-Phe had the lowest retention, but exhibited the best enantioseparation. The enhanced retention of the halogen species could be related to a higher π-acidity of the phenyl ring. It was known that a π-acid exhibited stronger retention on a quinine-based CSP, according to a π-acid and π-base interaction with the selector [54].

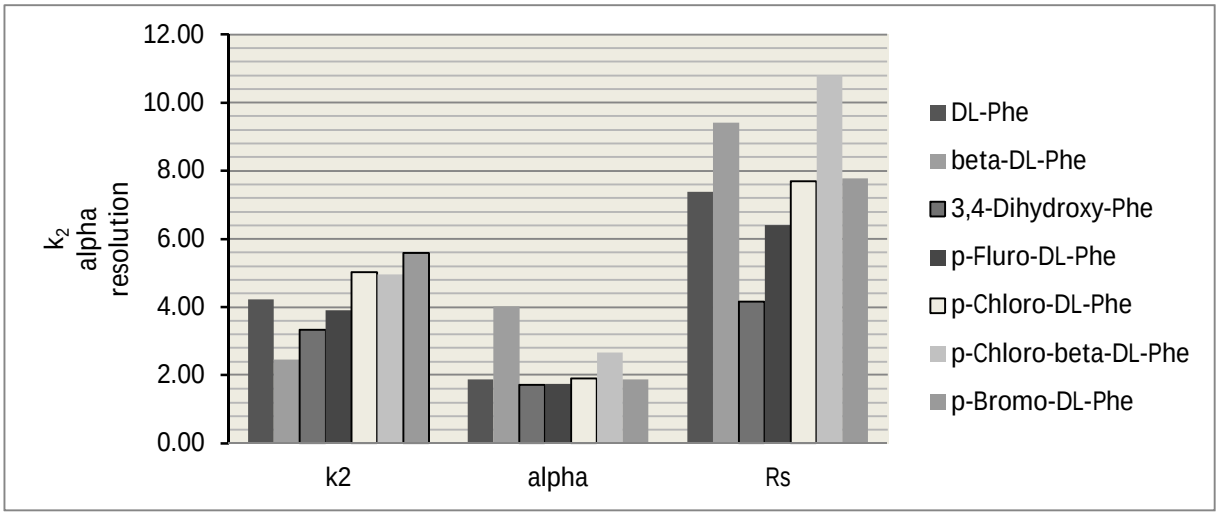


Figure 40: Phenylalanine analogous and their chromatographic parameters

Aminophosphonic acids were also successfully derivatized with AQC and enantioseparations were performed. The derivatization strategy was not suitable for secondary amino acids, such as pipecolinic acid, nipecotinic acid and 3-carboxy pyridinic acid. N-Methyl leucine could be derivatized with AQC, but an enantiodiscrimination was not capable using a quinine-based CSP. The reason for this miscarriage was not further investigated.

4.5 Separation of AQC-labeled oligopeptides via *tert*-butyl carbamate modified quinine/quinidine based chiral stationary phases

4.5.1 Separation of alanine peptides

The collected separation data for the enantioseparation of AQC-tagged alanine peptides were summarized in Table 18, Table 19, Table 20 and Table 21. The investigation of alanine peptides, namely all-D and all-L enantiomers up to the heptamer, was expected to provide a deeper insight into the chiral reorganization mechanism of the SA-SO interaction. Furthermore, the derivatization with AQC, with an urea linkage, might expand previous investigations for alanine peptides on a quinine-based WAX-type CSP [43, 45, 46]. Different types of protection groups were applied in the before mentioned studies. Separation of alanine peptides, tri- and tetrapeptides and their stereoisomers as well as di- and tripeptides containing one or more glycine moieties were investigated. The collected separation data for the AQC-tagged alanine peptides was provided in Table 18 and Table 20. The polar-organic mobile phase was not found to be suitable for the separation of alanine peptides. Previous studies had achieved better results with hydro-organic mobile phase, consisting of MeOH/H_2O 80/20 with 20 mM NH_4AcO pH_a 6,00 (method 2).

As a trend in the change in retention, enantioselectivity and resolution of the stereochemically pure peptide series from alanine up to its heptamer, it was found that the enantioselectivity was negligibly influenced by the growing peptide chain. Alanine and DD/LL-alanine showed an alpha >1,5, but beginning with the tripeptides and up to the heptamer, the alpha value was slightly lower than 1,3. Resolution and retention showed a different behavior, both were decreased with increasing peptide chain length (Figure 41). Note that retention and resolution was again quiet equal for the tripeptide up to the hexamer.

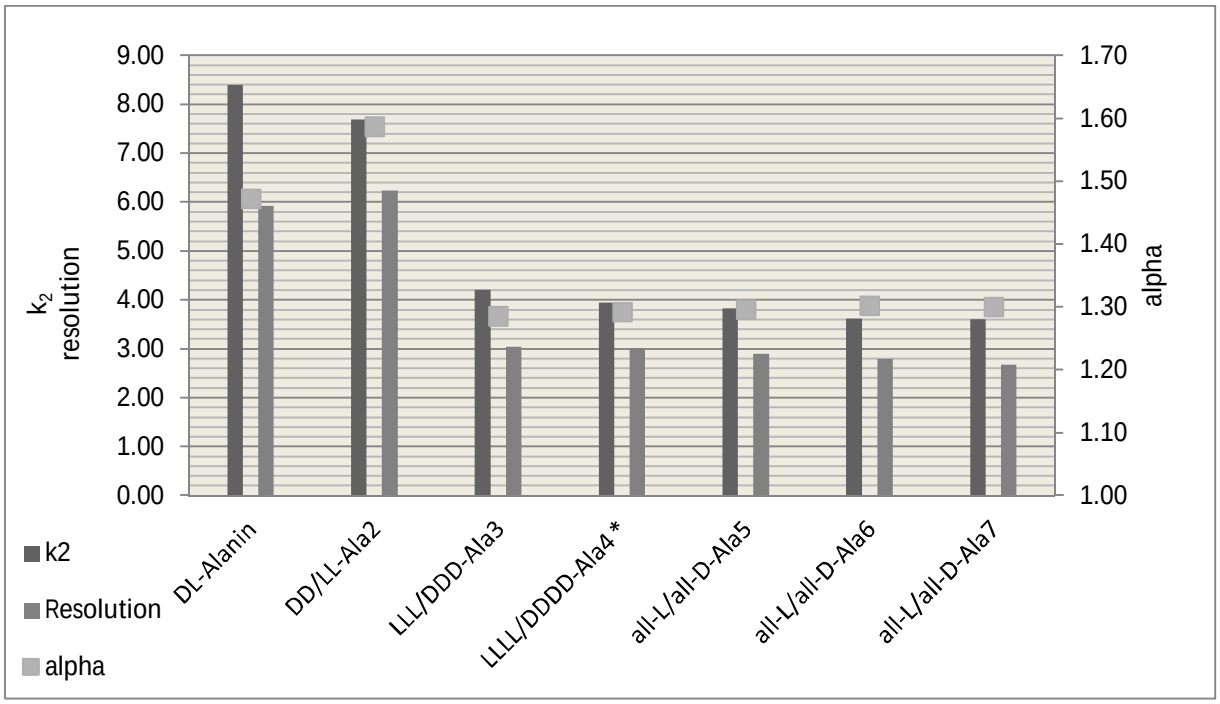


Figure 41: Comparison of separation parameters of an enantiomerically pure alanine peptide series using method 2. * flow rate was decreased to 0,4 ml/min

All sixteen stereoisomers of alanine tripeptides were derivatized with AQC and their chromatographic behavior was explored by applying two different flow rates of 1 ml/min and 0,4 ml/min (Figure 42). The results provided a mixed picture. The retention, expressed as k_2 value of the later eluting enantiomer, was quiet the same for all tripeptides. A low enantioselectivity <1,5, with low variation was found for the whole peptide series. The resolution underwent significant changes, because some enantiomer pairs were not separated at all. For the LDL/DLD- and the LLD/DDL-Ala_3 pairs, the Rs values were enhanced when the flow rate was decreased. The LDD/DLL-Ala_3 pair showed the reversed behavior and the Rs value was decreased when the flow rate was reduced.

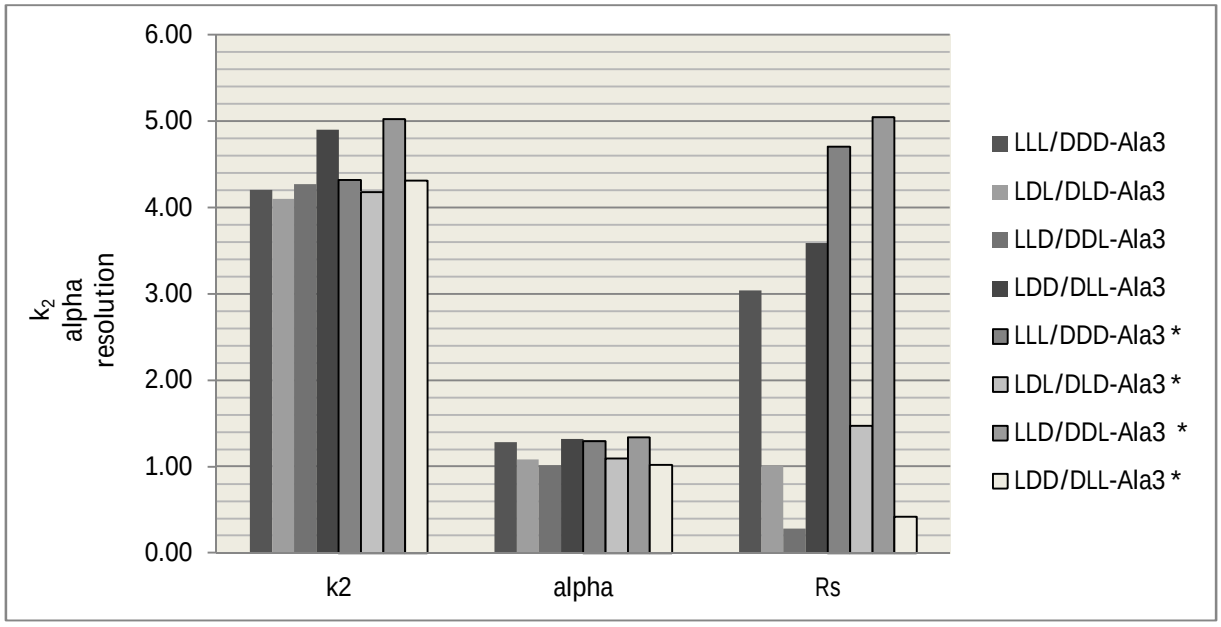


Figure 42: Stereoisomers of alanine tripeptides and their separation parameters

The investigation of Ala/Gly peptides served to provide a better insight regarding the influence of a non-stereogenic glycine moiety into close proximity of the stereogenic center. As illustrated in Figure 43, the performance of alanine and DD/LL-alanine was almost the same. The stereoisomers of the dipeptides, DL/LD-Ala_2 showed slightly lower values. All four stereoisomers could be separated if QD-AX column was applied, which produced better separations than QN-AX. QD-AX exhibited an elution order of DD<LD<DL<LL, in contrast the QN-AX had the elution order of DD<LD<LL<DL. A change in the elution order, by varying the N-protection group or by applying different mobile phase compositions were reported in literature [79]. Based on the "pseudo-enantiomeric" character of QN/QD-AX and keeping all other parameters constant, it could actually be expected that the elution order should be reversed. In case of the AQC-tagged alanine dipeptides, the elution order on QN/QD-AX was a surprising phenomenon.

Generally, for glycine/alanine peptides the resolution and enantioselectivity were decreased, especially when glycine was N-terminally located, compared to the alanine peptides. The tripeptides with two glycine moieties were hardly ever separated and exhibited reduced retention, compared to the alanine peptides. It did not matter, whether the two glycines were located on the N- or C- terminal end. The results for glycine up to the tripeptide, pointed out that the retention time decreased with increasing length of the peptide chain. The AlaGlyGly/GlyGlyAla tripeptides had nearly the same retention time as the GlyGlyGly species. The retention time was the same as observed for the DDD/LLL-peptides, but chiral recognition was totally diminished. The slightly higher retention of alanine and alanine dipeptides, compared to glycine and glycine dipeptides, could be explained with an additional hydrophobic retention increment, based on the alanine side chain. The additional interaction had led to a higher molecular interaction of alanine with the SO, compared to glycine.

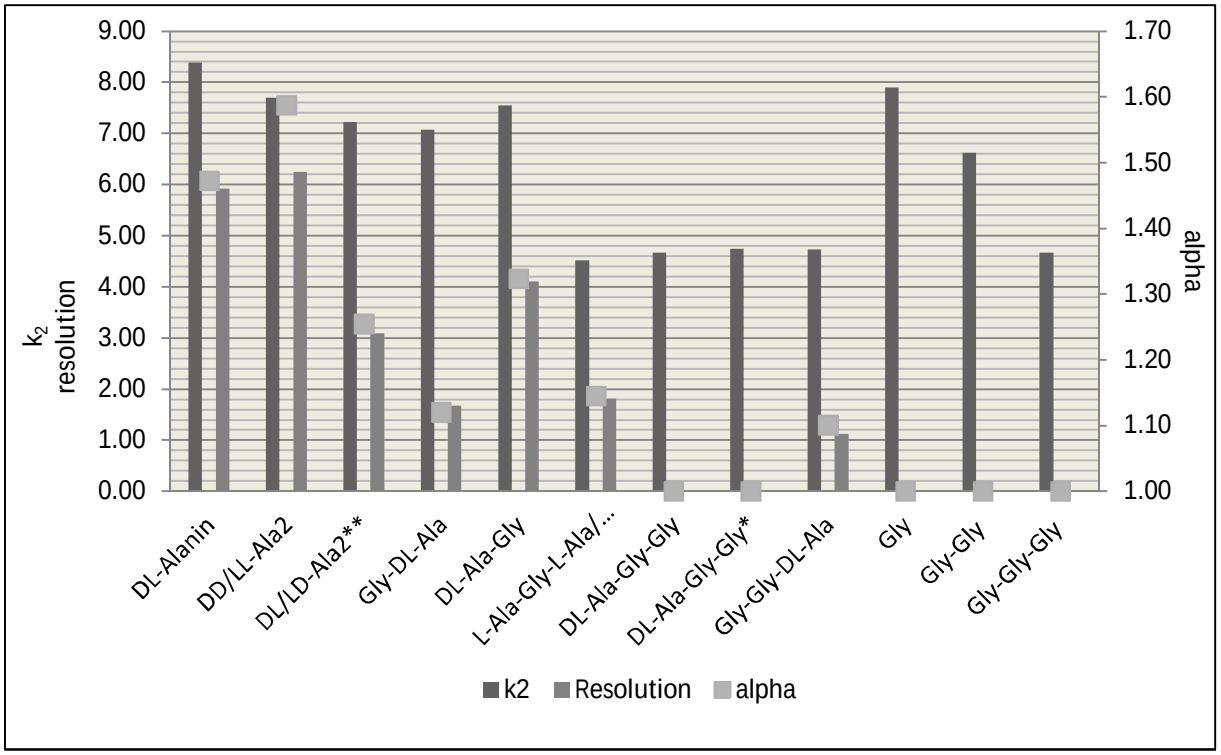


Figure 43: Comparison of chromatographic parameters of Ala/Gly peptides measured on a QD-AX column applying method 2. flow rate was 0,4 ml/min**

4.5.2 Separation of phenylalanine peptides

The separation of phenylalanine peptides, N-derivatized with the AQC tag, was investigated on QN/QD-AX columns. The hydrophobic character of the phenylalanine peptides led to their reduced solubility in a certain solvent mixture. Generally, a small amount of peptide was dissolved in a mixture of acetonitrile/water 40/60, better solubility were achieved with an solution containing 10 % (v/v) acetonitrile in 20 mM aqueous borate buffer, with pH 9,8 adjusted with 15 % (w/v) natrium hydroxide solution. The derivatization was carried out as described in chapter 3.4.1. It should be mentioned that the phenylalanine derivatives seemed less soluble in the derivatization mixture than alanine peptides or amino acids. A precipitate was often noticeable soon after derivatization of the phenylalanine peptides.

For the enantioseparation of phenylalanine peptides, it was expected that retention times should be increased relatively to the alanine peptides. On that account, a suitable mobile phase composition and optimized chromatographic parameters had to be obtained in order to yield the aspired chiral separation of these peptides. The measurements were performed in a hydro-organic mobile phase using method 3. It was found that the buffer concentration had to be ten times higher to reach acceptable retention times, compared to the mobile phase, which was used for the alanine peptide separation. The adjustment of the flow rate was an important parameter, because at 1 ml/min nearly no enantioseparation was observed. Therefore, it was decided to keep the flow rate constant at 0,5 ml/min for all phenylalanine investigations.

Table 22, Table 23, Table 24 and Table 25 represented the calculated separation data for the separation of AQC-tagged phenylalanine peptides. The series of all-D/all-L phenylalanine peptides from the amino acid up to the pentamer was derivatized and the chromatographic parameters were illustrated in Figure 44. The retention time, expressed as k_2 value, was increased with growing peptide chain length on a QD-AX CSP. This effect was more distinct on QD than on QN and stood in high contrast to the results obtained for the alanine peptides, where retention was decreased with growing chain length. Enantioselectivity was better on QD and the variation range was 2, 00 to 2, 24. For the QN, alpha was generally lower and varied from 1,41 to 1,91. Enantioselectivity of phenylalanine peptides were better than for alanine peptides, for which the range of enantioselectivity was between 1,29 and 1,59. On both columns the resolution exhibited a trend towards lower values when the chain length was increased.

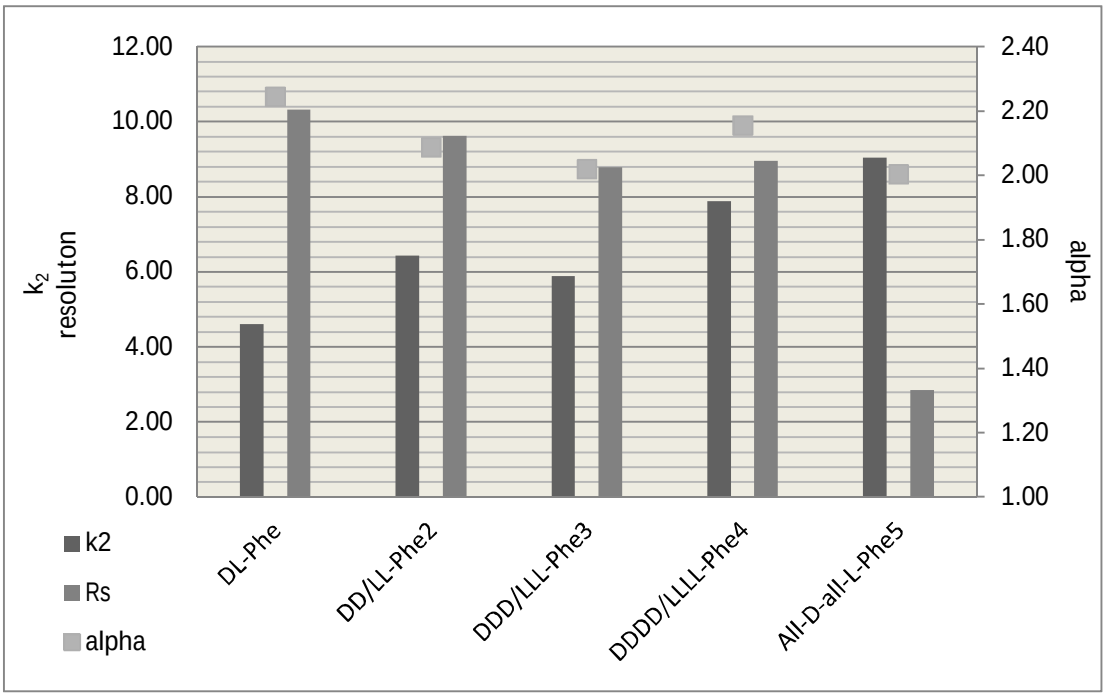


Figure 44: Comparison of chromatographic parameters of the phenylalanine peptide chain series derivatized with AQC. Separation was done with a QD-AX column using method 3.

The separation study for the phenylalanine tripeptides produced a mixed picture (Table 9). The elution order of these peptides was influenced by stereochemistry of the C-terminal moiety. For the observed QD-AX column, the first eluting enantiomer had in all cases a L-phenylalanine at the C-terminus. The enantioseparation decreased in the series of DDD/LLL>DLL/LLD>DLD/LDL>DDL/LDD. The DDL/LLD-Phe pair were be separated, for the DLD/LDL-Phe pair a partial separation was observed, and the DLL/LDD-Phe pair was fully enantioseparated with an alpha of 1,73. The mixed tripeptides had lower retention times and their resolution was decreased or diminished compared to the stereochemically pure DDD/LLL-tripeptide.

Table 9: Comparison of chromatographic parameters of AQC-tagged tripeptides of phenylalanine. They were separated on a QD-AX column using method 3.

Analytes		to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
DDD/LLL-Phe$_3$	QD-AX	3.10	12.13	21.33	2.91	5.88	2.02	8.79	4222	4020	LLL
DLL/LDD-Phe$_3$		3.10	12.77	19.91	3.12	5.42	1.74	6.94	4002	4074	DLL
DDL/LLD-Phe$_3$		3.10	12.66	12.66	3.08	3.08	1.00	\	3445	3445	n.a.
DLD/LDL-Phe$_3$		3.10	11.23	12.60	2.62	3.06	1.17	1.87	4294	4142	LDL
DDD/LLL-Phe$_3$	QN-AX	2.80	10.10	14.26	2.61	4.09	1.57	4.30	2538	2561	DDD
DLL/LDD-Phe$_3$		2.80	10.51	13.34	2.75	3.76	1.37	3.18	2887	2899	DLL
DDL/LLD-Phe$_3$		2.80	10.21	10.21	2.65	2.65	1.00	\	2645	2945	n.a.
DLD/LDL-Phe$_3$		2.80	9.25	10.19	2.30	2.64	1.15	1.31	2947	2933	DLD
n.a. not applicable											

The series of phenylalanine tetrapeptides showed a more plausible result. In Figure 45, the chromatographic parameters were juxtaposed. The enantioselectivity and resolution values were best for the all-D/all-L enantiomers and the retention was also the highest, followed by the DDLL/LLDD and the DLLL/LDDD-species, which exhibited nearly the same chromatographic behavior. The DLDD/LDLL-pair was only slightly worse separated and could be positioned in the before mentioned group of well separated phenylalanine tetrapeptides. This group fulfilled the same criteria, namely at least two stereochemically equivalent phenylalanines at the C-terminus. If this criteria was not matched, as for the DDDL/LLLD,- DDLD/LLDL,- DLLD/LDDL,- and DLDL/LDLD-pairs, enantioselectivity as well as resolution was almost diminished. The same behavior was obtained applying the QN-AX CSP. This effect was observed due to a generally better enantioselectivity for the phenylalanine peptides compared to the alanine peptides. Additionally, the phenylalanine peptides were available in all forms of diastereomers, which completed the set of obtainable data. The same effect was also observed by studying the tripeptides series of phenylalanines (Table 9), but in a less clarified way. These results explained bad enantioseparation performance of phenylalanine dipeptides stereoisomers on QD-AX. No separation could be achieved on QN-AX. The DL/LD-pair was in both cases inadequately separated (Table 25 and Table 23).

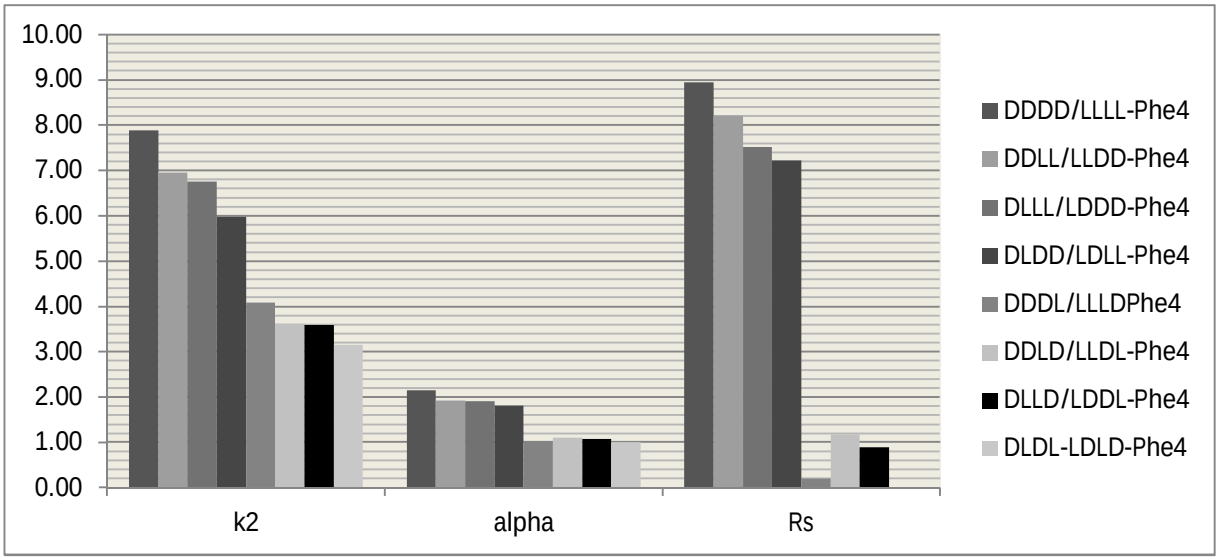


Figure 45: Chromatographic parameters compared of a series of phenylalanine tetrapeptides derivatized with AQC and separated with QD-AX

4.6 Evaluation of the separation performance of FITC-tagged amino acids on *tert*-butyl carbamate modified quinine/quinidine-based chiral separation columns

4.6.1 Optimization study for the enantioseparation of FTIC-labeled amino acids

The fluoresceine-label was frequently used to tag proteins, peptides and amino acids in biochemical assays, involving biological matrices, cells and cell compartments. Enantioseparations of racemic mixtures of amino acids, derivatized with FITC, were performed in this investigation and quinine/quinidine alkaloid-type chiral stationary phases were used (CSP 1 and 2). Identification of the (S/R)-enantiomer was performed by comparison of the peak areas of a racemic mixture, which were expected to be equal. Furthermore, the injection of a pure enantiomer allowed the identification of single enantiomers by comparison of the retention times. The reversal of the elution order on quinine and quinidine based CSPs provided a further identification possibility for the enantiomer peaks. Several mobile phase mixtures were tested to find optimized separation conditions. The obtained resolution, α and k_2 values were juxtaposed in Table 27 and Table 28.

First investigations aimed to find appropriate separation conditions, especially a suitable mobile phase providing acceptable run times and enantioselectivity. As shown in Figure 9, a successful enantioseparation of FITC-derivatives would challenge with two requirements. The fluoresceine label was a quiet huge molecule with a strong apolar and aromatic nature. Compared with the proteinogenic amino acids, the label would prevail the chemical properties of the whole derivative. The label has also a carboxylic function group and according to the enantioseparation on a WAX-type CSP, a strong

retention was expected. Method 1, used for the enantioseparations of AQC labels amino acids, did not match good chromatographic behavior, because of extra-ordinary extended retention times on a "normal loaded" quinine-based CSP (CSP 1). Hereby, normal loaded meant a coverage of >380 µmol selector per gram of silica gel. For the investigation of an adequate mobile phase, a so-called "low coverage" QN-AX column (CSP 6) was used, which had a loading of 142 µmol per gram silica gel. It was well known that a lower coverage of selector would lead to reduced retention times, but need not lead to lower enantioselectivity or resolution. The following mobile phase compositions were tested to find an optimized separation conditions, such as, retention time, enantioselectivity and resolution of the FITC-derivatives. The overlapping of analytes peaks with reagent related peaks was an important factor during the optimization process.

- ➔ a) MeOH 2 % (v/v) AcOH, 0,5 % (w/v) NH_4AcO
- ➔ b) MeOH 100 mM FA, 50 mM NH_4AcO
- ➔ c) MeOH/ACN 80/20, 100 mM FA, 50 mM NH_4AcO
- ➔ d) MeOH/ACN 70/30, 200 mM FA, 100 mM NH_4AcO
- ➔ e) MeOH/ACN 80/20, 150 mM FA, 75 mM NH_4AcO

The optimized mobile phase conditions for investigation of all FITC-AA derivatives on quinine/quinidine-base CSP 1 and 2 were MeOH/ACN 80/20, 150 mM FA, 75 mM NH_4AcO. In average, the chromatographic performance was regarded best for this mobile phase composition. Other parameters were applied according method 6.

Comparison of retention, expressed as k_2-value from the later eluting enantiomers is illustrated in Figure 46. For the mobile phase composition a) to c) a low coverage QN-AX column was used. The lower coverage of about 142 µmol/g selector, influenced the enantioselectivity to a minor degree, but the retention was reduced compared to CSPs of "normal" loadings. For the standard mobile phase a), retention was the highest. The change of the buffer type, from AcOH/NH_4AcO (350/65 mM) to formic acid/NH_4formate (100/50 mM), reduced retention. These results stood in agreement with later investigations regarding the elution strength of different type of acids and base additives to the mobile phase [56]. An added amount of 20 % (v/v) of acetonitrile to the mobile phase composition of b) increased the elution strength. It is known that acetonitrile inhibits the π/π-interactions and could be used as a retention time reducing additive [79]. After changing from a low coverage column to a column with normal selector coverage, the mobile phase composition e) was used. Compared to d), both mobile phases matched the requirements.

Aspartic acid as well as the more apolar amino acids like tryptophane was highly retained. Especially asparagine was unexpected to exhibit such high retention. Enantioselectivity was not influenced and was little changed for all analytes, when one of the five mobile phase compositions were used. Bigger

variability was obtained for lysine and aspartic acid, the first one was the least retained and the latter was the strongest retained. Asp could not been separated with any of the five tested mobile phases.

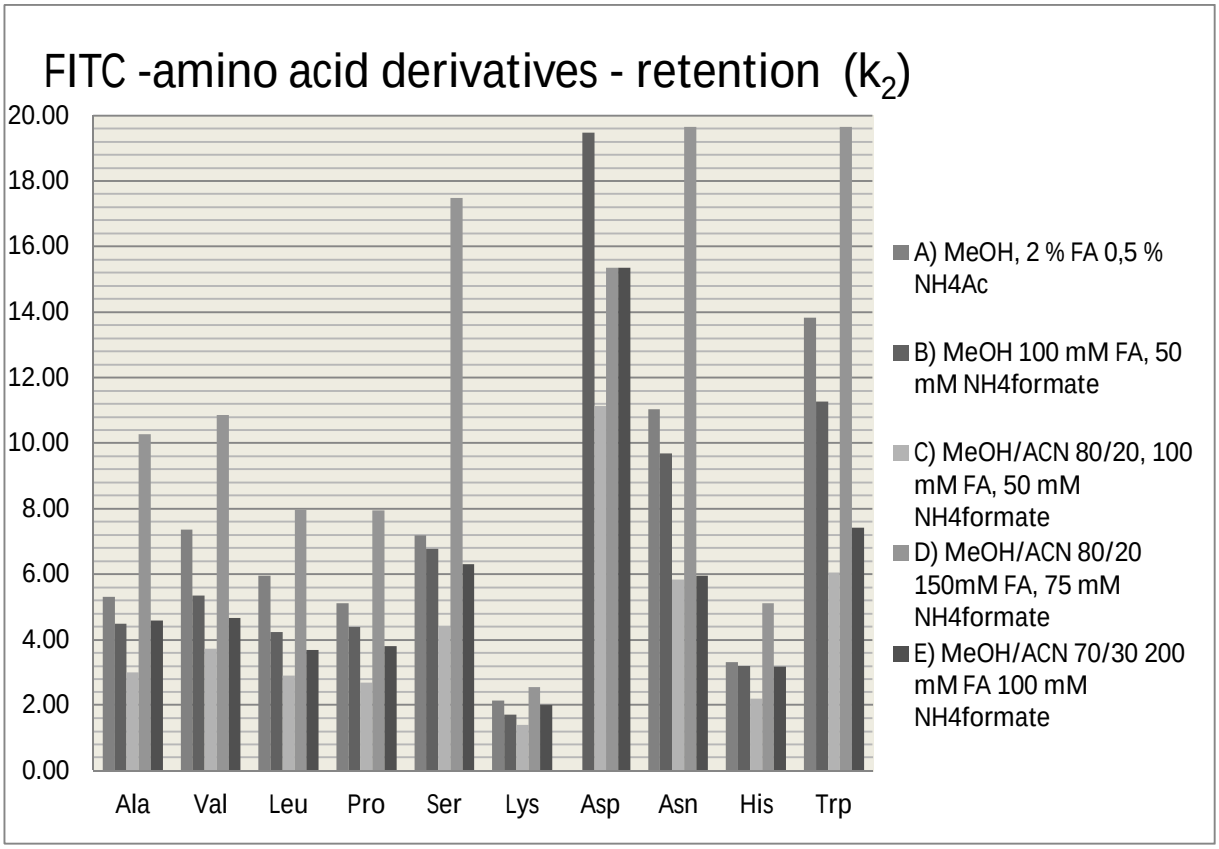


Figure 46: k_2-values of a set of amino acids, derivatized with FITC, for five different mobile phases. Data for the elution strength of the first three mobile phases, MeOH/AcOH/NH_4AcO 98/2/0,5 % (v/v/w), MeOH 100 mM FA, 50 mM NH_4 formate and MeOH/ ACN 80/20 100 mM FA, 50 mM NH_4 formate were done using a low coverage QN-AX column (loading of about 142 µmol/g silica gel). QN-AX was used with mobile phase consisting of MeOH/ACN 80/20 150 mM FA, 75 mM NH_4formate and QD-AX was applied, when mobile phase composition MeOH/ACN 70/30 200 mM FA, 100 mM NH_4formate was used.

The investigated resolution for a set of ten amino acids, derivatized with FITC and separated on different *tert*-butyl carbamate modified quinine-based CSPs with five different mobile phase compositions were illustrated in Figure 47. For alanine and leucine, mobile phase e) produced best resolution, whereas for valine and histidine the resolution was quiet unchanged. Proline was badly resolved and the alpha values were <1,5 for all five testes mobile phases. Asparagine, serine and tryptophane were best resolved with b) and had the worse resolution with d). Lysine and aspartic acid were not resolved with any of the tested mobile phases.

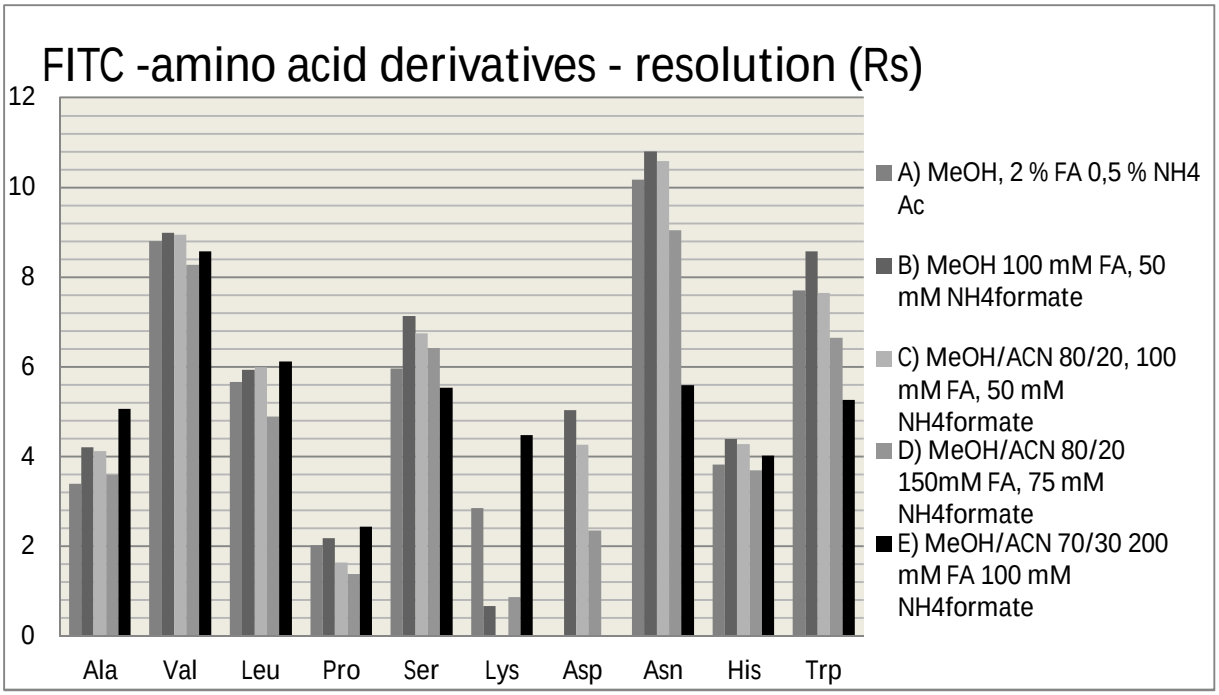


Figure 47: Resolution of a set of amino acids, derivatized with FITC, for five different mobile phases. Data for the resolution of separated enantiomer peaks of the first three mobile phases, MeOH/AcOH/NH_4AcO 98/2/0,5 % (v/v/w), MeOH 100 mM FA, 50 mM NH_4formate and MeOH/ ACN 80/20 100 mM FA, 50 mM NH_4formate were done using a low coverage QN-AX column (loading of about 142 µmol/g silica gel). QN-AX was used with mobile phase consisting of MeOH/ACN 80/20 150 mM FA, 75 mM NH_4formate and QD-AX was applied when mobile phase composition was MeOH/ACN 70/30 200 mM FA, 100 mM NH_4formate.

4.6.2 Enantioseparation of twenty proteinogenic amino acids labeled with FITC

Twenty proteinogenic amino acids were derivatized with FITC and enantioseparation was performed using the optimized chromatographic separation conditions as stated in chapter 4.6.1 (Table 27 and Table 28). Lysine had the first eluting pair of enantiomers and was badly separated. This diamino acid was surely monosubstituted and therefore the basic substance was eluted early using an anion-exchange type CPS. Proline was separated with an alpha of 1,13 and a resolution of 1,38, which was significantly below the average of the whole set of amino acids.

Comparison of enantioselectivity of the separations of proteinogenic amino acids, derivatized with the two investigated N-derivatization labels, AQC and FITC, is given in Figure 48. Generally, AQC-tagged amino acids exhibited higher enantioselectivity with the used QN-AX CSP. Aspartic acid was not separated employing both labels. Alanine, proline, glutamic acid and glutamine AQC derivatives were separated with an alpha below 1,5. Lysine, arginine, histidine, and cysteine were separated tightly above this critical alpha value, if AQC was used as label. Better enantioseparation for FITC-amino acid derivatives were only generated for alanine, glutamic acid and glutamine.

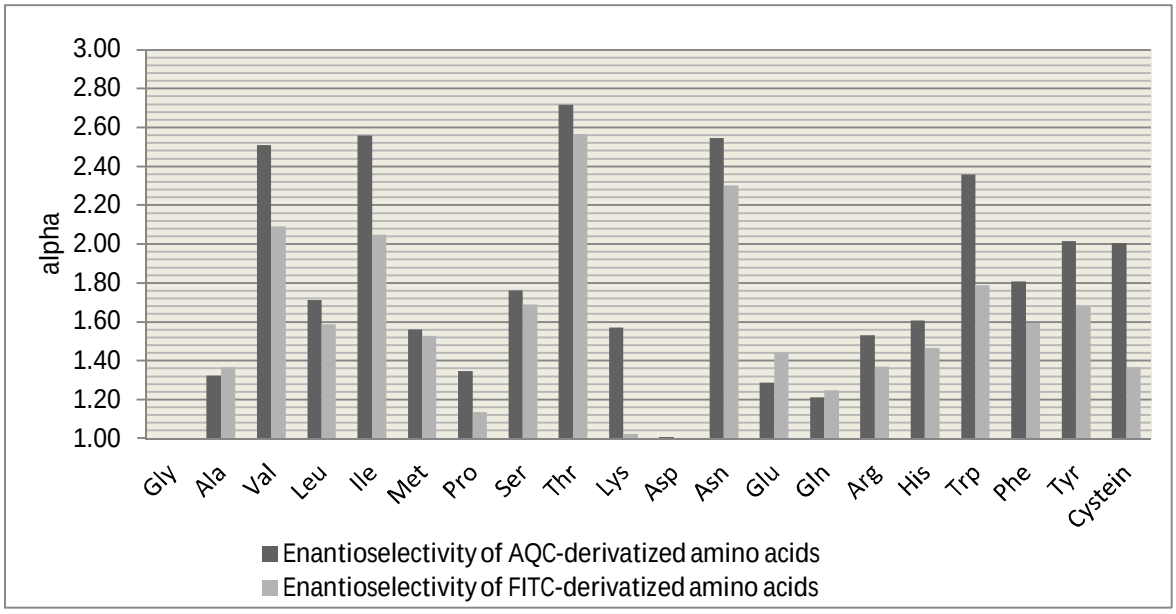


Figure 48: Comparison of enantioselectivity of N-derivatized proteinogenic amino acids, with AQC and FITC, both fluorescence active labels. AQC amino acid separations were done using method 1. FITC-AA analyses were done using method 6. QN-AX column was applied.

Resolution values for the enantioseparation of amino acids, derivatized with AQC or FITC, were given in Figure 49. Summarized, the FITC-amino acid derivatives were slightly better resolved than the AQC-tagged derivatives. Proline, lysine, phenylalanine, and tyrosine were better resolved with the AQC-tag.

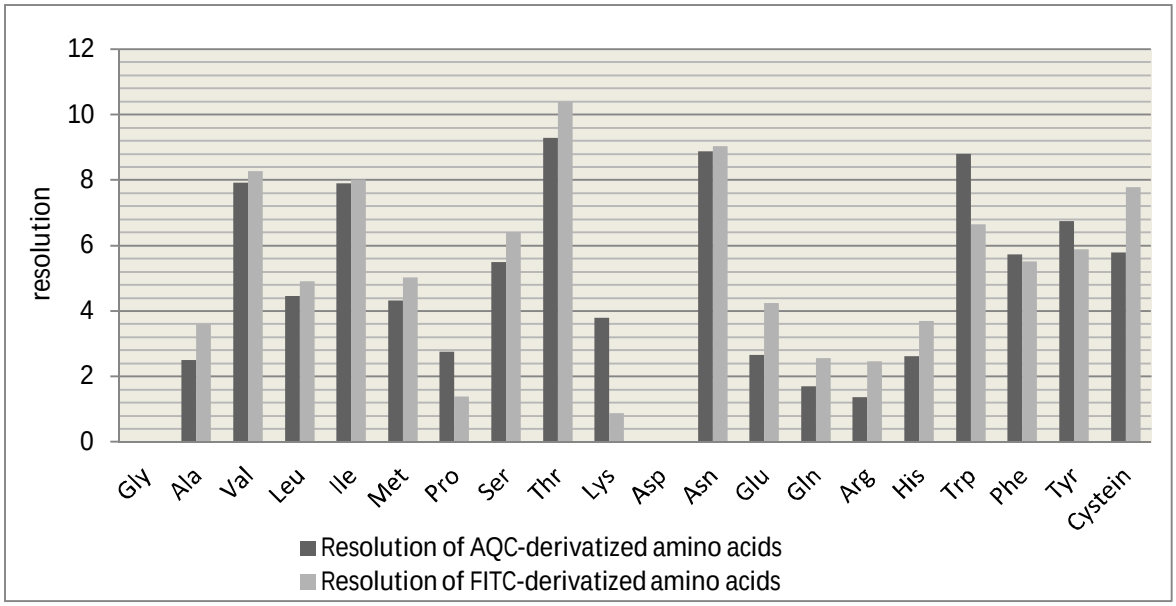


Figure 49: Comparison of resolution of N-derivatized proteinogenic amino acids, with AQC and FITC, both fluorescence active labels. AQC amino acid separations were done using method 1. FITC-AA analyses were done using method 6. QN-AX column was applied.

4.7 Evaluation of the sulfobetaine-type chiral stationary phases (CSP)

4.7.1 Screening for chiral recognition of sulfobetaine-quinine-based CSPs

The synthesis of quinine-based-zwitterionic CSPs was conducted as described in literature [80]. The combination of a strong acid and a weak base in one selector molecule was already investigated by Hoffmann et al. [56]. In previous studies, the introduction of a second charged moiety was carried out with a carbamate linkage at C9, the vinyl group at C3, or on the C6' position of the quinoline ring. Modifications at the two amino groups were done by a simple alkylation reaction. The addition of an acid functional group, connected to the nitrogen atoms, present near the chiral recognition center at C8/C9, were not investigated. A sulfobetaine moiety could easily be created, when a sultone species was used. Sultones were known as alkylating reagent and provided a modified quinine-type selector in a one step synthesis approach (Figure 50).

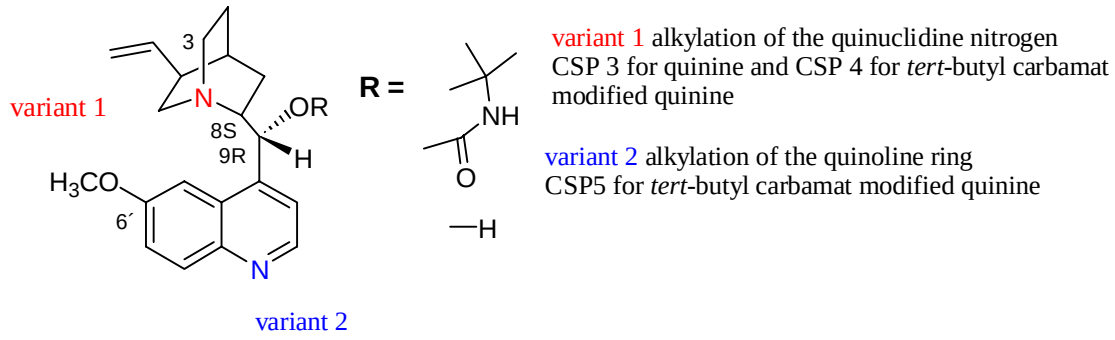


Figure 50: Illustration of the quinine selector and its modification possibilities for the alkylation reaction with a sultone

Summarized, all three sulfobetaine-type CSPs exhibited little to no enantiodiscrimination ability. A partial separation into two peaks was observed for the AQC derivatives of lysine, aspartic acid, cysteine, cystine, tyrosine and tryptophane. Some DNB derivatives were only partially separated on CSP 5. Note that CSP 5 delivered more often a partial enantioseparation than CSP 4 (Scheme 3). It also showed a minor difference in the retention behaviour compared to the QN/QD-AX columns. The retention of all analytes was reduced on the other sulfobetaine columns, CSP 3 and CSP 4. CSP 5 was able to separate DNB-Leu and AQC-cystine and AQC-Asp (Table 29).

The missing enantioseparation is not only based on the absence of chiral recognition, but is possibly influenced by the loss of enantiodiscrimination ability of the selector. In most cases, the two enantiomers showed exactly the same retention and therefore were not separated. In comparison to CSP 1 or 2, a difference in retention time was obvious. The sulfobetaine-type CSP showed a lower retention of the analytes. Another drawback could be based on the type of chemical functionalities combined in the selector. The combination of a strong acid, the sulfonic acid moiety and a quaternary amine, which

was altered to a strong base, was not yet investigated. In different studies, a strong (SCX) or weak (WCX) cation exchange moiety was introduced via the C9, or the C6´-position of the quinine molecule. These modifications were fully tested and were found to be applicable in AX, CX and ZX mode for separation of basic, acidic and zwitterionic compounds [56], [55]. In this study the zwitterionic CSPs were only used in an AX mode for the separation of acidic and zwitterionic analytes. An intermolecular counter ion (IMCI) effect was investigated in earlier studies [57]. The IMCI effect meant a shortened retention time for acidic analytes and reduced ion-exchange capacity due to intermolecular charge compensation. In the case of sulfobetaine, the two moieties were connected with a four atom-bond bridge. For CSP 3 and 4, the quinuclidine ring was altered from a weak to a strong AX site. It is well known that the attractive anion-exchange moieties were essential for the chiral recognition process, because of its closeness to the stereogenic centres at C8/C9 of the quinine structure [54]. It was supposed that the IMCI and the modification at the quinuclidine ring wiped away the enantiodiscrimination ability of the quinine-based selector, because of a counter ion effect, a strong repulsive force of the sulfonic acid group and the modification to a quaternary base.

If the two opposite charged intramolecular ions would shield each other, an ion pairing process would be prevented. The addition of a strong base or acid to the mobile phase would overwhelm this intermolecular process. A phosphate buffer with pH 2,0 was tested, but did not provide the aimed results.

4.7.2 Screening for separation performance in the HILIC mode of the sulfobetaine-type stationary phases

As it was mentioned in previous publications, a HILIC retention increment for the *tert*-butyl carbamate modified quinine-based CSPs, was expected [63].The sulfobetaine-based CPSs were studied, in course of their applicability for hydrophilic interaction chromatography (HILIC), because of the strongly more polar character of the sulfobetaine modified quinine-based columns, compared to the *tert*-butyl carbamate modified CSPs.

A screening study was set up, using method 7. A representative set of hydrophilic compounds containing fourteen analytes, acidic and basic compounds, purines and nucleosides were used for the investigation (Figure 22). The influence of mobile phase composition and pH was investigated. Special parameters for HILIC were recorded and compared, e.g. the peak shape, efficiency and selectivity to different but related compounds. The mobile phase was a mixture of ACN/aqueous buffer 9/1, containing 10 mM NH_4AcO with buffer pH 5,0 or 8,0 adjusted with acetic acid or ammonium hydroxide. In another experiment, the pH_a was adjusted to 5,0, which meant that a much bigger amount of acetic acid had to be added to the mobile phase.

The detection of analytes was carried out using different wavelengths, e.g. 215, 248, 280 and 360 nm and the highest absorbing wavelength was taken into account for data analysis. The retention factors and the number of plates per meter of column were juxtaposed in Table 30. As it was described in

literature, the pH influenced acidic HILIC compounds, thus exhibiting reduced retention. Whereas the same trend was observed for basic compounds, but was less obvious. The ln(k) values were calculated and a correlation plot was made for each of the three HILIC columns, for two different pH values. These plots illustrated the impact of different mobile phase pH on separation selectivity, in respect to the selectivity determined by the stationary phase [59]. Afterwards the selectivity coefficients s^2 were calculated. A correlation plot illustrated the change in selectivity and/or retention caused by an alteration of a chromatographic parameter e.g. the pH (Figure 51).

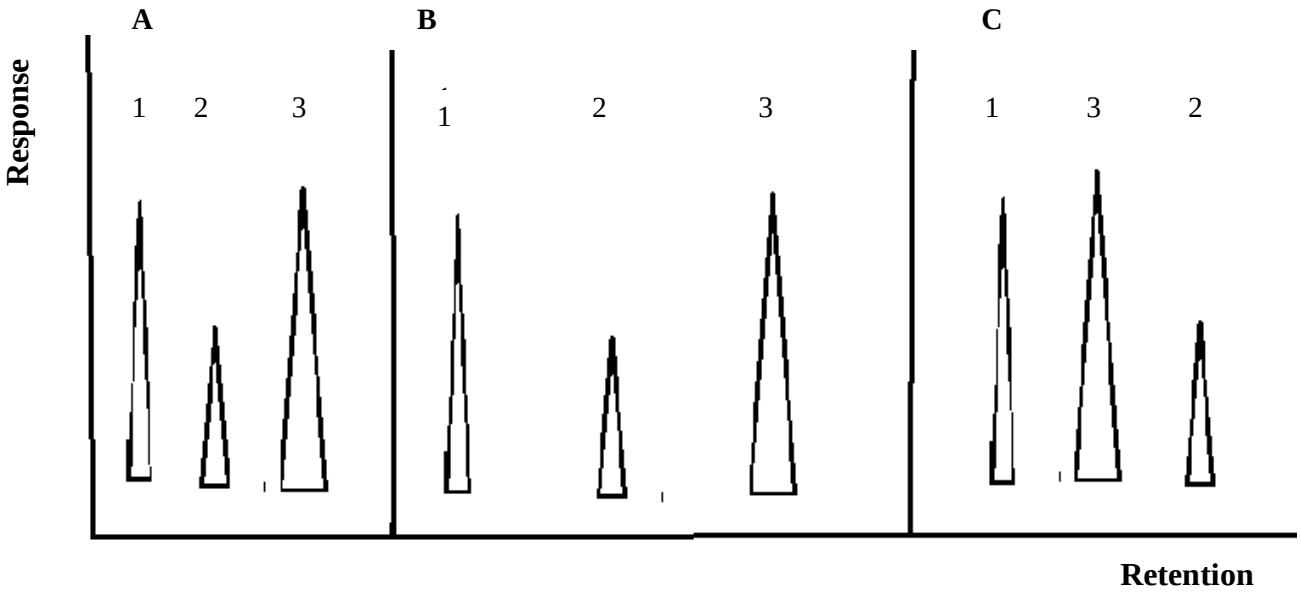


Figure 51: Illustration of the possible influence of mobile phase pH on analyte retention and selectivity applying a zwitterionic HILIC column. A) a retention behaviour and a selectivity is obtained for analyte 1,2 and 3. B) the retention of analyte 1,2 and 3 was changed by the change of mobile phase pH. A change in elution strength could also cause a altered retention C) the selectivity of analyte 1, 2 and 3 was changed by the mobile phase pH.

Firstly, the two mobile phases with pH 5,0 and 8,0 were compared for each of the tested columns. For CSP 3 a high degree of correlation of 0,98 was achieved. Correlation was expressed as the square of the correlation coefficient R^2. The selectivity coefficient s^2 was 0,019. Both examined mobile phases were compared and exhibited a pH depended linear relationship for the set of investigated analytes, regarding the retention. Obviously, the modification of the mobile phase pH did not led to a changed selectivity for CSP 3 and for this reason a pH alteration will not be useful to solve a specific separation problem (Figure 51).

CSP 4 showed a mixed picture concerning the selectivity correlation investigation.
R^2 was 0,46 and s^2 was 0,539, without any outlier eliminations (Figure 53). Compounds, which disturbed a linear correlation were the alkaloids, theobromine and theophylline as well as the basic

analytes, phenylephrine and thiamine. The basic compounds exhibited a higher retention, if the mobile phase with pH 5 was maintained and their selectivity could be significantly altered by a pH adjustment. For CSP 5 a similar behaviour could be achieved as for CSP 4 (Figure 54). The R^2 was 0,86 and s^2 was 0,144. If theobromine was excluded as an outlier, the correlation was raised to 0,96 with a selectivity coefficient of 0,040. It could be stated that theobromine was stronger influenced by changing the ionic strength of the mobile phase and this could be a hint for the selectivity enhancement in course of a suitable separation of theobromine and theophylline [81].

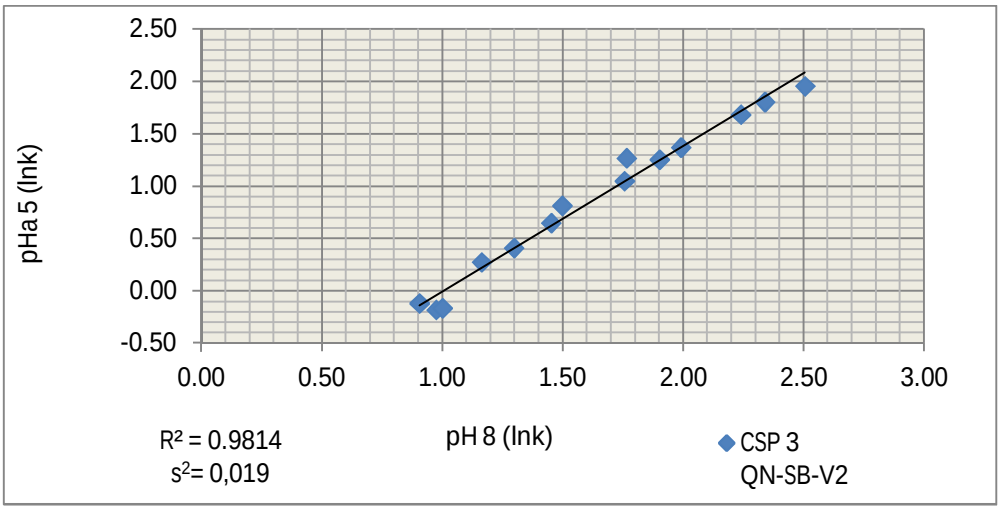


Figure 52: Correlation plot of two mobile phases with different adjusted pH value for a test set of typical HILIC compounds, and evaluation of the selectivity coefficient. The column was CSP 3.

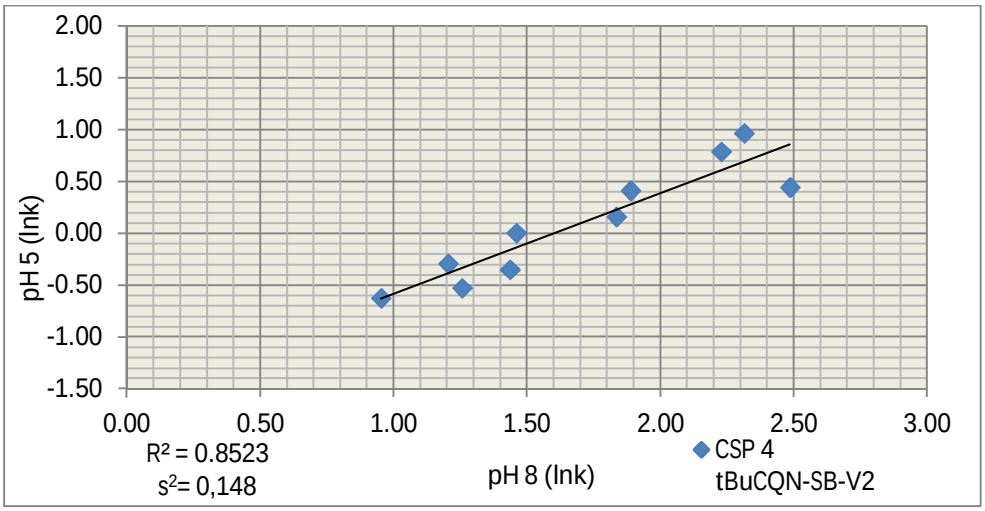


Figure 53: Correlation plot of two mobile phases with different adjusted pH value for a test set of typical HILIC compounds, and evaluation of the selectivity coefficient. The column was CSP 4. Theophylline, theobromine, thiamine and phenylephrine were possibly outliers and were eliminated.

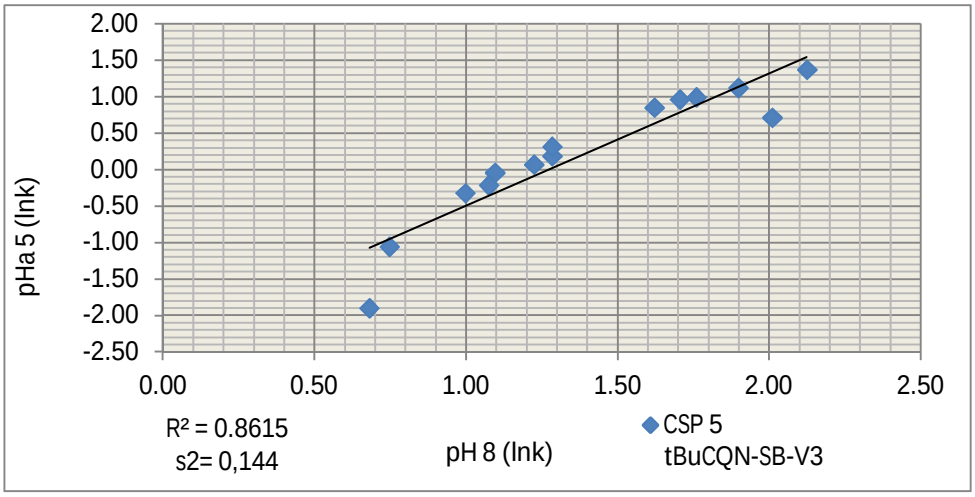


Figure 54: Correlation plot of two mobile phases with different adjusted pH value for a test set of typical HILIC compounds, and evaluation of the selectivity coefficient. The column was CSP 5 and for the plot the outliers were not eliminated.

Secondly, the more acidic mobile phase with pH_a 5,0 was compared to the mobile phase with pH 8,0. For the CSP 3, the s^2 value was 0,137 (Figure 55) and R^2 was 0,86. These values meant a reduced correlation of the retention regarding the influence of pH. The acids, ibuprofen, flurbiprofene and 4-OH-phenyl acetic acid were eliminated as outliers. These acidic compounds were not retained with the applied pH. It could be stated that a higher pH value would allow the consideration to place these compounds into the selectivity plot.

For the CSP 4, the selectivity plot is shown in Figure 56. Here, the selectivity coefficient was calculated, as 0,361 and R^2 was 0,64, theophylline was cancelled as outlier. If outliers were dismissed more restrictly, e.g. 4-OH-phenyl acetic acid and theobromine, the R^2 and s^2 values were 0,89 and 0,112, respectively. In contrast to CSP 3, the alkaloids were typical outliers and interrupted the linearity of the plot. The basic compounds did not give a clear trend, as mentioned in the literature [59]. Retention of every compound was decreased when increasing the pH of mobile phase. This trend was observed to be quite linear for the set of investigated analytes. This behaviour was not observed for the other two investigated columns, CSP 3 and 5.

For the third HILIC column, CSP 5, a selectivity coefficient of 0,142 and a R^2 of 0,85 were achieved (Figure 57). Ibuprofen, flurbiprofen and 4-hydoxy-phenyl acetic acid were dismissed, due to a lack of retention when applying the mobile phase at pH_a 5,0. For all other investigated analytes it could be stated that the retention showed a correlation for pH 5,0 to 8,0, where the retention was enhanced with increasing the pH value of the mobile phase. The generally low retention was strongly depended on the adjusted apparent pH. For that reason a well fitted selectivity plot could not be created. If the most influenced substances, the acidic compounds were cancelled, a plot could be produced, which showed the influence of the ionic strength of the mobile phase on the retention.

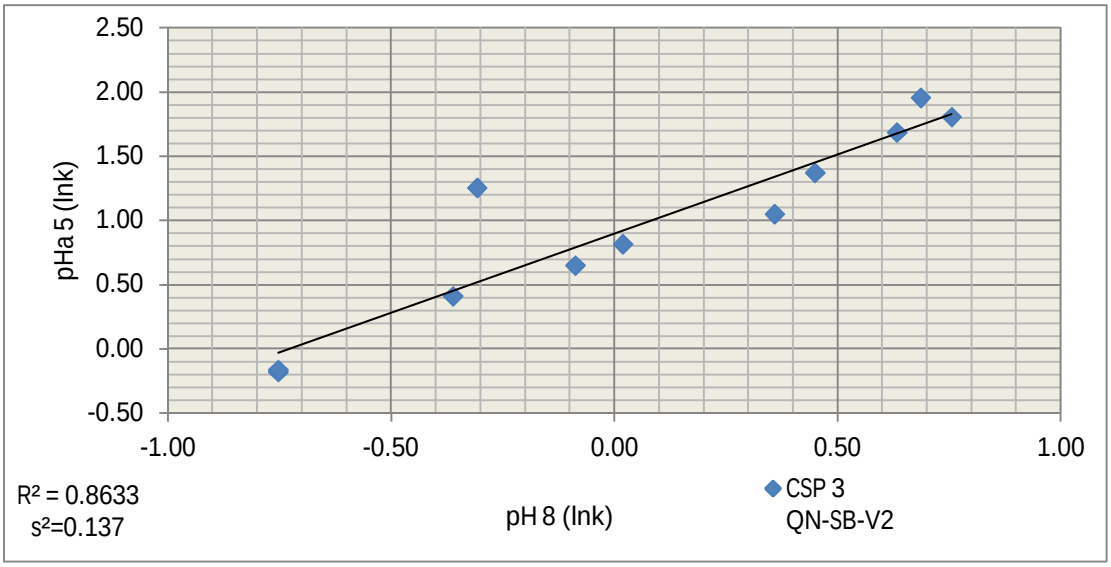


Figure 55: Selectivity plot of CSP 3, for two different pH values. Ibuprofen, flurbiprofene and 4-OH-phenyl acetic acid were eliminated, because they showed minimal retention.

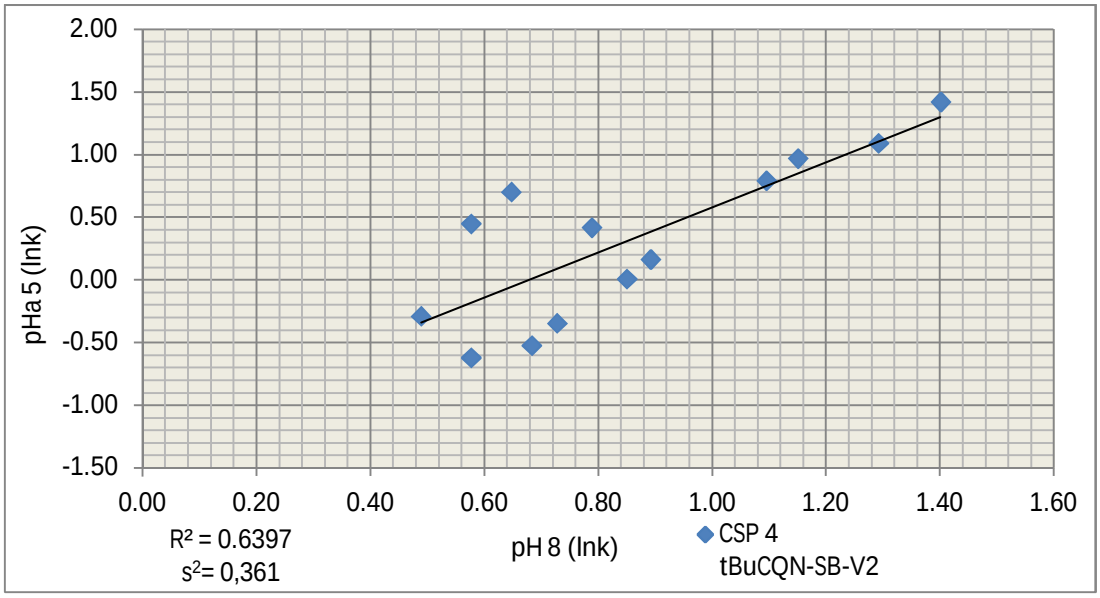


Figure 56: Selectivity plot of CSP 4, for two different pH values. Theophylline was eliminated as outlier.

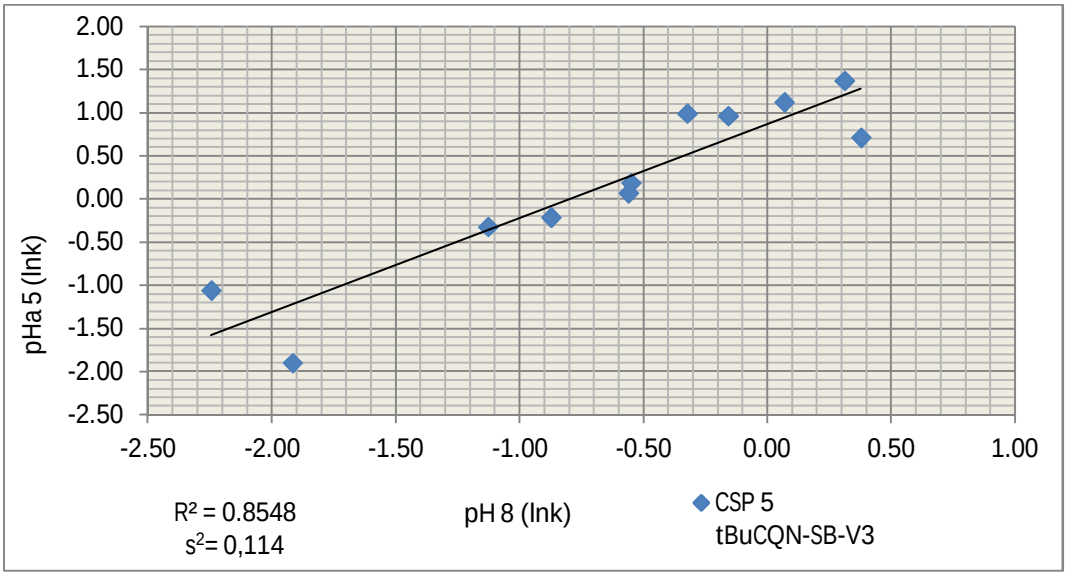


Figure 57: Selectivity plot of CSP 5, for two different pH values. Ibuprofen, flurbiprofen and 4-OH-phenyl acetic acid were eliminated, because they showed minimized retention.

Generally, with the use of a test set, containing acidic, basic, zwitterionic and polar analytes, it could be outlined that the three groups of analytes, with different chemical behaviour, showed retention on all three columns. The nucleosides, desoxynucleosides and the nucleobases, such as phenylephredine and thiamine had highest retention on the CSP 3, if the mobile phase with pH 5,0 was applied. The separations provided good column efficiencies, except thiamine which underlay peak broadening. Therefore it could be stated that CSP 3 would hit the prospects for separations of basic compounds at the best. Especially 4-OH-phenyl acetic acid and cytidine showed high retention on CSP 4.

In general, the mobile phase composition with pH_a 5,0 and 8,0 exhibited a worse separation performance than the "middle" pH of 5,0 for acidic compounds.

For acids it could be said that the retention was frequently the highest with pH 5,0, using CSP 3 for 4-OH-phenyl-acetic acid and CSP 5 for flurbiprofene and ibuprofen.

Zwitterionic compounds, such as nicotinic acid, alpha-methyl-tryptophane and tryptophane were highly retained on CSP 3 and CSP 5, and had their highest retention with mobile phase of pH 5,0.

The peak efficiencies were noticeably less satisfying with CSP 3 than with the two other columns. Zwitterionic analytes yielded the best retention behaviour with quiet good efficiency with the CSP 5 (Table 30). In general, CSP 5 provided the best performance in this study, especially at the middle pH of 5,0. However, with this mobile phase the highest peak efficiencies, expressed as number of theoretical plates were achieved. The combination of CSP 5 with mobile phase of pH 5,0 was remarkable for acidic compounds and good selectivity could be reached.

The alkaloids, theobromine and theophylline were not strongly retained on all three columns using any of the applied mobile phase compositions and therefore they were the most challenging compounds in

this study.

Concerning the peak shape, which was also influenced by the number of plates, it should be clarified that the zwitterionic compounds, such as tryptophane and alpha-methyl-tryptophane showed the broadest peaks with a significant tailing. This was truly based on their zwitterionic character and was also observed in reversed phase separations. Thiamine also exhibited low efficiencies on this type of sulfobetaine-HILIC columns (Table 30).

The chromatographic parameters for the investigated set of HILIC compounds were also investigated using a commercially available ZIC-HILIC column, sulfobetaine type Nucleodur® 100-3 (Table 31). The mobile phase was ACN/aqueous buffer, containing 10 mM NH_4AcO with pH 5,0 and method 7 was applied. In direct comparison with the data for the sulfobetaine columns (Table 30), some aspects should be mentioned. CSP 5 had frequently the highest efficiency. Actually, flurbiprofene and ibuprofene had highest retention on this column and lowest on the Nucleodur®. On CSP 4, 4-OH-phenyl acetic acid, flurbiprofene and ibuprofene were highly retained. CSP 3 showed high retention for adenine, adenosine, deoxyadenosine and phenylephrine, but did not exhibit the proper efficiency for these compounds compared to the other sulfobetaine columns. The Nucleodur® column gave the best retention for cytidine, thiamine, tryptophane, alpha-methyl-tryptophane and nicotinic acid, but efficiency was in most cases better on one of the sulfobetaine columns. On the other hand the Nucleodur® column had the worst separation performance for adenine, adenosine, deoxyadenosine, flurbiprofene and ibuprofene.

Figure 58 provided an overlay of chromatograms of single injections of HILIC compounds using method 7 and CSP 5. Theophyllin and theobromine eluted firstly, then the acidic compounds, 4-OH-ühenyl acetic acid, flurbiprofene and ibuprofene as well as nicotinic acid eluted. The zwitterionic tryptophane exhibited a high peak broadening because of its amphoteric nature.

Keeping these results in mind, it should be stated that the synthesized sulfobetaine-type quinine-based columns were capable for separations in the HILIC mode.

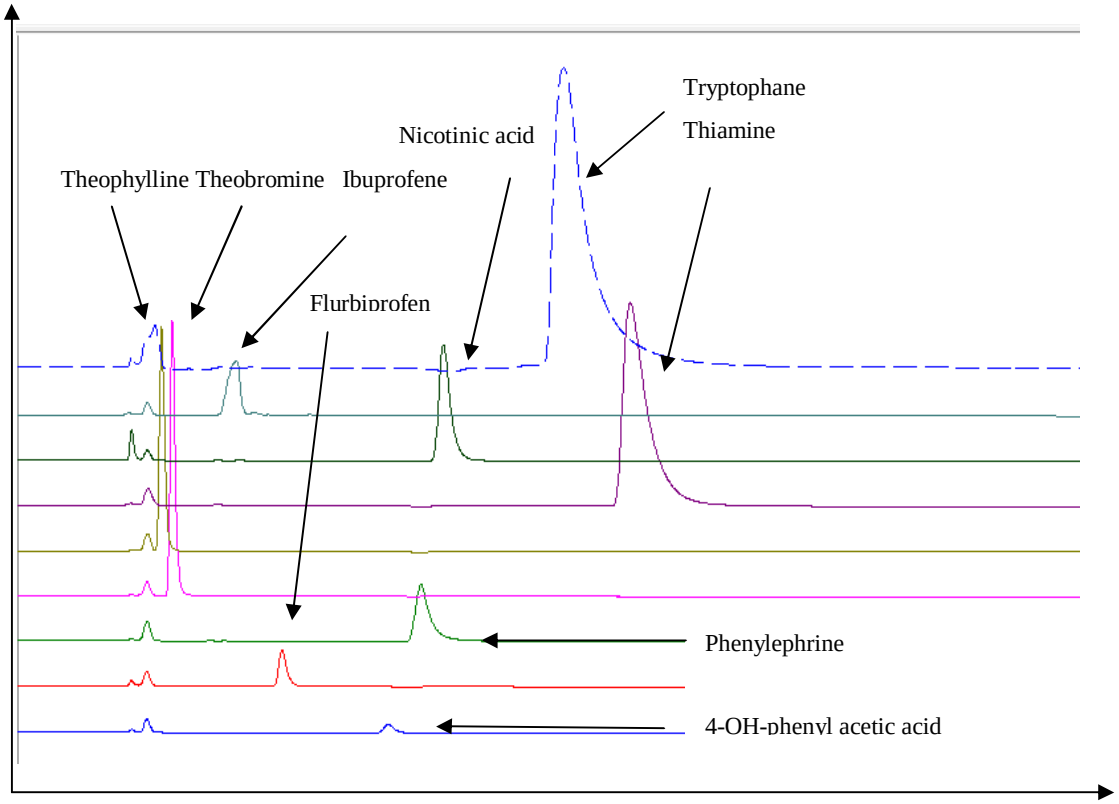


Figure 58: Overlay of the chromatogram of single injections of HILIC compounds using CSP 5 and method 7.

4.7.3 Examination of the retention mechanism of sulfobetaine-type quinine-based stationary phases

Representative for the three synthesized columns, CSP 3, was used for the investigation of the retention mechanism of sulfobetaine-type HILIC columns. Alpha-methyl-tryptophane, 4-hydroxy-acetic acid and deoxyadenosine were chosen as analytes for the investigation. The observed retention behaviour was exemplified using different mixtures of mobile phase, namely ACN/aqueous buffer 10 mM NH_4AcO pH 5,0, where the water content was increased from 10 to 90 % (v/v) (Figure 59). The buffer concentration was kept constant. Retention plots were generated, where the logarithm of the retention factor (log k) was plotted against the phase ratio of water. As illustrated, the retention plot showed a typically U-shape for all three tested substances. This indicated that a mixed retention mode was responsible for the observed elution dependency. Hydrophobic and hydrophilic retention increments accounted for the analyte retention [60].

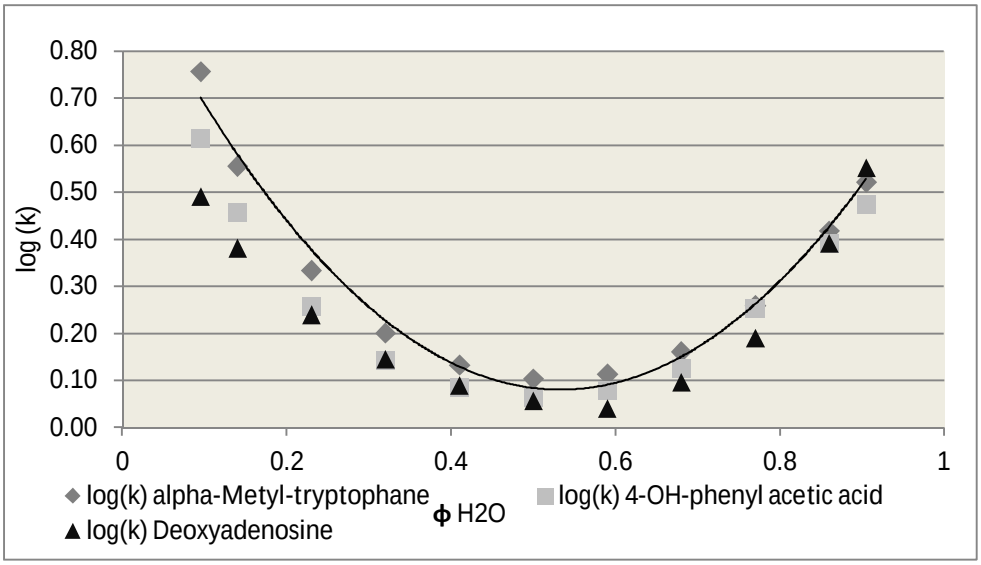


Figure 59: Investigation of the retention mechanism of QN-SB-V2, concerning three representative HILIC compounds and a mobile phase mixture of ACN/aqueous buffer, with varying content of H_2O from 10 to 90 % (v/v). A trendline was added for alpha-tryptophane to illustrate the typically U-shape for a mixed-mode retention behaviour.

4.7.3.1 Comparison of the retention mechanism of sulfobetaine- and tert-butyl carbamate modified type quinine-based selectors

Figure 60 illustrated the results of a comparison test. The same analytes and mobile phases were used, as for investigations of the retention mechanism of the sulfobetaine-type columns (CSP 3-5), but now CSP 2 was applied. The *tert*-butyl carbamate modified quinidine selector might exhibit a different retention mechanism compared to the sulfobetaine-type HILIC columns, although their quinidine moiety showed to a high degree of structural analogy. An oblated U-shape was observed in Figure 60 for deoxyadenosine and alpha-methyl-tryptophane. The retention behaviour 4-OH-phenyl acid did not follow this trend. This acidic compound was excluded, because it underwent a different retention mechanism on a WAX-type column. The subsequent change in buffer concentration during a series of injections would strongly influence the protonation of the acidic analyte as well as the opposite charged moiety of the quinidine. The zwitterionic alpha-methyl tryptophane seemed to be uninfluenced by the change of buffer concentration, as observed for deoxyadenosine. The latter two compounds exhibited a mixed mode retention mechanism on CSP 2. In direct comparison with Figure 59, the magnitude of the U-shape was lowered.

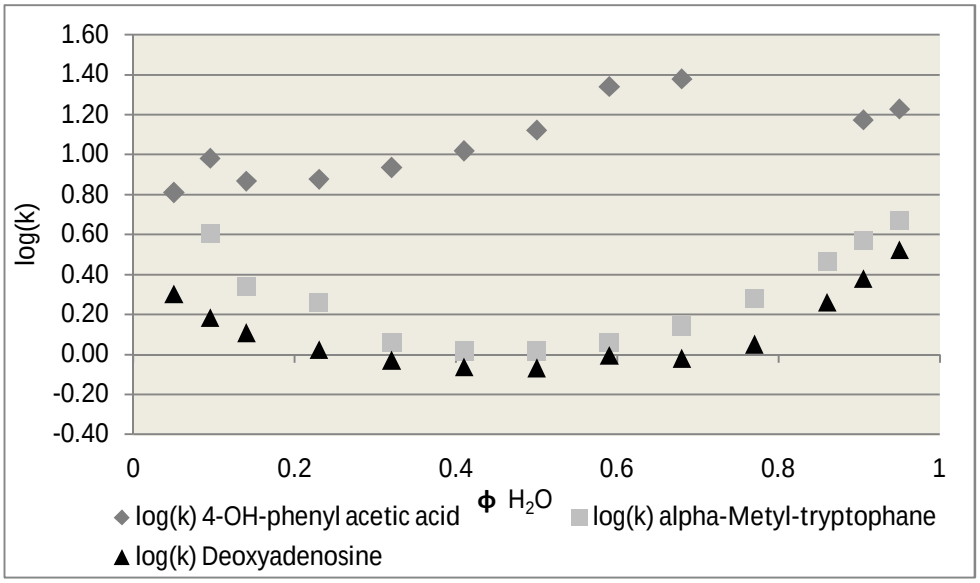


Figure 60: Evaluation of the retention mechanism of a *tert*-butyl-carbamate-quinine-based CSP with a mobile phase mixture of ACN/aqueous buffer. The content of buffer ranged from 5 to 95 % (v/v). Analytes were alpha-methyl-tryptophane, 4-OH-phenyl acetic acid and deoxyadenosine.

It was found that the reversed phase increment of CSP 3 and the *tert*-butyl modified quinine column was similar (Figure 61). This was confirmed, when the structure of the selectors was taken into consideration. The hydrophobic retention was caused by the linkage group or by an interaction site of the selector, like the quinoline ring. Both selectors had the similar reversed phase behavior, because of their structural analogy. Observing the typical HILIC mode with polar organic solvent and minor aqueous content, a difference in retention was observed. Surprisingly, the *tert*-butyl-carbamate-modified quinine CSP exhibited also HILIC character (Table 33). The sulfobetaine column showed a higher hydrophilic retention increment. It could be claimed, that the sulfobetaine-type columns exhibited stronger HILIC character than CSP 2. It should be mentioned that the selector loading was not equal, which would have some impact on the magnitude of the retention increments.

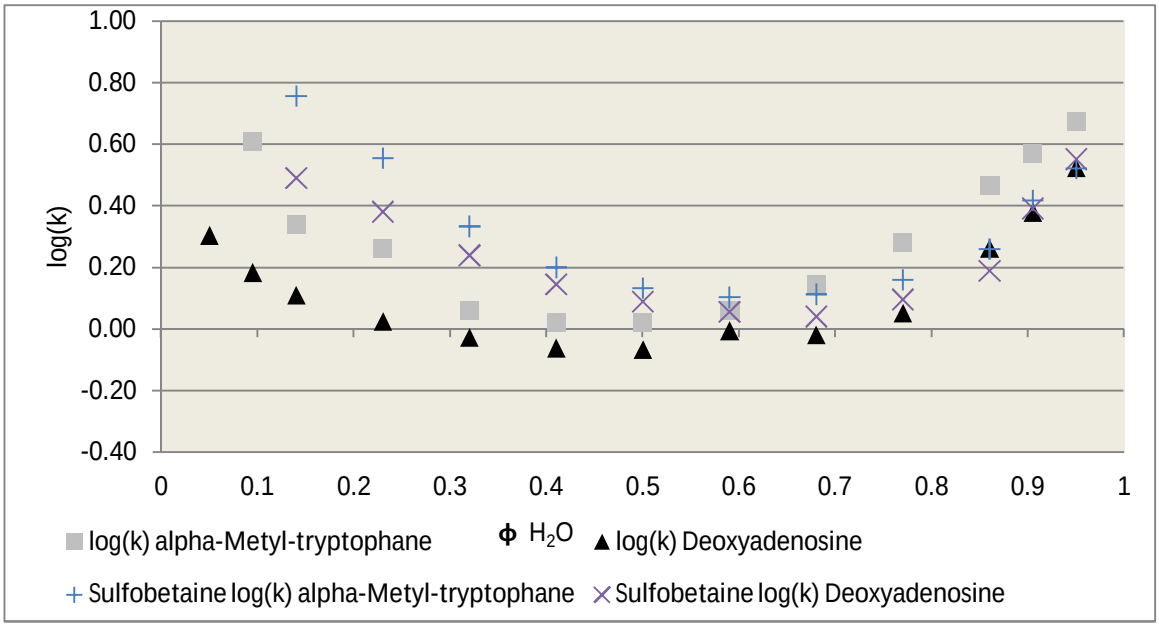


Figure 61: Comparison of the investigated retention mechanism of sulfobetaine-type, CSP 3 and *tert*-butyl carbamate modified quinine-based columns for deoxyadenosine and alpha-methyl-tryptophane. Mobile phase mixture of ACN/aqueous buffer was applied. The content of buffer was kept constant, but the amount of added water ranged from 5 to 95 % (v/v).

4.7.3.2 Comparison of the retention mechanism of sulfobetaine-type quinine-based with a commercial available zwitterionic sulfobetaine-type HILIC column

A typical zwitterionic HILIC column was tested with the same mobile phase and analytes as mentioned above (Figure 62). Deoxyadenosine was not retained if a water content of 50 % was exceeded, but for alpha-methyl-tryptophane and 4-OH-phenyl acetic acid a typical U-shape was observed in the retention plot. In direct comparison of Figure 59 and Figure 62, it could be seen that the Nucelodur® column exhibited a much stronger HILIC character. The U-shape of the log(k) *vs.* φ H_2O plot for the Nucleodur® column was steep in the HILIC phase mode and less strong pronounced in the reversed phase mode. It was found that the tested sulfobetaine column, CSP 3 exhibited a moderate HILIC character, compared to a commercial zwitterionic HILIC column (Table 32).

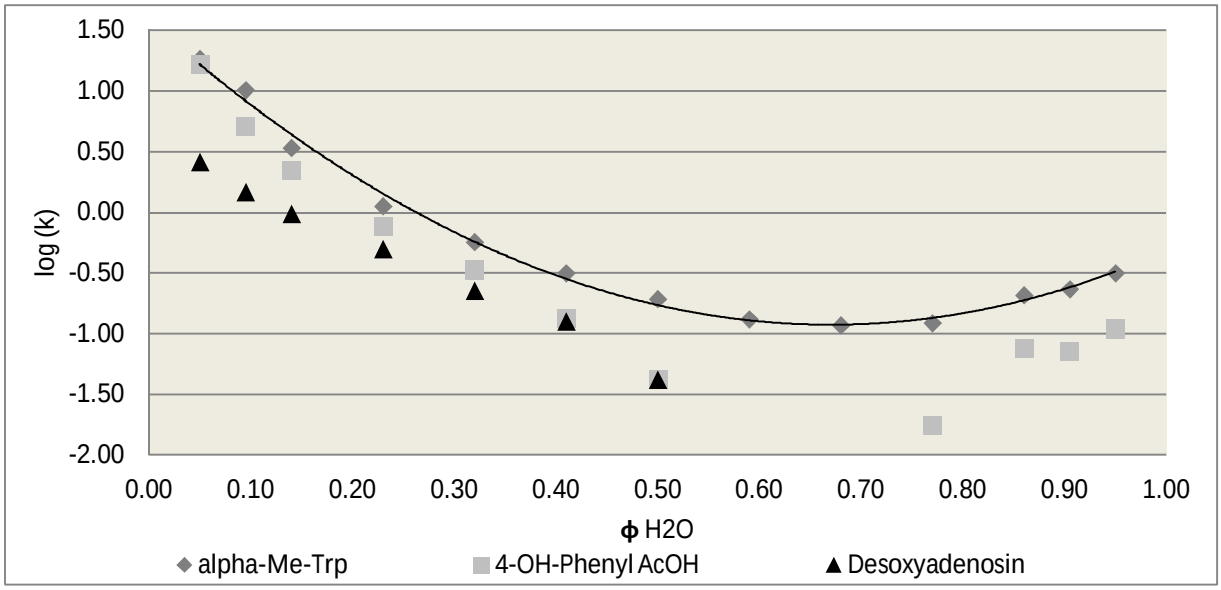


Figure 62: Investigation of the retention mechanism of a commercial HILIC phase. Nucleodur® 100-3 HILIC, from Macherey-Nagel AG, a sulfobetaine-type HILIC column. Mobile phase mixture of ACN/aqueous buffer was applied. The content of buffer ranged from 5 to 95 % (v/v). Analytes were alpha-methyl-tryptophane, 4-OH-phenyl acetic acid and deoxyadenosine. Deoxyadenosine eluted with the void volume if an aqueous content of 50 % was exceeded.

5 CONCLUSION AND OUTLOOK

The derivatization of amino acids with 6-aminoquinolyl-succinimidyl carbamate (AQC) was successfully introduced and optimized for all twenty proteinogenic amino acids and an extended range of non-proteinogenic amino acids as well as for alanine and phenylalanine peptides. The enantioseparation on *tert*-butyl carbamate modified quinine/quinidine based CSPs (QN/QD-AX) was successfully investigated. All proteinogenic amino acids were separated with a resolution >1,5, except for aspartic acid, which could not be separated on QN-AX and on QD-AX only a partial separation was achieved. As reported, biacidic analytes with two acidic interaction sites yielded worse separation results on the QN/QD-AX columns compared to mono acidic congeners [53]. In contrast, glutamic acid was well resolved, which might be a hint for the influence of the amino acid side chain on the chiral interaction mechanism and the reversal of the elution order. Other acidic amino acids were also resolved, but with somewhat higher retention times. The separation conditions, concentration, pH and ratio of buffer composition had to be well optimized, especially for acidic compounds. The separation of 4-hydroxyproline stereoisomers was successfully implemented. The threonine stereoisomers, with homologues of homoserine were not fully separated, the first eluting enantiomers of threonine and homoserine coeluted on both columns. The two coeluting compounds showed a lack of overall interaction with the selector and eluted very early. It was worthy to mention that the four stereoisomers of DL-threonine and DL-allo-threonine, respectively, could be enantioseparated. The four isoleucine stereoisomers were not fully separated. The first eluting stereoisomers coeluted and the later eluting ones were only partial resolved. The lack of selectivity was surely based on the small difference provided by the amino acid side chain. It was thought that the difference of the Gibbs free energy of the coeluting stereoisomers was too small to induce enantioseparation. The low energy amount of hydrophobic interactions confirmed this hypothesis. For hydrophobic interactions, the stereochemistry was of marginal importance. In addition, these hydrophobic interaction increments take to a different degree part in the chiral interaction mechanism. For 4-hydroxypoline and threonine isomers, the difference in configuration and comformation of each stereoisomer was remarkably high and besides that the interaction increments of their side chains were not hydrophobic in character.

The alanine peptide separations provided insight into the chiral recognition mechanism. The here presented results broadened the knowledge gathered during earlier alanine peptide investigations [43]. In comparison the investigated protection groups and AQC, the latter mentioned tag positioned itself in term of introduced enantioselectivity behind the DNB-derivatives, but before the bulk of the other applied protection groups [53]. With growing peptide chain length, the alanine peptides showed a loss of retention as well as in enantioselectivity and resolution. Diverse finding were adopted for phenylalanine peptides. The retention time increased with growing chain length and hence, the enantioselectivity was comparable for each of the peptides in the sequence up to the pentamer. The resolution was less reduced up to the tetrapeptide and surprisingly, a drop in resolution could be denoted

for the pentamer. The trend was not further investigated for higher peptides, because of strongly reduced solubility of longer phenylalanine peptides.

The use of fluoresceine isothiocyanate (FITC) for amino acid derivatization was successfully adopted. All twenty proteinogenic amino acid were derivatized and enantioseparation was compared to the performance of AQC derivatives. Generally, the enantioselectivity was for all observed derivatives marginally reduced, yet comparable. This can be explained by the use of different mobile phases, during the analysis. Comparison of the obtained resolution values gave a mixed picture. It was found that satisfactory resolution was accessible with one or the other of these two fluorescence labels, for all proteinogenic amino acids, besides aspartic acid. The achieved enantioseparation of FITC-labeled amino acids provided further evidence for the supreme chiral discrimination abilities of quinine-based CSPs. The fact that aspartic acid was not separated, neither with AQC or with FITC, was an obstacle and could be caused by the competing interaction of the two carboxylic groups. Empirically, Asp-derivatives were not separated, whereas glutamic acid was separated with moderate performance.
The achieved selectivity of enantioseparation of secondary and beta-amino acids, derivatized with AQC is worthy to mention. It is known that secondary amino acids like proline and hydroxyproline were only resolvable with a limited range of protection groups, whereas primary amino acids allowed the use of a broader range of N-derivatization reagents [53]. Especially the AQC reagent seemed to be a quiet good option for the enantioseparation of proline and proline analogues. Additionally, beta-amino acid showed shorter retention, but in contrast, better alpha and resolution values were obtained compared to their alpha counterpart. The urea type derivative of amino acids uses AQC as a reagent open up a new site for amide type hydrogen bonding compared to amide type protecting groups, which clearly causes differences.

The sulfobetaine-type quinine-based CSPs were found to lack in enantiodiscrimination. First, the combination of two strong ion-exchange groups in one selector molecule will overload the selector. As one charged group acts as a counterion for the oppositely charged ion-exchange site, and *vice versa*, the charged interaction sites are shielding each other. Furthermore, both modification positions, the quinuclidine and the quinoline nitrogen atom, seem to provide no alternative for modification of the quinine selector in order to alter or increase the enantiodiscrimination potential. Nevertheless, it was investigated that in some special cases some enantioselectivity was exhibited. In detail, the sulfobetaine type quinine based CSP (CSP 3) and the quinoline sulfobetaine type *tert*-butyl carbamate modified quinine based CSP (CSP 5) exhibited some enantiodiscrimination ability to the 3,5-dinitrophenyl derivatives of aspartic acid, leucine and cysteine as well as for the AQC derivatives of cysteine and cystine.
In addition these sulfobetaine columns were investigated using a hydrophilic interaction liquid chromatographic separation system. Generally, all classes of typical HILIC compounds with polar

character were retained to a certain degree on at least one of the sulfobetaine-type columns. They showed good selectivity and efficiency, especially for zwitterionic and charged compounds. Alkaloids, such as theobromine and theophylline, gave the lowest selectivity followed by the nucleosides and nucleobases. The best HILIC performance was reached with CSP 3. The hydroxyl group at position C9 was not modified, which provided a more polar character of the selector compared to *tert*-butyl carbamate modified quinine selector. Additionally, the higher selector loading of CSP 3 enhanced the polarity of the stationary phase. The highest efficiency was achieved with CSP 5. The enantioselectivity was also found to be not totally distinct in the HILIC mode. Alpha-methyl-tryptophane, phenylephrine, ibuprofene and DNB-aspartic acid were in some cases partially resolved.

The mode of retention was investigated and it was found that all three sulfobetaine modified quinine-based columns exhibited a mixed retention mode, containing hydrophobic as well as hydrophilic retention increments. In comparison with the *tert*-butyl carbamate modified quinine-based CSPs, the HILIC behaviour was moderately higher for the sulfobetaine columns. A further outlook for the investigation of the behaviour of sulfobetaine-type quinine-based HILIC columns is given to access better retention behaviour and selectivity. The selector loading, which will have an influence on the overall polarity of the stationary phase, should be increased. The alkylation of both nitrogen atoms provides another possibility to alter the polarity of the selector. However in the HILIC mode the enantioselectivity was widely diminished.

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7 APPENDIX

7.1 Tables

Table 10: Proteinogenic amino acids derivatized with AQC and column QN-AX (CSP1) was used. Method 1 was applied for the enantioseparations.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
Gly	1.40	5.65	5.65	3.04	3.04	1.00	0	2709	2709	n.a.
Ala	1.40	4.20	5.11	2.00	2.65	1.33	2.50	2354	2906	D
Val	1.40	3.40	6.42	1.43	3.59	2.51	7.93	2493	2755	D
Leu	1.40	3.56	5.10	1.54	2.64	1.71	4.45	2231	2809	D
Ile	1.40	3.33	6.34	1.38	3.53	2.56	7.91	2322	2814	D
Met	1.40	5.10	7.18	2.64	4.13	1.56	4.32	2402	2781	D
Pro	1.40	4.24	5.22	2.03	2.73	1.35	2.75	2677	2852	L
Ser	1.40	5.04	7.81	2.60	4.58	1.76	5.50	2348	2773	D
Thr	1.40	4.08	8.68	1.91	5.20	2.72	9.28	2376	2810	D
Lys	1.40	4.96	6.99	2.54	3.99	1.57	3.80	1779	2221	D
Asp	1.40	31.14	31.40	21.24	21.43	1.01	1.00	1716	2197	L
Asn	1.40	5.46	11.73	2.90	7.38	2.54	8.87	2147	2493	D
Glu	1.40	14.73	18.55	9.52	12.25	1.29	2.66	2026	2279	D
Gln	1.40	4.73	5.44	2.38	2.89	1.21	1.71	2131	2635	D
Arg	1.40	2.04	2.38	0.46	0.70	1.53	1.37	997	1679	D
Phe	1.40	4.99	7.89	2.56	4.64	1.81	5.73	2493	2716	D
His	1.40	2.60	3.33	0.86	1.38	1.61	2.63	1526	2071	D
Tyr	1.40	5.72	10.10	3.09	6.21	2.01	6.74	2302	2429	D
Trp	1.40	7.00	14.60	4.00	9.43	2.36	8.81	2410	2578	D
Cysteine	1.40	6.48	11.58	3.63	7.27	2.00	5.79	1796	2094	D
n.a. not applicable										

Table 11: Cystine derivatized with AQC, column was a QN-AX- low coverage (type CSP 1). Method 1 was applied for the enantioseparations.

Analytes	to	t_1	t_2	t_3	k_1 k_2 k_3	$\alpha_{2/1}$ $\alpha_{3/2}$ $\alpha_{3/1}$	$Rs_{1/2}$ $Rs_{2/3}$ $Rs_{1/3}$	N_1 N_2 N_3	e.o.
DL-Cystine	1.52	19.00	34.08	50.20	11.5	1.86	5.73	1757	DD-DL-LL
					21.42	1.45	3.68	1561	Ratio peak area
					31.03	2.70	8.82	1432	1:2:1

Table 12: Proteinogenic amino acids derivatized with AQC, column was QD-AX (CSP 2). Method 1 was applied for the enantioseparations.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
Gly	1.45	5.15	5.15	2.55	2.64	1.04	0	3137	5137	n.a.
Ala	1.45	3.70	4.84	1.55	2.42	1.56	4.63	5059	4705	L
Val	1.45	3.06	5.29	1.11	2.74	2.47	9.22	4927	4701	L
Leu	1.45	3.20	4.62	1.21	2.26	1.88	6.29	4900	4678	L
Ile	1.45	3.02	5.32	1.08	2.76	2.55	9.44	4826	4636	L
Met	1.45	4.60	6.85	2.17	3.86	1.78	6.71	4746	4600	L
Pro	1.45	4.28	6.10	1.95	3.32	1.70	6.04	4910	4660	D
Ser	1.45	4.48	6.29	2.09	3.46	1.65	5.63	4651	4446	L
Thr	1.45	3.79	6.29	1.61	3.46	2.14	8.35	4701	4443	L
Lys	1.45	4.75	6.19	2.28	3.39	1.49	4.44	3626	3444	L
Arg	1.45	2.00	2.28	0.38	0.59	1.56	1.24	753	2835	L
Asp	1.45	21.64	28.30	13.92	19.18	1.38	4.09	3793	3759	D
Asn	1.45	4.84	7.20	2.34	4.11	1.76	6.29	4134	4135	L
Glu	1.45	13.09	13.94	8.03	8.92	1.11	0.94	3602	3476	L
Gln	1.45	3.97	4.83	1.74	2.41	1.39	3.17	4338	4091	L
Arg	1.45	2.00	2.28	0.38	0.59	1.56	1.24	753	2835	L
Phe	1.45	4.70	7.36	2.24	4.22	1.88	7.38	4573	4443	L
His	1.45	2.55	3.08	0.76	1.16	1.53	1.83	2142	1187	L
Tyr	1.45	5.21	7.87	2.59	4.59	1.77	6.43	4170	3904	L
Trp	1.45	7.09	10.60	3.89	6.54	1.68	6.25	4117	3887	L
Cysteine	1.45	5.70	9.95	2.93	6.07	2.07	7.41	3385	2750	L

n.a. not applicable

Table 13: Separation data for the investigation of a set of proteinogenic amino acids, derivatized with AQC and separated on QD-AX using a hydro-polar mobile phase. Method 2 was applied for the enantioseparations.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
Val	1.5	9.30	23.90	5.20	14.93	2.87	14.47	4481	4453	L
Ile	1.5	9.89	27.45	5.59	17.30	3.09	15.7	4440	4483	L
Met	1.5	14.33	25.79	8.55	16.19	1.89	10.05	5036	4934	L
Pro	1.5	11.39	20.70	6.59	12.80	1.94	9.96	4674	4768	D
Ser	1.5	10.45	15.78	5.97	9.52	1.60	7.16	4965	5000	L
Phe	1.5	17.43	17.43	10.62	10.62	1.00	\	4781	4781	n.a.
His	1.5	2.86	3.60	0.91	1.40	1.54	2.95	2622	2696	L
Tyr	1.5	15.95	15.95	9.63	9.63	1.00	\	4355	4355	n.a.
Trp	1.5	29.56	29.56	18.71	18.71	1.00	\	4418	4418	n.a.

Table 14: Retention times of AQC-amino acid derivatives for the applied reversed phase system for optimization of derivatization procedure and further investigations. RP column Phenomenox Gemini 150x3,0 3µm.

Analytes	Retention time [min]	Analytes	Retention time [min]
Asp	15.3	**Met**	31,1
Ser	18,0	**Ile**	33.4
Gln	18,9	**Leu**	33.8
Asn	19,0	**Lys**	33.9
Glu	20.7	**Phe**	35.2
Gly	21.9	**alpha-Aminobuthyric acid**	27.8
NH_3 Peak	22.6	**Norvaline**	30.9
His	22.8	**Cysteine-acetamide**	24.6
Thr	24.1	**Cysteine-4-vinylpyridine**	31.5
Arg	24.3	**4-trans-L-Hydroxyproline**	13.2
Ala	25	**4-cis-L-Hydroxyproline**	24.5
Pro	26.4	**System peaks:**	
Cysteine	27.3	**AMQ**	11.8
Cystine	29.7	**DAD**	41.2; 42.1
Tyr	29.7	**FLD**	33.4; 34.4; 40.8; 40.9; 41.3; 43.8;
Val	30.3		

Table 15: Separation data for non-proteinogenic amino acids, stereoisomers, phosphoaminoacids and phenylalanine analoga. The column was a QN-AX (CSP 1). Method 1 was applied for the enantioseparations.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
trans-4-D or trans-4-L-Hydroxypro (2R, 4S and 2S, 4R)	1.50	4.37	5.78	1.91	2.85	1.49	2.34	3401	3678	D
cis-4-D or cis-4-L-Hydroxypro (2R, 4R allo and 2S, 4S)	1.50	5.20	6.30	2.47	3.20	1.30	1.59	3812	3886	D
trans-4-D or trans-4-L-Hydroxypro (2R, 4S and 2S, 4R)*	3.76	11.10	15.00	1.95	2.99	1.53	3.95	7526	7787	D
cis-4-D or cis-4-L-Hydroxypro (2R, 4R allo and 2S, 4S)*	3.76	13.30	16.20	2.54	3.31	1.30	4.41	7914	8232	D
beta-DL-Phe	1.50	2.42	5.20	0.61	2.47	4.02	9.41	2397	2838	D
Gly-DL-Phe	1.50	3.54	4.59	1.36	2.06	1.51	2.26	2579	2613	L
Gly-DL-Pro **	5.90	20.90	27.25	2.54	3.62	1.42	2.05	707	1281	L
DL-Phe-Gly	1.50	4.79	6.53	2.19	3.35	1.53	4.44	3210	3509	L
DL-Isoser	1.50	5.57	7.98	2.71	4.32	1.59	3.36	1324	1515	n.a.
DL-Homoser	1.50	3.96	5.53	1.64	2.69	1.64	4.97	3250	3626	L
DL-Thr	1.50	3.99	7.26	1.66	3.84	2.31	8.89	3513	3898	L
DL-allo-Thr	1.50	4.30	6.64	1.87	3.43	1.84	6.58	3639	3957	L
3,4-Dihydroxy-DL-	1.50	4.39	6.49	1.93	3.33	1.73	4.16	1881	1831	L

phenylalanine										
p-Fluro-DL-Phe	1.50	4.85	7.36	2.23	3.91	1.75	6.41	3712	4007	n.a.
p-Chloro-DL-Phe	1.50	5.47	9.04	2.65	5.03	1.90	7.69	3719	4048	L
p-beta-Chloro-DL-Phe	1.50	4.29	8.93	1.86	4.95	2.66	10.81	3502	3946	n.a.
p-Bromo-DL-Phe	1.50	5.95	9.89	2.97	5.59	1.89	7.78	3819	4015	n.a.
ZH555 (α-Amino-3-hydroxy-3-phenyl-ethyl-phosphon)	1.50	7.36	27.93	3.91	17.62	4.51	6.36	775	425	n.a.
FW460 (α -Amino-propyl-phosphon)	1.50	5.60	21.80	2.73	13.53	4.95	9.12	1029	936	n.a.
FW 133 (α -Amino-1-cycohexyl-methyl-phosphon)	1.50	5.54	5.54	2.69	2.69	1.00	0	1979	1979	n.a.
DL-Phospholeu	1.50	5.29	19.08	2.53	11.72	4.64	11.8	1692	1827	D
DL-Methioninsulfone	1.50	6.30	8.20	3.20	4.47	1.40	4.09	3826	3901	L
DL-Homocysteic acid	1.50	34.10	45.99	21.73	29.66	1.36	4.56	3750	3797	L
DL-Cys-S-acetamide	1.50	5.09	7.20	2.39	3.80	1.59	5.25	3532	3852	L
DL-Cys-S-AcOH	1.50	38.04	52.66	24.36	34.11	1.40	4.9	3645	3753	L
DL-Achsa carbonic acid	1.50	2.84	6.06	0.89	3.04	3.40	10.62	2654	3958	SS
DL-Achsa-sulfonic acid	1.50	5.27	9.77	2.51	5.51	2.19	9.62	3972	4249	SS
NVal	1.45	3.33	4.87	1.30	2.44	1.88	6.41	4882	4602	L
AABA (alpha -amino - butanoic acid)	1.45	3.39	4.92	1.34	2.48	1.85	6.48	4970	4933	L
DL-allo-Isoleu	1.50	2.07	3.73	0.38	1.49	3.91	7.13	2097	2730	L
Dichlorprop	1.50	3.55	4.33	1.37	1.89	1.38	3.48	4628	4519	n.a.
DL-Pipecolinic acid (Homoproline)	1.50	7.35	7.35	3.90	3.90	1.00	0	3896	3896	/
DL-Nipecotinic acid (β-Homoproline)	1.50	1.85	2.18	0.23	0.45	1.94	1.72	1891	1536	n.a.
Gly-DL-Pro*[a]	3.57	25.6	31.9	6.17	7.94	1.29	1.53	2816	416	D
Gly-Gly-DL-Pro[a]	1.43	7.2	7.6	4.03	4.31	1.07	0.54	958	2077	D
Gly-Gly-DL-Pro*[a]	3.57	18.6	19.9	4.21	4.57	1.09	0.68	1200	2785	D

n.a. not applicable

Back pressure ca. 60 bar

*** Flow 0.4 ml/min**

**** Flow 0.25 ml/min**

[a] Mobile phase: MeOH/$H_2$0 80/20, 20 mM NH_4AcO pH_a= 6,00

Table 16: Separation data for non-proteinogenic amino acids, stereoisomers, phosphoaminoacids and phenylalanine analoga. The column was a QD-AX (CSP 2). Method 1 was applied for the enantioseparations.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
trans-4-D or trans-4-L-Hydroxyproline (2R, 4S and 2S, 4R)	1.50	4.37	5.78	1.91	2.85	1.49	2.34	3401	3678	D
cis-4-D or cis-4-L-Hydroxyproline (2R, 4R allo and 2S,4S)	1.50	5.20	6.30	2.47	3.20	1.30	1.59	3812	3886	D
trans-4-D or trans-4-L-Hydroxyproline (2R 4S and 2S, 4R)*	3.76	11.10	15.00	1.95	2.99	1.53	3.95	7526	7787	D
cis-4-D or cis-4-L-Hydroxyproline (2R, 4R allo and 2S 4S)*	3.76	13.30	16.20	2.54	3.31	1.30	4.41	7914	8232	D
beta-DL-Phe	1.50	2.42	5.20	0.61	2.47	4.02	9.41	2397	2838	D
Gly-DL-Phe	1.50	3.54	4.59	1.36	2.06	1.51	2.26	2579	2613	L
Gly-DL-Pro **	5.90	20.90	27.25	2.54	3.62	1.42	2.05	707	1281	L
DL-Phe-Gly	1.50	4.79	6.53	2.19	3.35	1.53	4.44	3210	3509	L
DL-Isoser	1.50	5.57	7.98	2.71	4.32	1.59	3.36	1324	1515	n.a.
DL-Homoser	1.50	3.96	5.53	1.64	2.69	1.64	4.97	3250	3626	L
DL-Thr	1.50	3.99	7.26	1.66	3.84	2.31	8.89	3513	3898	L
DL-allo-Thr	1.50	4.30	6.64	1.87	3.43	1.84	6.58	3639	3957	L
3,4-Dihydroxy-DL-phenylalanine	1.50	4.39	6.49	1.93	3.33	1.73	4.16	1881	1831	L
p-Fluro-DL-Phe	1.50	4.85	7.36	2.23	3.91	1.75	6.41	3712	4007	n.a.
p-Chloro-DL-Phe	1.50	5.47	9.04	2.65	5.03	1.90	7.69	3719	4048	L
p-beta-Chloro-DL-Phe	1.50	4.29	8.93	1.86	4.95	2.66	10.81	3502	3946	n.a.
p-Bromo-DL-Phe	1.50	5.95	9.89	2.97	5.59	1.89	7.78	3819	4015	n.a.
ZH555 (1-Amino-3-hydroxy-3-phenyl-ethyl-phosphon)	1.50	7.36	27.93	3.91	17.62	4.51	6.36	775	425	n.a.
FW460 (2-Amino-propyl-phosphon)	1.50	5.60	21.80	2.73	13.53	4.95	9.12	1029	936	n.a.
FW 133 (1-Amino-1-cycohexyl-methyl-phosphon)	1.50	5.54	5.54	2.69	2.69	1.00	0	1979	1979	n.a.
DL-Phospholeu	1.50	5.29	19.08	2.53	11.72	4.64	11.8	1692	1827	D
DL-Methioninsulfone	1.50	6.30	8.20	3.20	4.47	1.40	4.09	3826	3901	L
DL-Homocysteic acid	1.50	34.10	45.99	21.73	29.66	1.36	4.56	3750	3797	L
DL-Cys-S-acetamide	1.50	5.09	7.20	2.39	3.80	1.59	5.25	3532	3852	L
DL-Cys-S-AcOH	1.50	38.04	52.66	24.36	34.11	1.40	4.90	3645	3753	L
DL-Achsa-carbonic acid	1.50	2.84	6.06	0.89	3.04	3.40	10.62	2654	3958	SS
DL-Achsa-sulfonic acid	1.50	5.27	9.77	2.51	5.51	2.19	9.62	3972	4249	SS
NVal	1.45	3.33	4.87	1.30	2.44	1.88	6.41	4882	4602	L
AABA (alpha -amino -butanoic acid)	1.45	3.39	4.92	1.34	2.48	1.85	6.48	4970	4933	L

DL-allo-Isoleu	1.50	2.07	3.73	0.38	1.49	3.91	7.13	2097	2730	L
Dichlorprop	1.50	3.55	4.33	1.37	1.89	1.38	3.48	4628	4519	n.a.
DL-Pipecolinic acid (Homoproline)	1.50	7.35	7.35	3.90	3.90	1.00	0	3896	3896	/
DL-Nipecotinic acid (β-Homoproline)	1.50	1.85	2.18	0.23	0.45	1.94	1.72	1891	1536	n.a.
Gly-DL-Pro[a]	1.5	11.49	14.44	6.66	8.63	1.30	1.17	1084	252	L
Gly-Gly-DL-Pro*[a]										
Gly-Gly-DL-Pro[a]	1.5	9.78	9.78	5.52	5.52	1.00	0.32	3228	3228	n.a.

n.a. not applicable

*** Flow 0.4 ml/min (better separation)**

**** Flow 0.25 ml/min (better separation)**

[a] Mobile phase: MeOH/$H_2$0 80/20, 20 mM NH_4AcO pH_a= 6,00

Table 17: Comparison of separation data of all four stereoisomers of hydroxyproline and isoleucine with three different mobile phase compositions a) flow rate 1 ml/min MeOH/H_2O 80/20, 20 mM NH_4AcO pH_a 6,0, b) MeOH/H_2O 80/20, 200 mM NH_4AcO pH_a 6,0 flow rate 1 ml/min and 0,5 ml/min for c). d) flow rate 1 ml/min MeOH/AcOH/NH_4AcO 98/2/0,5 % (v/v/w) and flow rate of 0,5 ml/min for e)

Analytes	k_1/k_2	k_3/k_4	$\alpha_{1/2}$ $\alpha_{2/3}$	$\alpha_{3/4}$ $\alpha_{1/4}$	$Rs_{1/2}$	$Rs_{2/3}$	$Rs_{3/4}$	N_1/N_2	N_3/N_4	e.o.
4-Hydroxy proline - four stereoisomers [a]	1.99	3.70	1.39	1.14	4.05	3.85	1.81	4740	4772	D<D-allo<L<L-allo
	2.77	4.21	1.34	2.12	\	\	\	5090	5342	
4-Hydroxy proline - four stereoisomers [b]	4.79	6.69	1.20	1.11	2.89	2.26	1.65	5580	4743	D<D-allo<L<L-allo
	5.77	7.41	1.16	1.55	\	\	\	5534	6180	
4-Hydroxy proline - four stereoisomers [c]	1.97	2.99	1.29	1.11	3.92	2.64	1.74	7526	7787	D<D-allo<L<L-allo
	2.55	3.32	1.18	1.69	\	\	\	7912	8832	
4-Hydroxy proline - four stereoisomers [d]	3.30	5.30	1.27	2.86	1.72	1.17	0.96	3442	1359	D<D-allo<L<L-allo
	4.20	15.16	1.26	4.59	\	\	\	1342	3886	
4-Hydroxy proline - four stereoisomers [d]	10.15	14.02	1.21	1.09	3.92	1.65	1.74	7525	7824	D<D-allo<L<L-allo
	12.33	15.23	1.14	1.50	\	\	\	7914	8232	
Isoleucine - four stereoisomers [d]	1.13	3.50	2.97	\	12.04	0.85	\	5748	5706	L=allo-L<D<D-allo
	3.36	\	1.04	\	\	\	\	4618	\	
Isoleucine - four stereoisomers [e]	2.22	8.37	3.55	\	14.53	0.84	\	4524	3611	L=allo-L<D<D-allo
	7.87	\	1.06	\	\	\	\	4312	\	

Table 18: Chromatographic parameters of the investigations of AQC-tagged alanine peptides, separated on QN-AX column. Method 2 was applied for the enantioseparations.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
DL-Ala	1.43	9.8	13.3	5.85	8.30	1.42	4.33	3205	3236	D
DD/LL-Ala$_2$	1.43	7.74	12.43	4.41	7.69	1.74	6.21	2722	2850	DD
LLL/DDD-Ala$_3$	1.43	5.5	6.2	2.85	3.34	1.17	1.65	2722	2719	DDD
LLL/DDD-Ala$_3$ *	3.57	14.16	16.19	2.97	3.54	1.19	2.42	4568	4486	DDD
LDL/DLD-Ala$_3$ *	3.57	15.9	16.8	3.45	3.71	1.07	0.9	4228	4434	DLD<LDL
LLD/DDL-Ala$_3$ *	3.57	16.4	16.4	3.59	3.59	1.00	\	2329	2329	n.a.
LDD/DLL-Ala$_3$ *	3.57	15.8	17.3	3.43	3.85	1.12	1.57	4741	4704	LDD<DLL
LLLL/DDDD-Ala$_4$ *	3.57	5.15	5.8	0.44	0.62	1.41	1.47	2608	2524	DDDD
LLLD/DDDL-Ala$_4$ *	3.57	13.07	15.5	2.66	3.34	1.26	2.25	4408	4450	DDDL<LLLD
LLDL/DDLD-Ala$_4$ *	3.57	13.44	14.02	2.76	2.93	1.06	\	4290	3347	DDLD<LLDL
LDLL/DLDD-Ala$_4$ *	3.57	13.48	13.48	2.78	2.78	1.00	\	3594	32594	n.a.
LDDD/DLLL-Ala$_4$ *	3.57	14.03	14.03	2.93	2.93	1.00	\	1836	1836	n.a.
all-L/all-D-Ala$_5$	1.43	4.94	5.49	2.45	2.84	1.16	1.25	2330	2356	DDDDD
all-L/all-D-Ala $_6$	1.43	4.7	5.2	2.29	2.64	1.15	1.15	2250	2264	DDDDDD
Gly-DL-Ala*	3.57	22.27	32.44	5.24	8.09	1.54	6.48	4996	4805	D
DL-Ala-Gly	1.43	8.6	12.4	5.01	7.67	1.53	4.94	3170	3020	D
DL-Ala-Gly-DL-Ala	1.43	6.48	6.8	3.53	3.76	1.06	0.61	2210	2403	D
DL-Ala-Gly-Gly*	3.57	18.07	18.77	4.06	4.26	1.05	0.13	6530	7117	D
Gly-Gly-DL-Ala*	3.57	17.23	17.42	3.83	3.88	1.01	0.22	5265	7702	D
Gly	1.43	12.57	12.57	7.79	7.79	1.00	0	3398	3398	n.a.
Gly-Gly	1.43	10.32	10.32	6.22	6.22	1.00	0	3118	3218	n.a.
Gly-Gly-Gly	1.43	6.98	6.98	3.88	3.88	1.00	0	2808	2808	n.a.

n.a. not applicable

***flow rate 0.4 ml/min**

Table 19: Chromatographic parameters of the investigations of AQC-tagged alanine dipeptides separated on QN-AX column. Method 2 was applied for the enantioseparations.

Analytes	to	t_1/t_2	t_3/t_4	k_1/k_2	k_3/k_4	$\alpha_{RR/SS}$ $\alpha_{SR/RS}$	Rs 1/2	Rs 2/3	Rs 3/4	N_1/N_2	N_3/N_4	e.o.
DL-Ala-DL-Ala	1.43	7.6 8.8	12.5 13.8	4.31 5.15	7.74 8.65	0.56 1.68	1.89	3.51	1.33	2904 3071	2901 2965	DD<DL<LL<LD

Table 20: Chromatographic parameters of the investigations of AQC-tagged alanine peptides, separated on QD-AX column. Method 2 was applied for the enantioseparations.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
DL-Ala	1.5	10.05	14.09	5.70	8.39	1.47	5.92	4993	5022	L
DD/LL-Ala$_2$	1.5	8.77	13.04	4.85	7.69	1.59	6.24	3970	3141	LL
DL/LD-Ala$_2$	1.5	10.13	12.33	5.75	7.22	1.25	3.09**			LD
LLL/DDD-Ala$_3$	1.5	6.4	7.8	3.27	4.20	1.29	3.04	3941	3452	LLL
LDL/DLD-Ala$_3$	1.5	7.16	7.65	3.77	4.10	1.09	1.01	4053	3542	LDL
LLD/DDL-Ala$_3$	1.5	7.79	7.9	4.19	4.27	1.02	0.28	7000	5783	DDL
LDD/DLL-Ala$_3$	1.5	7.07	8.85	3.71	4.90	1.32	3.59	4002	4226	DLL
LLLL/DDDD-Ala$_4$ *	1.5	6.08	7.42	3.05	3.95	1.29	3.01	3624	3681	LLLL
LLLD/DDDL-Ala$_4$ *	3.74	17.17	18.12	3.59	3.84	1.07	1.11	6716	7056	LLLD
LLDL/DDLD-Ala$_4$ *	3.74	15.67	17.11	3.19	3.57	1.12	1.95	7672	8013	LLDL
LDLL/DLDD-Ala$_4$ *	3.7	15.65	18.03	3.23	3.87	1.20	3.06	7396	7624	LDLL
LDDD/DLLL-Ala$_4$ *	3.74	15.61	17.91	3.17	3.79	1.19	2.82	6774	6797	LDDD
all-L/all-D-Ala$_5$	1.5	5.92	7.23	2.95	3.82	1.30	2.9	3377	3411	LLLLL
all-L/all-D-Ala$_6$	1.5	5.66	6.92	2.77	3.61	1.30	2.79	3216	3119	LLLLLL
all-L/all-D-Ala$_7$	1.5	5.66	6.91	2.77	3.61	1.30	2.67	2748	3037	LLLLLLL
Gly-DL-Ala	1.5	10.96	12.1	6.31	7.07	1.12	1.68	4692	4648	L
DL-Ala-Gly	1.5	10.06	12.83	5.71	7.55	1.32	4.11	4503	4728	L
DL-Ala-Gly-DL-Ala	1.5	7.42	8.28	3.95	4.52	1.15	1.81	3683	4550	L
DL-Ala-Gly-Gly	1.5	8.50	8.50	4.67	4.67	1.00	\	2695	2695	n.a.
DL-Ala-Gly-Gly*	3.74	21.47	21.47	4.74	4.74	1.00	\	3419	3149	n.a.
Gly-Gly-DL-Ala	1.5	7.94	8.59	4.29	4.73	1.10	1.12	2621	4160	L
Gly	1.5	13.35	13.35	7.90	7.90	1.00	\	5208	5208	n.a.
Gly-Gly	1.5	11.44	11.44	6.63	6.63	1.00	\	4797	4797	n.a.
Gly-Gly-Gly	1.5	8.50	8.50	4.67	4.67	1.00	\	4083	4083	n.a.
LLL/DDD-Ala$_3$ *	3.74	16.19	19.88	3.33	4.32	1.30	4.7	7656	9269	LLL
LDL/DLD-Ala$_3$ *	3.74	18.05	19.35	3.83	4.17	1.09	1.47	7993	6579	LDL
LLD/DDL-Ala$_3$ *	3.74	17.80	22.53	3.76	5.02	1.34	5.04	7655	8141	DLL
LDD/DLL-Ala$_3$ *	3.74	19.50	19.86	4.21	4.31	1.02	0.42	7486	9529	DDL

*** flow rate 0.4 ml/min**

n.a. not applicable

Table 21: Chromatographic parameters of the investigations of AQC-tagged alanine dipeptides, separated on QD-AX column. Method 2 was applied for the enantioseparations.

Analytes	to	t_1/t_2	t_3/t_4	k_1/k_2	k_3/k_4	$\alpha_{RR/SS}$ $\alpha_{SR/RS}$	Rs 1/2	Rs 2/3	Rs 3/4	N_1/N_2	N_3/N_4	e.o.
DL-Ala-DL-Ala	1.5	8.77	12.33	4.85	7.22	1.51	2.37	2.92	0.85	4310	3908	DD<L
		10.14	13.21	5.76	7.81	1.25				4307	4095	D<DL <LL

Table 22: Separation parameters of AQC-tagged phenylalanine peptides. Separation was carried out on a QN-AX CSP. Method 3 was applied for the enantioseparations.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
DL-Phe	2.80	9.17	14.94	2.28	4.34	1.91	7.01	3520	3381	D
DD/LL-Phe$_2$	2.80	10.80	17.80	2.86	5.36	1.88	6.73	3141	2962	DD
DL/LD-Phe$_2$	2.80	10.12	11.70	2.61	3.18	1.22	2.02	3146	3076	DL
DDD/LLL-Phe$_3$	2.80	10.10	14.26	2.61	4.09	1.57	4.30	2538	2561	DDD
DLL/LDD-Phe$_3$	2.80	10.51	13.34	2.75	3.76	1.37	3.18	2887	2899	DLL
DDL/LLD-Phe$_3$	2.80	10.21	10.21	2.65	2.65	1.37	\	2645	2945	n.a.
DLD/LDL-Phe$_3$	2.80	9.25	10.19	2.30	2.64	1.15	1.31	2947	2933	DLD
DDDD/LLLL-Phe$_4$	2.80	11.48	16.90	3.10	5.04	1.62	4.65	2285	2477	DDDD
DLDD/LDLL-Phe$_4$	2.80	10.90	14.75	2.89	4.27	1.48	3.63	2746	2410	DLDD
DLLL/LDDD-Phe$_4$	2.80	11.90	15.52	3.25	4.54	1.40	4.16	2399	2575	LDDD
DDLD/LLDL-Phe$_4$	2.80	10.25	10.25	2.66	2.66	1.00	\	2575	2575	n.a.
DDDL/LLLD-Phe$_4$	2.80	10.74	12.94	2.84	3.62	1.28	1.58	2667	788	DDDL
DDLL/LLDD-Phe$_4$	2.80	11.51	15.72	3.11	4.61	1.48	3.47	2518	2184	LLDD
DLLD/LDDL-Phe$_4$	2.80	10.20	10.82	2.64	2.86	1.08	0.65	1329	2465	LDDL
DLDL-LDLD-Phe$_4$	2.80	9.88	10.56	2.53	2.77	1.10	0.40	2067	6606	DLDL
All-D-all-L-Phe$_5$	2.80	12.70	17.54	3.54	5.26	1.49	2.39	443	2363	DDDDD

Table 23: Separation parameters of AQC-tagged phenylalanine dipeptides. Separation was carried out on a QN-AX. Method 3 was applied for the enantioseparations.

Analytes	to	t_1/t_2	t_3/t_4	k_1/k_2	k_3/k_4	$\alpha_{RR/SS}$ $\alpha_{SR/RS}$	Rs 1/2	Rs 2/3	Rs 3/4	N_1/N_2	N_3/N_4	e.o.
DL-Phe-DL-Phe	2.8	10.16	11.66	2.63	3.16	2.03	0.52	1.10	4.88	2753	3054	DL< DD< LD< LL
		10.70	17.74	2.82	5.34	1.12				1887	3053	

Table 24: Separation parameters of AQC-tagged phenylalanine peptides. Separation was carried out on a QD-AX CSP. Method 3 was applied for the enantioseparations.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
DL-Phe	3.10	9.46	17.37	2.05	4.60	2.24	10.33	5086	4916	L
DD/LL-Phe$_2$	3.10	12.66	23.05	7.38	14.26	1.93	9.63	4459	4368	LL
DL/LD-Phe$_2$	3.10	12.27	12.44	2.96	3.01	1.02	0.26	6735	4911	LD
DDD/LLL-Phe$_3$	3.10	12.13	21.33	2.91	5.88	2.02	8.79	4222	4020	LLL
DLL/LDD-Phe$_3$	3.10	12.77	19.91	3.12	5.42	1.74	6.94	4002	4074	DLL
DDL/LLD-Phe$_3$	3.10	12.66	12.66	3.08	3.08	1.00	\	3445	3445	n.a.
DLD/LDL-Phe$_3$	3.10	11.23	12.60	2.62	3.06	1.17	1.87	4294	4142	LDL
DDDD/LLLL-Phe$_4$	3.10	14.45	27.56	3.66	7.89	2.16	8.95	3236	3336	LLLL
DLDD/LDLL-Phe4	3.10	4.68	5.09	0.51	0.64	1.26	1.37	4150	4343	LDLL
DLLL/LDDD-Phe$_4$	3.10	14.08	24.03	8.32	14.91	1.79	7.52	3227	3387	DLLL
DLDD/LDLL-Phe$_4$	3.10	13.36	21.63	7.85	13.32	1.70	7.22	3677	3780	LDLL

DDLD/LLDL-Phe$_4$	3.10	13.27	14.30	7.79	8.47	1.09	1.17	3952	3837	LLDL
DDDL/LLLD-Phe$_4$	3.10	15.50	15.75	9.26	9.43	1.02	0.20	5571	5520	LLLD
DDLL/LLDD-Phe$_4$	3.10	14.33	24.67	8.49	15.34	1.81	8.21	3957	3789	DDLL
DLLD/LDDL-Phe$_4$	3.10	13.46	14.24	7.91	8.43	1.07	0.89	3949	3914	LDDL
DLDL-LDLD-Phe$_4$	3.10	12.87	12.87	7.52	7.52	1.00	\	3464	3464	n.a.
All-D-all-L-Phe$_5$	3.10	17.10	31.14	4.52	9.05	2.00	2.86	1986	227	LLLLL

Table 25: Separation parameters of AQC-tagged phenylalanine dipeptides. Separation was carried out on a QD-AX CSP. Method 3 was applied for the enantioseparations.

Analytes	to	t_1/t_2	t_3/t_4	k_1/k_2	k_3/k_4	$\alpha_{RR/SS}$ $\alpha_{SR/RS}$	Rs 1/2	Rs 2/3	Rs 1,2,3/4	N_1/N_2	N_3/N_4	e.o.
DL-Phe-DL-Phe	3.1	12.45	12.45	3.02	3.02	2.09	\	\	7.77	1624	1624	DL=LD=DD<LL
		12.45	22.64	3.02	6.30	1.00				1624	4597	

Table 26: Chromatographic parameters for the optimization of the retention for a set of FITC derivatized amino acids. Four different mobile phase compositions were applied, the parameters were kept constant such as column temperature 25°C, flow rate 1 ml/min and detection with DAD 235 nm.

MeOH/AcOH/NH$_4$formate 98/2/0,5 % (v/v/w) and CSP 6 was applied.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
Ala	1.55	7.74	9.79	3.99	5.32	1.33	3.4	3088	3648	\
Val	1.55	7.02	12.96	3.53	7.36	2.09	8.8	3676	3427	\
Leu	1.55	7.31	10.79	3.72	5.96	1.60	5.66	3334	3600	\
Pro	1.55	8.21	9.47	4.30	5.11	1.19	2.02	3168	3863	\
Ser	1.55	8.50	12.69	4.48	7.19	1.60	5.96	3602	3667	\
Lys	1.55	3.67	4.89	1.37	2.15	1.58	2.85	944	2754	\
Asp	1.55	\	\	\	\	\	\	\	\	\
Asn	1.55	9.08	18.65	4.86	11.03	2.27	10.17	3659	3395	\
His	1.55	4.98	6.68	2.21	3.31	1.50	3.82	2750	2749	\
Trp	1.55	13.56	23.00	7.75	13.84	1.79	7.71	3628	3540	\

MeOH 100mM FA, 50 mM NH$_4$formate and CSP 6column was applied.

Analytes	to	t_1	t_2	k_1	k_2	alpha	Rs	N_1	N_2	e.o.
Ala	1.57	6.67	8.62	3.25	4.49	1.38	4.21	4464	4267	\
Val	1.57	5.76	9.98	2.67	5.36	2.01	8.99	4379	3883	\
Leu	1.57	5.63	8.22	2.59	4.24	1.64	5.93	4472	3796	\
Pro	1.57	7.36	8.47	3.69	4.39	1.19	2.18	4040	3696	\
Ser	1.57	7.67	12.20	3.89	6.77	1.74	7.13	3748	4070	\
Lys	1.57	3.87	4.26	1.46	1.71	1.17	0.67	2892	392	\
Asp	1.57	23.13	32.14	13.73	19.47	1.42	5.04	3632	3679	\
Asn	1.57	7.93	16.78	4.05	9.69	2.39	10.8	3629	3716	\
His	1.57	4.81	6.60	2.06	3.20	1.55	4.39	3085	3192	\
Trp	1.57	11.13	19.27	6.09	11.27	1.85	8.58	42229	4046	\

MeOH/ACN 80/20 100 mM FA, 50 mM NH_4 formate and CSP 6was applied.										
Analytes	**to**	**t_1**	**t_2**	**k_1**	**k_2**	**alpha**	**Rs**	**N_1**	**N_2**	**e.o.**
Ala	1.53	4.84	6.14	2.16	3.01	1.39	4.13	4965	4885	\
Val	1.53	4.20	7.24	1.75	3.73	2.14	8.95	4879	4325	\
Leu	1.53	4.20	5.99	1.75	2.92	1.67	5.99	4908	4581	\
Pro	1.53	5.07	5.65	2.31	2.69	1.16	1.64	4335	4139	\
Ser	1.53	5.56	8.29	2.63	4.42	1.68	6.75	4913	4529	\
Lys	1.53	2.94	3.67	0.92	1.40	1.52	1..58	415	1805	\
Asp	1.53	14.08	18.55	8.20	11.12	1.36	4.27	3678	4121	\
Asn	1.53	5.40	10.46	2.53	5.84	2.31	10.59	4885	4196	\
His	1.53	3.70	4.90	1.42	2.20	1.55	4.28	3579	3805	\
Trp	1.53	6.80	10.79	3.44	6.05	1.76	7.65	4781	4466	\
MeOH/ACN 70/30 200 mM FA, 100 mM NH_4 formate and QD-AX column was applied.										
Analytes	**to**	**t_1**	**t_2**	**k_1**	**k_2**	**alpha**	**Rs**	**N_1**	**N_2**	**e.o.**
Ala	1.48	6.02	8.27	3.07	4.59	1.50	5.07	4266	4166	\
Val	1.48	4.81	8.39	2.25	4.67	2.08	8.58	4261	3889	\
Leu	1.48	4.76	6.93	2.22	3.68	1.66	6.12	4155	3849	\
Pro	1.48	6.06	7.10	3.09	3.80	1.23	2.44	3930	3741	\
Ser	1.48	7.51	10.80	4.07	6.30	1.55	5.54	3496	4052	\
Lys	1.48	3.17	4.44	1.14	2.00	1.75	4.48	2862	2962	\
Asp	1.48	22.54	22.54	14.23	14.23	1.00	0	2044	2044	\
Asn	1.48	7.10	10.29	3.80	5.95	1.57	5.59	3806	3648	\
His	1.48	4.66	6.19	2.15	3.18	1.48	4.03	3234	3209	\
Trp	1.48	8.82	12.46	4.96	7.42	1.50	5.27	3828	3791	\

Table 27: Chromatographic parameters of FITC derivatized amino acids. Separation was carried out with a QN-AX column and method 6 was applied.

Analytes	**to**	**t_1**	**t_2**	**k_1**	**k_2**	**alpha**	**Rs**	**N_1**	**N_2**	**e.o.**
Gly	1.4	18.06	18.06	11.90	11.90	1.00	\	2918	2918	n.a.
Ala	1.4	11.92	15.77	7.51	10.26	1.37	3.6	2631	2746	D
Val	1.4	8.67	16.60	5.19	10.86	2.09	8.28	2786	2792	D
Leu	1.4	8.42	12.55	5.01	7.96	1.59	4.9	2651	2523	D
Pro	1.4	11.22	12.54	7.01	7.96	1.13	1.38	2439	2562	L
Ser	1.4	15.88	25.87	10.34	17.48	1.69	6.42	2801	2952	D
Lys	1.4	4.89	4.97	2.49	2.55	1.02	0.87	1842	1923	D
Asp	1.4	29.58	29.58	20.13	20.13	1.00	\	2080	2080	n.a.
Asn	1.4	13.34	28.90	8.53	19.64	2.30	9.04	2540	2361	D
His	1.4	6.29	8.56	3.49	5.11	1.46	3.69	2240	2315	D
Trp	1.4	16.79	28.90	10.99	19.64	1.79	6.65	2541	2683	D
Tyr	1.4	16.20	26.20	10.57	17.71	1.68	5.89	2564	2452	D
Ile	1.4	8.19	15.30	4.85	9.93	2.05	8.01	2714	2859	D

Met	1.4	14.65	21.65	9.46	14.46	1.53	5.02	2761	2812	D
Thr	1.4	11.60	27.57	7.29	18.69	2.57	10.38	2380	2711	D
Arg	1.4	4.60	5.78	2.29	3.13	1.37	2.47	1881	1926	D
Glu	1.4	30.88	43.85	21.06	30.32	1.44	4.24	2348	2443	D
Gln	1.4	13.50	16.50	8.64	10.79	1.25	2.56	2632	2627	D
Phe	1.4	14.25	21.88	9.18	14.63	1.59	5.51	2725	2741	D
DL-allo-Thr	1.4	16.57	26.11	10.84	17.65	1.63	5.88	2776	2777	D
DL-Cysteine	1.4	13.89	18.47	8.92	12.19	1.37	7.78	1574	2683	D

n.a. not applicable

Table 28: Chromatographic parameters of FITC derivatized amino acids. Separation was carried out with a QD-AX column and method 6 was applied.

Analytes	**to**	**t_1**	**t_2**	**k_1**	**k_2**	**alpha**	**Rs**	**N_1**	**N_2**	**e.o.**
Gly	1.4	10.71	10.71	6.65	6.65	1.00	\	4291	4291	n.a.
Ala	1.4	7.10	9.96	4.07	6.11	1.50	5.42	4233	4168	L
Val	1.4	5.44	9.81	2.89	6.01	2.08	9.08	4181	3942	L
Leu	1.4	5.27	8.07	2.76	4.76	1.72	6.61	3944	4011	L
Pro	1.4	7.36	8.75	4.26	5.25	1.23	2.66	3957	3693	D
Ser	1.4	8.92	13.04	5.37	8.31	1.55	5.62	3218	3886	L
Lys	1.4	3.21	4.75	1.29	2.39	1.85	4.85	2605	3298	D
Asp	1.4	19.57	22.89	12.98	15.35	1.18	2.35	3610	3637	L
Asn	1.4	8.52	12.20	5.09	7.71	1.52	5.3	3412	3684	L
His	1.4	4.99	6.71	2.56	3.79	1.48	4.14	3026	3335	L
Trp	1.4	11.14	16.16	6.96	10.54	1.52	5.67	3964	3733	L
Tyr	1.4	9.70	15.45	5.93	10.04	1.69	6.34	2536	3533	L
Ile	1.4	5.25	9.27	2.75	5.62	2.04	8.58	3920	3830	L
Met	1.4	8.68	13.82	5.20	8.87	1.71	7.15	3890	3964	L
Thr	1.4	6.99	12.28	3.99	7.77	1.95	8.68	4108	3945	L
Arg	1.4	4.01	4.84	1.86	2.46	1.32	1.78	1557	1335	L
Gln	1.4	8.01	9.69	4.72	5.92	1.25	2.94	4038	3752	L
Glu	1.4	14.65	17.92	9.46	11.80	1.25	3.01	3705	3565	L
Phe	1.4	8.97	14.40	5.41	9.29	1.72	7.58	4233	4107	L
DL-allo-Thr	1.4	8.93	13.52	5.38	8.66	1.61	6.25	3406	3971	L
DL-Cysteine	1.4	8.04	9.3	4.74	5.64	1.19	1.21	523	2997	L

n.a. not applicable

Table 29: Excerpt of the investigation for chiral discrimination potential of the synthesized sulfobetaine-modified quinine-based stationary phases. Results just for the analytes, for which an enantioseparation or partial resolution could be achieved, were shown. Determination of the elution order was not performed.

QN-SB-V2										
Analytes	**to**	**t_1**	**t_2**	**k_1**	**k_2**	**alpha**	**Rs**	**N_1**	**N_2**	**e.o.**
DNB-Asp [a]	1.46	13.74	14.30	8.41	9.17	1.09	0.51	1674	1735	\
AQC-DL-Lys [d]	1.47	5.00	5.38	2.40	2.66	1.11	0.65	1471	1141	\
AQC-DL-Tyr [d]	1.47	3.02	3.27	1.05	1.22	1.16	0.64	1005	1023	\
AQC-DL-Cys [d]	1.47	9.03	10.20	5.14	5.94	1.15	0.95	1208	938	\
AQC-DL-Trp [d]	1.47	4.50	4.84	2.06	2.29	1.11	0.62	1717	1144	\
AQC-DL-Tyr [b]	1.47	3.53	3.76	1.40	1.56	1.11	0.61	1598	1446	\
DNB-Leu [c]	1.47	1.95	2.01	0.33	0.39	1.18	0.42	2254	3330	\
tBuCQN-SB-V2										
AQC-DL-Cys [b]	1.47	1.54	1.79	0.05	0.22	4.57	0.59	292	233	\
tBuCQN-SB-V3										
AQC-DL-Cysteine [e]	1.60	20.00	20.70	11.50	11.94	1.04	0.39	2003	2222	\
AQC-DL-Cystine [d]	1.60	15.80	16.00	8.88	9.00	1.01	0.25	2560	2652	\
DNB-DL-Leu [d]	1.60	2.10	2.20	0.31	0.38	1.20	0.38	5462	5556	\
DL-DNB-Leu [e]	1.60	2.48	2.57	0.55	0.61	1.10	0.45	4555	4956	\
AQC-DL-Cystine [b]	1.60	39.77	43.61	23.86	26.26	1.10	0.74	1582	1586	\
DNB-Leu [b]	1.60	13.20	13.90	7.25	7.69	1.06	0.45	2568	2544	\
AQC-DL-Thr [b]	1.60	14.90	23.60	8.31	13.75	1.65	0.85	3524	3222	\
DNB-DL-Leu [f]	1.60	4.96	5.25	2.10	2.28	1.09	0.94	4685	4068	\
DNB-Glu [a]	1.60	6.60	6.76	3.13	3.23	1.03	0.42	8150	3420	\
Ac-Phe [a]	1.60	4.04	4.28	1.53	1.68	1.10	0.91	4106	3769	\
DNB-Ile [a]	1.60	4.07	4.23	1.54	1.64	1.06	0.60	3643	2835	\
[a] **ACN/H_2O 9/1, 10mM NH_4AcO pH 5.0**										
[b] **MeOH 20mM AcOH**										
[c] **ACN/H_2O 9/1 10mM NH_4AcO pH 8.1**										
[d] **MeOH/AcOH/NH_4AcO 98/2/0.5 % (v/v/w)**										
[e] **MeOH/AcOH/NH_4AcO 99/1/0.25 (v/v/w)**										
[f] **MeOH/H_2O 20 mM NH_4AcO pH 6.0 with AcOH**										

Table 30: Comparison of the retention factors and the theoretical plate numbers per meter of column for the three tested sulfobetaine-type columns. Two different pH values in the mobile phase were applied, composition was ACN/H_2O 9/1, 10 mM NH_4AcO, pH was adjusted to pH=8,0 or 5,0 in the aqueous buffer or the apparent pH was adjusted to 5,0, with acetic acid or ammonia hydroxid. Other parameter according method 7.

	CSP 3 QN-SB-V2						CSP 4 tBuCQN-SB-V2						CSP 5 tBuCQN-SB-V3					
	pHa 5.0		pH 5.0		pH 8.0		pHa 5.0		pH 5.0		pH .0		pHa 5.0		pH 5.0		pH 8.0	
Analytes	**k**	**N [m^{-1}]**	**k**	**N [m^{-1}]**	**k**	**N [m^{-1}]**	**k**	**N [m^{-1}]**	**k**	**N [m^{-1}]**	**k**	**N [m^{-1}]**	**k**	**N [m^{-1}]**	**k**	**N [m^{-1}]**	**k**	**N [m^{-1}]**
Adenine	3.13	20587	4.47	17347	2.27	15513	2.34	28660	4.30	20800	1.01	30313	2.67	33333	3.40	33707	1.07	5007
Adenosine	2.97	26133	4.27	24513	1.92	24700	2.07	30287	4.20	27093	0.71	34660	2.41	36500	2.93	36207	0.81	1553
Deoxyadenosine	2.63	22580	3.66	23267	1.51	24073	1.98	12393	3.51	27447	0.59	28240	2.25	28707	2.71	24980	0.72	5160
Cytidine	3.77	30073	5.79	25367	2.86	24860	2.44	28287	6.26	26500	1.18	35053	2.68	36500	3.61	38580	1.20	2433
Ibuprofene	1.55	23227	2.47	12213	0.89	22493	1.78	27820	2.59	18773	0.54	33533	1.7	30140	2.99	9167	0.96	18560
Flurbiprofen	1.61	25973	3.20	27207	1.32	26687	1.63	28380	3.33	29027	0.75	35593	1.84	19947	3.61	41073	1.37	6093
4-OH-Phenyl acetic acid	1.92	20260	5.84	25600	3.56	24587	1.78	26740	12.00	19440	1.56	43813	1.92	32140	5.06	46227	2.34	14320
Theophylline	2.28	24867	2.65	25373	0.84	21053	1.86	28240	3.60	24033	0.36	31327	1.88	23973	2.11	32667	0.35	10153
Theobromine	2.28	23080	2.72	22653	0.85	20620	1.91	30047	2.60	24893	2.01	27867	1.95	25667	1.97	25087	0.15	1060
Phenylephrine	3.98	29993	7.32	17193	3.95	21113	3.64	41367	6.49	11967	2.97	30267	3.15	32480	5.50	30960	2.62	27080
Nicotinic acid	2.69	23653	6.70	21867	3.51	21900	2.20	34580	6.60	25600	1.52	38800	2.93	39613	5.81	48667	2.69	13853
Thiamine	4.63	7767	12.26	8000	7.08	8380	4.06	19587	9.09	15767	4.14	18627	4.02	13953	8.37	23820	3.94	13713
α-Methyl-tryptophane	4.47	15460	9.39	10647	5.40	14187	2.99	15020	9.27	11320	2.20	31200	3.52	27107	6.67	5040	3.07	8340
Tryptophane	4.85	19567	10.37	14733	6.10	17813	3.16	5820	10.10	17593	2.63	14240	4.18	21180	7.45	10707	2.04	7053

Table 31: Chromatographic separation data for a set of HILIC representative compounds were presented. Mobile phase was a mixture of ACN/H_2O 90/10, 10 mM NH_4AcO, where the pH of the buffer was adjusted to 5.0 with acetic acid. Data were investigated by Georg Schuster.

Nucleosid® - Zwitterionic HILIC column		
Analytes	**k**	**N [m^{-1}]**
Adenine	1.98	12419
Adenosine	2.45	12633
Deoxyadenosine	1.66	11958
Cytidine	7.43	13553
Ibuprofene	0.56	9136
Flurbiprofen	0.73	9667
4-OH-Phenyl acetic acid	6.9	13661
Theophylline	0.42	9120
Theobromine	0.44	9153
Phenylephrine	6.5	13304
Nicotinic acid	7.58	13496
Thiamine	14.98	11242
α-Methyl-tryptophane	10.79	11999
Tryptophane	13.37	11198

Table 32: Evaluation data of the investigation of retention mode for a commercial zwitterionic HILIC column, EC 150/4,6 mm Nucleodur® 100-3. Three analytes were compared. Mobile phase was a mixture of ACN/H_20 with 10 mM NH_4AcO pH 5,0, where the ratio of ACN/buffer was altered in a range of 5-95 % H_20.

NUCLEOSID® - Zwitterionic HILIC column						
	Retention factor k			**log(k)**		
φ H2O	**α-Methyl-tryptophane**	**4-OH-phe-AcOH**	**Deoxyadenosine**	**α-Methyl-tryptophane**	**4-OH-phe-AcOH**	**Deoxyadenosine**
0.05	18.44	16.53	2.63	1.27	1.22	0.42
0.10	10.22	5.17	1.48	1.01	0.71	0.17
0.14	3.41	2.24	0.97	0.53	0.35	-0.01
0.23	1.13	0.77	0.50	0.05	-0.11	-0.30
0.32	0.57	0.34	0.23	-0.24	-0.47	-0.64
0.41	0.32	0.14	0.13	-0.50	-0.87	-0.90
0.50	0.19	0.04	0.04	-0.71	-1.37	-1.37
0.59	0.13	0.00	0.00	-0.88	\	\
0.68	0.12	-0.02	0.00	-0.93	\	\
0.77	0.12	0.02	0.00	-0.91	-1.76	\
0.86	0.21	0.08	0.00	-0.68	-1.12	\
0.91	0.23	0.07	0.00	-0.63	-1.15	\
0.95	0.32	0.11	0.00	-0.50	-0.96	\

Table 33: Comparison of data for the evaluation of retention mode for two columns, QN-SB-V2 and tBuCQD. Three representative analytes were chosen. The mobile phase was a mixture of ACN/H_20 with 10 mM NH_4AcO pH 5,0, where the ratio of ACN/buffer was altered in a range of 5-95 % H_20.

QN-SB-V2							tBuCQD						
	k			log(k)				k			log(k)		
φ H2O	α-Methyl-tryptophane	4-OH-phe-AcOH	Deoxyadenosine	α-Methyl-tryptophane	4-OH-phenyl acetic acid	Deoxyadenosine	φ H2O	α-Methyl-tryptophane	4-OH-phe-AcOH	Deoxyadenosine	α-Methyl-tryptophane	4-OH-phe-AcOH	Deoxyadenosine
0.95	\	\	\	\	\	\	**0.95**	4.72	17.00	3.35	0.67	1.23	0.53
0.91	3.33	2.99	3.57	0.52	0.48	0.55	**0.91**	3.72	15.00	2.40	0.57	1.18	0.38
0.86	2.62	2.47	2.47	0.42	0.39	0.39	**0.86**	2.94	\	1.83	0.47	\	0.26
0.77	1.82	1.80	1.55	0.26	0.26	0.19	**0.77**	1.92	\	1.13	0.28	\	0.05
0.68	1.45	1.34	1.25	0.16	0.13	0.10	**0.68**	1.40	24.00	0.96	0.15	1.38	-0.02
0.59	1.30	1.20	1.10	0.11	0.08	0.04	**0.59**	1.15	22.00	0.99	0.06	1.34	0.00
0.50	1.27	1.16	1.14	0.10	0.06	0.06	**0.50**	1.05	13.30	0.86	0.02	1.12	-0.07
0.41	1.36	1.22	1.23	0.13	0.09	0.09	**0.41**	1.05	10.50	0.87	0.02	1.02	-0.06
0.32	1.59	1.39	1.40	0.20	0.14	0.15	**0.32**	1.15	8.66	0.94	0.06	0.94	-0.03
0.23	2.16	1.81	1.74	0.33	0.26	0.24	**0.23**	1.83	7.57	1.06	0.26	0.88	0.03
0.14	3.60	2.87	2.41	0.56	0.46	0.38	**0.14**	2.20	7.40	1.29	0.34	0.87	0.11
0.10	5.72	4.12	3.10	0.76	0.61	0.49	**0.10**	4.07	9.62	1.53	0.61	0.98	0.18
0.05	\	\	\	\	\	\	**0.05**	\	6.50	2.02		0.81	0.31

7.2 Summary

The enantioselective separation of fluorescent labelled amino acids and peptides was investigated. A set of amino acids, all proteinogenic, many non-proteinogenic and dipeptides of biological, pharmaceutical or chemical interest were selected for the investigation. *tert*-Butyl carbamate-modified quinine/quinidine based chiral stationary phases (CSPs) were used for enantioseparations. Most of the analytes were N-terminal labelled with 6-aminoquinolyl-succinimidyl carbamate (AQC), a common fluorescent tag for quantitative amino acid analysis. As an easy derivatization procedure allowing sensitive quantification, AQC was often used in chiral separation studies. A comparison with fluoresceine-isothiocyanate (FITC) was conducted in the present study, concerning their chromatographic performance on an anion-exchanger-type CSP. FITC was found to yield equal chiral separation potential compared to AQC. Besides the proteinogenic amino acids, the separation of AQC-tagged stereoisomers of 4-hydroxyproline, threonine and isoleucine, of dipeptides like phenyalaninoylglycine and glycoylphenylalanine or prolinoylglycine and glycoylproline was investigated. Further, the separation performance of the AQC reagent was explored using a set of alanine peptides. All-D/all-L peptides up to the hexamer were separated. The investigation of the influence on chiral recognition of one or two non-chiral linkers in di- and tripeptides, namely glycine subunits, either N- or C-terminal located was also part of this study. Enantiomerically pure phenylalanine peptides up to pentamers were also separated. These very lipophilic compounds were known to exhibit an unfavourable character related to chiral separations. The separation of stereoisomers of tri- and tetrapeptides of the before mentioned alanine and phenylalanine peptides was also investigated. The performance of the *tert*-butyl carbamate modified quinine/quinidine based CSPs was explored, as well as the influence of the stereochemistry on the N- or C-terminus of peptides on chiral recognition.

The second part of the master thesis was the synthesis and application of sulfobetaine-type quinine-based chiral stationary phases. A set of native or derivatized amino acids was analyzed with different types of mobile phase. It was found that the alkylation of the nitrogen atoms, either on the quinuclidine or the quinoline ring, had led to strongly reduced or even diminished chiral discrimination behaviour of the quinine selector. The sulfobetaine-type columns were also investigated concerning their applicability in the hydrophilic interaction liquid chromatography (HILIC) mode. A set of typical HILIC compounds, polar and/or charged substances were separated using mobile phases with different compositions and adjusted pH values. The retention mechanism of the columns was found to be a mixed mode, containing hydrophobic and hydrophilic retention increments. The study also showed that the *tert*-butyl carbamate quinine-based CSPs has also a hydrophilic retention increment. It was shown that the synthesized sulfobetaine columns can be used as HILIC columns for the separation of highly polar substances.

The present master thesis showed the applicability of a fluorescent tag, based on a carbamate linkage group, for the enantioselective separation of amino acids and related compounds on a quinine-based anion exchange-type chiral stationary phase.

7.3 Zusammenfassung

Im Zuge der Masterarbeit wurde die Enantiomerentrennung von Aminosäuren (AS) unter Verwendung von auf Chinin/Chinidin-basierenden chiralen stationären Phasen untersucht. Die AS wurden N-terminal funktionalisiert, um auf einer Anionenaustauschersäule retardiert zu werden. Dafür wurde ein weit verbreiteter Fluoreszenztag, 6-Aminoquinolin succinimid carbamate (AQC), verwendet. Demgegenüber wurde ein weiteres Label, Fluoresceinisothiocyanat (FITC), eingesetzt und die Ergebnisse bezüglich ihrer chromatographischen Leistung, wie Enantioselektivität, Auflösung und Retentionsverhalten, verglichen. AQC wurde über eine Harnstoffgruppe und FITC über eine Thioharnstoffgruppe an ein primäres oder sekundäres Amin des Analyten gebunden. Diese Arten von chemischen Brückengruppen wurde bislang noch nicht in Kombination mit Chinin/Chinidin basierenden chiralen stationären Phasen zur enantioselektiven Trennung untersucht. Die verwendeten Substanzen, Aminosäuren, sind mit Ausnahme von Glycin von Natur aus chiral, und kommen beinahe ausschließlich in der (S)- bzw. L-Form vor. Die Spanne von Analyten umfasste die proteinogenen AS, sowie eine Reihe von nicht proteinogenen AS, wie Aminophosphonsäuren, Phenylalaninderivaten- bzw deren Analoga, Sulfon,- als auch Phosphonsäuren, Dipeptide, Phenylalanine- und Alaninpeptide. Die chirale Trennung wurde über Hochleistungsflüssigchromatographie (HPLC) durchgeführt. Der chirale Selektor, Chinin/Chinidine, wurde an der C9 Position mit einer *tert*-Butylcarbamatgruppe versehen. Diese Modifikation führte zu einer sehr hohen Enantioselektivität gegenüber chiralen Molekülen. Betreffend der Wahl eines N-terminalen AS-tags kann das AQC Reagenz als sehr brauchbar eingestuft werden. Mit Ausnahme von Asparagrinsäure, wurden alle proteinogenen AS aufgetrennt. Spezielle Trennungsprobleme, wie die Separation von Stereoisomeren, wurden untersucht. So konnten alle vier Stereoisomere des 4-Hydroxyprolins basislinien rein getrennt werden. Die Threonin Stereoisomere, Threonine, allo-Threonin und Homoserin, wurden aufgelöst, mit der Ausnahme von den ersten beiden eluierenden Enantiomeren, welche kaum retardiert wurden und als eine Analytbande eluierten. Isoleucin Stereoisomere wurden dagegen viel schlechter aufgelöst. Dies kann darauf zurück geführt werden, dass das zweite Stereozentrum in der hydrophoben Seitenkette lokalisiert ist. Die hydrophoben Wechselwirkungen mit dem chiralen Selektor haben aber keinen Anteil an der chiralen Erkennung. Dem zugrunde liegend kann erklärt werden wieso für Isoleucin keine Basislinientrennung bewerkstelligt werden konnte. Schwer zu trennende Dipeptide wie Glycylprolin und Glycylphenylalanine konnten basislinienrein aufgelöst werden. Die Untersuchung von Alaninpeptiden sollte weiteren Aufschluss über die Leistungskraft der AS-label geben und auch über den chiralen Erkennungmechanismus weitere Erkenntnisse liefern. Die erzielten Ergebnisse ergänzen frühere Untersuchungen zu Alaninpeptiden und vervollständigen das erarbeitete Konzept der chiralen Erkennung von Peptiden mit mehreren Stereozentren. Die Abhängigkeit der Enantiomerentrennungen bezüglich des Abstandes der Schutzgruppe zu dem chiralen Zentrum wurden untersucht, ebenso der Einfuss einer achiralen Brücke, wie der eines Glycinrestes in solch einem Peptid. Die Auftrennung der

Phenylalaninpeptide war ein weiterer Leistungstest für das angewandte Label. Aufgrund von durchgehend erzielten Basislinientrennungen, mit Ausnahme einiger Stereoisomer der Tri- und Tetrapeptiden, konnte dieser Derivatisierungsreagenz große Komplementariät zu dem *tert*-butyl-carbamate Chinineselektor zugesprochen werden. Generell stellt die AQC Gruppe, aufgrund von guten Trennleistungen, eine Erweiterung der möglichen N-terminalen Schutzgruppen dar. FITC konnte ebenfalls alle proteinogenen Aminoäuren derivatisieren und die Enantiomere wurden mit ähnlicher Trennleistung wie für die AQC-Derivate aufgetrennt. Die Enantioselekitivität war durschnittlich etwas geringer als bei AQC. Aufgrund von der weitaus kostengünstigeren Verfügbarkeit von FITC, war auch diese fluoreszenz aktive Gruppe sehr attraktiv.

Ein weiterer wesentlicher Bestandteil dieser Masterarbeit war die Synthese und Charakterisierung von auf Chinin basierenden Sulfobetain modifizierten chiralen Selektoren. Drei chirale stationäre Phasen dieses Typus wurden mittels einer Alkylierung unter Verwendung eines Propylsultons hergestellt.
Die Alkylierung am Quinucelodinring des nativen Chinins führte zum Selektor der Säule CSP 3. Die gleiche Modifikation wurde an einem *tert*-butyl carbamate modifizierten Chininanalogon durchgeführt und führte zu CSP 4. Die Alkylierung am Quinolinring des *tert*-butyl carbamate Analogons ergab CSP 5. Die Säulen wurden auf ihre Fähigkeit zur Enantiomerentrennung getested. Es wurde festgestellt, das die Modifikation an einem der beiden enthaltenen Stickstoffatome unter Einführung einer Sulfonsäuregruppe die Fähigkeit zur Enantiodiskriminierung stark verringert, beziehungsweise völlig auslöscht. Nur für einige Analyte konnte eine Antrennung der Enantiomere erzielt werden. Grundsätzlich kann der Ort der Modifkation, als auch die eingeführte Gruppe dafür verantwortlich gemacht werden. Die Sulfonsäuregruppe und das quaternäre Amin stellen intramolekulare Gegenionen dar und ihre Ladungen können sich gegenseitig abdecken.
Die Säulen wurden daraufhin auf ihre Anwendbarkeit in den hydrophilen Interaktionsmodus (HILIC) untersucht. Generell wurden dem Selektormolekül zwei Ladungen zugefügt, wodurch die Polarität des Gesamtmoleküls wesentlich gesteigert wurde. In Bezug auf die Anwendung als HILIC Säulen konnte ermittelt werden, dass eine grundsätzliche Retention von polaren und geladenen Analyten, Nukleoside, Nukleotide, Alkaloide, Säuren, Basen und amphotere Verbindungen, stattfindet. Die Selektivität bezüglich Nukleosiden und Nukleotiden war begrenzt, wogegen geladene und zwitterionische Verbindugen mit hoher Selektivität als auch Effizienz getrennt wurden.
Die Modifikation an den Stickstoffatomen des Chinins, unter Einführung einer zweiten starken Ionenaustauschgruppe, führte zu einer Verminderung des chiralen Erkennungssystems. Diese Modifizerung des Chinins war daher nicht zielführend um die chirale Erkennung zu verändern oder zu verstärken. Als HILIC Säulen konnten die Sulfobetainphasen Verwendung finden. Eine höhere Selektorbeladung, sowie die Einführung einer zweite Alkylierung mit einem Akylsulfon, oder einer anderen, mehr HILIC taugliche Gruppe am C9-Atom des Chinins könnte zu mehr Effizienz der Auftrennung von HILIC Verbindungen führen. Die Retention von Analyten auf CSP 3 wurde durch

hydrophobe als auch hydrophile Inkremente hervorgerufen. Es wurde auch hydrophiles Retentionsinkrement des *tert*-butyl carbamat Chininselektors festgestellt, das zu moderaten Retentionen von hydrophilen Substanzen im HILIC Modus führte.

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