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„Zwitterionic Chiral Stationary Phases for HPLC based on Taurine-Homologues and Cinchona Alkaloids - Synthesis and Evaluation“

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List of Abbreviations

AA	amino acid
AcOEt	ethyl acetate
AE	active (activated) ester
AGP	α_1-acid glycoprotein
AIBN	azobis(isoburyro)nitrile (2,2′-Azobis(2-methylpropionitrile))
Ala	alanine
APOC	1-(9-anthyryl)-2-propyl chloroformate
Asp	aspartic acid
AMSA	aminomethanesulfonic acid
(W)AX	(weak) anion exchanger
BSA	N,O-bis(trimethylsilyl) acetamide
CAD	charged aerosol detector
CD	cyclodextrin
CDA	chiral derivatising agent
CE	capillary electrophoresis
CEC	capillary electrochromatography
CSP	chiral stationary phase
DAD	diode array detector
DCM	dichloromethane
DEA	diethylamine
EO	elution order
EtOH	ethanol
FA	formic acid
FDAA	1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey´s reagent)
FLEC	1-(9-fluorenyl)ethyl chloroformate
fig	figure
GC	gas chromatography
Glu	glutamic acid
hhtau	homohomotaurine (4-aminobutanes. acid)
HPLC	high performance liquid chromatography
hr(s)	hour(s)
HSA	human serum albumine
htau	homotaurine (3-aminopropanesulfonic acid)
IMCI	intramolecular counterion
MeOH	methanol
min	minute(s)
MIP	molecularly imprinted polymer
MW	molecular weight
MWD	multiple wavelength detector
MS	mass spectrometry
N	(theoretical) plate number (chromatography)
NEt_3	triethylamine
n.d.	not determined/not determinable
OPA	*ortho*-phthaldialdehyde

Phe	phenylalanine
pI	isoelectric point
QN	quinine
QD	quinidine
RBF	round-bottomed flask
RP	reversed phase (chromatography)
r.t.	room temperature
s	second
SA	selectand (analyte in chiral separations)
(S)CX	(strong) cation exchanger
Ser	serine
SO	selector
(*S*)-TBMB-COOH	2-*tert*-butyl-2-methyl-1,3-benzoxadiazole-4-carboxylic acid
(*S*)-MNB-COOH	2-β-naphthyl-2-methyl-1,3-benzoxadiazole-4-carboxylic acid
(*S*)-NIFE	(S)-N-(4-nitrophenoxycarbonyl)-Phe methoxy ethyl ester
tau	taurine (2-aminoethanesulfonic acid)
TAG	Teicoplanin Aglycone (CSP)
TLC	thin layer chromatography
TMCS	trimethylchlorosilane
Trp	tryptophane
V	Vancomycin (CSP)
Val	valine
WAX	weak anion exchanger
ZWIX	zwitterionic ion exchanger

Introduction

1.) The Importance of Separating Enantiomers

Chiral compounds – molecules that have no internal symmetry plane or centers of inversion and are non-superimposable on their mirror images - constitute a large part of modern drugs:

According to Caner, more than half of all drugs approved by the FDA in 1991-2002 are chiral and are administered as racemates or single enantiomers,[1] with the latter gaining importance rapidly.[2]

Nowadays, it is well known that the enantiomers of a chiral compound can differ dramatically in their respective effects on the human body.

Usually one enantiomer (the eutomer) of a chiral compound has the desired therapeutic effect, while the other (the distomer) can have no effect at all, a therapeutic effect smaller or in other ways different from that of the eutomer, or even an undesired effect. A prominent example of the latter is the chiral drug Thalidomide (Figure 1).

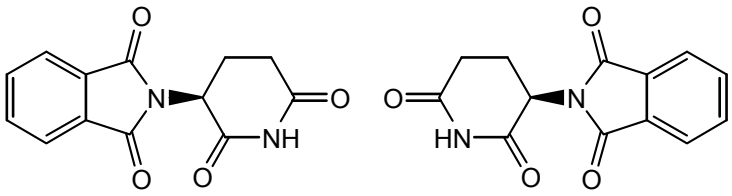


Fig.1: Enantiomers of Thalidomide. The R enantiomer has a sedating effect, while the S enantiomer causes severe deformities in unborn children.

Its racemic mixture enantiomer was administered to pregnant women in the 1950s and 1960s as a mild sedative and a remedy against morning sickness. Even if only the *R* enantiomer had been used, the tragedy would not have been prevented because, unfortunately, inside the human body it can be converted into the *S* enantiomer which has severe teratogenic effects. As a result, more than 10 000 babies in 46 countries were born with serious physical deformities.[3]

Nevertheless, drugs are frequently administered as racemates or mixtures of stereoisomers, especially if no appropriate asymmetric synthesis protocol is available and enantiomer separation is unreasonably labour-intensive or expensive.

In those cases, regulatory laws require that the manufacturer determine the enantiomeric composition of the product (enantiomeric excess levels of > 99,9 %) . Furthermore, the distomer must be proven to be non-hazardous.
It is obvious that to fulfil these requirements, enantiomerically pure samples of both enantiomers are necessary. This is when enantioselective analysis techniques (also termed chiral separation methods) are called for.

Since the 1990s, rigorous regulatory demands have further enhanced the need for reproducible, efficient and highly selective enantiomer separation methods.
Scientists dealing with the development of such techniques had to face a challenging task:
As two enantiomers of a chiral compound consist of exactly the same atoms, bonded in the same way and with the same interatomic distances, their *scalar* physical properties such as molecular weights, melting points and internal energies are exactly alike. This makes them indistinguishable with “classical” achiral detectors such as UV, fluorescence or MS.
The only way to differentiate between enantiomers is to create a chiral environment, meaning interaction sites where the analyte can get in contact with another chiral compound. Besides, circularly polarised light can be used to create optical rotation whose orientation is different for the two enantiomers of a chiral molecule. Thus, it can be seen as a chiral source and environment.

2.) Enantiomer Separation Methods

In stereochemical analysis it is often required to transform enantiomers (the selectands, SA) by using chiral selectors (SO) to create diastereomeric complexes which can be separated and detected with conventional methods (Figure 2).[4]

$$(R)-SO+(S)-SA \xleftrightarrow{K_S} \left[(R)-SO-(S)-SA)\right]$$
$$(R)-SO+(R)-SA \xleftrightarrow{K_R} \left[(R)-SO-(R)-SA)\right]$$

Fig. 2: Formation of diastereomeric complexes from an enantiomerically pure selector ((*R*) –SO) and enantiomers of a selectand ((*S*)-SA, (*R*)-SA).

The formation of diastereomeric entities can be covalent or due to number of different non-covalent interaction forces.
A large number of chiral separation techniques have been developed to separate the enantiomers of diverse groups of chemical compounds such as amines, alcohols, acids and bases.

The methods are classified primarily as “direct” or “indirect” separation techniques, according to the type of diastereomeric complexes that are formed.

2.1) The Indirect Approach

In the course of indirect chiral separations, the selectand enantiomers are converted into covalently bonded SO-SA diastereomers with an enantiomerically pure auxiliary (chiral derivatising agent, CDA). The resulting molecules are diastereomers of each other and can be separated relatively easily, for example with conventional achiral chromatographic methods such as RP-HPLC.

Indirect separation techniques have a long-standing tradition and therefore, chiral derivatising agents for all kinds of derivatisable functional groups are available.[5]
They must fulfil certain requirements concerning their chemical and physical stability. Besides, they should be commercially available with very high enantiomeric purity, preferably in both enantiomeric forms to enable the reversal of elution order. They should contain appropriate functional groups to facilitate the chosen separation method (e.g. hydrophobic groups for RP-HPLC) and chromophoric or fluorophoric groups for easy detection.

Figure 3 shows two commercially available chiral derivatising agents and their applications.

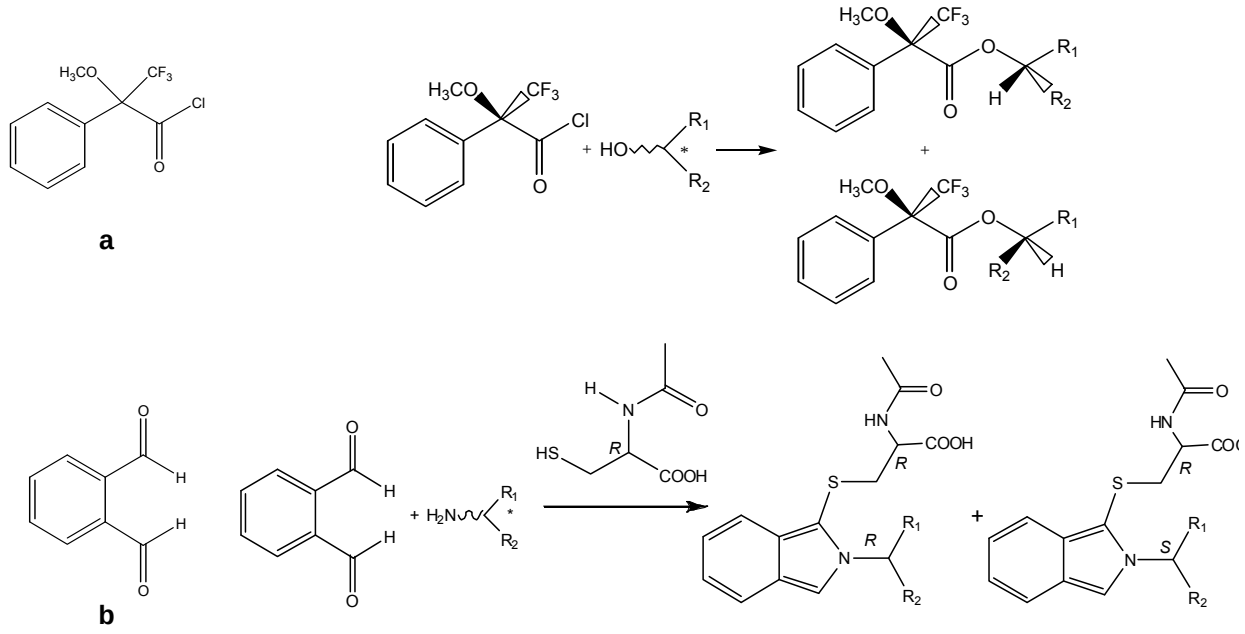


Fig. 3: Chiral derivatising agents (CDAs) **a** Mosher´s reagent for amines and alcohols **b** OPA (ortho-phthaldialdehyde) with chiral thiol for primary amines and amino acids

However, there are also several drawbacks to the indirect approach, e.g. the required existence of derivatisable functional groups (hydroxyl, amine, carbonyl, etc.) in the selectand.

The derivatising agent must be of particularly high enantiomeric purity, otherwise a total of 4 stereoisomers will be formed, of which two are enantiomers of the other two. This means that they would not be separable with classical methods and thus, the analytical results would be compromised.

If the reaction rates of the enantiomers for the derivatisation reaction are different, kinetic racemate solution can occur. Therefore, it is of utmost importance to use a CDA that allows reasonably fast conversion and to wait for the completion of the reaction.

The detector response might be different for the diastereomers, which can lead to problems with quantitative analyses.[6]

Due to these restrictions, indirect chiral separation methods are of minor importance compared to direct ones.

2.2) The Direct Approach

A reversible formation of diastereomeric molecule associates based on non-covalent interactions is the principle of the direct approach to the separation of enantiomers.
Again, the selectand enantiomers are brought into contact with a chiral auxiliary, the selector (SO). The reaction partners can interact in different ways according to their functional groups and their stereochemistries, which will lead to the discrimination of one enantiomer.
Chiral recognition is based on electrostatic (ionic or dipole-dipole) interactions, hydrogen bonds, hydrophobic interactions or π–π interactions between the selector and the enantiomers of the selectand. Frequently, a combination of some or all of the interactions mentioned is responsible for the separation.

As indicated above, the enantiomers of a chiral compound will interact differently with a chiral selector due to their stereochemical properties.
The 3-point interaction model created by K. Dalgliesh is most suitable to illustrate enantiodiscrimination (Figure 4).
The spatial arrangement of the molecule on the left matches the interaction sites A´, B´and C´ of the selector, whereas its enantiomer on the right cannot interact as effectively with the selector. The molecule associates that are formed differ in their stability and thus, enantiodiscrimination is achieved.

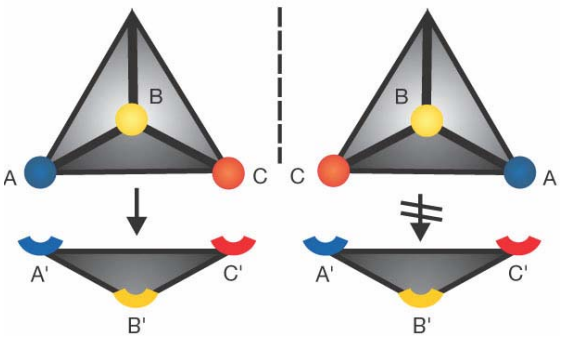


Fig. 4: 3-point interaction model. Left: The molecule matches the active sites of the selector. Right: Its enantiomer does not match the selector and is therefore discriminated. [7]

Even though some GC applications exist, the direct approach to enantiomer separation is most often realised in liquid chromatographic methods, such as chiral HPLC.
The selector can either be added to the mobile phase (chiral mobile phase mode, CMP) or it can be covalently liked to or otherwise integrated into the stationary phase (chiral

stationary phase mode, CSP). The latter is by far the most used chiral separation technique today, with more than 100 chiral stationary phases for all kinds of analytes commercially available from a number of suppliers. [4]

Figure 5 provides an overview of modern chiral stationary phases.

Type of selector	Examples	Analytes
Polysaccharides	cellulose amylose chemically modified polysaccharides	acidic, basic, neutral broadest applicability!
Synthetic polymers	poly(triphenylmethacrylate)	
Proteins	HSA AGP ovomucoid	acidic broad applicability
Cyclodextrines	α-CD β-CD γ-CD chemically modified CDs	smaller hydrophobic analytes substituted phenyl, naphthyl, heteroaromatic rings steroids
Macrocyclic antibiotics	vancomycin teicoplanine ristocetin A aglycones	amines amino acids, peptides bases
Chiral crown ethers	aryl and tartaric acid-derived crown ethers	1° and 2° amines

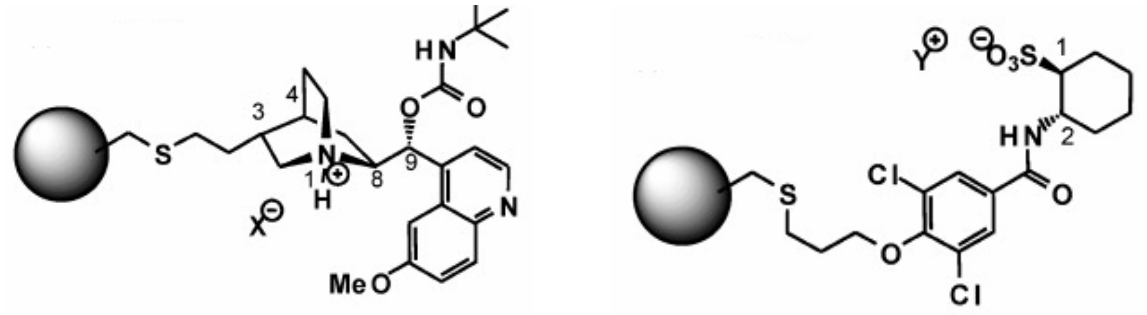
Fig. 5: A selection of modern chiral stationary phases and their applications

Type of selector	Examples	Analytes
Donor-acceptor phases (brush-type or Pirkle-type phases)	Whelk-O1™ (4-(3,5-dinitrobanzamido)-1,2,3,4-tetrahydrophenanthrene)	Naproxen, pharmaceuticals
	ULMO™ (11-[2-(3,5-dinitrobenzamido)-1,2-diphenylethylamino]-11-oxoundecyl-silica[8]	aryl carbinols and many other compounds
Chiral ion exchangers	cinchona alkaloid derivatives	chiral acids
	carboxylic and sulfonic acid derivatives	chiral bases

Fig. 5 (continued): A selection of modern chiral stationary phases and their applications

3.) Separation of Amino Acids and Peptides

The separation of chiral acids and bases can be achieved most elegantly with chiral ion exchangers based, for example, on cinchona alkaloids and sulfonic acids, respectively (Figure 6).[9]



a **b**

Fig. 6: a Anion exchange CSP based on cinchona alkaloid quinine for the enantiomer separation of chiral acids X **b** cation exchange CSP based on a chiral sulfonic acid for the separation of chiral bases Y.

But what do chemists do when the analyte that they want to separate into its enantiomers has both acidic and basic functional groups, as is the case with several drugs, amino acids and peptides?

The latter two are essential ingredients of life: 22 so-called proteinogenic amino acids consisting of a carboxyl function, an α-amino group and a variable side chain (Figure 7) are the main building blocks of the proteins in our bodies. Interestingly, they all are α-amino acids and they all have L-configuration (except for glycine, which is achiral).

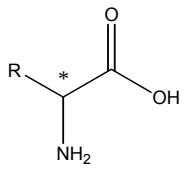


Fig. 7: α-amino acid structure. The asterisk marks the α position which is asymmetric in most amino acids.

Apart from the proteinogenic amino acids, a number of other amino acids are to be found in plants and microorganisms. These amino acids can have D configuration (often found in bacteria cell walls) or an amino group in β or γ position.

Proteinogenic amino acids can be classified by the properties of their side chains: [10]

aliphatic amino acids	glycine (Gly), alanine (Ala), Valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro)
aliphatic amino acids containing sulphur	methionine (Met), Cysteine (Cys)
aromatic amino acids	phenylalanine (Phe), tyrosine (Tyr), tryptophane (Trp)
polar uncharged amino acids	serine (Ser), threonine (Thr), asparagine (Asn), glutamine (Gln)
basic amino acids (positively charged)	lysine (Lys), arginine (Arg), histidine (His)
acidic amino acids (negatively charged)	aspartic acid (Asp), glutamic acid (Glu)

Amino acids can undergo condensation reactions involving the carboxyl function of one and the amino group of another to form a peptide (amide) bond (Figure 8):

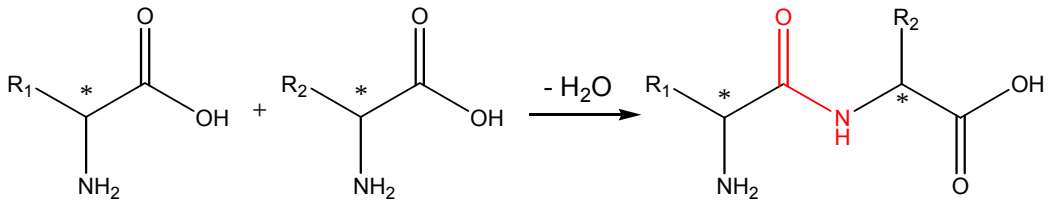


Fig. 8: Formation of a peptide bond between amino acids.

By condensation of 2 or more amino acids, peptides (2-100 amino acid residues) and proteins (> 100 amino acid residues) are formed.

Chiral separation techniques for amino acids and peptides are required to determine the enantiomeric purities of synthetic peptides and other asymmetric synthesis products. Under acidic or basic conditions, racemisation may occur. These processes, too, must be monitored by enantioselective analysis since D and L enantiomers of amino acids can differ in their biological activities and sensoric properties. For example, many L amino acids taste bitter, while their D enantiomers have a sweet taste. [11]

Due to the presence of both acidic (the carboxylic function) and basic (the amino group) moieties in amino acids and, therefore, peptides and proteins, these molecules are amphoteric. Depending on the pH of the surrounding medium, they can exist as acids or bases, but most of the time the carboxylic function will be deprotonated and the amino function will be protonated. Thus, they will be outwardly neutral despite the presence of both positive and negative charges in the molecule. Such a compound is called zwitterionic.

The challenge of the enantioseparation of zwitterionic analytes is that it cannot be achieved by a conventional chiral anion or cation exchanger. The second charge present in the analyte is of the same name as the ion exchanger and therefore prevents retention. To be precise, in ion exchange chromatography repulsion between charges of the same name leads to elution before the void volume marker.

However, several other methods for the separation of amino acid and peptide enantiomers have been developed. The discussion will be limited to chromatographic applications as they represent the most successful approach to chiral separations.

3.1) Indirect Separation Methods for Amino Acids and Peptides

Indirect enantioseparation aims at the conversion of enantiomers into diastereomeric molecules which are separable on an achiral stationary phase (see 2.1).

The indirect approach to enantioseparation of amino acids is relatively popular due to a number of advantages[12]: Suitable chiral derivatising agents are available and the choice of chromatographic conditions to separate the resulting diastereomers ranges from RP to NP and GC applications. Besides, achiral columns can be used.
Often both enantiomeric forms of a CDA can be purchased, which allows the reversal of elution orders – this can be of special interest if enantiomeric impurities (e.g. the D enantiomer of an amino acid) are to be determined – in chromatography, the impurity (usually the smaller peak) should be eluted from the column before the main analyte.
Predicting elution orders is often easier when using indirect methods. Generally, method development may be achieved more quickly than with chiral stationary phases which often require laborious mobile phase optimisation.

In addition to the drawbacks that have been discussed briefly in section 2.1., it should be mentioned here that the hydrolysis of peptides, as it is done to assess their chiral amino acid composition, can lead to racemisation (3.3) which might compromise the outcome of the analysis.

For indirect enantioseparation, amino acids are most often converted into amides, carbamates, ureas and thioureas. Acid chlorides, chloroformates isocyanates and isothiocyanates are frequently used as CDAs.
Figure 9 provides examples for the chiral derivatisation of proteinogenic amino acids.

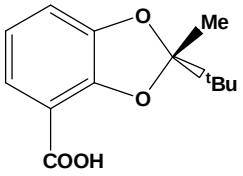
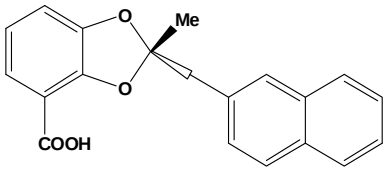
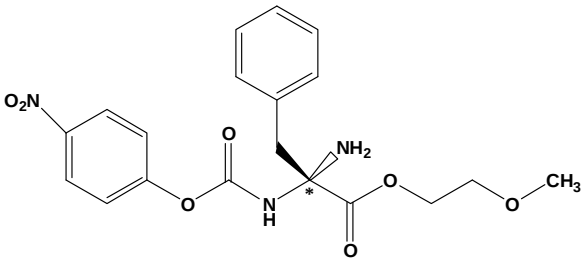
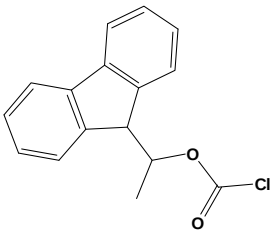
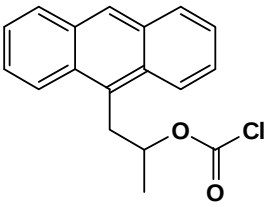
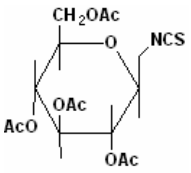
	chiral derivatising agent	CDA structure	Ref.
activated carboxylic acids	(S)-TBMB-COOH		13
	(S)-MNB-COOH		
	(*S*)-NIFE		14
chloroformates	FLEC		15
iso(thio)cyanates	APOC		16
	GITC		17

Fig. 9: Chiral derivatising agents for amino acids

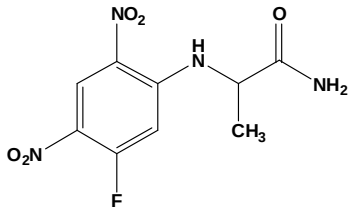
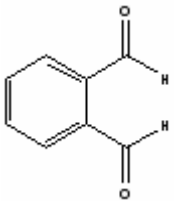
chiral derivatising agent		CDA structure	Ref.
N-haloarylamino acid derivatives	FDAA (Marfey´s reagent)		[12]
chiral thiol reagents	OPA + chiral thiol		[18], Fig. 3b

Fig. 9 (continued): Chiral derivatising agents for amino acids

It was mentioned before that it constitutes one of the major advantages of the indirect enantioseparation methods that by derivatising them, the scientist can introduce functional groups which facilitate sensitive detection (a concept widely known as “labelling”). Therefore, many of the CDAs presented in Fig. 8 have aromatic substituents to enhance UV or fluorescence detection.

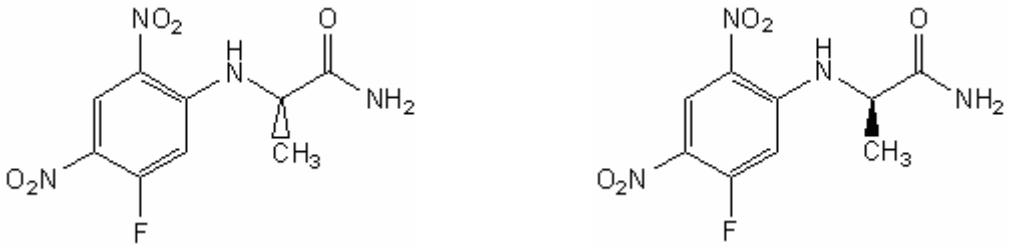
The CDAs based on carboxylic acids usually need to be activated by conversion into their acid chlorides which then react with the amino acid analytes to form diastereomers.
The chloroformate reagent FLEC is suitable for the chiral separation of primary and secondary amino acids and can also be used for chiral α-hydroxy acids. [19]

Derivatisation of chiral amino acids with chiral isocyanantes and isothiocyanates to give diastereomeric carbamates and thiourea derivatives is not carried out very often due to their requiring relatively high temperatures and longer reaction times than other CDAs.[18]

Marfey´s reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide), widely used for the *N*-derivatisation of amino acids, is a chiral derivative of 1-fluoro-2,4-dinitrobenzene. In addition to high yields of the resulting diastereomers, the application of Marfey´s reagent leads to products that are easily detectable with UV.

After derivatisation with FDAA (L enantiomer) and separation of the resulting diastereomers on RP-HPLC, the original L amino acid is often eluted prior to its D enantiomer.

To enhance selectivity and to improve the predictabilities of the elution order, several variants of Marfey´s reagent were created by replacing Ala with other chiral entities such as valine or leucine (Figure 10)[20]:



FDAA: R = CH_3 D-FDAA

FDVA: R = $CH(CH_3)_2$
FDPA: R = CH_2-Ph
FDIA: R = $CH(CH_3)CH_2CH_3$
FDLA: R = $CH2CH(CH_3)_2$

Fig. 10: Variations of Marfey´s reagent (adapted from Ref. [20])

Scientists also modified the aromatic group and replaced it with other chromophoric substituents.

OPA and chiral thiols (*N*-acetyl-D-penicillamine, *N*-protected cysteine) are routinely used to derivatise chiral primary amino acids to yield highly fluorescent diastereomeric isoindol derivatives.

Indirect enantioseparation of chiral amino acids can also be achieved by gas chromatography. Due to its good separation performance, GC is a particularly interesting tool for analytical chemists. However, in order to analyse polar compounds such as amino acids by GC, they need to be derivatised to become less polar and more volatile. Therefore, several methods for derivatisation of amino acids to give volatile diastereomers have been reported, e.g. in 2008 by Bertrand et al.[21]. It is based on ethyl chloroformate and 2-chloropropanol and the resulting diastereomers can be separated on an achiral gas chromatography column.

3.2) Direct Separation of Amino Acids and Peptides

The direct approach to enantioseparation of amino acids and peptides is usually realised in a separation of underivatised compounds on chiral stationary phases (3.2.1).
However, in order to perform chiral GC or to separate the enantiomers of originally zwitterionic (amphoteric) compounds on chiral anion or cation exchangers, derivatisation with an achiral reagent may be required (3.2.2 and 3.2.3)

3.2.1) Chiral Liquid Chromatography

As indicated in section 2.2, the separation of enantiomers with chiral stationary phases is by far the most commonly used chiral separation technique today.
Chiral stationary phases for liquid chromatography may have the selector immobilised onto the silica surface (mostly employing a covalent linkage) or the stationary phase itself may be chiral (chiral monolithic columns). Sometimes, chiral molecularly imprinted polymers are employed.

a) CSPs Based on Macrocyclic Antibiotics

Macrocyclic glycopeptide antibiotics were first introduced by Armstrong in 1994.[22] The most important chiral selectors based on macrocyclic antibiotics are vancomycin, teicoplanin and ristocetin A (Figure 11), which are fermentation products of fungi. Their biological activity is based on their affinity towards the D-Ala-D-Ala moieties of the cell walls of gram-positive bacteria.[23]
Other antibiotics-based CSPs are based on ansamycines or aminoglycosides, although they are of far less importance than those mentioned above.

Due to their structures, which feature a hydrophobic basket of macrocyclic rings linked by peptide and ether bonds and 2-6 carbohydrate moieties, glycopeptide antibiotics offer various possible interactions with the selectand: ionic, H-bond, $\pi-\pi$ interactions, dipole-dipole, hydrophobic and steric interactions contribute to retention and enantioselectivity.
Vancomycin, teicoplanin and ristocetin A are commercially available as Chirobiotic™ CSPs with various binding chemistries. Today, their application for chiral separations is

common in many fields such as drug discovery or organic synthesis. They are the method of first choice when enantiomers of both free and N-protected amino acids and peptides are to be separated.

For example, Berthod et al. reported the separation of 54 free amino acids (among them all proteinogenic amino acids) on a teicoplanin-based CSP employing an aqueous-organic mobile phase. [24]

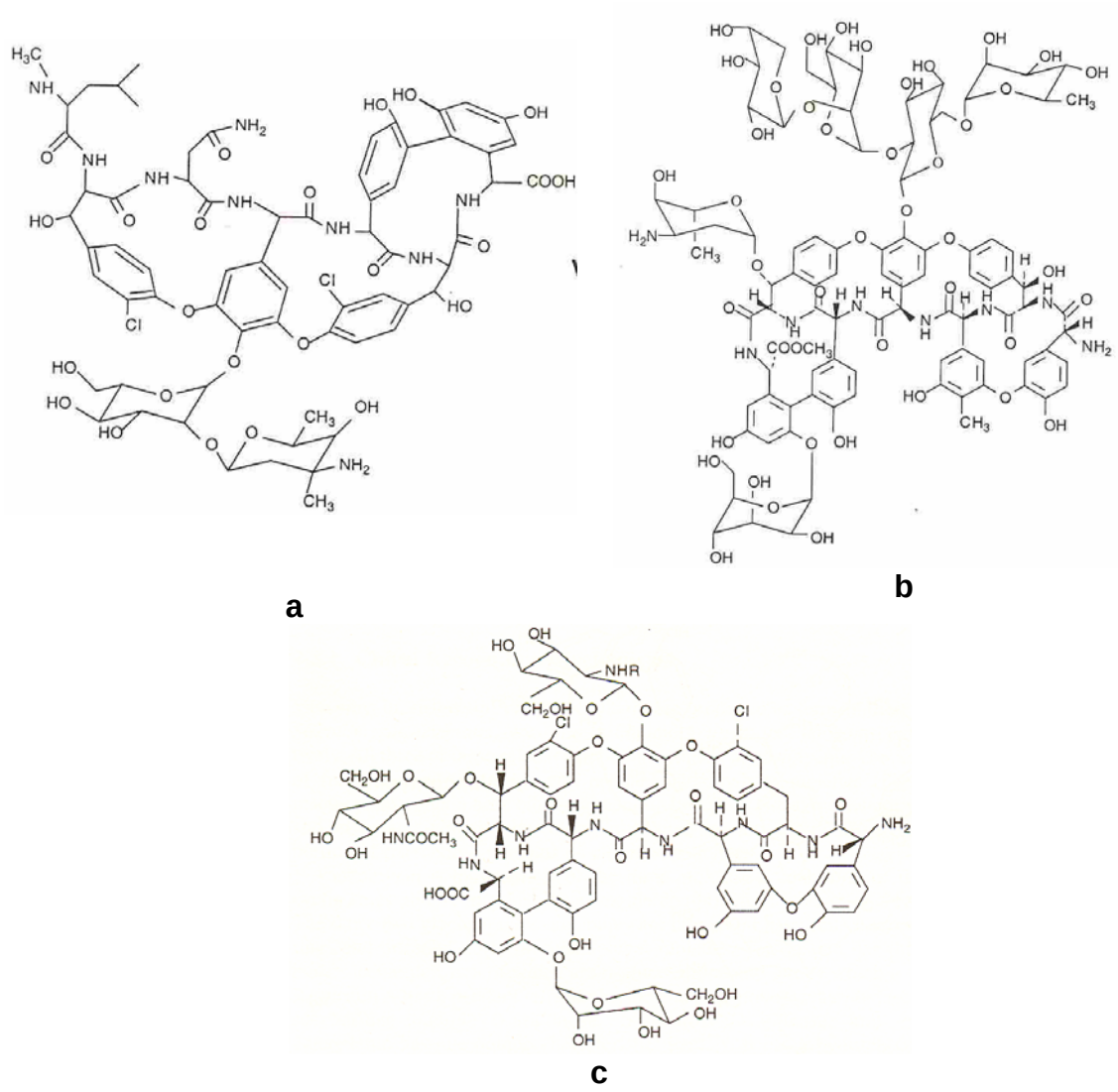


Figure 11 (continued): Macrocyclic glycopeptide antibiotics as chiral selectors for HPLC. **a** Vancomycin **b** Ristocetin A **c** Teicoplanin

Macrocyclic antibiotics offer several advantages, one of them being their applicability in various mobile phase modes. According to the mobile phase mode, the selectivities of the CSPs can vary, which is used in method development (Table 3.1).

Polar organic mode (methanol with or without acidic/basic modifier)	ionic interaction hydrogen bonding steric interaction
Reversed phase (buffers)	ionic interaction hydrophobic inclusion hydrogen bonding steric interaction
Normal phase	hydrogen bonding p-p interaction dipole stackig steric interaction
Table 3.1: Possible retention mechanisms for three mobile phase compositions on glycopeptide CSPs. (*adapted from reference* [23])	

Enantioselectivity can often be predicted for compounds with similar structural features – if one compound is separable on a certain CSP with a certain mobile phase then others with the same functional groups attached to the steric center will most likely be separable with the same system.

Macrocyclic antibiotics are highly selective for D-Ala-D-Ala peptide sequences. Therefore, the L enantiomers of amino acids and peptides are often eluted previously to the D enantiomers. Especially high retention is to be expected for peptides with D amino acids at the C terminus.[24] Elution order is normally the same on all 3 columns (V, R and T), so that column coupling is possible. In some cases though, elution order may be reversed between R and T and between R and V.

On the other hand, the principle of complementary separation says that by switching from one Chirobiotic™ column to another, selectivity can often be increased (but the reverse is also possible).

Even though several protocols for method development for the separation of diverse racemates exist[25], method development and optimisation on glycopeptide-based stationary phases may be very labour-intensive as there are so many parameters to be considered, among them the operating temperature and the type and amount of modifier. Due to the large number of papers dealing with chiral separations on these stationary phases, literature research may be particularly time-consuming.

b) Molecular Imprinted Polymers

MIPs are prepared by polymerisation of suitable functional monomers and cross-linkers in the presence of a molecular template. The latter is removed after polymerisation to leave a cavity which can incorporate molecules of the same structure as the template due to its shape.
By using an enantiomerically pure template, enantioselective MIPs can be prepared for use in chromatographic applications (among others).

Enantioseparation of N-protected amino acids and peptides on MIPs was reported as early as 1995.[26] The predictable selectivites and elution orders are of great value, especially in preparative chromatography. Besides, the stability of the MIP stationary phases is superior to that of CSPs that have a selector immobilised onto solid support.
However, the fact that a MIP CSP can only separate a very limited number of analytes (often only the one that was used as a template) and therefore have to be tailor-made for every application is a major drawback.
Resolution and peak-shape are often poor, which is thought to be a result of limited mass transfer and heterogeneous binding sites.[27]
Use of unsuitable mobile phases can cause swelling of the polymer, which further impairs separation performance.

3.2.2) Chiral Ligand Exchange Electrochromatography

Electrochromatography is considered a hybrid of electrophoresis and chromatography. Depending on the stationary phase that is used (coating or monoliths), scientists can use it to perform ligand exchange experiments:

Complexes consisting of metal ions such as Cu^{2+} and organic (often multidentate) ligands comprise the selectors in chiral ligand exchange chromatography. The chiral analytes can substitute one of the ligands of the complex and thereby form mixed copper complexes as depicted in Figure 12. The diastereomeric complexes are of different stability and therefore suitable for enantiomer separation. Elution in electrochromatography is achieved by applying a voltage to the capillary.

Figure 12: Principle of chiral electrochromatography (*adapted from reference [28])*

Ligand exchange chromatography was initially performed on polymer resins containing amino acids complexed with metal ions.[28] Later, the method was transformed to HPLC, with silica-based stationary phases onto which amino acids were immobilised. Cu^{2+} was used to facilitate complex-forming.
The method was subsequently transferred to capillary electrochromatography (CEC) with monolithic phases and used for the enantioseparation of amino acids.[29]

E. Pittler and her coworkers used silica-based stationary phases with covalently bonded or dynamically coated L-4-hydroxyproline as a chiral selector to separate amino acids.[30] They achieved baseline separation and acceptable resolution of phenylalanine and tyrosine isomers, although retention times were relatively long. (This can be overcome by increasing the temperature which might lead to formation of air bubbles in the system and an instable baseline). Some dipeptides such as Gly-Ala or Gly-Phe were also enantioseparated, but with weaker results.

Elution order can sometimes be reversed by switching from a column that has the selector covalently linked to the silica to a column where the selector is dynamically coated onto the stationary phase.
Mobile phases in CEC should contain metal ions to prevent column bleeding. Optimisation parameters should include temperature and mobile phase pH.

3.2.3) Chiral Gas Chromatography

In addition to the applications presented above, direct chromatographic enantioseparation can also be facilitated with gas chromatography if the analytes are volatile enough or can be derivatised with a suitable non-chiral derivatising agent.

Even though it is not quite as common as chiral (HP)LC, chiral GC columns are commercially available from a variety of suppliers.
They are usually based on various cyclodextrines or their derivatives (Astec CHIRALDEX^{TM}, Supelco DEX^{TM} columns), which exhibit enantioselectivites based on the formation of inclusion complexes and polar interactions.
The most widespread application of chiral GC makes use of the Chirasil-(L)-Val or -(D)-Val columns, which has a chiral amino acid (derivative) immobilised onto the polysiloxane surface of the GC capillary.
In 2009, H. Zahradnickova et al. reported the enantioseparation of a number of proteinogenic amino acids on such a GC CSP. [31] However, the GC standard procedure of silylating polar groups is not a suitable derivatisation for chiral GC, so the scientists had to employ a technique which is significantly more laborious and time-consuming.
This is a particularly important disadvantage of gas chromatography compared to chiral LC, especially if the analytes are as polar as amino acids and peptides.

3.2.4) Chiral Ion Exchange Chromatography

The underlying principle of ion exchange chromatography is, as the name suggests, the ionic interaction of a charged analyte with a stationary phase of the opposite charge. In chiral separations, these long-range interactions serve to bring the analyte into close contact with a chiral ion exchanging CSP, where enantioseparation is enhanced by non-covalent interaction such as $\pi-\pi$ stacking, hydrogen bonding between polar functional goups and steric interaction which in combination lead to the discrimination of one enantiomer.

At the beginning of section 3 it was mentioned that easy separation of zwitterionic compounds into their enantiomers is not possible on chiral ion exchangers due to the presence of an intramolecular counterion in the selectand.
It is possible to circumvent this by derivatising either the carboxylic (this is rarely done) or the amine functionality as it is routinely done in peptide synthesis. By creating an either solely basic or solely acidic molecule, chiral ion exchange methods can be applied to amino acids and peptides. Besides, UV or fluorescence detection can be facilitated by introducing e.g. aromatic protective groups such as Fmoc or benzoyl.

Figure 13 depicts *N*-protective groups often used for the derivatisation of amino acids and peptides.
The carboxylic function can be derivatised by creating esters or amides. After the separation, the protective groups can be removed by well-known protocols.

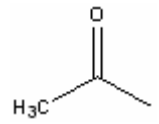
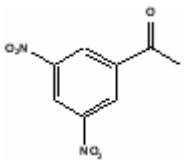
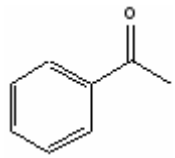
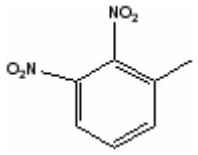
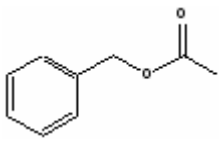
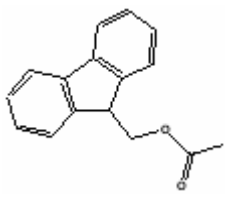
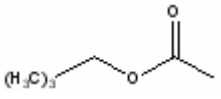
Chemical Structure	Chemical Name	Abbr.	Chemical Structure	Chemical Name	Abbr.
	acetyl	Ac		3,5-dinitrobenzoyl	DNB
	benzoyl	Bz		2,4-dinitrophenyl	DNP
	benzyloxy-carbonyl	Z (Cbz)		9-fluorenyl-methoxy-carbonyl	Fmoc
	*t*-Butyl-oxy-carbonyl	Boc			

Fig. 13: Examples of *N*-protective groups for the derivatisation of amino acids.

Fig. 6a (p. 14) presents the structure of a commercially available chiral anion exchanger based on *tert*-butyl-cabamoylated quinine.
When the CSP is operated under buffered hydro-organic conditions, the tertiary amine of the quinuclidine moiety is protonated (positively charged).[32] Ionic interactions with the negatively charged acidic analytes are the basis of retention, but enantioselectivity is introduced by additional interactions such as hydrogen bonding, $\pi-\pi$ interaction between aromatic groups and steric interactions. The carbamate substituent proved to be of special interest to the latter as bulky substituents resulted in especially high enantioselectivities for N-protected amino acids.[32]
Anion exchanging CSPs have also been employed successfully for the enantioseparation of N-protected peptides[33] and other chiral acids such as sulfonic acids.

Chiral cation exchangers are based on an acid function (e.g. a sulfonic acid, see Figure 6, p. 14). To achieve enantiodiscrimination, a chiral center close to the acid moiety is crucial. CSPs like the one shown in Figure 5 offer additional functional moieties for $\pi-\pi$, hydrogen bond or van der Waals interactions to enhance selectivity.
Chiral cation exchangers can serve for the enantioseparation of chiral amines such as Clenbuterol using nonaqueous polar organic mobile phases based on methanol or acetonitrile. Acidic and basic mobile phase additives serve as co- and counterions which balance the strong ionic interactions and facilitate elution of the analytes.[34]

3.3) Drawbacks of Conventional Separation Methods with Regard to Amino Acids and Peptides

It was mentioned before that the indirect method of enantiomer separation has several drawbacks such as the need for an enantiomerically pure derivatisation reagent, the required presence of derivatisable groups in the selectand (no cause for concern with amino acids and peptides) or the possibly different dectector responses to the diastereomers which are formed in the process.
Racemisation may occur under the derivatisation conditions and compromise the outcome of the analysis.
As mentioned in section 3.2.1, peptides (especially synthetic ones) are frequently hydrolised and the free amino acids are converted into diastereomeric derivatives with

appropriate CDAs to determine their amino acid composition. In the course of this process, racemisation may occur. It is very intricate to differentiate between racemisation that occurred during peptide synthesis and racemisation during peptide hydrolysis or other stages of the synthesis (e.g. also during labelling). Sophisticated methods involving deuterated hydrolysis solvents and subsequent MS analysis may be required.[18]

The direct approach to enantioseparation of chiral compounds can overcome several of the drawbacks attached to the indirect enantioseparation methods.
However, the diverse techniques are still not perfectly suitable for amino acids and peptides:
Chiral GC, like GC in general, is best suited for apolar molecules. Polar compounds such as amino acids and peptides always have to be derivatised, which is often time-consuming and can be a source of errors in an analytical method, if, for example, the derivatisation reaction is not quantitative. Often, not all amino acids can be derivatised without degradation.

Chiral ligand exchange liquid chromatography requires a relatively sophisticated set-up and an experienced experimenter. Columns are not commercially available and the loadability is usually very low. Optimisation is time-consuming and retention times for amino acids are often unfavourably high.
HPLC with enantioselective molecular imprinted polymers suffers from low loadabilities of the columns which, besides, have to be custom-made for every application.

Today, the separation of amino acids and peptides is mainly carried out using CSPs based on glycopeptide antibiotics. The columns are commercially available. However, extensive optimisation may be required to solve a certain separation problem and the optimisation protocols usually rely on the use of at least 2 different columns. Elution order reversal can only be achieved by switching to a column with a different CSP, which can result in changed separation characteristics.

Zwitterionic compounds such as amino acids and peptides cannot be separated on chiral anion exchangers due to the presence of an intramolecular counterion in the selectand. Achiral derivatisation of the *N* terminus of the analyte results in chiral acids which can be separated on cinchona-based chiral anion exchangers.

However, it is preferable to separate analytes without the need for a derivatisation reaction, which always bears the risk of introducing errors into the analysis.

4.) Zwitterionic chiral stationary phases (ZWIX CSPs)

To overcome the limitations of enantioseparation of zwitterionic compounds on cation and anion exchanging CSPs, a novel type of zwitterionic chiral stationary phases was introduced by Professor Lindner´s research group in 2008.[35]

Zwitterionic selectors for HPLC were prepared by fusing weak anion exchanging sites (WAX) based on cinchona alkaloids and sulfonic acids as strong cation exchanging sites (SCX), zwitterionic selectors for HPLC were prepared (Figure 14).

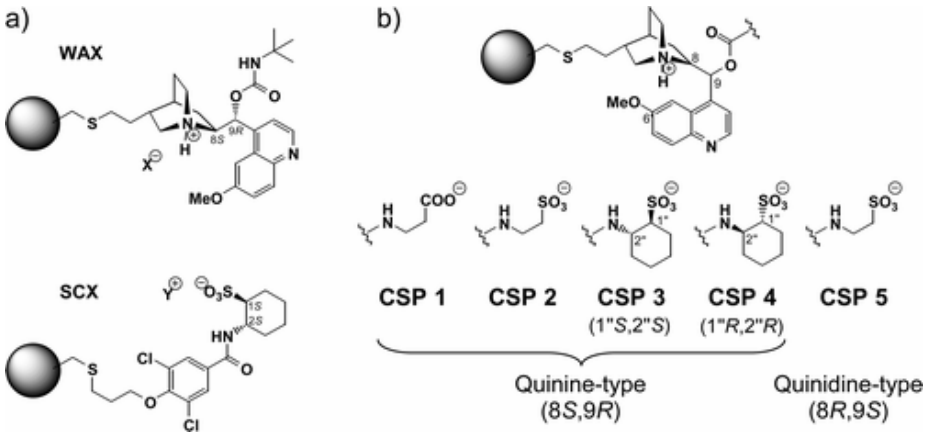


Fig. 14: Zwitterionic chiral stationary phases (ZWIX CSPs) for HPLC enantioseparation (*from reference* [35]).

The resulting ZWIX CSPs combined the applicability of WAX CSPs for chiral acids and of SCX CSPs for chiral bases and, in addition, offered the possibility to separate enantiomers of amphoteric compounds.

ZWIX CSPs based on cinchona alkaloids and sulfonic acids are operated in polar-organic mobile phases with acidic and basic additives (typically in a 2:1 ratio), which modulate elution.

4.1) Separation of Acidic and Basic Analytes on ZWIX CSPs

As indicated above, the ability of the WAX site to enantioseparate chiral acids is preserved in the novel ZWIX CSPs, with the added benefit of reduced retention times compared to the underlying anion exchanger: the AX site in the selectors is a carboxylic (weaker) or sulfonic (stronger) acid which acts as an intramolecular counterion (IMCI) to the analyte. Retention can be further adjusted by the type and concentration of the acidic counterion in the mobile phase.
By using quinine and its pseudo-enantiomer quinidine as chiral scaffolds for ion exchangers, it is possible to reverse the elution order of the analyte enantiomers[34], which was found to be also true for the zwitterionic CSPs. This means that a chiral SCX site attached to the C-9 position (CSPs 3 and 4) is not predominant the enantiorecognition. In these systems, the latter is obviously determinded by the QN/QD-derived chiral scaffold.

The scientists also investigated the separation of chiral amines (bases) on the ZWIX CSPs depicted in Figure 14. ZWIX CSPs with a chiral cation exchanging site were able to separate the enantiomers of the drugs tested with significantly shorter retention times than on a SCX CSP, which was ascribed to the IMCI activity of the protonated quinuclidine moiety.
From their results, the scientists concluded that the presence of a stereogenic center in the SCX site of the ZWIX selector is not necessary to achieve enantioseparation of chiral bases such as Mefloquine, but that it is highly beneficial.
Elution orders can be reversed by switching from quinine to quinidine-based CSPs and also (for some analytes) by employing QN-based ZWIX CSPs with opposite configuration of their SCX sites only.

4.2) Separation of Zwitterionic Analytes on ZWIX CSPs

In their 2008 publication, Hoffmann et al. enantioseparated a large set of chiral zwitterionic compounds such as amino acids and mixed dipeptides and established that the retention mechanism of zwitterionic compounds on ZWIX CSPs is based on simultaneous double ion pairing.[35]

The ZWIX CSPs that were evaluated showed different enantioselectivities towards groups of structurally similar analytes. With amphoteric analytes, the stronger sulfonic acid moiety led to higher retention factors than the weaker carboxylic acid due to a stronger SO-SA interaction.
Apparently, elution orders for amphoteric analytes are determined mainly by the cinchona alkaloid scaffold, as they were found to be reversed on quinine and quinidine-based ZWIX CSPs.

Obviously, this type of CSPs presents a particularly interesting approach to the enantioseparation of amino acids and peptides with all the benefits of direct enantioseparation techniques in HPLC.
The aim of the present work was to prepare a series of three ZWIX CSPs with homologous SCX sites based on CSP 1 from Figure 14 (so-called Tau-QN, from the underlying taurine and quinine building blocks) and to evaluate the ZWIX CSPs for their enantioseparation capabilities towards N-protected amino acids, free amino acids and peptides. Besides, their quinidine-based pseudo-enantiomers were to be synthesised, evaluated in the same way and compared to the quinine-based CSPs.

Experimental – Materials and Methods

1) Materials

1.1) Syntheses

Syntheses procedures for the individual selectors are described in detail in the following chapter.

Synthesis reagents were of reagent grade or higher purity and were purchased from Sigma-Aldrich (Vienna, Austria) unless stated otherwise. They were used without further purification.
All reaction steps were performed under nitrogen and with oven-dried glassware.
Technical Grade solvents (dichloromethane, ethylacetate, methanol, acetone, toluene) were purchased from VWR (via Merck, Darmstadt, Germany).
HPLC grade solvents were from Sigma-Aldrich (acetone, water), Fluka (dichloro-methane, via Sigma-Aldrich Austria), VWR (HiPerSolv methanol) and Carl Roth GmbH (n-heptane).

For purification of synthesis products by column chromatography, Kieselgel 60 (40-63 µm) was purchased from Merck (Darmstadt, Germany).

Quinine and quinidine were purchased from Buchler (Braunschweig, Germany).

Aminomethanesulfonic acid and taurine were from Sigma-Aldrich Austria and EGA Chemie Gesellschaft (Steinheim, Germany), respectively.

1,3-Propanesultone and 1,4-butanesultone were purchased from Sigma-Aldrich and from Tokyo Chemical Industries (via TCI Deutschland, Eschborn, Germany).
7N solutions of NH_3 in methanol were from Sigma Aldrich Austria and Fisher Scientific (Schwerte, Germany).

AIBN was purchased from Merck (Darmstadt, Germany).

N,N-Diethyl trimethylsilylamine was purchased from Bucher via Merck (Darmstadt, Germany).
N,N-Diisopropylethylamine and triethylamine were from Fluka via Sigma-Aldrich Austria.

Silica gel for selector immobilisation (Daisogel 120-5P, 5µm particles, pore diameter 120 Å) was purchased from Daiso Co., Ltd., Düsseldorf, Germany and mercaptopropyl-modified in-house.

1.2) Analyses

NMR solvents were purchased from Deutero (Kastellaun, Germany).

HPLC grade solvents (water, acetone) were purchased from Sigma-Aldrich (Vienna, Austria) and VWR (HiPerSolv methanol).

Mobile phase additives (diethylamine, formic acid, ammonium acetate, formic acid, acetic acid) were purchased from Fluka and Sigma-Aldrich.

The chiral zwitterionic analytes and acidic drugs used for the evaluation of the ZWIX CSPs were either commercially available or gifts from research partners of our group.
Protected amino acids had been synthesised in-house according to established protocols.
Peptide enantiomers were either commercially available from Sigma-Aldrich and Bachem (Weil am Rhein, Germany) or custom-synthesised by Genecust Europe (Dudelange, Luxembourg).

2) Methods

2.1) Instrumentation

Thin layer chromatography of synthesis products was performed on Kieselgel 60 F_{254} TLC plates (Merck, Darmstadt, Germany) with UV detection at 254 nm in a cabinet from Camag (Berlin, Germany).

NMR analyses were carried out on a Bruker DRX 400 MHz NMR spectrometer. Spectra were recorded in D_2O, MeOH-d_4 and $CDCl_3$ (see individual spectra). Chemical shifts are stated in ppm (parts per million) with tetramethylsilane as internal standard. Solvent signals were used as reference signals. NMR spectra were processed with SpinWorks 2.2.5.

Mass Spectrometry experiments were performed on a PE Sciex API 365 triple-quadrupol mass spectrometer equipped with an electrospray source and an Agilent 1100 Series CL/MSD Trap ion-trap MS system.

Elemental Analysis (CHNS) of immobilised selector was performed on an EA 1108 CHNS-O Element Analyser (Carlo Erba, now Thermo Scientific, Germany) and selector coverage of the silica was calculated from the nitrogen content.

HPLC experiments were performed on an Agilent 1100 HPLC system with MWD (multiple wavelength detector) and an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany), equipped with a DAD (diode array detector).
Detection was at 254 nm for UV active analytes.
Analytes with low or no absorption at 254 nm and peptides were detected with a Corona Charged Aerosol Detector (ESA Biosciences, Inc., Chelmsford, MA, USA).

Methanol (50 mmol/L formic acid and 25 mmol/L diethylamine as additives) was used as the standard mobile phase - it had previously been established as a particularly versatile mobile phase for cinchona-based ZWIX applications.
For the experiments with a mixture of water and methanol as mobile phase, the solvents were mixed previously and allowed to cool to ambient temperature. After the addition of

25 mmol/L of diethylamine, apparent pH was set to 5,4 with formic acid (pH meter 540 GLP from WTW via Aigner-Unilab, Vienna, Austria).

Mobile Phase flow rate was usually 1 mL/min and analysis was carried out at room temperature (22°C) unless stated otherwise.

Stainless steel HPLC columns (150 x 4 mm) for the preparation of ZWIX columns were from Bischoff and were slurry-packed in-house. Chirobiotic columns (Chirobiotic V, Chirobiotic TAG; column dimensions: 250 x 4,6 mm, 5 µ material) were purchased from Astec (via Sigma-Aldrich, Vienna, Austria).
Void volumes of the columns were determined by injection of 10-50 µL of a 1:9 solution of acetone in MeOH.

HPLC samples were prepared by dissolution of the analytes in either methanol or a mixture of water and methanol (1:1, v/v) at a concentration of 1 mg/mL. 10 µL of these solutions were injected into the HPLC system.
Elution orders were determined by injection of a single enantiomer of the analyte if available.

Data acquisition was achieved with Agilent ChemStation Software and Evaluation of the Data was carried out with Microsoft Excel.

2.2) ZWIX Selector Syntheses

2.2.1) Tau-QN and Tau-QD

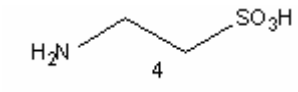


Fig. 1: Taurine (2-aminoethanesulfonic acid)

The synthesis concept of the taurine selectors was in accordance with Figure 2:

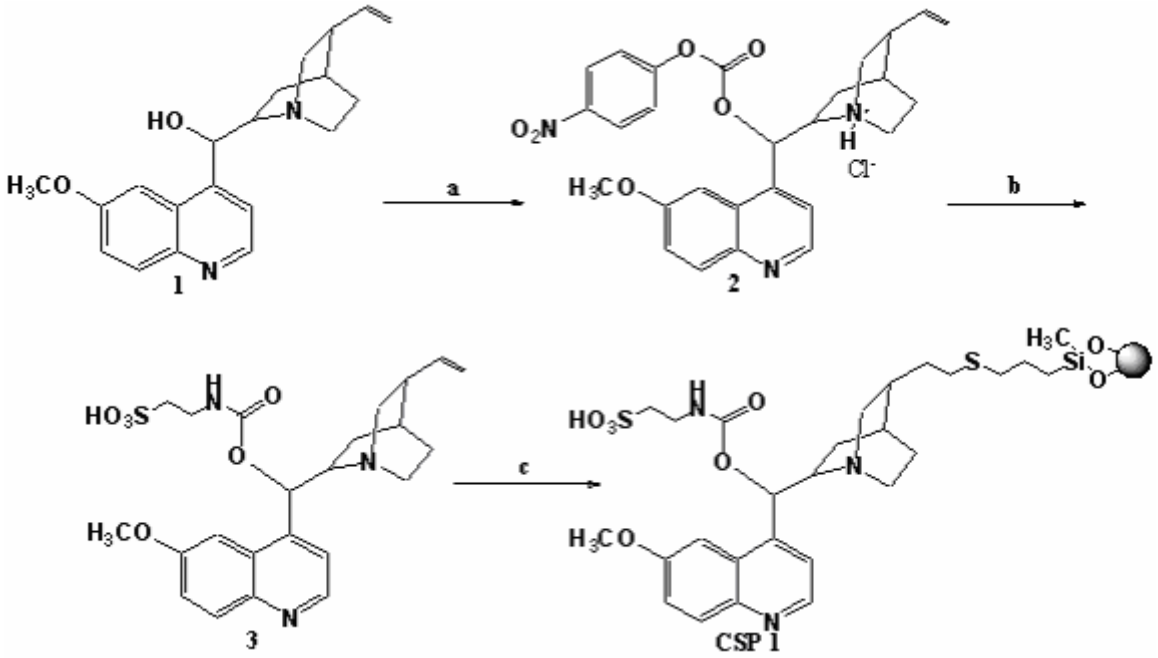


Fig. 2: Synthetic route from quinine to ZWIX CSP 1. *Conditions:* ***a*** *4-nitrophenyl chloroformate, toluene$_{dest}$, r.t., 24 hrs,* ***b*** *taurine, BSA, DCM$_{HPLC}$, reflux, 24 hrs,* ***c*** *AIBN, mercaptopropylsilane-modified silica gel, MeOH$_{HPLC}$, reflux, 5 hrs*

Quinine (**1**) and quinidine, respectively, were converted into their activated ester hydrochlorides **2** by addition of an equimolar amount of 4-nitrophenyl chloroformate in toluene. The reaction mixture was refluxed for 24 hrs and the activated ester was obtained by filtration (**a**).

Finely ground taurine (**4**) was silylated with BSA in dried boiling dichloromethane and converted into tau-QN and tau-QD selectors **3** by addition of equimolar amounts of the respective activated esters of quinine and quinidine (**b**).

After quenching of the reaction by addition of methanol, the selectors were purified by column chromatography with a stationary phase of 30 g silica for every g of theoretical yield. Apolar nitrophenol was eluted with DCM/MeOH 9:1 (v/v), and then the mobile phase was changed to DCM/MeOH 1:1 to facilitate elution of the polar product. The fractions containing product (control: TLC with DCM/MeOH 9:1) were pooled, solvent removed by evaporation and the product was dried in vacuum.

ESI-MS (positive and negative mode) and NMR analysis were employed for characterisation.

N-[[[(8S, 9R)-6´-methoxycinchonan-9-yl]oxy]carbonyl]-aminoethanesulfonic acid (Tau-QN):

86%, light yellow crystals. ^{1}H NMR [CD_3OD]: δ = 8.73 (d, 1H), 8.00 (d, 1H), 7.64 (d, 1H), 7.50 (s, 1H), 7.48 (d, 1H), 6.94 (s, 1H), 5.78 (m, 1H), 5.14 (d, 1H), 5.05 (d, 1H), 4.05 (s, 3H), 3.87-3.74 (m, 2H), 3.65 (m, 2H), 3.50 (m, 1H), 3.37 (m, 2H), 3.01 (m, 2H), 2.84 (m, 1H), 2.30-2.19 (m, 2H), 2.13 (m, 1H), 1.99 (m, 1H), 1.75 (m, 1H).

^{13}C NMR: δ = 160.5 (C_{ar}), 155.9 (C=O), 147.8 (C_{ar}H), 144.4 (C_{ar}), 143.6 (C_{ar}), 138.9 (CH=), 131.4 (C_{ar}H), 127.4 (C_{ar}H), 124.3 (C_{ar}H), 119.5 (C_{ar}H), 117.4 (CH_2=), 102.3 (C_{ar}H), 71.1 (CH), 60.0 (CH), 57.1 (OMe), 55.8 (CH_2), 51.5 (CH_2), 45.5 (CH_2), 38.4 (CH_2), 38.3 (CH), 28.2 (CH), 25.0 (CH_2), 20.7 (CH_2).

MS (ESI, positive): 476.4 $[M+H]^+$, 498.4 $[M+Na]^+$. MS (ESI, negative): 474.2 $[M-H]^-$.

N-[[[(8R, 9S)-6´-methoxycinchonan-9-yl]oxy]carbonyl]-aminoethanesulfonic acid (Tau-QD):

58%, yellow crystals. ^{1}H NMR [CD_3OD]: δ = 8.79 (d, 1H), 7.96 (d, 1H), 7.78 (d, 1H), 7.56-7.50 (m, 2H), 7.11 (s, 1H), 6.14 (m, 1H), 5.33-5.24 (m, 2H), 4.01 (s, 3H), 3.87 (m, 1H), 3.65-3.51 (m, 5H), 3.37 (m, 1H), 3.02 (m, 2H), 2.77 (m, 1H), 2.42 (m, 1H), 2.06 (m, 1H), 2.02-1.83 (m, 2H), 1.47 (m, 1H).

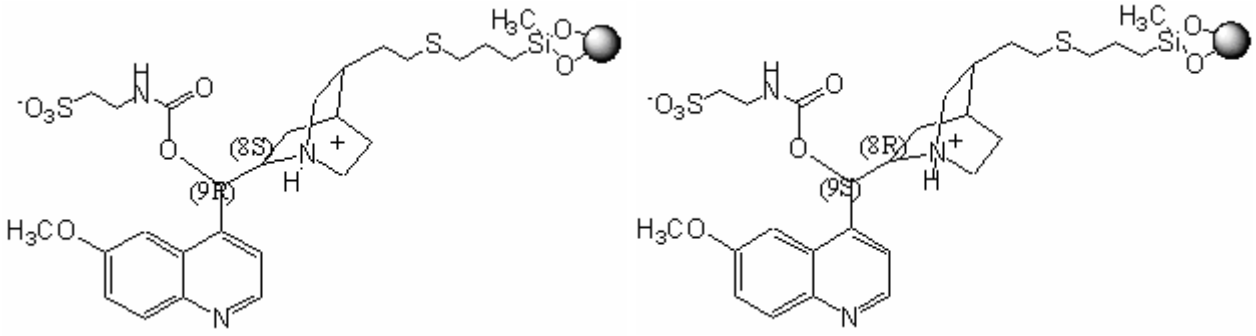
^{13}C NMR: δ = 161.2 (C_{ar}), 155.7 (C=O), 146.8 (C_{ar}), 145.8 (C_{ar}H), 141.2 (C_{ar}), 137.9 (CH=), 128.8 (C_{ar}H), 127.9 (C_{ar}), 126.1 (C_{ar}H), 120.1 (C_{ar}H), 118.4 (CH2=), 102.6

($C_{ar}H$), 71.4 (CH), 59.8 (CH), 57.4 (OMe), 51.5 (CH_2), 51.0 (CH_2), 50.1 (CH_2), 38.5 (CH_2), 38.2 (CH), 28.7 (CH), 23.7 (CH_2), 20.4 (CH_2).
MS (ESI, positive): 476.2 $[M+H]^+$, 498.2 $[M+Na]^+$, 951.4 $[2M+H]^+$, 973.4 $[2M+Na]^+$.

For immobilisation, the Tau-QN selector was added to a suspension of 3 µm mercaptopropyl-modified silica gel in methanol (1 mmol SO, 3 g silica). By refluxing of the reaction mixture with the radical initiator AIBN (40 mg of initiator per mmol SO) for 5 hrs, the selector was covalently bound to the surface (Figure 2, **c**).

After drying the CSP was subjected to CHNS elemental analysis which gave a surface coverage of 224 µmol selector/g silica.
Tau-QD was not immobilised as two CSPs with the selector existed already.

CSP 1 ***CSP 2***



QN-based **QD-based**

Fig. 3: Tau-QN and Tau-QD CSPs

For the evaluation and comparative experiments, Tau-QN and Tau-QD columns with 5 µm material that had been prepared by members of our group were used.

2.2.2) Homotau-QN and Homotau-QD

For the synthesis of the homotaurine-selectors homotau-QN and homotau-QD, homotaurine (**6**) was prepared by ring opening of 1,3-propanesultone (**5**) with ammonia[36] according to Figure 4:

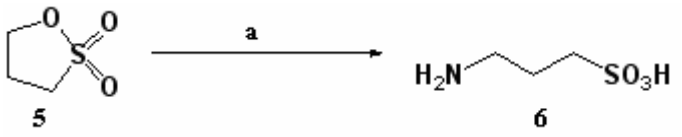


Fig. 4: Preparation of homotaurine (3-aminopropanesulfonic acid, **6**) by ring opening of 1,3-propanesultone (**5**) *Conditions: **a**: MeOH, NH_3, -21°C to r.t, 18 hrs.*

1,3-propanesultone (**5**) was filled into a reaction flask and cooled with a mixture of ice and NaCl. A three-fold excess of NH_3 (as a 7N methanolic solution) was added slowly, while the reaction mixture was stirred continuously.

After completion of the addition, the mixture was cooled for another two hours and then the ice was allowed to melt and the solution was stirred overnight.

Homotaurine was obtained as a white precipitate which can be recrystallised from a water/ethanol (1:1 v/v) mixture.

Conversion was always quantitative throughout an upscaling process from 4 to 200 mmol.

Homotau-QN and Homotau-QD selectors were prepared as depicted in Figure 5 (the procedure is analogous to the synthesis described in detail in section 2.2.1 for Tau-QN and Tau-QD).

Homotaurine from the ring opening was finely ground and silylated with BSA in dried boiling dichloromethane. Conversion with the activated esters of quinine and quinidine, respectively, was carried out in toluene. Selector synthesis was quenched by addition of methanol and the concentrated mixture was subjected to column chromatography on 30 g of silica per g of theoretical yield to remove nitrophenol (mobile phase: DCM/MeOH 9:1 v/v). The product was eluted with DCM/MeOH 1:1 (monitoring by TLC), dried in vacuum and characterised by MS and NMR analysis.

The respective selectors, Homotau-QN and Homotau-QD, were immobilised onto mercapto-modified silica by radical addition (AIBN) in methanol to give CSPs 3 and 4 (Fig. 6)

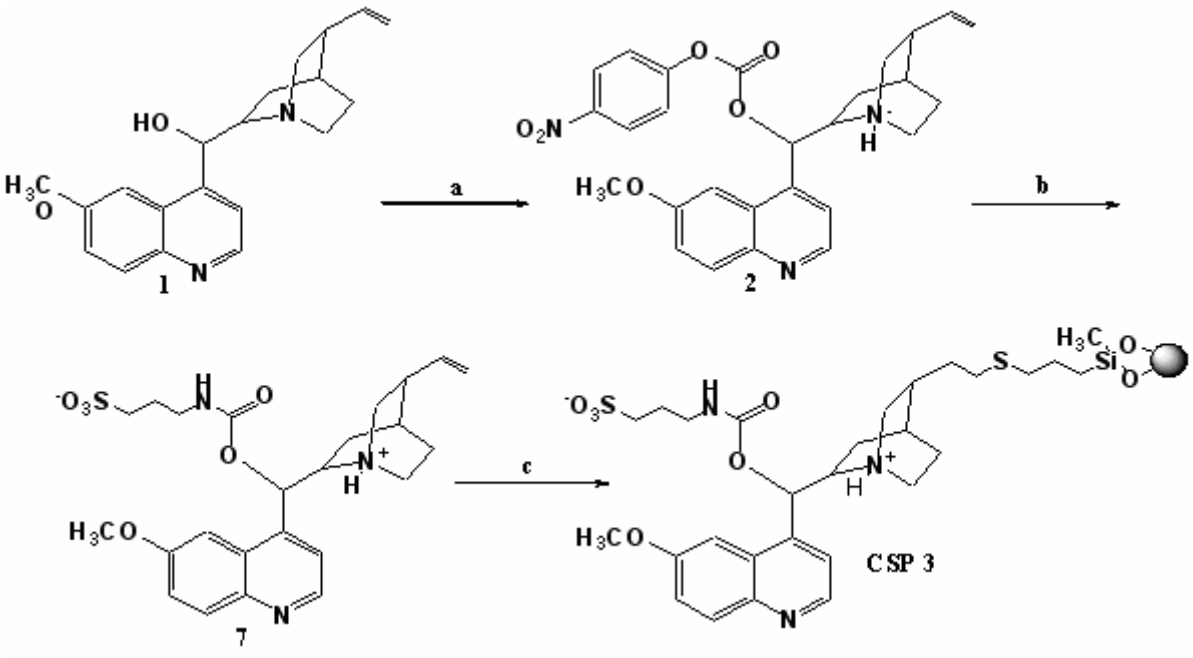


Fig. 5: Synthetic route from quinine to ZWIX CSP 3. *Conditions:* ***a*** *4-nitrophenyl chloroformate, toluene$_{dest}$, r.t., 24 hrs,* ***b*** *homotaurine, BSA, DCM$_{HPLC}$, reflux, 24 hrs,* ***c*** *AIBN, mercaptopropylsilane-modified silica gel, MeOH$_{HPLC}$, reflux, 5 hrs*

CSP 3 ***CSP 4***

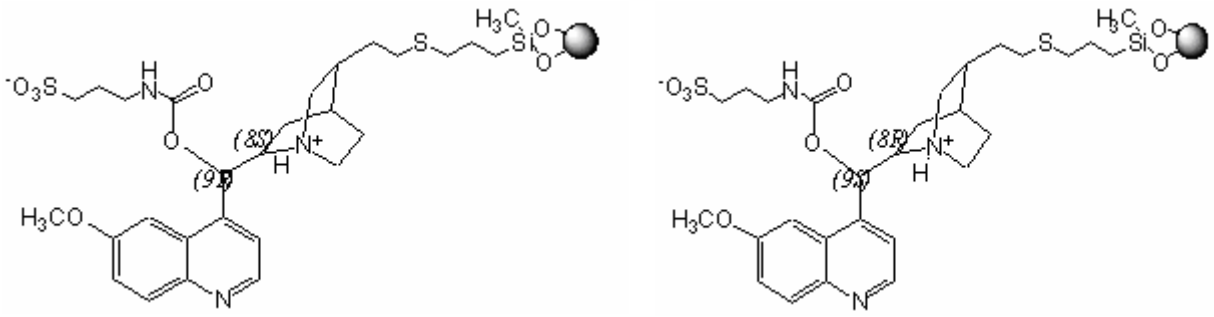


Fig. 6: Homotau-QN (CSP 3) and Homotau-QD (CSP 4)

N-[[[(8S, 9R)-6´-methoxycinchonan-9-yl]oxy]carbonyl]-aminopropanesulfonic acid (Homotau-QN):

70%, light yellow crystals.

MS (ESI, negative): 488.0 $[M-H]^-$; MS (ESI, positive): 490,2 $[M+H]^+$

N-[[[(8R, 9S)-6´-methoxycinchonan-9-yl]oxy]carbonyl]-aminopropanesulfonic acid (Homotau-QD):

50%, yellow crystals. ^{1}H NMR [CD_3OD]: δ = 8.79 (d, 1H), 7.88 (d, 1H), 7.81 (d, 1H), 7.55 (m, 2H), 7.00 (s, 1H), 6.11-6.0 (m, 1H), 5.2-5.1 (m, 2H), 3.94 (s, 3H), 3.76 (m, 1H), 3.49 (m, 3H), 3.27 (s, 1H), 3.13 (m, 2H), 2.84-2.61 (m, 3H), 2.31 (m, 1H), 2.08-1.70 (m, 5H), 1.36 (m, 1H).

MS (ESI, negative): 488.1 $[M-H]^-$

CHNS elemental analysis gave a surface coverage of 280 µmol selector/g silica for CSP 3 and 188 µmol selector/g silica for CSP 4.

2.2.3) Homohomotau-QN and Homohomotau-QD

Synthesis procedures for Homohomotau-QN and Homohomotau-QD were carried out in analogy to those described in detail above.

Homohomotaurine (**9**) was prepared from commercially available 1,4-butanesultone by ring opening with NH_3 ([36],[37]) with a yield of over 85% and recrystallised from water/ethanol (1:1 v/v) (Figure 7).

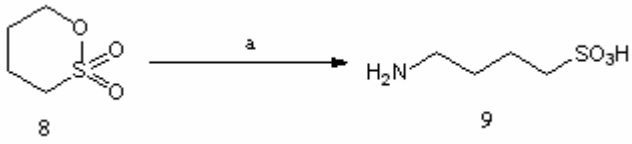


Fig. 7: Preparation of Homohomotaurine (4-aminobutanesulfonic acid, **9**) by ring opening of 1,4-butanesultone (**8**). *Conditions: **a**: MeOH, NH_3, -21°C to r.t, 18 hrs.*

1,4-Butanesultone was cooled with ice/NaCl (3:1) and a three-fold excess of NH_3 (methanolic solution) was added slowly. The ice was allowed to melt after ca. 2 hrs of stirring, and stirring was continued overnight. Homohomotaurine, which precipitated from the solution, was obtained by filtration and dried in vacuum.

The aminobutanesulfonic acid was finely ground and silylated with BSA in dried boiling dichloromethane for 24 hrs (Figure 8).

Reaction of the silylated homohomotaurine with the activated esters of quinine and quinidine (**2**) gave the homohomotau-QN and homohomotau-QD selectors (**10**). After purification of the selectors by column chromatography (30 g of silica for every g of theoretical yield, mobile phase DCM/MeOH 9:1 (v/v) for the elution of nitrophenol and DCM/MeOH 1:1 for the elution of the product), they were characterised by MS and NMR analysis.

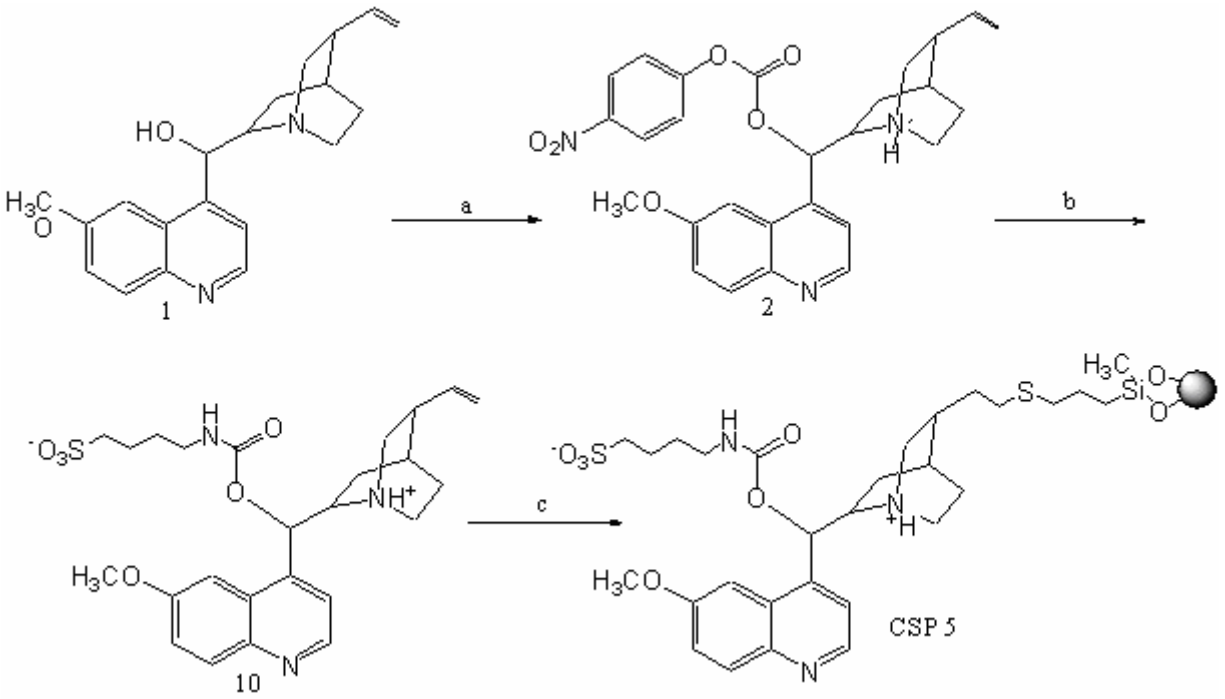


Fig. 8. Synthetic route from quinine to ZWIX CSP 5. *Conditions:* ***a*** *4-nitro-phenyl chloroformate, toluene$_{dest}$, r.t., 24 hrs,* ***b*** *homohomotaurine, BSA, DCM$_{HPLC}$, reflux, 24 hrs,* ***c*** *AIBN, mercaptopropylsilane-modified silica gel, MeOH$_{HPLC}$, reflux, 5 hrs*

N-[[[(8S, 9R)-6´-methoxycinchonan-9-yl]oxy]carbonyl]-aminobutanesulfonic acid (Homohomotau-QN):

40%, light yellow crystals. ^{1}H NMR [CD_3OD]: δ = 7.88 (d, 1H), 7.48 (d, 1H), 7.39 (m, 2H), 6.69 (s, 1H), 5.74-5.62 (m, 1H), 5.04-4.98 (d, 1H), 4.95-4.90 (d, 1H), 3.93 (s, 3H), 3.62-3.57 (s, 1H), 3.54-3.48 (s, 1H), 3.15-3,04 (m, 3H), 3.04-2.98 (m, 1H), 2.87-2.83 (d, 1H), 2.78-2.68 (m, 3H), 2.61 (s, 1H), 2.10-1.90 (m, 3H), 1.83-1.48 (m, 6H).

MS (ESI, negative): 502,8 $[M-H]^-$

N-[[[(8R, 9S)-6´-methoxycinchonan-9-yl]oxy]carbonyl]-aminobutanesulfonic acid (Homohomotau-QD):

20%, yellow crystals.

MS (ESI, negative): 502,6 $[M-H]^-$

Homohomotau-QN and homohomotau-QD were immobilised onto mercaptopropylsilyl modified silica gel in a radical addition reaction with AIBN as described in 2.2.1 for the taurine selectors.

CHNS elemental analysis gave a surface coverage of 254 µmol selector/g silica for CSP 5 and 187 µmol selector/g silica for CSP 6.

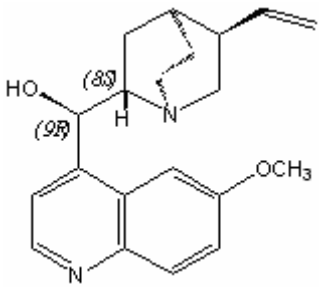
RESULTS & DISCUSSION

A) Synthesis of ZWIX Selectors

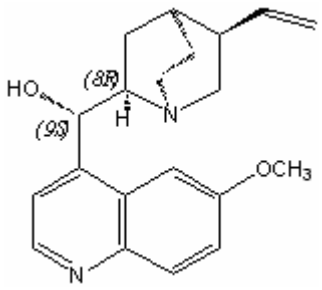
1) Standard Procedure & ZWIX CSP Characteristics

In the course of this work, 3 quinine-based zwitterionic CSPs with homologous aminoalkanesulfonic acids as SCX part and their pseudo-enantiomeric quinidine-based analogues were synthesised.

It has been shown before that quinine ((8α, 9*R*)-6´-methoxy-Cinchonan-9-ol, Figure 1a) and its pseudo-enantiomer quinidine ((9S)-6´-methoxy-Chinconan-9-ol, Figure 1b) as chiral backbones for CSPs can be very useful as they usually achieve comparable selectivity, but reversed elution order.[34] This is particularly important when dealing with enantiomeric impurities.
In synthesising and evaluating both pseudo-enantiomeric forms of each CSP, this behaviour was to be further investigated.



1a



1b

Fig. 1: Pseudo-enantiomeric cinchona alkaloids quinine (**1a**) and quinidine (**1b**) which form the chiral backbone of ZWIX selectors.

Some ZWIX CSPs included in this investigation (Tau-QN and Tau-QD) have been synthesised and evaluated before [35] and showed a great potential for the separation of ionisable, especially zwitterionic, chiral compounds.
It was to be expected that CSPs including homologous acids, namely aminomethanesulfonic acid (AMSA), aminopropanesulfonic acid (homotaurine), and aminobutanesul-

fonic acid (homohomotaurine), as their cation exchanger sites would be similarly useful in chiral separations of zwitterionic compounds.
The “distance” between the anion and cation exchanger sites of the selector – summarising all directly associated conformational variabilities of the zwitterionic selectors and thus their special arrangements - is, of course, crucial to the enantioseparation of zwitterionic analytes. This aspect was going to be investigated by the separation of homologous peptide enantiomers on the homologous CSPs.

It has been mentioned that some of the CSPs have already been synthesised before, whereas others (those featuring homotaurine and homohomotaurine as the cation exchanging sites) were novel.
Syntheses of CSPs 3-6 included a ring opening reaction of 1,3-propanesultone and 1,4-butanesultone to yield homotaurine and homohomotaurine, as those compounds were not easily commercially available.
Ring opening was achieved by treatment of the respective sultone with a methanolic solution of ammonia at reduced temperature (for details, see synthesis of the homotau and homohomotau selectors). These reactions yielded the internal salts of the aminoalkane sulfonic acids.

Table 1.1 shows the individual CSPs with their full and short names:

CSP	Alkaloid (WAX part)	Sulfonic Acid (SCX part)	Full Name	Short Name
CSP 1	quinine	taurine	taurine-quinine	Tau-QN
CSP 2	quinidine	taurine	taurine-quinidine	Tau-QD
CSP 3	quinine	homotaurine	homotaurine-quinine	Htau-QN
CSP 4	quinidine	homotaurine	homotaurine-quinidine	Htau-QD
CSP 5	quinine	homohomotaurine	homohomotaurine-quinine	Hhtau-QN
CSP 6	quinidine	homohomotaurine	homohomotaurine-quinidine	Hhtau-QD
CSP 7*	quinine	aminomethanesulfonic acid	aminomethanesulfonic acid-quinine	AMSA-QN

Table 1.1: Zwitterionic chiral stationary phases based on taurine-homologues and cinchona alkaloids (* CSP 7: not realised)

The selector syntheses were carried out according to Figure 2 (for details, see Experimental 2.2.1).

Quinine and quinidine (**1**), respectively, were converted into their activated ester hydrochlorides (QN-AE/QD-AE, **2**) by addition of 4-nitrophenyl chloroformate (**a**).
The products are prone to hydrolysis and therefore have to be stored under nitrogen.
The aminoalkane sulfonic acid was silylated with N,O-bis(trimethylsilyl)acetamide (BSA) to increase its solubility in apolar dichloromethane (DCM) which was used as solvent for the subsequent conversion of the active ester and the acid into the zwitterionic selector **3** (**b**).

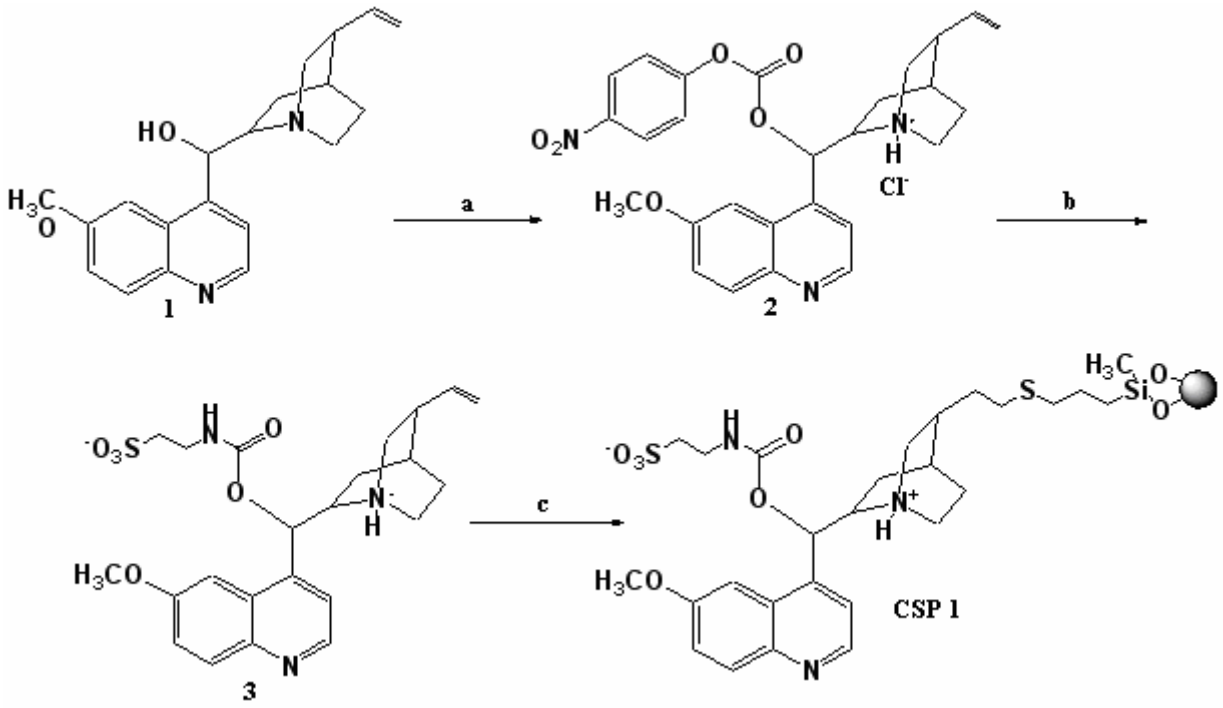


Fig. 2: Synthetic route from quinine to ZWIX CSP 1. *Conditions: **a** 4-nitrophenyl chloroformate, toluene$_{dest}$, r.t., 24 hrs, **b** aminoalkanesulfonic acid, BSA, DCM$_{HPLC}$, reflux, 24 hrs, **c** AIBN, mercaptopropylsilane-modified silica gel, MeOH$_{HPLC}$, reflux, 5 hrs.*

Completion of the silylation step (indicated by a clear solution of the aminoalkanesulfonic acid in DCM) is crucial for an acceptable selector yield.

After completion of the reaction, remaining activated ester was quenched with methanol and the product was purified by column chromatography to remove byproducts (nitrophenol and, most critically, polar acetamide from the silylation) and reagent residues.

For selector immobilisation by radical addition, dried mercaptosilane-modified silica gel was suspended in methanol and, by means of the radical initiator AIBN, the selector was covalently bound to the silica surface as depicted in Figure 2 (**c**).

In this step, particular attention has to be paid to the reaction conditions and the set-up of the apparatus because the radical initiator is only effective at temperatures above 60°C and grinding of the stirrer against the glassware can impair immobilisation.

Overall yields decreased from Tau- over Homotau- to Homohomotau-QN and QD selectors and selector loadings varied from 150-280 µmol selector/g silica (Table 1.2). It is worth mentioning that generally, ionic selectors are more difficult to immobilise than non-charged ones.

CSP Number	Short Name	Selector Coverage (µmol SO/g silica)
CSP 1	tau-QN	205
CSP 2	tau-QD	150
CSP 3	htau-QN	280
CSP 4	htau-QD	188
CSP 5	hhtau-QN	254
CSP 6	hhtau-QD	187

Table 1.2: Selector coverages of ZWIX CSPs used in this study.

The selector loadings of the individual ZWIX CSPs are sufficient for chiral separation and for comparison of the separation capabilities of the various CSPs: Previous studies have established that (above a certain value) selector loading only affects retention, but not selectivity.

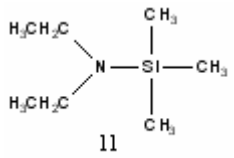
2) Variation of Standard Synthesis Procedure

The standard procedure for the synthesis of cinchona-based ZWIX selectors as described above has been carried out numerous times, mostly with selector yields between 60-80%. Higher yields are needed in order to facilitate upscaling procedures.
Therefore, a number of synthesis steps were systematically altered to achieve more satisfactory results:

2.1) Silylating Agent N,N-diethyltrimethylsilylamine

It has been stated before that in order to achieve a high selector yield, the silylation of the aminoalkanesulfonic acid has to be quantitative.

BSA has been found to be unsuitable for the silylation of AMSA (see 2.2.5). Therefore it is obvious that a silylating agent other than BSA has to be included in the attempts to improve the outcome of the tau-QN synthesis. N,N-diethyltrimethylsilylamine (**11**, Figure 3) was the reagent of choice:



11

Fig. 3: *N,N*-diethyltrimethylsilylamine (TMSDEA)

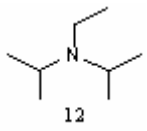
Whereas BSA´s relatively polar silylation by-product acetamide turned out to be very difficult to remove from the selector, diethylamine, the by-product of TMSDEA, is very volatile.[38] This was supposed to be beneficial to the time-and solvent consuming selector purification by column chromatography.
There are many examples for successful silylation of acids and amino acids with TMSDEA but it was obviously unsuitable for the silylation of taurine: even though the silylation was continued for 48 hrs, the reaction mixture never turned clear. As mentioned above, this indicates that the silylation was not quantitative.

2.2) Hünig´s Base (*N,N*-diisopropylethylamine) as HCl Scavenger

Following the standard selector synthesis protocol as described in 2.2, the activated ester is added to the silylated aminoalkanesulfonic acid in its hydrochloride form.
We can assume that the HCl will compromise the conversion of the AE into the ZWIX selector - at least to some extent – due to its ionic properties.

Converting the active ester into its free base before using it for the selector synthesis would be impractical due to its instability towards hydrolysis; therefore an HCl scavenger was employed:

N,N-diisopropylethylamine (Hünig´s base, **12**, Figure 4) was added directly to the reaction mixture in substoichiometric amount prior to the addition of the activated ester.



12

Fig. 4: *N,N*-diisopropylethylamine
(Hünig´s base)

The rest of the procedure was carried out according to the established method. It turned out that Hünig´s base did not bring about the expected improvements in selector yield.

2.3) Product Purification by Precipitation

Although it is often the most suitable way to purify synthesis products, column chromatography is known to affect adversely the yield of synthetic procedures.
This holds true especially in the case of zwitterionic selector synthesis where, in order to separate the selector from the polar silylation by-product acetamide, only the main fractions containing product can be used.
Besides, column chromatography is very time-consuming and uses up large quantities of organic solvents.
For these reasons, alternative purification routes were also included in the efforts to make the selector synthesis route suitable for up-scaling.

Precipitation of products is often used in synthetic chemistry and was thought to be a suitable approach for the zwitterionic selector molecule as well.
Solubility experiments were conducted with the components of the reaction mixture (namely quinine, activated ester, selector, acetamide, nitrophenol) and revealed that the selector was practically insoluble in ethyl acetate, whereas the most unwanted reaction by-product acetamide was readily dissolved in this medium.

Therefore, selector synthesis was carried out according to the standard protocol described above, but instead of perfoming a separation of reaction products by chromatography, the reaction mixture was left standing for several days.
After completion of the reaction, the mixture was concentrated and the residue taken up in ethyl acetate.
Insoluble components were filtrated off and the filtrate analysed by RP-HPLC with UV detection. The process was repeated twice, but separation of acetamide, selector and taurine was not satisfactory.

2.4) One-pot Reaction

In an attempt to reduce time and solvent consumption and thereby make the selector synthesis fit for upscaling, the reaction was carried out as a one-pot synthesis in a minimum of solvent (ca. 15 % of normal amount) with consecutive addition of taurine, activated ester and BSA.
After stirring for 48 hrs, TLC showed product formation.
Nevertheless, after column chromatography the yield was only 12%, which is significantly lower than that achieved with the standard synthesis protocol and does not justify the reduction of solvent consumption.

3) Unrealised Selector: AMSA-QN

3.1) AMSA-QN Synthesis

Synthesis of AMSA-QN was to be carried out in analogy to the selector syntheses described above:
Quinine activated ester (**2**) was prepared by reaction of the cinchona alkaloid with 4-nitrophenyl chloroformate (Figure 5).
AMSA (**13**, Figure 6) was finely ground and silylated with BSA, but, in contrast to the other aminoalkane sulfonic acids, silylation was not complete after 20 hrs, which was indicated by a cloudy (or, at another attempt, even dark brown) mixture instead of a clear solution in the reaction flask.

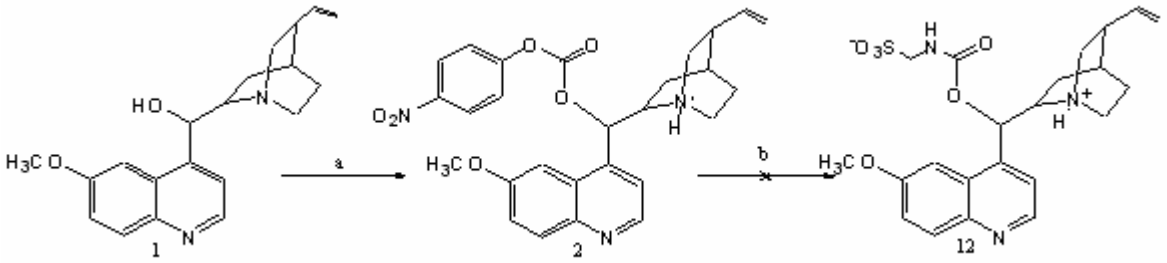


Fig. 5: Synthesis of AMSA-QN (unsuccessful). *Conditions: **a** 4-nitrophenyl chloroformate, toluene$_{dest}$, r.t., 24 hrs, **b** aminomethanesulfonic acid, BSA, DCM$_{HPLC}$, reflux, 20 hrs*

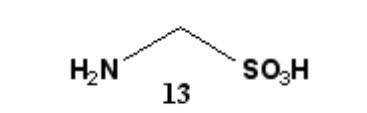


Fig. 6: Aminomethanesulfonic acid (AMSA)

Suspicions that the subsequent conversion with the activated ester had not been successful were confirmed by MS analysis where no molecule peak was found.

The silylation step is known to be crucial to the outcome of the experiment and was therefore repeated with freshly purchased silylating agent and prolonged reaction time (48 hrs) but again, no product was formed according to TLC and MS analysis.
It can be assumed that the silylation of AMSA with BSA is sterically hindered. Therefore, an alternative route to AMSA-QN had to be looked for.

3.2) The Search for an Alternative Route to AMSA-QN

3.2.1) O-iPr as Protective Group

As steric hindrance was suspected to be reason for the failure of the standard synthesis procedure with aminomethanesulfonic acid, it was decided that an alternative reagent for the introduction of a protective group was to be employed.
Based on [39] and [40], aminomethanesulfonic acid was supposed to react with triisopropyl orthoformate according to Figure 7:

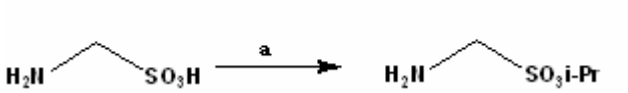


Fig. 7: Introduction of protective group into aminomethanesulfonic acid with triisopropyl orthoformate (not successful). *Conditions:* ***a*** *: $CH(O\text{-}iPr)_3$, DCM_{HPLC}, reflux, 36 hrs*

Reaction control by TLC with permanganate coloring reagent showed that the derivatisation had not been successful.

3.2.2) Silylation with Chlorotrimethylsilane

According to [41] and [42], silyl protective groups can be introduced into amino acids with chlorotrimethylsilane.
Therefore, AMSA was supposed to react with chlorotrimethylsilyl according to Figure 8:

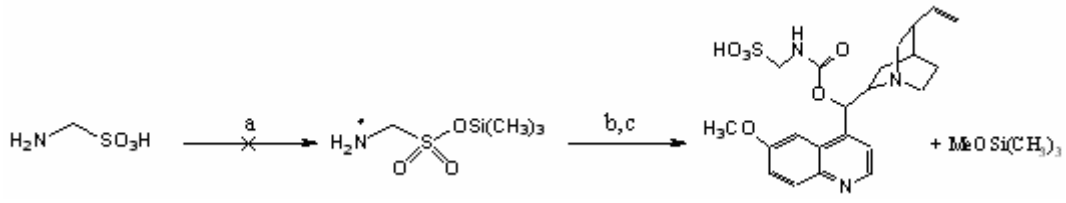


Fig. 8: Synthesis of AMSA-QN with chlorotrimethylsilane as silylating agent (not successful). *Conditions:* ***a*** *$(CH_3)_3SiCl$, DCM_{HPLC} (distilled over $CaCl_2$), reflux (overnight),* ***b*** *NEt_3, QN-AE, r.t., 48 hrs* ***c*** *MeOH*

Even though TLC analyis before quenching (**c**) and during column chromatography purification suggested product formation, MS analysis proved that the synthesis had not been successful.

Apparently, it is due to its small size that AMSA cannot be derivatised and converted into a cinchona-based sulfonic acid-type ZWIX selector by the selector synthesis protocols that were used for its homologues taurine, homotaurine and homohomotaurine.

Finding a synthetic route to AMSA-QN to complete the series of homologous ZWIX selectors exceeds the scope of this work and may be the subject of further experiments.

B) CSP Evaluation

In this chapter, the evaluation of the quinine- and quinidine-based zwitterionic selectors with a set of chiral acidic and zwitterionic analytes is discussed.
Chromatographic parameters are compared for QN- and QD-based CSPs and for CSPs incorporing homologous sulfonic acid side chains.

A separate section is dedicated to the enantioseparation of peptides on the ZWIX CSPs.
Homologous all-L and all-D alanine and valine peptides and several mixed di- and tripeptides were analysed and the results are presented with reference to the separation of peptides on Chirobiotic™ CSPs.
The influence of the water content of the bulk solvent on peptide retention and separation on QN-based ZWIX CSPs was also investigated and the results are presented in the last section of this chapter.

The following chromatographic parameters were determined to evaluate the performance of the individual CSPs:

- **Retention factors k_i**
  The retention factor is calculated from the void time of the column, t_0, and the retention time of the compound:

$$k_i = \frac{t_R - t_0}{t_0}.$$

  In this study, k_1 and k_2 represent the retention factors of the first and second eluted enantiomers, respectively.
  k_i reflects the strength of the interactions between stationary phase and selectand: The stronger the interactions, the longer the retention time and the larger the retention factor. A chiral stationary phase is expected to interact differently with the two enantiomeric forms of an analyte and therefore lead to different retention factors for the R and S enantiomer (amino acids and peptides: D and L enantiomers).

In ZWIX applications, the number of possible selector-selectand interactions (and hence, the retention time) is heavily dependent on the selector loading of the CSP (given as amount of selector/ g stationary phase or µmol selector / m^2 of surface).

Retention factors between 1 and 5 are most desirable for a chromatographic separation, because *k* values below 1 indicate weak selector-selectand interactions while *k* values above 5 lead to impracticably long retention times.

- **Selectivity factors α_{ij}**

α values are descriptive of the separation capabilities of a stationary phase. They are calculated from the retention factors of the analytes:

$$\alpha_{12} = \frac{k_2}{k_1},$$

with k_1 and k_2 representing the retention factors of the first and second eluted enantiomers.

A difference in the strength of the interactions between the stationary phase and the two analytes involved (here: the two enantiomeric forms of a chiral molecule) leads to different retention factors and, therefore, to a selectivity factor greater than 1. An α value of 1 means that there is no (enantio)selectivity of the CSP for this analyte.

High selectivity factors are especially desirable for preparative applications or if one is dealing with unfavourable peak-shapes (e.g. tailing).

To demonstrate enantioselectivity, we define an elution-order dependent selectivity factor α_{EO}: $\alpha_{EO} = \frac{k_L}{k_D}$, with k_L and k_D being the respective retention factors of the L and D enantiomer. Here, $\alpha > 1$ means that the D enantiomer is eluted first, while $\alpha < 1$ means the L enantiomer is eluted first. $\alpha = 1$ indicates that there is no enantioselectivity and both enantiomers have the same retention times.

- **Theoretical plate numbers N_i**

In chromatography, a theoretical plate (originally an expression from the theory of distillation) is a (virtual) section of the column in which equilibrium is established

between mobile and stationary phase. As the analyte travels through the system, this process occurs a number of times – referred to as theoretical plate number N.

N can be calculated from the retention time of the analyte and the peak width at the base (w) or at half maximum ($w_{0,5}$):

$$N = 16\left(\frac{t_R}{w}\right)^2 = 5{,}54\left(\frac{t_R}{w_{0,5}}\right)^2$$

The theoretical plate number serves as a measure for the separation performance of a chromatographic system.

The height of a theoretical plate is calculated from the plate number and the length of the chromatographic column.

- **Chromatographic resolution *R***

  While the selectivity factor reflects the chromatographic separation performance on the grounds of the phase system, the chromatographic resolution takes into account the number of theoretical plates (the efficiency) as well.

$$R = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2}{1 + k_2}.$$

  R is the basis for the optimisation of a chromatographic separation: α and k can be modified by mobile phase variation, N by changing the column length, the mobile phase flow rate or the particle size of the stationary phase.[43]

1.) Comparison of Quinine- and Quinidine-Based ZWIX CSPs

It has been stated before that quinine and quinidine-based selectors can act as pseudo-enantiomers. As CSPs, they often show comparable enantioselectivities for individual analytes, but reversed elution order.

Sets of acidic and zwitterionic analytes and peptides were enantioseparated on tau-QN, homotau-QN and homohomotau-QN and on their quinidine-based homologues to further investigate the pseudo-enantiomeric nature of these zwitterionic selectors.

The mobile phase employed was methanol with 50 mM formic acid and 25 mM diethylamine, which corresponds to an apparent mobile phase pH of 5,4.

Elution of zwitterionic analytes was carried out in isocratic mode - the mobile phase additives act as co- and counterions simultaneously and render gradient elution unnecessary.

1.1) Acidic Analytes

In addition to their obvious capacity for the enantioseparation of zwitterionic compounds, the ZWIX CSPs presented here can also be employed for the enantioseparation of acids or bases. Compared to “classical” anion or cation exchangers, ZWIX CSPs have the advantage of reduced retention times due to the presence of a charge of the same name as the analyte´s on the selector, which is referred to as an intramolecular counterion (IMCI).
N-protected amino acids with diverse protective groups comprise the largest part of acidic analytes. In addition, a chiral drug (Naproxen) and two chiral herbicides (dichlorprop and mecoprop) are included in the acidic analyte set (Figure 9).

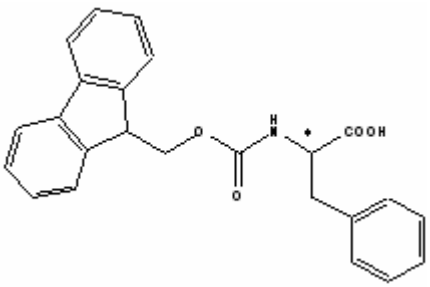
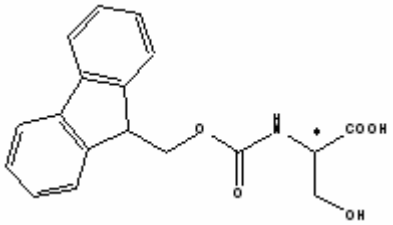
Amino Acid	Symbol	Structural Formula
Fmoc-Phenylalanine	Fmoc-Phe	
Fmoc-Serine	Fmoc-Ser	

Fig. 9: Acidic analytes for the evaluation of zwitterionic chiral stationary phases

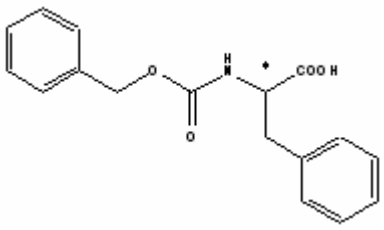
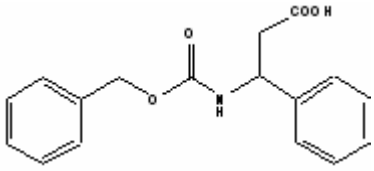
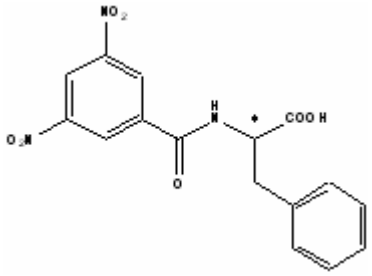
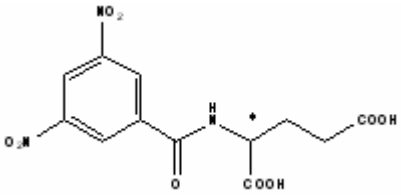
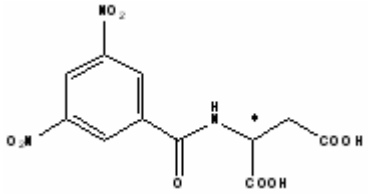
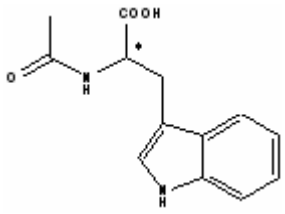
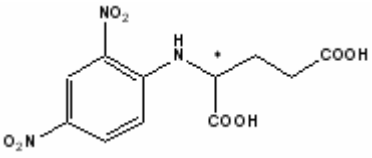
Benzyloxycarbonyl-Phenylalanine	Z-Phe	
Benzyloxycarbonyl-β-Phenylalanine	Z-β-Phe	
Dinitrobenzoyl-Phenylalanine	DNB-Phe	
DNB-Glutamic Acid	DNB-Glu	
DNB-Aspartic Acid	DNB-Asp	
N-Acetyltryptophane	Ac-Trp	
Dinitrophenyl-Glutamic acid	DNP-Glu	

Fig. 9 (continued): Acidic analytes for the evaluation of zwitterionic chiral stationary phases.

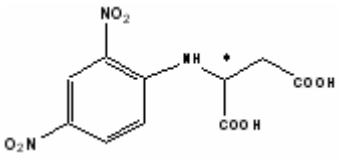
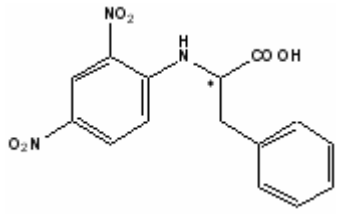
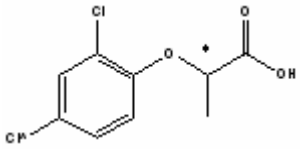
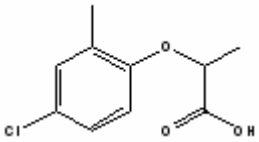
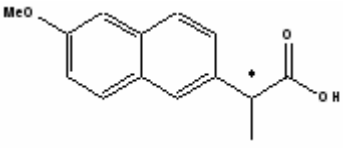
Dinitrophenyl-Aspartic acid	DNP-Asp	
Dinitrophenyl-Phenylalanine	DNP-Phe	
2-(2,4-dichlorophenoxy) propionic acid	Dichlorprop	
2-(4-chloro-2-methylphenoxy)propionic acid	Mecoprop	
Naproxen		

Fig. 9 (continued): Acidic analytes for the evaluation of zwitterionic chiral stationary phases

1.1.1) Tau-QN and Tau-QD

Both tau-QN and tau-QD were able to enantioseparate all of the acidic analytes employed in this study with the exception of Naproxen (not separable on any of the CSPs tested) and Z-β-phenylalanine (not separable on Tau-QD):

It has been mentioned before that selector density on the CSP surface was significantly higher for Tau-QN than for Tau-QD. This was reflected in the retention factors k_1 and k_2, which were always smaller on tau-QD. Nevertheless, enantioselectivites α of the two pseudo-enantiomeric CSPs are comparable and obviously not dependent on the retention time. For example, the overall highest enantioselectivity and resolution were

achieved for DNB-phenylalanine on Tau-QD, where the retention factors of the two enantiomers are less than 50 % (k_1) and 60 % (k_2) of those of Tau-QN (see Table 1.3).

Acidic	Tau-QN (205 µmol SO/g silica)						Tau-QD (150 µmol SO/g silica)					
Analytes	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$
Fmoc-Phe	0,83	1,08	**1,31**	**1,69**	19513	16413	0,41	0,54	**1,33**	**1,52**	23427	36460
Fmoc -Ser	0,72	0,98	**1,35**	**1,86**	21387	18413	0,36	0,55	**1,54**	**1,89**	14333	35893
Z-Phe	0,48	0,56	**1,16**	**0,62**	12193	20287	0,23	0,28	**1,23**	**0,65**	24200	30053
Z-beta-Phe	0,19	0,28	**1,48**	**1,28**	30520	32180	0,15	0,15	**1,00**			
Ac-Trp	0,53	0,93	**1,75**	**3,48**	29013	21760	0,27	0,52	**1,96**	**3,53**	40193	38893
DNB-Glu	1,06	4,20	**3,95**	**8,44**	16673	8660	0,44	1,73	**3,93**	**10,39**	32500	29160
DNB-Asp	2,60	4,90	**1,88**	**4,41**	9467	8613	1,63	2,18	**1,34**	**2,99**	28427	25747
DNB-Phe	1,42	7,62	**5,36**	**9,55**	13073	6820	0,63	4,13	**6,61**	**16,31**	37240	23967
DNP-Glu	1,89	2,48	**1,31**	**1,83**	11127	10160	0,80	1,01	**1,25**	**1,85**	34313	31880
DNP-Asp	2,53	3,62	**1,43**	**2,44**	9460	8560	0,99	1,33	**1,34**	**2,74**	34713	31400
Dichlorprop	0,41	0,56	**1,36**	**1,34**	20667	18913	0,17	0,32	**1,96**	**2,49**	43947	38367
Mecoprop	0,26	0,33	**1,28**	**0,87**	25907	24620	0,11	0,18	**1,68**	**1,34**	37327	54253
Naproxen	0,31	0,31	**1,00**				0,19	0,19	**1,00**			

Table 1.3: Results of Tau-QN and Tau-QD evaluation with acidic analytes.

As depicted in Figure 10, selectivities of Tau-QN and Tau-QD, respectively, are of comparable magnitude for most acidic analytes, but many enantiomers are slightly better separated on Tau-QD:

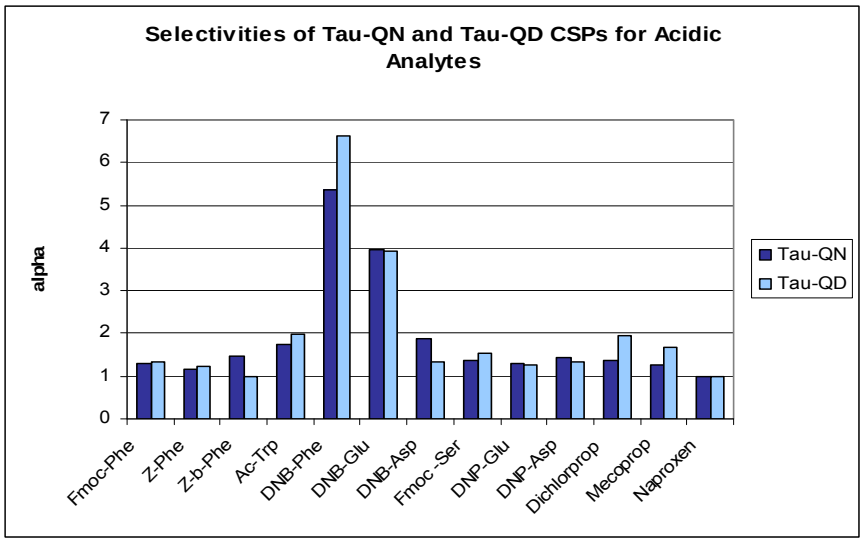


Figure 10: Selectivities of pseudo-enantiomeric tau-QN and tau-QD ZWIX CSPs for acidic analytes

In part, this can be attributed to the fact that the Tau-QN column that was used in these experiments had also been subjected to stress-tests and other demanding analyses

before, which could have led to column bleeding and thus impaired the column´s separation performance. The fact that the Tau-QN column performance obtained in the experiments for this thesis was significantly poorer than its performance in earlier analyses further confirms this suspicion.
(Nevertheless, Homotau-QD and Homohomotau-QD, too, exhibited slightly (or, in the case of DNB-Phe, significantly) better separation capabilities for acidic analytes than their pseudo-enantiomeric counterparts (see Figures 16 and 18).)

DNB protected amino acids were particularly well separated. According to [32], the reason for this is a combination of ionic interactions and the extremely advantageous $\pi-\pi$ interaction of the electron-poor (π-acidic) aromatic substituent (DNB) of the selectand and the electron-rich (π-basic) quinoline moiety of the selector. Hydrogen bonding between the carbamate function of the selector and the amide of the DNB-protected acid is crucial for the enantiodiscrimination.
In contrast, the enantioseparation of amino acids with a DNP protective group was not nearly as successful, which is due to the lack of an amide bond in the analyte.

As a rule of thumb, on QN-based CSPs the D enantiomer of a chiral analyte is eluted before the L enantiomer (there are some exceptions to this, such as DNP-glutamic acid and DNP-aspartic acid).
Elution order is usually inverted with QD-based chiral stationary phases, where with most analytes the L enantiomer is eluted before the D enantiomer.
These presuppositions were confirmed for all acidic analytes on Tau-QN and Tau-QD. Fig. 11 illustrates the reversal of elution order for the acidic analytes Ac-Trp and DNP-Glu. Elution orders were determined by injection of an enantiomerically pure (either D or L) sample of the analyte.

Table 1.4 presents the elution orders of acidic analytes on Tau-QN and Tau-QD.

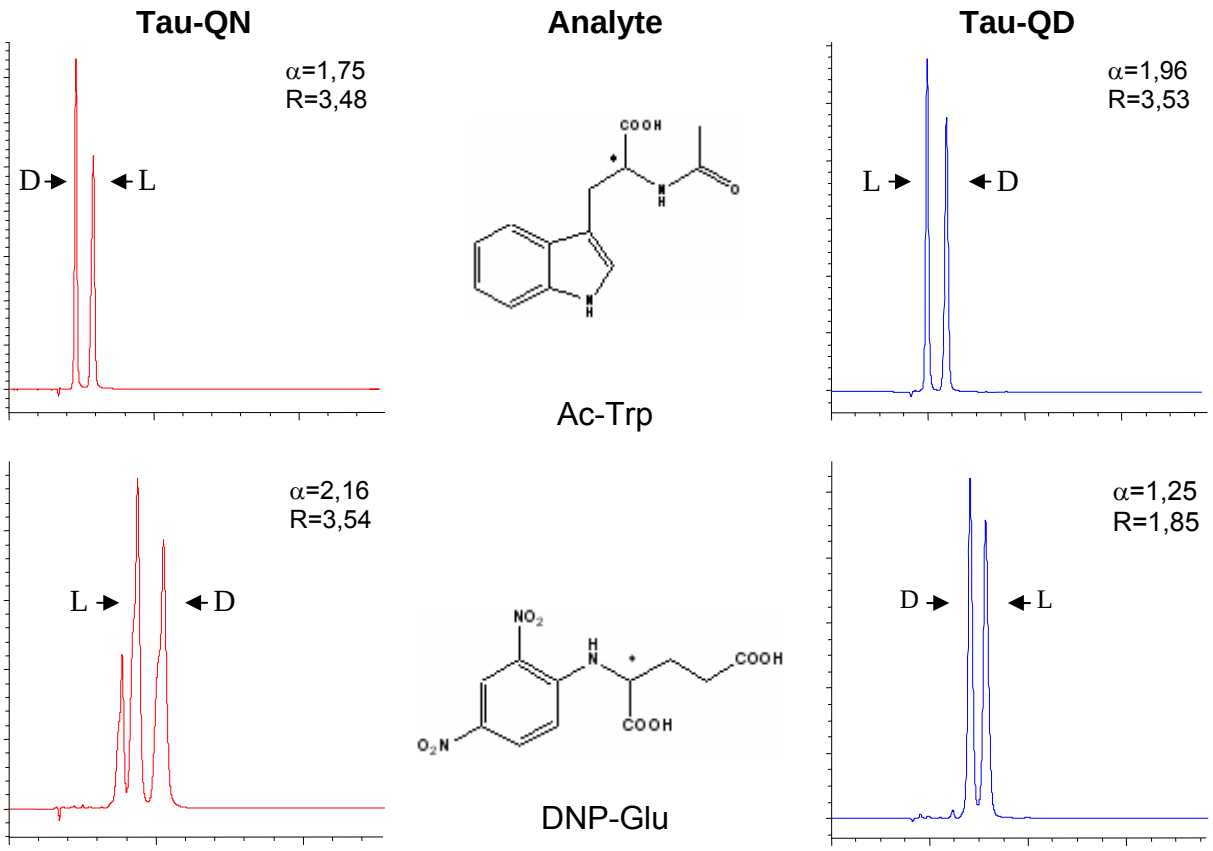


Fig. 11: Reversal of elution order for acidic analytes on Tau-QN and Tau-QD CSPs.

Analyte	**EO* (Tau-QN)**	**EO* (Tau-QD)**
Fmoc-Phe	D	L
Z-Phe	D	L
Z-β-Phe	D	no sep.
Ac-Trp	D	L
Fmoc-Ser	D	L
DNB-Phe	D	n.d.
DNP-Glu	L	D
DNP-Asp	L	D

Table 1.4: Elution orders of acidic analytes on Tau-QN and Tau-QD. (* EO: Konfiguration of first eluted enantiomer)

1.1.2) Homotau-QN and Homotau-QD

All acidic analytes with the exception of Naproxen could be enantioseparated on Homotau-QN and Homotau-QD.

Acidic	Homotau-QN (280 µmol SO/g silica)						Homotau-QD (188 µmol SO/ g silica)					
Analytes	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$
Fmoc-Phe	1,37	1,79	**1,30**	**2,80**	32960	32093	0,56	0,71	**1,28**	**1,52**	25720	29960
Fmoc -Ser	1,30	1,69	**1,30**	**2,81**	35820	33727	0,50	0,66	**1,34**	**1,50**	16513	28273
Z-Phe	0,86	0,99	**1,14**	**1,17**	35773	36160	0,33	0,39	**1,19**	**0,60**	22347	16700
Z-beta-Phe	0,40	0,76	**1,90**	**3,99**	24467	23033	0,12	0,20	**1,62**	**1,20**	40660	34067
Ac-Trp	1,01	1,88	**1,87**	**6,77**	41987	36293	0,37	0,69	**1,87**	**3,76**	33193	34667
DNB-Glu	2,44	11,48	**4,70**	**16,91**	29780	22400	0,87	3,48	**3,99**	**11,77**	19287	23233
DNB-Asp	7,41	>12	**>1,62**	**n.d.**	n.d.	n.d.	3,26	4,01	**1,23**	**1,98**	15460	17080
DNB-Phe	2,70	15,29	**5,67**	**18,29**	35453	35467	1,04	5,09	**4,87**	**15,19**	24933	24993
DNP-Glu	4,93	6,14	**1,25**	**2,78**	26293	22193	1,61	1,81	**1,13**	**1,11**	24607	22147
DNP-Asp	6,83	10,04	**1,47**	**5,19**	25107	24893	2,18	2,67	**1,23**	**1,87**	16807	18820
Dichlorprop	0,86	1,08	**1,25**	**2,21**	44033	41727	0,30	0,49	**1,64**	**2,56**	39093	35733
Mecoprop	0,53	0,64	**1,20**	**1,44**	47600	46860	0,20	0,28	**1,44**	**1,25**	34780	34713
Naproxen	0,43	0,43	**1,00**				0,22	0,22	**1,00**			

Table 1.5: Results of Homotau-QN and Homotau-QD CSP evaluation with acidic analytes.

Even though there was a significant difference in the retention factors of the individual analyte enantiomers on Homotau-QN and Homotau-QD, the respective selectivity coefficients α were almost always very similar (Table 1.4)

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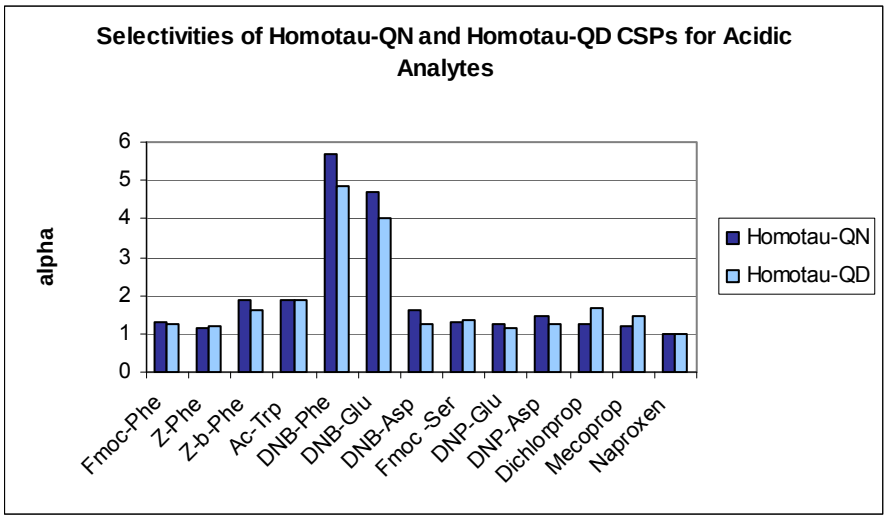


Fig. 12: Selectivities of pseudo-enantiomeric Homotau-QN and Homotau-QD ZWIX CSPs for acidic analytes

With the exception of DNP-glutamic acid and DNP-aspartic acid, the L enantiomers of the acidic analytes were eluted from the Homotau-QN column after the D enantiomers (elution order of Z-β-phenylalanine could not be determined due to lack of an enantiomerically pure sample).

In all cases, elution order was reversed on Homotau-QD with L eluting before D (D before L for DNP-glutamic acid and DNP-aspartic acid) (Figure 13).

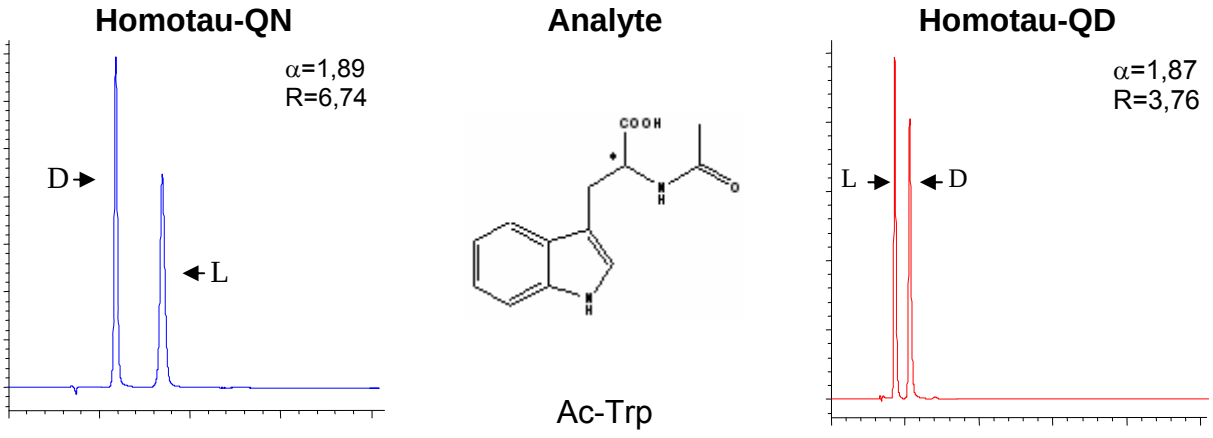


Fig. 13: Reversal of elution order for an acidic analyte on Homotau-QN and Homotau-QD CSPs.

Table 1.6 presents the elution orders of chiral acidic analytes on Homotau-QN and Homotau-QD.

Analyte	EO* (Homotau-QN)	EO* (Homotau-QD)
Fmoc-Phe	D	L
Z-Phe	D	L
Ac-Trp	D	L
Fmoc-Ser	D	L
DNP-Glu	L	D
DNP-Asp	L	D

Table 1.6: Elution orders of acidic analytes on Homotau-QN and Homotau-QD. (* EO: Konfiguration of first eluted enantiomer)

1.1.3) Homohomotau-QN and Homohomotau-QD

Homohomotau-QN and Homohomotau-QD were able to separate all chiral acidic analytes except for Naproxen (Table 1.7).

Acidic	Homohomotau-QN (254 µmol SO / g silica)						Homohomotau-QD (187 µmol SO / g silica)					
Analytes	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$
Fmoc-Phe	1,40	1,86	**1,33**	**2,74**	28007	25527	0,44	0,59	**1,34**	**1,50**	22507	26927
Fmoc -Ser	1,35	1,77	**1,31**	**2,39**	26693	19940	0,39	0,54	**1,38**	**1,36**	15027	24867
Z-Phe	0,93	1,07	**1,15**	**1,22**	34887	32400	0,25	0,31	**1,25**	**0,69**	21467	20613
Z-beta-Phe	0,30	0,41	**1,34**	**1,52**	63667	32247	0,11	0,17	**1,59**	**1,03**	41507	35193
Ac-Trp	1,03	1,70	**1,65**	**4,86**	32927	30653	0,30	0,49	**1,64**	**2,28**	26900	31793
DNB-Glu	2,61	10,71	**4,10**	**15,07**	24453	21027	0,60	2,53	**4,23**	**11,71**	27727	24960
DNB-Asp	8,51	11,87	**1,40**	**3,66**	14180	17300	2,08	2,74	**1,32**	**2,73**	21540	20333
DNB-Phe	2,74	12,95	**4,73**	**n.d.**	n.d.	n.d.	0,77	4,22	**5,49**	**12,79**	14640	19347
DNP-Glu	5,57	6,53	**1,17**	**1,90**	21240	20773	1,20	0,14	**1,14**	**1,17**	27673	26193
DNP-Asp	8,39	11,57	**1,38**	**3,88**	19193	19360	1,50	1,87	**1,25**	**2,11**	25520	24633
Dichlorprop	1,04	1,26	**1,21**	**1,77**	33440	32733	0,22	0,37	**1,65**	**1,95**	32280	32640
Mecoprop	0,68	0,78	**1,16**	**1,14**	38847	15393	0,14	0,21	**1,43**	**0,89**	30780	30787
Naproxen	0,43	0,43	**1,00**				0,20	0,20	**1,00**			

Table 1.7: Results of Homohomotau-QN and Homohomotau-QD CSP evaluation with acidic analytes.

Again, due to the lower surface coverage of the QD selector, enantiomer retention factors k_1 and k_2 were significantly smaller on Homohomotau-QD than on Homohomotau-QN. Nevertheless, enantioselectivities of the two CSPs were comparable or even higher on Homohomotau-QD, which further supports the statement made above that selectivity is not necessarily dependent on the retention times of the analytes (Figure 14).

Resolution, though, was almost always higher on Homohomotau-QN which can be attributed to the higher selector coverage of this CSP.

Similarly to the taurine and homotaurine selectors, the best results were obtained for DNB protected amino acids. This illustrates the importance of H-bonding between the carbamate moiety of the selector and the amide of the selectand.

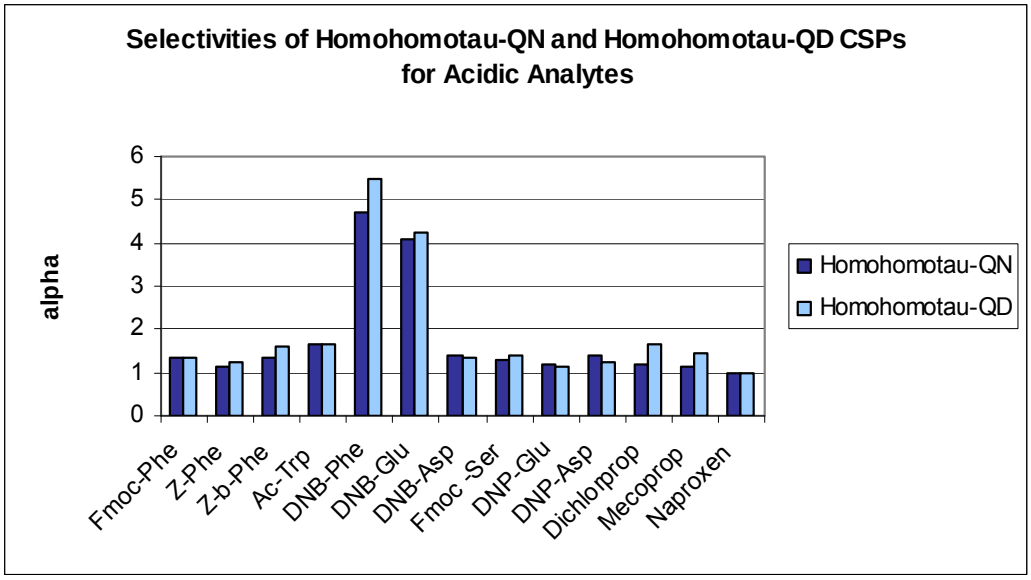


Fig. 14 : Selectivities of pseudo-enantiomeric Homohomotau-QN and Homohomotau-QD ZWIX CSPs for acidic analytes

Elution order for acidic analytes on Homohomotau-QN was usually D before L. This order was inverted in all cases on Homohomotau-QD (Figure 15, Table 1.8).

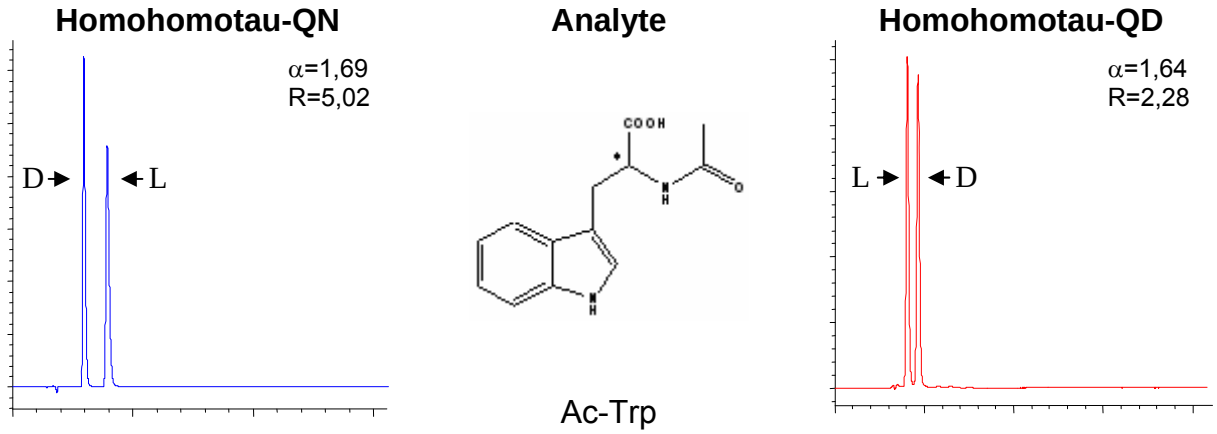


Fig. 15: Reversal of elution order for an acidic analyte on Homohomotau-QN and Homohomotau-QD CSPs.

Table 1.8 presents elution orders of acidic analytes on Homohomotau-QN and Homohomotau-QD.

Analyte	**EO* (Homohomotau-QN)**	**EO* (Homohomotau-QD)**
Fmoc-Phe	D	L
Z-Phe	D	L
Z-β-Phe	n.d.	n.d.
Ac-Trp	D	L
Fmoc-Ser	D	L
DNB-Phe	D	n.d.
DNP-Glu	L	D
DNP-Asp	L	D

Table 1.8: Elution orders of acidic analytes on Homohomotau-QN and Homohomotau-QD. (* EO: Konfiguration of first eluted enantiomer)

1.2) Amphoteric (Zwitterionic) Analytes

In amphoteric molecules, both acidic and basic functional groups are present. These functional groups are protonated or deprotonated according to the pH of their surrounding environment. At a certain pH which is characteristic for every amphoteric compound,

the net charge of the molecule will be zero. This pH is called the isoelectric point of the substance and can be calculated from the pk_a values of the functional groups. Above and below this pH, the compound has a positive or negative net charge resulting from the predominant protonation of basic or deprotonation of acidic functional groups, respectively. This means that in most of the pH range, amphoteric compounds exist as zwitterions (positive and negative charges within one molecule).

Zwitterionic analytes cannot be separated on conventional cation or anion exchangers. This is due to the second charge present in the selectand which has the same name as the ion exchanger and acts as an intramolecular counterion that prevents retention.

Therefore it is necessary to use zwitterionic chiral ion exchangers (ZWIX) such as the ones presented here.

Figure 16 shows the chiral amino acids used as zwitterionic analytes in this study and their structures.

All experiments were carried out with a mobile phase of methanol with 50 mM formic acid and 25 mM DEA, which corresponds to a slightly acidic apparent pH of 5,4.

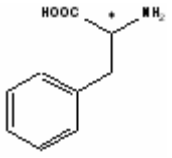
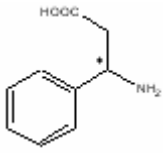
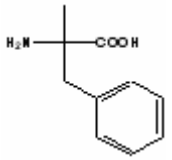
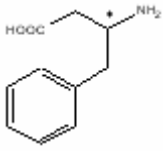
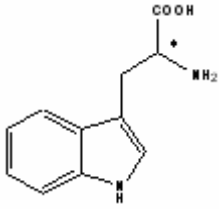
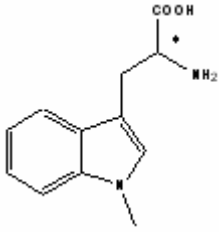
Amino Acid	Symbol	Structural Formula	Amino Acid	Symbol	Structural Formula
Phenyl-alanine	Phe		β-Phenyl-alanine	β-Phe	
α-Methyl-phenylalanine	α-Me-Phe		β-Homo-Phenyl-alanine	*β–Homo-Phe*	
Tryptophane	Trp		1-Methyl-tryptophane	1-Me-Trp	

Fig. 16: Chiral amphoteric (zwitterionic) analytes for ZWIX evaluation

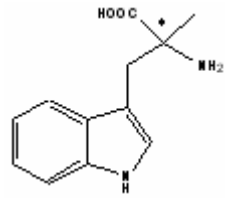
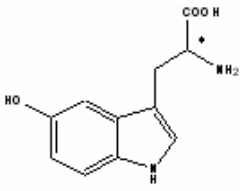
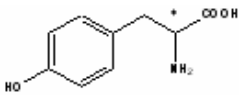
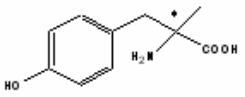
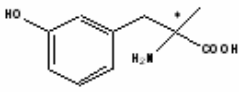
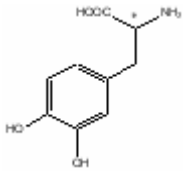
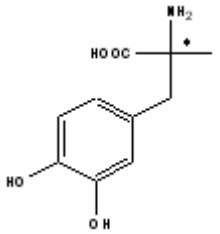
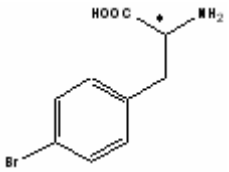
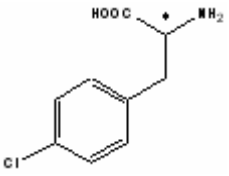
α-Methyl-tryptophane	α-Me-Trp		5-Hydroxy-tryptophane	5-OH-Trp	
Tyrosine	Tyr		α-Methyl-tyrosine	α-Me-Tyr	
α-Methyl-*meta*-Tyrosine	α-Me-m-Tyr		3,4-Dihydroxy-phenyla-lanine	DOPA	
α-Methyl-3,4-dihydroxy-phenylalanine	α-Me-DOPA		*para*-Bromo-phenyla-lanine	p-Br-Phe	
			para-Chloro-phenylala-nine	p-Cl-Phe	

Fig. 16 (continued): Chiral amphoteric (zwitterionic) analytes for ZWIX evaluation

1.2.1) Tau-QN and Tau-QD

Enantioseparation of several chiral zwitterionic (amphoteric) compounds on both Tau-QN and Tau-QD was not satisfactory: Tau-QN could only separate 5 out of 10 analytes with relatively small resolution values, Tau-QD separated 7 out of 10.

Even though the selector surface coverage of Tau-QD is significantly lower and therefore retention times are shorter, higher resolution and higher plate numbers were achieved with the latter CSP compared to tau-QN (Table 1.6).

Zwitterionic	Tau-QN (205 µmol SO / g silica)						Tau-QD (150 µmol SO / g silica)					
Analytes	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$
Phe	0,72	0,76	**1,04**	**0,42**	73713	42367	0,46	0,46	**1,00**			
beta–Phe	1,35	1,35	**1,00**				0,93	1,01	**1,09**	**0,78**	47640	33000
alpha-Me-Phe	0,03	0,03	**1,00**				0,38	0,47	**1,25**	**1,37**	49433	43993
Trp	1,43	2,18	**1,53**	**4,31**	24887	29847	0,80	1,10	**1,39**	**3,01**	40280	38573
alpha-Me-Trp	1,33	3,22	**2,42**	**10,34**	34853	33900	0,72	1,68	**2,35**	**8,38**	41547	37440
DOPA	1,46	1,46	**1,00**				0,72	0,72	**1,00**			
alpha-Me-DOPA	1,09	1,57	**1,44**	**3,36**	27000	29113	0,56	0,88	**1,57**	**3,51**	39893	37813
p-Br-Phe	1,02	1,02	**1,00**				0,11	0,61	**5,45**	**7,38**	50000	39760
p-Cl-Phe	0,9	0,9	**1,00**				0,09	0,54	**6,28**	**6,40**	30407	41793
Tyr	0,91	0,97	**1,07**	**0,46**	38093	16787	0,50	0,50	**1,00**			

Table 1.9: Results of Tau-QN and Tau-QD CSP evaluation with zwitterionic analytes

DOPA was the only compound that could neither be separated on Tau-QN nor on Tau-QD. Interestingly, all other analytes not enantioseparated by Tau-QN were separable on tau-QD and vice versa (Figure 17).

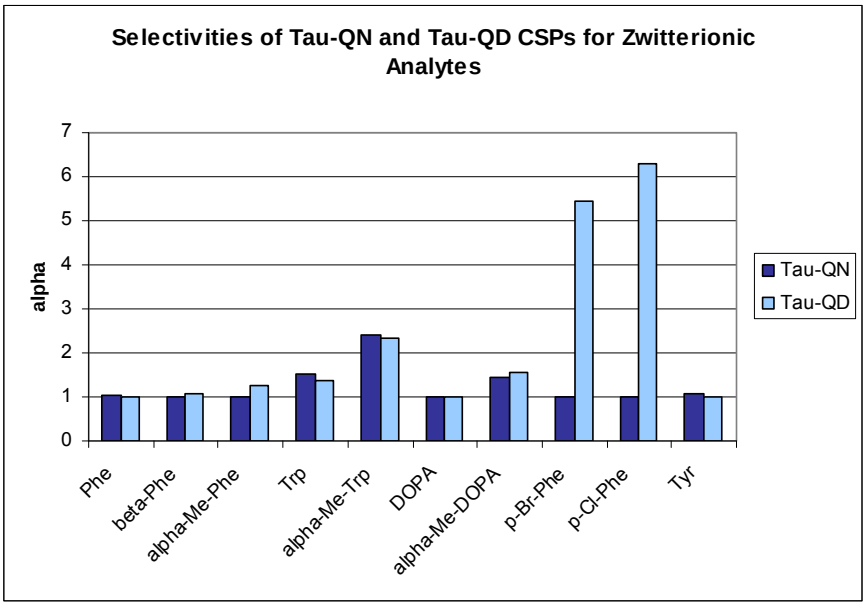


Figure 17: Selectivities of pseudo-enantiomeric Tau-QN and Tau-QD ZWIX CSPs for zwitterionic analytes

Analyte	EO* (Tau-QN)	EO* (Tau-QD)
Phe	D	no separation
Trp	D	n.d.
Tyr	D	no separation

Table 1.10: Elution orders of amphoteric analytes on Tau-QN and Tau-QD. (* EO: Konfiguration of first eluted enantiomer

Nevertheless, enantioselectivites were generally small, especially for the unprotected amino acids phenylalanine, β-phenylalanine and tyrosine. Eye-catching exceptions are the *para* substituted aromatic amino acids *p*-Br-Phe and *p*-Cl-Phe, whose selectivity coefficients on Tau-QD are higher than 5. However, the high enantioselectivity values are due to particularly short retention times. It remains to be investigated in more detail if there exists a beneficial influence of the substituents on the π-acidity of the analytes.

It is also remarkable that a methyl substituent in α position to the carboxyl group significantly enhances enantioselectivity (see Figure 17: Phe – α-Me-Phe, Trp – α-Me-Trp, DOPA – α-Me-DOPA).

On Tau-QN, the D enantiomers of phenylalanine, tryptophane and tyrosine were eluted before their respective L enantiomers (Phe and Tyr could not be separated on Tau-QD).

1.2.2) Homotau-QN and Homotau-QD

Homotau-QN and Homotau-QD were able to enantioseparate, albeit with rather low selectivities, the majority of amphoteric (zwitterionic) analytes (Table 1.11).The relatively short retention times (< 10 min) reflected in retention factors below 6 produced acceptable resolution values nevertheless.
Enantioselectivities for zwitterionic analytes were mostly between 1 and 2, with the striking exception of β-Phe (Figure 18). Apparently, in this case the proportions of the selector and the selectand are particularly favourable to achieve discrimination of one enantiomer.

Zwitterionic	Homotau-QN (280 µmol SO/ g silica)						Homotau-QD (188 µmol SO / g silica)					
Analytes	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$
Phe	0,89	1	**1,12**	**1,14**	47047	43173	0,45	0,45	**1,00**			
beta–Phe	1,92	3,6	**4,73**	**14,01**	108787	43333	0,12	0,75	**6,45**	**8,21**	43240	33613
alpha-Me-Phe	0,79	1,05	**1,32**	**2,50**	37713	37973	0,38	0,48	**1,28**	**1,31**	34453	33093
Trp	1,91	3,55	**1,86**	**7,95**	35760	34107	0,83	1,25	**1,50**	**3,41**	30900	29267
alpha-Me-Trp	1,83	5,65	**3,10**	**14,31**	36540	32420	0,87	0,96	**1,11**	**0,73**	23540	23607
DOPA	1,62	1,96	**1,21**	**2,03**	28980	28620	0,66	0,73	**1,10**	**0,56**	37307	16453
alpha-Me-DOPA	1,49	2,28	**1,54**	**4,84**	35207	31527	0,60	0,85	**1,42**	**2,39**	29333	28740
p-Br-Phe	1,25	1,35	**1,08**	**0,71**	27780	23840	0,62	0,62	**1,00**			
p-Cl-Phe	1,09	1,18	**1,08**	**0,67**	33213	26293	0,55	0,55	**1,00**			
Tyr	1,15	1,34	**1,16**	**1,35**	30613	27927	0,23	0,55	**2,39**	**2,69**	17553	12860

Table 1.11: Results of Homotau-QN and Homotau-QD evaluation with zwitterionic (amphoteric) analytes.

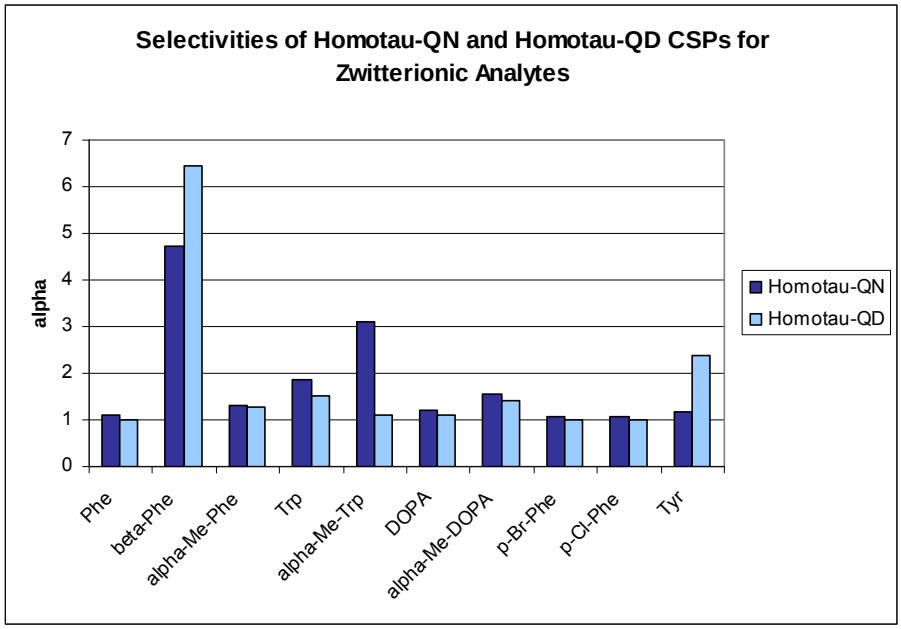


Fig. 18: Selectivities of pseudo-enantiomeric Homotau-QN and Homotau-QD ZWIX CSPs for zwitterionic analytes

Reversal of elution order of amphoteric analytes was achieved on Homotau-QN and Homotau-QD (Figure 19, Table 1.12).

Elution orders were determined by injection of enantiomerically pure samples of the analyte.

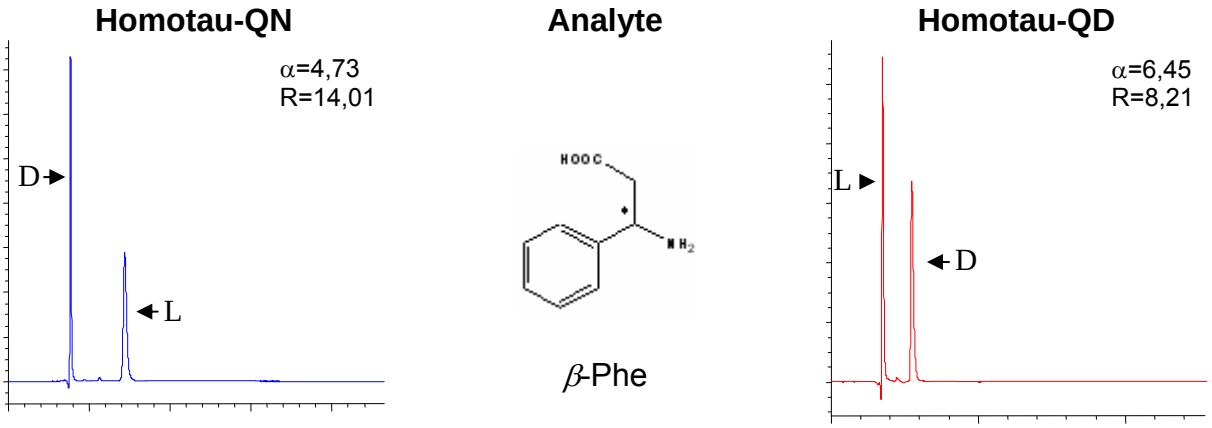


Fig. 19: Reversal of elution order for an amphoteric analyte on Homotau-QN and Homotau-QD CSPs.

Analyte	EO* (Homotau-QN)	EO* (Homotau-QD)
Phe	D	no sep.
Trp	D	n.d.
Tyr	D	n.d.

Table 1.12: Elution orders of amphoteric analytes on Homotau-QN and Homotau-QD. (* EO: Konfiguration of first eluted enantiomer)

1.2.3) Homohomotau-QN and Homohomotau-QD

Whereas half of the zwitterionic chiral analytes remained unseparated on Homohomotau-QD, enantioseparation was achieved in all cases on Homohomotau-QN (Table 1.13).

Zwitterionic Analytes	Homohomotau-QN (254 µmol SO / g silica)						homohomotau-QD (187 µmol SO / g silica)					
	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$
Phe	0,56	0,62	**1,11**	**0,63**	31980	23187	0,44	0,44	**1,00**			
beta–Phe	0,87	0,93	**1,07**	**0,55**	49933	27033	0,11	0,85	**7,99**	**9,24**	41060	33420
alpha-Me-Phe	0,47	0,63	**1,32**	**1,78**	34280	35187	0,38	0,46	**1,23**	**1,02**	31507	29280
Trp	1,19	1,98	**1,67**	**5,04**	29800	28307	0,83	1,06	**1,27**	**1,75**	24460	23360
alpha-Me-Trp	1,11	2,96	**2,67**	**9,81**	30680	26233	0,83	0,89	**1,07**	**0,48**	44860	18560
DOPA	1,00	1,21	**1,21**	**1,50**	24067	23053	0,74	0,74	**1,00**			
alpha-Me-DOPA	0,95	1,44	**1,53**	**3,28**	23200	21847	0,62	0,84	**1,36**	**1,84**	22780	21147
p-Br-Phe	0,76	0,83	**1,09**	**0,61**	34913	22393	0,59	0,59	**1,00**			
p-Cl-Phe	0,68	0,74	**1,09**	**0,58**	41400	22587	0,53	0,53	**1,00**			
Tyr	0,75	0,87	**1,15**	**0,77**	17600	14860	0,55	0,55	**1,00**			

Table 1.13: Results of Homohomotau-QN and Homohomotau-QD CSP evaluation with zwitterionic analytes

The respective chromatographic resolution was acceptable and led to a baseline separation for many of the analytes.

Even though the selectivity of homohomotau-QD was low or even resulted in an α value of 1 (no separation) in most cases, the overall highest enantioselectivity was obtained on this CSP, namely for β-phenylalanine (Figure 20).

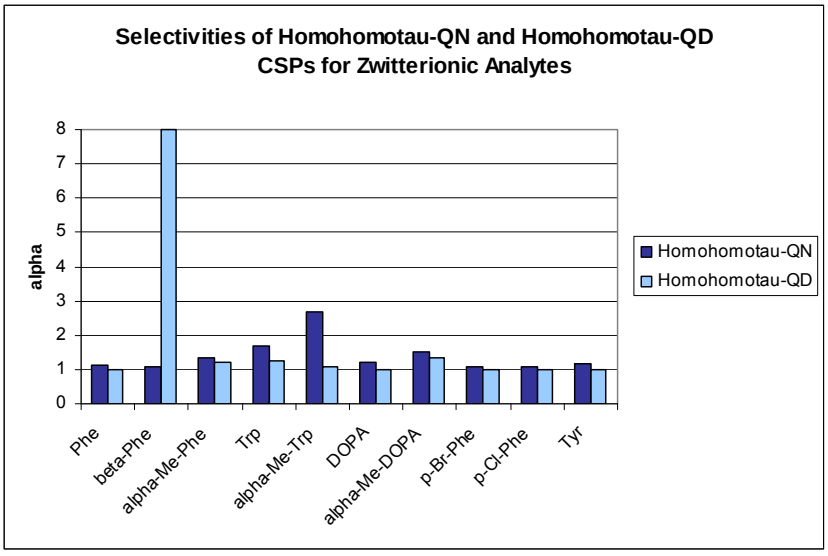


Fig. 20: Selectivities of pseudo-enantiomeric Homohomotau-QN and Homohomotau-QD ZWIX CSPs for zwitterionic analytes

Elution orders on Homohomotau-QN were determined for Phe, Trp and Tyr by injection of an enantiomerically pure sampe into the HPLC system. In all three cases, the D enantiomers were eluted first (Figure 21).

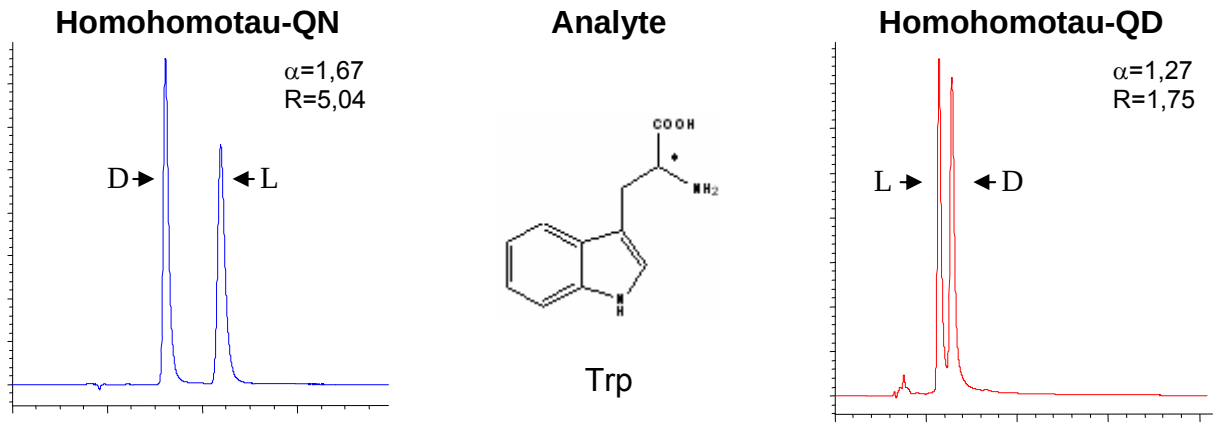


Fig. 21: Reversal of elution order for an amphoteric analyte on Homohomotau-QN and Homohomotau-QD CSPs.

Table 1.14 depicts the elution orders obtained for zwitterionic analytes on Homohomotau-QN and Homohomotau-QD CSPs.

Analyte	**EO*** **(Homohomotau-QN)**	**EO*** **(Homohomotau-QD)**
Phe	D	no sep.
Trp	D	n.d.
Tyr	D	n.d.

Table 1.14: Elution orders of amphoteric analytes on Homohomotau-QN and Homohomotau-QD. (* EO: Konfiguration of first eluted enantiomer)

1.3) Peptide Enantiomers

Due to their amphoteric nature and chirality, peptides represent promising targets for enantioseparation on zwitterionic chiral stationary phases, especially since the ZWIX CSPs offer an opportunity to separate peptides without the need for derivatisation.

Figure 22 presents the amino acids that comprise the building blocks of the peptides investigated as well as their pK_a values.

In this study, different groups of peptides were investigated: Mixed peptides consisting of two or three amino acids as well as homologous peptides on the basis of amino acids alanine and valine.
The analytes to be separated on the zwitterionic chiral stationary phases were all-L and all-D enantiomers of the individual peptides shown in Figure 23.

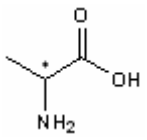
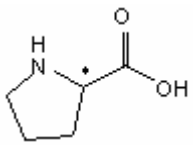
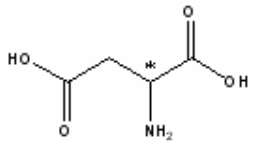
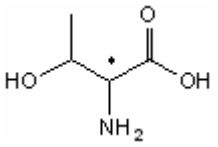
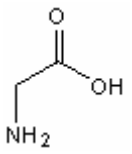
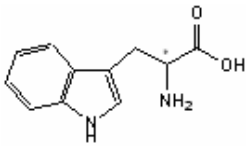
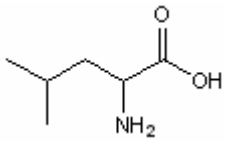
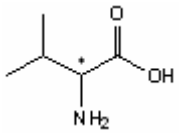
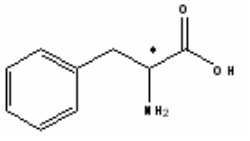
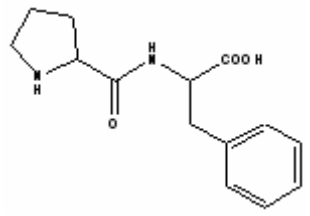
Amino Acid	Structure	Sym-bol	Amino Acid	Structure	Sym-bol
Alanine pK_a=2,35/9,87		Ala	Proline pK_a=1,95/10,64		Pro
Aspartic Acid pK_a=1,99/3,90/9,90		Asp	Threonine pK_a=2,17/9,00		Thr
Glycine pK_a=2,35/9,78		Gly	Tryptophane pK_a=2,38/9,44		Trp
Leucine pKa=2,33/9,74		Leu	Valine pK_a=2,29/9,72		Val
Phenylalanine pK_a=1,83/9,13		Phe			

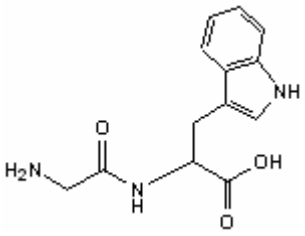
Fig. 22: Amino acid building blocks for peptides investigated in this work

Mixed Dipeptides

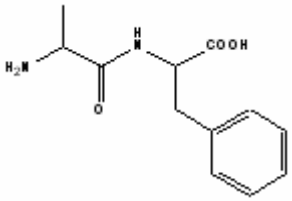
Pro-Phe



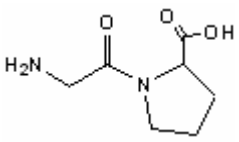
Gly-Trp



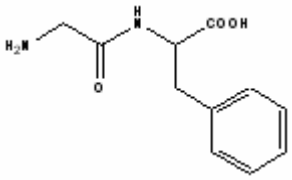
Ala-Phe



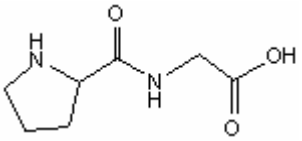
Gly-Pro



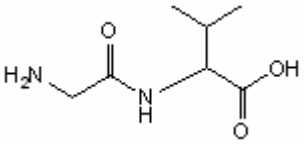
Gly-Phe



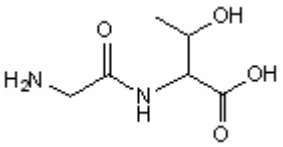
Pro-Gly



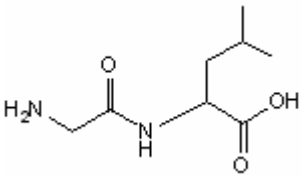
Gly-Val



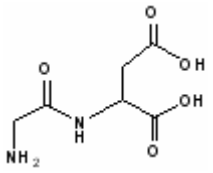
Gly-Thr



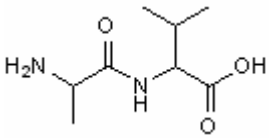
Gly-Leu



Gly-Asp

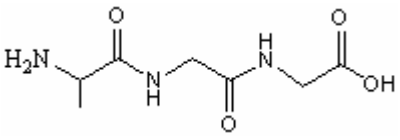


Ala-Val



Tripeptides

Ala-Gly-Gly



Gly-Gly-Ala

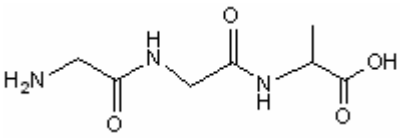
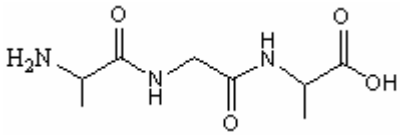


Fig. 23: Di-, tri- and oligopeptides used in the evaluation of ZWIX CSPs.

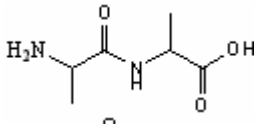
Tripeptides (continued)

Ala-Gly-Ala

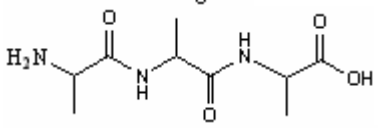


Homologous Ala Peptides

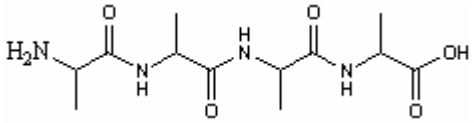
Ala-2



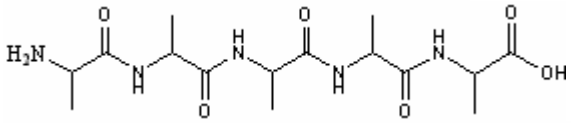
Ala-3



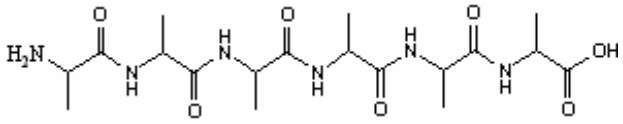
Ala-4



Ala-5

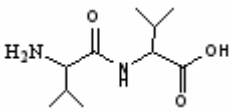


Ala-6

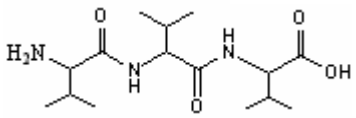


Homologous Valine Peptides

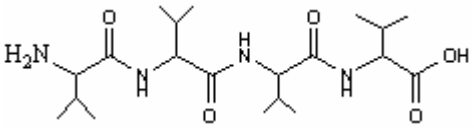
Val-2



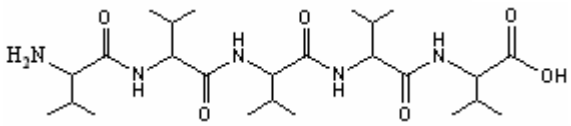
Val-3



Val-4



Val-5



Val-6

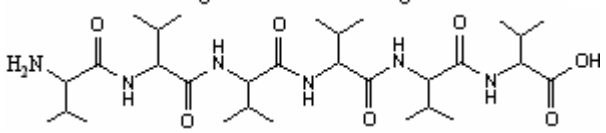


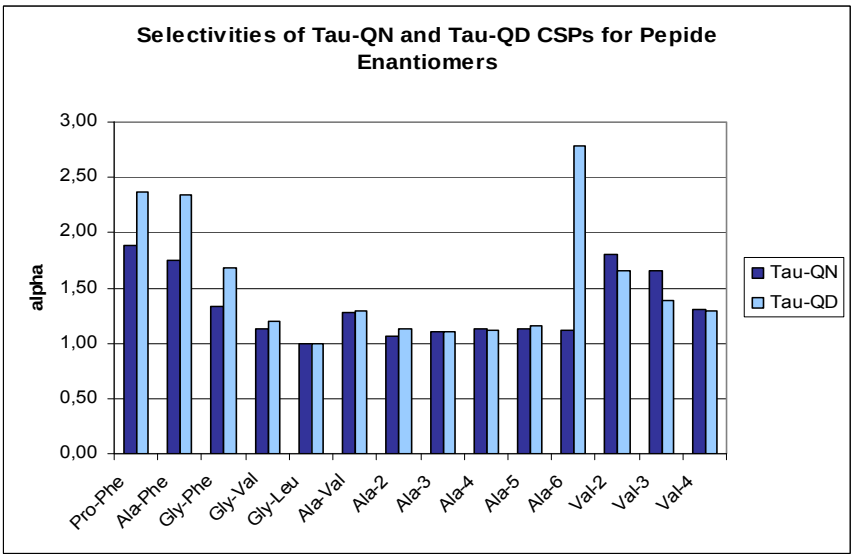
Fig. 23 (continued): Di-, tri- and oligopeptides used in the evaluation of ZWIX CSPs.

Even though the retention of peptides was significantly higher on the QN CSPs than on the QD-based ones due to the lower selector loadings of the latter, mixed dipeptides were better separable on QD CSPs (Figure 24).

In contrast, QN CSPs often exhibited better separation capabilities for homologous valine peptides.

Alanine peptides, too, were slightly better separated on QD-based CSPs.

Similar to acidic and other zwitterionic analytes, the elution order for peptides on QN-based CSPs was usually all-D before all-L and the other way around for QD-based stationary phases.



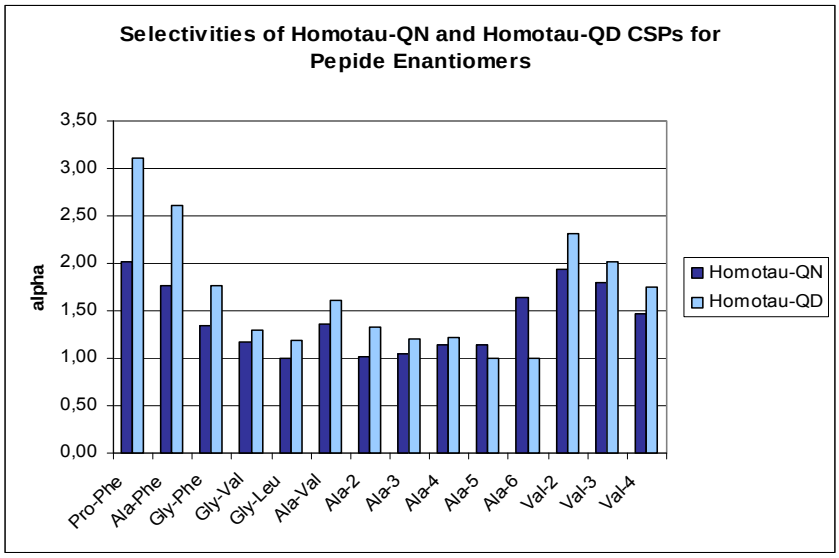


Fig. 24: Selectivity factors of ZWIX CSPs for peptide enantiomers (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min, CAD detection).

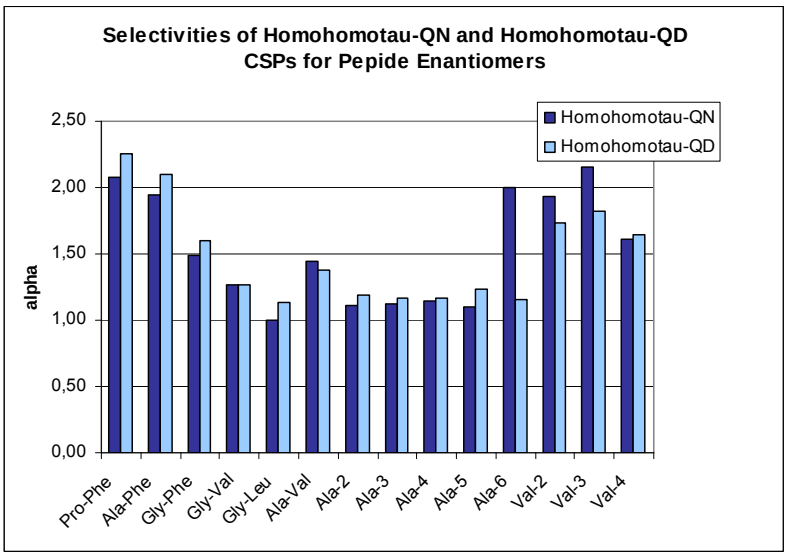
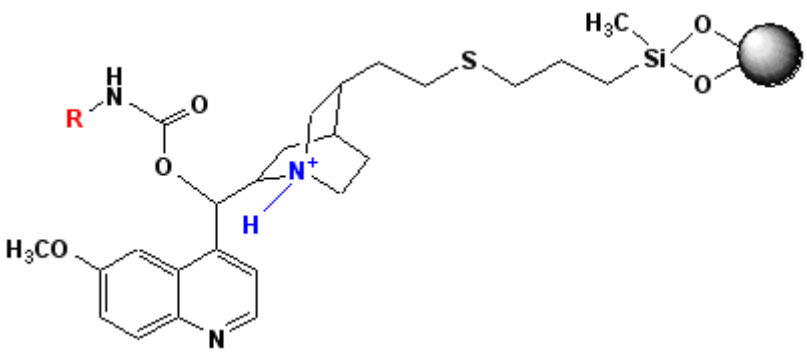


Fig. 24 (continued): Selectivity factors of ZWIX CSPs for peptide enantiomers enantiomers (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min, CAD detection)

2.) Comparison of Homologous ZWIX Selectors



R	*selector*	*aminosulfonic acid*
$(CH_2)_2SO_3^-$	*tau-QN, tau-QD*	*taurine*
$(CH_2)_3SO_3^-$	*homotau-QN homotau-QD*	*homotaurine*
$(CH_2)_4SO_3^-$	*homohomotau-QN, homohomotau-QD*	*homohomotaurine*

Fig. 25: Structure of zwitterionic selectors. Red: Cation exchanging site (sulfonic acid), blue: anion exchanging site (protonated quinuclidine moiety)

Figure 25 shows a ZWIX selector based on a sulfonic acid cation exchanging site (R) and an amine anion exchanging site (protonated quinuclidine nitrogen atom):

In the course of this work, selectors with homologous sulfonic acid cation exchanging sites R were prepared.
These “homologous” selectors were evaluated for their enantioseparation capabilities with acidic and zwitterionic chiral analytes (for structural formulae, see 1.1. and 1.2) and the results were compared for the three QN-based selectors tau-QN, homotau-QN and homohomotau-QN as well as for tau-QD, homotau-QD and homohomotau-QD.

2.1) Comparison of Quinine-Based ZWIX selectors

The tau-QN, homotau-QN and homohomotau-QN CSPs employed in this study possess comparable selector loadings between 250 and 280 µmol selector/g silica.
The mobile phase used was methanol with formic acid and diethylamine as additives and with a pH of 5,4.
Detection was achieved by a UV detector for acidic and zwitterionic analytes and by a CAD detector for peptides.

Elution order on QN-based CSPs is usually D before L. This aspect was discussed in the previous section and will therefore not be regarded here.

2.1.1) Acidic Analytes

As shown in Figure 26 (more detailed results: appendix, Table A1), most acidic analytes could be enantioseparated on all 3 quinine-based stationary phases. Only very few, namely the protected amino acid Z-proline and the acidic anti-inflammatory drug Naproxen, however, could not be separated into their respective enantiomers on either tau-QN, homotau-QN or homohomotau-QN.

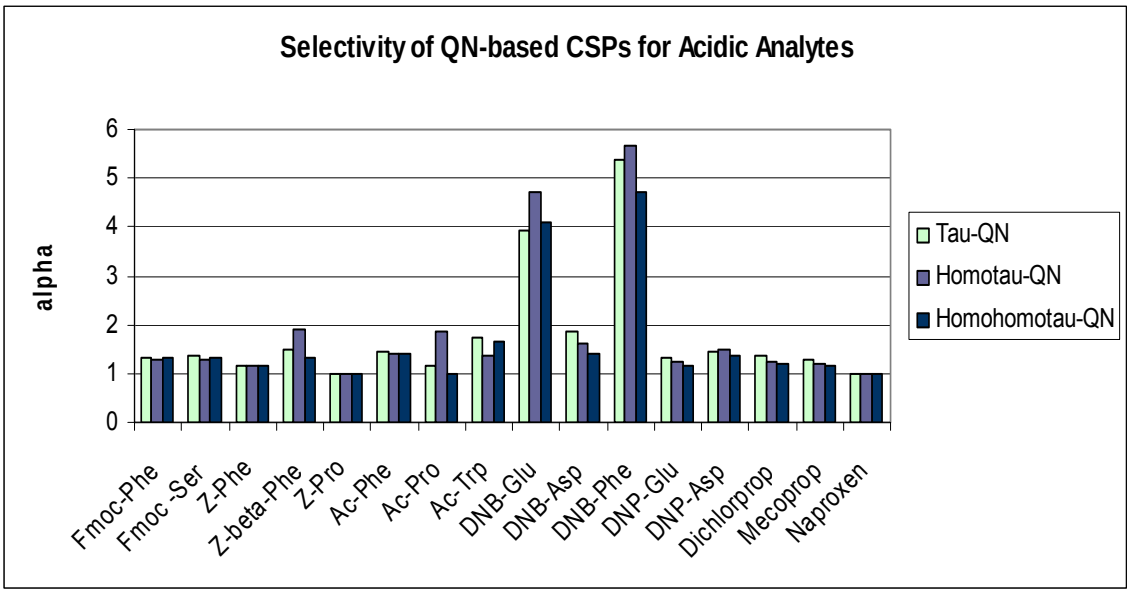


Fig. 26: Enantioselectivity of QN-based CSPs for acidic analytes (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min, CAD detection).

Even though retention times for the individual analytes varied between the three quinine-based CSPs, enantioselectivities were almost always of comparable magnitude on all three. The corresponding resolutions are shown in Figure 27.

With acidic analytes, high enantioselectivity is always connected with good resolution.

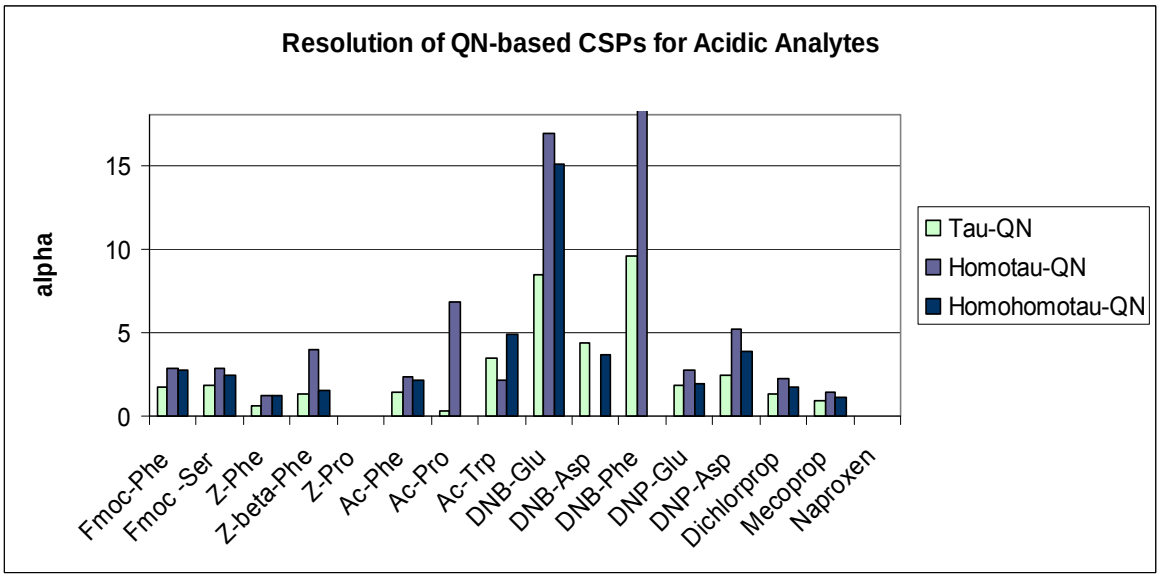


Fig. 27: Resolution (R) obtained for acidic analytes on QN-based ZWIX CSPs (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min). *Note: Resolution of DNB-Phe on Hhtau-QN was not determined.*

There is no recognisable trend along the line of tau-QN, homotau-QN and homohomotau-QN as to which CSP is the most capable one for the separation of acidic analytes, although homotau-QN appears very promising.

2.1.2) Zwitterionic Analytes

Compared to the acidic analytes, a relatively large number of zwitterionic compounds could not be separated on either of the QN-based ZWIX CSPs. The reason for this is probably the fact that the selectors used in this study lack a stereogenic center in the vicinity of the SO_3^- moiety (SCX site). From this, one may deduct that the separation of free amino acids on ZWIX selectors with a chiral cation exchanging site will have a higher chance of success.

The best enantioseparations were obtained for α-methyl substituted amino acids, which indicates the importance of the substituents at the chiral center (Figure 28).

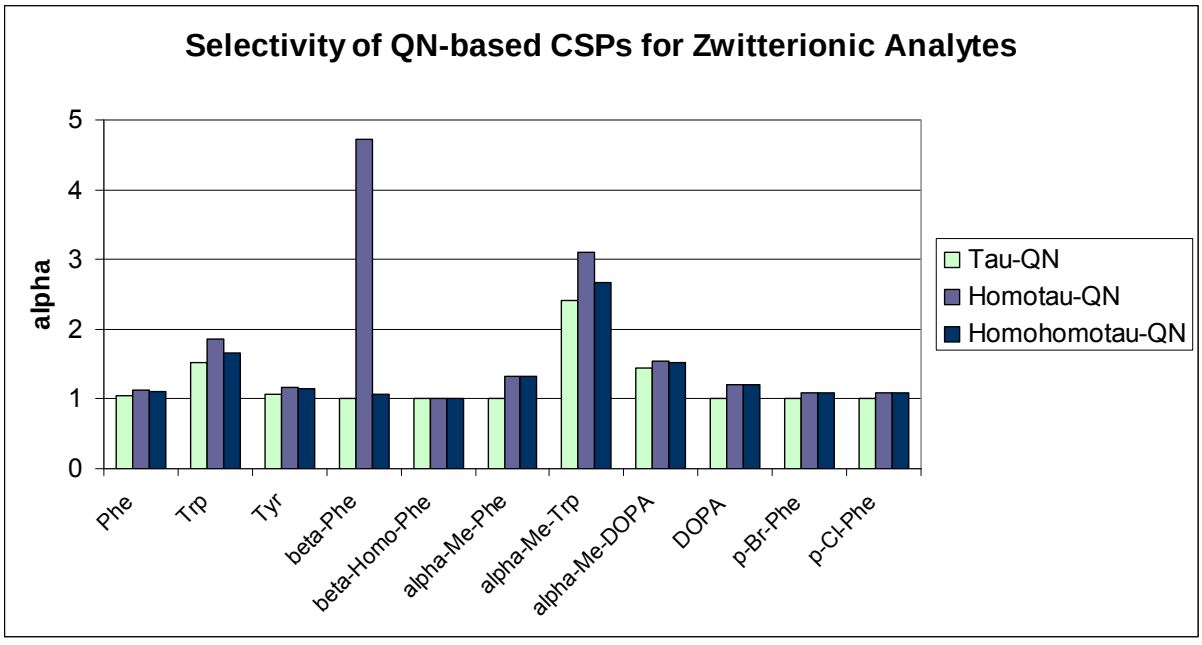


Fig. 28: Enantioselectivity of QN-based CSPs for zwitterionic (amphoteric) analytes (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min).

The corresponding resolutions are depicted in Figure 29:

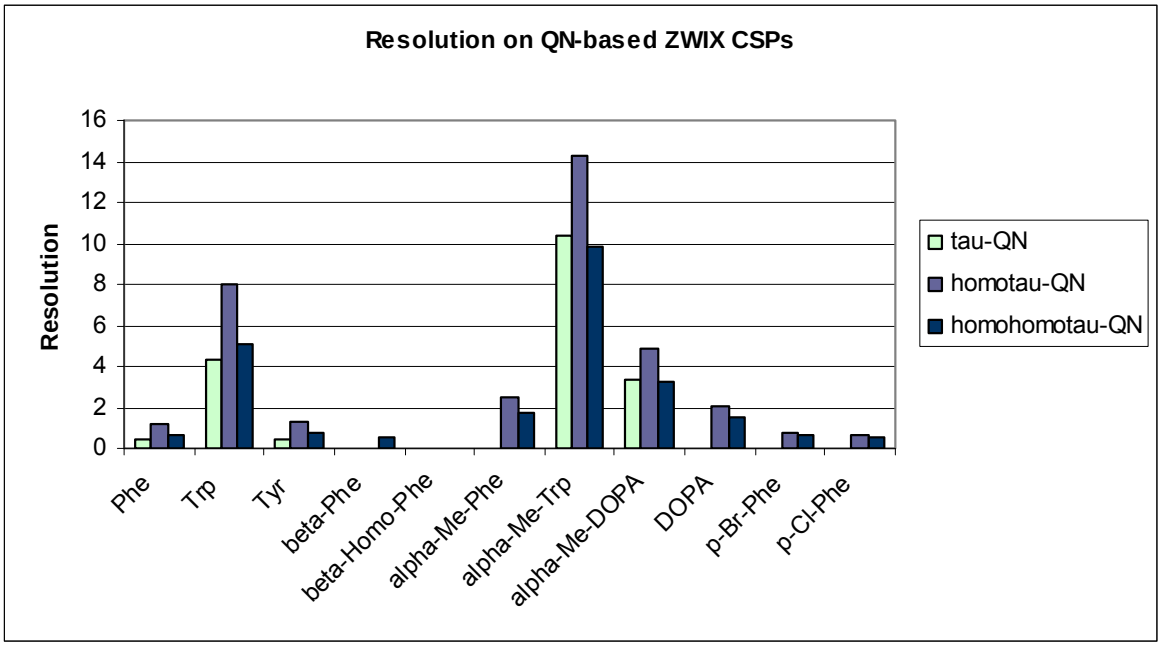


Fig. 29: Resolution (R) obtained for zwitterionic analytes on QN-based ZWIX CSPs (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min).

Of the three CSPs investigated here, homotau-QN seems more suitable for the separation of amphoteric compounds than its “homologues”, but certainly both homotau-QN and homohomotau-QN performed better than the tau-QN CSP.

2.1.3) Mixed Di- and Tripeptides

Even though retention times were shortest on homohomotau-QN due to its comparatively low selector loading, it performed especially well in the peptide separation experiment and was able to separate all but 2 of the 14 mixed di- and tripeptides investigated (Figure 30). It achieved higher selectivities for most of the analytes than its shorter homologues (details: see table A2, appendix).

The corresponding R (resolution) values obtained for peptides on QN-based ZWIX CSPs are shown in Figure 31.

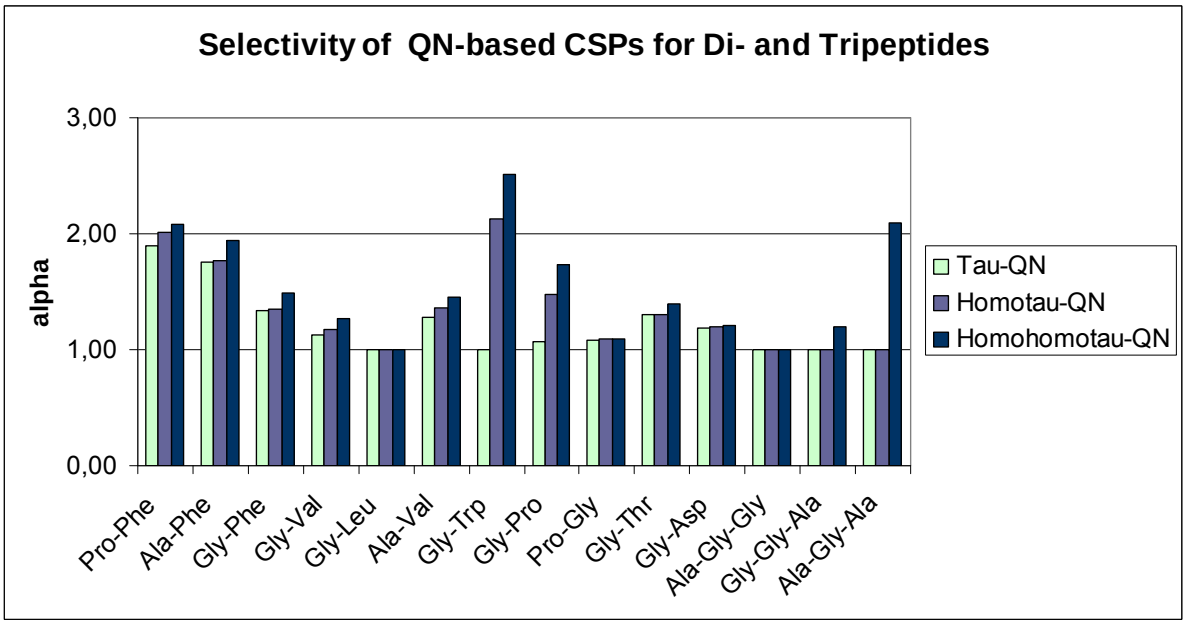


Fig. 30: Selectivity of QN-based ZWIX CSPs for all-D and all-L enantiomers of mixed di- and tripeptides (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min).

It is probably due to the length of the alkanesulfonic acid moiety (and hence, the distance and flexibility between the cation and anion exchanging sites) in the selector that homohomotau-QN was able to separate two out of three tripeptides that were inseparable on the two other CSPs.

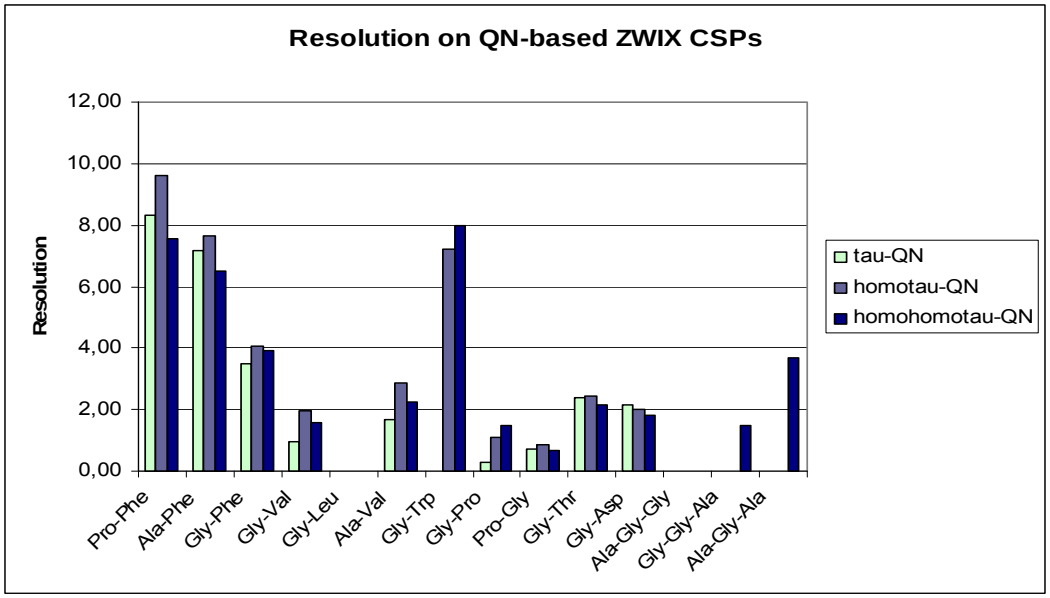


Fig. 31: Resolution (R) obtained for peptides on QN-based ZWIX CSPs (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min).

2.1.4) Homologous Peptides

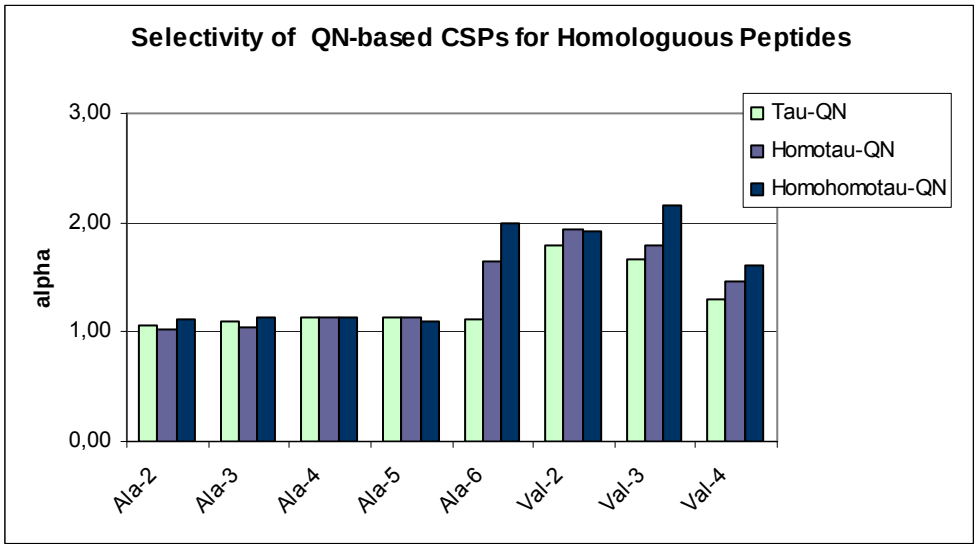


Fig. 32: Selectivity of QN-based ZWIX CSPs for all-D and all-L enantiomers of homologous all-L and all-D alanine and valine peptides (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min, CAD detection).

Separation was accomplished for the all-L and all-D enantiomers of all alanine and valine peptides investigated, and again, the enantioselectivity of homohomotau-QN stood out (Table 2, appendix).

Resolution values for homologous peptides are shown in Figure 33.

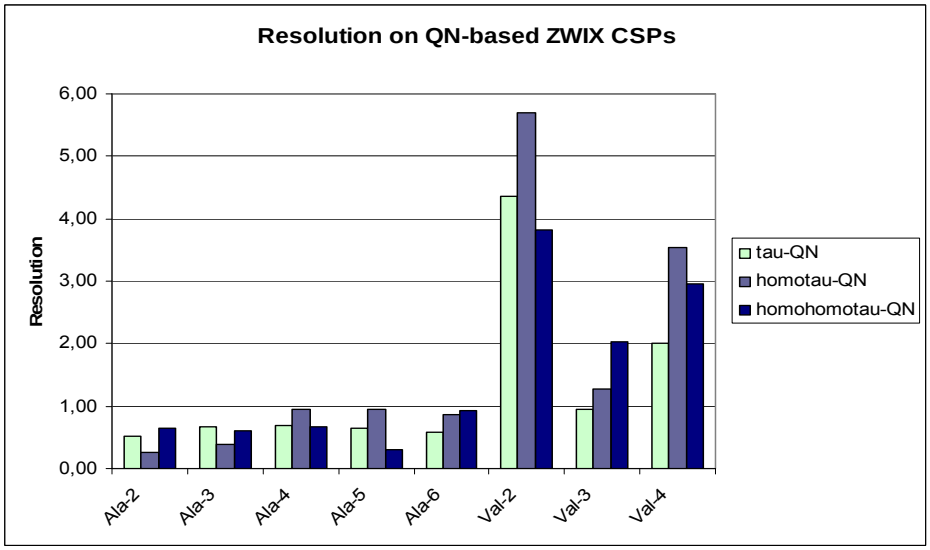


Fig. 33: Resolution (R) obtained for homologous peptides on QN-based ZWIX CSPs (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min, CAD detection).

The peptide enantioseparation results are discussed in more detail in section 3.2.1.

2.2) Comparison of Quinidine-Based ZWIX selectors

The quinidine-based selectors tau-QD, homotau-QD and homohomotau-QD investigated in this study possess selector loadings between 150 (tau-QD) and 188 (homohomotau-QD) µmol selector /g silica.

As pseudo-enantiomers of the quinine-based selectors, their usual elution order of enantiomers is L before D.

The comparative experiments were carried out with a mobile phase of methanol with 50 mM of formic acid and 25 mM of diethylamine (pH = 5,4) at room temperature and with UV (acidic and zwitterionic analytes and aromatic peptides) and CAD (peptides) detection.

2.2.1) Acidic Analytes

Most analytes could be separated on all 3 quinidine-based CSPs. Tau-QD offered the highest enantioselectivities (alpha), especially for DNB-protected acids (Figure 34).

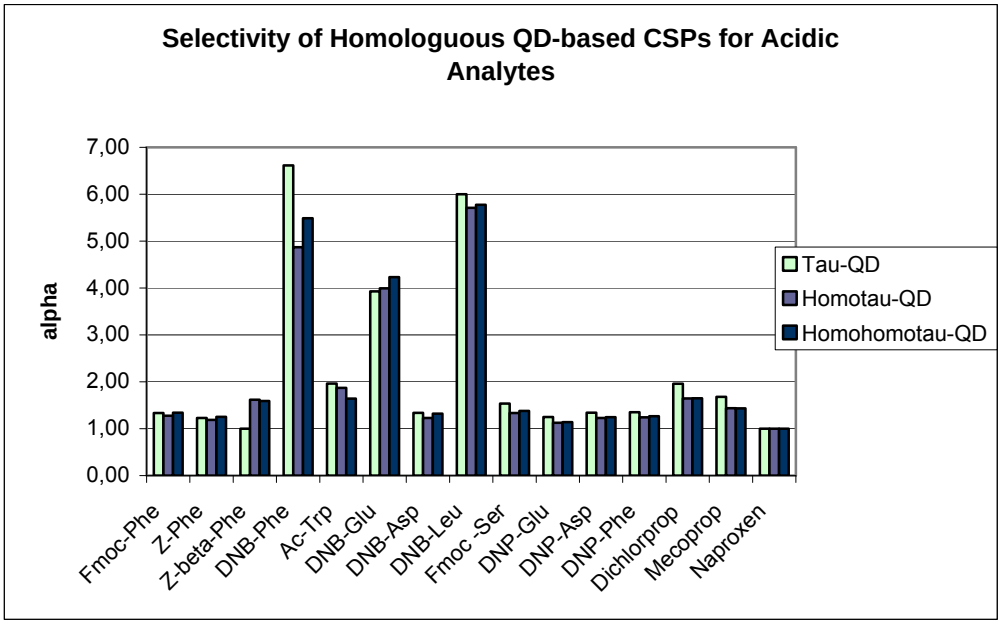


Fig. 34: Enantioselectivities of tau-QD, homotau-QD and homohomotau-QD for acidic analytes (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min).

Interestingly, tau-QD was not able to separate Z-β-Phe whereas the two other CSPs achieved reasonable separation of this compound. Presumably the position of the aromatic protective group (α and β, respectively, in Phe and β-Phe) is of crucial importance to the enantioselective interaction between selecand and selector.

Figure 35 depicts the corresponding resolutions.

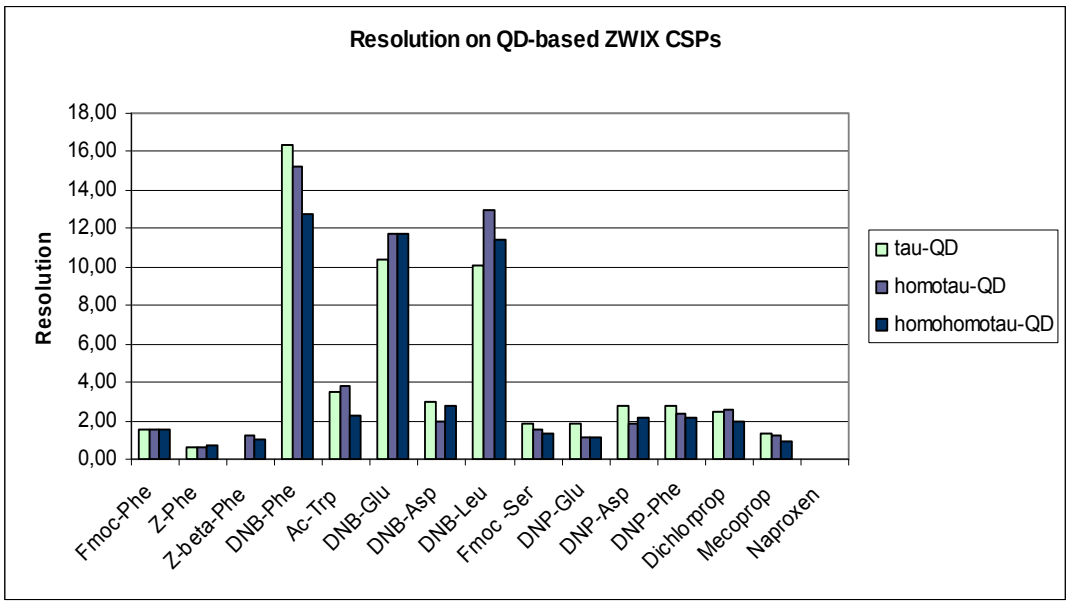


Fig. 35: Resolution (R) obtained for acidic analytes on QD-based ZWIX CSPs.

2.2.2) Zwitterionic (Amphoteric) Analytes

Enantioseparation performance of the quinidine-based stationary phases for the amphoteric analytes was rather low, although homotau-QD was the only CSP that was able to enantioseparate free tyrosine (Figure 36).
In comparison, tau-QD provided the most satisfactory (or rather, least disappointing) results.

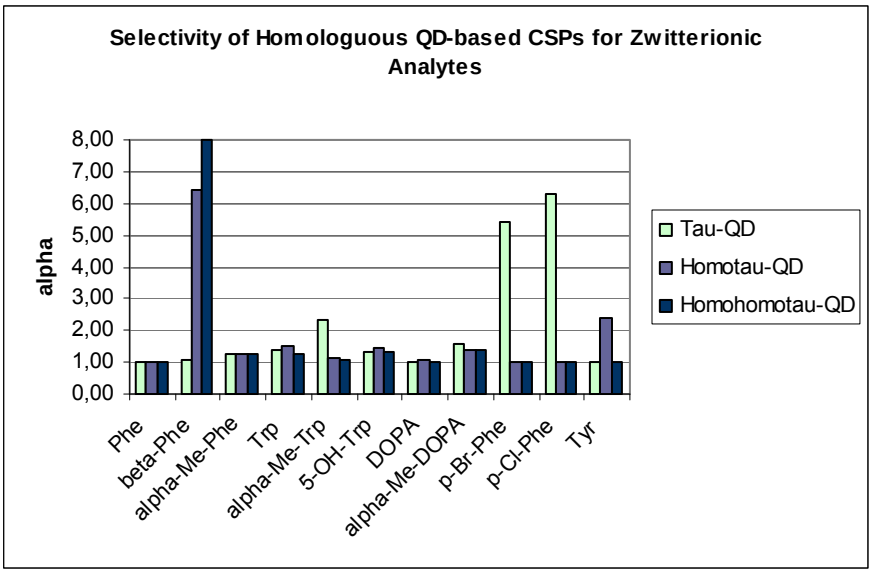


Fig. 36: Enantioselectivities of tau-QD, homotau-QD and homohomotau-QD for zwitterionic analytes. (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min).

It is remarkable though that no baseline separation of β-phenylalanine enantiomers could be achieved with tau-QD, whereas this analyte was particularly well separated on the two homologous QD CSPs.

Figure 37 shows the resolution values obtained for zwitterionic analytes on the QD-based ZWIX CSPs.

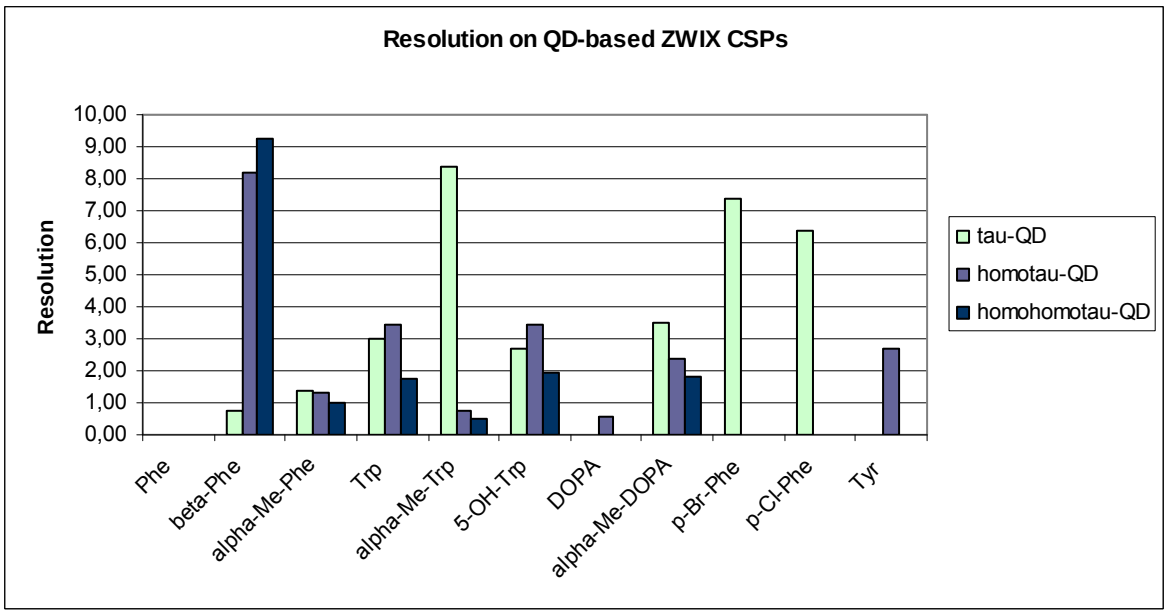


Fig. 37: Resolution (R) obtained for zwitterionic analytes on QD-based ZWIX CSPs.

2.2.3) Peptides

Whereas homohomotau-QD was able to separate the largest number of peptide enantiomers (13 out of 14), the highest overall selectivities and best resolutions were obtained with homotau-QD (Figures 38 and 39).

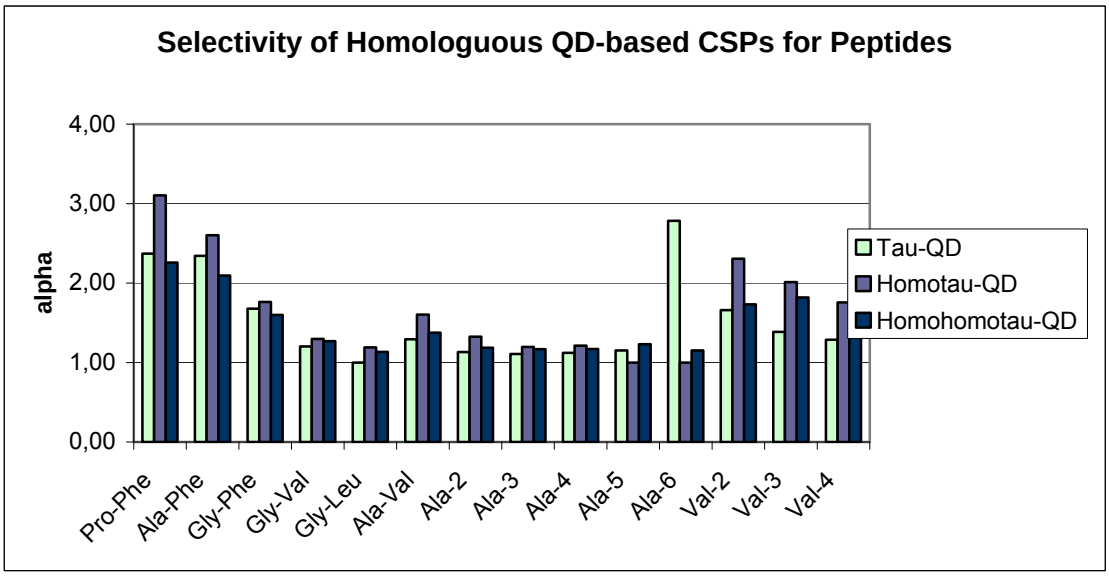


Fig. 38: Enantioselectivities of tau-QD, homotau-QD and homohomotau-QD for all-L and all-D peptide enantiomers (mobile phase: methanol, 50 mM FA, 25 mM DEA, 1 mL/min, CAD detection).

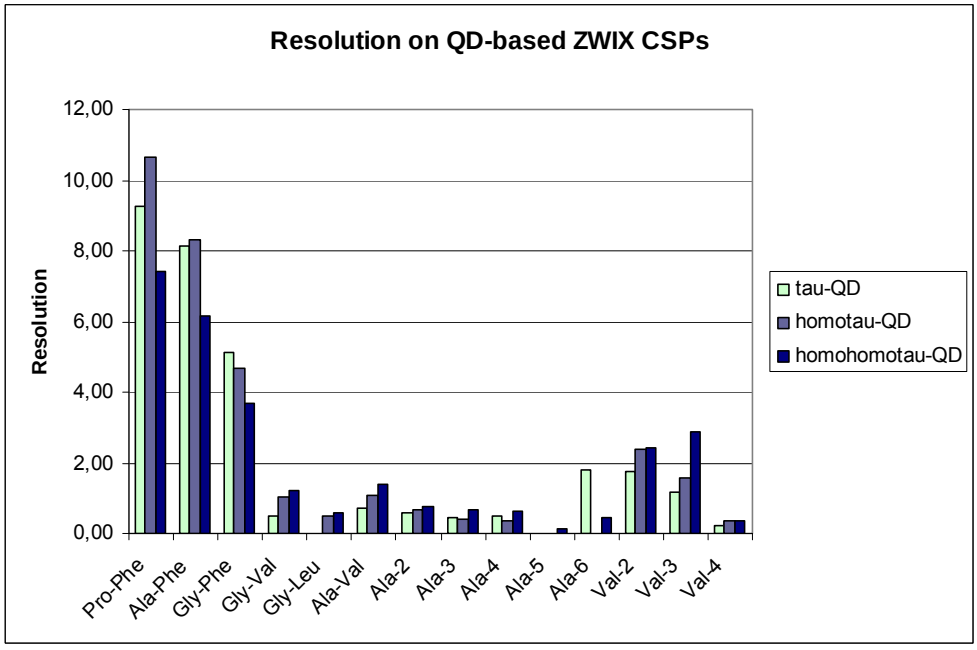


Fig. 39: Resolution (R) obtained for peptides on QD-based ZWIX CSPs.

3) Peptide Enantioseparation

Today, the standard stationary phases for enantioselective HPLC separations of peptides are macrocyclic antibiotics such as vancomycin (V), teicoplanine (T) or teicoplanin aglycone (TAG) (for structures, see introduction).
As chiral amphoteric compounds though, peptides represent a class of analytes predestined for separation on chiral zwitterionic stationary phases.
Therefore, a set of all-L and all-D peptides (Figure 31) was subject to analysis on Chirobiotic ^{TM} stationary phases and the separation results were compared to those of the same peptides separated on our ZWIX stationary phases.

Furthermore, the influence of peptide size (number of amino acids) on the retention and separation of homologous alanine and valine peptides was investigated.

ZWIX stationary phases are normally operated with a mobile phase of methanol with acidic and basic additives such as formic acid and diethylamine at a ratio of 2:1. The addition of water to the mobile phase is expected to interfere with the ionic interactions of the analyte and the stationary phase and therefore to influence retention and separation. This aspect was investigated in mobile phases with water content ranging from 0-100 % and pH values from 3,4 to 5,4.

3.1) Peptide Enantioseparation on Chirobiotic^{TM} CSPs

D and L enantiomers of Gly-Val were analysed on Chirobiotic V with the mobile phases given in Fig. 40.
In all cases, only one single peak was detected. Judging from the retention times of single enantiomers, it is improbable that enantioseparation was achieved, although it has to be said that analysis time was limited to 60 minutes, which was regarded as the upper limit for a feasible separation.
Peak-shapes were frequently irregular even though only 15 µL of the analyte solutions were injected into the HPLC system. Ionic additives (formic acid, diethylamine) did not improve separation.

Mobile Phase #	methanol (%)	water (%)	mobile phase additive(s)
1	100	0	-
2	90	10	-
3	50	50	-
4	10	90	-
5	0	100	-
6	90	10	50 mM FA, 25 mM DEA

Fig. 40: Mobile phase compositions for peptide enantiomer separation on CSPs based on macrocyclic antibiotics.

Results were similarly dissatisfactory for Gly-Leu, Ala-Val and homologous Ala (Ala-2 to Ala-6) and Val (Val-2 to Val-4) peptides. In those cases where both enantiomer peaks were found, the most striking results were the rapidly decreasing numbers of theoretical plates N_1 and N_2. As an example, Figure 41 depicts the chromatogram of Ala-3 on Chirobiotic V with mobile phase MP3.

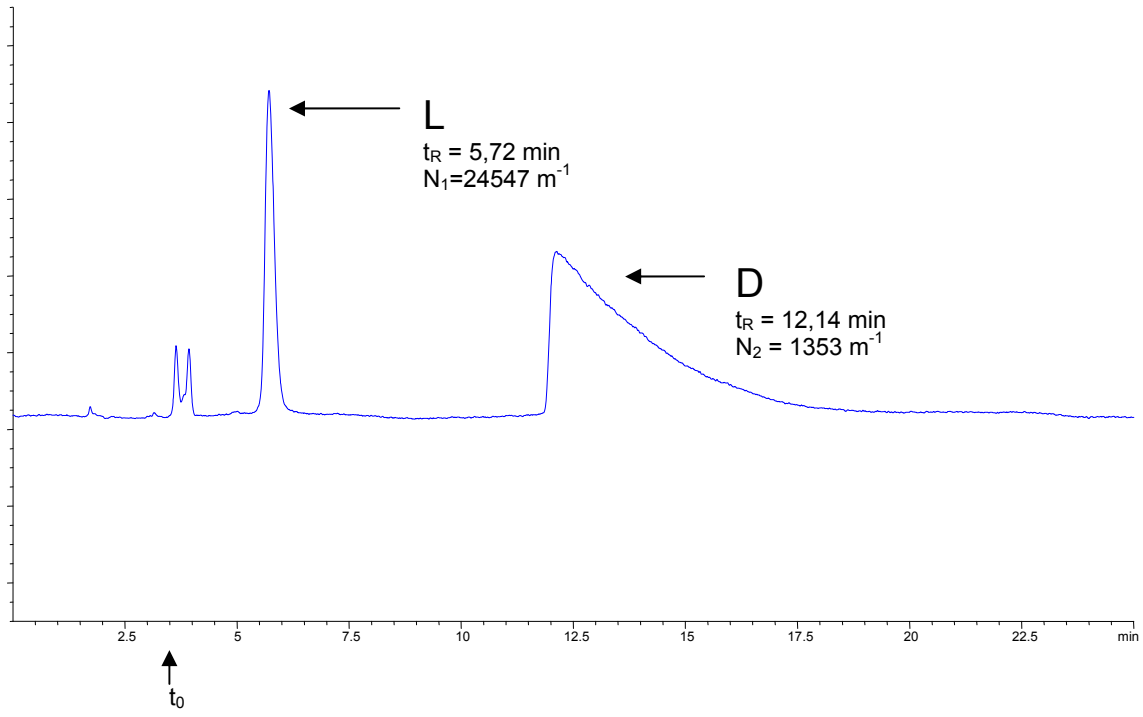


Fig. 41: Enantioseparation of Ala-3 on Chirobiotic V.

Peptide elution orders on the Chirobiotic columns should be all-L before all-D as the biological activity of the chiral antibiotics used in these CSPs is based on a particularly strong interaction with terminal D-Ala-D-Ala moieties of bacteria cell walls (introduction 3.2.1 a). This was confirmed in those (few) cases where both enantiomer peaks could be detected (Figure 41).

On Chirobiotic TAG, satisfactory separation of peptide enantiomers within run times of 60 minutes was never achieved. Peak-shapes were irregular despite small amounts of analytes injected and frequently, only one peak was detected instead of two.
Here, as with Chirobiotic V, separation performance of the system was not be significantly improved by acidic (FA) or basic (DEA) additives.

Altogether, separation of peptides on macrocyclic antibiotic-based chiral stationary phases was very time-consuming and did not yield particulary satisfactory results.
Optimisation of the separation systems with stationary phases Chirobiotic V and Chirobiotic TAG would have exceeded the scope of this work – with modifications regarding the mobile phase composition (water content, additives, pH) better separation with shorter run times should be feasible.

3.2) Peptide Enantioseparation on Cinchona Alkaloid-Based ZWIX CSPs

According to the results reported in section B (1.3, 2.1.3 and 2.2.3), zwitterionic chiral stationary phases offer relatively short retention time for peptides (thereby reducing solvent consumption) and, in addition, provide the possibility of reversing the elution order of chiral analytes by switching from quinine-based to quinidine-based CSPs.

The following sections report the outcome of several experiments intended to deepen our understanding of peptide enantioseparation on ZWIX CSPs.
Most of the peptides of Figure 31 were successfully enantioseparated on ZWIX selectors, as reported above. Enantioselectivity of zwitterionic stationary phases was significantly higher for valine peptides than for alanine peptides, suggesting a crucial influence of the amino acid side-chains on selector-selectand interaction.

As was the case for other zwitterionic compounds, switching from QN-based (all-D enantiomer is eluted first) to QD-based CSPs (all-L enantiomer first) led to a reversal of peptide enantiomer elution order in all cases (Figure 42).

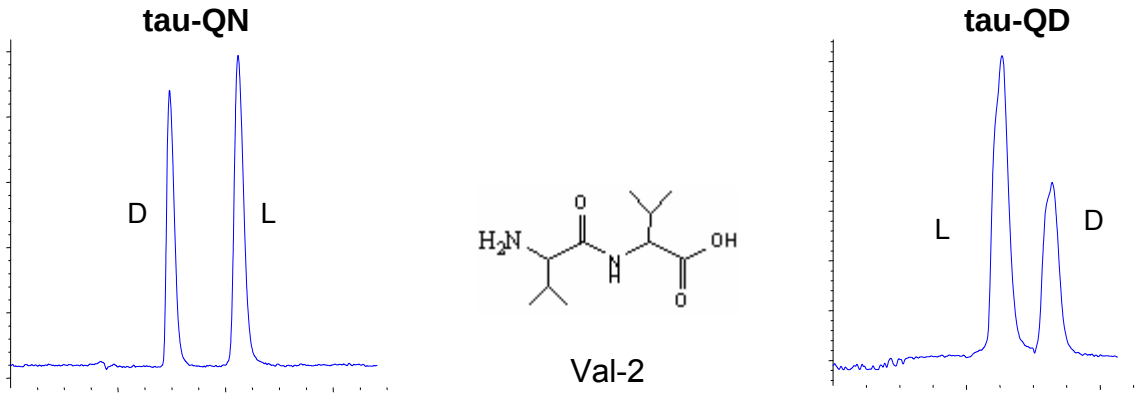


Fig. 42: Reversal of elution order for all-L and all-D peptide enantiomers on ZWIX CSPs.

3.2.1) Influence of Peptide Size

Homologous alanine and valine peptides (with 2 to 6 amino acid residues) were subject to enantiomer separation on tau-QN, homotau-QN and homohomotau-QN (see 2.2.4). The aim of the experiment was to evaluate the influence of the distances between the SCX and WAX sites of the selector with regard to the length of the peptide.

Figure 43 shows the correlation between peptide size (number of amino acid residues) and retention time for homotau-QN.

All-D and all-L alanine peptides both experienced decreasing retention for Ala-3 compared to Ala-2 and a slightly increasing retention when moving further along from Ala-3 to Ala-6.

Retention of all-L-valine decreased with peptide length, but both more rapidly and to a larger extent than that of the Ala peptides.

This suggests that under the conditions of the experiment, especially the longer Val-peptides retain a conformation (possibly a helical structure) that is less favourable for interactions with the zwitterionic selector than that of the shorter peptides.

A similar decline in retention is to be expected for the all-D enantiomers of Val-5 and Val-6 which were not included in this study (not available).

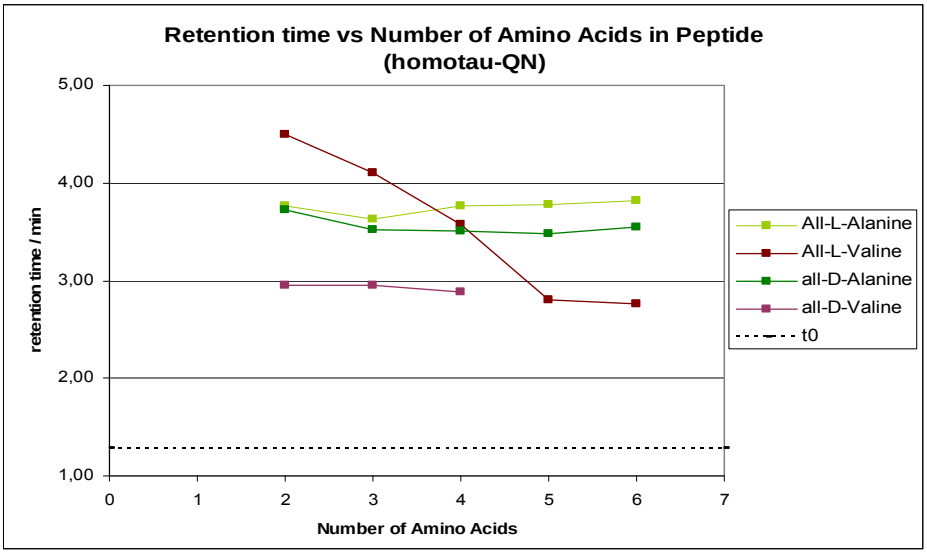


Fig. 43: Influence of peptide size on retention time of homologous peptides. CSP: homotau-QN, MP: methanol, 50 mM FA, 25 mM DEA, flow rate: 1 mL/min

The retention of the homologous peptides on quinine-based stationary phases is shown in Figure 37, where the increase in selectivity for Ala-6 and the longer valine peptides when switching from tau-QN to homotau-QN and homohomotau-QN is clearly visible.

Although the correlation is not as distinctive as expected, the separation of peptides of variable length on ZWIX selectors is definitely dependent on the relative distances between SCX/WAX sites of the selector and the respective peptide interaction sites.

Further investigation of this aspect of peptide enantiomer separation of ZWIX selectors will be carried out in the future.

3.2.2) Influence of Mobile Phase Composition

It has been shown before that retention of zwitterionic compounds on ZWIX CSPs is pH dependent as the protonation of the basic moieties and the dissociation of the acidic site depend on the pH. The same is of course valid for the analytes. Usually, weakly acidic conditions are optimal for the operation of ZWIX CSPs.

Employing ZWIX CSPs under reversed-phase conditions is likely to change their retention characteristics so that they can be characterised as mixed-mode RP/ion exchanger CSPs.[44]

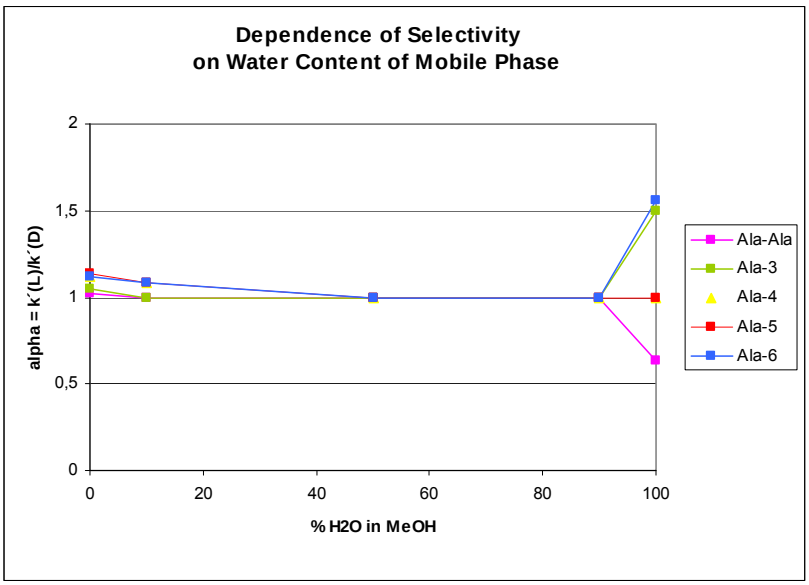
First the retention of peptides on ZWIX CSPs with mobile phases of decreasing pH was investigated. Mobile phases were prepared by dissolving 50 mM of formic acid and 25 mM of diethylamine in water/methanol mixtures of different composition (Fig. 44):

Mobile Phase #	methanol (%)	water (%)	Mobile phase additive(s)	apparent pH
1	100	0	50 mM FA, 25 mM DEA	5,4
2	90	10	50 mM FA, 25 mM DEA	5,25
3	50	50	50 mM FA, 25 mM DEA	4,25
4	10	90	50 mM FA, 25 mM DEA	3,63
5	0	100	50 mM FA, 25 mM DEA	3,60

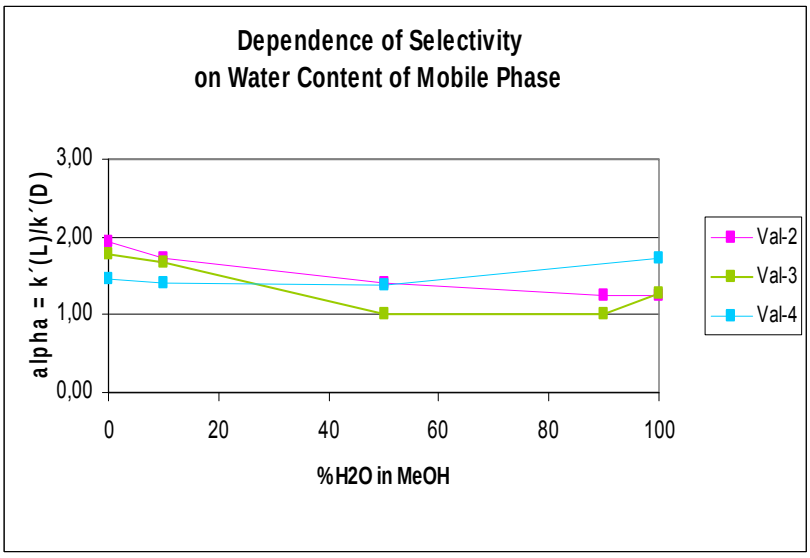
Fig. 44: Mobile phases for evaluation of pH influence on peptide enantioseparation.

Retention of peptides on tau-QN, homotau-QN and homohomotau-QN decreased along with the pH of the mobile phase. In most cases, enantioselectivity was reduced to α=1 with mobile phases 3 or 4. Interestingly, at the lowest water content (mobile phase 5), enantioselectivity increased again, but elution order was reversed for some analytes. It is likely that protonation of the peptides´ carboxyl moieties generated a change in the retention mechanism.

Figure 45 depicts the elution order-dependent selectivity coefficients (here: $\alpha_{EO} = \frac{k(L)}{k(D)}$) of alanine and valine peptides on homotau-QN as a function of mobile phase composition:



a



b

Fig. 45: Decrease of selectivity and eventual reversal of peptide enantiomer elution order as a function of mobile phase composition. CSP: homotau-QN.

To assess whether the reversal of elution order was really a result of decreasing pH (modulation of the charge of the peptide) or rather due to a change in the retention mechanism towards a mixture of RP and ion exchange, mobile phases with a water content of 0-100% and 25 mM of DEA with a pH of 5,4 (FA) were prepared.

All-L and all-D peptide enantiomers were subject to investigation on tau-QN, homotau-QN and homotau-QN.

The retention time of Ala peptides on the QN-based CSPs decreased considerably when the amount of water in the mobile phase was increased (Figure 46). This is due to a solvatation effect of the ion exchanging sites of the zwitterionic selector.

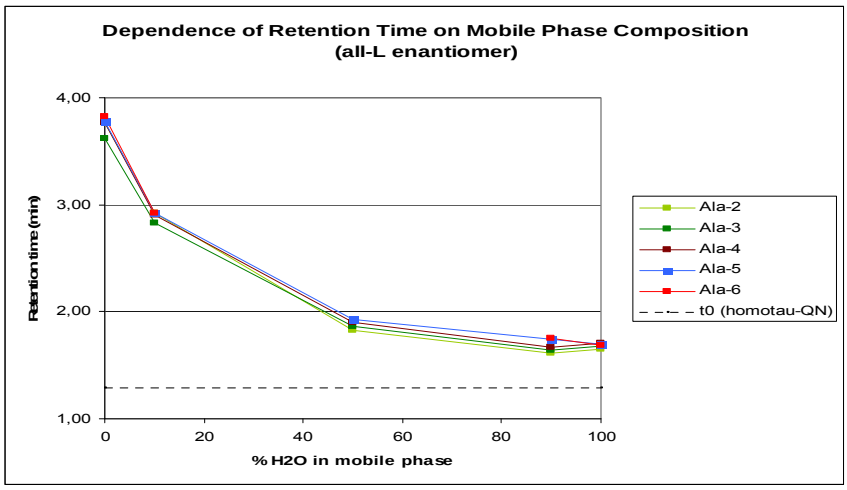


Fig. 46: Retention time of homologous Ala peptides as a function of mobile phase composition. CSP: homotau-QN.

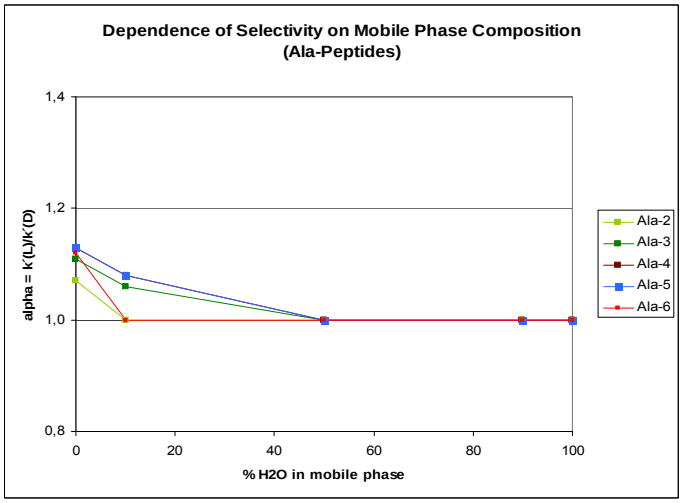
This phenomenon was of similar magnitude for all five Ala peptides on all 3 QN-based CSPs tested (data for tau-QN and homohomotau-QN are not shown).
Apparently, ionic interactions and hydrogen bonds between the zwitterionic selector and the peptides, which are the basis of retention in ion exchange mode, are severely compromised by hydratation of the ionic interaction sites.

In some cases (that is, Ala-3 on tau-QN, Ala-2, Ala-3 and Ala-4 on homotau-QN, and Ala-5 and Ala-6 on homohomotau-QN), retention times rose slightly when switching from 90% to 100% water indicating a change in retention mechanism. Possibly weak RP-related hydrophobic interactions are responsible for this effect.

It was mentioned before that shorter retention times on ZWIX CSPs do not necessarily mean lower selectivity, but in the case of tau-QN, enantioselectivity was quickly reduced and eventually cancelled out completely by the addition of water to the mobile phase. This was especially true for relatively short (Ala-2) and long (Ala-6) peptides, which could not be separated on tau-QN with a mobile phase water content of more than 10% (Figure 47).

Analysis results were similar for Ala peptides on homotau-QN and homohomotau-QN, but whereas the enantioselectivity of homotau-QN for the longer peptide enantiomers (Ala-4, Ala-5, Ala-6) decreased more slowly than that for the shorter ones, homohomotau-QN´s enantioselectivity for all 5 peptides investigated collapsed at 10 % mobile phase water content.

Interestingly, at 100% water content the two smaller peptides, Ala-2 and Ala-3 became separable again, which is probably due to a change in the retention mechanism.



a (tau-QN)

b (homotau-QN)

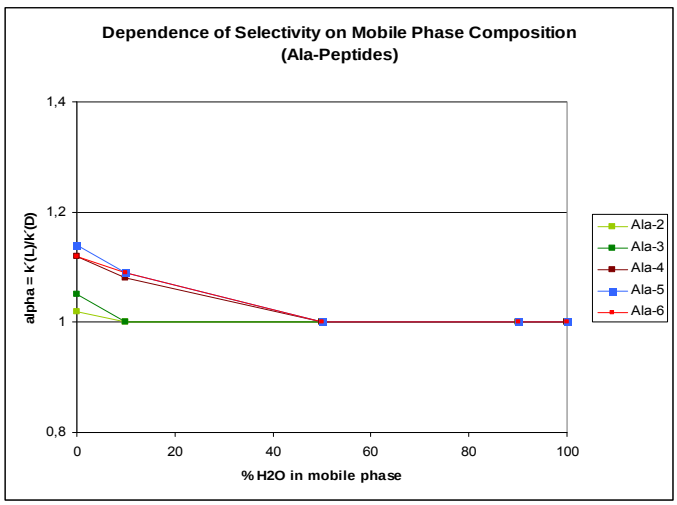
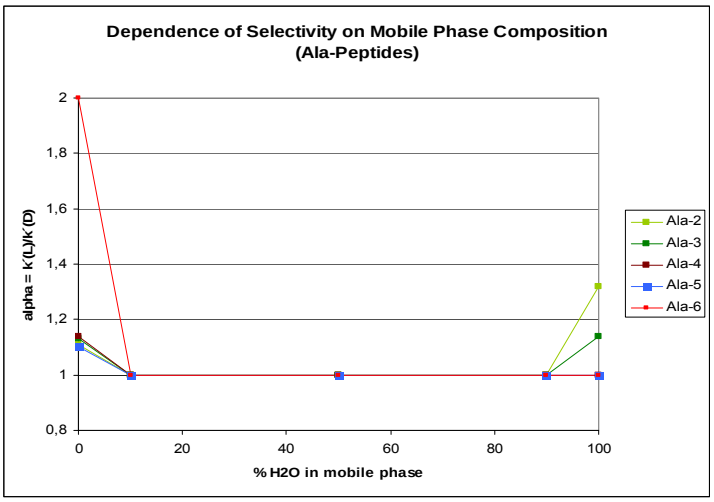


Fig. 47: Influence of mobile phase composition on selectivity of QN-based CSPs for homologous Ala peptides. **a** tau-QN **b** homotau-QN **c** homohomotau-QN



c (homohomotau-QN)

Fig. 47 (continued): Influence of mobile phase composition on selectivity of QN-based CSPs for homologous Ala peptides. **a** tau-QN **b** homotau-QN **c** homohomotau-QN

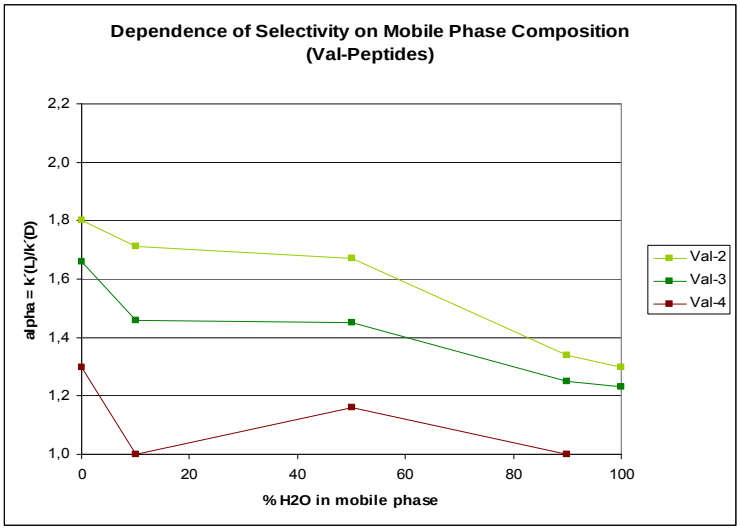
Enantioselectivities of QN-based CSPs for valine peptides were always higher than those for alanine peptides. This remained true throughout the mobile phase variation experiments, even though enantioselecitivity for Val-peptides also decreased with increasing water content of the mobile phase.

On tau-QN and homotau-QN (Figure 48 a, b), selectivities were especially low at 10%, 90% and 100% water content, whereas at a mobile phase composition of 50/50 (v/v) of water and methanol a slight increase in enantioselectivity was observed.

Val-2 was particularly well separated compared to its homologues, whereas enantioselectivity was weakest for Val-4.

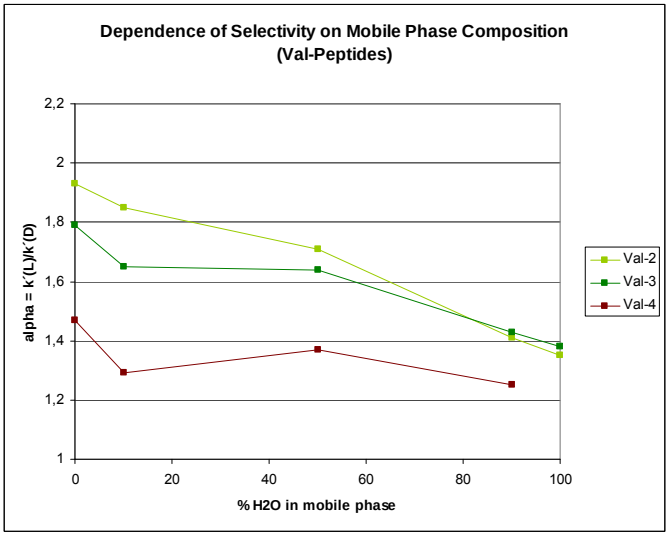
On homohomotau-QN, however (Figure 48 c), Val-3 was significantly better separated than its homologues with all mobile phases.

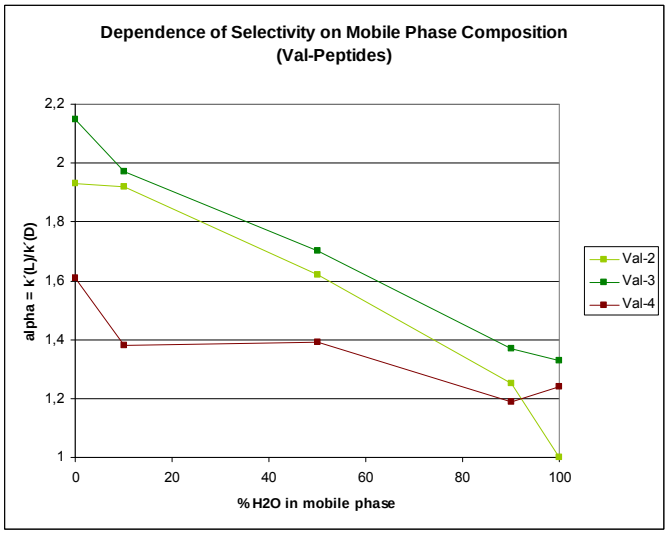
Besides, the decrease in Val peptide enantioselectivity with increasing water content was more constant than for the other two CSPs.



a (tau-QN)

b (homotau-QN)





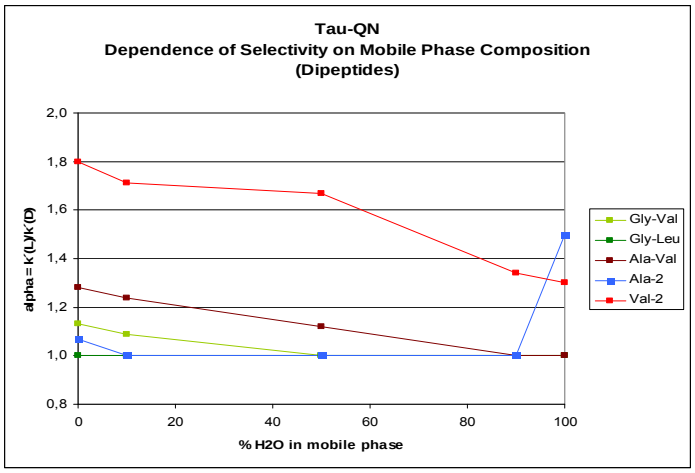
c (homohomotau-QN)

Fig. 48: Influence of mobile phase composition on selectivity of QN-based CSPs for homologous Val peptides. **a** tau-QN **b** homotau-QN **c** homohomotau-QN

Mixed dipeptides Gly-Val, Gly-Leu and Ala-Val were also analysed on tau-QN, homo-tau-QN and homohomotau-QN with various mobile phases.

As observed for the homologous peptides, selectivity for those dipeptides decreased with increasing water content of the mobile phase on all 3 CSPs.

The only exception to that was Gly-Leu, which was inseparable with all mobile phases on tau-QN and homohomotau-QN but could be enantioseparated on homotau-QN with mobile phases with more than 50 % water (Fig. 49 b).



a (tau-QN)

b (homotau-QN)

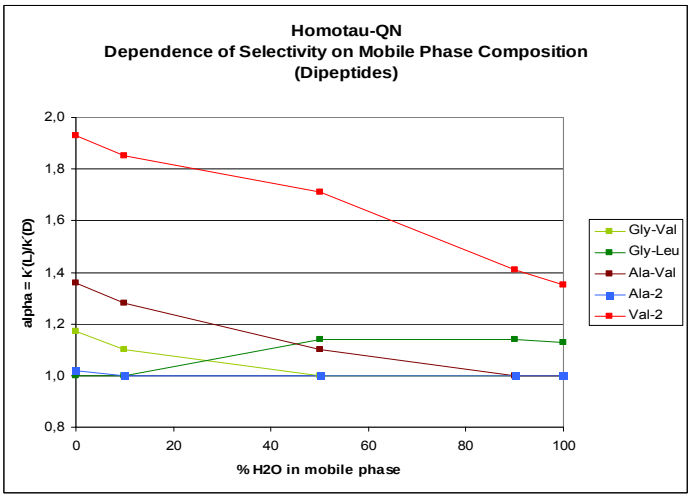
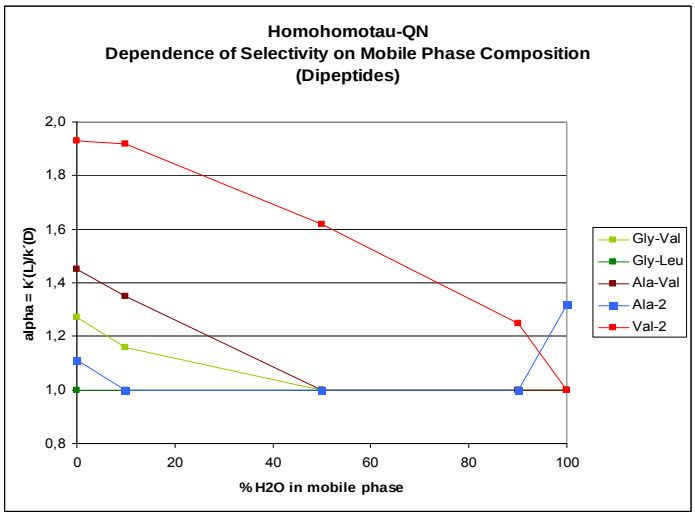


Fig. 49: Influence of mobile phase composition on the enantioseparation of mixed dipeptides. CSPs: **a** tau-QN **b** homotau-QN **c** homohomotau-QN



c (homohomotau-QN)

Fig. 49 (continued): Influence of mobile phase composition on the enantioseparation of mixed dipeptides. CSPs: **a** tau-QN **b** homotau-QN **c** homohomotau-QN

4) Conclusion & Outlook

Preparation of zwitterionic chiral stationary phases (ZWIX CSPs) based on the fusion of cinchona alkaloids and taurine-homologues yielded tau-QN, homotau-QN and homohomotau-QN and their pseudo-enantiomers tau-QD, homotau-QD and homohomotau-QD.
Selectors with aminomethanesulfonic acid (AMSA) as the SCX site (AMSA-QN and AMSA-QD) remain yet to be synthesised, as the protocols employed for the synthesis of the other selectors were not successful with AMSA – most probably, the reason for the failure seems to be the small size of the aminomethanesulfonic acid. Systematic modification of individual synthesis steps also did not bring about the desired results.

The chiral zwitterionic stationary phases prepared in the course of this work were successfully employed for the enantioseparation of acidic (e.g. N-protected amino acids, acidic drugs) as well as amphoteric (zwitterionic) compounds such as free amino acids and peptides.
Therefore, ZWIX CSPs represent a powerful alternative to chiral stationary phases based on macrocyclic antibiotics which to date are the most widely used system for this kind of enantioseparation. In addition to relatively short run times, the most advantageous characteristic of ZWIX CSPs is their straightforward application which does not require a great deal of mobile phase optimization. Besides, cinchona-based ZWIX CSPs offer the potential of reversing the elution order of enantiomers by switching from quinine to quinidine-based phases. There is often no such possibility with the macrocyclic antibiotics-based CSP, although sometimes the elution order can be reversed by switching from e.g. a Ristocetin A to a Vancomycin or Teicoplanin column.

The comparison of quinine- and quinidine-based stationary phases showed that enantioselectivites on the pseudo-enantiomeric stationary phases were comparable, whereas the elution order was inverted. This is especially beneficial for the separation of enantiomeric impurities or for preprarative applications where a reversal of elution order may be required.

Separation capabilities of tau-QN, homotau-QN and homohomotau-QN were investigated with a special focus on mixed di- and tripeptides and homologous alanine and

valine peptides. Even though tau-QN delivered satisfactory results for acidic analytes and unprotected amino acids, homotau-QN and homohomotau-QN performed significantly better in the peptide separations.
Enantioseparation of free amino acids was often not satisfactory. This is most probably due to the lack of a chiral center in the vicinity of the sulfonic acid SCX site in the tau-, homotau- and homohomotau selectors. ZWIX CSPs which incorporated a chiral sulfonic acid (aminocyclohexanesulfonic acid, see introduction, section 4) obtained better enantioselectivity of free amino acids than those which an achiral sulfonic acid side chain.[35]

To fully understand the retention mechanism and enantioselectivity of ZWIX CSPs for peptides, an extended set of peptides sets will have to be analysed. The influence of the α-amid binding on chiral recognition can be investigated by analysis of peptides based on α- and β-amino acids.

Currently, the Lindner Group is working on the widening of the spectrum of zwitterionic chiral stationary phases based on diverse chiral cationic and anionic scaffolds and synthons, among them cinchona-based CSPs with chiral anion and cation exchanging moieties.

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Appendix

Abstract **A3**

Zusammenfassung **A4**

Results of ZWIX CSP Evaluation – Tables **A6**

Curriculum Vitae **A10**

Abstract

The separation of chiral compounds into their enantiomers has been gaining importance over the last two decades, mostly due to rigorous regulatory demands concerning chiral drugs and also chiral intermediates.

In this work, chiral separation techniques for amino acids and peptides with their advantages and drawbacks are discussed.

Depending on the surrounding environment, amino acids and peptides can be present in ionic form and therefore represent natural targets for HPLC separation methods based on an ion exchange mechanism. However, they cannot be enantioseparated on chiral anion or cation exchanging stationary phases because a second charge, acting as an intramolecular counterion, is present in these amphoteric compounds.

For these analytes, zwitterionic ion exchange chiral stationary phases (ZWIX CSPs) have to be employed in order to achieve retention based on ionic interaction.

In the course of this work, zwitterionic chiral stationary phases based on the fusion of cinchona alkaloid anion exchanging sites and homologous achiral sulfonic acids as cation exchanging sites were synthesised. The CSPs were evaluated for their enantioseparation capabilities with a set of acidic and amphoteric analytes and peptides under polar-organic mobile phase conditions.

The CSPs performed satisfactorily in the enantioseparation of acidic compounds and, compared to a chiral anion exchanger, yielded shorter retention times. Especially high enantioselectivites (> 5) were obtained for halide-substituted phenylalanines and DNB-protected amino acids.

Zwitterionic compounds, however, were often not baseline separated, which may be attributed to the absence of a chiral center in the vicinity of the sulfonic acid moieties.

The enantioseparation of all-L and all-D enantiomers of dipeptides was accomplished for most of the peptides analysed, and particularly good results were obtained for peptides containing aromatic side chains.

Homologous peptides based on alanine and valine were employed to investigate the influence of peptide length on enantioseparation with ZWIX CSPs which had homologous sulfonic acid moieties.

Adding water to the methanolic mobile phase severely decreased both retention and enantioselectivity for peptides on the CSPs, indicating a solvatation effect of the ion exchanging sites which significantly reduces ionic selector-selectand interactions.

Zusammenfassung

Die Bedeutung von Enantiomerentrennungen hat in den letzten Jahrzehnten stark zugenommen, was vor allem den immer strenger werdenden Anforderungen der Regulierungsbehörden in Bezug auf die Charakterisierung chiraler Arzneistoffe zuzuschreiben ist.

In der vorliegenden Arbeit wird eine Auswahl chiraler Trenntechniken für Aminosäuren und Peptide vorgestellt und ihre Vor- und Nachteile kurz beschrieben.

Aufgrund ihrer ionisierbaren funktionellen Gruppen stellen Aminosäuren und Peptide nahe liegende Zielanalyten für HPLC-Enantiomerentrennungen mittels chiraler Ionenaustauscherphasen dar. Allerdings sind die Verbindungen amphoter (zwitterionisch), können also gleichzeitig positive und negative Ladungen tragen. Diese können ihrerseits mit weiteren Ladungsträgern interagieren.

Solche Verbindungen können nicht mit reinen Anionen- oder Kationenaustauschern getrennt werden, da die zweite Ladung als intramolekulares Gegenion wirkt und damit eine Retention verhindert.

Für derartige Trennungen werden zwitterionische chirale Ionenaustauscherphasen benötigt, welche ebenso saure und basische Gruppen tragen, sodass eine simultane kooperative Ionenpaarbildung möglich wird.

Im Laufe der vorliegenden Diplomarbeit wurden 6 derartige Phasen für die HPLC hergestellt, indem homologe Sulfonsäuren als Kationenaustauscher und Chinin sowie sein Pseudoenantiomer Chinidin als Anionenaustauscher miteinander verbunden wurden.

Diese chiralen Phasen wurden mit einem Analytenset bestehend aus chiralen Säuren und amphoteren Verbindungen sowie Peptiden hinsichtlich ihrer Trennleistung im polar-organischen Modus charakterisiert.

Obwohl für saure Analyte wie N-geschützte Aminosäuren zufrieden stellende Ergebnisse erzielt wurden, konnten freie Aminosäuren oft nicht basisliniengetrennt werden. Die Gründe hierfür liegen zum Teil im Fehlen eines chiralen Zentrums in der Nähe der Kationenaustauscherseite, bemerkenswerte Ausnahmen waren aber Halogen-substituierte Aminosäuren (z.B. Brom-Phenylalanin), für die teilweise Enantioselektivitäten über 5 erzielt werden konnten

Die zugrunde liegenden molekularen Erkennungsmechanismen dieses Verhaltens bedürfen noch näherer Betrachtung.

Peptidenantiomere konnten in vielen Fällen basisliniengetrennt werden, wobei die funktionellen Gruppen der Seitenkette eine entscheidende Rolle bei der Trennung spielten.

Weiters wurde der Einfluss der mobilen Phase auf die Retention und Enantioselektivität von homologen Peptiden getestet, indem der methanolischen mobilen Phase unterschiedliche Anteile von Wasser zugefügt wurden. Dies beeinträchtigte die Trennung der Peptide massiv, was mit der zunehmenden Solvatisierung der ionischen Gruppen des Selektors und den damit abnehmenden polaren Wechselwirkungen zwischen Selektor und Analyten erklärt werden kann.

	Chiral Analytes	tau-QN						homotau-QN						homohomotau-QN					
		k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$	k_1	k_2	alpha	Res.	$N_1[m^{-1}]$	$N_2[m^{-1}]$
Acidic	Fmoc-Phe	0,83	1,08	**1,31**	1,69	19513	16413	1,37	1,79	**1,3**	**2,8**	32960	32093	1,40	1,86	**1,33**	**2,74**	28007	25527
	Fmoc -Ser	0,72	0,98	**1,35**	1,86	21387	18413	1,3	1,69	**1,3**	**2,81**	35820	33727	1,35	1,77	**1,31**	**2,39**	26693	19940
	Z-Phe	0,48	0,56	**1,16**	0,62	12193	20287	0,86	0,99	**1,14**	**1,17**	35773	36160	0,93	1,07	**1,15**	**1,22**	34887	32400
	Z-beta-Phe	0,19	0,28	**1,48**	1,28	30520	32180	0,4	0,76	**1,9**	**3,99**	24467	23033	0,30	0,41	**1,34**	**1,52**	63667	32247
	Z-Pro	0,25	0,25	**1,00**				0,41	0,41	**1,00**				0,48	0,48	**1,00**			
	Ac-Phe	0,21	0,3	**1,45**	1,4	43693	35913	0,39	0,54	**1,4**	**2,29**	55307	47380	0,47	0,65	**1,39**	**2,12**	31267	38667
	Ac-Pro	0,13	0,15	**1,16**	0,32	36873	28493	1,01	1,88	**1,87**	**6,77**	41987	36293	0,36	0,36	**1,00**			
	Ac-Trp	0,53	0,93	**1,75**	3,48	29013	21760	0,57	0,77	**1,37**	**2,14**	30573	32213	1,03	1,70	**1,65**	**4,86**	32927	30653
	DNB-Glu	1,06	4,2	**3,95**	8,44	16673	8660	2,44	11,48	**4,7**	**16,91**	29780	22400	2,61	10,71	**4,10**	**15,07**	24453	21027
	DNB-Asp	2,6	4,9	**1,88**	4,41	9467	8613	7,41	12	**1,62**				8,51	11,87	**1,40**	**3,66**	14180	17300
	DNB-Phe	1,42	7,62	**5,36**	9,55	13073	6820	2,15	2,87	**1,34**	**3,54**	35453	35467	2,44	2,73	**1,12**	**1,41**	35713	27820
	DNP-Glu	1,89	2,48	**1,31**	1,83	11127	10160	4,93	6,14	**1,25**	**2,78**	26293	22193	5,57	6,53	**1,17**	**1,90**	21240	20773
	DNP-Asp	2,53	3,62	**1,43**	2,44	9460	8560	6,83	10,04	**1,47**	**5,19**	25107	24893	8,39	11,57	**1,38**	**3,88**	19193	19360
	Dichlorprop	0,41	0,56	**1,36**	1,34	20667	18913	0,86	1,08	**1,25**	**2,21**	44033	41727	1,04	1,26	**1,21**	**1,77**	33440	32733
	Mecoprop	0,26	0,33	**1,28**	0,87	25907	24620	0,53	0,64	**1,2**	**1,44**	47600	46860	0,68	0,78	**1,16**	**1,14**	38847	15393
	Naproxen	0,31	0,31	**1,00**				0,43	0,43	**1,00**				0,43	0,43	**1,00**			
Zwitterionic	Phe	0,72	0,76	**1,04**	0,42	73713	42367	0,89	1	**1,12**	**1,14**	47047	43173	0,56	0,62	**1,11**	**0,63**	31980	23187
	Trp	1,43	2,18	**1,53**	4,31	24887	29847	1,91	3,55	**1,86**	**7,95**	35760	34107	1,19	1,98	**1,67**	**5,04**	29800	28307
	Tyr	0,91	0,97	**1,07**	0,46	38093	16787	1,15	1,34	**1,16**	**1,35**	30613	27927	0,75	0,87	**1,15**	**0,77**	17600	14860
	beta-Phe	1,35	1,35	**1,00**				1,5	1,5	**1,00**				0,87	0,93	**1,07**	**0,55**	49933	27033
	beta-Homo-Phe	1,53	1,53	**1,00**				1,68	1,68	**1,00**				1,00	1,00	**1,00**			
	alpha-Me-Phe	0,03	0,03	**1,00**				0,79	1,05	**1,32**	**2,5**	37713	37973	0,47	0,63	**1,32**	**1,78**	34280	35187
	alpha-Me-Trp	1,33	3,22	**2,42**	10,34	34853	33900	1,83	5,65	**3,1**	**14,31**	36540	32420	1,11	2,96	**2,67**	**9,81**	30680	26233
	alpha-Me-DOPA	1,09	1,57	**1,44**	3,36	27000	29113	1,49	2,28	**1,54**	**4,84**	35207	31527	0,95	1,44	**1,53**	**3,28**	23200	21847
	DOPA	1,46	1,46	**1,00**				1,62	1,96	**1,21**	**2,03**	28980	28620	1,00	1,21	**1,21**	**1,50**	24067	23053
	p-Br-Phe	1,02	1,02	**1,00**				1,25	1,35	**1,08**	**0,71**	27780	23840	0,76	0,83	**1,09**	**0,61**	34913	22393
	p-Cl-Phe	0,9	0,9	**1,00**				1,09	1,18	**1,08**	**0,67**	33213	26293	0,68	0,74	**1,09**	**0,58**	41400	22587

Table A1: *Results of evaluation of QN-based CSPs with acidic and zwitterionic chiral compounds.*

	Peptides	tau-QN						homotau-QN						homohomotau-QN					
		k1	k2	alpha	Res.	N1[m-1]	N2[m-1]	k1	k2	alpha	Res.	N1[m-1]	N2[m-1]	k1	k2	alpha	Res.	N1[m-1]	N2[m-1]
Di- and Tripeptides	Pro-Phe	1,86	3,52	1,89	8,31	32467	39673	2,10	4,22	2,01	9,63	39727	37460	1,11	2,32	2,08	7,55	32887	29853
	Ala-Phe	1,55	2,71	1,75	7,15	34980	42747	1,85	3,27	1,77	7,65	40407	38393	0,97	1,88	1,95	6,52	34827	30127
	Gly-Phe	1,90	2,54	1,33	3,47	29067	37153	2,22	3,00	1,35	4,07	37887	38067	1,11	1,65	1,49	3,93	32733	30827
	Gly-Val	1,60	1,81	1,13	0,95	14980	16847	1,75	2,05	1,17	1,94	64227	24353	1,00	1,27	1,27	1,60	17073	17773
	Gly-Leu	1,68	1,68	1,00				1,84	1,84	1,00	0,00			1,12	1,12	1,00	0,00		
	Ala-Val	1,32	1,69	1,28	1,67	11087	16387	1,46	1,99	1,36	2,85	20853	24393	0,89	1,28	1,45	2,25	13627	15980
	Gly-Trp	4,18	4,18	1,00				2,42	5,16	2,13	7,21	15127	18333	2,73	6,88	2,52	7,98	10933	14780
	Gly-Pro	1,81	1,93	1,07	0,31	n.d.	n.d.	1,29	1,91	1,47	1,09	10080	1147	0,67	1,15	1,73	1,47	19933	1693
	Pro-Gly	2,04	2,21	1,08	0,74	25673	15447	2,35	2,56	1,09	0,86	25013	16373	1,43	1,57	1,10	0,68	32060	10327
	Gly-Thr	1,86	2,42	1,30	2,37	16900	20640	2,19	2,85	1,30	2,45	17680	18853	1,29	1,81	1,40	2,13	11173	12333
	Gly-Asp	3,16	3,74	1,18	2,13	38460	22087	4,44	5,33	1,20	2,00	19320	17960	2,61	3,16	1,21	1,80	22147	13747
	Ala-Gly-Gly	2,30	2,30	1,00				2,73	2,73	1,00				1,62	1,62	1,00			
	Gly-Gly-Ala	2,23	2,23	1,00				2,49	2,49	1,00				1,22	1,45	1,19	1,50	43527	15407
	Ala-Gly-Ala	1,76	1,76	1,00				2,04	2,04	1,00				0,59	1,23	2,09	3,70	23807	9133
Homologues	Ala-2	1,57	1,67	1,07	0,51	30767	11980	1,88	1,92	1,02	0,25	36820	25467	1,08	1,20	1,11	0,65	17173	12173
	Ala-3	1,48	1,63	1,11	0,66	9380	16867	1,73	1,81	1,05	0,38	13887	28707	1,02	1,15	1,13	0,60	7873	13733
	Ala-4	1,46	1,65	1,13	0,68	9800	8293	1,67	1,90	1,13	0,94	19847	17160	1,00	1,15	1,14	0,67	10493	9800
	Ala-5	1,45	1,65	1,13	0,65			1,66	1,89	1,14	0,94	13293	14220	1,00	1,10	1,10	0,30	2260	8180
	Ala-6	1,52	1,69	1,12	0,58	8633	6860	0,14	0,23	1,64	0,86	n.d.	n.d.	0,51	1,02	2,00	0,93	733	1587
	Val-2	1,18	2,12	1,80	4,35	13420	18313	1,29	2,49	1,93	5,70	21107	19333	0,80	1,53	1,93	3,82	13380	13393
	Val-3	1,17	1,94	1,66	0,94	12747	447	1,29	2,32	1,79	1,27	16880	580	0,81	1,74	2,15	2,03	15233	1360
	Val-4	1,09	1,42	1,30	2,01	19200	21253	1,21	1,78	1,47	3,54	24433	27307	0,76	1,23	1,61	2,95	16007	18053

Table A2: Results of evaluation of QN-based CSPs with all-L and all-D peptide enantiomers.

	Chiral Analytes	tau-QD						homotau-QD						homohomotau-QD					
		k1	k2	alpha	Res.	N1[m-1]	N2[m-1]	k1	k2	alpha	Res.	N1[m-1]	N2[m-1]	k1	k2	alpha	Res.	N1[m-1]	N2[m-1]
Acidic	Fmoc-Phe	0,41	0,54	1,33	1,52	23427	36460	0,56	0,71	1,28	1,52	25720	29960	0,44	0,59	1,34	1,50	22607	26927
	Z-Phe	0,23	0,28	1,23	0,65	24200	30053	0,33	0,39	1,19	0,60	22347	16700	0,25	0,31	1,25	0,69	21467	20613
	Z-beta-Phe	0,15	0,15	1,00		0	0	0,12	0,20	1,62	1,20	40660	34067	0,11	0,17	1,59	1,03	41507	35193
	DNB-Phe	0,63	4,13	6,61	16,31	37240	23967	1,04	5,09	4,87	15,19	24933	24993	0,77	4,22	5,49	12,79	14640	19347
	Ac-Trp	0,27	0,52	1,96	3,53	40193	38893	0,37	0,69	1,87	3,76	33193	34667	0,30	0,49	1,64	2,28	26900	31793
	DNB-Glu	0,44	1,73	3,93	10,39	32500	29160	0,87	3,48	3,99	11,77	19267	23233	0,60	2,53	4,23	11,71	27727	24960
	DNB-Asp	1,63	2,18	1,34	2,99	28427	25747	3,26	4,01	1,23	1,98	15460	17080	2,08	2,74	1,32	2,73	21540	20333
	DNB-Leu	0,22	1,33	6,00	10,08	23940	30480	0,38	2,14	5,71	13,01	29187	29807	0,28	1,62	5,78	11,39	27960	30020
	Fmoc -Ser	0,36	0,55	1,54	1,89	14333	35893	0,50	0,66	1,34	1,50	16513	28273	0,39	0,54	1,38	1,36	15027	24867
	DNP-Glu	0,80	1,01	1,25	1,85	34313	31880	1,61	1,81	1,13	1,11	24607	22147	1,20	1,37	1,14	1,17	27673	26193
	DNP-Asp	0,99	1,33	1,34	2,74	34713	31400	2,18	2,67	1,23	1,87	16807	18820	1,50	1,87	1,25	2,11	25620	24633
	DNP-Phe	0,84	1,13	1,35	2,75	36873	36573	1,36	1,69	1,24	2,36	33147	33607	1,07	1,35	1,27	2,20	32113	30860
	Dichlorprop	0,17	0,32	1,96	2,49	43947	38367	0,30	0,49	1,64	2,56	39093	35733	0,22	0,37	1,65	1,95	32280	32640
	Mecoprop	0,11	0,18	1,68	1,34	37327	54253	0,20	0,28	1,44	1,25	34780	34713	0,14	0,21	1,43	0,89	30780	30787
	Naproxen	0,19	0,19	1,00				0,22	0,22	1,00				0,20	0,20	1,00			
Zwitterionic	Phe	0,46	0,46	1,00				0,45	0,45	1,00				0,44	0,44	1,00			
	beta--Phe	0,93	1,01	1,09	0,78	47640	33000	0,12	0,75	6,45	8,21	43240	33613	0,11	0,85	7,99	9,24	41060	33420
	alpha-Me-Phe	0,38	0,47	1,25	1,37	49433	43993	0,38	0,48	1,28	1,31	34453	33093	0,38	0,46	1,23	1,02	31507	29280
	Trp	0,80	1,10	1,39	3,01	40280	38573	0,83	1,25	1,50	3,41	30900	29267	0,83	1,06	1,27	1,75	24460	23360
	alpha-Me-Trp	0,72	1,68	2,35	8,38	41547	37440	0,87	0,96	1,11	0,73	23540	23607	0,83	0,89	1,07	0,48	44860	18560
	5-OH-Trp	1,01	1,33	1,32	2,71	35687	34960	1,02	1,51	1,47	3,46	28053	28367	1,04	1,35	1,29	1,95	21027	21000
	DOPA	0,72	0,72	1,00				0,66	0,73	1,10	0,56	37307	16453	0,74	0,74	1,00			
	alpha-Me-DOPA	0,56	0,88	1,57	3,51	39893	37813	0,60	0,85	1,42	2,39	29333	28740	0,62	0,84	1,36	1,84	22780	21147
	p-Br-Phe	0,11	0,61	5,45	7,38	50000	39760	0,62	0,62	1,00				0,59	0,59	1,00			
	p-Cl-Phe	0,09	0,54	6,28	6,40	30407	41793	0,55	0,55	1,00				0,53	0,53	1,00			
	Tyr	0,50	0,50	1,00				0,23	0,55	2,39	2,69	17553	12860	0,55	0,55	1,00			

Table A3: Results of evaluation of QD-based CSPs with acidic and zwitterionic analytes

	Peptides	tau-QD						homotau-QD						homohomotau-QD					
		k1	k2	alpha	Res.	N1[m-1]	N2[m-1]	k1	k2	alpha	Res.	N1[m-1]	N2[m-1]	k1	k2	alpha	Res.	N1[m-1]	N2[m-1]
Peptides	Pro-Phe	0,84	2,00	**2,37**	**9,24**	44200	37787	0,82	2,54	**3,10**	**10,63**	33253	27520	0,82	1,86	**2,26**	**7,42**	31347	29240
	Ala-Phe	0,62	1,45	**2,34**	**8,13**	46167	39940	0,64	1,67	**2,60**	**8,33**	33220	32260	0,66	1,36	**2,09**	**6,14**	31547	31587
	Gly-Phe	0,77	1,29	**1,68**	**5,11**	43993	41440	0,77	1,35	**1,76**	**4,67**	30293	28180	0,74	1,18	**1,60**	**3,69**	29527	27360
	Gly-Val	1,00	1,20	**1,20**	**0,48**	7007	1500	0,66	0,86	**1,30**	**1,03**	9340	8947	0,73	0,93	**1,27**	**1,21**	13587	13500
	Gly-Leu	1,13	1,13	**1,00**				0,64	0,77	**1,19**	**0,51**	13847	3000	0,74	0,83	**1,13**	**0,58**	13240	10707
	Ala-Val	0,91	1,17	**1,29**	**0,74**	3340	3573	0,50	0,80	**1,60**	**1,06**	3200	4020	0,66	0,90	**1,37**	**1,41**	9540	12873
	Ala-2	1,07	1,21	**1,13**	**0,58**	7413	9433	0,59	0,78	**1,33**	**0,69**	4787	3427	0,74	0,87	**1,19**	**0,78**	11380	11293
	Ala-3	1,00	1,11	**1,11**	**0,43**	5547	9080	0,53	0,63	**1,20**	**0,42**	9487	2627	0,70	0,82	**1,17**	**0,66**	10060	10640
	Ala-4	0,95	1,06	**1,12**	**0,48**	5467	10963	0,49	0,59	**1,21**	**0,38**	5087	2487	0,65	0,76	**1,17**	**0,61**	7860	11340
	Ala-5	0,97	1,12	**1,15**	**n.d.**	n.d.	n.d.	0,81	?	**1,00**				0,75	0,92	**1,23**	**0,12**	11153	53
	Ala-6	0,36	0,99	**2,78**	**1,78**	1347	3833	0,59	?	**1,00**				0,64	0,74	**1,15**	**0,44**	6820	5840
	Val-2	0,82	1,36	**1,66**	**1,75**	3733	6200	0,44	1,01	**2,31**	**2,36**	2320	10507	0,60	1,03	**1,73**	**2,42**	6713	17353
	Val-3	0,77	1,06	**1,39**	**1,17**	6687	5733	0,40	0,80	**2,01**	**1,57**	2820	6167	0,58	1,05	**1,82**	**2,89**	10200	16413
	Val-4	0,70	0,90	**1,29**	**0,24**	3940	200	0,38	0,67	**1,76**	**0,34**	907	200	0,52	0,85	**1,64**	**0,36**	1340	187

Table A4: *Results of evaluation of QD-based CSPs with all-L and all-D peptide enantiomers*

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