**University of Szeged**
**Faculty of Pharmacy**
**Institute of Pharmaceutical Chemistry**

# Liquid and supercritical fluid chromatographic enantioseparation of $N^{\alpha}$-Fmoc proteinogenic amino acids on *Cinchona* alkaloid-based chiral stationary phases

**Ph.D. Thesis**

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# Tartalom

## List of publications and lectures

### Papers related to the thesis

**I.** **Lajkó G.**, Ilisz I., Tóth G., Fülöp F., Lindner W., Péter A.
Application of *Cinchona* alkaloid-based zwitterionic chiral stationary phases in supercritical fluid chromatography for the enantioseparation of $N^{\alpha}$-protected proteinogenic amino acids, In: Journal of Chromatography A, 1415 (2015) 134–145.

**if: 3.981**

**II.** **Lajkó G.**, Grecsó N., Tóth G., Fülöp F., Lindner W., Péter A., Ilisz I.
A comparative study of enantioseparations of $N^{\alpha}$-Fmoc proteinogenic amino acids on Quinine-based zwitterionic and anion exchanger-type chiral stationary phases under hydro-organic liquid and subcritical fluid chromatographic conditions, In: Molecules, 21 (11) (2016) 1579.

**if: 2.861**

**III.** **Lajkó G.**, Grecsó N., Tóth G., Fülöp F., Lindner W., Péter A., Ilisz I.
Liquid and subcritical fluid chromatographic enantioseparation of $N^{\alpha}$-Fmoc proteinogenic amino acids on quinidine-based zwitterionic and anion-exchanger type chiral stationary phases. A comparative study, In: Chirality, 29 (2017) 225–238.

**if: 1.956**

**Sum of impact factors: 8.798**

### Other papers

**IV.** Ilisz I., Gecse Z., **Lajkó G**., Nonn M., Fülöp F., Lindner W., Péter A.
Investigation of the structure-selectivity relationships and van't Hoff analysis of chromatographic stereoisomer separations of unusual isoxazoline-fused 2-aminocyclopentanecarboxylic acids on *Cinchona* alkaloid-based chiral stationary phases, In: Journal of Chromatography A, 1384 (2015) 67–75.

**if: 3.981**

**V.** Ilisz I., Gecse Z., **Lajkó G.**, Forró E., Fülöp Z., Lindner W., Péter A.
High-performance liquid chromatographic enantioseparation of cyclic $\beta$-amino acids applying zwitterionic chiral stationary phases based on *Cinchona* alkaloids, In: Chirality, 27 (2015) 563–570.

**if: 1.956**

**VI.** **Lajkó G.**, Orosz T., Kiss L., Forró E., Fülöp F., Péter A., Ilisz I.
High-Performance liquid chromatographic enantioseparation of fluorinated cyclic $\beta^3$–amino acid analogs on polysaccharide-based chiral stationary phases. Comparison with nonfluorinated counterparts, In: Biomedical Chromatography, 30 (2016) 1441–1448.

**if:1.613**

**VII.** **Lajkó G.**, Orosz T., Grecsó N., Fekete B., Palkó M., Fülöp F., Lindner W., Péter A., Ilisz I.
High-Performance liquid chromatographic enantioseparation of cyclic *β*-aminohydroxamic acids on zwitterionic chiral stationary phases based on *Cinchona* alkaloids, In: Analytica Chimica Acta, 921 (2016) 84–94.

**if:4.950**

**VIII.** **Lajkó G.**, Grecsó N., Megyesi R., Forró E., Fülöp F., Wolrab D., Lindner W., Péter A., Ilisz, I.
Enantioseparation of *β*-carboline derivatives on polysaccharide- and strong cation exchanger-based chiral stationary phases. A comparative study, In: Journal of Chromatography A, 1467 (2016) 188–198.

**if: 3.981**

**IX.** Orosz T., Grecsó N., **Lajkó G.**, Szakonyi Z., Fülöp F., Armstrong D.W., Péter A., Ilisz I.
Liquid chromatographic enantioseparation of carbocyclic *β*-amino acids possessing limonene skeleton on macrocyclic glycopeptide-based chiral stationary phases. In: Journal of Pharmaceutical and Biomedical Analysis, 145 (2017) 119–126.

**if:3.255**

**X.** **Lajkó G.**, Orosz T., Ugrai I., Szakonyi Zs., Fülöp F., Lindner W., Péter A., Ilisz I.
Liquid chromatographic enantioseparation of limonene-based carbocyclic *β*–amino acids on zwittterionic *Cinchona* alkaloid-based chiral stationary phases. In: Journal of Separation Science, 40 (2017) 3196–3204.

**if:2.557**

**Sum of impact factors: 22.293**

**Total impact factor: 31.091**

## Abbreviations and symbols

| | |
|---|---|
| (*v*/*v*) | volume to volume ratio |
| ACHSA | *trans*-2-aminocyclohexanesulfonic acid |
| AcOH | glacial acetic acid |
| BA | butylamine |
| CDA | chiral derivatizing agent |
| CMPA | chiral mobile phase additive |
| CSP | chiral stationary phase |
| DEA | diethylamine |
| EA | ethylamine |
| *ee*% | enantiomeric excess [*ee*% = (*R*-*S*) / (*R*+*S*)]×100 (amount of *R* and *S* configuration of enantiomers) |
| FA | formic acid |
| $H_2O$ | Milli-Q water |
| HO | hydro-organic |
| HPLC | high-performance liquid chromatography |
| LC | liquid chromatography |
| MeCN | acetonitrile |
| MeOH | methanol |
| NP | normal-phase |
| PA | propylamine |
| PI | polar-ionic |
| PO | polar organic |
| QD | quinidine |
| QN | quinine |
| RP | reversed-phase |
| SA | selectand |
| SFC | supercritical fluid chromatography |
| SO | selector |
| TEA | triethylamine |
| TEAA | triethylammonium acetate |
| TFA | trifluoroacetic acid |
| TLC | thin layer chromatography |
| ZWIX | zwitterionic chiral stationary phase |
| $t_R$ | retention time |
| $t_0$ | column dead-time |
| $k$ | retention factor; defined as $(t_R - t_0)/t_0$; |
| $\alpha$ | selectivity; defined as $k_2/k_1$; 1: first- and 2: second-eluting peak |
| $R_S$ | resolution; defined as $[(t_{R2} - t_{R1})/(w_1 + w_2)]\times 2$ ; *w*, peak width measured on the baseline |

# 1 Introduction

It is broadly accepted, that chirality is a universal phenomenon and chirality at the molecular level plays an essential role in biological systems. In this context, proteins, peptides, canonical amino acids (except glycine), saccharides, enzymes, and many metabolites are chiral products being present in living organisms. Under "chiral conditions" (*e.g.*, in a living organism), enantiomeric compounds may behave in different ways. They may differ in their type and range of biological effects as well as their utilization, distribution, metabolism, *etc*. It is well established that pharmacological activity is mostly restricted to one of the enantiomers (eutomer). In several cases, unwanted side effects or even toxic effects may occur with the inactive enantiomer (distomer). Even if the side effects are not that drastic, the inactive enantiomer has to be metabolized; this, however, represents an unnecessary burden for the organism. An example of this is thalidomide, which was introduced as a sedative drug and painkiller in the late of 1950s. Another example is amphetamine, where the *S*-(+)-isomer is a few times more potent in central nervous system stimulation than *R*-(–)-amphetamine. The latter, in turn, is slightly more potent in the peripheral system, for example, in cardiovascular action. The administration of pure, pharmacologically active enantiomers is therefore of great importance. Therefore, regulatory authorities in Europe (EMA), USA (FDA), and Japan (PMDA) nowadays impose strict guidelines for the commercialization of chiral drug substances. Enantioselective identification and quantification methods should be developed for each active pharmaceutical ingredient with chiral properties. In addition, pharmacokinetic and toxicological assays should be executed with both pure enantiomers and racemates. Based on these fundamental findings, the life science industry has to pay attention to chirality-related phenomena when developing, *e.g.*, biologically active chiral pharmacons.

The separation of enantiomers is among the more challenging chromatographic modalities due to the fact that conventional strategies employed to separate achiral analytes are ineffective when applied to enantiomers. Chromatographic methods, including gas chromatography (GC), thin-layer chromatography (TLC), capillary electrophoresis (CE), capillary electrochromatography (CEC), supercritical fluid chromatography (SFC), and high-performance liquid chromatography (HPLC) are the most popular techniques. Researchers have explored various column screening methods to reduce method development time, and they utilized smaller particle sizes of fully porous particles (FPPs) and superficially porous particles (SPPs) to improve efficiencies and analysis times, which are typical constraints in enantiomeric separations. The last quarter of the century has seen a vast growth of diverse chiral technologies,

including stereocontrolled synthesis and enantioselective separation and analysis concept. As the introduction of effective, new classes of chiral selectors has slowed, other important factors such as efficiency and analysis time have started to garner attention from the chromatography community.

Recently, SFC has become an alternative technique to HPLC for routine applications in enantioresolution of pharmaceutical compounds, because it may offer several advantages over HPLC in certain circumstances, including improved resolution, faster separations, and higher throughput. These benefits arise from the characteristics of supercritical fluids (SCFs), which are considered green mobile phases. Characteristic features are limited environmental impact, low disposal costs, reduced consumption of toxic solvents and additives, lack of toxicity (in most cases), residue-free removal of the solvent from the extract and the raffinate, and the ability to recover the solvent almost completely. The reduction in the use of organic solvents results in cost, health, and safety benefits, and faster, cleaner sample recovery during experimental procedures. Moreover, SFC is suitable for non-polar pharmaceuticals but cannot be applied to very polar compounds. However, the addition of an organic modifier into the mobile phase (possibly, with the addition of a third component at low concentration) may afford elution of polar drugs. Furthermore, compression of solvents requires elaborate recycling measures to reduce energy costs and high capital investment for equipments.

### *1.1 The aim of this work*

The primary aim of this work was to develop chiral separation methods for 19 $N^{\alpha}$-Fmoc-protected protein amino acids on *Cinchona* alkaloid-based zwitterionic and anion-exchanger type chiral stationary phases (CSPs). Two different types of techniques were used for separation. One of them is the well-known high-performance liquid chromatography (HPLC) technique, which is the most straightforward and efficient mode used widely. The other separation technique, which uses supercritical fluid as the main component of the mobile phase, is supercritical fluid chromatography (SFC).

The effect of the nature and concentration of bulk solvent components, the role of water content in the mobile phase, the nature and concentration of base and acid additives, and the temperature on chromatographic parameters were investigated applying *Cinchona* alkaloid-based chiral stationary phases (CSPs). Thermodynamic parameters were calculated utilizing temperature dependence studies.

# 2 Literature Review

Various methods are available for chiral separation. Direct crystallization affords high optical purity (in cases, up to 90%), but development time is long. Enzymatic reactions destroying the unwanted enantiomer are difficult, because the appropriate enzyme, which should be available on a large scale, need to be identified. Finally, high-performance liquid chromatography (HPLC) represents the most popular, rapid, and highly applicable technology in the field of varied chiral analyses of racemic and scalemic mixtures.

While HPLC has long been in the lead, now supercritical fluid chromatography (SFC) is gaining ground and is progressively becoming the first choice in enantioseparation and purification in the pharmaceutical industries.

## *2.1 Chiral chromatography*

Chiral recognition and enantiomer distinction are significant phenomena in both nature and chemical systems. It has high impact in various fields dealing with bioactive compounds, in particular, in drug discovery, development of agrochemicals, research on food additives, chiral pollutants, *etc.* However, the most significant developments in chiral recognition were triggered by demand of drug discovery in pharmaceutical industries. Among the analytical techniques, the most important chromatographic methods are thin layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) as well as supercritical fluid chromatography (SFC).

In chiral chromatography, two different procedures can be used for the separation of enantiomers: indirect and direct methods. Each of these techniques has advantages and drawbacks. Historically, indirect methods were developed first. This separation was based on the formation of diastereoisomeric complexes between the enantiomers and one of the antipodes of the chiral derivatizing agents (CDAs) followed by their subsequent separation by an achiral liquid chromatographic method. Numerous CDAs are available on the market with a comparatively wide selection of chromatographic conditions *[1]*. This method is not very practical, because derivatization is an additional step, which can involve undesirable side reactions, formation of decomposition products, racemization, and kinetic resolution. Furthermore, the chiral derivatization reagent has to be of high enantiomeric purity and the presence of derivatizable groups in the analyte is a prerequisite.

The other and, in fact, one of the best methods of enantioseparation is the direct chromatographic method, which involves two modes. One of them is the chiral mobile phase

additive (CMPA) mode, when a chiral compound is added to the mobile phase in an appropriate concentration. Separation can be achieved on an achiral stationary phase through the formation of a diastereomeric complex. The other mode is the direct liquid chromatographic enantioseparation with chiral stationary phases (CSP). Nowadays, this is the most straightforward and efficient mode used widely *[2–4]*.

### *2.2 Chiral stationary phases*

In separation science, the reversible formation of diastereomers between the enantiomers of analyte (*R/S*)-A and the chiral selector (*R*)-S or (*S*)-S is the basis for direct enantioseparation. The equilibria can be characterized by the equations assuming (*R*)-configuration of the selector S:

$$(R)\text{–A} + (R)\text{–S} \overset{K_R}{\leftrightarrows} [(R)\text{–A --- (}R\text{)–S}]$$

$$(S)\text{–A} + (R)\text{–S} \overset{K_S}{\leftrightarrows} [(S)\text{–A --- (}R\text{)–S}]$$

The association constants $K_R$ and $K_S$ represent the physico-chemical basis for the stereoselective recognition of enantiomers by chiral selectors *[5]*.

For CSPs the degree of separation depends on different interactions of the enantiomers with the chiral selector (*e.g.*, hydrogen bonding, *π–π*, dipole–dipole, ionic, electrostatic, hydrophobic or hydrophilic interactions, steric effects, *etc.*). The most widespread structural model to explain the stereoselective binding of chiral molecules and selector is the three-point interaction model, which was postulated by *Easson* and *Stedman* in 1933 *[6]*. It was later adapted by *Ogsten* for enzyme–substrate interaction *[7]*. *Dalgliesh* has interpreted this model for the separation of amino acids by thin layer chromatography (TLC) *[8]*. He found that it is necessary to have three attractive interaction sites between the selector (SO) and selectand (SA). *Pirkle* and *Pochapsky [9]*, and then *Davankov [10]* in their milestone announcements in this area refined the model with various additions through a summary of the results. In addition to attractive interactions, they also recognized the possible role of repulsive interactions (*e.g.*, steric inhibition) and determined, that at least one of the three necessary interactions must be stereoselective. The three-point interaction model (***Figure 1***) is the most reliable model and it is still utilized frequently to explain the process of chiral recognition *[11,12]*.

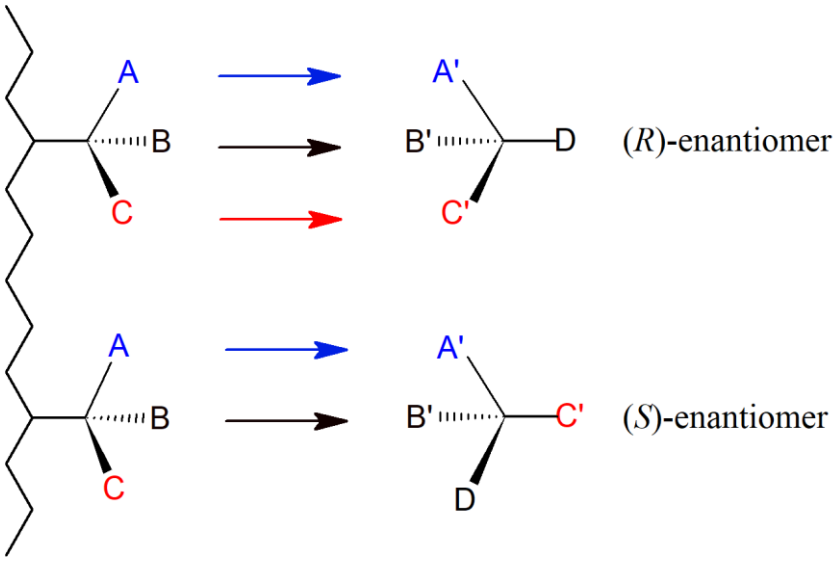



***Figure 1.*** The three-point-interaction model

Effective binding can be achieved if there is:

- *steric fit* – size and shape complementarity; the binding guest sterically fit to the binding site of the chiral selector,
- *electrostatic fit* – favorable geometric and spatial orientation of complementary functional groups,
- *hydrophobic fit* – if hydrophobic regions of both binding partners can spatially match each other,
- *dynamic fit* and *induced fit* – maximize binding interactions by dynamic and conformational adaptation in the course of complex formation.

CSPs can be grouped in several ways, depending on their separation principle. The most frequent stationary phases are summarized by their selector and major interactions (***Table 1***).

***Table 1.*** *Groups of chiral stationary phases*

| | **Stationary phases** | **Selector** | **Major interaction** |
|---|---|---|---|
| **I** | Ligand-exchange (Davankov) | amino acid–metal complexes | complex formation |
| **II** | Donor–acceptor (Pirkle-type CSP) | $\pi$-acidic and $\pi$-basic groups | H-bonding, $\pi$–$\pi$, and dipole–dipole |
| **III** | Polysaccharide-based | modified cellulose and amylose | polar, $\pi$–$\pi$, and steric |
| **IV** | Inclusion complexes | cyclodextrin<br>chiral crown ether<br>cyclofructan | complexation, $\pi$–$\pi$, H-bonding, and steric effects |

| | | | |
|---|---|---|---|
| **V** | Macrocyclic antibiotics | macrocyclic glycopeptides | electrostatic, H-bonding, hydrophobic, $\pi$–$\pi$ interaction, steric effects |
| | **Stationary phases** | **Selector** | **Major interaction** |
| **VI** | Ion-exchanger | anion-, cation-, and zwitter-ion-based selectors | ionic, polar, $\pi$–$\pi$, and steric |
| **VII** | Protein | natural proteins | ionic and hydrophobic |
| **VIII** | Molecular imprinted | selective sorbents (*e.g.*, macromolecules, organic molecules) | steric |

### *2.2.1 Ligand-exchange CSPs*

In the late 1960s, the first full separation of a racemic amino acid by chiral ligand-exchange chromatography (CLEC) could be achieved by *Davankov [13,14]*. He used the amino acid proline as SO immobilized onto a polystyrene support in combination with a metal ion ($Cu^{2+}$). The technique is based on the formation of a reversible ternary diastereomeric coordination complex between SO, a metal ion, and SA. During the chromatographic process, the coordinated ligands are reversibly replaced by other ligands from the mobile phase such as water, ammonia or other components of the eluent. The resulting diastereomeric chelate possesses a different thermodynamic stability.

This CSP was used not only for the separation of both $\alpha$-amino acids and $\beta$-amino acids *[15,16]*. Nowadays, the commercially available ligand-exchange CSPs include immobilized derivatives of proline *[17]*, hydroxyproline *[18]* or penicillamine *[19]*. For a long time, CLEC was the only separation method that enabled the direct enantiomer separation of amino acids without derivatization. Nowadays, it is less important due to attractive alternatives.

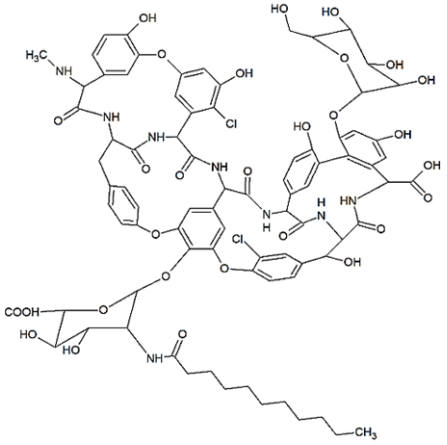
### *2.2.2 Macrocyclic antibiotics CSPs*

Macrocyclic antibiotics have been introduced as chiral selectors for HPLC in 1994 by *Armstrong* and coworkers *[20,21]*. They used many macrocyclic antibiotic compounds as chiral selectors in HPLC, including glycopeptides vancomycin (Chirobiotic V) *[20]*, teicoplanin (Chirobiotic T) *[22]*, teicoplanin aglycon (Chirobiotic TAG) *[23]*, ristocetin A (Chirobiotic R) *[24]*, and avoparcin *[25]*, the polypeptide thiostrepton, as well as ansamycin and rifamycins (***Figure 2***). The common structural feature of these selectors is a set of interconnected amino acid-based macrocycles, each macrocycle containing two aromatic rings and a peptide sequence. Vancomycin contains three macrocycles, while teicoplanin and ristocetin A are composed of four. The macrocycles form a three-dimensional, C-shaped basketlike structure.

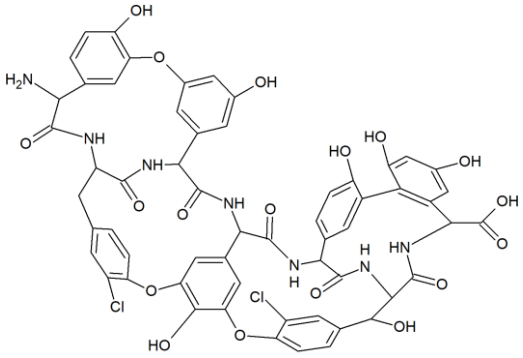
The carbohydrate moieties are positioned at the surface and ionizable groups such as a carboxylic acid group or amino groups are also present. Thus, a large number of interactions between analyte molecules and glycopeptide antibiotics are possible including hydrogen bonds, $\pi$–$\pi$, dipole–dipole, and ionic interactions depending on the experimental conditions. The main reasons of the versatility of CSPs are their multi-modal applicability in normal phase (NP), polar organic (PO), polar ionic (PI), and reversed phase (RP) modes. Macrocyclic glycopeptide CSPs are also used for chiral separation in SFC *[26]*. *Armstrong et al.* had compared the chiral recognition capabilities of three glycopeptide-based columns (Chirobiotic T, Chirobiotic TAG, and Chirobiotic R) in SFC for a set of 111 chiral compounds, including heterocycles, analgesics (nonsteroidal anti-inflammatory compounds), *β*-blockers, sulfoxides, as well as *N*-protected and native amino acids *[27]*.

Dozens of papers have demonstrated their capability of enantiomeric separation and their broad applicability profiles, comprising chiral acids, bases, amphoteric, and neutral compounds, as well as small peptides *[28–32]*.

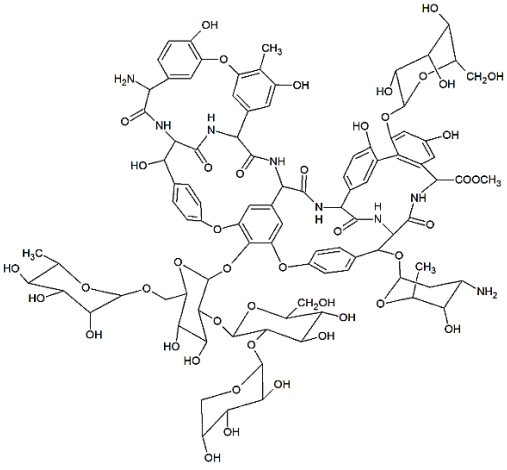
***A,*** *Teicoplanin*



***B,*** *Teicoplanin aglycon*



***C,*** *Ristocetin A*



***D,*** *Vancomycin*

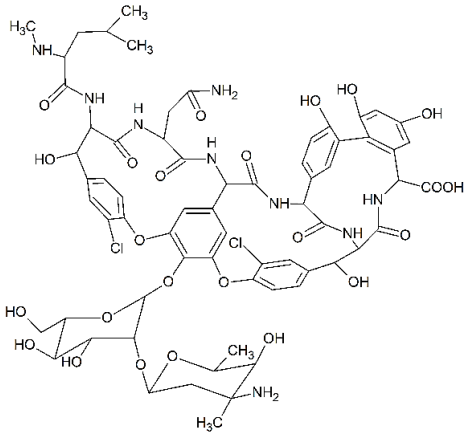


***Figure 2****. Structures of teicoplanin and its structurally related analogs*

### *2.2.3 Polysaccharide-based CSPs*

Polysaccharide selectors have a long tradition in enantioselective liquid chromatography. In the 1970s *Hessel* and *Hagel* applied microcrystalline cellulose triacetate (MTCA) as a polymeric selector material *[33]*. *Okamoto et al.* in 1984 coated macroporous aminopropyl-silanized silica gel with cellulose triacetate *[34]*. Such coated polysaccharide CSPs based on cellulose and amylose (***Figure 3***) derivatives (carbamates and esters) have set the state-of-the-art for several decades and have since been available from several suppliers.

The immobilized polysaccharide CSPs have further expanded the versatility and application area via their extended choice of mobile phases. It can be operated in NP mode, PO mode, RP mode, and SFC mode. This widespread applicability offers the possibility to develop more complex systematic methods and automated screening procedures. It should be emphasized that polysaccharide CSPs are also a good choice for preparative enantiomer separation, because they have the highest loadabilities *[35]*.

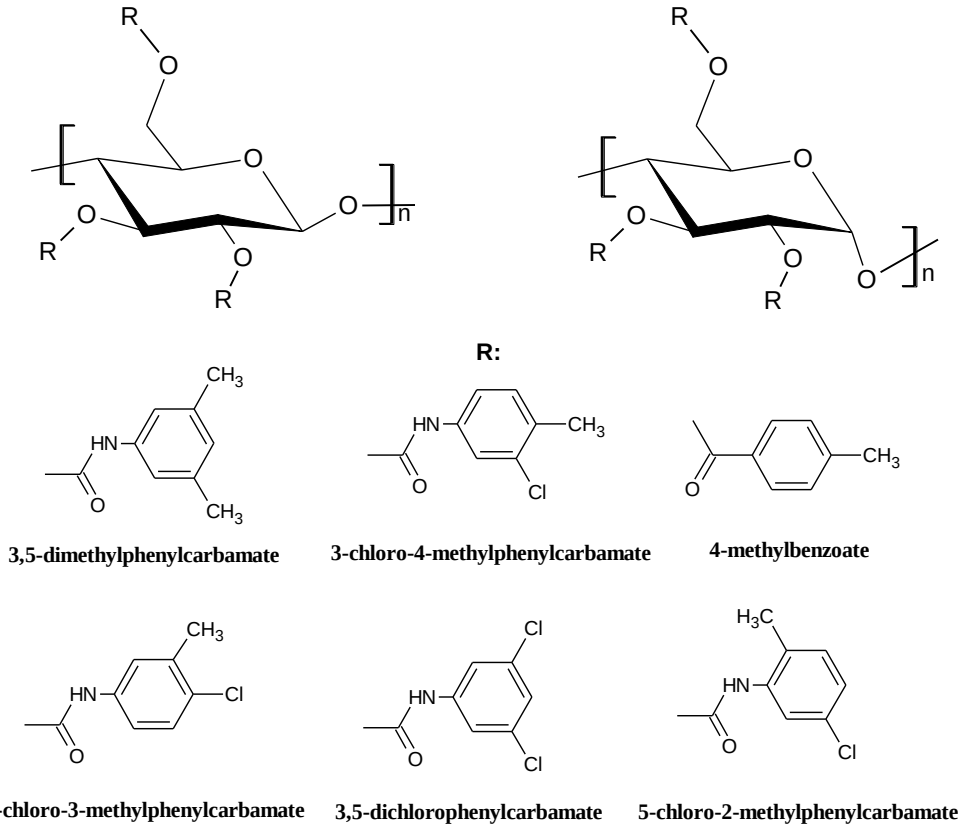



***Figure 3.*** *Structures of cellulose (left) and amylose (right) and some coating structure moieties*

The exceptional chiral recognition properties of polysaccharide CSPs originate from a number of structural peculiarities:

- *molecular chirality* – due to the presence of several stereogenic centers of the glucopyranose units,
- *conformational chirality* – due to the helical twist of the polymer backbone, and
- *supramolecular chirality* – resulting from the alignment of adjacent polymer chains forming ordered regions *[36]*.

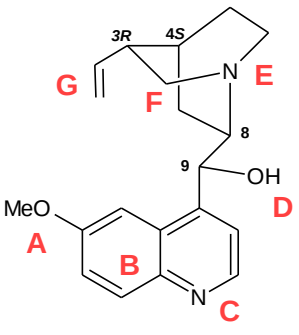
Recently, *West et al.* investigated chiral recognition mechanisms in SFC with tris-(3,5-dimethyphenylcarbamate) amylose and cellulose CSPs by quantitative structure–retention relationships *[37,38]*.

### *2.2.4 Chiral ion-exchange CSPs*

Chiral ion-exchange stationary phases are often considered as a subgroup of donor–acceptor (Pirkle-type) phases. These selectors interact with ionizable analytes via ionic interactions, but $\pi$–$\pi$ interactions and hydrogen bonding also contribute to the stabilization of the complex. Popular chiral ion-exchange stationary phases for separation of anionic racemates are based on *Cinchona* alkaloids. The native *Cinchona* alkaloids, *quinine* (*QN*) and its pseudo-enantiomeric isomer *quinidine* (*QD*), are the most significant representatives of alkaloids. They were isolated from the bark of the cinchona tree (*Cinchona ledgeriana*) by Pelletier in 1820 *[39]*. They have five stereogenic centers both with (1*S*, 3*R*, 4*S*) configurations and opposite configurations at carbons 8 and 9, which are (8*S*, 9*R*) for *QN* and (8*R*, 9*S*) for *QD* ***(Figure 4)***. Although they are actually diastereomers, *QN* and *QD* in chromatographic systems behave like enantiomers, that is they are called „pseudo-enantiomers". It means that, in separation technologies, they show reversed affinity towards the enantiomers of an analyte, which then translates into reversed elution orders. In most cases, the stereoselectivity is under C-8 and C-9 control *[40]*.

**QN** (8*S*,9*R*)

**QD** (8R,9S)




**A**: *methoxy group*
**B**: *quinoline ring*
**C**: *amine nitrogen*
**D**: *hydroxy group*
**E**: *amine nitrogen*
**F**: *quinuclidine ring*
**G**: *vinyl group*

***Figure 4.*** *Structure of Cinchona alkaloids*

The first silica-supported CSP with *Cinchona* alkaloids was applied in the 1980s for enantiomer separation by Rosini *et al. [41]*. They immobilized native *QN* and *QD* via a spacer at the vinyl group of the quinuclidine ring. *Cinchona* alkaloids have a unique combination of characteristics of structural features. Due to the combination of numerous functional groups, the application of *Cinchona* alkaloids are potentially unlimited in chiral recognition systems. The vinyl group (**G**) is often used for immobilization. The aromatic heterocycle quinoline (**B**) may participate in $\pi$–$\pi$ and steric interactions. The methoxy group (**A**) is sometimes used for immobilization. The secondary OH group (**D**) at C-9 can act as a H-bond donor or a metal coordination site. The bulky quinuclidine ring system (**F**) containing a basic nitrogen atom (**E**), when protonated, can be involved in electrostatic interactions *[42]*.

In the 1990s, *Lindner et al.* modified the secondary hydroxyl group at C-9 with the *tert*-butyl-carbamoyl moiety (***Figure 5***). This newly created H-bonding site resulting from carbamate modification significantly enhanced the enantiorecognition capabilities of the weak anion-exchange-type CSPs. These new chiral SOs are classified as anion-exchanger CSPs, due to the presence of the basic amino group of the quinuclidine ring *[40,43]*.

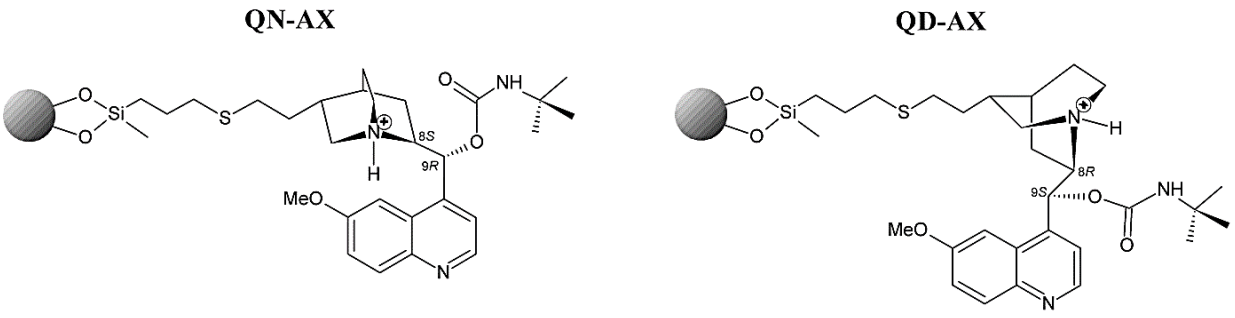
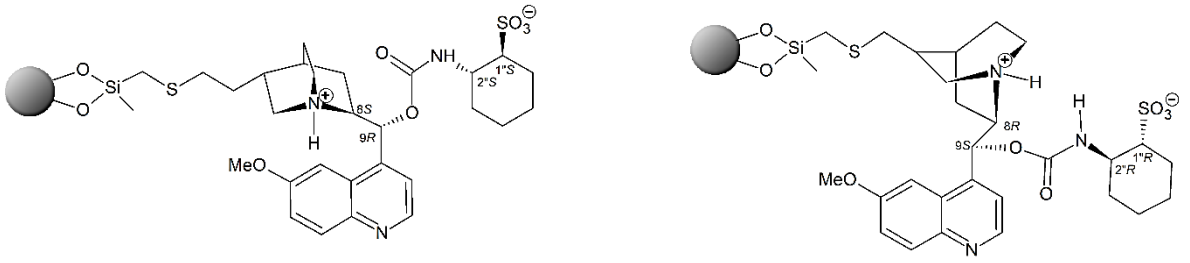



***Figure 5.*** *The structure of anion-exchanger Chiralpak QN-AX and QD-AX CSPs*

*Hoffmann* and *Lindner* synthesized a new selector by the fusion of quinine or quinidine with enantiomerically pure *trans*-2-aminocyclohexanesulfonic acid [(*R*,*R*)- or (*S*,*S*)-ACHSA] through a carbamoyl group at the C-9 position. This modification gave new zwitterionic chiral selectors Chiralpak ZWIX(+)™ and ZWIX(–)™ *[44,45]* (***Figure 6***).



**ZWIX(+)™**
(8*S*,9*R*)
(1"*S*,2"*S*)

**ZWIX(-)™**
(8*R*,9*S*)
(1"*R*,2"*R*)

***Figure 6.*** *The structure of zwitterionic Chiralpak ZWIX(+)™ and ZWIX(–)™ CSPs*

In the case of ion-exchange separation, the retention is primarily based on ionic interactions between the ions in solution and the fixed charged functional groups of the stationary phase (***Figure 7***). In addition to ionic interaction, chiral discrimination is promoted by H-bonding, *π–π*, dipole–dipole, and other van der Waals interactions. In order to the SO and SA to be charged, acid and base modifiers should be added to the mobile phase.

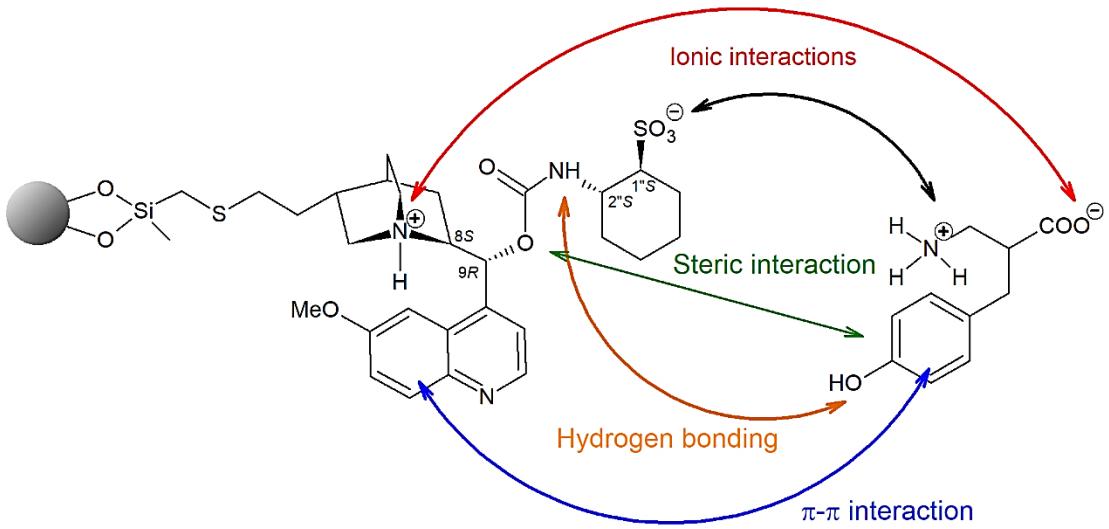



***Figure 7****. Chiral interactions between the zwitterionic CSP and analyte*

### *2.3 Supercritical fluid chromatography*

The separation technique, using supercritical fluid as the main component of the mobile phase, is widely accepted as SFC, despite the fact that the majority of SFC separations take place in the subcritical region due to the addition of organic modifiers. This technique uses pressurized liquid carbon dioxide ($CO_2$) as mobile phase component together with organic co-

solvent. Light hydrocarbons, $N_2O$, ammonia, and chlorofluorocarbons have been quite successfully used as supercritical mobile phases. Nowadays, however, $CO_2$ is the most commonly applied supercritical mobile phase, because of its numerous positive features, such as low cost, non-flammability, abundance, adequate purity, inertness toward most compounds, moderate critical pressure and temperature values, and weak UV absorbance at low wavelength. Methanol, ethanol, 2-propanol or acetonitrile are polar modifiers used most frequently. The mobile phase enables high flow rates and, therefore, rapid analyses. A supercritical fluid is a physico-chemical state of a substance that occurs when temperature and pressure are elevated above their thermodynamic critical point. In the case of $CO_2$, the critical point is above $T_C$ = 31 °C and $P_C$ = 73.8 bar (***Figure 8***).

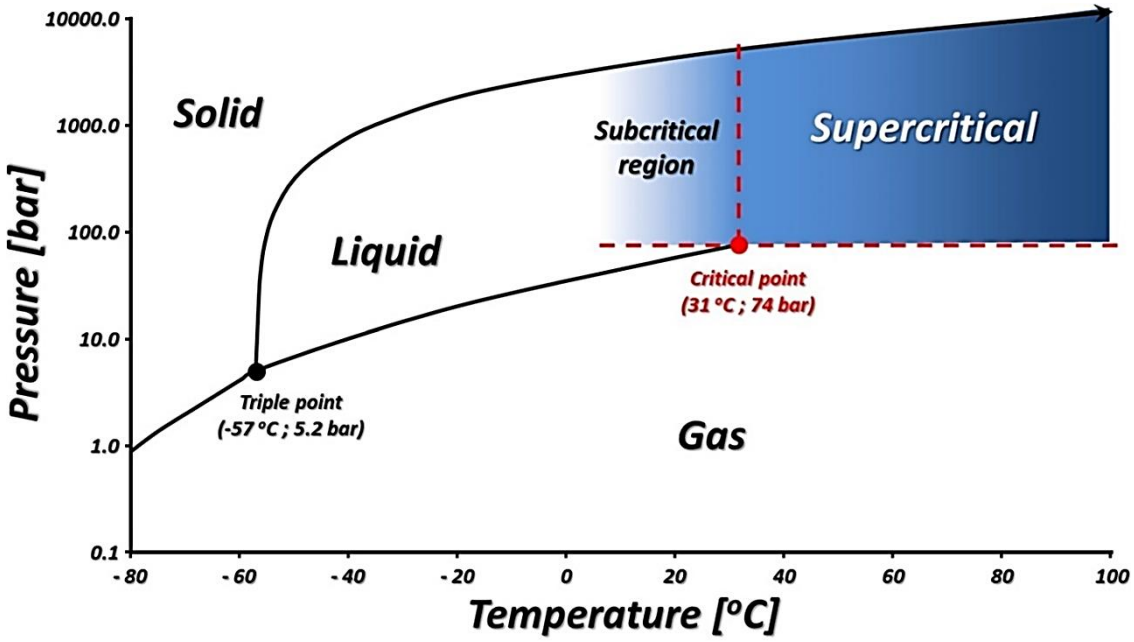



***Figure 8.*** *Phase diagram of pure carbon dioxide [46]*

SFC was introduced more than 50 years ago, but only a few papers were published in the two early decades. Unfortunately, the development of SFC was shaded by the rapid development of HPLC taking place in the late 1960s and early 1970s. Klesper *et al.* were the first to propose the use of supercritical fluids as eluents for chromatographic separation in 1962. They described the separation of thermo-labile porphyrin derivatives using supercritical chlorofluoromethanes as the mobile phase *[47]*. SFC attracted attention in the 1980s thanks to its recognized benefits for enantioseparation often providing improved resolution at higher rate than in HPLC. In 1982, *Gere et al.* modified a Hewlett-Packard (HP) HPLC system to operate

as an SFC system *[48]*. *Mourier et al.* were the first, who separated enantiomers by SFC in 1985 *[49]*. In 1986, *Hara et al.* demonstrated the chiral separation of D,L-amino acid derivatives on a chiral diamide stationary phase *[50]*. *Röder* and co-workers reported the first example of a chiral separation performed by an open tubular SFC column in 1987. Recently, *Guiochon* and *Tarafder* summarized the history of supercritical fluids and thoroughly described the physical characteristics of these fluids *[51]*. They focused maily on pure carbon dioxide, which allowed a good modeling of their physical properties, especially for preparative chromatography. In another paper, *Saito* reviewed the history of the instrumental development of SFC, from capillary to modern packed columns *[52]*. They developed an electronically controlled backpressure regulator, which allows pressure control independent of mobile phase flow rate *[53]*. While open tubular capillary column SFC was a GC-like application, packed-column SFC is more similar to LC. In 2013 a new SFC apparatus was introduced by Waters as ultra-performance convergence chromatography UPC$^2$, which opened a new dimension of analytical instrumentation. SFC has become a widely accepted and used technique in both academic and commercial spheres.

Nowadays, packed-column SFC is widely accepted. It uses the same configuration (injector and packed column) applied in HPLC. The advantages of packed-column SFC over HPLC methodologies are clear:

- supercritical mobile phases have relatively lower viscosity and higher diffusivity than liquids resulting in faster and more efficient separations per unit time and shorter turnaround times between injections,
- carbon dioxide is an inert, environmentally “green”, and volatile mobile phase for large-scale separations and energy-efficient isolation of the desired product,
- adaptable longer, stacked columns with the same or multiple phases with total theoretical plates excessing 100,000,
- HPLC applications can be run on SFC instrumentation *[38,54–56]*.

Growing popularity of SFC in both chiral and achiral analyses comes towards faster, more economic, and greener separations. This growing trend is shown in the number of related scientific publications (***Figure 9***). For example, some applications are enantioselective separation (*Kalikova et al. [57], West [58], Klerck et al. [59]*), metabolite analysis (*Taguchi et al. [60], Matsubara et al. [61]*), food analysis (*Bernal et al. [62]*), polymer analysis (*Takahashi [63]*), peptide and ionic analyte analysis (*Taylor [64,65]*), clinical analysis (*Abbott et al. [66]*), carbohydrate analysis *[67]*, *etc.*

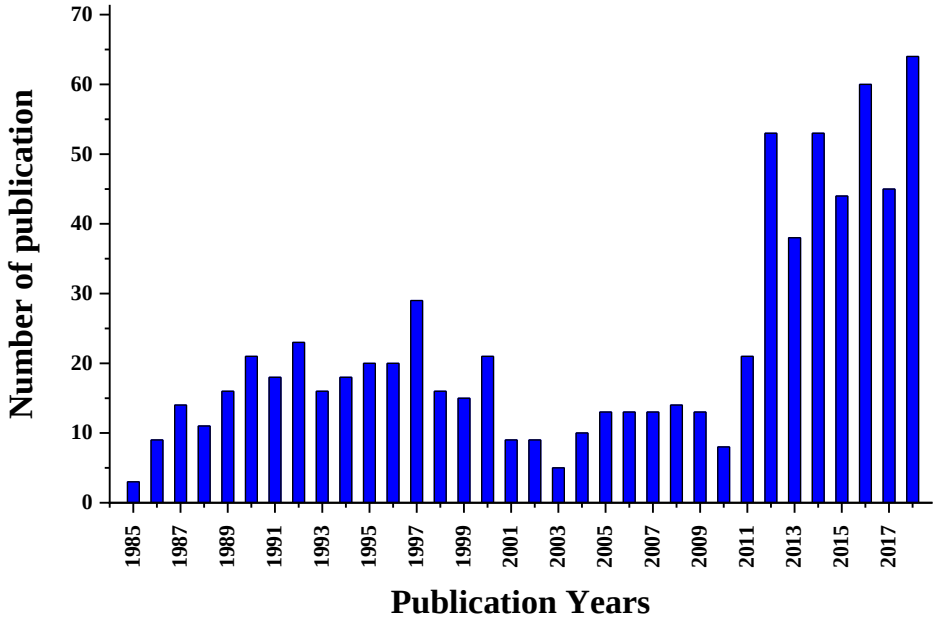



***Figure 9.*** *Number of scientific publications related to SFC between 1985 and 2018*

*Articles searched in ScienceDirect containing (in keywords, abstract or title) the words referred to "supercritical fluid" or "SFC" and "Chromatography" in Review articles, Research articles and Short Communications.*

### *2.4 Thermodynamic considerations*

Enantiomeric separation by chromatography is only possible, when the difference in the Gibbs energy of the diastereomeric complexation equilibria between the SO–SA is not zero.

The equilibrium constant $K_i$ of the SA–SO association is related to the standard Gibbs energy according to the following equation:

$$\Delta G° = \Delta H° - T\Delta S° = -RTlnK_i \quad (1)$$

where $\Delta H°$ is the standard change of enthalpy, $\Delta S°$ is the standard change of entropy, $R$ is the universal gas constant, and $T$ is the absolute temperature in K.

The relationship between retention factor $k$ and $K_i$ is:

$$k = K_i\phi \quad (2)$$

$$\phi = V_s/V_m \quad (3)$$

where $k$ is the retention factor and $\phi$ is the phase ratio [the ratio of the volumes of the stationary ($Vs$) and the mobile phase ($Vm$)].

The dependence of the retention of the SA on temperature can be expressed by the van't Hoff equation, which may be interpreted in terms of the mechanistic aspect of chiral recognition.

$$lnk = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + ln\phi$$

The difference in the change of standard free energy of the two enantiomers can be written as:

$$\Delta(\Delta G^\circ)_{2,1} = \Delta G^\circ{}_2 - \Delta G^\circ{}_1 = ln\frac{k_2}{k_1} = -RTln\alpha \quad (4)$$

$$ln\,\alpha = -\frac{\Delta(\Delta H)^\circ}{RT} + \frac{\Delta(\Delta S)^\circ}{R} \quad (5)$$

This expression relates the temperature and the experimentally easily available $\alpha$ value to the molar differential enthalpy and entropy of enantioselective adsorption. Provided that these quantities are temperature independent, which is usually the case, graphical analysis of *ln* $\alpha$ vs. *1/T* gives linear plots, from which $\Delta(\Delta H)^\circ$ and $\Delta(\Delta S)^\circ$ can be extracted from the slope and intercept, respectively.

### *2.5 $N^{\alpha}$-Fmoc proteinogenic amino acids*

The 19 proteinogenic $\alpha$-amino acids are the building blocks of the proteomes found in mammals *[68,69]*. These are organic compounds belonging to carboxylic acids, in which a hydrogen atom in the side chain (usually at the $\alpha$-carbon) has been replaced by an amino group. On the basis of the number of carboxylic groups (COOH) as acidic and amino groups ($NH_2$) as basic in the molecule, amino acids are divided into three groups: neutral (*e.g.*, serine), acidic (*e.g.*, glutamic acid), and basic (*e.g.*, arginine). An asymmetric carbon atom in amino acids plays a role of a chiral center. For this reason, amino acid molecules are optically active and exist in the form of respective enantiomers, which are designated by the symbols D and L (nomenclature developed by *Fischer* and determined on the basis of D-glyceralaldehyde structure) *[70,71]*. The natural protein amino acids are generally L-enantiomers. D-Enantiomers can be found in plants, bacterial cells or in several antibiotics *[72]*. All of them, except glycine, contain at least one stereogenic center. Amino acid enantiomers have identical chemical and physical properties (except the direction of the rotation of plane polarized light), but possess different biological activities in living systems *[73]*. Therefore, the separation of enantiomers is important for pharmaceutical (*e.g.*, drugs, antibiotics), industrial (*e.g.*, chiral catalysts), and toxicological (*e.g.*, xenobiotics) applications *[74]*.

The 9-fluorenylmethoxycarbonyl moiety (Fmoc) is widely used as an amine-protecting group in peptide synthesis. It is well-known, that the intrinsic hydrophobicity and aromaticity of the Fmoc group affect the hydrophobic and $\pi$–$\pi$ stacking interaction of the fluorenyl rings. That is the reason why many Fmoc amino acids and short peptides possess relatively rapid self-assembly kinetics and remarkable physicochemical properties along with wide application

potentials in many fields *[75–78]*. An increasing number of Fmoc-modified amino acids have been reported to be able to self-assemble and some of them, mostly those with aromatic side chains, can even form extended three-dimensional networks, trapping solvent molecules and forming gels.

Using Fmoc-based synthesis, long peptides can be prepared in high yields from micromolar (mg) up to molar scale (kg). As the number of amino acid residues increases, the final purity and overall yield of the peptide produced depend on the chemical and chiral purity of the protected amino acids used. Currently, for the most common commercially available Fmoc-protected α-amino acids, the expected enantiomeric purity is > 99.0% for the L form. Moreover, sometimes the purity required must be higher than 99.8% enantiomeric excess (*ee*) *[79]*. This level of precision can only be achieved by very few analytical techniques and chiral HPLC is one of them.

# 3 Experimental

## *3.1 Apparatus and chromatography*

Measurements were carried out on two HPLC systems and one SFC system.

**System I:**

Liquid chromatographic experiments were performed on a Waters Breeze system containing a 1525 binary pump, a 2487 dual-channel absorbance detector, a 717 plus autosampler, and Empower 2 data manager software (Waters Chromatography, Milford, MA, USA).

**System II:**

A 1100 Series HPLC system consisted of a solvent degasser, a pump, an autosampler, a column thermostat, and a multiwavelength UV-Vis detector from Agilent Technologies (Waldbronn, Germany) as well as a corona-charged aerosol detector from ESA Biosciences, Inc. (Chelmsford, MA, USA). Data acquisition and analysis were carried out with Chemstation chromatographic data software from Agilent Technologies.

Both chromatographic systems were equipped with Rheodyne Model 7125 injectors (Cotati, CA, USA) with 20 µl loops. The columns were thermostated in a Spark Mistral column thermostat (Spark Holland, Emmen, The Netherlands) or Lauda Alpha RA8 thermostat (Lauda Dr. R. Wobser Gmbh, Lauda-Königshofen, Germany). The precision of temperature adjustment was ±0.1 °C. For determination of the columns' dead-times ($t_0$), a methanolic solution of acetone was applied.

**System III:**

The Waters Acquity Ultra Performance Convergence Chromatography™ ($UPC^2$, Waters Chromatography) system was equipped with a binary solvent delivery pump, an autosampler with a partial loop volume injector system, a backpressure regulator, a column oven, and a PDA detector. The system control and data acquisition Empower 2 software (Waters Chromatography) was used. Experiments were executed with mobile phases composed of liquid $CO_2$/MeOH in different ratios with various additives. The outlet pressure was maintained at 150 bar. The dead time ($t_0$) was determined by injecting a solution of acetone in MeOH.

### *3.2 Applied columns*

The four *Cinchona* alkaloid-based CSPs ZWIX(+)™, ZWIX(–)™, QN-AX, and QD-AX were provided by Chiral Technologies Europe (CTE, Illkirch, France). All CSPs comprised 3 μm particles packed into 150 x 3.0 mm I.D. columns.

### *3.3 Chemicals and reagents*

The applied methanol (MeOH) and acetonitrile (MeCN) of HPLC grade, ammonia ($NH_3$), ethylamine (EA), diethylamine (DEA), triethylamine (TEA), propylamine (PA), butylamine (BA), glacial acetic acid (AcOH), and formic acid (FA) of analytical reagent grade were purchased from VWR International (Arlington Heights, IL, USA) and Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained from the Ultrapure Water System, Puranity TU UV/UF (VWR International bvba, Leuven, Belgium).

All eluents were degassed in an ultrasonic bath, and helium gas was purged through them during HPLC analysis. Stock solutions of analytes (1.0 mg/mL) were prepared by dissolution in the mobile phase, or MeOH in the case of SFC.

### *3.4 Investigated analytes*

Besides $N^{\alpha}$-Fmoc protection, the other reactive site of proteinogenic amino acids possesses additional protecting groups to make them the most appropriate for peptide synthesis protocol: *tert*-butyloxycarbonyl (Boc) for Lys, *tert*-butyl (*t*Bu) for Ser, Thr, and Tyr, *O*-*tert*-butyl (O*t*Bu) for Asp and Glu, triphenylmethyl (trityl, Trt) for Cys and His, and *Nω*-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg (***Figure 10***). The protected amino acid derivatives were obtained from different sources. L-amino acids **1** and **2** were purchased from Reanal (Budapest, Hungary), **3**–**14** and **16** from Orpegen Pharma Gmbh (Heidelberg, Germany), **15** from GL Biotech Gmbh (Marktredwitz, Germany), and **18** from Merck (Darmstadt, Germany). D-amino acids **3**, **4**, **6**, **9**, **10**, **12**–**14**, **16**, **18**, and **19** were obtained from Bachem AG (Bubendorf, Switzerland), **1**, **2**, **7**, **8**, **11**, **15**, and **17** from AK Scientific, Inc (Union City, CA, USA), and **5** from Advanced ChemTech (Louisville, KY, USA).

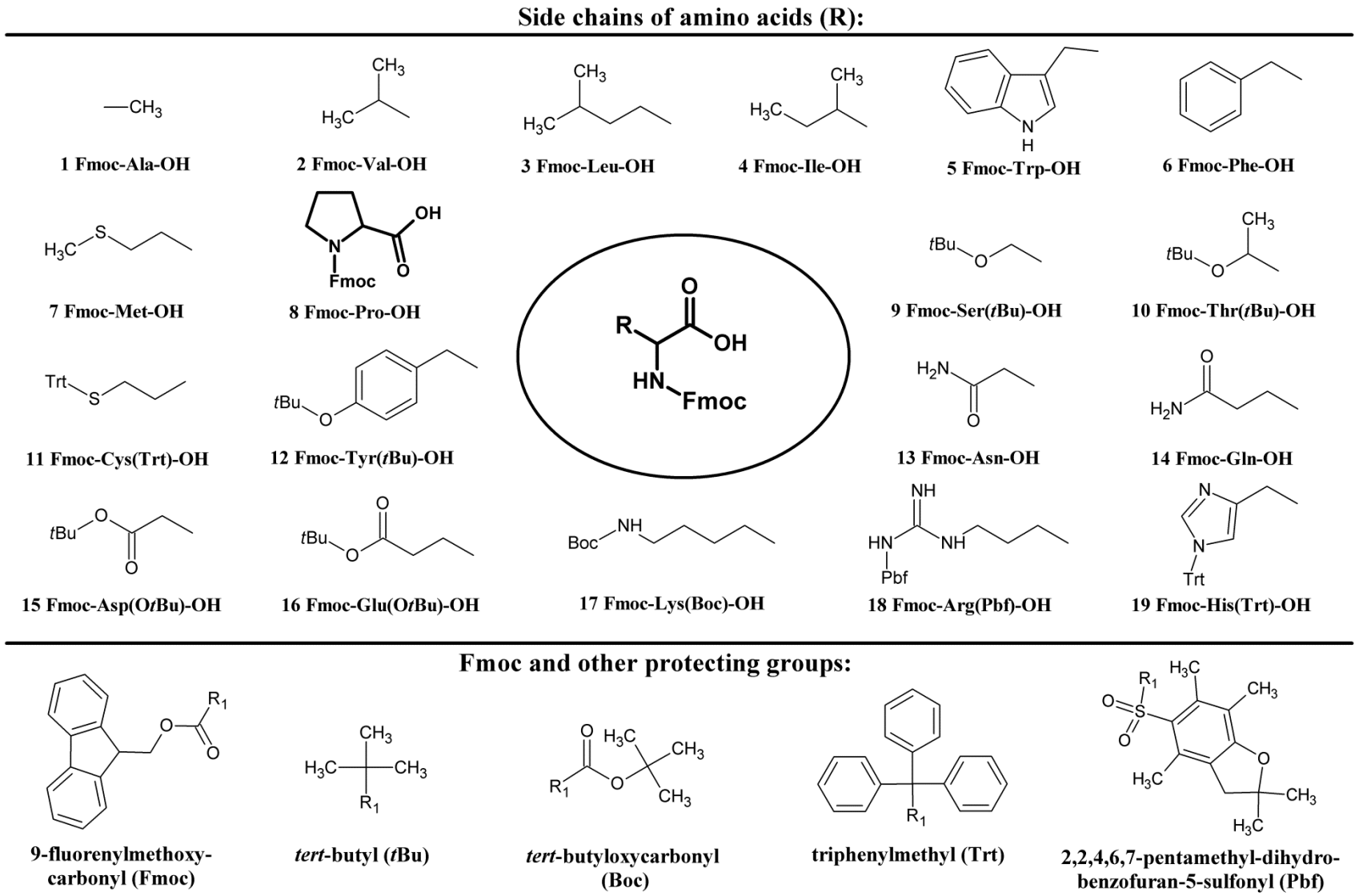



***Figure 10.*** *Structure of $N^{\alpha}$-Fmoc-protected proteinogenic amino acids*

# 4 Results and Discussion

In my thesis, chromatographic results for 19 Fmoc-protected protein amino acids on *Cinchona* alkaloid-based zwitterionic [ZWIX(+)™, ZWIX(-)™] and anion-exhanger type (QN-AX, QD-AX) chiral stationary phases in HPLC and SFC technics are presented and discussed.

To study the effect of experimental conditions, of the investigated 19 $N^{\alpha}$-Fmoc protein amino acids with an overall acidic character, five analytes representing the spectrum of acidic [Fmoc-Asp(O*t*Bu)-OH (**15**)], basic [Fmoc-Lys(Boc)-OH (**17**)], aliphatic [Fmoc-Leu-OH (**3**)], aromatic [Fmoc-Phe-OH (**6**)], and polar [Fmoc-Tyr(*t*Bu)-OH (**12**)] $\alpha$-amino acids have been selected.

## *4.1 Influence of mobile phase composition on chromatographic parameters*

Variation of the mobile-phase composition is always the first choice to achieve resolution in the method development. In most cases, *Cinchona* alkaloid-based CSPs afforded an excellent separation ability in PIM (polar ionic mode), when using a mixture of MeOH as a protic solvent (which can suppress H-bonding interaction) and MeCN as an aprotic, but polar bulk solvent component (which supports ionic interaction, but interfere with $\pi$–$\pi$ interaction). In order to promote ionic interaction and constant ionic strength, acid and base additives are needed in the mobile phase. The acid-to-base ratio was kept at a constant value of 2:1 providing weak acidic conditions. A slight excess of acids ensures that the quinuclidine moiety of the SO is protonated and the carboxyl group of the SA is deprotonated to some extent. In this way the ionizable state of both the SO and SA may facilitate the ion-pairing process.

### *4.1.1 Effect of bulk solvent composition in LC mode*

In LC mode, a mixture of MeOH/MeCN (50/50, 75/25, and 85/15 *v/v*) as the bulk solvent containing 25 mM TEA and 50 mM FA on anion-exchanger CSPs was used. The corresponding solvent composition applied on zwitterionic CSPs is MeOH/MeCN (75/25, 50/50, and 25/75 *v/v)* containing 30 mM TEA and 60 mM FA. The effect of the bulk solvent on chromatographic parameters on *quinine*-based zwitterionic ZWIX(+)™ and anion-exchanger type QN-AX CSP on selected five model componds is depicted in (***Figure 11***).

Applying the MeOH/MeCN mobile phase on ZWIX-type CSPs gave very low *k* values. Furthermore, $k_1$ varied between 0.16 and 0.56 and it increased with increasing MeCN content. In the case of the studied model compounds, the primary interaction, decisive in retention, is

the ionic interaction between the cation site of the SO and anion site of the SA, with additional intermolceular SO–SA interaction responible for chiral discrimination.

Due to the Fmoc-protection of the amino group, only a single ion-pair process is active. For this reason, the double ion-paring process is not possible, resulting in rather low retention. However, at least partial resolution could be obtained in many cases with $R_S$ values lower than 1.0, with the exception Fmoc-Phe-OH.

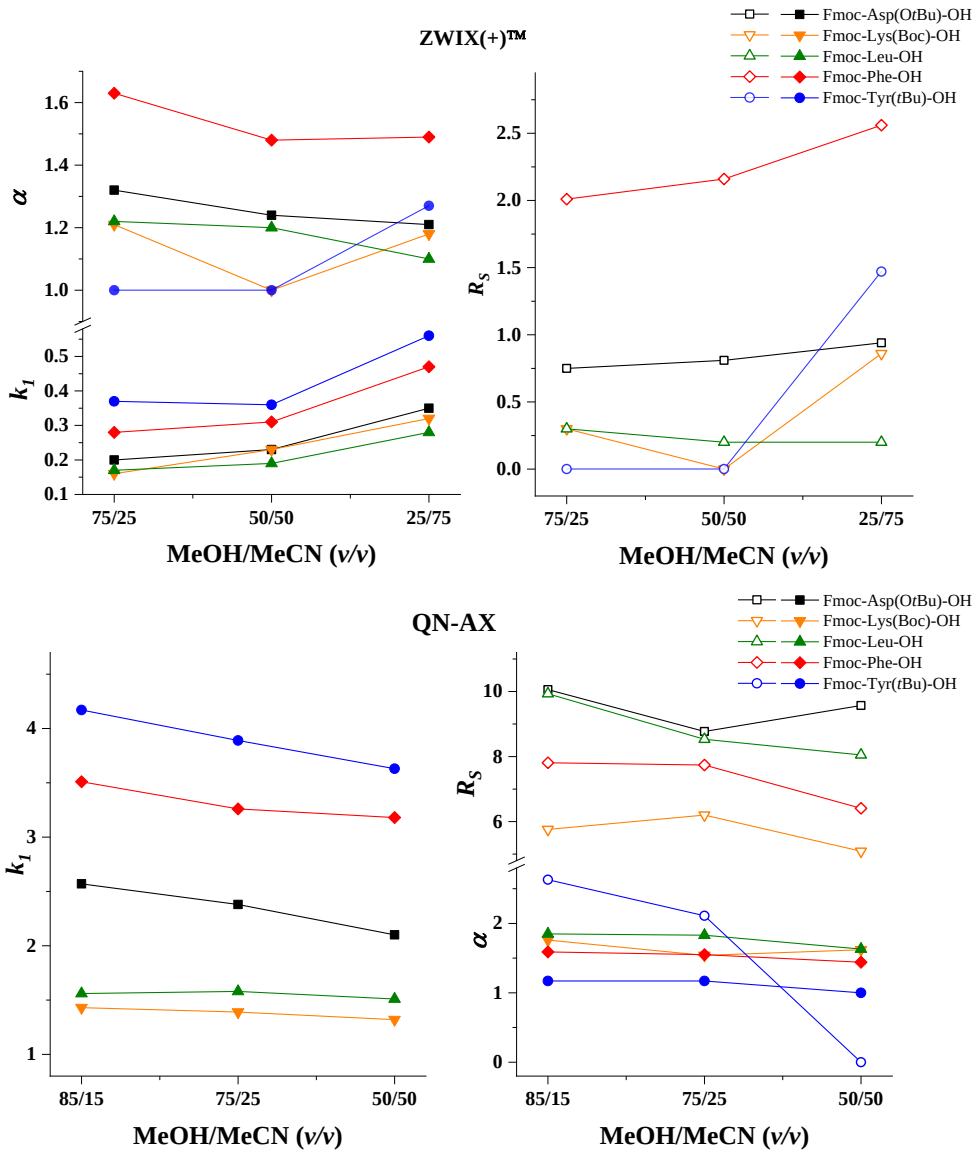



***Figure 11.*** *The effect of bulk solvent composition on $k_1$, α, and $R_S$ in LC mode*

*Chromatographic conditions: mobile phase on ZWIX(+)™ MeOH/MeCN (75/25, 50/50, and 25/75 v/v) containing 25 mM TEA and 50 mM FA; mobile phase on QN-AX MeOH/MeCN (85/15, 75/25, and 50/50 v/v) containing 30 mM TEA and 60 mM FA; flow rate: 0.6 mL/min; detection: 262 nm*

On aninon-exchanger CSPs, separations were carried out in MeOH/MeCN (85/15, 75/25, and 50/50 *v/v*) mobile phase containing 30 mM TEA and 60 mM FA. The $k_1$ values ranged between 1.4–4.2 and retention slightly decreased for each of the selected analytes with

increasing MeCN content. The selectivity changed between 1.4–1.9 and it also decreased with increasing MeCN content in the mobile phase. Resolution in most cases also decreased with increasing MeCN concentrations. However, for Fmoc-Phe-OH and Fmoc-Lys(Boc)-OH, maximum values were registered. In summary, $k$, $\alpha$, and $R_S$ values decreased slightly with increasing MeCN content in contrast to the tendency observed on zwitterionic selectors.

### *4.1.2 Effect of bulk solvent composition in SFC mode*

In the SFC mode the polarity and elution strenght of liquid $CO_2$ is varied most significantly by the addition of the MeOH as organic modifier. However, it should be noted that in most cases subcritical conditions rather than supercritical state were applied, because of added MeOH. SFC experiments were accomplished with mobile phases containing liquid $CO_2$/MeOH in different ratios (*v/v*) with additives (acid or base) at a flow rate of 2.0 mL/min. The outlet pressure was maintained at 150 bar and the colomn temperature was 40 °C.

Depending on the concentration of MeOH in the mobile phase, several comments should be added:

- at SFC conditions, the more polar mobile phase components adsorb on the surface of the stationary phase and, consequently, the co-solvent concentration can be significantly higher in this adsorbed layer than in bulk solvent,
- increasing the polar MeOH content in the apolar $CO_2$ solvent promotes the interaction between the polar SAs and the mobile phase,
- reaction between pressurized $CO_2$ and the added MeOH leads to the formation of methyl hydrogen carbonate and carbonic acid, which permits the use of chiral ion-exchange type CSPs under SFC conditions even without addition of buffers,
- the increase in co-solvent concentration affects fluid viscosity by increasing fluid density, which contributes to the enhanced elution strength,
- although it is less significant, but the co-solvent concentration also influences critical temperature and pressure values.

In all cases, retentions decreased drastically on the increase of the MeOH content from 10 to 20 *v*%, especially for Fmoc-Phe and Fmoc-Lys(Boc)-OH. Further increase in MeOH content (from 30 to 40 *v*%), however, was accompanied by lower decrease in retention. These results can be attributed to the more efficient solvation of the SAs in a mobile phase at a higher MeOH content and, therefore, the retention is significantly reduced. Similar to retention, $R_S$ also decreased with increasing MeOH content on both types of CSPs. In contrast, α increased slightly with higher MeOH content (***Figure 12***).

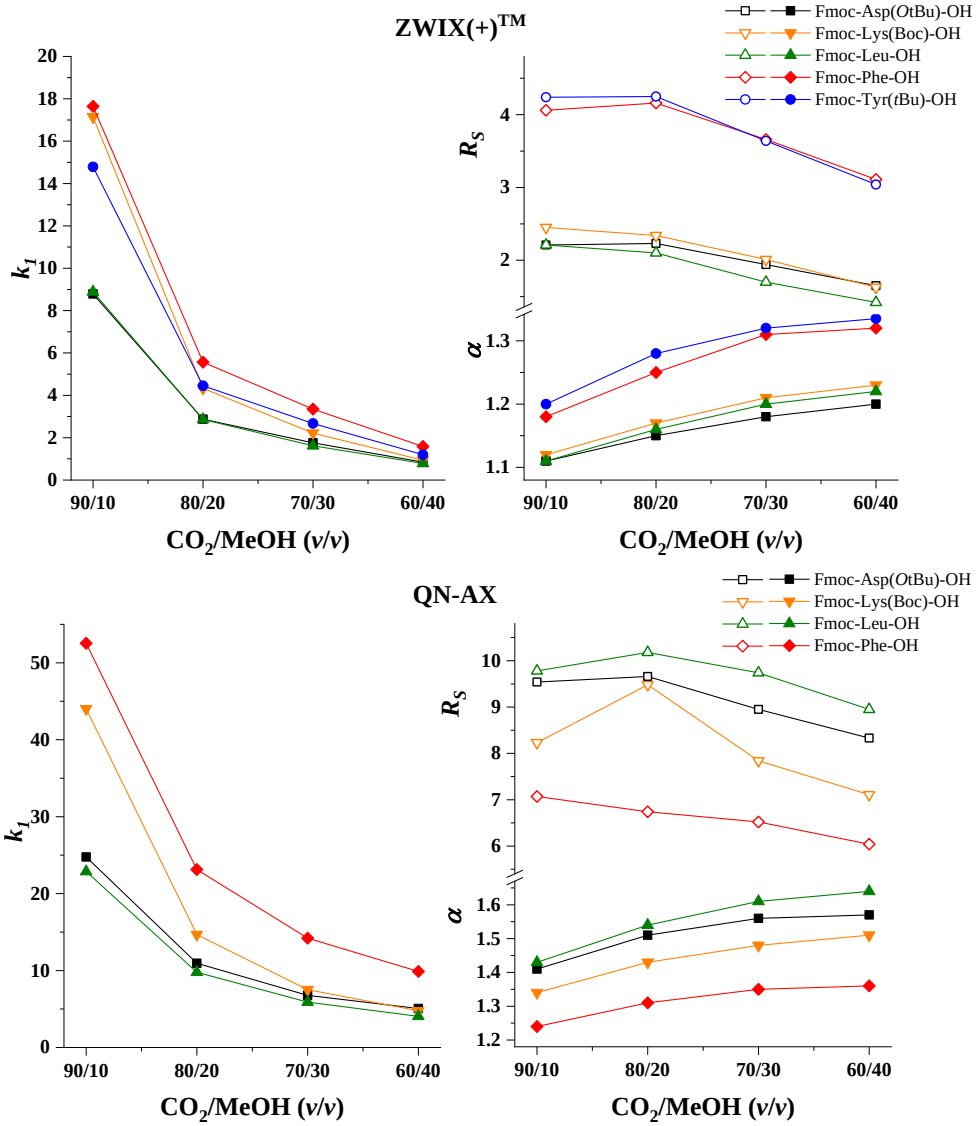



***Figure 12.*** *The effect of bulk solvent composition on $k_1$, α, and $R_S$ in SFC mode*

*Chromatographic conditions: mobile phase on ZWIX(+)™ $CO_2$/MeOH (90/10–60/40 v/v) containing 25 mM TEA and 50 mM FA; mobile phase on QN-AX $CO_2$/MeOH (90/10–60/40 v/v) containing 25 mM TEA and 50 mM FA; flow rate: 2.0 mL/min; $T_{col}$: 40 °C; back pressure: 150 bar; detection: 262 nm*

### *4.2 Role of water content of the mobile phase*

Most chiral separations on *Cinchona* CSPs were carried out by using water-free polar organic mobile phases, consisting of MeOH or a mixture of MeOH/MeCN as bulk solvent. However, the presence of water in a low percentage is beneficial for peak shape, resolution, analysis time as well as sample and solubility performance. Water is on the top of the protic solvent list due to its powerful proton activity. *Hoffmann et al.* investigated the chromatographic behavior of zwitterionic CSPs in RP mode for aromatic amino acids *[80]*. *Zhang et al.* used high water (2–20%) content for separation of free amino acids *[45]*. Based on these results, our invetigations were extended to hydro-organic (HO) mode, because solvation of the ionic

compounds in the HO mobile phase can play an important role in enantioseparations on ion-exchange type CSPs.

### *4.2.1 Effect of water content in mobile phase in LC mode*

The addition of small amounts of water into the polar ionic mobile phase shifts the elution system from a nonaquesous PI mode to an HO mode. A few percentage points of $H_2O$ affect solvation of both SO and SA and might reduce the strength of the ionic interactions.

Studies were carried out on zwitterionic CSPs in HO mobile phase with $H_2O$ contents varying between 1.0–5.0 *v*%. The changes of chromatographic parameters were not significant (data not shown). In most cases 1.0–2.0% $H_2O$ in the eluent was advantageous, yielding better peak shapes and higher resolution. Utilizing the mobile phase $H_2O$/MeOH (1/99 *v/v*) containing 30 mM TEA 60 mM FA, $k_1$ values were slightly higher than in the MeOH/MeCN mobile phase.

### *4.2.2 Effect of water content in mobile phase in SFC mode*

Water as a polar modifier in SFC separation was first used in the late 1980s to promote elution of polar compounds and to improve peak shapes. *Geiser et al*. reported the separation of underivatized fatty acids using water as modifier in $CO_2$ *[81]*. *Taylor* and co-workers investigated four nucleobases via SFC with $CO_2$ modified with alcohol and water *[82]*. *Welch et al*. used a water-rich modifier for the separation of several hydrophilic compounds by SFC *[83]*.

To investigate the effect of water content, mobile phases composed of $CO_2$/MeOH/$H_2O$ in different ratios were applied by keeping the amount of $CO_2$ constant and varying the water content in the MeOH phases. On zwitterionic columns, the mobile phase composition of $CO_2$/MeOH (70/30 *v/v*) containing 30 mM TEA and 60 mM FA, whereas on anion-exchanger columns, $CO_2$/MeOH (60/40 *v/v*) containing 30 mM TEA and 60 mM FA were applied. The concentraiton of $H_2O$ in the MeOH part of the mobile phase was varied between 0.0–8.0 *v*% (***Figure 13***). Upon increasing the water content, $k_1$ values of selected analytes decreased slightly. On ZWIX(+)™ $k_1$ values varied between 1.32–3.35, while the changes on QN-AX were between 3.18–9.89. The observed decrease in retention time can be partially explained by the increased formation of the counter-ion via the reaction of $CO_2$ and $H_2O$, yielding carbonic acid, which dissociates to hydrogen carbonate and proton. The latter species act as additional counter-ions in anion chromatography systems. Similar to this behaviour, both α and $R_S$ decreased slightly with increasing water content.

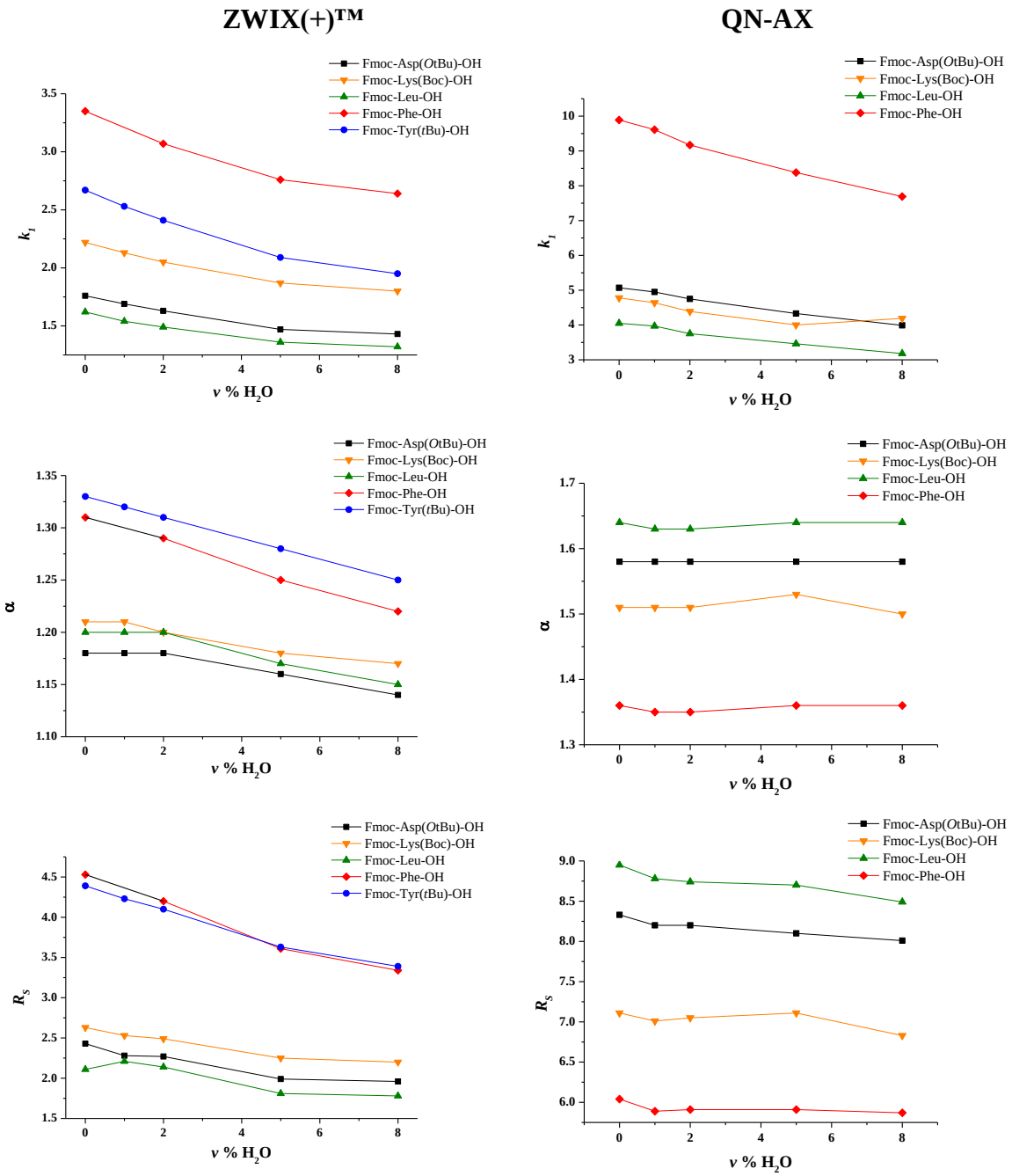



***Figure 13.*** *The effect of water content in mobile phase on $k_1$, $\alpha$, and $R_S$ on quinine-based CSPs*

*Chromatographic conditions: mobile phase on ZWIX(+)™ $CO_2$/MeOH/$H_2O$ containing 30 mM TEA and 60 mM FA; and mobile phase on QN-AX $CO_2$/MeOH/$H_2O$ containing 30 mM TEA and 60 mM FA; flow rate: 2.0 mL/min; detection: 262 nm*

### *4.3 Role of the nature of base and acid as mobile phase additives*

The nature of various acid and base additives in the mobile phase may significantly influence the chromatographic parameters and play an important role in the optimization of enantioseparations on *Cinchona* alkaloid-based CSPs. In zwitterionic CSPs, both anion- and cation-exchange phenomena occur in the ion-exchange process. Therefore, both acid and base

additives act as competitors for the ion pairing of SA and SO in the ion-exchange equilibrium. The additives in nonaqueous polar organic solvents greatly influence the solvation of both SO and SA. Consequently, the anions and cations of acid and base additives have great effects on the elution strength of the mobile phase, inversely acting as displacers at the cation- and anion-exchanger sites. For this reason, acid and base additives (counter-ions) in the mobile phase will play a crucial role. A number of studies demonstrated that the nature of co- and counter-ions have opposite effects at the anionic and cationic exchanger parts of the zwitterionic CSPs (***Figure 14***) *[45,80,84]*.

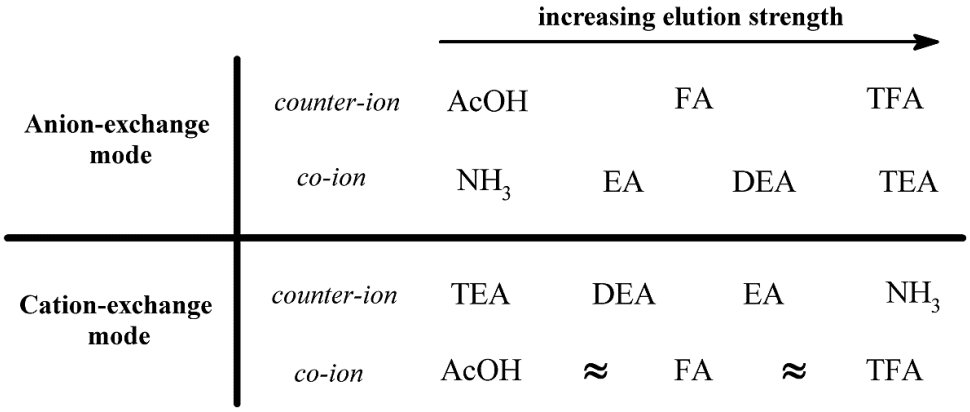



***Figure 14.*** *Elution strength of co- and counter-ion additives on retention applying single ion-exchange-type CSPs [80]*

### *4.3.1 Effect of base and acid as mobile phase additives in LC mode*

To study the effect of the nature of acid and base additives on the chromatographic performance of zwitterionic CSPs in LC mode, FA or AcOH as acid additives as well as EA, DEA, TEA, PA, and BA as base additives were selected. Experiments were carried out in $H_2O$/MeOH mobile phases containing 3.75 mM base and 7.5 mM FA, keeping the acid-to-base ratio at 2:1. Acid excess in the mobile phase guaranteed that the bases (amines) were present in their protonated „ammonium ion" form. The amines differed in the degree and nature of their alkyl substitution on the nitrogen atom, while the acids (FA and AcOH) had different strengths. The experimental results revealed that for the same analyte, $k_1$ values differ only slightly on varying the base additives (***Figure 15***). For Fmoc-Asp(O*t*Bu)-OH, $k_1$ varied between 0.47–0.56, for Fmoc-Lys(Boc)-OH between 0.32–0.37, for Fmoc-Leu-OH between 0.28–0.38, for Fmoc-Phe-OH between 0.59–0.70, and for Fmoc-Tyr(*t*Bu)-OH between 0.53–0.64. However, no general trend was observed in the variation of the nature of the base. It was also found that slightly higher retentions were obtained with the application of DEA or TEA. The nature of the

acids have a slight effect on retention. Comparing the effect of FA and AcOH, with a few exceptions, slightly smaller $k_1$ values were obtained for AcOH (data not shown). In summary, optimization of the base and acid additives, DEA, TEA, and FA appear to be more favorable concerning retention and selectivity.

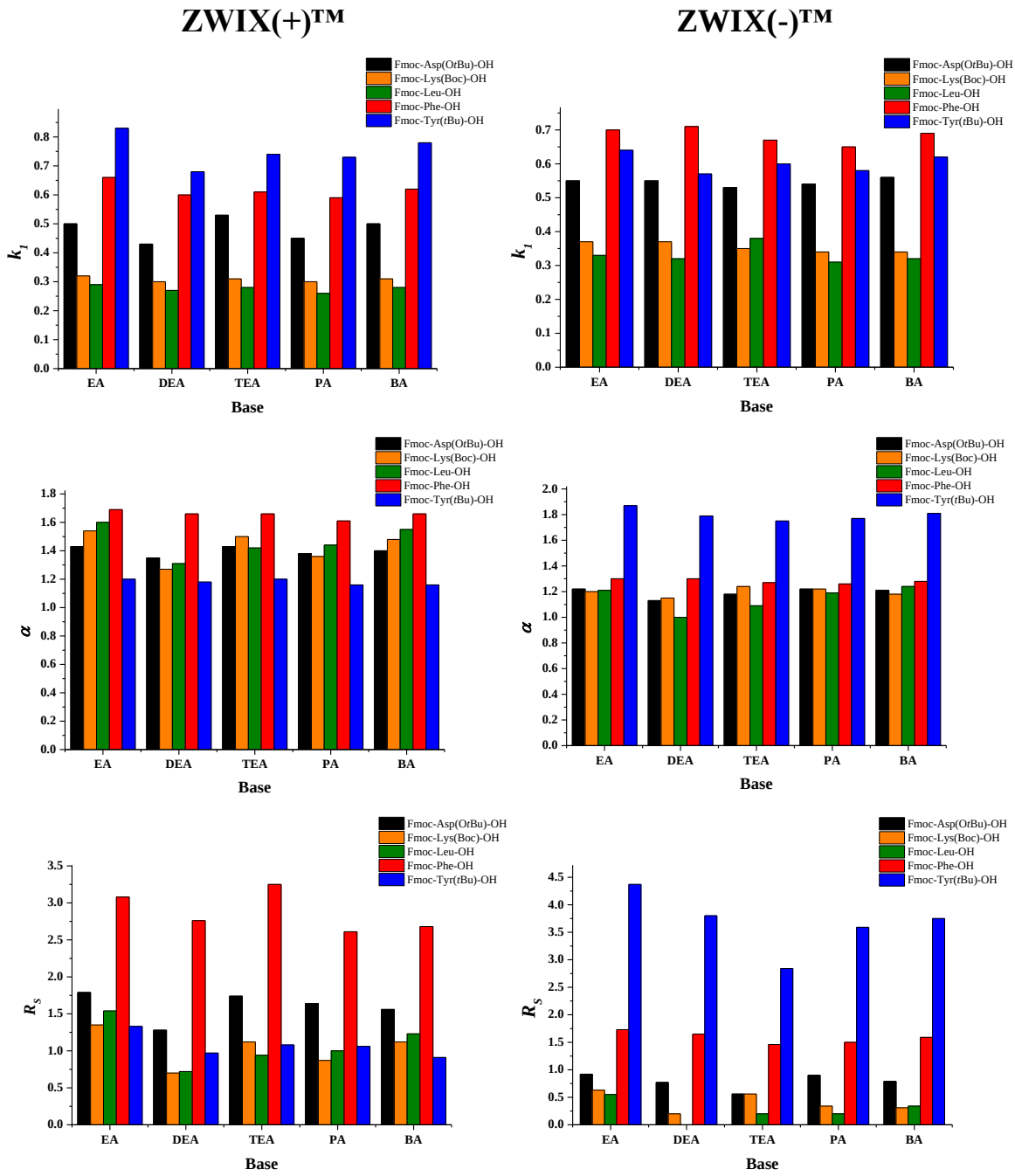
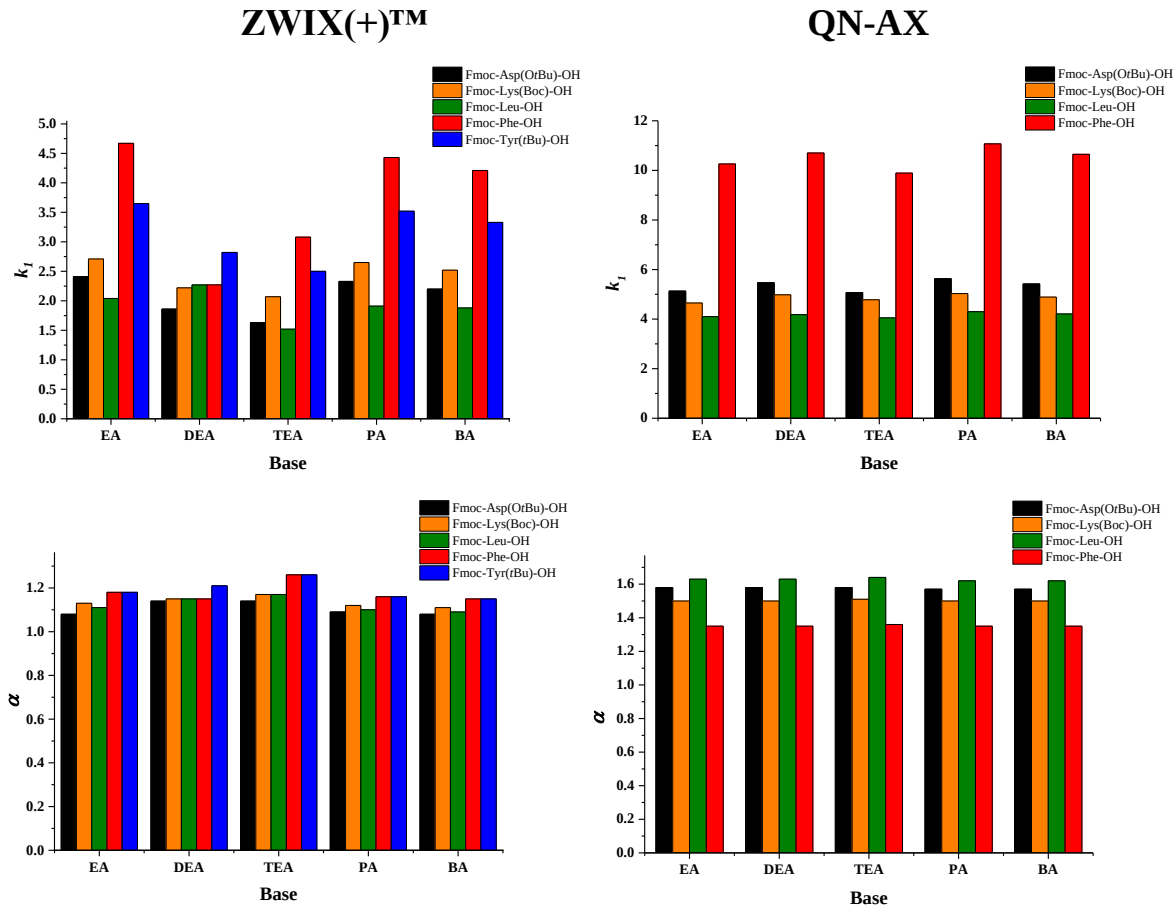



***Figure 15.*** *The effect of nature of base additives on chromatographic parameters in LC mode*
*Chromatographic conditions: columns, ZWIX(+)™ and ZWIX(-)™; mobile phase, $H_2O$/MeOH (1/99 v/v) containing 3.75 mM Base and 7.5 mM FA; flow rate: 0.6 mL/min; detection: 262 nm*

### *4.3.2 Effect of base and acid as mobile phase additives in SFC mode*

For the studies of the effects of acid and base additives in SFC modalities on ZWIX(+)™ and QN-AX phases, the bulk solvent composition was $CO_2$/MeOH (60/40 *v/v*) containig the same FA or AcOH as acid additives, and EA, DEA, TEA, PA, and BA as base additives were selected (***Figure 16***), similar to those of LC modalities. For the same analytes, the nature of the base had a slight effect on $k_1$ values. They were the highest for Fmoc-Phe-OH and Fmoc-Tyr(*t*Bu)-OH. Regarding selectivity, they varied in a relatively narrow range, while for $R_S$ values, the application of DEA or TEA was most promising. Similar to the bases, acid additives (FA or AcOH) had a slight effect on the chromatographic parameters.



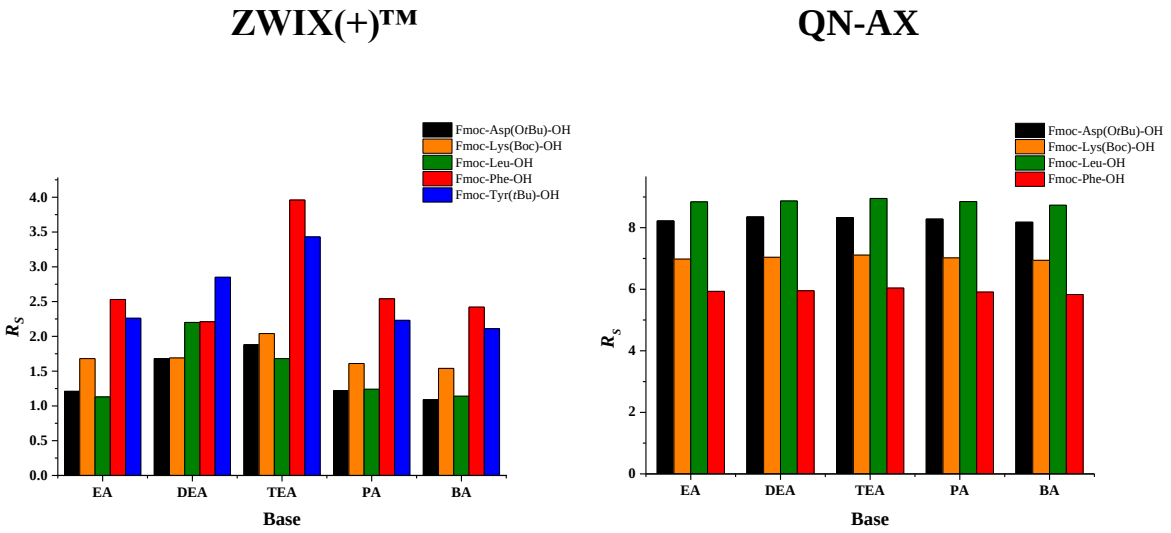



***Figure 16.*** *The effect of nature of base additives on chromatographic parameters in SFC mode*

*Chromatographic conditions: columns, ZWIX(+)™ and QN-AX; mobile phase, $CO_2$/MeOH (60/40 v/v) containing 30 mM Base and 60 mM FA; flow rate: 0.6 mL/min; detection: 262 nm*

### *4.4 The role of counter-ion concentration*

On the *Cinchona* alkaloid-based CSPs in PIM and RPM, an ion-pairing process between the SO and SA dominates the retention. This means that retention can be greatly influenced by changing the amounts of co-ions and counter-ions present in the mobile phase. The application of a higher counter-ion concentration should result in lower retention, according to the stoichiometric displacement model. As shown by *Kopaciewicz et al*., linear relationship can be found between the logarithm of the retention factor of the first-eluted enantiomer (log $k_1$) and the logarithm of the counter-ion concentration (log $c_{counter\text{-}ion}$) *[85,86]*. The slopes of these plots are given by the ratio of the effective charges of SA and counter-ion. This slope was close to 1.0 in the case of the single cation-exchanger type CSPs *[87]*.

#### *4.4.1 Effect of counter-ion concentration in LC mode*

In LC mode, the effect of concentration of the counter-ion was investigated for selected Fmoc amino acids on zwitterionic based CSPs. The mobile phase system was $H_2O$/MeOH (1/99 *v/v*) containing FA and TEA. Keeping the acid-to-base ratio at 2:1, concentrations were varied in the range 3.75–120 mM FA and 1.87–60 mM TEA. Under these conditions, zwitterionic CSPs should work in a „single cation-exchange mode". Values of *log* $k_1$ and *log* $c_{counter\text{-}ion}$ gave straight lines with slopes varying between 0.29–0.36 on ZWIX(+)™ and between 0.19–0.28 on ZWIX(-)™. Experimental results are depicted in ***Figure 17***.

On anion-exchanger QN-AX and QD-AX CSPs, we also suppose a „single ionic" ion-exchange mechanism. Under the studied conditions, linear relationships were found between *log* $k_1$ and *log* $c_{FA}$. The slopes of the *log k* vs. *log c* plots were found around 0.7 for Fmoc-amino-acids samples in LC mode (data from ref. *[88]*).

**ZWIX(+)™** **ZWIX(-)™**

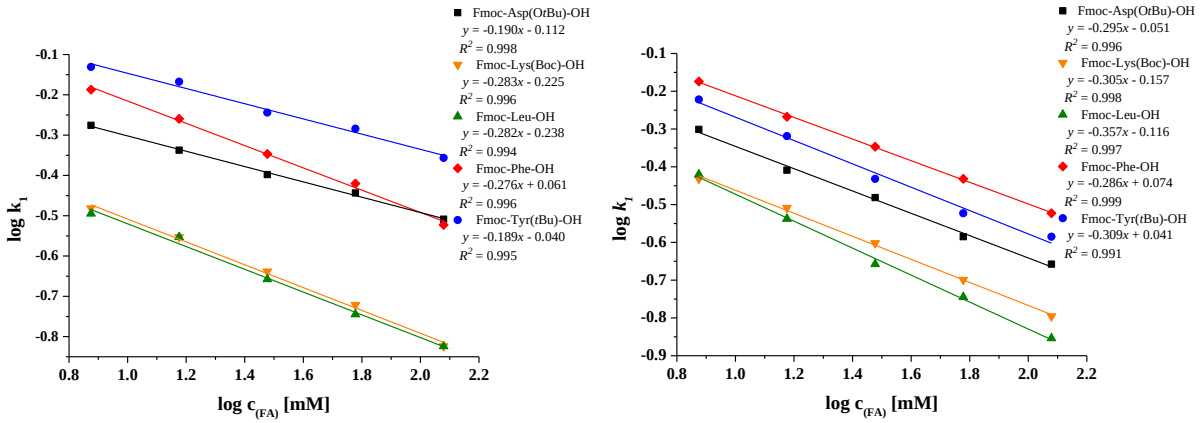



***Figure 17.*** *The effect of counter-ion concentration in LC mode*

*Chromatographic conditions: columns, ZWIX(+)™ and ZWIX(-)™; mobile phase, $H_2O$/MeOH (1/99 v/v) containing TEA/FA in conentrations 1.87/3.75, 3.75/7.5, 7.5/15, 15/30, 30/60, and 60/120 mM/mM; flow rate: 0.6 mL/min; detection: 262 nm*

### *4.5 Effect of counter-ion concentration in SFC mode*

As described earlier, on the *Cinchona* alkaloid-based zwitterionic CSPs in LC mode in PI and HO mobile phases, an ion-pairing displacement process between the SO and SA dominates retention. To prove the ion-exchange mechanism in SFC mode, the displacement model was also applied.

In SFC mode, liquid $CO_2$ mobile phase with MeOH co-solvent was applied. The counter-ion concentration was varied in the range 15–120 mM, while the acid-base ratio was kept constant to ensure similar ionization states for the SO and SA. It was demonstrated that the displacement model exists in SFC conditions and, similar to LC conditions, higher slopes were registered on anion exchanger column than on zwitterionic phases (***Figure 18***).

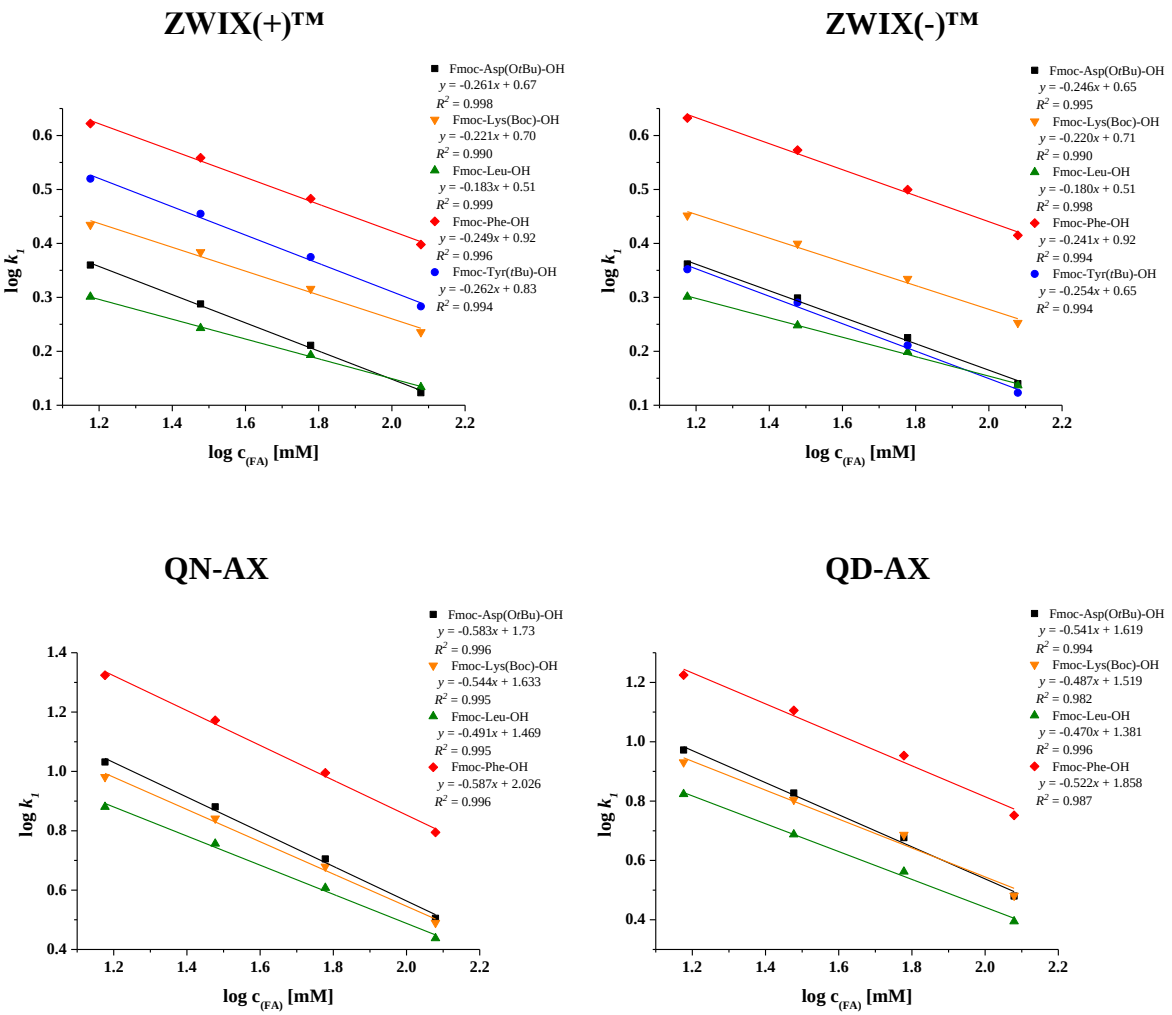



***Figure 18.*** *The effect of counter-ion concentration in SFC mode*

*Chromatographic conditions: columns, ZWIX(+)™, ZWIX(-)™ QN-AX and QD-AX; mobile phase on ZWIX columns, $CO_2$/MeOH (70/30 v/v) and on anion-exchanger columns, $CO_2$/MeOH (60/40 v/v) containing TEA/FA in conentrations 7.5/15, 15/30, 30/60, and 60/120 mM/mM; flow rate: 2.0 mL/min; detection: 262 nm; $T_{COL}$: 40 °C; back pressure: 150 bar*

### *4.6 Enantioseparation of $N^{\alpha}$-Fmoc proteinogenic amino acids*

***Table 2*** and ***Table 3*** present a comprehensive set of data of the 19 $N^{\alpha}$-Fmoc-protected amino acids on all four *Cinchona* alkaloid-based CSPs in LC and SFC mode. On ZWIX(+)™ and ZWIX(-)™, $H_2O$/MeOH (1/99 *v/v*) as mobile phase was used in LC mode, and the mobile phase applied in SFC mode was $CO_2$/MeOH (70/30 *v/v*). On QN-AX and QD-AX columns in LC MeOH/MeCN (75/25 *v/v*) and in SFC mode $CO_2$/MeOH (60/40 *v/v*) mobile phases were used.

The mobile phase, in all cases, contained FA (60 mM) as acid and TEA (30 mM) as base additive keeping the constant acid-base ratio of 2:1. It should be noted that the same acid/base concentration level does not mean the same ionic strength, because of the different activity coefficients existing in $H_2O$/MeOH and MeOH/MeCN mobile phases.

### *4.6.1 LC separation of $N^{\alpha}$-Fmoc proteinogenic amino acids*

The experimental results in ***Table 2*** show that the separation performance of the zwitterionic CSPs ZWIX(+)™ and ZWIX(-)™ was unsatisfactory under HO mobile phase conditions. Of the 19 $N^{\alpha}$-Fmoc-proteinogenic amino acids, only a few exhibited just partial or, in some cases, baseline separations. $k_1$, $\alpha$, and $R_S$ values were much lower than those obtained under SFC conditions (data shown in ***Table 3***). $k_1$ ranged between 0.18– 0.99 and higher values ($k_1 > 0.4$) were obtained for polar amino acids. Exceptions are Fmoc-Thr(*t*Bu)-OH**)** and for SAs and compounds possessing additional aromatic rings (Fmoc-Trp-OH; Pbf or Trt protecting group). Selectivity and resolution also changed in parallel with $k_1$ values. Fmoc-Asp(O*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, and Fmoc-Ser(*t*Bu)-OH were separated only on ZWIX(+)™, and the separation efficiency was better on ZWIX(+)™ than on ZWIX(-)™. Interestingly, despite lower $k_1$ values, better resolution was obtained for Fmoc-Cys(Trt)-OH under LC conditions than at SFC conditions. On the other hand, Fmoc-Pro-OH and Fmoc-Thr(*t*Bu)-OH were not separable on ZWIX phases.

The anion-exchanger type QN-AX and QD-AX CSPs in PIM mode exhibited very different separation performances. Under LC condition, the parameters were much higher than in zwitterion mode with $k_1$ ranging between 1.32–6.77 and $\alpha$ between 1.05–2.22. For most amino acids, *Rs* was higher than 5.0 and only Fmoc-Pro-OH and Fmoc-Thr(*t*Bu)-OH exhibited partial resolution.

As mentioned earlier, the primary interaction is the ionic interaction between the cationic site of the SO and the anionic site of the SA with additional intermolecular SO–SA interaction responsible for chiral discrimination. Because of the presence Fmoc-protection, the cation site of the amino acid is blocked shifting the double ion pairing notion towards an anion exchanger (mono ion pairing) concept. This is the possible reason of the poor separation ability of zwitterionic CSPs using the HO mobile phase.

***Table 2.*** *Chromatographic data, separation factor (k), selectivity factor ($\alpha$), resolution ($R_S$), and elution sequence of $N^{\alpha}$-Fmoc proteinogenic amino acids at LC condition*

| | Compound | | ZWIX(+)™ | ZWIX(-)™ | QN-AX | QD-AX |
|---|---|---|---|---|---|---|
| ***Acidic*** | **Fmoc-Asp(O*t*Bu)-OH** | $k_1$ | 0.36 (*D*) | 0.22 (*L*) | 2.38 (*D*) | 2.45 (*L*) |
| | | $\alpha$ | 1.32 | 1.00 | 1.82 | 1.78 |
| | | $R_S$ | 1.16 | 0.00 | 8.77 | 9.26 |
| | **Fmoc-Glu(O*t*Bu)-OH** | $k_1$ | 0.22 (*D*) | 0.24 (*L*) | 1.97 (*D*) | 2.02 (*L*) |
| | | $\alpha$ | 1.32 | 1.00 | 1.88 | 1.66 |
| | | $R_S$ | 0.64 | 0.00 | 9.54 | 7.86 |
| ***Basic*** | **Fmoc-Lys(Boc)-OH** | $k_1$ | 0.19 (*D*) | 0.20 (*L*) | 1.32 (*D*) | 1.54 (*L*) |
| | | $\alpha$ | 1.43 | 0.00 | 1.80 | 1.39 |
| | | $R_S$ | 1.01 | 0.00 | 6.20 | 5.00 |

***Table 2.*** (*continued*) *Chromatographic data, separation factor (k), selectivity factor (α), resolution ($R_S$), and elution sequence of $N^{\alpha}$-Fmoc proteinogenic amino acids at LC condition*

| | Compound | | ZWIX(+)™ | ZWIX(-)™ | QN-AX | QD-AX |
|---|---|---|---|---|---|---|
| ***Basic*** | **Fmoc-Arg(Pbf)-OH** | $k_1$ | 0.99 (*D*) | 0.71 (*L*) | 2.74 (*D*) | 2.74 (*L*) |
| | | $\alpha$ | 1.91 | 1.50 | 1.59 | 1.42 |
| | | $R_S$ | 4.67 | 2.52 | 7.87 | 4.00 |
| | **Fmoc-His(Trt)-OH** | $k_1$ | 0.63 (*D*) | 0.43 (*L*) | 1.85 (*D*) | 2.09 (*L*) |
| | | $\alpha$ | 1.66 | 1.47 | 1.99 | 1.62 |
| | | $R_S$ | 2.89 | 1.50 | 10.49 | 6.27 |
| ***Hydrophobic*** | **Fmoc-Ala-OH** | $k_1$ | 0.25 (*D*) | 0.27 (*L*) | 2.18 (*D*) | 2.10 (*L*) |
| | | $\alpha$ | 1.36 | 1.00 | 1.50 | 1.59 |
| | | $R_S$ | 0.88 | 0.00 | 6.67 | 6.89 |
| | **Fmoc-Val-OH** | $k_1$ | 0.20 (*D*) | 0.21 (*L*) | 1.88 (*D*) | 1.79 (*L*) |
| | | $\alpha$ | 1.54 | 1.13 | 2.06 | 2.06 |
| | | $R_S$ | 0.77 | 0.20 | 12.66 | 10.78 |
| | **Fmoc-Leu-OH** | $k_1$ | 0.18 (*D*) | 0.18 (*L*) | 1.58 (*D*) | 1.56 (*L*) |
| | | $\alpha$ | 1.36 | 1.00 | 1.83 | 1.82 |
| | | $R_S$ | 0.70 | 0.00 | 8.53 | 9.73 |
| | **Fmoc-Ile-OH** | $k_1$ | 0.18 (*D*) | 0.22 (*L*) | 1.80 (*D*) | 1.55 (*L*) |
| | | $\alpha$ | 1.43 | 1.00 | 2.05 | 2.22 |
| | | $R_S$ | 0.69 | 0.00 | 10.62 | 12.3 |
| | **Fmoc-Phe-OH** | $k_1$ | 0.38 (*D*) | 0.40 (*L*) | 3.26 (*D*) | 3.46 (*L*) |
| | | $\alpha$ | 1.63 | 1.31 | 1.55 | 1.62 |
| | | $R_S$ | 2.11 | 1.09 | 7.74 | 8.47 |
| | **Fmoc-Trp-OH** | $k_1$ | 0.80 (*D*) | 0.65 (*L*) | 4.11 (*D*) | 4.50 (*L*) |
| | | $\alpha$ | 2.36 | 1.67 | 1.64 | 1.50 |
| | | $R_S$ | 6.82 | 3.15 | 8.70 | 7.02 |
| | **Fmoc-Met-OH** | $k_1$ | 0.33 (*D*) | 0.32 (*L*) | 2.84 (*D*) | 2.90 (*L*) |
| | | $\alpha$ | 1.41 | 1.19 | 1.67 | 1.78 |
| | | $R_S$ | 1.31 | 0.30 | 9.83 | 10.22 |
| | **Fmoc-Pro-OH** | $k_1$ | 0.27 (*D*) | 0.28 (*L*) | 1.88 (*D*) | 1.61 (*L*) |
| | | $\alpha$ | 1.00 | 1.00 | 1.06 | 1.15 |
| | | $R_S$ | 0.00 | 0.00 | 1.06 | 2.03 |
| ***Polar*** | **Fmoc-Ser(*t*Bu)-OH** | $k_1$ | 0.22 (*D*) | 0.23 (*L*) | 2.14 (*D*) | 1.97 (*L*) |
| | | $\alpha$ | 1.41 | 1.00 | 1.35 | 1.37 |
| | | $R_S$ | 0.87 | 0.00 | 5.42 | 5.02 |
| | **Fmoc-Thr(*t*Bu)-OH** | $k_1$ | 0.19 (*D*) | 0.18 (*L*) | 1.72 (*D*) | 1.31 (*L*) |
| | | $\alpha$ | 1.00 | 1.00 | 1.05 | 1.11 |
| | | $R_S$ | 0.00 | 0.00 | 0.52 | 1.42 |
| | **Fmoc-Cys(Trt)-OH** | $k_1$ | 0.59 (*D*) | 0.41 (*D*) | 6.77 (*D*) | 4.26 (*L*) |
| | | $\alpha$ | 1.49 | 1.63 | 1.14 | 1.33 |
| | | $R_S$ | 2.36 | 2.29 | 2.68 | 5.26 |
| | **Fmoc-Tyr(*t*Bu)-OH** | $k_1$ | 0.52 (*D*) | 0.48 (*L*) | 3.89 (*D*) | 2.88 (*L*) |
| | | $\alpha$ | 1.00 | 1.92 | 1.17 | 2.11 |
| | | $R_S$ | 0.00 | 3.58 | 2.11 | 12.14 |
| | **Fmoc-Asn-OH** | $k_1$ | 0.73 (*D*) | 0.48 (*L*) | 3.45 (*D*) | 3.32 (*L*) |
| | | $\alpha$ | 2.06 | 1.84 | 1.33 | 1.26 |
| | | $R_S$ | 5.50 | 3.19 | 5.47 | 4.37 |
| | **Fmoc-Gln-OH** | $k_1$ | 0.45 (*D*) | 0.46 (*L*) | 2.75 (*D*) | 3.70 (*L*) |
| | | $\alpha$ | 1.54 | 1.08 | 1.98 | 1.39 |
| | | $R_S$ | 2.11 | 0.20 | 10.61 | 5.64 |

*Chromatographic conditions: column, ZWIX(+)™, ZWIX(-)™,QN-AX and QD-AX; mobile phase, on ZWIX(+)™ and ZWIX(-)™ $H_2O$/MeOH (1/99 v/v) containing 30 mM TEA and 60 mM FA and on QN-AX and QD-AX MeOH/MeCN (75/25 v/v) containing 30 mM TEA and 60 mM FA; flow rate, 0.6 mL/min; detection, 262 nm; temperature, ambient; configuration (D and L) in parenthesis represents the configuration of the first eluting enantiomer*

### *4.6.2 SFC separation of $N^{\alpha}$-Fmoc proteinogenic amino acids*

***Table 3*** depicts the enantioseparation of 19 $N^{\alpha}$-Fmoc-protected amino acids under SFC conditions on all four *Cinchona*-based CSPs. In SFC mode both zwitterionic and anion-exchanger type CSPs are suitable for enantioseparation. The only exception is Fmoc-Pro-OH, which can be separated only on QD-AX column. The most obvious phenomeon is that in all cases higher $k_1$ values were registered under SFC conditions. Despite the higher MeOH content applied on anion-exchanger column [$CO_2$/MeOH (60/40 *v/v*)], higher $k_1$ values were obtained in all cases, except for Fmoc-Thr(*t*Bu)-OH. In most cases $\alpha$ and $R_S$ values were also higher than on zwitterionic CSPs. High retentions were registered – especially on anion exchangers – for the so-called polar amino acids like Fmoc-Asn-OH and Fmoc-Gln-OH, and also for amino acids containing additional large or aromatic protecting groups (*t*Bu, Pbf, and Trt), such as Fmoc-Arg(Pbf)-OH, Fmoc-His(Trt)-OH, Fmoc-Cys(Trt)-OH, and Fmoc-Tyr(*t*Bu)-OH. This is also valid for amino acids containing an aromatic side chain (*e.g.*, Fmoc-Phe-OH and Fmoc-Trp-OH). It is probable, that the presence of the additional aromatic group (Trt) on Cys and His contributes to higher retention.

***Table 3.*** *Chromatographic data, separation factor (k), selectivity factor ($\alpha$), and resolution ($R_S$) of $N^{\alpha}$-Fmoc proteinogenic amino acids at SFC conditions*

| Compound | | | ZWIX(+)™ | ZWIX(-)™ | QN-AX | QD-AX |
|---|---|---|---|---|---|---|
| ***Acidic*** | **Fmoc-Asp(O*t*Bu)-OH** | $k_1$ | 1.76 (*D*) | 1.63 (*L*) | 5.07 (*D*) | 4.75 (*L*) |
| | | $\alpha$ | 1.18 | 1.15 | 1.58 | 1.58 |
| | | $R_S$ | 2.43 | 1.88 | 8.33 | 7.95 |
| | **Fmoc-Glu(O*t*Bu)-OH** | $k_1$ | 1.66 (*D*) | 1.69 (*L*) | 5.01 (*D*) | 4.84 (*L*) |
| | | $\alpha$ | 1.17 | 1.12 | 1.64 | 1.48 |
| | | $R_S$ | 2.13 | 1.50 | 8.86 | 6.72 |
| ***Basic*** | **Fmoc-Lys(Boc)-OH** | $k_1$ | 2.22 (*D*) | 2.07 (*L*) | 4.78 (*D*) | 4.86 (*L*) |
| | | $\alpha$ | 1.21 | 1.18 | 1.51 | 1.42 |
| | | $R_S$ | 2.63 | 2.04 | 7.11 | 5.79 |
| | **Fmoc-Arg(Pbf)-OH** | $k_1$ | 24.39 (*D*) | 23.31 (*L*) | 27.50 (*D*) | 23.74 (*L*) |
| | | $\alpha$ | 1.65 | 1.54 | 1.33 | 1.38 |
| | | $R_S$ | 9.95 | 7.78 | 5.26 | 5.71 |
| | **Fmoc-His(Trt)-OH** | $k_1$ | 5.19 (*D*) | 4.48 (*L*) | 8.20 (*D*) | 7.90 (*L*) |
| | | $\alpha$ | 1.31 | 1.26 | 1.47 | 1.48 |
| | | $R_S$ | 4.36 | 3.53 | 4.64 | 5.43 |
| ***Hydrophobic*** | **Fmoc-Ala-OH** | $k_1$ | 2.20 (*D*) | 2.03 (*L*) | 5.86 (*D*) | 5.12 (*L*) |
| | | $\alpha$ | 1.15 | 1.15 | 1.40 | 1.47 |
| | | $R_S$ | 2.03 | 1.87 | 6.49 | 6.89 |
| | **Fmoc-Val-OH** | $k_1$ | 1.60 (*D*) | 1.51 (*L*) | 4.46 (*D*) | 3.81 (*L*) |
| | | $\alpha$ | 1.24 | 1.21 | 1.71 | 1.75 |
| | | $R_S$ | 2.79 | 2.22 | 9.87 | 9.59 |
| | **Fmoc-Leu-OH** | $k_1$ | 1.62 (*D*) | 1.52 (*L*) | 4.05 (*D*) | 3.94 (*L*) |
| | | $\alpha$ | 1.20 | 1.18 | 1.64 | 1.68 |
| | | $R_S$ | 2.11 | 1.68 | 8.95 | 8.89 |

***Table 3.*** (*continued*) *Chromatographic data, separation factor (k), selectivity factor (α), and resolution ($R_S$) of $N^{\alpha}$-Fmoc proteinogenic amino acids at SFC conditions*

| Compound | | | ZWIX(+)™ | ZWIX(-)™ | QN-AX | QD-AX |
|---|---|---|---|---|---|---|
| *Hydrophobic* | **Fmoc-Ile-OH** | $k_1$ | 1.42 (*D*) | 1.43 (*L*) | 4.20 (*D*) | 3.60 (*L*) |
| | | $\alpha$ | 1.21 | 1.19 | 1.72 | 1.77 |
| | | $R_S$ | 1.49 | 1.33 | 9.82 | 9.67 |
| | **Fmoc-Phe-OH** | $k_1$ | 3.35 (*D*) | 3.08 (*L*) | 9.89 (*D*) | 8.98 (*L*) |
| | | $\alpha$ | 1.31 | 1.27 | 1.36 | 1.45 |
| | | $R_S$ | 4.53 | 3.96 | 6.04 | 6.91 |
| | **Fmoc-Trp-OH** | $k_1$ | 16.40 (*D*) | 14.10 (*L*) | 26.98 (*D*) | 23.05 (*L*) |
| | | $\alpha$ | 2.02 | 1.99 | 1.45 | 1.39 |
| | | $R_S$ | 10.70 | 9.30 | 7.42 | 6.43 |
| | **Fmoc-Met-OH** | $k_1$ | 2.95 (*D*) | 2.74 (*L*) | 8.55 (*D*) | 7.54 (*L*) |
| | | $\alpha$ | 1.23 | 1.21 | 1.52 | 1.62 |
| | | $R_S$ | 3.41 | 3.17 | 8.13 | 8.77 |
| | **Fmoc-Pro-OH** | $k_1$ | 1.66 (*D*) | 1.59 (*L*) | 4.04 (*D*) | 3.47 (*L*) |
| | | $\alpha$ | 1.00 | 1.00 | 1.00 | 1.11 |
| | | $R_S$ | 0.00 | 0.00 | 0.00 | 1.70 |
| *Polar* | **Fmoc-Ser(*t*Bu)-OH** | $k_1$ | 1.26 (*D*) | 1.23 (*L*) | 3.98 (*D*) | 3.47 (*L*) |
| | | $\alpha$ | 1.13 | 1.11 | 1.23 | 1.23 |
| | | $R_S$ | 0.96 | 0.99 | 3.64 | 3.51 |
| | **Fmoc-Thr(*t*Bu)-OH** | $k_1$ | 3.67 (*D*) | 3.52 (*L*) | 2.73 (*D*) | 2.45 (*L*) |
| | | $\alpha$ | 1.95 | 1.92 | 1.17 | 1.00 |
| | | $R_S$ | 9.42 | 9.04 | 2.70 | 0.00 |
| | **Fmoc-Cys(Trt)-OH** | $k_1$ | 7.92 (*D*) | 6.95 (*L*) | 15.84 (*D*) | 10.29 (*L*) |
| | | $\alpha$ | 1.07 | 1.06 | 1.73 | 1.99 |
| | | $R_S$ | 1.20 | 1.13 | 10.49 | 12.20 |
| | **Fmoc-Tyr(*t*Bu)-OH** | $k_1$ | 2.67 (*D*) | 2.50 (*L*) | 10.63 (*D*) | 7.08 (*L*) |
| | | $\alpha$ | 1.33 | 1.27 | 1.94 | 3.27 |
| | | $R_S$ | 4.39 | 3.43 | 12.36 | 19.82 |
| | **Fmoc-Asn-OH** | $k_1$ | 8.95 (*D*) | 7.42 (*L*) | 14.54 (*D*) | 11.65 (*L*) |
| | | $\alpha$ | 1.35 | 1.54 | 1.23 | 1.29 |
| | | $R_S$ | 5.73 | 7.58 | 3.94 | 4.69 |
| | **Fmoc-Gln-OH** | $k_1$ | 6.57 (*D*) | 7.41 (*L*) | 13.04 (*D*) | 14.86 (*L*) |
| | | $\alpha$ | 1.49 | 1.32 | 2.31 | 1.97 |
| | | $R_S$ | 7.41 | 5.27 | 15.10 | 11.89 |

*Chromatographic conditions: column, ZWIX(+)™, ZWIX(-)™, QN-AX and QD-AX; mobile phase, on ZWIX(+)™ and ZWIX(-)™ $CO_2$/MeOH (70/30 v/v) containing 30 mM TEA and 60 mM FA and on QN-AX and QD-AX $CO_2$/MeCN (60/40 v/v) containing 30 mM TEA and 60 mM FA; flow rate, 2.0 mL/min; detection, 262 nm; $T_{col}$, 40 °C; back pressure, 150 bar; configuration (D and L) in parenthesis represents the configuration of the first eluting enantiomer*

### *4.7 Influence of temperature on the separation of $N^{\alpha}$-Fmoc proteinogenic amino acids*

The effect of temperature on the separation mechanism is complex. Thermodynamic studies are often performed in order to understand the mechanistic aspect of chiral recognition. Enantioselective retention mechanisms are sometimes influenced by temperature to a greater extent when compared to non-chiral separations. Accordingly, the effect of column temperature has often been investigated and optimized in enantioselective chromatographic separations. In the past decade several paper were published addressing the effect of temperature in enantioseparations on *Cinchona* alkaloid-based CSPs *[89–94]*.

There are two main temperature effects governing the chromatographic performance of a CSP. One is the *thermodynamic effect*. This effect is the changes in the selectivity ($\alpha$), which is related to the peak-to-peak separation distance. The selectivity usually decreases with increasing temperature. This occurs because of the partition coefficient; therefore, the free energy change ($\Delta G^{o}$) of the transfer of the analyte between the mobile phase and the stationary phase varies with temperature. The effect of temperature on selectivity is controversial. It is, in part, due to the lack of our understanding how the $\Delta G°$ of the compound would change in the course of the mass-transfer process. Another completely different effect is the influence on viscosity and diffusion coefficient of the analyte in the two phases. This is called *kinetic effect*. At higher temperature viscosity decreases. However, the diffusion coefficient of the solute increases, thereby affecting the mass transfer between the mobile and stationary phases. As a result of this two effects, the temperature increase often produces a trade-off for resolution. Namely, the increased efficiency is good for resolution, while the decreasing peak-to-peak separation is disadvantageous for resolution *[95–97]*.

For the investigation of the temperature effect, the van't Hoff plots approach was applied. The differences of the standard enthalpy and entropy changes calculated from the ln α vs. 1/*T* curves give $\Delta(\Delta H^{o})$ as the slope and $\Delta(\Delta S^{o})$ as the intercept (Eq. 5). The $\Delta(\Delta H^{o})$ values present the difference of enthalpy changes accompanying the transfer of the analytes from the mobile to the stationary phase. High negative $\Delta(\Delta H^{o})$ values indicate an exothermic process, *i.e.* a stronger interaction of the enantiomers with the stationary phase or more efficient enantiomer transfer between the mobile and the stationary phases. The trend in the change of $\Delta(\Delta S^{o})$ is similar as in $\Delta(\Delta H^{o})$. If $\Delta(\Delta H^{o})$ values are negative, $\Delta(\Delta S^{o})$ values are also negative and the largest negative $\Delta(\Delta H^{o})$ values are accompanied by the largest negative $\Delta(\Delta S^{o})$ values. The more negative $\Delta(\Delta S^{o})$ values suggest an enhanced increase of order or stronger interactions of the SA–SO complex resulting in a significant loss of freedom. In chiral separation – similar to achiral chromatography – retention and selectivity decrease with increasing temperature,

*Δ(ΔH°)* and *Δ(ΔS°)* values are negative, *i.e.* the enantioseparation is enthalpically controlled. However, in some cases, retention decreases but selectivity increases with increasing temperature. In that case, both *Δ(ΔH°)* and *Δ(ΔS°)* exhibit positive values, *i.e.* the enantioseparation is entropically controlled *[90,94,98,99]*.

### *4.7.1 Influence of temperature on the separation of N$^{\alpha}$-Fmoc proteinogenic amino acids on quinine-based CSPs in HO, PI, and SFC mode*

In order to investigate the effect of temperature on chromatographic parameters, a variable temperature study was carried out for selected *N*$^{\alpha}$-Fmoc amino acids on both *quinine*- and *quinidine*-based CSPs in HO, PI, and SFC modes. The temperature range varied between 5–40 ºC and 20–50 ºC, respectively. The applied mobile phases for HO-LC contained $H_2O$/MeOH (1/99 *v/v*), 3.75 mM TEA, and 7.5 mM FA on ZWIX(+)™. For PIM, MeOH/MeCN (75/25 *v/v*) contained 30 mM TEA and 60 mM FA on QN-AX. For SFC we chose the condition $CO_2$/MeOH (70/30 *v/v*) containing 30 mM TEA and 60 mM FA for ZWIX(+)™, and $CO_2$/MeOH (60/40 *v/v*) containing 30 mM TEA and 60 mM FA for QN-AX. Chromatographic data received at various temperatures are depicted in Appendix ***Table S1***.

Increasing temperature, in all cases, resulted in a decrease in $k_1$ and $\alpha$ values, while $R_S$ in most cases also decreased. However, minimum or maximum values of $R_S$ were registered in a few cases. All thermodynamic data are presented in ***Table 4***.

***Table 4.*** *Thermodynamic parameters, Δ(ΔH°), Δ(ΔS°), TxΔ(ΔS°), Δ(ΔG°), and Q values of N$^{\alpha}$-Fmoc-protected proteinogenic amino acids on ZWIX(+)$^{TM}$ and QN-AX CSP in liquid chromatographic and SFC mode*

| Compound | Column | Mode/ mobile phase | -Δ(Δ*H°*) (kJ mol$^{-1}$) | -Δ(Δ*S°*) (J mol$^{-1}$ K$^{-1}$) | -TxΔ(Δ*S°*)$_{298K}$ (kJ mol$^{-1}$) | -Δ(Δ*G°*)$_{298K}$ (kJ mol$^{-1}$) | *Q* |
|---|---|---|---|---|---|---|---|
| **Fmoc-Asp(O*t*Bu)-OH** | ZWIX(+)™ | HO | 5.6 | 16.7 | 5.0 | 0.6 | 1.1 |
| | QN-AX | PIM | 5.5 | 13.5 | 4.0 | 1.5 | 1.4 |
| | ZWIX(+)™ | SFC | 0.8 | 1.3 | 0.4 | 0.4 | 2.0 |
| | QN-AX | SFC | 3.6 | 7.5 | 2.2 | 1.4 | 1.6 |
| **Fmoc-Lys(Boc)-OH** | ZWIX(+)™ | HO | 3.5 | 10.1 | 3.0 | 0.5 | 1.2 |
| | QN-AX | PIM | 3.8 | 7.8 | 2.3 | 1.5 | 1.7 |
| | ZWIX(+)™ | SFC | 1.4 | 2.7 | 0.8 | 0.6 | 1.8 |
| | QN-AX | SFC | 1.9 | 2.5 | 0.7 | 1.2 | 2.7 |
| **Fmoc-Leu-OH** | ZWIX(+)™ | HO | 2.8 | 7.0 | 2.1 | 0.7 | 1.2 |
| | QN-AX | PIM | 4.2 | 9.1 | 2.7 | 1.5 | 1.6 |

***Table 4.*** (*continued*) *Thermodynamic parameters, Δ(ΔH°), Δ(ΔS°), TxΔ(ΔS°), Δ(ΔG°), and Q values of N$^{\alpha}$-Fmoc-protected proteinogenic amino acids on ZWIX(+)$^{TM}$ and QN-AX CSP in liquid chromatographic and SFC mode*

| Compound | Column | Mode/ mobile phase | -Δ(Δ*H*°) (kJ mol$^{-1}$) | -Δ(Δ*S*°) (J mol$^{-1}$ K$^{-1}$) | -TxΔ(Δ*S*°)$_{298K}$ (kJ mol$^{-1}$) | -Δ(Δ*G*°)$_{298K}$ (kJ mol$^{-1}$) | *Q* |
|---|---|---|---|---|---|---|---|
| **Fmoc-Leu-OH** | ZWIX(+)$^{TM}$ | SFC | 0.8 | 1.1 | 0.3 | 0.5 | 2.7 |
| | QN-AX | SFC | 5.1 | 12.1 | 3.6 | 1.5 | 1.4 |
| **Fmoc-Phe-OH** | ZWIX(+)$^{TM}$ | HO | 7.1 | 19.7 | 5.9 | 1.2 | 1.2 |
| | QN-AX | PIM | 4.0 | 9.6 | 2.9 | 1.1 | 1.4 |
| | ZWIX(+)$^{TM}$ | SFC | 2.0 | 4.2 | 1.3 | 0.7 | 1.5 |
| | QN-AX | SFC | 2.1 | 4.3 | 1.3 | 0.8 | 1.6 |
| **Fmoc-Tyr(*t*Bu)-OH** | ZWIX(+)$^{TM}$ | HO | 7.5 | 23.7 | 7.1 | 0.4 | 1.1 |
| | QN-AX | PIM | 2.6 | 7.3 | 2.2 | 0.4 | 1.2 |
| | ZWIX(+)$^{TM}$ | SFC | 2.7 | 6.2 | 1.8 | 0.9 | 1.5 |
| | QN-AX | SFC | 2.4 | 2.4 | 0.7 | 1.7 | 3.4 |

*Chromatographic conditions: column, ZWIX(+)$^{TM}$ and QN-AX; mobile phase in HO-LC mode, H$_2$O/MeOH (1/99 v/v) containing 3.75 mM TEA and 7.5 mM FA and in PIM MeOH/MeCN (75/25 v/v) containing 30 mM TEA and 60 mM FA, in SFC mode CO$_2$/MeOH (70/30 v/v) containing 30 mM TEA and 60 mM FA on ZWIX(+)$^{TM}$; and CO$_2$/MeOH (60/40 v/v) containing 30 mM TEA and 60 mM FA on QN-AX; flow rate, 0.6 mL/min or 2.0 mL/min (SFC); detection, 262 nm; Q = Δ(ΔH°) / 298 × Δ(ΔS°)*

According to data summarized in ***Table 4***, all *Δ(ΔH°)* values are negative ranging from –0.8 to –7.5 kJ mol$^{-1}$. This reflects the relative ease of the transfer of SAs from the mobile to the stationary phase, and a negative *Δ(ΔH°)* value relates to a favorable exothermic process. It was generally observed that *Δ(ΔH°)* values were more negative on both ZWIX(+)™ and QN-AX under LC condition than under SFC condition (except for Fmoc-Leu-OH). The trend in the change in *Δ(ΔS°)* was found to be similar to that in *Δ(ΔH°)*. Under the applied conditions, *Δ(ΔS°)* ranged from –1.1 to –23.7 J mol$^{-1}$ K$^{-1}$. In most cases the *Δ(ΔS°)* values on ZWIX(+)™ and QN-AX were more negative under LC condition, than under SFC condition, again with the exception of Fmoc-Leu-OH. In the case of negative *Δ(ΔS°)*, the adsorbed enantiomers exhibited increased order of the SO–SA complex formed on the stationary phase resulting in a significant loss of freedom, indicating a thermodynamically unfavorable process. A comparison of *Δ(ΔH°)* and *Δ(ΔS°)* in LC condition revealed that they are more negative on ZWIX(+)™ (HO modality) than on QN-AX. In conrast, the values at SFC condition show a reverse order, *i.e.* more negative values were obtained on QN-AX than on ZWIX(+)™. The more negative *Δ(ΔG°)* values calculated at 298 K were generally obtained on QN-AX rather than on ZWIX(+)™ working either in liquid chromatographic or SFC mode.

The relative contribution of the enthalphic and entropic terms to the free energy of adsorption can be visualized through the enthalpy/entropy ratio $Q$ {$Q = \Delta(\Delta H^\circ) / [298 \times \Delta(\Delta S^\circ)]$} calculated at 298 K. A comparison of the $Q$ values for the individual analytes revealed that, in all cases, the enantioselective discrimination was enthalpically driven ($Q$ > 1.0) and, with a few exceptions, the highest $Q$ values were most often obtained on QN-AX.

### *4.7.2 Influence of temperature on the separation of $N^{\alpha}$-Fmoc proteinogenic amino acids on quinidine-based CSPs in LC and SFC mode*

To explore the temperature effect of selected $N^{\alpha}$-Fmoc amino acids on both *quinidine*-based CSPs, the same mobile phases as on *quinine*-based CSPs (***4.6.1***) in LC and SFC mode were chosen. We used also the same temperature range as before. The chromatographic data obtained at varied temperatures are depicted in Appendix ***Table S2***.

All $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$ data are presented in ***Table 5***. The $\Delta(\Delta H^\circ)$ values measured on ZWIX(-)™ and QD-AX ranged from –1.0 and –3.7 kJ mol$^{-1}$ and between –2.7 and –6.6 kJ mol$^{-1}$, respectively. It was generally observed that $\Delta(\Delta H^\circ)$ values were more negative on both columns under LC condition compared to SFC condition (the only exception was Fmoc-Phe-OH on ZWIX(-)™). Comparing the $\Delta(\Delta H^\circ)$ values on the studied columns, in all cases, more negative values were registered on anion-exchanger type QD-AX than on ZWIX(-)™. Regarding the $\Delta(\Delta S^\circ)$ values, they ranged on ZWIX(-)™ between –1.1 and –9.2 J mol$^{-1}$ K$^{-1}$ and on QD-AX between –5.8 and –16.6 J mol$^{-1}$ K$^{-1}$. The trend was similar for $\Delta(\Delta H^\circ)$ values: more negative values were observed on QD-AX than on ZWIX(-)™ and under LC condition compared to SFC condition. $\Delta(\Delta G^\circ)$ values calculated at 298 K were more negative on QD-AX CSP in both LC and SFC modalities.

***Table 5.*** *Thermodynamic parameters, $\Delta(\Delta H^\circ)$, $\Delta(\Delta S^\circ)$, $Tx\Delta(\Delta S^\circ)$, $\Delta(\Delta G^\circ)$, and Q values of $N^{\alpha}$-Fmoc-protected proteinogenic amino acids on ZWIX(-)$^{TM}$ and QD-AX CSP in liquid chromatographic and SFC mode*

| **Compound** | **Column** | **Mode/ mobile phase** | **$-\Delta(\Delta H^\circ)$ (kJ mol$^{-1}$)** | **$-\Delta(\Delta S^\circ)$ (J mol$^{-1}$ K$^{-1}$)** | **$-T\times\Delta(\Delta S^\circ)_{298K}$ (kJ mol$^{-1}$)** | **$-\Delta(\Delta G^\circ)_{298K}$ (kJ mol$^{-1}$)** | ***Q*** |
|---|---|---|---|---|---|---|---|
| **Fmoc-Asp(O*t*Bu)-OH** | ZWIX(-)$^{TM}$ | HO | 2.4 | 6.6 | 2.0 | 0.4 | 1.2 |
| | QD-AX | PIM | 6.0 | 15.0 | 4.5 | 1.5 | 1.3 |
| | ZWIX(-)$^{TM}$ | SFC | 1.0 | 2.0 | 0.6 | 0.4 | 1.7 |
| | QD-AX | SFC | 3.9 | 8.6 | 2.6 | 1.3 | 1.5 |

***Table 5.*** (*continued*) *Thermodynamic parameters, $\Delta(\Delta H^\circ)$, $\Delta(\Delta S^\circ)$, $Tx\Delta(\Delta S^\circ)$, $\Delta(\Delta G^\circ)$, and Q values of $N^{\alpha}$-Fmoc-protected proteinogenic amino acids on ZWIX(-)™ and QD-AX CSP in liquid chromatographic and SFC mode*

| Compound | Column | Mode/ mobile phase | $-\Delta(\Delta H^\circ)$ (kJ mol$^{-1}$) | $-\Delta(\Delta S^\circ)$ (J mol$^{-1}$ K$^{-1}$) | $-Tx\Delta(\Delta S^\circ)_{298K}$ (kJ mol$^{-1}$) | $-\Delta(\Delta G^\circ)_{298K}$ (kJ mol$^{-1}$) | *Q* |
|---|---|---|---|---|---|---|---|
| **Fmoc-Lys(Boc)-OH** | ZWIX(-)™ | HO | 3.1 | 9.2 | 2.7 | 0.4 | 1.1 |
| | QD-AX | PIM | 4.3 | 10.8 | 3.2 | 1.1 | 1.3 |
| | ZWIX(-)™ | SFC | 2.0 | 5.2 | 1.6 | 0.4 | 1.3 |
| | QD-AX | SFC | 2.7 | 5.8 | 1.7 | 1.0 | 1.6 |
| **Fmoc-Leu-OH** | ZWIX(-)™ | HO | 1.9 | 5.4 | 1.6 | 0.3 | 1.2 |
| | QD-AX | PIM | 6.6 | 16.6 | 4.9 | 1.7 | 1.3 |
| | ZWIX(-)™ | SFC | 1.4 | 3.1 | 0.9 | 0.5 | 1.6 |
| | QD-AX | SFC | 4.3 | 9.7 | 2.9 | 1.4 | 1.5 |
| **Fmoc-Phe-OH** | ZWIX(-)™ | HO | 1.0 | 1.1 | 0.3 | 0.7 | 3.3 |
| | QD-AX | PIM | 5.0 | 12.6 | 3.8 | 1.2 | 1.3 |
| | ZWIX(-)™ | SFC | 2.4 | 5.8 | 1.7 | 0.7 | 1.4 |
| | QD-AX | SFC | 3.1 | 6.7 | 2.1 | 1.1 | 1.5 |
| **Fmoc-Tyr(*t*Bu)-OH** | ZWIX(-)™ | HO | 3.7 | 7.5 | 2.2 | 1.5 | 2.5 |
| | QD-AX | PIM | 6.6 | 16.0 | 4.8 | 1.8 | 1.4 |
| | ZWIX(-)™ | SFC | 2.9 | 7.3 | 2.2 | 0.7 | 1.3 |
| | QD-AX | SFC | 5.7 | 8.1 | 2.4 | 3.3 | 2.4 |

*Chromatographic conditions: column, ZWIX(-)™ and QD-AX; mobile phase in LC mode, $H_2O$/MeOH (1/99 v/v) containing 3.75 mM TEA and 7.5 mM FA and MeOH/MeCN (75/25 v/v) containing 30 mM TEA and 60 mM FA, in SFC mode $CO_2$/MeOH (70/30 v/v) containing 30 mM TEA and 60 mM FA; and $CO_2$/MeOH (60/40 v/v) containing 30 mM TEA and 60 mM FA; flow rate, 0.6 mL/min or 2.0 mL/min (SFC); detection, 262 nm; $Q = \Delta(\Delta H°) / 298 \times \Delta(\Delta S°)$*

### *4.8 Determination of elution sequences on Cinchona alkaloid-based zwitterionic and anion-exchanger type CSPs*

In chromatography, it is important to determine the elution sequence of enantiomers, in particular, to reveal impurity profile and enantiomer excess. *Cinchona* alkaloids (*QN* and *QD*) and *trans*-2-aminocyclohexanesulfonic acid-based chiral SOs and CSPs behave as pseudoenantiomeric CSPs; actually, they are like diastereomeres. Therefore, switching from ZWIX(+)™ to ZWIX(-)™ or from QN-AX to QD-AX, the sequence of the enantiomers of the SAs might be reveresed (***Figure 19***). The molecular part of the anion-exchanger site of the ZWIX(+)™ selector is based on *QN*, while the cation-exchange site is based on (1*S*,2*S*)-cyclohexyl-1-amino-2-sulfonic acid, and the two charged molecular moieties are bridged via

the carbamoyl group. According to the pseudo-enantiomer concept outlined above, the ZWIX (-)™ selector is composed of *QD* and (1*R*,2*R*)-cyclohexyl-1-amino-2-sulfonic acid.

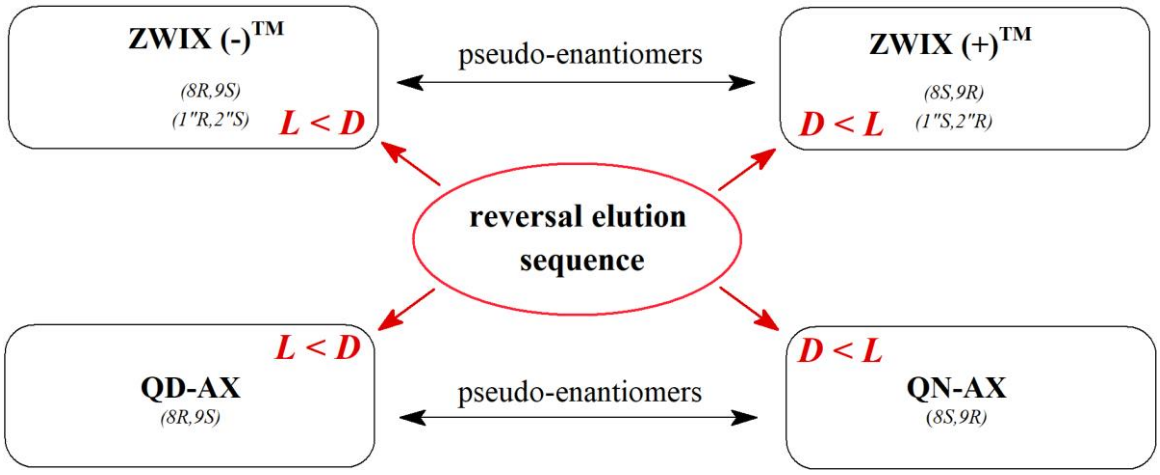



***Figure 19.*** *The relationship between the elution sequence and the structure of selectors*

Elution sequences were determined in all cases. On ZWIX(+)™ and QN-AX, the elution order was *D* < *L* (Fmoc-Pro-OH was not separable on ZWIX(+)™), while on ZWIX(-)™ and QD-AX, a reversed elution order *L* < *D* was obtained. Exceptions were Fmoc-Cys(Trt)-OH and Fmoc-Asn-OH on QD-AX CSP, where the elution order was *D* < *L*.

It seems that the configuration of carbon atoms C-8 and C-9 in the core of *quinine* and *quinidine* determine the elution sequence. If the configuration of C-8 and C-9 is 8*S*,9*R*, the elution sequence is *D* < *L*, while the 8*R*,9*S* configuration gives the elution sequence *L* < *D*. In any other combinations, 8*S*,9*R* and 8*R*,9*S* resulted in a reversal of the elution sequence, *i.e.* selectors indeed work like pseudo-enantiomers.

### *4.9 Analysis of minor components in the presence of major one on Cinchona alkaloid-based zwitterionic CSPs*

The enantiomeric purity of $N^{\alpha}$-Fmoc-protected amino acids is crucial from the viewpoint of peptide synthesis. The enantiomeric excess is a measurement used in chemistry to characterize the composition of enantiomeric mixtures, that is it shows the purity of a substance. The determination of the enantiomeric excess (*ee* values) of the starting free amino acids and of the *N*-protected derivatives is carried out mainly with gas and liquid chromatographic methods. However, various modalities are available for the enantioseparation of free and *N*-

protected proteinogenic and unusual amino acids and results have been summarized in numerous review articles.

A fast and sensitive chromatographic protocol was developed for the identification and quantitation of enantiomeric impurities of commercially available $N^{\alpha}$-Fmoc-protected amino acids on *Cinchona* alkaloid *QN*- and *QD*-based zwitterionic stationary phases. Selected chromatograms collected in ***Figure 20*** exhibit examples for the enantioseparation of $N^{\alpha}$-Fmoc proteinogenic amino acids containing 0.1% chiral impurity in the presence of the major one. To quantify the amount of enantiomeric impurities, analyses were performed for the five selected analytes. Measurements were carried out in SFC modality with a mobile liquid phase of $CO_2$/MeOH (70/30 *v/v*) containing 30 mM TEA and 60 mM FA. Determination of the minor D-enantiomer component was performed on ZWIX(+)™, whereas ZWIX(-)™ CSP was applied for the minor L-enantiomer. ***Figure 20*** depicts the chromatograms and peak areas of the minor components of the selected Fmoc amino acids. The concentration level of minor component, D-amino acids (on ZWIX(+)™) or L-amino acids (on ZWIX(-)™) was 10.0 *μg/mL* (with 7 μL injected volume, *70 ng, ca. 0.1–0.2* nmol). Chromatograms demonstrate that 0.1% of the minor enntiomer can be detected in the presence of the major one. One year later, on the basis of these results, the same research group successfully determined 0.01% of the minor component in the presence of the major one under LC conditions applying anion-exchanger CSP QN-AX for determination of the D-enantiomer and QD-AX for determination of the L-enantiomer *[88]*. The determination levels (LOQ values) ranged between 3.0–5.4 pmol.

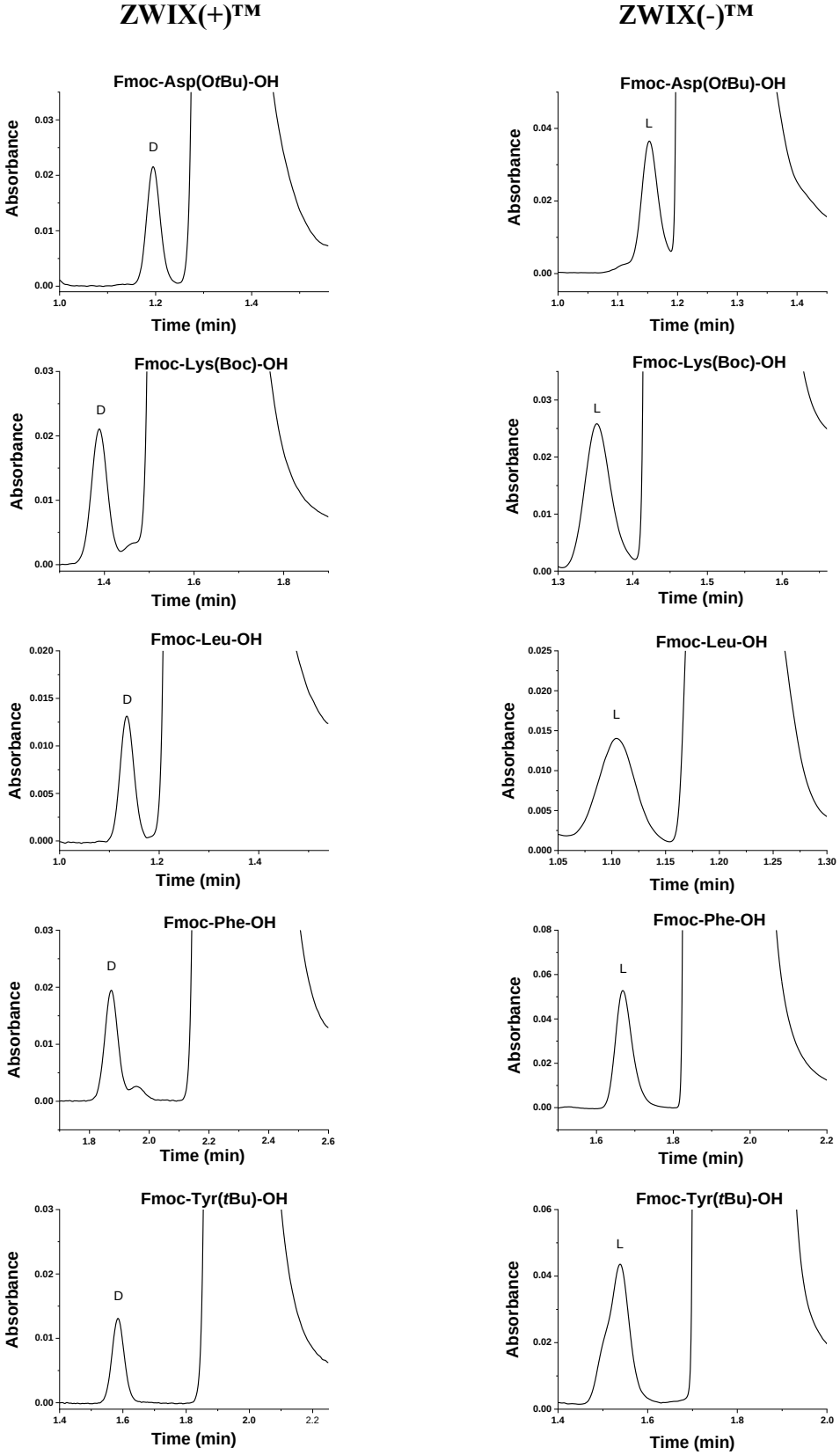
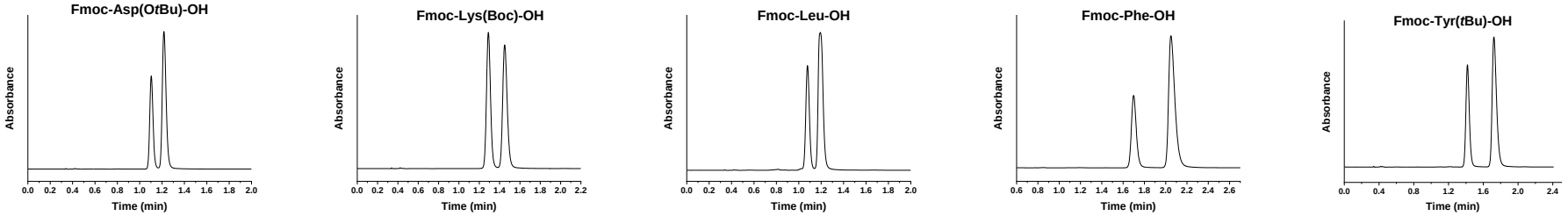



***Figure 20.*** *Chromatograms of selected SAs when present in an excess of the major isomer*
*Chromatographic conditions: column, ZWIX(+)™ or ZWIX(−)™; mobile phase,$CO_2$/MeOH (70/30 v/v) containing 30 mM TEA and 60 mM FA; flow rate, 2.0 mL/min; detection, 262 nm; Tcol, 40 °C; back pressure, 150 bar; concentration of minor component,10.0 μg/mL*

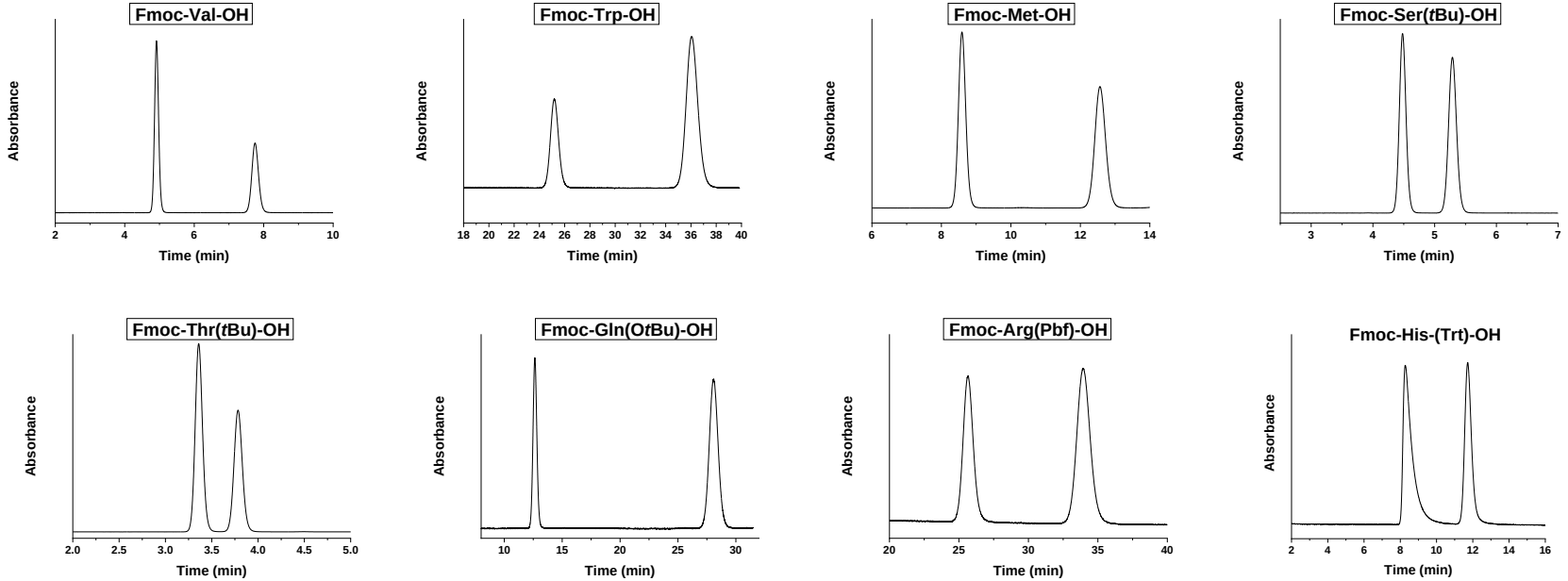
### *4.10 Selected Chromatograms*

Selected chromatograms of $N^{\alpha}$-Fmoc proteinogenic amino acids on ZWIX(+)™ and QN-AX column under SFC and LC conditions are collected in Figure 21.

**SFC condition:** **ZWIX(+)™**




**SFC condition:** **QN-AX**



**LC condition:** **ZWIX(+)™**

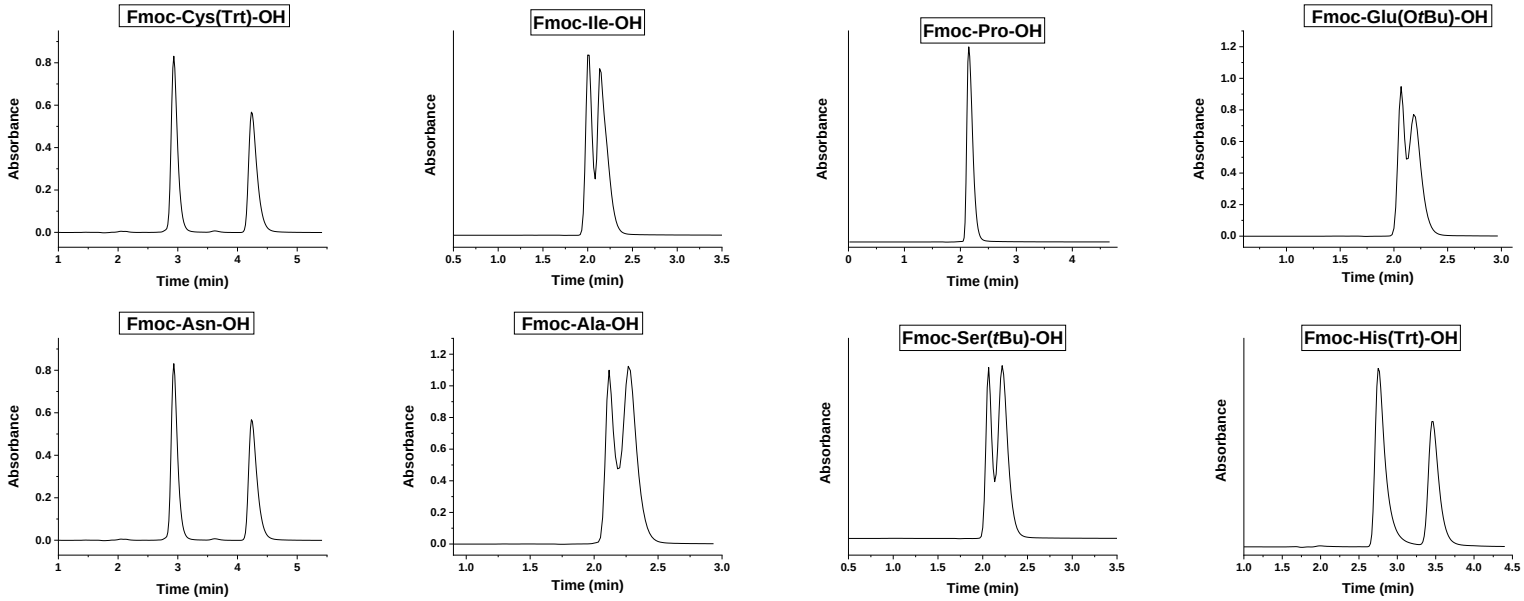



***Figure 21.*** *Selected chromatograms of $N^{\alpha}$-Fmoc proteinogenic amino acids*

*Chromatographic conditions: column, ZWIX(+)™ and QN-AX; mobile phase in LC mode, $H_2O$/MeOH (1/99 v/v) containing 30 mM TEA and 60 mM FA mobile phase in SFC mode, $CO_2$/MeOH (70/30 v/v) containing 30 mM TEA and 60 mM FA on ZWIX(+)™ and $CO_2$/MeOH (60/40 v/v) containing 30 mM TEA and 60 mM FA on QN-AX; flow rate, 2.0 mL/min; detection, 262 nm; Tcol, 40 °C; back pressure, 150 bar*

# 5 Summary

In my research work, methods were developed utilizing zwitterionic and anion-exchanger type chiral CSPs based on *Cinchona* alkaloids for the separation of $N^{\alpha}$-Fmoc proteinogenic amino acid enantiomers in LC and SFC mode. In the course of method optimization, several mobile phase compositions and conditions as well as different temperatures affecting the chromatographic parameters were investigated.

*1) Effect of mobile phase composition*

Experiments were performed at various mobile phase compositions, involving mixtures of MeOH and MeCN in LC mode, and liquid $CO_2$ and MeOH in the SFC mode with constant ionic strength (the acid-to-base ratio was kept at a constant value of 2:1). The presence of a polar solvent had a strong effect on retention, selectivity, and resolution. These values changed (generally decreased) significantly at higher MeOH content in MeCN or $CO_2$ as bulk solvent. With increasing MeOH content in the mobile phase, the polarity of the mobile phase increased promoting the interaction between the mobile phase and SA; therefore, retentions decreased in all cases. It is important to note that under all chromatographic conditions applied, ionic interactions between the SO and SA have a decisive role regarding retention. Enantioselectivity is influenced by additional hydrogen bonding as well as aromatic $\pi$–$\pi$ and van der Waals interactions.

*2) Role of water content of the mobile phase*

The effects of the water content in the mobile phase on the retention, selectivity, and resolution were investigated. The influence of water applied in lower concentrations (≤10 *v*%) in the eluent turned out to be favorable on separation factors. Water in a low percentage is beneficial for peak shape, resolution, analysis time, sample, and solubility performance. The addition of small amounts of water to the polar ionic mobile phase shifts the elution system from a nonaquesous PI mode to a HO mode. A few percentage points of $H_2O$ affect solvation of both SO and SA and might reduce the strength of ionic interactions. In LC mode, in most cases, 1.0–2.0% $H_2O$ content in the eluent was advantageous, yielding better peak shapes and higher resolution. In SFC mode, increasing the water content resulted in slightly decreased rentention times. This can be partially explained by the increase in the formation of counter-ions via the reaction of $CO_2$ and $H_2O$, yielding carbonic acid, which dissociates to hydrogen carbonate and proton. Formed hydrogen carbonate and proton act as additional counter-ions in

anion chromatographic system. Similar to this behaviour, both $\alpha$ and $R_S$ decreased slightly with increasing water content.

*3) Role of nature of base and acid as mobile phase additives*

To investigate the effect of the nature of acid and base additives, separations are generally carried out with constant bulk solvent composition and using an excess of the acid to the base component in the mobile phase ensuring that the base is present in its protonated „ammonium ion" form.To study the effects of acid and base additives, FA and AcOH were selected as acid additives, and EA, DEA, TEA, PA, and BA served as base additives. The amines differed in the degree and nature of their alkyl substitution on the nitrogen atom, while acids FA and AcOH have different strength. The nature of the various acid and base additives in the mobile phase may affect chromatographic parameters and play an important role in the optimization of the enantioseparation on *Cinchona* alkaloid-based CSPs.

*4) Effect of the counter-ion concentration*

Retention can be controlled by the type of the counter-ion, but the concentration of the counter-ion can also affect chromatographic behavior. This means that retention can be greatly influenced by the amounts of co-ions and counter-ions present in the mobile phase. The application of a higher counter-ion concentration should result in lower retention, as the stoichiometric displacement model described. According to this model, linear relationship was found between the logarithm of the retention factor of the first-eluted enantiomer (log $k_1$) and the logarithm of the counter-ion concentration (log $c_{counter\text{-}ion}$). On anion-exchanger QN-AX and QD-AX CSPs, a „single ionic" ion-exchange mechanism was also suggested. Under the studied conditions, linear relationships were found between *log* $k_1$ and *log* $c_{FA}$.

*5) Temperature dependence and thermodynamic parameters*

The influence of temperature on the separation mechanism is complex. Thermodynamic studies are often performed in order to understand the mechanistic aspects of chiral recognition. There are two completely different effects governing the chromatographic performance of CSPs. One of these is the *thermodynamic effect*, and the other is the *kinetic effect*.

For the investigation of the temperature effect, the van't Hoff plots approach was applied. The temperature range varied between 5–40 ºC in LC mode and between 20–50 ºC in SFC mode. The differences of the standard enthalpy and entropy changes were calculated from the ln α vs. 1/*T* curves, where the slope gives $\Delta(\Delta H^o)$ and the intercept gives $\Delta(\Delta S^o)$ values. It

should be noted, that in all cases the selectivity decreased with increasing temperature and both $\Delta(\Delta H^{o})$ and $\Delta(\Delta S^{o})$ exhibited negative values, *i.e.* the enantioseparation was enthalpically driven. The relative contribution of the enthalpic and entropic terms to the free energy of adsorption can be visualized through the enthalpy/entropy ratio $Q$ {$Q = \Delta(\Delta H^{o}) / [298 \times \Delta(\Delta S^{o})]$} calculated at 298 K. A comparison of the $Q$ values for the individual analytes revealed that the enantioselective discrimination was enthalpically driven ($Q > 1.0$) in all cases and, with a few exceptions, the highest $Q$ values were most often obtained on QN-AX CSP.

6) *Determination of elution sequence on different types of Cinchona alkaloids*

*Cinchona* alkaloids (*QN* and *QD*) and *trans*-2-aminocyclohexanesulfonic acid-based chiral SOs and CSPs behave as pseudoenantiomeric CSPs. In fact, they are diastereomers. Elution sequences were determined for all studied analytes and a general rule could be observed: D enantiomers eluted first before the L ones on *QN*-based ZWIX(+)™ and QN-AX CSPs in both LC and SFC modes. On the other hand, on *QD*-based ZWIX(-)™ and QD-AX CSPs, the L enantiomers eluted first before D ones in both chromatographic modes.

*7) Method development for the enantiomeric purity determination of $N^{\alpha}$-Fmoc proteinogenic amino acids*

Determination of the enantiomeric excess (*ee* values) of the starting free amino acids and of the *N*-protected amino acids is highly important in peptide synthesis. Nowadays various methods are available in the literature. The enantiomeric excess used in chemistry is a measurement to characterize the composition of mixtures of enantiomers indicating the enantiomeric purity of a substance. A rapid and sensitive chromatographic method protocol was developed for the identification and quantitation of enantiomeric impurities of commercially available $N^{\alpha}$-Fmoc-protected amino acids on *Cinchona* alkaloid *QN*- and *QD*-based zwitterionic stationary phases. To quantify the amount of enantiomeric impurities, analyses were carried out for the determination of the minor enantiomers in the case of five selected analytes.

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# APPENDIX

## Table S1

Temperature dependence of retention factor of first eluting enantiomer ($k_1$), separation factor ($\alpha$) and resolution ($R_S$) of $N^{\alpha}$-Fmoc-protected proteinogenic amino acids on ZWIX(+)™ and QN-AX CSPs in liquid chromatographic conditions

| *Compound* | *Column* | *Eluent* | $k_1$, $\alpha$, $R_S$ | *Temperature (°C)* | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | **5** | **10** | **20** | **30** | **40** |
| **Fmoc-Asp(O*t*Bu)-OH** | ZWIX(+)™ | **a** | $k_1$ | 0.46 | 0.45 | 0.44 | 0.41 | 0.37 |
| | | | $\alpha$ | 1.50 | 1.40 | 1.32 | 1.22 | 1.14 |
| | | | $R_S$ | 1.30 | 1.04 | 1.04 | 1.08 | 0.91 |
| | ZWIX(-)™ | | $k_1$ | 0.57 | 0.55 | 0.50 | 0.44 | 0.40 |
| | | | $\alpha$ | 1.26 | 1.24 | 1.20 | 1.16 | 1.13 |
| | | | $R_S$ | 0.92 | 0.83 | 0.78 | 0.68 | 0.42 |
| | QN-AX | **b** | $k_1$ | 3.27 | 2.67 | 2.57 | 2.36 | 2.16 |
| | | | $\alpha$ | 2.15 | 2.03 | 1.89 | 1.76 | 1.63 |
| | | | $R_S$ | 11.50 | 11.33 | 10.88 | 10.39 | 9.25 |
| | QD-AX | | $k_1$ | 2.56 | 2.50 | 2.49 | 2.24 | 2.08 |
| | | | $\alpha$ | 2.16 | 2.08 | 1.91 | 1.76 | 1.63 |
| | | | $R_S$ | 10.43 | 9.90 | 7.83 | 8.18 | 7.50 |
| **Fmoc-Lys(Boc)-OH** | ZWIX(+)™ | **a** | $k_1$ | 0.30 | 0.30 | 0.30 | 0.29 | 0.26 |
| | | | $\alpha$ | 1.37 | 1.33 | 1.27 | 1.21 | 1.15 |
| | | | $R_S$ | 1.03 | 0.93 | 0.94 | 0.65 | 0.76 |
| | ZWIX(-)™ | | $k_1$ | 0.44 | 0.42 | 0.37 | 0.28 | 0.25 |
| | | | $\alpha$ | 1.25 | 1.23 | 1.16 | 1.13 | 1.08 |
| | | | $R_S$ | 0.57 | 0.53 | 0.27 | 0.44 | 0.20 |
| | QN-AX | **b** | $k_1$ | 1.85 | 1.69 | 1.48 | 1.35 | 1.25 |
| | | | $\alpha$ | 1.99 | 1.95 | 1.86 | 1.76 | 1.66 |
| | | | $R_S$ | 10.11 | 10.95 | 10.53 | 7.29 | 7.91 |
| | QD-AX | | $k_1$ | 1.39 | 1.38 | 1.35 | 1.33 | 1.28 |
| | | | $\alpha$ | 1.72 | 1.67 | 1.59 | 1.48 | 1.41 |
| | | | $R_S$ | 5.90 | 6.08 | 5.85 | 5.00 | 4.29 |
| **Fmoc-Leu-OH** | ZWIX(+)™ | **a** | $k_1$ | 0.30 | 0.30 | 0.30 | 0.29 | 0.26 |
| | | | $\alpha$ | 1.47 | 1.43 | 1.38 | 1.33 | 1.27 |
| | | | $R_S$ | 0.57 | 0.53 | 0.27 | 0.44 | 0.20 |
| | ZWIX(-)™ | | $k_1$ | 0.29 | 0.29 | 0.28 | 0.26 | 0.24 |
| | | | $\alpha$ | 1.19 | 1.17 | 1.14 | 1.12 | 1.08 |
| | | | $R_S$ | 0.35 | 0.31 | 0.36 | 0.38 | 0.20 |

**Table S1** (*continued*) Temperature dependence of retention factor of first eluting enantiomer ($k_1$), separation factor ($\alpha$) and resolution ($R_S$) of $N^{\alpha}$-Fmoc-protected proteinogenic amino acids on ZWIX(+)™ and QN-AX CSPs in liquid chromatographic conditions

| *Compound* | *Column* | *Eluent* | $k_1$, $\alpha$, $R_S$ | *Temperature (°C)* | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | **5** | **10** | **20** | **30** | **40** |
| **Fmoc-Leu-OH** | QN-AX | **b** | $k_1$ | 2.04 | 1.85 | 1.63 | 1.55 | 1.41 |
| | | | $\alpha$ | 2.06 | 1.98 | 1.88 | 1.77 | 1.68 |
| | | | $R_S$ | 9.33 | 9.25 | 10.00 | 7.62 | 7.57 |
| | QD-AX | | $k_1$ | 1.38 | 1.38 | 1.36 | 1.35 | 1.30 |
| | | | $\alpha$ | 2.38 | 2.29 | 2.10 | 1.89 | 1.75 |
| | | | $R_S$ | 10.00 | 10.24 | 9.67 | 8.95 | 6.59 |
| **Fmoc-Phe-OH** | ZWIX(+)™ | **a** | $k_1$ | 0.61 | 0.59 | 0.55 | 0.53 | 0.49 |
| | | | $\alpha$ | 2.02 | 1.90 | 1.73 | 1.55 | 1.43 |
| | | | $R_S$ | 3.15 | 3.06 | 2.62 | 1.75 | 2.05 |
| | ZWIX(-)™ | | $k_1$ | 0.74 | 0.72 | 0.66 | 0.59 | 0.52 |
| | | | $\alpha$ | 1.35 | 1.35 | 1.32 | 1.31 | 1.29 |
| | | | $R_S$ | 1.75 | 1.76 | 1.67 | 1.49 | 1.28 |
| | QN-AX | **b** | $k_1$ | 5.03 | 3.54 | 3.44 | 3.20 | 2.88 |
| | | | $\alpha$ | 1.75 | 1.72 | 1.60 | 1.53 | 1.45 |
| | | | $R_S$ | 8.76 | 9.29 | 8.85 | 8.16 | 7.33 |
| | QD-AX | | $k_1$ | 3.50 | 3.43 | 3.22 | 3.05 | 2.70 |
| | | | $\alpha$ | 1.94 | 1.87 | 1.74 | 1.63 | 1.53 |
| | | | $R_S$ | 9.53 | 9.43 | 8.62 | 8.33 | 6.71 |
| **Fmoc-Tyr(*t*Bu)-OH** | ZWIX(+)™ | **a** | $k_1$ | 0.77 | 0.74 | 0.66 | 0.59 | 0.57 |
| | | | $\alpha$ | 1.47 | 1.38 | 1.26 | 1.12 | 1.02 |
| | | | $R_S$ | 1.49 | 1.21 | 1.06 | 0.20 | 0.20 |
| | ZWIX(-)™ | | $k_1$ | 0.65 | 0.64 | 0.61 | 0.52 | 0.44 |
| | | | $\alpha$ | 1.98 | 1.91 | 1.82 | 1.73 | 1.66 |
| | | | $R_S$ | 4.00 | 3.92 | 3.36 | 3.79 | 3.30 |
| | QN-AX | **b** | $k_1$ | 5.33 | 4.21 | 4.06 | 3.68 | 3.23 |
| | | | $\alpha$ | 1.27 | 1.25 | 1.20 | 1.16 | 1.12 |
| | | | $R_S$ | 3.87 | 3.58 | 3.31 | 2.75 | 2.18 |
| | QD-AX | | $k_1$ | 2.94 | 2.89 | 2.72 | 2.57 | 2.36 |
| | | | $\alpha$ | 2.54 | 2.42 | 2.23 | 2.04 | 1.83 |
| | | | $R_S$ | 17.65 | 12.74 | 11.67 | 10.00 | 9.29 |

Chromatographic conditions: column, ZWIX(+)™, ZWIX(-)™, QD-AX and QN-AX; mobile phase, **a**, $H_2O$/MeOH (1/99 *v/v*) containing 3.75 mM TEA and 7.5 mM FA, **b**, MeOH/MeCN (75/25 *v/v*) containing 30 mM TEA and 60 mM FA; flow rate, 0.6 mL/min; detection, 262 nm

**Table S2**
Temperature dependence of retention factor of first eluting enantiomer ($k_1$), separation factor ($\alpha$) and resolution ($R_S$) of $N^{\alpha}$-Fmoc-protected proteinogenic amino acids on ZWIX(-)™ and QD-AX CSPs in supercritical fluid chromatographic conditions

| *Compound* | *Column* | *Eluent* | $k_1$, $\alpha$, $R_S$ | *Temperature (°C)* | | | |
|---|---|---|---|---|---|---|---|
| | | | | **20** | **30** | **40** | **50** |
| **Fmoc-Asp(O*t*Bu)-OH** | ZWIX(+)™ | **a** | $k_1$ | 1.74 | 1.75 | 1.76 | 1.77 |
| | | | $\alpha$ | 1.21 | 1.19 | 1.18 | 1.17 |
| | | | $R_S$ | 2.54 | 2.59 | 2.43 | 2.26 |
| | ZWIX(-)™ | | $k_1$ | 1.64 | 1.63 | 1.63 | 1.62 |
| | | | $\alpha$ | 1.18 | 1.16 | 1.15 | 1.13 |
| | | | $R_S$ | 1.84 | 1.87 | 1.88 | 1.68 |
| | QN-AX | **b** | $k_1$ | 5.83 | 5.41 | 5.06 | 4.68 |
| | | | $\alpha$ | 1.72 | 1.67 | 1.59 | 1.50 |
| | | | $R_S$ | 8.61 | 8.59 | 8.03 | 7.36 |
| | QD-AX | | $k_1$ | 5.11 | 4.82 | 4.48 | 4.20 |
| | | | $\alpha$ | 1.74 | 1.68 | 1.60 | 1.50 |
| | | | $R_S$ | 8.76 | 8.48 | 8.01 | 7.33 |
| **Fmoc-Lys(Boc)-OH** | ZWIX(+)™ | **a** | $k_1$ | 2.17 | 2.20 | 2.22 | 2.26 |
| | | | $\alpha$ | 1.25 | 1.23 | 1.21 | 1.19 |
| | | | $R_S$ | 2.82 | 2.83 | 2.63 | 2.51 |
| | ZWIX(-)™ | | $k_1$ | 2.04 | 2.05 | 2.07 | 2.09 |
| | | | $\alpha$ | 1.23 | 1.21 | 1.18 | 1.14 |
| | | | $R_S$ | 2.01 | 2.12 | 2.04 | 1.81 |
| | QN-AX | **b** | $k_1$ | 5.65 | 5.35 | 5.06 | 4.78 |
| | | | $\alpha$ | 1.59 | 1.57 | 1.51 | 1.49 |
| | | | $R_S$ | 7.17 | 7.19 | 6.83 | 6.31 |
| | QD-AX | | $k_1$ | 5.07 | 4.69 | 4.48 | 4.22 |
| | | | $\alpha$ | 1.50 | 1.47 | 1.42 | 1.35 |
| | | | $R_S$ | 6.54 | 6.16 | 5.74 | 5.25 |
| **Fmoc-Leu-OH** | ZWIX(+)™ | **a** | $k_1$ | 1.65 | 1.64 | 1.62 | 1.60 |
| | | | $\alpha$ | 1.23 | 1.22 | 1.20 | 1.19 |
| | | | $R_S$ | 2.48 | 2.56 | 2.11 | 2.25 |
| | ZWIX(-)™ | | $k_1$ | 1.56 | 1.54 | 1.52 | 1.49 |
| | | | $\alpha$ | 1.22 | 1.20 | 1.18 | 1.15 |
| | | | $R_S$ | 1.76 | 1.87 | 1.68 | 1.61 |

**Table S2** (*continued*) Temperature dependence of retention factor of first eluting enantiomer ($k_1$), separation factor ($\alpha$) and resolution ($R_S$) of $N^{\alpha}$-Fmoc-protected proteinogenic amino acids on ZWIX (-)$^{TM}$ and QD-AX CSPs in supercritical fluid chromatographic conditions

| *Compound* | *Column* | *Eluent* | $k_1$, $\alpha$, $R_S$ | *Temperature (°C)* | | | |
|---|---|---|---|---|---|---|---|
| | | | | **20** | **30** | **40** | **50** |
| **Fmoc-Leu-OH** | QN-AX | **b** | $k_1$ | 5.51 | 5.07 | 4.68 | 4.14 |
| | | | $\alpha$ | 1.57 | 1.55 | 1.54 | 1.53 |
| | | | $R_S$ | 8.99 | 9.21 | 8.76 | 8.02 |
| | QD-AX | | $k_1$ | 4.05 | 3.75 | 3.52 | 3.29 |
| | | | $\alpha$ | 1.81 | 1.77 | 1.61 | 1.56 |
| | | | $R_S$ | 9.88 | 9.21 | 8.63 | 7.83 |
| **Fmoc-Phe-OH** | ZWIX(+)$^{TM}$ | **a** | $k_1$ | 3.39 | 3.37 | 3.35 | 3.32 |
| | | | $\alpha$ | 1.37 | 1.34 | 1.31 | 1.27 |
| | | | $R_S$ | 4.83 | 4.83 | 4.53 | 4.26 |
| | ZWIX(-)$^{TM}$ | | $k_1$ | 3.17 | 3.13 | 3.08 | 3.01 |
| | | | $\alpha$ | 1.34 | 1.30 | 1.27 | 1.23 |
| | | | $R_S$ | 3.64 | 3.82 | 3.96 | 3.43 |
| | QN-AX | **b** | $k_1$ | 12.07 | 11.12 | 10.16 | 9.03 |
| | | | $\alpha$ | 1.39 | 1.37 | 1.33 | 1.29 |
| | | | $R_S$ | 5.99 | 5.76 | 5.58 | 5.19 |
| | QD-AX | | $k_1$ | 10.19 | 9.54 | 8.63 | 7.72 |
| | | | $\alpha$ | 1.56 | 1.50 | 1.45 | 1.39 |
| | | | $R_S$ | 7.95 | 7.39 | 6.92 | 6.43 |
| **Fmoc-Tyr(*t*Bu)-OH** | ZWIX(+)$^{TM}$ | **a** | $k_1$ | 2.64 | 2.65 | 2.67 | 2.68 |
| | | | $\alpha$ | 1.41 | 1.37 | 1.33 | 1.28 |
| | | | $R_S$ | 4.68 | 4.72 | 4.39 | 4.10 |
| | ZWIX(-)$^{TM}$ | | $k_1$ | 2.44 | 2.47 | 2.50 | 2.52 |
| | | | $\alpha$ | 1.35 | 1.31 | 1.27 | 1.21 |
| | | | $R_S$ | 3.39 | 3.54 | 3.43 | 3.08 |
| | QN-AX | **b** | $k_1$ | 13.32 | 12.22 | 10.72 | 9.31 |
| | | | $\alpha$ | 1.92 | 1.91 | 1.90 | 1.89 |
| | | | $R_S$ | 10.98 | 11.25 | 11.88 | 12.32 |
| | QD-AX | | $k_1$ | 7.59 | 7.27 | 6.73 | 6.36 |
| | | | $\alpha$ | 3.86 | 3.60 | 3.43 | 3.08 |
| | | | $R_S$ | 20.37 | 19.95 | 20.08 | 20.28 |

Chromatographic conditions: column, ZWIX(+)$^{TM}$, ZWIX(-)$^{TM}$, QD-AX and QN-AX; mobile phase, **a**, $CO_2$/MeOH (70/30 *v/v*) containing 30 mM TEA and 60 mM FA, **b**, $CO_2$/MeOH (60/40 *v/v*) containing 30 mM TEA and 60 mM FA; 2 ml/min; detection, 262 nm; back pressure, 150 bar