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Faculty of Pharmacy

Institute of Pharmaceutical Chemistry

Investigation of Novel *Cinchona* Alkaloid-based Zwitterionic Chiral Stationary Phases in Cation- and Zwitterion-Exchange Modes

Ph.D. Thesis

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Contents

List of publications and lectures

Papers related to the thesis

I. Ilisz, **N. Grecsó**, A. Aranyi, P. Suchotin, D. Tymecka, B. Wilenska, A. Misicka, F. Fülöp, W. Lindner, A. Péter, Enantioseparation of *β*2-amino acids on cinchona alkaloid-based zwitterionic chiral stationary phases. Structural and temperature effects, Journal of Chromatography A 1334 (2014) 44-54. **if: 3.924**

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III. I. Ilisz, **N. Grecsó**, F. Fülöp, W. Lindner, A. Péter, High-performance liquid chromatographic enantioseparation of cationic 1,2,3,4-tetrahydroisoquinoline analogs on Cinchona alkaloid-based zwitterionic chiral stationary phases, Analytical and Bioanalytical Chemistry 407 (2015) 961-972. **if: 3.125**

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I. A. Aranyi, I. Ilisz, **N. Grecsó**, A. Misicka, D. Tymecka, S. Wernisch, W. Lindner, A. Péter, ß2-aminosav enantiomerek folyadékkromatográfiás elválasztása új, zwitterion típusú királis állófázisokon, Vegyészkonferencia 26-28. April 2013. (poster)

II. **N. Grecsó**, β2-Aminosavak nagyhatékonyságú folyadékkromatográfiás elválasz-tása új, zwitterion típusú királis állófázisokon, XXXVI. Kémiai Előadói Napok, 28-30 October 2013. (oral presentation)

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V. **N. Grecsó**, F. Fülöp, A. Péter, I. Ilisz, Ioncserélő királis állófázisok tanulmányozása szelektív szerotoninújrafelvétel-gátló antidepresszáns modellvegyület alkalmazásával, Elválasztástudományi Vándorgyűlés 2016. Kecskemét, Magyarország, 9-11 November 2016. (poster)

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VI. **N. Grecsó**, Z. Gecse, M. Nonn, F. Fülöp, A. Péter, Izoxazolin gyűrűvel kondenzált 2-amino-ciklopentán karbonsav analógok sztereoizomrjeinek elválasztása ligandumcserés királis állófázison, Szeged, XXXV. Kémiai Előadói Napok, 29-31 October 2012. (oral presentation)

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XVI. I. Ilisz, **N. Grecsó**, F. Fülöp, I. Szatmári, A. Péter, Capillary electrophoretic enantioseparation of unusual amino acids and aminonaphthol analogues, Olomouc, 10-14 February 2014. (poster)

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XXIII. **N. Grecsó**, F. Fülöp, A. Péter, I. Ilisz, Ioncserélő királis állófázisok tanulmányozása szelektív szerotoninújrafelvétel-gátló antidepresszáns modellvegyület alkalmazásával, Elválasztástudományi Vándorgyűlés 2016. Kecskemét, 9-11 November 2016. (poster)

Abbreviations and symbols

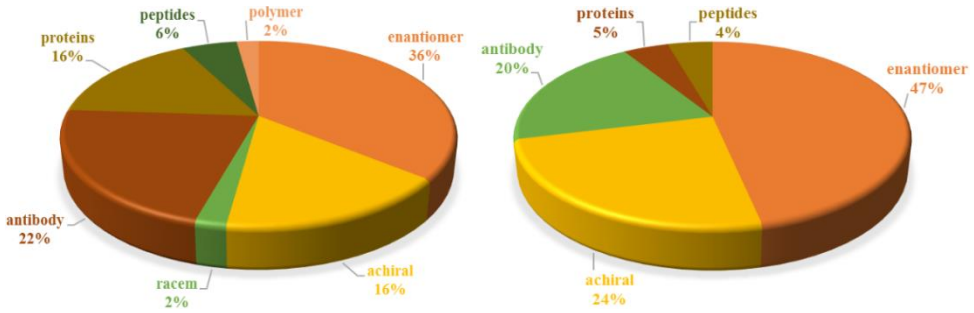
(*v*/*v*)	volume to volume ratio
ACHSA	*trans*-2-aminocyclohexanesulfonic acid
AcOH	glacial acetic acid
BA	butylamine
CDA	chiral derivatizing agents
CMPA	chiral mobile phase additive
CSP	chiral stationary phase
DEA	diethylamine
EA	ethylamine
FA	formic acid
H_2O	Milli-Q water
HO	hydro-organic
HPLC	high-performance liquid chromatography
MeCN	acetonitrile
MeOH	methanol
NP	normal-phase
PA	propylamine
PI	polar-ionic
QD	quinidine
QN	quinine
RP	reversed-phase
SA	selectand
SO	selector
TBA	tributylamine
TEA	triethylamine
TEAA	triethylammonium acetate
TFA	trifluoracetic acid
THF	tetrahydrofuran
TIQ	1,2,3,4-tetrahydroisoquinoline
TLC	thin layer chromatography
TPA	tripropylamine
ZWIX	zwitterionic chiral stationary phase

k	retention factor; defined as $\frac{(t_R - t_0)}{t_0}$; t_R, retention time; t_0, column dead-time
α	selectivity; defined as $\frac{k_2}{k_1}$; 1: first- and 2: second-eluting peak
R_S	resolution; defined as $\frac{(t_{R2} - t_{R1})}{(w_1 + w_2)} * 2$; w, peak width measured on the baseline

1. Introduction

1.1. The importance of chirality and the enantiomer separation

Chirality and the associated phenomena have a great interest in biology, medicine, pharmaceutical industry, food and agricultural industry. In 2015, small-molecule chiral pharmaceuticals comprised about 38% of the sales of top 100 global pharmaceutical products (***Figure 1a.***) and these biggest selling pharmaceutical products generated combined sales of $265 billion[1]. The distribution in percentage of the 45 novel innovative drugs have approved by FDA's Center for Drug Evaluation and Research, are depicted on ***Figure 1b***[2].



a) **b**)

Figure 1. *Distribution in percentage of* ***a****) the 100 best selling drugs in 2015 and* ***b****) the approved novel drugs in 2015*

The important purpose of the modern analytical chemistry is the separation and identification of chiral compounds. In living organisms the chiral bioorganic molecules, such as amino acids, enzymes, nucleic acids, sugars, proteins *etc*. have a great interest. The importance of chirality in the modern pharmaceutical industry has been demonstrated in several papers[3, 4]. In the living system the enatiomers of a racemic drug are essentially involved in different biological processes, like absorption, secretion, metabolism, allosteric control, protein binding, receptor-ligand interactions and others[5-8]. One of the enantiomers (eutomer) often possesses the optimum therapeutic effect, while the other isomer (distomer) may be inactive, or in worse cases toxic. An example ethambutol, whose active form is *D*-ethambutol is antituberculosis drug, while *L*-ethambutol has been found to cause blindness[9]. An other example Prozac, whose active substance is fluoxetine, a racemic mixture of the (*R*)- and (*S*)-enantiomers and have equivalent pharmacological activity[10, 11]. A negative example is Thalidomide, which was marketed in racemic form in the 1960s. Its teratogenic effect was discovered only after several thousands of infants born with malformed, deformed eyes and hearts, deformed alimentary and urinary tracts, blindness and deafness[12]. This tragedy definitely contributed to change the attitude of the

modern pharmaceutical industry and strict guide-lines were issued by the Food and Drug Administration (FDA) followed by the Japanese and Chinese authorities and the European Medicine Agency (EMA). The guide-lines can be summarized in two main points:

I. „Appropriate manufacturing and control procedures should be used to assure stereoisomeric composition of a product with respect to identity, strength, quality and purity. Manufacturers should notify compendia of these specifications and tests."

II. „Pharmacokinetic evaluations that do not use a chiral assay will be misleading if the disposition of the enantiomers is different. Therefore, techniques to quantify individual stereoisomers in pharmacokinetic samples should be available early. If the pharmacokinetics of the enantiomers are demonstrated to be the same or to exist as a fixed-ratio in the target population, an achiral assay or an assay that monitors one of the enantiomers may be used, subsequently."[13]

From the 1970s chirality has become of special importance in the drug safety[14, 15]. Several methods were developed to produce pure enantiomers such as asymmetric synthesis[16], crystallization, enantioselective biotransformation[17, 18], nonstereoselective chromatography or distillation. On the other hand, nowadays the enantiomerically pure substances have yielded by modern technologies involving biosensors, membranes, electrophoretic and chromatographic methods (HPLC and GC)[19-21]. To control the chiral purity of starting materials and products, well reproducible, reliable, accurate, highly sensitive and stereoselective analytical methods are required in the industrial and pharmaceutical research.

1.2. The aims of this work

The primary aim of this work was to develop chiral HPLC methods for the separation of the enantiomers of racemic *β*-amino acids and secondary amines on *cinchona* alkaloid-based zwitterionic chiral stationary phases.

Investigated compounds were:

- aliphatic and aromatic *β*-2- and *β*-3-amino acid enantiomers,
- various 1,2,3,4-tetrahydroisoquinoline enantiomers,
- *trans*-paroxetine enantiomers.

The influence of the nature and concentration of bulk solvent components, the nature and concentration of base and acid additives, the structure of analytes, and the temperature on

chromatographic parameters were investigated applying *cinchona* alkaloid-based chiral stationary phases (CSPs). From temperature dependence studies thermodynamic parameters were calculated. In all cases, the sequence of elution was determined.

2. Literature Review

2.1. Chiral chromatography

It is well known that the physical and chemical properties of the *R* and *S* forms of amino acids and other chiral compounds are identical, and their discrimination requires chiral environment. In chiral chromatography, two main strategies have been applied: an indirect and a direct approach. The reaction of enantiomers (selectands, SAs) with enantiomerically pure reagent (chiral derivatizing agents, CDAs) form diastereomeric derivatives that can be separated on achiral columns is called an indirect approach. Numerous CDAs are commercially available and have comparatively wide alternatives of chromatographic conditions. The classical CDAs are based on chloroformates, isocyanates, isothiocyanates, activated carboxylic acids and their derivatives, *N*-haloarylamino acid derivatives and *o*-phthalaldehyde with chiral thiol[22]. A critical requisite of the indirect method is that the analyte should possess a selectively derivatizable functional group, such as amino, hydroxyl, phenolic, carboxylic, thiol or carbonyl moiety. The derivatizing agent must be chemically and enantiomerically pure, and chemically and stereochemically stable. In addition, the reaction should be fast in order to avoid potential kinetic resolution.

The second way and one of the best methods to achieve enantioseparation is the direct chromatographic approach, which involves two methods. One of them is the chiral mobile phase additive (CMPA) mode or simply „additive mode": a chiral compound is added to the mobile phase in an appropriate concentration. In this case the separation of diastereomeric complex can be achieved on an achiral stationary phase (with a normal (NP)- or a reversed-phase (RP) column). Enantiomer separation with a CSP is nowadays the most straightforward and convenient, widely used mode. Generally, chiral selectors (SO) are chemically (covalently) linked or alternatively physically adsorbed onto spherical porous silica particles.

2.2. The chiral stationary phases

Understanding the chiral recognition mechanisms and the retention of the diastereomers is extremely important for many fields, like drug discovery, proteins and biomimetic receptor design or the development of CSPs. The most widely accepted and the

most reliable theory for the chiral recognition process is known as the „three-point" rule (***Figure 2.***), which was elaborated by *Easson and Stedman*[23] in 1933. This work inspired *Dalgliesh*[24] to investigate chromatographic enantiomer separation by thin layer chromatography (TLC). According to this model, it is absolutely necessary to have at least three attractive interaction sites between the SA and SO. In 1979 *Pirkle* and *Pochapsky*[25] revised and modified Dalgliesh's model: at least one of these three interactions must be stereospecific.

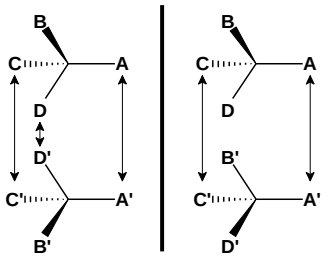


Figure 2*. The „three-point" rule*

Effective interaction (ideal fit) can be described as:

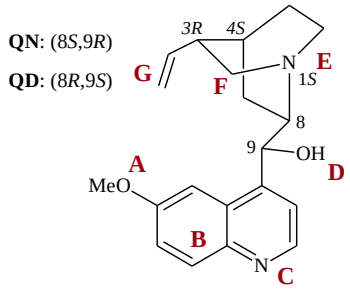
- *steric fit* – size and shape complementary, the SA sterically fits to the binding site of the SO (pocket or cleft),
- *functional fit* – a favorable geometric and spatial orientation of complementary functional groups, so that attractive noncovalent intermolecular interactions can become active. Through these interactions the SA and the SO enantiomers associate, they include: H–bonding, ionic, dipole-dipole, π–π, π–cation and π–anion interactions, *etc*,
- *hydrophobic fit* – hydrophobic regions of the SA and the SO match spatially each other so enable binding by hydrophobic interactions (only in aqueous or hydro-organic eluents),
- *dynamic fit* and *induced fit* – in the course of complexation, the flexibility and conformational change of SA and SO molecules may further enhance the complex stability.

Table 1. *The CSPs fall into the following classes:*

Stationary phases	***Selector***	***Main interactions***
protein-based	proteins or enzymes[26, 27]	ionic and hydrophobic
ligand-exchange (Davankov)	complex formed of amino acid and metal ion (Cu^{2+}, Zn^{2+}, Ni^{2+})[28, 29]	complex formation
Pirkle- or „brush-type”	π-electron acceptor	π-π-, dipole-dipole interactions, H-bonding and steric effects
	π-electron donor	
	π-electron acceptor/π-electron donor[30]	
	anion-[31], cation-[32] zwitterionic-exchange[33]	ionic interaction
polysaccharide-based	cellulose[34, 35]	π-π, dipole-dipole, H-bonding and steric effects
	amylose[36, 37]	
macrocyclic antibiotic-based	macrocyclic glycopeptide[38, 39]	ionic, H-bonding, hydrophobic, dipole-dipole, π-π-interaction, steric effects
inclusion complexes	modified cyclodextrin[40, 41]	complexation, π-π, ionic interaction and steric effects
	crown ethers[42, 43]	
CSPs derived from synthetic polymers	polymers including vinyl, aldehyde, isocyanide, and acetylene polymers, condensation polymers including polyamides and polyurethanes, or cross-linked gels[44, 45]	steric effects
molecularly imprinted polymers	synthetic antibodies, enzymes, nucleic acid, hormones or glycoproteins [46, 47]	steric effects

2.2.1. Cinchona alkaloid-based CSPs

Chiral ion exchangers with low-molecular weight selectors covalently immobilized on spherical silica support were recently marketed. The first silica-supported CSP with *cinchona* alkaloid (***Figure 3.***), which was extracted from the bark of *Cinchona ladgeriana,* was applied in the 1980s for enantiomer separation by *Rosini et.al*.[48]. These CSPs are based on *quinine* (QN) and its pseudo-enantiomer, *quinidine* (QD) as starting materials, which are cheap natural sources. The native QN and QD were immobilized via a spacer at the vinyl group of quinuclidine ring.



A	**Methoxy group**	*polar*
B	**Quinoline**	*aromatic heterocycle*
C	**Amine**	$pK_a = 9.28 \pm 0.70$
D	**Hydroxy group**	
E	**Amine**	$pK_a = 12.80 \pm 0.20$
F	**Quinuclidine**	
G	**Vinyl group**	*used for immobilization*

Figure 3. *Structure of cinchona alkaloids*

Cinchona alkaloids have unique combination of characteristics of structural features. Due to the combination of so many 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 π-π 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 system (**F**) containing a basic nitrogen atom (**E**) in a protonated form can be involved in electrostatic interactions[49]. The structure of QN and QD consists of a planar quinoline and a rigid quinuclidine ring, which are connected by a secondary methyl alcohol bridge. Moreover, QN and QD have five stereogenic centers, which may provide an excellent basis for effective chiral recognition. The difference lies only in the absolute configuration of C-8 and C-9. Although they are actually diastereomers, QN and QD in chromatographic systems behave like enantiomers, so they are called „pseudo-enantiomers". In most cases the stereoselectivity is under C-8 and C-9 control[50].

Native *cinchona* alkaloid-based CSPs were derivatized or esterified at C-9-hydroxyl moiety by various groups, but these CSPs suffered from low enantioselectivities and limited stability[51-53]. In the 1990s, the secondary hydroxyl group at C-9 were modified with *tert*-butyl-carbamoyl moiety (***Figure 4.***). This newly created H-bonding site of the carbamate modification significantly enhanced the enantiorecognition capabilities of the weak anion exchange-type CSPs[50, 54].

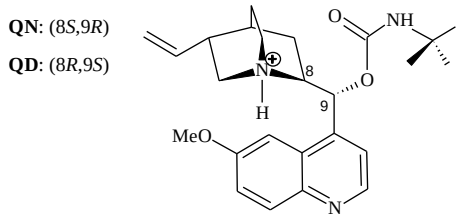


Figure 4. *Structure of the modified cinchona alkaloid*

These new chiral SOs are classified as anion-exchanger CSPs, due to the presence of the basic nitrogen group of the quinuclidine ring ($pK_a \approx 9$).

Cinchona alkaloid-based anion- and aminosulfonic acid-based cation-exchange moieties have been fused in a combinatorial synthesis approach into a new zwitterionic chiral selector as Chiralpak ZWIX(+)™, ZWIX(–)™, ZWIX(+A) and ZWIX(–A). ***Figure 5.*** illustrates the concept of the fusion of anion- and cation-exchange selectors.

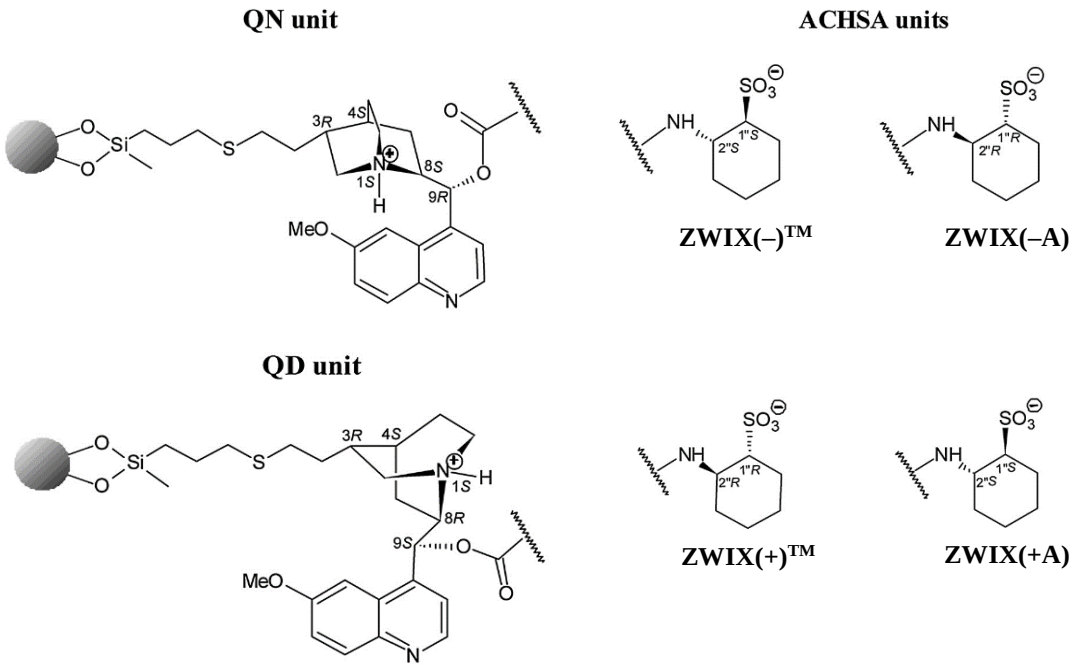


Figure 5. *The structure of cinchona alkaloid-based zwitterionic CSPs*

The main, dominant molecular interaction between the SOs and SAs is double ion pairing. The protonated amine and the dissociated acid of the zwitterionic SA are recognized simultaneously by both charged sites of the zwitterionic SO. The intramolecular counter-ion effect leads to short retention times and facilitates elution of the charged SAs in mobile phase with low amounts of base and acidic modifiers in the mobile phase. The simultaneous double ion pairing process can be supported by additional interactions such as H-bonding and polar, steric (attraction or repulsion), π–π, dipole-dipole and other van der Waals interactions, which can lead to chiral discrimination of the analyte[55]. ***Figure 6.*** depicts the most important interactions between the SAs and zwitterionic SOs.

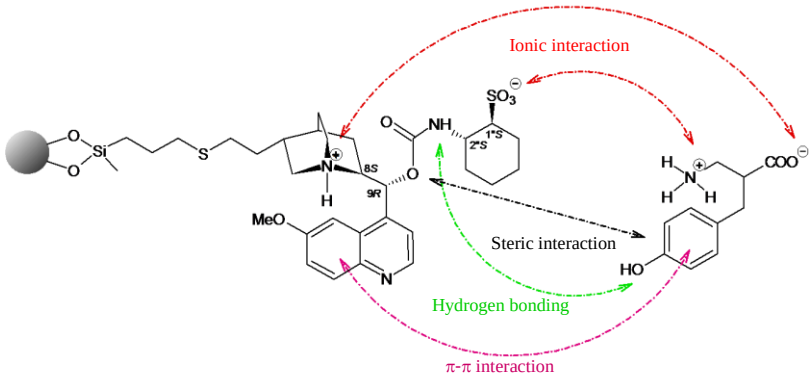


Figure 6. *Chiral interactions between the ZWIX(+)^{TM} CSP and a zwitterionic analyte*

The fusion of anion-exchange and cation-exchange moieties in ZWIX CSPs is a good choice to facilitate the enantiomer discrimination of chiral acids, amines, amino acids and peptides[56, 57].

2.3. Thermodynamics

There are two main temperature effects governing the chromatographic performance of a CSP[58]. The first effect is the influence of temperature on selectivity (α). This is a *thermodynamic effect*. *Chen et.al.*[59] and *Zhu et.al.*[60] have pointed out, that the selectivity generally decrease as the temperature is increased. This occurs because of the partition coefficient, therefore the free energy change ($\Delta G°$) of transfer of the analyte between the mobile and the stationary phase varies with temperature. This effect is controversial, it is unknown how the $\Delta G°$ of the compound would change in the course of the mass transfer process[59, 60]. On the other hand temperature changes the viscosity and the diffusion coefficients of the analyte in both phases, this is called *kinetic effect*. At higher temperatures viscosity decreases, which results in increasing diffusion of solute from the mobile to the stationary phase and it can favour mass transfer.

The equilibrium constant K_i of the SA-SO association is releated to the standard Gibbs energy according to the following equation

$$\Delta G° = \Delta H° - T\Delta S° = -RT \ln K_i \quad \textbf{(1)}$$

where

$\Delta H°$ = standard change of enthalphy, $\Delta S°$ = standard change of entropy, R = universal gas contant, T = absolute temperature in K.

The relationship between retention factor (k) and K_i is:

$$k = K_i \, \phi \qquad (2)$$

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

where:

k = retention factor, ϕ = the phase ratio [the ratio of the volumes of the stationary (V_s) and the mobile phase (V_m)].

In order to understand the mechanism of the separation, the determination of molar enthalphy and molar entropy changes is needed. In thermodynamic analysis, van't Hoff plots are generally applied[61], which can be given by further manipulation of Eq (**1**)

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln\phi \qquad (4)$$

In chiral chromatography, the difference in the change of standard free energy of the two enantiomers can be written as:

$$\Delta(\Delta G^\circ)_{2,1} = \Delta G^\circ_2 - \Delta G^\circ_1 = -RT \ln \frac{k_2}{k_1} = -RT \ln \alpha \qquad (5)$$

and

$$\ln \alpha = \frac{\Delta(\Delta H^\circ)}{RT} + \frac{\Delta(\Delta S^\circ)}{R} \qquad (6)$$

The van't Hoff plot curves, ln α *vs.* 1/T give the opportunity to estimate the corresponding enthalpic and entropic contributions ($\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$) to enantiomer discrimination (enantioselectivity). If ln α *vs.* 1/T is linear, the slope provides -$\Delta(\Delta H^\circ)$ and the y-intercept provides $\Delta(\Delta S^\circ)$ values, respectively. These values indicate, that the separation is enthalpically or entropically controlled.

2.4. Chemical and biological importance of the investigated compounds

2.4.1. β-Amino acids

β-Amino acids are currently a hot topic because of numerous pharmaceutical applications such as their neurological, enzyme inhibitor, receptor agonist-antagonist and antitumor activities[62-65]. In the β-amino acids there is an extra carbon atom between the amino and carboxylic groups, ensuring the availability of a number of stereo- and regioisomers, together with the possibility for further functionalization. Monosubstituted β-amino acids can be subdivided into β-2- and β-3-amino acids, depending upon the position of the side-chain on the 3-aminoalkanoic acid skeleton. Moreover, β-amino acids are generally more stable against hydrolysis or enzymatic degradation than their α analogs and incorporated into peptides the stability increases. β-Amino acids are used as starting

substances for the synthesis of heterocyclic compounds, potential pharmacons and analogs of natural products. Therefore, *β*-amino acids are important in the development of drugs[66]. In general, *β*-3-amino acids are commercially available, whereas most of the *β*-2-amino acids are not, and the syntheses of *β*-2-amino acids in enantiomerically pure form are quite a challenging task, since these amino acids must be prepared through multistep procedures[67].

2.4.2. Analogs possessing 1,2,3,4-tetrahydroisoquinoline skeleton

Tetrahydroisoquinoline (TIQ) derivatives are important members of the family of naturally occurring alkaloids. The naphthyridinomycin alkaloid TIQ, which have antitumor activities, have been isolated and this recognition inspired medicinal chemists to synthesize further TIQ compounds in order to obtain an increased number of novel medicinal agents[68]. The broad spectrum of biological activities of TIQ have been reported such as the anti-HIV activity of michellamine B or antiplasmodial activity of dioncophylline C, naphthylisoquinolines, naturally occurring alkaloids isolated from *Ancistrocladus korupensis*. Naphthylisoquinoline alkaloid containing compounds, for example dioncophylline E, exhibit curative, strong and highly specific effects against malaria[69]. *Hanna et.al.* have revealed that 1-aryl-6-hydroxy-1,2,3,4-tetrahydroisoquinoline and 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolines display anti-HIV and bronchodilator properties[70], the antiplasmodial activity of 1-aryl-1,2,3,4-tetrahydroisoquinoline analogues[71] were also investigated by them. Some commercially available drugs such as the antitussive noscapin and the antitumor agent trabectedin (as Yondelis®) contain enantiomerically pure TIQ as a key structural unit. Moreover, tetrahydroisoquinolines based sulfonamide and thiosemicarbazone[72] play important role in anticancer activity. TIQ compounds, *e.g.*, [(*R*)-salsolinol], have been detected in the human brain and intraventricular fluid, and their possible roles in the pathogenesis of Parkinson's disease have been discussed[73]. 1-Methyl- and 1-phenyl-TIQ are of importance in the prevention of Parkinson's and other neurological diseases[74]. TIQs may be important intermediates in the preparation of the expectorant emetin[75], and potential intermediates for the preparation of crispine A, which displays high biological activity against the human cancer cell lines SKOV3, KB, and HeLa[76].

2.4.3. Trans-paroxetine

Trans-paroxetine contains two chiral centres on the piperidine ring, in positions 3 and 4, respectively (***Figure 10.***). Of the four possible isomers, only one has pharmacologically active features with absolute configuration of 3*S*,4*R*, known as 4*R-trans*-(−)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine.

Therefore, the processes for *trans*-paroxetine synthesis must result in the formation of the piperidine moiety exclusively in the above mentioned configuration (–)-3*S*,4*R* (or 4*R-trans*). *Trans*-paroxetine, also known by the trade names Paxil, Pexeva, Seroxat, Brisdelle and Rexetin among others, belongs to one class of an orally administered antidepressant agents. These medicines are known as selective serotonin-reuptake inhibitors (SSRIs). *Trans*-paroxetine has no active metabolites and has the highest specificity for serotonin receptors of all the SSRIs. It is used to treat depression complicated by anxiety, panic disorder, social and general anxiety disorder, obsessive-compulsive disorder (OCD), premenstrual dysphoric disorder, premature ejaculation, and hot flashes of menopause in women[77-79].

3. Experimental

3.1. Apparatus

Measurements were carried out on two HPLC systems.

System I: 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: 1100 Series HPLC system from Agilent Technologies (Waldbronn, Germany): a solvent degasser, a pump, an autosampler, a column thermostat, a multiwavelength UV-Vis detector and a corona-charged aerosol detector (ESA Biosciences, Inc., Chelmsford, MA, USA).

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). The precision of temperature adjustment was ±0.1 °C.

3.2. Applied columns

Of the four zwitterionic CSPs ZWIX(+)™ and ZWIX(–)™ are commercially available from Chiral Technologies Europe (CTE, Illkirch, France), while the synthesis of ZWIX(–A) and ZWIX(+A) were described previously[80]. Each of these three CSPs comprised 3 μm particles packed into 150 x 3.0 mm I.D. columns and ZWIX(+A) comprised 5 μm packed into 150 x 4.0 mm I.D., respectively. All columns were provided by CTE (Illkirch, France).

All chromatographic experiments were carried out in isocratic mode at a flow rate of 0.6 ml min^{-1}, with Corona detection or UV detection at 215, 230, 260 and 295 nm. The void volume of the columns (t_0) was determined by injecting a solution of acetone in MeOH.

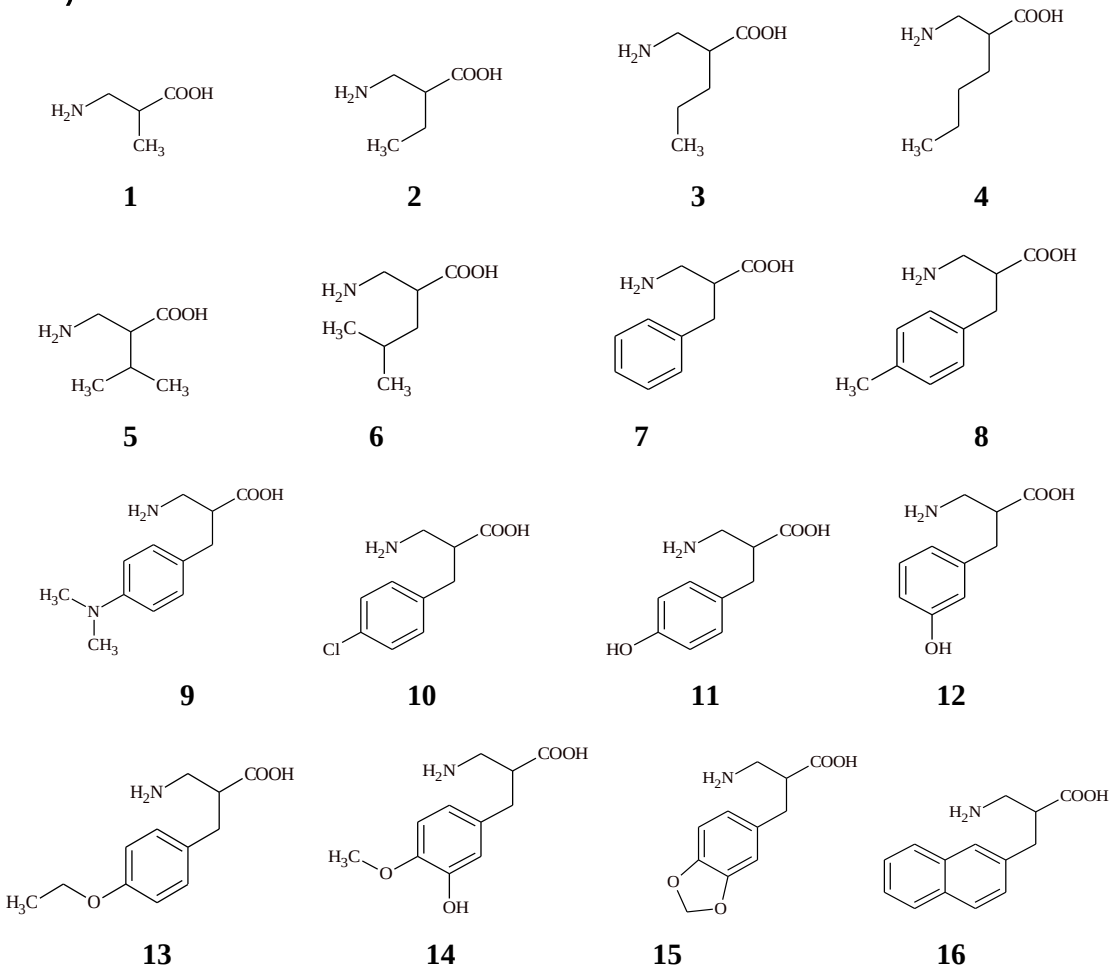
3.3. Chemicals and reagents

The applied methanol (MeOH), acetonitrile (MeCN) and tetrahydrofuran (THF) of HPLC grade and ammonia (NH_3), ethylamine (EA), diethylamine (DEA), triethylamine (TEA), propylamine (PA), tripropylamine (TPA), butylamine (BA), tributylamine (TBA), 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). The 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 the HPLC analysis. Stock solutions of analytes (1 mg mL^{-1}) were prepared by dissolution in the mobile phase.

3.4. Investigated analytes

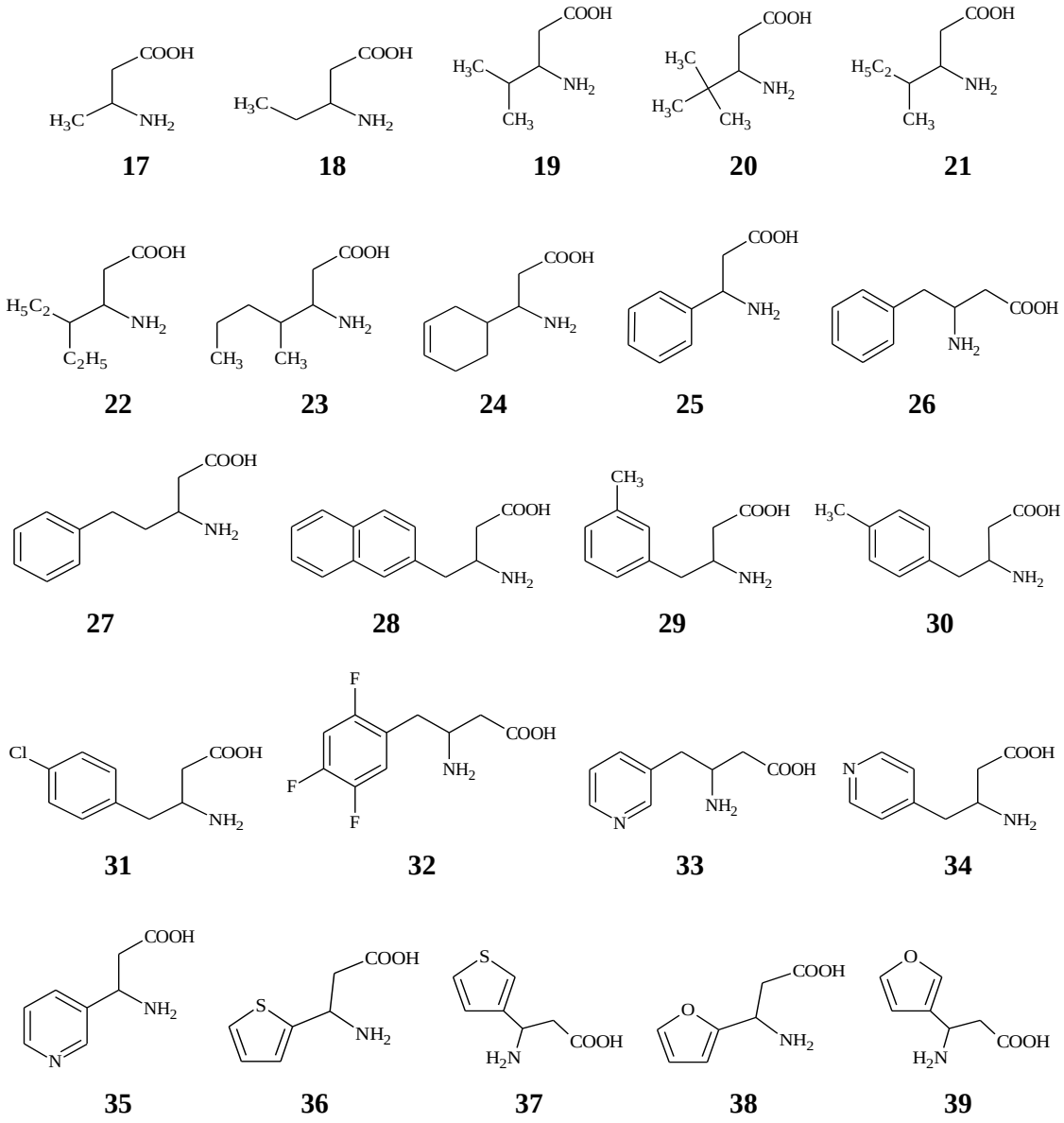
- ✓ **β-2-Amino acids:**



***1**: 3-amino-2-methylpropionic acid; **2**: 2-aminomethylbutanoic acid; **3**: 2-aminomethylpentanoic acid; **4**: 2-aminomethylhexanoic acid; **5**: 2-aminomethyl-3-methylbutanoic acid; **6**: 2-amino-methyl-4-methylpentanoic acid; **7**: 3-amino-2-benzylpropionic acid; **8**: 3-amino-2-(4-methylbenzyl)propionic acid; **9**: 3-amino-2-(4-dimethylaminobenzyl)propionic acid; **10**: 3-amino-2-(4-chlorobenzyl)propionic acid; **11**: 3-amino-2-(4-hydroxybenzyl) propionic acid; **12**: 3-amino-2-(3-hydroxybenzyl)propionic acid; **13**: 3-amino-2-(4-ethoxybenzyl)propionic acid; **14**: 3-amino-2-(3-hydroxy-4-methoxybenzyl)propionic acid; **15**: 3-amino-2-benzo[1,3]dioxol-5-yl-methylpropionic acid and **16**: 3-amino-2-(naphthalen-2-ylmethyl)propionic acid.*

***Figure 7.** The structure of β-2-amino acids*

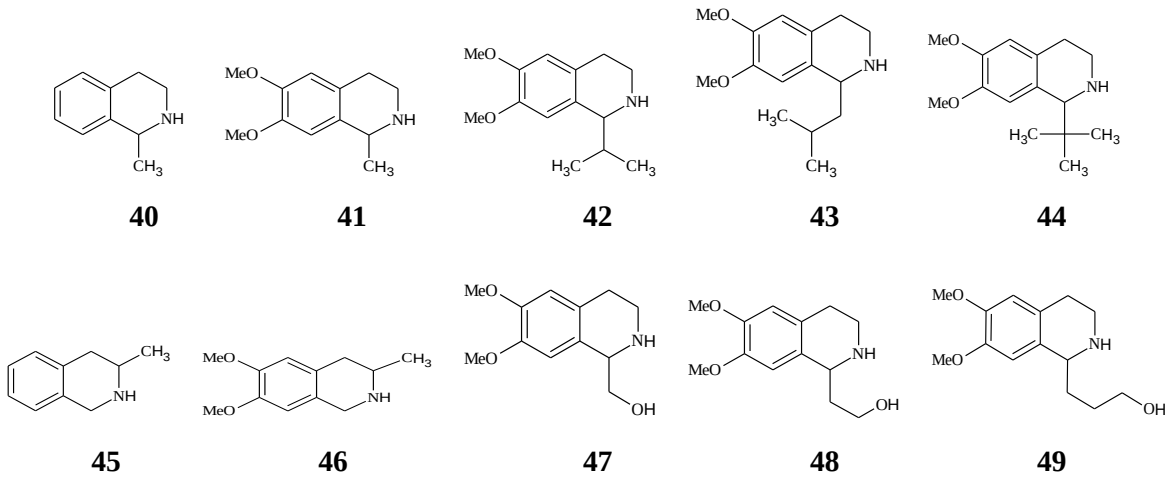
✓ ***β-3-Amino acids:***



***17**: 3-aminobutanoic acid; **18**: 3-aminopentanoic acid; **19**: 3-amino-4-methylpentanoic acid; **20**: 3-amino-4,4-dimethylpentanoic acid; **21**: 3-amino-4-methylhexanoic acid; **22**: 3-amino-4-ethylhexanoic acid; **23:** 3-amino-3-cyclohexylpropanoic acid; **24**: 3-amino-3-(3-cyclohexen-1-yl)propanoic acid; **25**: 3-amino-3-phenylpropanoic acid; **26**: 3-amino-3-phenylbutanoic acid; **27**: 3-amino-5-phenylpentanoic acid; **28**: 3-amino-4-(2-naphthyl)butanoic acid; **29**: 3-amino-4-(3-methylphenyl)-butanoic acid; **30**: 3-amino-4-(4-methylphenyl)butanoic acid; **31**: 3-amino-4-(4-chlorophenyl)butanoic acid; **32**: 3-amino-4-(3,4,6-trifluorophenyl)butanoic acid; **33**: 3-amino-4-(3-pyridyl)butanoic acid; **34**: 3-amino-4-(4-pyridyl)butanoic acid; **35**: 3-amino-3-(3-pyridyl)propanoic acid; **36**: 3-amino-3-(2-thienyl)propanoic acid; **37**: 3-amino-3-(3-thienyl)propanoic acid; **38**: 3-amino-3-(2-furyl)propanoic acid; **39**: 3-amino-3-(3-furyl)propanoic acid.*

***Figure 8.** The structure of β-3-amino acids*

✓ ***Analogs possessing 1,2,3,4-tetrahydroisoquinoline skeleton (TIQ):***



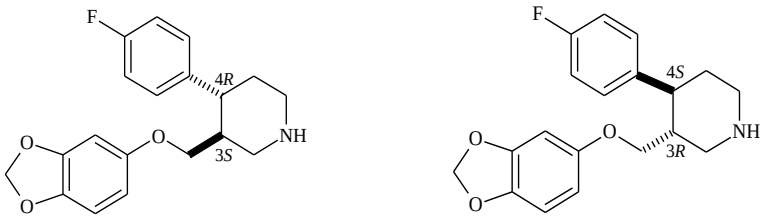
40 **41** **42** **43** **44**

45 **46** **47** **48** **49**

40: *1-methyl-1,2,3,4-tetrahydroisoquinoline;* ***41****: 6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline;* ***42****: 6,7-dimethoxy-1-(propan-2-yl)-1,2,3,4-tetrahydroisoquinoline;* ***43****: 6,7-dimethoxy-1-(2-methylpropyl)-1,2,3,4-tetra-hydroisoquinoline;* ***44****: 1-tert-butyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline;* ***45****: 3-methyl-1,2,3,4-tetrahydroisoquinoline;* ***46****: 6,7-dimethoxy-3-methyl-1,2,3,4-tetrahydroisoquinoline;* ***47****: (6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)methanol;* ***48****: 2-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)ethanol and* ***49****: 3-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)propan-1-ol.*

Figure 9. *The structure of derivatives of 1,2,3,4-tetrahydroisoquinoline*

✓ ***trans-Paroxetine enantiomers***



50a **50b**

trans-(–)-paroxetine *trans*-(+)-paroxetine

50a*: [(3S,4R)-3-[(1,3-benzodioxol-5-yloxy)-methyl]-4-(4-fluorophenyl)piperidine]and* ***50b****: [(3R,4RS)-3-[(1,3-benzodioxol-5-yloxy)-methyl]-4-(4-fluorophenyl)piperidine]*

Figure 10. *The structure of trans-paroxetine enantiomers*

4. Results and Discussion

The zwitterionic CSPs can work as ion-exchangers in different ways: for chiral acids they work as anion-exchangers, while for the separation of chiral amines they are applied as cation-exchangers. In case of amphoteric compounds (especially amino acids and peptides, without derivatization) a zwitterion-exchange mechanism exists. Zwitterionic phases exhibits multimodal behavior, they can be used in various operational modes: hydro-organic (HO), polar-ionic (PI) and normal-phase (NP) mode.

4.1. Influence of mobile phase composition on chromatographic parameters

4.1.1. Effect of bulk solvent composition

The effect of bulk solvent composition on chromatographic parameters was investigated applying β-2- and β-3-amino acids, TIQ analogs and *trans*-paroxetine enantiomers. The separation of cationic- and amphoteric analytes was investigated in PI mode with nonaqueous polar organic solvents containing acid and base additives (acid-to-base ratio was kept at 2:1). To optimize the bulk solvent composition MeOH as a protic solvent and MeCN as an aprotic solvent were applied in different ratios containing 25 mM base and 50 mM acid. The chromatographic parameters (retention factor (k_1), selectivity (α) and resolution (R_S)) were determined in the mobile phase containing increasing amount of MeCN in MeOH. For comparison purposes and to simplify the presentation, ***Table 2.*** lists only the chromatographic data obtained at some mobile phase compositions for selected aliphatic β-2- and aromatic β-3-amino acids and cationic analytes (TIQ) on ZWIX(–)^{TM} CSP. Regardless of the structure of investigated analytes (β-amino acids and TIQ) the retention increased with increasing MeCN content in the mobile phase. Furthermore, the prescence of the high MeCN content in the mobile phase usually led to enhanced enantioselectivity and resolution. These results are in contrast with earlier studies obtained for α-amino acids, where selectivity and resolution decreased in MeCN-rich mobile phases[55, 57]. The best column performance, which means short analysis time, high peak efficiency and enantioselectivity was usually obtained in zwitterionic and cation-exchange mode at MeOH/MeCN 50/50 (*v*/*v*) composition.

***Table 2**. Chromatographic data, retention factor (k_1), selectivity (α), resolution (R_S) on ZWIX(–)^{TM} and ZWIX(+)^{TM} CSPs for the separation of β-amino acids and TIQ analogs*

Compound	Column	Eluent *MeOH/MeCN (v/v%)*	k_1	α	R_S	*Elution sequence*
β-2-**1**	**ZWIX(–)™**	80/20 [a]	2.38	1.24	1.40	*S<R*
		70/30 [a]	2.90	1.28	1.17	
		60/40 [a]	3.80	1.28	1.70	
		50/50 [a]	5.50	1.31	2.00	
	ZWIX(+)™	70/30 [a]	3.60	1.13	0.85	*R<S*
		50/50 [a]	7.38	1.12	1.72	
β-2-**7**	**ZWIX(–)™**	70/30 [a]	4.21	1.14	1.60	-
		50/50 [a]	7.59	1.18	2.40	
	ZWIX(+)™	70/30 [a]	4.70	1.06	0.80	
		50/50 [a]	8.73	1.07	1.29	
β-2-**10**	**ZWIX(–)™**	70/30 [a]	4.58	1.15	1.55	-
		50/50 [a]	9.13	1.18	2.00	
β-3-**17**	**ZWIX(–)™**	75/25 [a]	2.27	1.14	1.37	*S<R*
		50/50 [a]	4.18	1.21	1.12	
		40/60 [a]	6.71	1.25	1.65	
β-3-**25**	**ZWIX(–)™**	75/25 [a]	2.73	1.46	4.55	*R<S*
		50/50 [a]	5.14	1.60	5.73	
		40/60 [a]	7.93	1.76	6.17	
TIQ-**41**	**ZWIX(–)™**	75/25 [b]	1.80	1.17	0.83	-
		50/50 [b]	3.75	1.40	4.91	
		25/75 [b]	6.73	1.48	3.10	
	ZWIX(+)™	75/25 [b]	3.46	1.14	2.17	-
		50/50 [b]	4.51	1.19	3.02	
		25/75 [b]	8.02	1.25	4.17	
TIQ-**44**	**ZWIX(–)™**	75/25 [b]	1.25	1.00	0.00	-
		50/50 [b]	1.95	1.08	1.20	
		25/75 [b]	2.24	1.12	0.50	
	ZWIX(+)™	75/25 [b]	2.50	1.04	0.71	-
		50/50 [b]	2.94	1.05	0.83	
		25/75 [b]	3.85	1.07	1.17	
TIQ-**45**	**ZWIX(–)™**	75/25 [b]	1.56	1.00	0.00	-
		50/50 [b]	2.61	1.00	0.00	
		25/75 [b]	2.84	1.12	0.50	
	ZWIX(+)™	75/25 [b]	3.11	1.01	0.20	-
		50/50 [b]	3.81	1.03	0.28	
		25/75 [b]	5.93	1.03	0.53	
TIQ-**47**	**ZWIX(–)™**	75/25 [b]	1.05	1.00	0.00	*S<R*
		50/50 [b]	2.07	1.08	0.83	
		25/75 [b]	4.04	1.09	1.00	
	ZWIX(+)™	75/25 [b]	3.06	1.06	0.86	*R<S*
		50/50 [b]	3.23	1.07	0.81	
		25/75 [b]	5.33	1.08	1.29	

Chromatographic conditions: mobile phase, [a]25 mM TEA and 50 mM AcOH, [b]12.5 mM TEA and 25 mM AcOH temperature, ambient; flow rate, 0.6 mL min^{-1}; detection, 215, 230 and 258 nm or Corona detector

Similar trends could be noticed with increasing MeCN content on ZWIX(+)^{TM} CSP, but the k_1 values were higher than those obtained on ZWIX(–)^{TM} CSP, while in most cases the α and R_S values were significantly higher on ZWIX(–)^{TM} CSP [exception was TIQ-**43**, data not shown]. Mechanistically in zwitterionic and cation-exchange mode, the observed chromatographic behavior can be summarized as follows:

- The increase of aprotic MeCN content led to increased retention. It is probably due to the decreasing solvation effect in the mobile phase, therefore strengthen the electrostatic interactions between the SA and the SO;
- The trend of increasing selectivity in MeCN-rich mobile phase can be explained by promotion of electrostatic and H-bonding interactions, enhancing chiral recognition. In MeOH-rich mobile phase, the extent of solvation of SA is more pronounced than in the aprotic MeCN. Solvation in the protic MeOH can inhibit the main electrostatic interaction and chiral recognition by blocking the easy access of the SA to the SO and can suppress the H-bonding.

4.1.2. Effect of THF content in the separation of trans-paroxetine enantiomers

Interesting results were observed in case of the separation of *trans*-paroxetine enantiomers. Mobile phase combination of MeOH/MeCN containing 25 mM DEA and 50 mM FA provided unfavorable results, no enantiomer separation was observed. On ZWIX(–A) CSP addition of THF in 1, 2, 5, 10, 50 and 90 *v*% to the MeOH/MeCN bulk solvent significantly influenced the separation characteristics. The obtained results are shown in ***Table 3***. With increasing THF content in the MeOH/MeCN mobile phase, a minimum curve was obtained for retention [the minimum value was obtained at MeOH/MeCN/THF (47.5/47.5/5 *v*/*v*/*v*) mobile phase composition]. In contrast to the retention factor, the selectivity exhibited a maximum curve. For the resolution, no general trend was observed.

Table 3. *Chromatographic data: retention factor (k_1), selectivity (α), resolution (R_S) on ZWIX(–A) column for the enantioseparation of trans-paroxetine enantiomers*

Column	**Eluent** *(+25 mM DEA and 50 mM FA)*	k_1	α	R_S
ZWIX(–A)	49.5/49.5/1 MeOH/MeCN/THF	1.86	1.15	1.10
	49/49/2 MeOH/MeCN/THF	1.72	1.17	1.78
	47.5/47.5/5 MeOH/MeCN/THF	1.41	1.19	1.35
	45/45/10 MeOH/MeCN/THF	1.58	1.16	1.68
	25/25/50 MeOH/MeCN/THF	1.96	1.13	1.47
	5/5/90 MeOH/MeCN/THF	3.58	1.02	<0.20

Chromatographic conditions: flow rate, 0.6 ml min^{-1}; detection, 295 nm

The separation of *trans*-paroxetine enantiomers were also improved by the change of MeOH or MeCN to THF in MeOH/MeCN bulk solvent. On ZWIX(–)™, ZWIX(+)™, ZWIX(–A) and ZWIX(+A) CSPs with MeOH/THF mobile phase system containing 25 mM DEA and 50 mM FA, the k_1 values increased significantly with increasing THF content, while an opposite trend was registered using MeCN/THF; namely k_1 values decreased significantly with increasing THF content (***Table 4***.) As regards selectivity, it changed from 1.00 to 1.17, while no general trend was observed in respect to the resolution.

Table 4. *Chromatographic data: retention factor (k_1), selectivity (α), resolution (R_S) and elution sequence of trans-paroxetine enantiomers on ZWIX(+)™, ZWIX(–)™, ZWIX(+A) and ZWIX(–A) columns*

Column	**Eluent** *(+25 mM DEA and 50 mM FA)*	k_1	α	R_S	***Elution sequence***
ZWIX(–)™	10/90 MeOH/THF	16.24	1.14	1.39	(–)<(+)
	20/80 MeOH/THF	9.58	1.16	1.18	
	50/50 MeOH/THF	5.40	1.07	<0.20	
	80/20 MeOH/THF	3.42	1.00	0.00	
	10/90 MeCN/THF	7.35	1.04	1.07	(–)<(+)
	20/80 MeCN/THF	8.80	1.10	0.87	
	50/50 MeCN/THF	13.76	1.13	1.47	
	80/20 MeCN/THF	17.54	1.10	1.14	
ZWIX(+)™	10/90 MeOH/THF	15.84	1.00	0.00	(–)<(+)
	20/80 MeOH/THF	9.42	1.07	<0.20	
	50/50 MeOH/THF	8.83	1.08	1.06	
	80/20 MeOH/THF	5.54	1.09	1.17	
	10/90 MeCN/THF	4.97	1.00	0.00	-
	20/80 MeCN/THF	7.98	1.00	0.00	
	50/50 MeCN/THF	8.54	1.00	0.00	
	80/20 MeCN/THF	18.11	1.03	0.28	
ZWIX(+A)	10/90 MeOH/THF	2.96	1.10	1.38	(+)<(–)
	20/80 MeOH/THF	2.53	1.11	1.18	
	50/50 MeOH/THF	1.71	1.07	1.19	
	80/20 MeOH/THF	2.22	1.14	1.15	
ZWIX(+A)	10/90 MeCN/THF	3.22	1.05	0.98	(+)<(–)
	20/80 MeCN/THF	4.20	1.06	0.76	
	50/50 MeCN/THF	3.90	1.06	0.80	
	80/20 MeCN/THF	5.09	1.07	0.85	
ZWIX(–A)	10/90 MeOH/THF	3.43	1.06	<0.20	(–)<(+)
	20/80 MeOH/THF	2.57	1.10	1.10	
	50/50 MeOH/THF	1.69	1.12	1.20	
	80/20 MeOH/THF	1.50	1.17	1.57	

Table 4.*(continued)*

Column	**Eluent** *(+25 mM DEA and 50 mM FA)*	k_1	α	R_S	***Elution sequence***
ZWIX(–A)	10/90 MeCN/THF	5.43	1.04	<0.20	(–)<(+)
	20/80 MeCN/THF	6.01	1.04	<0.20	
	50/50 MeCN/THF	6.15	1.06	0.88	
	80/20 MeCN/THF	8.62	1.06	0.60	

Chromatographic conditions: flow rate, 0.6 ml min^{-1}; detection, 295 nm

4.1.3. Role of water content of the mobile phase

Most of the chiral separation on ZWIX CSPs were carried out with application of polar-organic (PO) or PI mobile phases consisting of MeOH or a mixture of MeOH/MeCN with acid and base additives[54-56, 80, 81]. The investigations were extended by application of HO mobile phase, because solvation of the ionic compounds can play an important role in enantioseparations on ZWIX CSPs[57, 82, 83]. Water is on the top of the protic solvent list due to its powerful proton activity. Applying water in the eluent system might result in reduced strength of the ionic interactions between SO and SA due to the strong solvation of SO and SA, and since desolvation is energetically unfavorable the diastereomeric SO–SA complex formation can be hindered. However, the presence of water may improve the peak shape and column efficiency leading to improved separation efficiency. The effect of water content was investigated on ZWIX CSPs in the separation of β-3-amino acids and *trans*-paroxetine enantiomers.

4.1.3.1. Effect of water content and pH on the separation of β-3-amino acids enantiomers

To illustrate the possibilities applying ZWIX CSPs in HO mobile phase separations of all β-3-amino acids were carried out on ZWIX(–)^{TM} and ZWIX(+)^{TM} CSPs in H_2O/MeOH (10/90 *v*/*v*) mobile phases containing 25 mM TEA and 50 mM AcOH (***Table 5.***). Using HO mobile phase, lower k_1 values were obtained than in PI mobile phase (MeOH/MeCN 50/50 *v*/*v*) containing 25 mM TEA and 50 mM AcOH; data not shown). The α values were also higher in PIM, but in some cases with ZWIX(+)^{TM} CSP higher α values were obtained in HO mobile phase, mainly for SAs containing an aromatic ring.

Comparison of the ZWIX(+)^{TM} and ZWIX(–)™ CSPs in both PIM and HO mobile phase revealed that, the k_1 values were usually higher on ZWIX(+)™ CSP than on ZWIX(–)™ CSP (exceptions were β-3-**21**, β-3-**28** and β-3-**32** in the PIM), but the α values were

generally higher on ZWIX(–)™ CSP (exceptions were *β*-3-**23**, *β*-3-**28** and *β*-3-**32** in HO mobile phase).

Table 5. *Chromatographic data: retention factor (k_1), selectivity (α), resolution (R_S) and elution sequence of β-3-amino acids on ZWIX(+)™ and ZWIX(–)™ column in hydro-organic mobile phase*

Compound	Column	k_1	α	R_S	Elution sequence
β-3-**17**	**ZWIX(+)™**	6.12	1.02	<0.20	*R*<*S*
	ZWIX(–)™	3.32	1.05	0.40	*S*<*R*
β-3-**18**	**ZWIX(+)™**	5.65	1.11	1.01	-
	ZWIX(–)™	3.03	1.13	1.36	-
β-3-**19**	**ZWIX(+)™**	4.74	1.19	1.81	*S*<*R*
	ZWIX(–)™	2.69	1.21	3.11	*R*<*S*
β-3-**20**	**ZWIX(+)™**	3.63	1.37	3.28	-
	ZWIX(–)™	2.21	1.44	4.27	-
β-3-**21**	**ZWIX(+)™**	4.46	1.22	1.44	-
	ZWIX(–)™	2.62	1.28	2.72	-
β-3-**22**	**ZWIX(+)™**	4.38	1.27	2.53	-
	ZWIX(–)™	2.56	1.37	3.03	-
β-3-**23**	**ZWIX(+)™**	5.82	1.96	1.78	*S*<*R*
	ZWIX(–)™	3.18	1.17	1.89	*R*<*S*
β-3-**24**	**ZWIX(+)™**	5.31	1.16	0.81	-
	ZWIX(–)™	3.07	1.23	2.04	-
β-3-**25**	**ZWIX(+)™**	5.19	1.15	2.43	*S*<*R*
	ZWIX(–)™	3.29	1.19	2.46	*R*<*S*
β-3-**26**	**ZWIX(+)™**	5.79	1.19	2.28	*R*<*S*
	ZWIX(–)™	3.24	1.28	3.19	*S*<*R*
β-3-**27**	**ZWIX(+)™**	5.72	1.11	1.23	*R*<*S*
	ZWIX(–)™	3.49	1.15	1.55	*S*<*R*
β-3-**28**	**ZWIX(+)™**	7.59	1.28	4.26	*R*<*S*
	ZWIX(–)™	4.35	1.23	2.80	*S*<*R*
β-3-**29**	**ZWIX(+)™**	5.70	1.25	3.77	*R*<*S*
	ZWIX(–)™	3.27	1.27	3.18	*S*<*R*
β-3-**30**	**ZWIX(+)™**	5.81	1.26	3.97	*R*<*S*
	ZWIX(–)™	3.33	1.30	3.80	*S*<*R*
β-3-**31**	**ZWIX(+)™**	6.66	1.27	4.28	*R*<*S*
	ZWIX(–)™	4.05	1.31	4.14	*S*<*R*
β-3-**32**	**ZWIX(+)™**	4.70	1.35	5.45	*R*<*S*
	ZWIX(–)™	3.03	1.33	4.10	*S*<*R*

Table 5. *(continued)*

Compound	Column	k_1	α	R_S	*Elution sequence*
β-3-**33**	**ZWIX(+)™**	9.96	1.14	1.74	*R*<*S*
	ZWIX(–)™	5.10	1.15	1.37	*S*<*R*
β-3-**34**	**ZWIX(+)™**	12.70	1.12	2.03	*R*<*S*
	ZWIX(–)™	6.68	1.12	1.35	*S*<*R*
β-3-**35**	**ZWIX(+)™**	8.78	1.08	1.15	-
	ZWIX(–)™	4.55	1.11	1.12	-
β-3-**36**	**ZWIX(+)™**	5.26	1.12	1.93	*S*<*R*
	ZWIX(–)™	3.07	1.14	1.77	*R*<*S*
β-3-**37**	**ZWIX(+)™**	6.60	1.08	1.57	*S*<*R*
	ZWIX(–)™	3.91	1.12	1.58	*R*<*S*
β-3-**38**	**ZWIX(+)™**	4.37	1.09	1.52	*S*<*R*
	ZWIX(–)™	2.40	1.11	1.23	*R*<*S*
β-3-**39**	**ZWIX(+)™**	5.78	1.07	1.27	*S*<*R*
	ZWIX(–)™	3.20	1.10	1.24	*R*<*S*

Chromatographic conditions: mobile phase: H_2O/MeCN (10/90 v/v) containing 25 mM TEA and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215, 230 and 260 nm

Ion-pairing processes are expected to dominate in the retention of zwitterionic analytes in the HO mobile phase. Under slightly acidic conditions the tertiary amino group within the quinuclidine ring of SO is protonated, while the negatively charged sulfonic acid moiety is deprotonated. The effect of pH on the separation was investigated in the acidic pH range from 6.00 to 4.00 using 0.1% aqueous TEAA/MeOH (10/90 *v*/*v*) eluent system with alphatic *β*-3-**20** and aromatic *β*-3-**32** amino acids (***Figure 11.***). With increasing pH, the k_1, α and R_S values decreased on ZWIX(–)^{TM} and ZWIX(+)^{TM} CSPs. At low pH higher k_1, α and R_S values were observed, the proton concentration obviously influences the protonation and the solvation of both the quinuclidine ring of SO and SAs.

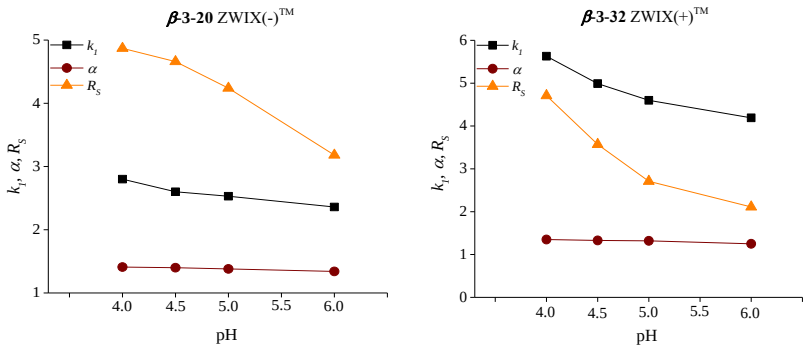


Figure 11. *The effect of pH on the separation*
Chromatographic conditions: column, ZWIX(–)™ and ZWIX(+)™; mobile phase, aqueous TEAA (pH 4.0, 4.5, 5.0, 6.0)/MeCN (10/90 v/v) containing 25 mM TEA (the pH of the aqueous phase was adjusted by the addition of AcOH); flow rate, 0.6 ml min^{-1}; detection, 215, 230 and 260 nm

4.1.3.2. Effect of water content in separation of trans-paroxetine enantiomers

Effect of water content on ZWIX(–)^{TM} CSP was monitored in MeCN/THF mobile phase containing 25 mM DEA and 50 Mm FA by variation of H_2O content between 1 – 5 (*v*%). With increasing water content the k_1 values first decreased than increased, while the α values remained almost constant (α=1.06 – 1.10), and the highest R_S value was obtained at 2.0 (*v*%) H_2O content (***Figure 12***). Since the mobile phase containing 2.0 (*v*%) H_2O was the most promising the separation of *trans*-paroxetine enantiomers applying this eluent composition was carried out on ZWIX(–)^{TM}, ZWIX(+)^{TM}, ZWIX(–A) and ZWIX(+A) CSPs.

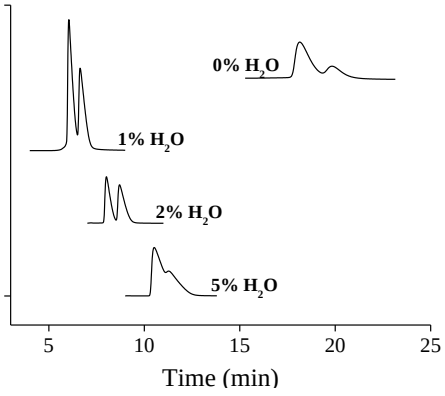


Figure 12. *Enantiomer separations of trans-paroxetine with and without water in the mobile phase*
Chromatrographic conditions: column, ZWIX(–)^{TM}; mobile phase, MeCN/THF (20/80/0, 20/79/1, 20/78/2 and 20/75/5 v/v/v) ; flow rate, 0.6 ml min^{-1}, detection, 295 nm

Comparison of the obtained chromatoghraphic parameters in the absence and presence of water revealed that in general, addition of 2.0 (*v*%) water to the mobile phase yielded shorter retention and lower resolution (***Table 6.***). However, on ZWIX(–)^{TM} and ZWIX(+A) CSPs the enantioresolution was higher in MeCN/THF/H_2O eluent than in MeCN/THF

eluent. In the presence of water the observed higher R_S values were due to the improved peak symmetry. Water acts as ion-pairing breaker because of its strong solvation power.

Table 6. *Chromatographic data: retention factor (k_1), selectivity (α), resolution (R_S) of trans-paroxetine enantiomers on ZWIX(–)™, ZWIX(+)™, ZWIX(–A) and ZWIX(+A) CSPs*

Column	Eluent (+25 mM DEA and 50 mM FA)	k_1	α	R_S
ZWIX(–)™	20/80 MeOH/THF	9.58	1.16	1.18
	20/78/2 MeOH/THF/H_2O	3.34	1.10	<0.20
	20/80 MeCN/THF	8.80	1.10	0.87
	20/78/2 MeCN/THF/H_2O	4.07	1.06	1.23
ZWIX(+)™	20/80 MeOH/THF	9.42	1.07	<0.20
	20/78/2 MeOH/THF/H_2O	6.97	1.06	0.76
	20/80 MeCN/THF	7.98	1.00	0.00
	20/78/2 MeCN/THF/H_2O	5.44	1.00	0.00
ZWIX(+A)	20/80 MeOH/THF	2.53	1.11	1.18
	20/78/2 MeOH/THF/H_2O	2.07	1.10	1.27
	20/80 MeCN/THF	4.20	1.06	0.76
	20/78/2 MeCN/THF/H_2O	3.44	1.05	0.85
ZWIX(–A)	20/80 MeOH/THF	2.57	1.10	1.10
	20/78/2 MeOH/THF/H_2O	1.71	1.07	<0.20
	20/80 MeCN/THF	6.01	1.04	<0.20
	20/78/2 MeCN/THF/H_2O	4.07	1.00	0.00

Chromatrographic conditions: flow rate, 0.6 ml min^{-1}, detection, 295 nm

4.2. Effect of the nature of base and acid additives

In zwitterionic mode both anion- and cation-exchange phenomena occur, therefore both acid and base additives in the mobile phase will play crucial role. They act as a competitor in the ion-exchange equilibrium. The addition of acid and base to the nonaqueous polar organic solvents greatly influences the solvation of both SO and SA, and 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 of the zwitterionic CSP, as summarized in ***Figure 13***[57, 84-86].

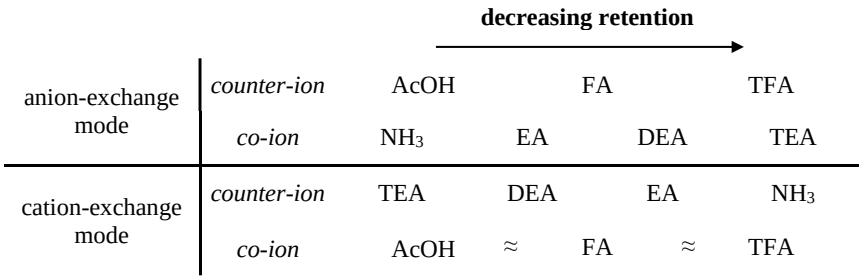


Figure 13. *Effect of elution strengths of co- and counter-ion additives on retention applying single ion-exchange-type CSPs*

According to literature reports in single anion-exchange mode the base additives (co-ions) show low effect on enantioselectivity and resolution, these additives decrease retention of analytes in the order NH_3 > EA > DEA > TEA[50, 86]. The opposite trend was found for pure enantioselective cation-exchanger-type CSPs, where the retention decreased, in the order TEA < DEA < EA < NH_3, and base additive served as the counter-ions[50, 57, 84, 85]. However, the literature data indicate that the elution strenghts of various amines are not directly related to their pK_a values (basicity)[87] (***Table 7.***).

Table 7. *The pK_a values of the applied base and acid modifiers*[87]

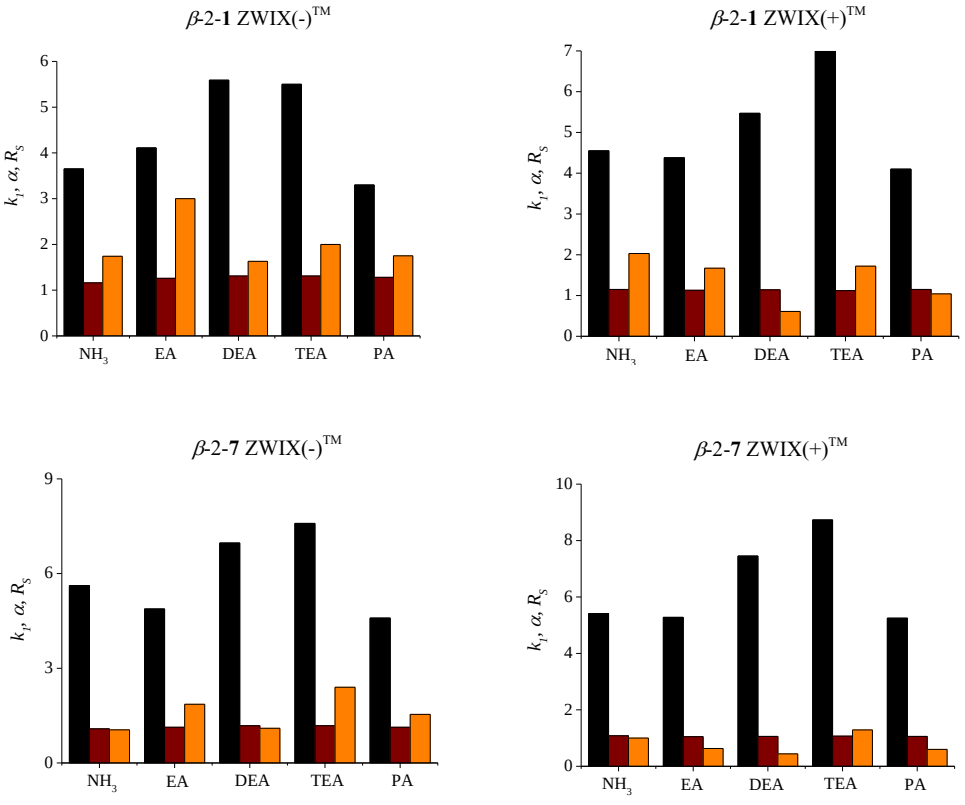
Base modifiers	**pK_a**
Ammonia	9.25
EA	10.65
DEA	10.84
TEA	10.75
PA	10.54
TPA	10.64
BA	10.60
TBA	10.68
Acid modifiers	
FA	3.75
AcOH	4.76

To investigate the effect of acid and base additives in an earlier study the acid-to-base ratio was varied between 4:1 to 1:4 (from acid to base excess)[57, 88]. The effect of acid and base additive on the separation of *β*-2-amino acid enantiomers were investigated on ZWIX(–)™ and ZWIX(+)™ CSPs and of *β*-3-amino acid enantiomers on ZWIX(–)™ CSP with MeOH/MeCN (50/50 *v*/*v*) containing 25 mM base and 50 mM AcOH. As base, NH_3, EA, DEA, TEA, PA, TPA, BA and TBA were selected which differ in the degree and nature of their alkyl substitution on *N* atom. AcOH and FA were used as acid additivies. The acid-to-base ratio was kept at ~2, ensuring that all the bases were present in their protonated „ammonium-ion" form. The selected *β*-2-amino acids contained one aliphatic (linear) side-chain (*β*-2-**1**), and two aromatic side-chains bearing benzyl (*β*-2-**7**) or halobenzoyl (*β*-2-**10**) groups, while *β*-3-amino acids contained one aliphatic (linear) side-chain (*β*-2-**17**) and one aromatic side-chain bearing benzyl (*β*-2-**25**) moiety. The experimental results depicted in ***Figures 14-15.*** reveal that in general, the k_1 values increased as the degree of alkyl substitution on the *N* atom increased (EA < DEA < TEA; PA < TPA and BA < TBA). As compared with the monosubstituted bases the presence of more apolar trisubstituted bases probably influenced the solvation of the ionic analytes disadvantageously, resulting in increased retention. In summary, ZWIX(–)™ and

ZWIX(+)^{TM} CSPs in case of *β*-amino acids operated as zwitterionic phases. Presence of amines influences retention of SAs through their competition for the acidic sites of SO and deprotonated carboxylic groups of SA. This competitive effect, through the strong electrostatic interaction between SO and SA is balanced, which is important in the view of chiral discrimination and column efficiency[89].

The nature of the base slightly affects the *α* values. For aliphatic *β*-2-**1** with the applied mobile phase system the *α* values ranged between 1.12–1.15, so for aromatic *β*-2-**7** and *β*-2-**10** between 1.05–1.08 and 1.00–1.07 on ZWIX(+)^{TM} CSP. On ZWIX(−)^{TM} CSP their values ranged between 1.16–1.31, 1.08–1.18 and 1.07–1.18. For aliphatic *β*-3-**17** the *α* values ranged between 1.18–1.22, and for aromatic *β*-2-**7** and *β*-3-**25** between 1.56–1.65 on ZWIX(−)^{TM} CSP. The *α* values were generally higher on ZWIX(−)^{TM} CSP than on ZWIX(+)^{TM} CSP. Enantioselective recognition in the zwitterionic mode requires both anion- and cation-exchanger sites, and at least a minor influence of the nature of the base additive on the enantioselectivity can therefore be expected. In zwitterionic mode, the effects due to the nature of the base additives possibly balance each other.

The applied amines influenced the resolution and these values were also generally higher on ZWIX(–)^{TM} than on ZWIX(+)^{TM}, but general trend was not obtained (***Figures 14-15.***).



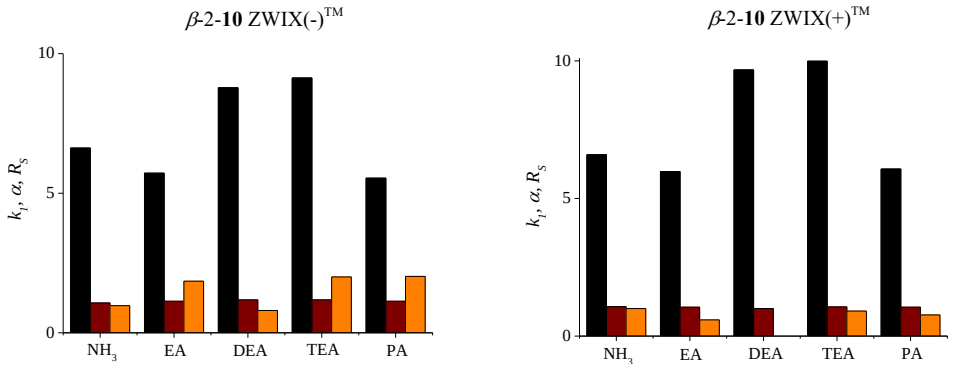


Figure 14. *Effect of the nature of base additives on the separation of β-2-amino acids*
Chromatographic conditions: column, ZWIX(–)™, ZWIX(+)™; mobile phase, MeOH/MeCN (50/50 v/v) containing 25 mM base and 50 mM AcOH,; flow rate, 0.6 ml min-1; detection, 230 nm or Corona, symbol, k1: ■, α: ■ and RS: ■

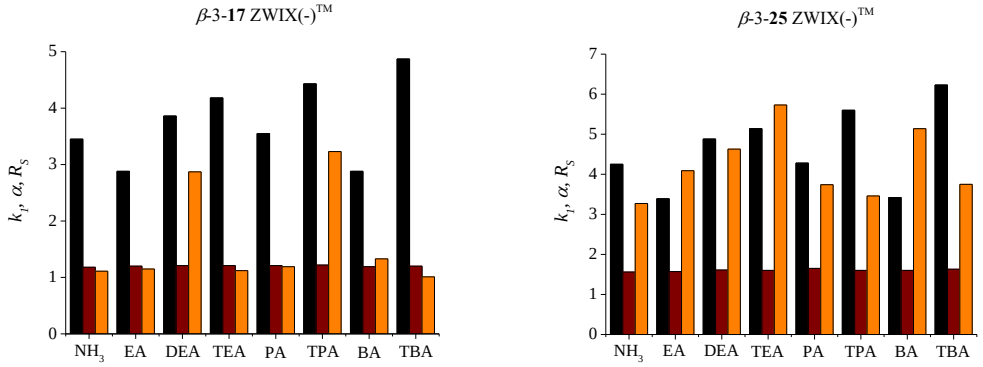


Figure 15*. Effect of the nature of base additives on the separation of β-3-amino acids*
Chromatographic conditions: column, ZWIX(–)™; mobile phase, MeOH/MeCN (50/50 v/v) containing 25 mM base and 50 mM AcOH; flow rate, 0.6 ml min-1; detection, 230 nm; symbol, k1: ■, α: ■ and RS: ■

In cation-exchange mode under acidic conditions, TIQ analogs and *trans*-paroxetine enantiomers contain protonated secondary amino group which interact electrostatically with the sulfohexyl moiety of the SO, primarily through an ion-pairing/ion-exchange retention mechanism. The protonated alkylamines (in the presence of an excess of AcOH or FA) in the mobile phase may compete for the sulfohexyl moiety of the SO with basic compounds and this may influence the band width and peak symmetry.

Experimental results obtained with mobile phase composed of MeOH/MeCN (50/50 *v*/*v*) containing 12.5 mM AcOH and 25 mM base for the TIQ analogs are shown in ***Figures 16-17.*** The k_1 values increased as the degree of alkyl substitution on the *N* atom increased [EA (≤ PA) < DEA < TEA], similarly to the *β*-amino acids. For the *trans*-paroxetine enantiomers in MeOH/THF (20/80 *v*/*v*) mobile phase system containing 50 mM FA and 25 mM base, in most cases the retention also increased in order PA < TPA; BA < TBA; EA < PA > BA and TEA < TPA < TBA. The larger the cationic component of

the mobile phase additive, the less effective it is in displacing a protonated SA from the SO-SA ion-pair complex. As a result, the k_1 values increase. In order to facilitate the enantioselectivity, additional SO–SA binding events are necessary, which can be of π-π or H-bonding character, for instance. The nature of the base had a slight effect on the selectivity, it changed in the narrow range between 1.04–1.10 on ZWIX(–)™ CSP and between 1.06–1.07 on ZWIX(+)™ CSP for TIQ-**47**, while for *trans*-paroxetine enantiomers 1.11–1.15 on ZWIX(–)™ CSP. The highest R_S values were obtained with the application of DEA, TEA and BA.

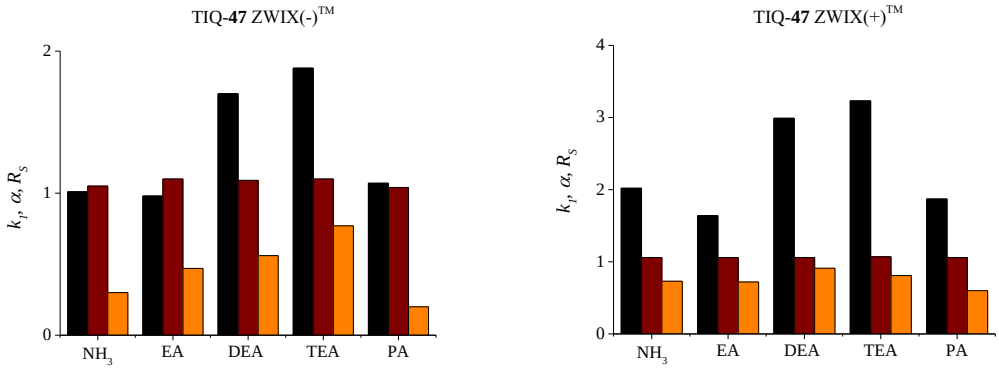


Figure 16. *Effect of the nature of base additives on the separation of TIQ analogs*
Chromatographic conditions: column, ZWIX(–)™ and ZWIX(+)™; mobile phase, MeOH/MeCN (50/50 v/v) containing 12.5 mM base and 25.0 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 230 and 258 nm; symbol, k_1: ■, α: ■ and R_S: ■

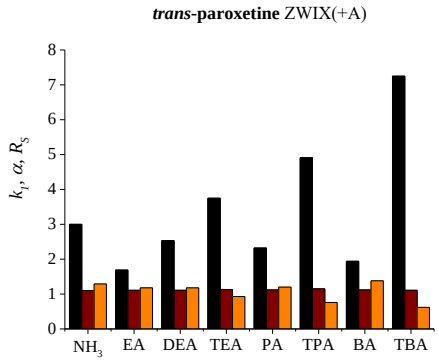


Figure 17. *Effect of the nature of base additives on the separation of trans-paroxetine enantiomers*
Chromatographic conditions: column, ZWIX(+A); mobile phase, MeOH/THF (20/80 v/v) containing 25 mM base and 50 mM FA; flow rate, 0.6 ml min^{-1}; detection, 295 nm; symbol, k_1: ■, α: ■ and R_S: ■

4.3. Effect of the counter-ion concentration

Under slightly acidic conditions, where ion-pairing takes place between SA and SO, long-range ionic interaction occures between the cationic site of the SA and the anionic site of SO as primary interaction. If the ZWIX CSPs are operated in zwitterionic

mode, the primary interaction is supported by the second ion-pairing process leading to the formation of sterically defined intermediate SO-SA complex. The counter-ions present in the mobile phase act as competitors for the interaction sites with the analyte, *i.e.* the application of higher counter-ion concentration should result in lower retention. According to the stoichiometric displacement model[90-92], linear relationship is to 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 $c_{counter\text{-}ion}$). 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 in the case of the single cation-exchange type CSPs[32, 93].

The effects of concentration of counter-ion were investigated for aliphatic *β*-3-**20** and aromatic *β*-3-**32** in HO mobile phase on both ZWIX(–)™ and ZWIX(+)™ CSPs. The mobile phase was aqueous TEAA (pH=4)/MeCN (10/90 *v*/*v*) and the concentration of TEA was 5, 12.5, 25 or 50 mM (the acid-to-base ratio was kept at 2:1 by the addition of AcOH). The slope of the log k_1 *vs* log c_{TEA} plots for *β*-3-**20** were -0.29 on both ZWIX(–)™ and ZWIX(+)™ CSPs, while for *β*-3-**32** were slightly lower -0.21 (***Figure 18.***). Similar results were reported by *Hoffmann et.al.*[56] for zwitterionic analytes on ZWIX column working in zwitterionic mode, where the slopes varied from 0.1 to 0.2. The enantioselectivity and the resolution remained almost constant with increasing concentration of TEA (data not shown) indicating that the change of counter-ion concentration has a slight effect on enantioselectivity.

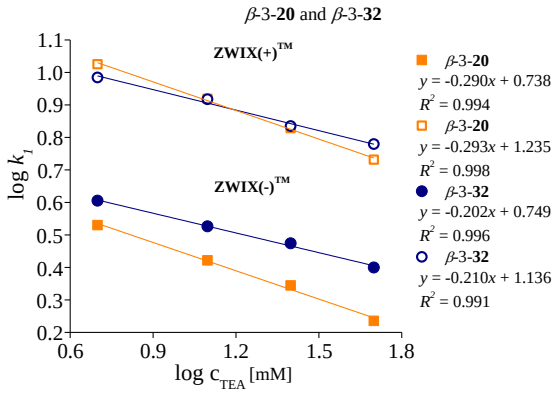


Figure 18. *Effects of the counter-ion concentration in zwitterionic mode*
Chromatographic conditions: mobile phase, aqueous TEAA (pH 4.0)/MeCN (10/90 v/v) containing 5, 12.5, 25 or 50 mM TEA (the pH of the aqueous phase was adjusted by the addition of AcOH); flow rate, 0.6 ml min^{-1}; detection, 215, 230 and 260 nm

The effects of the variation of the concentrations of basic counter-ion additives (DEA) were also studied by separation of *trans*-paroxetine enantiomers (***Figure 19.***). The concetration of DEA was changed between 6.125 – 100 mM (the acid-to-base ratio was

kept at 2:1 by the addition of AcOH). The slopes of the plots fit in the range of -0.7 to -0.8, an exception was found for the ZWIX(–A) CSP with the mobile phase MeOH/MeCN/THF (49/49/2 *v*/*v*/*v*), where the slope was -0.37. These slopes differ considerably from the slopes obtained on ZWIX CSPs working in zwitterionic mode and are close to the slope obtained on CSP working in cation-exchange mode[32, 93]. These different slopes indicate the difference in separation mechanism existing in zwitterionic or single ion-exchange mode.

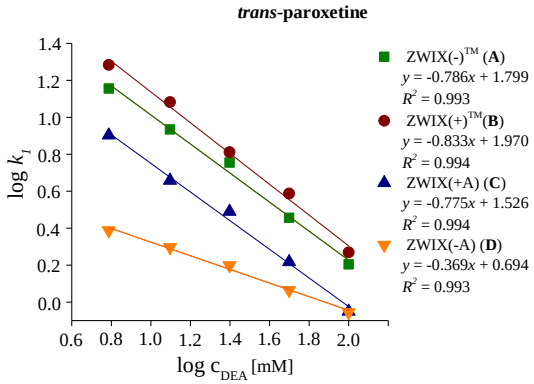


Figure 19. *Effects of the counter-ion concentration in cation-exchange mode Chromatographic condition: mobile phase, (**A**) MeCN/THF/H_2O (20/78/2 v/v/v) on ZWIX(–)^{TM}; (**B**) MeOH/THF/H_2O (20/78/2 v/v/v) on ZWIX(+)^{TM}; (**C**) MeOH/THF (20/80 v/v) on ZWIX(+A) and (**D**) MeOH/MeCN/THF (49/49/2 v/v/v) on ZWIX(–A) containing 6.125, 12.5, 25, 50 or 100 mM DEA; flow rate, 0.6 ml min^{-1}; detection, 295 nm*

4.4. Structure-retention (selectivity) relationships

The sterically demanding structures of the constrained SAs influence the retention and the chiral recognition. The k_1 values may vary with the structures of the SAs.

4.4.1. β-2-Amino acids

The structure-retention relationships for *β*-2-amino acids were investigated in MeOH/MeCN (50/50 *v*/*v*) mobile phase containing 25 mM TEA and 50 mM AcOH for aliphatic analytes (***Table 8.***). For *β*-2-amino acids the retention factor varied slightly with the length of the alkyl chain in the molecule. In case of molecules *β*-2-**1** – *β*-2-**4**, increasing chain length probably sterically favor the stabilization of SO-SA complex especially on ZWIX(–)^{TM}, therefore the retention factor slightly increased in parallel with the selectivity. For the bulkier *β*-2-**5** and *β*-2-**6** slightly smaller retention factor was registered but especially for *β*-2-**5** relatively higher α and R_S values were obtained. The presence of an extra CH_2 group in *β*-2-**6** as compared with *β*-2-**5** increases the flexibility and lipophilicity of the molecule: the retention factor increased, but the selectivity decreased. The aromatic

SAs are bulkier and sterically more constrained. The k_1 values proved to be considerably higher, whereas the selectivity was generally lower [especially on ZWIX(+)^{TM} CSP] as compared with alphatic SAs. The π character of the analytes contributes to the retention through the interaction with the quinoline moieties of the SO, but this was not manifested in difference in the degree of stabilization of SO–SA complex. A $–CH_3$ or $–N(CH_3)_2$ group on the benzyl ring (*β*-2-**8** and *β*-2-**9** vs. *β*-2-**7**) exhibited small effects on the retention and selectivity. The presence of the –Cl group, –OH group or –O– (*β*-2-**10** – *β*-2-**15**) may improve the interaction with the SO through H-bonding, which was resulted in higher k_1 values, but generally not in higher α values. The presence and position of the –OH group (*β*-2-**11**, *β*-2-**12** and *β*-2-**14**) exhibit a slight effect on the retention and selectivity. The significantly higher k_1 values of *β*-2-**16** may be attributed to the enhanced π–π interactions of quinoline moiety through the naphthyl ring.

Table 8. *Chromatographic data: retention factor (k_1), selectivity (α) and resolution (R_S) of β-2-amino acids on ZWIX(+)^{TM} and ZWIX(–)^{TM} CSPs*

Compound	**Column**	k_1	α	R_S	***Elution sequence***
β-2-**1**	**ZWIX(+)™**	7.38	1.12	1.72	*R*<*S*
	ZWIX(–)™	5.50	1.31	2.00	*S*<*R*
β-2-**2**	**ZWIX(+)™**	6.93	1.28	3.65	-
	ZWIX(–)™	5.59	1.38	2.90	-
β-2-**3**	**ZWIX(+)™**	7.07	1.26	3.67	-
	ZWIX(–)™	6.11	1.33	2.66	-
β-2-**4**	**ZWIX(+)™**	7.05	1.26	3.75	-
	ZWIX(–)™	6.11	1.34	3.00	-
β-2-**5**	**ZWIX(+)™**	5.58	1.5	8.38	*R*<*S*
	ZWIX(–)™	6.06	1.30	2.75	*S*<*R*
β-2-**6**	**ZWIX(+)™**	6.19	1.18	2.86	*R*<*S*
	ZWIX(–)™	6.18	1.20	1.60	*S*<*R*
β-2-**7**	**ZWIX(+)™**	8.73	1.07	1.29	-
	ZWIX(–)™	7.59	1.18	2.40	-
β-2-**8**	**ZWIX(+)™**	8.87	1.06	0.91	-
	ZWIX(–)™	7.51	1.16	1.90	-
β-2-**9**	**ZWIX(+)™**	9.29	1.08	1.30	-
	ZWIX(–)™	7.90	1.16	2.10	-
β-2-**10**	**ZWIX(+)™**	9.99	1.06	0.91	-
	ZWIX(–)™	9.13	1.18	2.00	-

Table 8. *(continued)*

Compound	Column	k_1	α	R_S	*Elution sequence*
β-2-**11**	**ZWIX(+)™**	10.47	1.07	1.21	-
	ZWIX(–)™	8.72	1.17	1.60	-
β-2-**12**	**ZWIX(+)™**	11.37	1.06	1.01	-
	ZWIX(–)™	9.21	1.15	1.80	-
β-2-**13**	**ZWIX(+)™**	8.20	1.07	1.20	-
	ZWIX(–)™	7.16	1.17	1.60	-
β-2-**14**	**ZWIX(+)™**	10.73	1.04	0.40	-
	ZWIX(–)™	8.09	1.16	1.90	-
β-2-**15**	**ZWIX(+)™**	9.39	1.04	0.47	-
	ZWIX(–)™	7.32	1.17	1.70	-
β-2-**16**	**ZWIX(+)™**	12.07	1.00	0.00	-
	ZWIX(–)™	9.73	1.11	1.50	-

Chromatographic conditions: flow rate 0.6 ml min^{-1}; 215, 230 and 260 nm detection

4.4.2. β-3-Amino acids

The structural effects of β-3-amino acids were investigated in MeOH/MeCN (50/50 *v*/*v*) mobile phase containing 25 mM TEA and 50 mM AcOH in the PIM on ZWIX(+)™ and ZWIX(–)™ columns. In the case of β-3-amino acid possessing an aliphatic side-chain, the k_1 and α values depend strongly on the chain length of the side-chain. According to *Meyer*, the steric effect of a substituent on the reaction rate is characterized by the size-descriptor V^a[94]. The data in ***Figure 20.*** reveal that the retention factor depended strongly on the volume of the alkyl group: via steric effects, a bulkier substituent slightly inhibited the overall interaction with the SO, and the retention decreased. However, the molecular structure has a significant effect on the enantiorecognition too; a longer chain length and a bulkier molecular structure resulted in improved chiral recognition (data not shown). Our results demonstrated that the steric effects of the alkyl side-chains exerted a considerable influence on retention (and chiral discrimination) of primary β-3-amino acids.

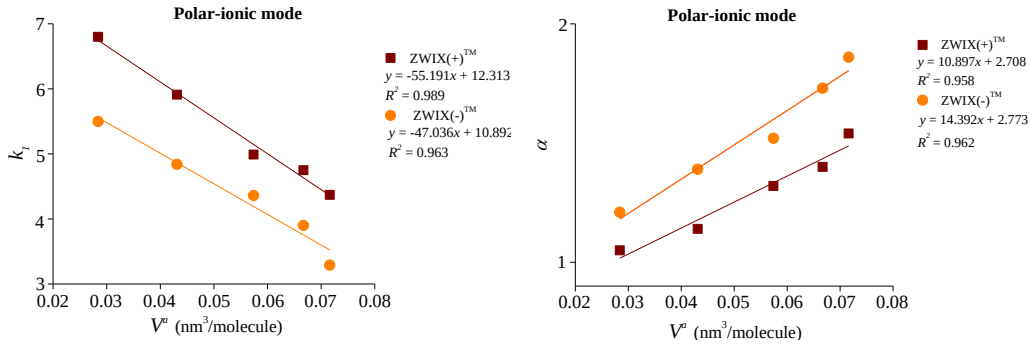


Figure 20. *Dependence of retention factors and separation factors of β-3-**17**, β-3-**18**, β-3-**19**, β-3-**20** and β-3-**21** on the Meyer substituent parameter (V^a)*
Chromatographic conditions: column, ZWIX(+)™ or ZWIX(–)™; mobile phase, MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM AcOH; flow rate, 0.6 ml min-1; detection 215 and 230 nm or corona detector

For aromatic *β*-3-**25** –*β*-3-**28**, the k_1 values proved to be considerably higher, whereas the enantioselectivity generally remained at the same level as seen for alphatic *β*-3-amino acids (***Table 9.***). The *π* character of the analytes may contribute to the retention through interactions with the quinoline moieties of the SO; an especially enhanced interaction was observed for the naphthalene ring-containing *β*-3-**28** as compared with *β*-3-**26**, but this was not manifested in markedly improved enantioselectivity. A –CH_3 group on the benzyl ring, independently of its position slightly affects the retention and selectivity. The presence of the –Cl or –F group in *β*-3-**31** and *β*-3-**32** may improve the interaction with the SO through H-bonding, this is manifested in most cases in higher k_1 and *α* values on both columns. However, the presence and position of the –N–, –S– and –O– groups (*β*-3-**33** – *β*-3-**39**) are associated with only moderate effects on the retention and enantioselectivity. The larger k_1 values of *β*-3-**33** – *β*-3-**35** as compared with *β*-3-**25** and *β*-3-**26** may be attributed to the difference in the effects of the pyridinium and the benzyl ring. The *ortho* or *meta* position of the –S– and –O– groups in *β*-3-**36** *vs.* *β*-3-**37** and *β*-3-**38** *vs.* *β*-3-**39** exerts a steric influence on the interaction, the *meta* position of the heteroatoms resulting in higher k_1 and *α* values, supporting a favored SA-SO interaction.

Table 9. *Chromatographic data, retention factor (k_1), selectivity (α), resolution (R_S) and elution sequence of aromatic β-3-amino acids on ZWIX(+)™ and ZWIX(–)™ CSPs*

Compound	Column	k_1	α	R_S	Elution sequence
β-3-**25**	**ZWIX(+)™**	6.70	1.25	3.69	*S*<*R*
	ZWIX(–)™	5.14	1.60	5.73	*R*<*S*
β-3-**26**	**ZWIX(+)™**	7.62	1.10	1.35	*R*<*S*
	ZWIX(–)™	6.92	1.36	3.57	*S*<*R*

Table 9. *(continued)*

Compound	Column	k_1	α	R_S	*Elution sequence*
β-3-**27**	**ZWIX(+)™**	6.99	1.16	1.88	*R<S*
	ZWIX(–)™	6.70	1.47	3.76	*S<R*
β-3-**28**	**ZWIX(+)™**	9.50	1.14	2.04	*R<S*
	ZWIX(–)™	9.64	1.22	2.08	*S<R*
β-3-**29**	**ZWIX(+)™**	7.23	1.10	1.36	*R<S*
	ZWIX(–)™	7.08	1.30	2.75	*S<R*
β-3-**30**	**ZWIX(+)™**	7.45	1.13	1.87	*R<S*
	ZWIX(–)™	7.03	1.36	3.47	*S<R*
β-3-**31**	**ZWIX(+)™**	8.97	1.17	2.63	*R<S*
	ZWIX(–)™	8.97	1.36	4.13	*S<R*
β-3-**32**	**ZWIX(+)™**	6.19	1.31	4.77	*R<S*
	ZWIX(–)™	6.66	1.46	5.06	*S<R*
β-3-**33**	**ZWIX(+)™**	8.38	1.19	1.64	*R<S*
	ZWIX(–)™	6.83	1.34	2.58	*S<R*
β-3-**34**	**ZWIX(+)™**	11.12	1.09	1.17	*R<S*
	ZWIX(–)™	8.67	1.25	2.09	*S<R*
β-3-**35**	**ZWIX(+)™**	7.34	1.20	2.29	-
	ZWIX(–)™	5.51	1.58	2.73	-
β-3-**36**	**ZWIX(+)™**	5.64	1.14	2.22	*S<R*
	ZWIX(–)™	5.04	1.34	3.43	*R<S*
β-3-**37**	**ZWIX(+)™**	7.64	1.17	2.36	*S<R*
	ZWIX(–)™	6.41	1.53	5.17	*R<S*
β-3-**38**	**ZWIX(+)™**	4.50	1.10	1.34	*S<R*
	ZWIX(–)™	3.73	1.32	3.09	*R<S*
β-3-**39**	**ZWIX(+)™**	7.08	1.10	1.80	*S<R*
	ZWIX(–)™	5.77	1.46	5.23	*R<S*

Chromatographic conditions: mobile phase, MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM AcOH; flow rate, 0.6 ml min^{-1}*; detection, 215, 230 and 260 nm*

4.4.3. Enantioseperation of various analogs possessing 1,2,3,4-tetrahydroisoquinoline skeleton (TIQ)

Structure-retention relationships in case of TIQ analogs were also studied. ***Table 10.*** reports the k_1 and α values on ZWIX(–)™ and ZWIX(+)™ CSPs in MeOH/MeCN (75/25 *v*/*v*) mobile phase containing 12.5 mM TEA and 25 mM AcOH. Methoxy-substitution in TIQ-**41** and TIQ-**46** resulted in higher k_1 and α values. The increased retention and selectivity may be explained by the presence of methoxy group, which can possible form H-bond interaction with selector. Comparing chromatographic behavior of

TIQ-**40** and TIQ-**45**, which differ in the position of methyl group, revealed that enantiomers of TIQ-**45 (**methyl group is in 3 position) were not or only slightly separable on ZWIX(–)™ and ZWIX(+)™ CSPs. Similar results were obtained for TIQ-**41** and TIQ-**46**. It seems that the methyl group (in position 3) sterically hinders the effective interaction between SO and SA. For analytes TIQ-**41** – TIQ-**44**, k_1 values decreased slightly with the length of the alkyl chain; increasing chain length and the bulkier molecular structure probably sterically hinder the stabilization of SO-SA complex, therefore the retention decreased slightly in parallel with the selectivity. In analytes TIQ-**47** – TIQ-**49,** the hydroxyalkyl-substitution hinders the SA-SO complexation, therefore a decrease in the k_1 and α values were registered, but for TIQ-**48** higher k_1 and α values were obtained on both CSPs.

Table 10. *Chromatographic parameters, retention factor (k_1), selectivity (α), resolution (R_S) and elution sequence of TIQ analogs on ZWIX(+)^{TM} and on ZWIX(–)^{TM} CSP*

Compound	**Column**	k_1	α	R_S	***Elution sequence***
TIQ-**40**	**ZWIX(+)™**	3.07	1.12	1.25	*n.d.*
	ZWIX(–)™	1.45	1.24	1.26	*n.d.*
TIQ-**41**	**ZWIX(+)™**	3.46	1.14	2.17	*R<S*
	ZWIX(–)™	1.80	1.17	0.83	*S<R*
TIQ-**42**	**ZWIX(+)™**	2.57	1.04	0.53	*n.d.*
	ZWIX(–)™	1.27	1.11	0.55	*n.d.*
TIQ-**43**	**ZWIX(+)™**	3.18	1.08	1.27	*n.d.*
	ZWIX(–)™	2.04	1.23	1.50	*n.d.*
TIQ-**44**	**ZWIX(+)™**	2.50	1.04	0.71	*n.d.*
	ZWIX(–)™	1.25	1.00	0.00	*n.d.*
TIQ-**45**	**ZWIX(+)™**	3.11	1.01	<0.20	*n.d.*
	ZWIX(–)™	1.56	1.00	0.00	*n.d.*
TIQ-**46**	**ZWIX(+)™**	3.14	1.03	0.53	*n.d.*
	ZWIX(–)™	1.70	1.00	0.00	*n.d.*
TIQ-**47**	**ZWIX(+)™**	3.16	1.05	0.67	*R<S*
	ZWIX(–)™	1.29	1.00	0.00	*n.d.*
TIQ-**48**	**ZWIX(+)™**	2.81	1.09	1.45	*R<S*
	ZWIX(–)™	1.09	1.13	0.80	*S<R*
TIQ-**49**	**ZWIX(+)™**	3.06	1.06	0.86	*R<S*
	ZWIX(–)™	1.49	1.05	0.30	*S<R*

Chromatographic conditions: column, Chiralpak ZWIX(+)™; mobile phase, MeOH/MeCN (75/25 v/v) containing 12.5 mM TEA and 25 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 or 230 nm; n.d.: not determined

4.5. Determination of elution sequence on ZWIX(–)™, ZWIX(+)™, ZWIX(–A) and ZWIX(+A) CSPs

The determination of elution sequence of enantiomers is important in revealing the impurity profile and enantiomer excess. The chiral selectors of Chiralpak ZWIX(+)™, ZWIX(–)™, ZWIX(+A) and ZWIX(–A) CSPs (***Figure 5.***) are actually diastereomeric to each other, but behave in most cases like pseudo-enantiomers. The *cinchona* alkaloids QN and QD are diastereomers and the configurations of the QN (8*S*,9*R*) and QD (8*R*,9*S*) molecular moieties are different and the resulting steric environment largely determine the chiral recognition abilities of these SOs. ZWIX(+)™ and ZWIX(−)™ are therefore frequently referred to as "pseudo-enantiomers" because they behave as quasi-enantiomeric CSPs. As a result, on change from QN to QD, the elution sequence of enantiomers should be reversed. (***Figure 21.***)

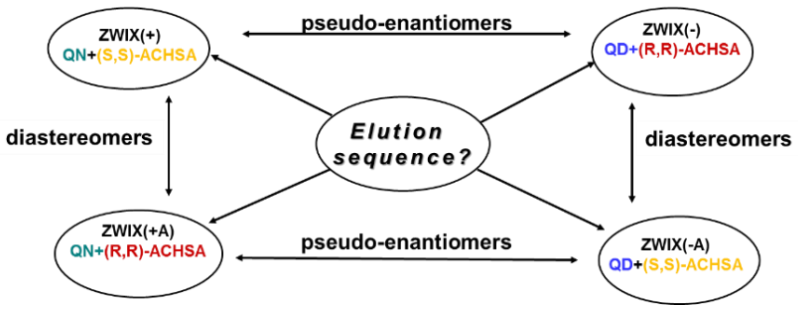


Figure 21*. The possible relationships between the elution sequence and the structure of selector*

In most cases the *S* configuration of *β*-amino acids and TIQ analogs on ZWIX(+)™ formed more stable complexes with the *cinchona*-based backbone, therefore the elution sequence was *R* < *S*, with some exceptions like *β*-3-**19**, *β*-3-**23**, *β*-3-**25** and *β*-3-**36** – **39**. On ZWIX(–)™ CSP, opposite sequence was observed. Namely, amino acids and TIQ analogs with *R* configuration formed more stable complexes (exception were *β*-3-**19**, *β*-3-**23**, *β*-3-**25** and *β*-3-**36**-**39)**. Nevertheless, the paroxetine did not fit this pattern fully. The elution sequence of paroxetine enantiomers on the pseudo-enantiomeric ZWIX(+)™ and ZWIX(–)™ CSPs was the same [(–) < (+)], whereas on the other pseudo-enantiomeric pair of ZWIX(+A) CSP [(+) < (–)] and ZWIX(–A) CSP [(–) < (+)] it was reversed. This inconsistency was unexpected. For a better understanding of the interactions of the paroxetine enantiomers with the four SOs, *in silico* simulations of the eight SO–SA systems were computed at a MeOH/THF (80/20 *v*/*v*) virtual eluent system by researchers of the University of Perugia (Italy). These results are not shown in this thesis, but are available in the original article[95].

4.6. Temperature dependence and thermodynamic parameters

Temperature influences chromatogarphic parameters in different ways[96, 97]. A detailed study to describe the temperature dependence was carried out for *β*-2-amino acids (*β*-2-**1**, *β*-2-**6**, *β*-2-**7**, *β*-2-**8**, *β*-2-**10**, *β*-2-**11** and *β*-2-**16**) between -5 – 55°C, for *β*-3-amino acids (*β*-3-**20**, *β*-3-**26** and *β*-3-**32**) and TIQs (TIQ-**40** – **41,** TIQ-**44** – **49**) between 10 – 50°C on ZWIX(–)^{TM} and ZWIX(+)^{TM} CSPs, while for *trans*-paroxetine between 0 – 50°C on ZWIX(–)^{TM}, ZWIX(+)^{TM}, ZWIX(–A) and ZWIX(+A) CSPs. A part of the experimental data is presented in ***Table 11a-d***. Comparison of the k_1 values reveals that most of the recorded k_1 values decreased with increasing temperature. The internal energy of the system is higher at higher temperature, consequently the transfer of the SA from the mobile phase to the stationary phase is faster, resulting in decreased k_1 values. However, regarding k_1 values an interesting effect has also been observed by increasing the column temperature, *i.e.* the k_1 values increased with increasing temperature. This behavior was earlier registered in nonchiral separations[98-100] and for enantiomer separations[37, 88]. This phenomenon was observed on ZWIX(–)^{TM} CSP for *β*-3-**20**, *β*-3-**26** and *β*-3-**32**, for TIQ-**40,** TIQ-**41**, TIQ-**44** – **49**, on ZWIX(+)^{TM} CSP for TIQ-**48** and last but not least for *trans*-paroxetine enantiomers on ZWIX(+A) CSP in the mobile phase MeOH/THF (20/80 *v*/*v*) containing 25 mM DEA and 50 mM FA between 0 – 40 °C (only a part of these data are depicted in ***Tables 11a-d***). The α values usually decreased with increasing temperature, while in some cases they increased with increasing temperature. Such behavior was observed on ZWIX(–)^{TM} CSP for *β*-2-**7**, *β*-2-**8**, *β*-2-**10**, *β*-2-**16** and TIQ-**44**, and on ZWIX(+)^{TM} CSP for *trans*-paroxetine enantiomers. The change in structure (conformational flexibility of the semi-rigid SO units) or solvation of the SO due to the elevated temperature should contribute to this unusual effect. Further studies are required for a better understanding of the mechanism behind this behavior.

The dependence of the resolution on temperature was more complex. The data presented in ***Table 11a-d*** show, that in most cases the R_S values decreased with increasing temperature, however, there were few exceptions also, where the R_S values exhibited a maximum curve (mostly in the case of *β*-2-amino acids and TIQ analogs) or increased continuously (*β*-3-amino acids, TIQ-**48** on ZWIX(–)^{TM} CSP, *trans*-paroxetine enantiomers on ZWIX(+)^{TM} CSP) with increasing temperature. Increasing temperature may improve the peak symmetry and efficiency, and therefore the resolution may also improve.

Table 11a. *Temperature dependence of the retention factor (k_1), the selectivity (α) and the resolution (R_S) of β-2-amino acids*

Compound / k_1, α, R_S	Column	Temperature (°C)					
		-5	15	25	35	45	55
β-2-**7**							
k_1	ZWIX(–)™	5.48	4.35	4.21	3.74	3.43	3.20
α		1.13	1.14	1.14	1.14	1.14	1.14
R_S		1.41	1.60	1.60	1.45	1.33	0.84
k_1	ZWIX(+)™	6.06	5.41	4.70	4.11	3.47	3.04
α		1.09	1.08	1.06	1.05	1.04	1.03
R_S		1.00	0.94	0.80	0.67	0.52	0.32
β-2-**8**							
k_1	ZWIX(–)™	4.69	4.00	3.82	3.68	3.51	3.33
α		1.10	1.12	1.13	1.13	1.13	1.14
R_S		0.87	1.38	1.35	1.33	1.08	0.73
k_1	ZWIX(+)™	6.15	5.47	4.81	4.18	3.53	3.15
α		1.09	1.07	1.06	1.05	1.04	1.03
R_S		1.00	0.77	0.83	0.54	0.43	0.34
β-2-**10**							
k_1	ZWIX(–)™	5.75	4.88	4.58	4.44	4.17	3.95
α		1.12	1.14	1.15	1.15	1.15	1.15
R_S		1.60	1.55	1.50	1.47	0.83	1.04
k_1	ZWIX(+)™	6.97	6.25	5.43	4.64	4.09	3.51
α		1.11	1.07	1.05	1.04	1.02	1.00
R_S		0.85	0.87	0.76	0.51	0.47	0.00

Chromatographic conditions: mobile phase, MeOH/MeCN (70/30 v/v) containing 25 mM TEA and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 and 258 nm

Table 11b. *Temperature dependence of the retention factor (k_1), the selectivity (α) and the resolution (R_S) of β-3-amino acids*

Compound / k_1, α, R_S	Column	Temperature (°C)				
		10	20	30	40	50
β-3-**20**						
k_1	ZWIX(–)™	2.51	2.57	2.63	2.69	2.74
α		1.39	1.37	1.36	1.35	1.33
R_S		4.15	4.33	4.60	4.79	4.56
k_1	ZWIX(+)™	4.11	4.06	4.02	3.96	3.91
α		1.42	1.40	1.38	1.35	1.33
R_S		6.17	6.36	6.25	5.72	5.61
β-3-**32**						
k_1	ZWIX(–)™	4.22	4.33	4.44	4.54	4.62
α		1.29	1.28	1.27	1.26	1.25
R_S		3.92	4.05	4.07	4.09	4.15
k_1	ZWIX(+)™	7.64	7.54	7.43	7.36	7.28
α		1.30	1.28	1.26	1.24	1.22
R_S		4.80	4.70	4.55	4.40	4.19

Chromatographic conditions: mobile phase, 25 mM aqueos TEAA (pH 4.0)/MeCN/ (10/90 v/v), pH set by AcOH addition; flow rate, 0.6 ml min^{-1}; detection, 215, 230 and 260 nm

Table 11c. *Temperature dependence of the retention factor (k_1), the selectivity (α) and the resolution (R_S) of TIQ analogs*

Analyte / k_1, α, R_S	Column	*Temperature (°C)*				
		10	***20***	***30***	***40***	***50***
TIQ-**41**						
k_1	**ZWIX(–)™**	2.90	3.50	3.95	4.13	4.19
α		1.49	1.43	1.37	1.32	1.27
R_S		4.85	4.89	4.96	4.37	3.71
k_1	**ZWIX(+)™**	4.20	4.45	4.59	4.39	4.01
α		1.24	1.20	1.17	1.14	1.12
R_S		3.54	3.00	3.00	2.20	1.14
TIQ-**44**						
k_1	**ZWIX(–)™**	1.58	1.84	2.10	2.16	2.13
α		1.08^{7}	1.09^{2}	1.09^{6}	1.09^{3}	1.09^{0}
R_S		1.05	1.16	1.24	1.18	0.82
k_1	**ZWIX(+)™**	2.88	2.94	2.94	2.76	2.45
α		1.05	1.05	1.05	1.03	1.02
R_S		0.77	0.77	0.88	0.57	0.20
TIQ-**45**						
k_1	**ZWIX(–)™**	2.43	2.54	2.65	2.67	2.69
α		1.00	1.00	1.00	1.00	1.00
R_S		0.00	0.00	0.00	0.00	0.00
k_1	**ZWIX(+)™**	3.87	3.84	3.77	3.57	3.19
α		1.02	1.03	1.04	1.02	1.01
R_S		0.20	0.26	0.26	0.24	<0.20
TIQ-**47**						
k_1	**ZWIX(–)™**	1.12	1.46	1.78	2.03	2.15
α		1.12	1.11	1.10	1.08	1.07
R_S		<0.20	<0.20	0.31	0.34	0.30
k_1	**ZWIX(+)™**	3.97	3.98	3.92	3.78	3.56
α		1.08	1.07	1.07	1.06	1.08
R_S		1.29	1.19	0.96	0.88	0.77

Chromatographic conditions: mobile phase, MeOH/MeCN=50/50 (v/v) containing 12.5 mM TEA and 25 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 and 230 nm

Table 11d. *Temperature dependence of the retention factor (k_1), the selectivity (α) and the resolution (R_S) of trans-paroxetine enantiomers*

Compound / k_1, α, R_S	Column	*Temperature (°C)*					
		0	***10***	***20***	***30***	***40***	***50***
trans-paroxetine enantiomers							
k_1	***ZWIX(–)™**	11.88	11.41	10.97	10.04	9.04	8.06
α		1.28	1.26	1.23	1.17	1.13	1.00
R_S		1.92	1.91	1.76	1.48	0.96	0.00
k_1	****ZWIX(+)™**	5.61	5.44	4.99	4.13	3.42	2.90
α		1.04	1.05	1.07	1.09	1.11	1.13
R_S		0.38	0.68	0.85	1.04	1.33	0.69

Table 11d. (*continued)*

Compound / k_1, α, R_S	Column	*Temperature (°C)*					
		0	***10***	***20***	***30***	***40***	***50***
k_1	*ZWIX(+A)	2.47	2.49	2.51	2.53	2.54	2.53
α		1.14	1.13	1.12	1.11	1.10	1.09
R_S		1.39	1.42	1.50	1.25	1.12	1.06
k_1	***ZWIX(–A)	1.65	1.62	1.62	1.59	1.56	1.54
α		1.28	1.25	1.23	1.19	1.16	1.12
R_S		2.26	2.27	2.09	1.75	1.55	1.21

*Chromatographic conditions: column, *ZWIX(–)^{TM} and ZWIX(+A): 20/80 MeOH/THF containing 25 mM DEA and 50 mM, **ZWIX(+)^{TM}: 80/20 MeOH/THF containing 25 mM DEA and 50 mM, ***ZWIX(–A): 49/49/2 MeOH/MeCN/THF containing 25 mM DEA and 50 mM; flow rate, 0.6 ml min^{-1}; detection, 295 nm*

Since the effect of temperature on the separation was complex, a detailed thermodynamic study may contribute to understand the mechanistic aspects of chiral recognition [97, 101-103]. Thermodynamic parameters were obtained by the application of van't Hoff plots (Eq. (5)). The differences of the standard enthalpy and entropy changes calculated from the ln α *vs* 1/*T* curves, are presented in ***Table 12a-d.*** When a linear van't Hoff plot applies the terms of $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ can be obtained from the slope and the y-intercept, respectively. The $\Delta(\Delta H°)$ values reflect to the difference of enthalpy changes accompanying the transfer of the analytes from the mobile to the stationary phase. High negative $\Delta(\Delta H°)$ values refer to exothermic process *i.e.* stronger interaction of the enantiomers with the stationary phase or more efficient enantiomer transfer between the mobile and the stationary phase. The trend in the change of $\Delta(\Delta S°)$ is similar as in $\Delta(\Delta H°)$, if $\Delta(\Delta H°)$ values are negative $\Delta(\Delta S°)$ values are also negative and the largest $\Delta(\Delta H°)$ values are accompanied by the largest $\Delta(\Delta S°)$ values. The lower $\Delta(\Delta S°)$ values suggest an enhanced increase of order and stronger interactions of SA-SO complex resulting in a significant loss of freedom.

In most cases, the thermodynamic parameters were negative. It should be noted when the selectivity increased with increasing temperature both $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ exhibited positive values.

According to literature data[102, 104, 105], the separation of enantiomers is enthalpy controlled or enthalpically driven when the $\Delta(\Delta H°)$ dominates over the $\Delta(\Delta S°)$ or $298*\Delta(\Delta S°)$. In this case, decrease of the temperature improves enantioselectivity and thus, the enantioseparation.

However, on ZWIX(–)^{TM} CSP, the Δ(Δ*H*°) and the 298*Δ(Δ*S*°) values for *β*-2-**7**, *β*-2-**8**, *β*-2-**10**, *β*-2-**16**, TIQ-**44**, and on ZWIX(+)^{TM} CSP for TIQ-**45** and *trans*-paroxetine enantiomers were positive. In this case the increasing temperature had positive effect for enantioseparation, as the selectivity increased with increasing temperature, in parallel with decreasing retention. This is called entropy controlled or entropically driven enantioseparation, where Δ(Δ*S*°) or 298*Δ(Δ*S*°) dominates over Δ(Δ*H*°). The entropically driven enantioseparation is demonstarted on ***Figure 22.*** for *trans*-paroxetine enantiomers.

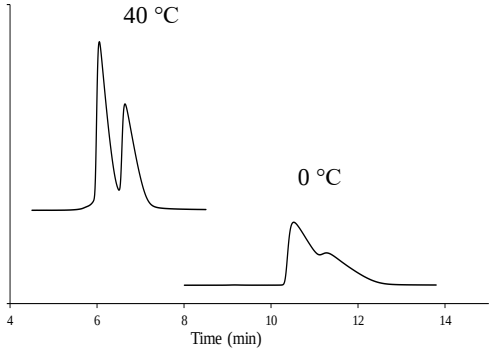


Figure 22. *Entropically driven enantioseparation of trans-paroxetine enantiomers on ZWIX(+)^{TM}*

Chromatographic conditions: mobile phase, MeOH/THF (20/80 v/v) containing 25 mM DEA and 50 mM FA; flow rate, 0.6 ml min^{-1}; detection, 295 nm

Regarding free energy, larger Δ(Δ*G*°)$_{298K}$ means that the binding of SA to the SO was more efficient and SO sterically ensured more interaction on the CSP. In all cases the Δ(Δ*G*°)$_{298K}$ values were negative and generally lower values were observed on ZWIX(–)^{TM} and ZWIX(–A) CSPs than on ZWIX(+)^{TM} and ZWIX(+A) CSPs, as depicted in ***Tables 12a-d***.

The relative contributions of the enthalpic and entropic terms to the free energy of adsorption can be visualized through the enthalpy/entropy ratio *Q* [*Q* = *Δ*(*ΔH*°) / [298 x Δ(Δ*S*°)] (***Table 12a-d***). Comparison of the *Q* values for the individual analytes revealed that in most cases, the enantioselective discrimination was enthalpically driven (*Q* > 1.0) while *Q* < 1.0 indicates that enantioseparation was entropically driven.

The slope of ln α vs. *1/T* curves may reflect to the mechanism of enantioseparation. If ln α vs. *1/T* curves in the investigated temperature range: (i) are linear indicating the same mechanism is valid over the investigated temperature range, (ii) are nonlinear (has a curvature) meaning that the separation mechanism changes by changing the temperature, (iii) are linear with different slopes indicating that below and above of a breaking point the mechanism may be different.

According to our findings in most cases the ln α *vs 1/T* curves were linear as depicted in ***Figure 23a.*** However, in some cases on ZWIX(–)^{TM} CSP for TIQ-**44** and ZWIX(+)^{TM} CSP for TIQ-**44**, TIQ-**45**, and TIQ-**46** the ln α *vs* 1/*T* curves could be divided into two regions, which means that van't Hoff plots are linear but with different slopes. As ***Figure 23b*** depicts in the case of TIQ-**45** temperature range can be divided into two regions: **region I** between 10 – 30°C and **region II** between 30 – 40°C. The values of Δ(Δ*H*°), Δ(Δ*S*°) and 298*Δ(Δ*S*°) in **region I** were positive, indicating an entropically driven enantioseparation, while in **region II** these values were negative, suggesting an enthalpically driven enantioseparation. This type of ln α *vs* 1/*T* curve typically reflects the changes of retention mechanism (or conformation of the stationary phase).

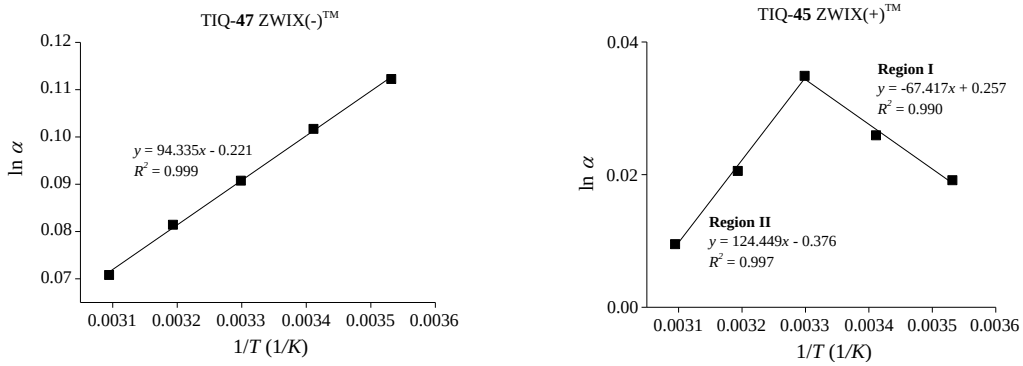


Figure 23. *Different type of van't Hoff plot curves*
Chromatographic conditions: mobile phase, MeOH/MeCN (50/50 v/v) containing 12.5 mM TEA and 25 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 and 230 nm

Table 12a. *Thermodynamic parameters, Δ(ΔH°), Δ(ΔS°), TxΔ(ΔS°), Δ(ΔG°), correlation coefficients (R^2) and Q of selected β-2-amino acids on ZWIX(–)^{TM} and ZWIX(+)^{TM} CSPs*

Column	Corr. coeff.	−Δ(Δ*H*°) (kJ/mol)	−Δ(Δ*S*°) (J/mol*K)	−*T*xΔ(Δ*S*°) (kJ/mol)	−Δ(Δ*G*°)$_{298K}$ (kJ/mol)	*Q*
			β-2-**7**			
ZWIX(–)^{TM}	0.994	-0.1	-1.4	-0.4	0.3	0.2
ZWIX(+)^{TM}	0.998	0.9	2.6	0.7	0.2	1.3
			β-2-**8**			
ZWIX(–)^{TM}	0.992	-0.3	-2.1	-0.6	0.3	0.5
ZWIX(+)^{TM}	0.992	0.8	2.3	0.7	0.1	1.1
			β-2-**10**			
ZWIX(–)^{TM}	0.994	-0.3	-2.1	-0.6	0.3	0.5
ZWIX(+)^{TM}	0.995	1.4	4.4	1.3	0.1	1.1

Chromatographic conditions: mobile phase, MeOH/MeCN (70/30 v/v) containing 25 mM TEA and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 and 258 nm; R^2, correlation coefficient of van't Hoff plot, ln α – 1/T curves; Q, Δ(ΔH°) / T_{298K}xΔ(ΔS°)

Table 12b. *Thermodynamic parameters, Δ(ΔH°), Δ(ΔS°), TxΔ(ΔS°), Δ(ΔG°), correlation coefficients (R^2) and Q of selected β-3-amino acids on ZWIX(–)™ and ZWIX(+)™ CSPs*

Column	Corr. coeff.	−Δ(Δ*H*°) (kJ/mol)	−Δ(Δ*S*°) (J/mol*K)	−*T*xΔ(Δ*S*°) (kJ/mol)	−Δ(Δ*G*°)$_{298K}$ (kJ/mol)	*Q*
β-3-**20**						
ZWIX(–)™	0.998	0.8	0.2	0.1	0.7	8.0
ZWIX(+)™	0.992	1.3	1.7	0.5	0.8	2.6
β-3-**32**						
ZWIX(–)™	0.999	0.6	0.03	0.01	0.6	60
ZWIX(+)™	0.993	1.3	2.3	0.7	0.6	1.9

Chromatographic conditions: mobile phase, 25 mM aqueos TEAA (pH 4.0)/MeCN/ (10/90 v/v), pH set by AcOH addition; flow rate, 0.6 ml min^{-1}, detection 215, 203 nm; R^2, correlation coefficient of van't Hoff plot, ln α – 1/T curves; Q, Δ(ΔH°) / T_{298K}xΔ(ΔS°)

Table 12c. *Thermodynamic parameters, Δ(ΔH°), Δ(ΔS°), TxΔ(ΔS°), Δ(ΔG°), correlation coefficients (R^2) and Q of selected TIQ analogs on ZWIX(–)™ and ZWIX(+)™ CSPs*

Column	Corr. coeff.	−Δ(Δ*H*°) (kJ/mol)	−Δ(Δ*S*°) (J/mol*K)	-*T*xΔ(Δ*S*°) (kJ/mol)	−Δ(Δ*G*°)$_{298K}$ (kJ/mol)	*Q*
TIQ-**41**						
ZWIX(–)™	0.999	3.0	7.2	2.2	0.8	1.4
ZWIX(+)™	0.993	2.0	5.2	1.5	0.5	1.3
TIQ-**44**						
ZWIX(–)™	0.999	-0.3*	-1.7*	-0.5*	0.2*	0.6
	0.999	0.3**	0.2**	0.1**	0.2**	3.0
ZWIX(+)™	0.997	0.1*	<<0.1*	<<0.1*	0.1*	1.0
	0.996	1.2**	3.6**	1.1**	0.1**	1.1
TIQ-**45**						
ZWIX(–)™	-	-	-	-	-	-
ZWIX(+)™	0.990	-0.6*	-2.1*	-0.6*	<<0.1*	1.0
	0.997	1.0**	3.1**	0.9**	0.1**	1.1
TIQ-**48**						
ZWIX(–)™	0.999	0.8	1.8	0.5	0.3	1.6
ZWIX(+)™	0.999	0.4	0.8	0.2	0.2	2.0

*Chromatographic conditions: mobile phase, MeOH/MeCN (50/50 v/v) containing 12.5 mM TEA and 25 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 or 230 nm; R^2, correlation coefficient of van't Hoff plot, ln α – 1/T curves, *temperature range: 10-30 °C, **temperature range 30-50 °C*

Table 12d. *Thermodynamic parameters, Δ(ΔH°), Δ(ΔS°), TxΔ(ΔS°), Δ(ΔG°), correlation coefficients (R^2) and Q of trans-paroxetine enantiomers on ZWIX(–)™, ZWIX(+)™, ZWIX(–A) and ZWIX(+A) CSPs*

Column	Corr. coeff.	−Δ(Δ*H*°) (kJ/mol)	−Δ(Δ*S*°) (J/mol*K)	−*T*xΔ(Δ*S*°) (kJ/mol)	−Δ(Δ*G*°)$_{298K}$ (kJ/mol)	*Q*
***trans*-paroxetine enantiomers**						
***ZWIX(–)™**	0.997	8.8*	3.0*	2.6*	0.4*	1.2
****ZWIX(+)™**	0.998	-5.9	-1.5	-1.8	0.2	0.8
***ZWIX(+A)**	0.991	1.4	0.7	0.4	0.3	1.8
*****ZWIX(–A)**	0.991	6.3	2.3	1.9	0.4	1.2

*Chromatographic conditions: column, *ZWIX(–)™ and ZWIX(+A): 20/80 MeOH/THF containing 25 mM DEA and 50 mM, **ZWIX(+)™: 80/20 MeOH/THF containing 25 mM DEA and 50 mM, ***ZWIX(–A): 49/49/2 MeOH/MeCN/THF containing 25 mM DEA and 50 mM; flow rate, 0.6 ml min^{-1}; detection, 295 nm; R^2, correlation coefficient of van't Hoff plot, ln α vs 1/T curves; * temperature range 0–30 °C; Q = Δ(ΔH°) / 298 x Δ(ΔS°)*

5. Summary

Direct liquid chromatographic methods were developed for the separation of the enantiomers of *β*-amino acids and secondary amines on a novel class of zwitterionic ion-exchange-type chiral columns based on *cinchona* alkaloids.

Enantiomer separation on ZWIX chiral stationary phases

1) Effects of polar organic bulk solvents

The effects of the eluent composition and the nature of the polar alcohol on the separations were studied. With increasing MeOH content in the mobile phase the retention decreased in all cases. In presence of MeOH, being a polar protic solvent, the ionic interaction between SO and SA is interfered, therefore retention is decreased. The presence of polar solvent had a strong effect on the selectivity and the resolution, these values decreased significantly at higher MeOH content in MeCN bulk solvent. The higher MeCN content is always accompanied by higher retention, and in most cases higher enantioselectivity and enantioresolution were obtained for cationic (exception was *trans*-paroxetine enantiomers) and zwitterionic compounds.

In MeOH/MeCN-based mobile phase *trans*-paroxetine enantiomers were not separable, therefore MeOH or MeCN was replaced by THF. With MeOH/THF mobile phase system containing 25 mM DEA and 50 mM FA, the retention increased significantly with increasing THF content, while the opposite trend was registered with MeCN/THF. The selectivity enhanced slightly when THF content was increased, while for resolution, no general trend was observed.

2) Role of water content of the mobile phase

The effects of the H_2O content in the mobile phase on the retention, selectivity and resolution were investigated. In cation-exchange mode for *trans*-paroxetine enantiomers retention exhibited a minimum curve with increasing H_2O content, while selectivity and resolution decreased in most cases. The highest resolution values were obtained at 2.0 *v*% H_2O content on ZWIX(–)™ and ZWIX(+A) CSPs when MeCN/THF/H_2O or MeOH/THF/H_2O eluent composition was used.

In zwitterionic mode in the HO mobile phase, lower k_1 values were obtained than in PIM on both ZWIX(–)™ and ZWIX(+)™ CSPs. The α values were also usually lower in

the HO mobile phase than in PIM, but in some cases the α and R_S values were higher, mainly for analytes containing aromatic ring applying ZWIX(–)™ and ZWIX(+)™ CSPs.

3) Effect of the nature of base and acid additives

Eight different base modifiers were applied for the separation of β-amino acid and secondary amines. The nature of base in the mobile phase influenced the retention. In zwitterionic and cation-exchange mode, the retention factor increased as the degree of alkyl substitution or the bulkiness of aliphatic moiety on the *N* atom increased (EA (≤ PA) < DEA < TEA; PA < TPA; BA < TBA). The nature of the base had a slight effect on the selectivity but no general trend was obtained. In most cases the best R_S values were obtained with application of DEA, TEA and BA, but application of BA was disadvantageous due to its high viscosity.

4) Effects of the counter-ion concentration

The retention can be controlled by the type of the counter-ion, but the concentration of the counter-ion also can affect the chromatographic behavior. The influence of the counter-ion concentration was investigated on ZWIX CSPs operated in zwitterionic and cation-exchange modes. In the mobile phase the counter-ions act as competitor at the interaction site with the analyte. According to the stoichiometric displacement model, a linear relationship is found between the plot of the logarithm of the retention factor of the first-eluted enantiomer (log k_1) and the logarithm of the counter-ion concentration (log $c_{counter\text{-}ion}$). The different slopes of log k_1 *vs.* log $c_{counter\text{-}ion}$ curves obtained for CSPs operating in zwitterionic or single ion-exchange mode indicate different mechanisms operating in two chromatographic modes.

5) Structure-retention relationships

Structure – retention (selectivity) relationships were studied on four ZWIX CSPs to demonstrate the effects of structure of SAs (and SOs) on chromatographic parameters.
It was expected that the different nature and the position of the substituent exert a significant influence on the solute polarity, the geometrical structure and hence the retention and selectivity.

For β-2- and β-3-amino acids a direct relationship was registered between the alkyl chain length of the SA and the k_1 or α values. The volume of alkyl chains through the steric interactions directly affects these values. The aromatic substitution has a strong

effect on retention. Probably due to the enhanced π-π interactions, the k_1 values were much higher on both ZWIX CSPs as compared with aliphatic ones. Especially high *k* values were obtained for β-2-amino acid containing a naphthol ring. The $-CH_3$ or $-N(CH_3)$ groups on the benzyl ring exhibited small effect on the retention and selectivity. The presence of the –Cl, –F, –OH group or –O– (or in aromatic moity) may improve the interaction with the SO through the H-bonding, which resulted higher retention, while the α values changed differently. The position of –OH and $-CH_3$ group exhibits a slight effect on the retention and selectivity. The presence of heteroatoms in the aromatic ring in β-3-amino acids such as –N–, –S– and –O– moieties showed slight effect on the retention and enantioselectivity, but their *meta* position resulted in higher k_1 and α values, indicating a favored SA-SO interaction.

In case of TIQ analogs the methoxy-substitution resulted in higher k_1 and α values comparing to the non-substituted ones. The presence of methoxy group may improve the chiral recognition through additional H-bond interactions. The SAs containing methyl group in position 1 were not or only partly separable on ZWIX(–)™ and ZWIX(+)™ CSPs comparing to the SAs with methyl group in position 3. This indicates the importance of steric arrangement and/or steric contribution to chiral recognition. The length of the alkyl chain of tetrahydroisoqunoline skeleton influences both k_1 and α values, they decreased slightly with increasing chain length of the hydroxyalkyl-substitutions. The increasing chain length was unfavorable regarding chiral recognition.

6) Relationships between elution sequence and the type of the cinchona alkaloids

Although *quinine* and *quinidine* are diastereomers, they often display quasi-enantiomeric behavior. Therefore they are called pseudo-enantiomers. This specific feature is very advantageous in trace analyses. On *quinine*- and *quinidine*-based CSPs opposite elution sequence could be obtained. The elution sequence was determined in most cases for β-amino acids and secondary amines. In case of the alphatic β-2- and β-3-amino acids and TIQ analogs, the elution sequence was found to be (*R*) < (*S*) on ZWIX(+)^{TM} CSP and (*S*) < (*R*) on ZWIX(–)^{TM} CSP. However, the elution sequence of β-3-amino acids containing aromatic moiety or heteroatoms was (*S*) < (*R*) on ZWIX(+)^{TM} CSP and (*R*) < (*S*) on ZWIX(–)^{TM} CSP. The sequence of elution of *trans*-paroxetine enantiomers on ZWIX(+)^{TM}, ZWIX(–)^{TM} and ZWIX(–A) CSPs was the same [(–) < (+)], whereas on ZWIX(+A) CSP it was opposite [(+) < (–)].

7) Temperature dependence and thermodynamic parameters

The effect of temperature was investigated between -5 °C and 50 °C on ZWIX(–)^{TM}, ZWIX(+)^{TM}, ZWIX(–A) and ZWIX(+A) CSPs. In most cases, the chromatographic parameters (k_1, α and R_S) decreased with increasing temperature, but some exceptions were registered (*e.g.* for β-2-amino acids, TIQ analogs and *trans*-paroxetine enantiomers). From van't Hoff plots, entropy and enthalpy changes were calculated. In most cases, enthalpy-controlled separations were achieved, but for *trans*-paroxetine enantiomers on ZWIX(+)^{TM} CSP and for some β-amino acids entropy-controlled separations were also observed. (In entropy-controlled separations, higher selectivity can be achieved at higher temperature). For TIQ analogs in some cases, both enthalpy- and entropy-controlled separations were observed with increasing temperature.

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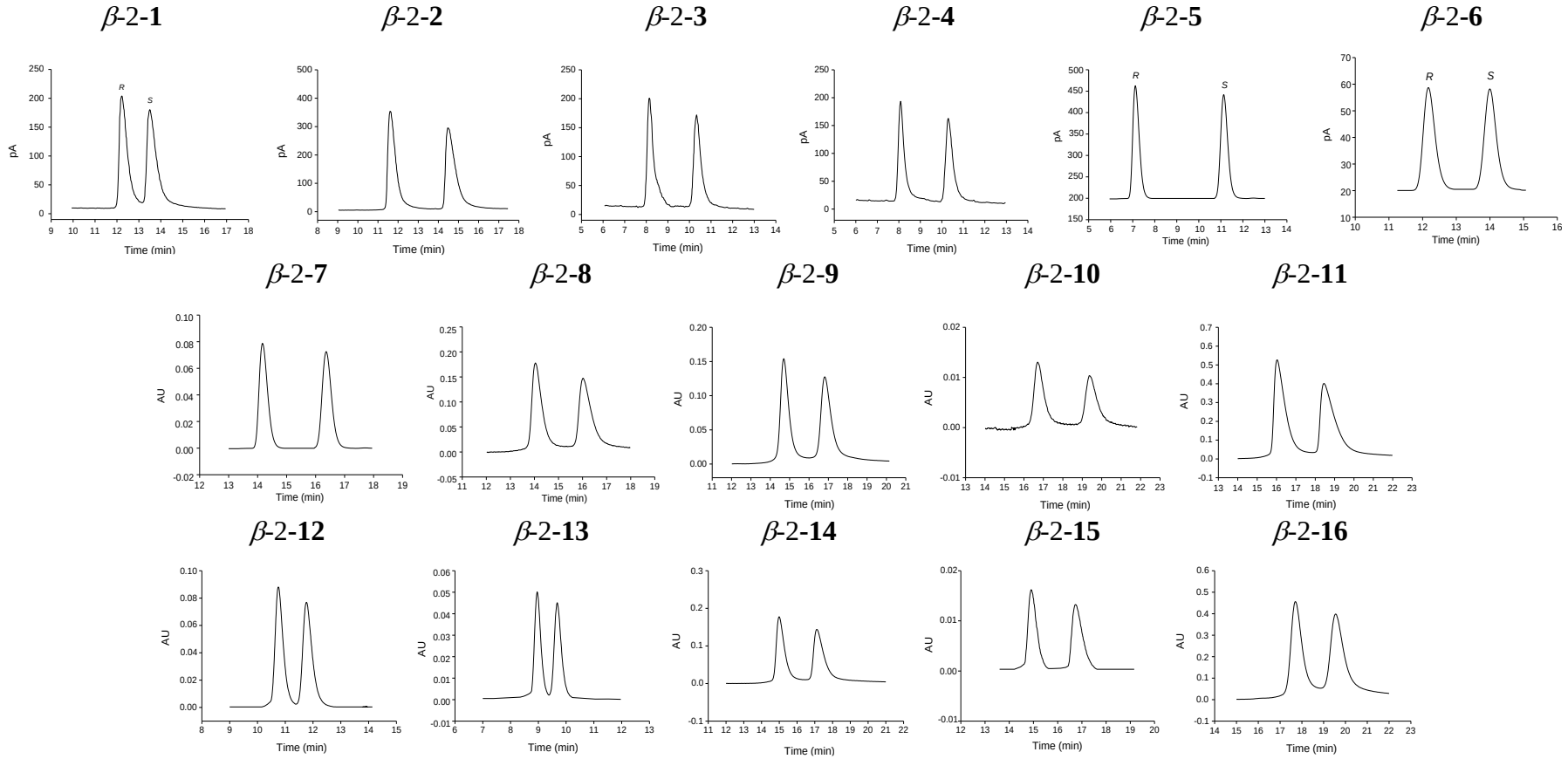
APPENDIX

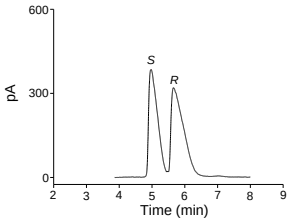
Selected chromatograms of β-2-amino acids



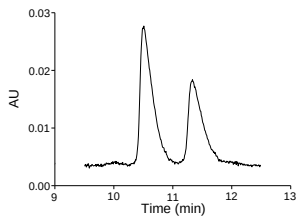
*Chromatographic conditions: column, ZWIX(+)^{TM} for analytes **1**, **2**, **4**–**6** and **15**, ZWIX(−)^{TM} for analytes **3**, **7**–**14** and **16**; mobile phase, MeOH/MeCN (50/50, v/v) containing 25 mM NH3 and 50 mM AcOH for analytes **4**, **5** and **12**, MeOH/MeCN (50/50, v/v) containing 25 mM TEA and 50 mM AcOH for analytes **1**–**3**, **6**–**11**, **14** and **16**, MeOH/MeCN (50/50, v/v) containing 25 mM TEAP for analytes **13** and **15**; flow rate, 0.6 ml min^{-1}; detection 230 nm or Corona detector; temperature, 25 °C*

Selected chromatograms of β-3-amino acids

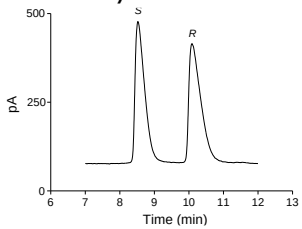
β-3-17



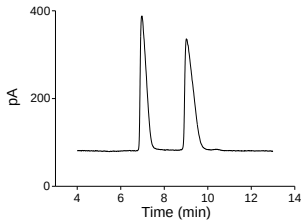
β-3-18



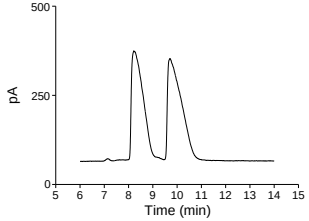
β-3-19



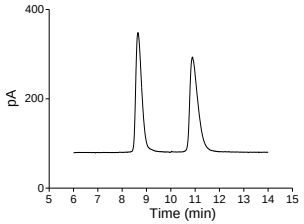
β-3-20



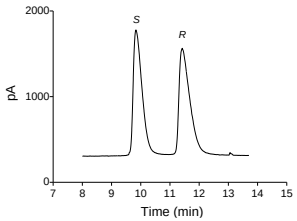
β-3-21



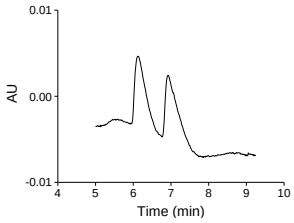
β-3-22



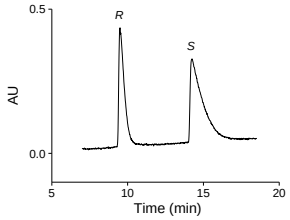
β-3-23



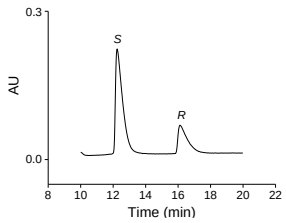
β-3-24



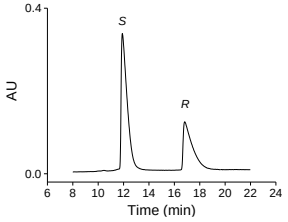
β-3-25



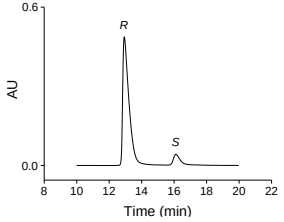
β-3-26



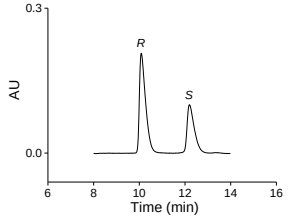
β-3-27



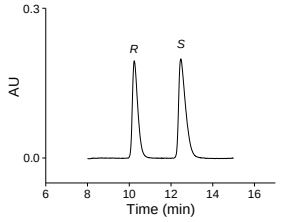
β-3-28



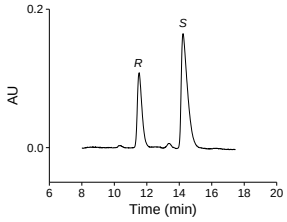
β-3-29



β-3-30

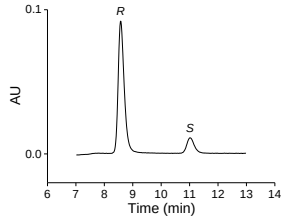


β-3-31

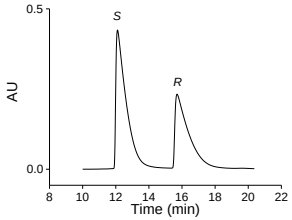


Selected chromatograms of β-3-amino acids (continued))

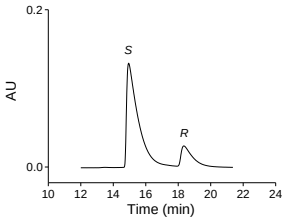
β-3-**32**



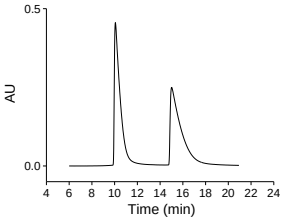
β-3-**33**



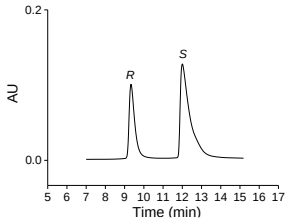
β-3-**34**



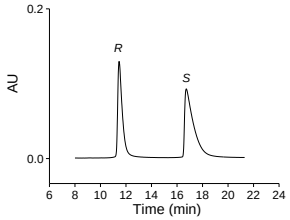
β-3-**35**



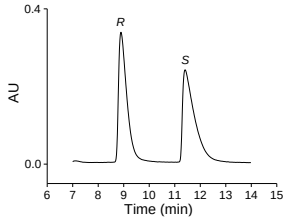
β-3-**36**



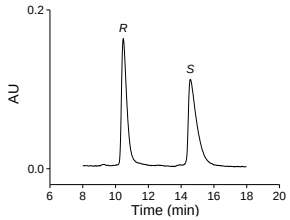
β-3-**37**



β-3-**38**



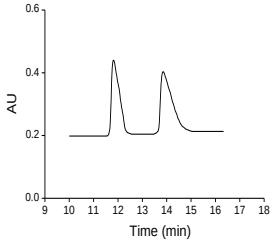
β-3-**39**



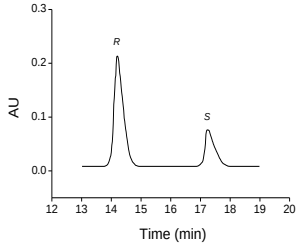
*Chromatographic conditions: column, ZWIX(−)^{TM} for analytes **17**, **24**−**27**, **33**−**39**, ZWIX(+)^{TM} for analytes **18-22**, **23**, **28**−**32**; mobile phase, MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM AcOH for analytes **17**−**20**, **22**, **23**, **25**−**27**, **33**−**39**, H_2O/MeCN (10/90 v/v) containing 25 mM TEA and 50 mM AcOH for analytes **21, 24**, **29**−**32**; flow rate, 0.6 ml min^{-1}; detection 215, 230 or 260 nm; temperature, 25 °C*

Selected chromatograms of TIQ analogs

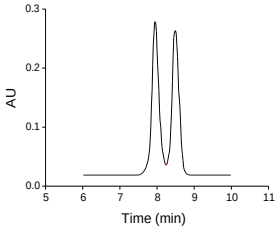
TIQ-**40**



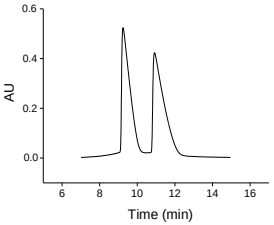
TIQ-**41**



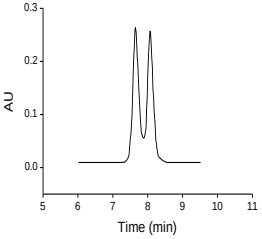
TIQ-**42**



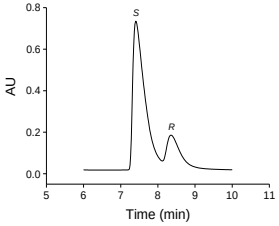
TIQ-**43**



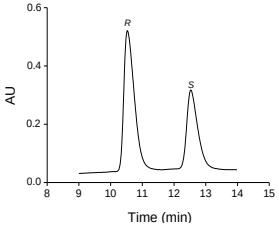
TIQ-**44**



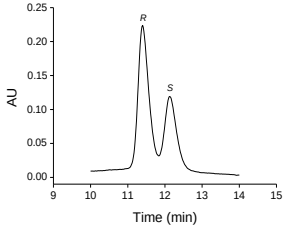
TIQ-**47**



TIQ-**48**

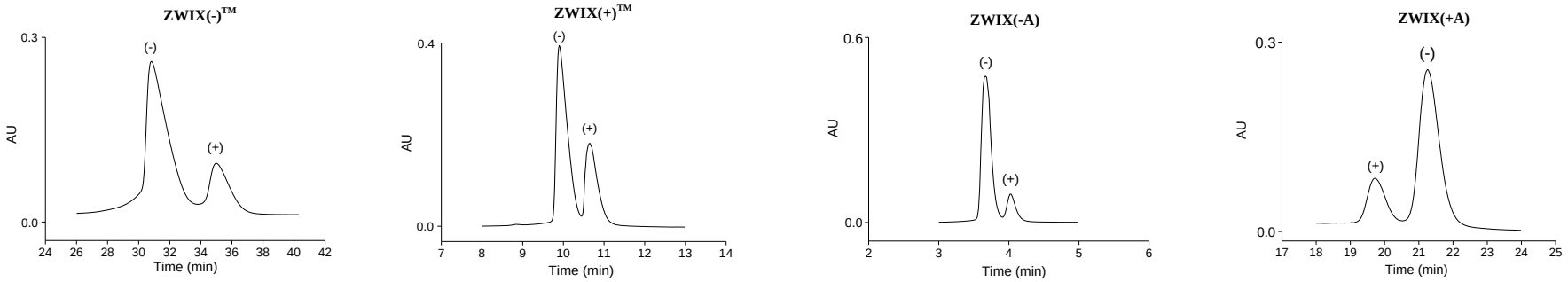


TIQ-**49**



Chromatographic conditions: column, ZWIX(+)™ for ***40-42***, ***44***, ***48-49*** *and ZWIX(-)™ for* ***43*** *and* ***47****; mobile phase, for* ***40-44***, ***47-49*** *MeOH/MeCN (25/75 v/v) containing 12.5 mM TEA and 25 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 230 nm and 258 nm; temperature, 25 ºC; peaks on chromatograms for* ***41*** *and* ***47-49*** *are the mixture of the racemic compound and enantiomer*

Selected chromatograms of trans-paroxetine enantiomers



Chromatographic conditions: ZWIX(–)^{TM} MeOH/THF (10/90, v/v) containing 25 mM DEA and 50 mM FA, ZWIX(+)^{TM} and ZWIX(–A)^{TM} MeOH/THF (80/20, v/v) containing 25 mM DEA and 50 mM FA, ZWIX(+A)^{TM} MeOH/THF (20/80, v/v) containing 25 mM DEA and 50 mM FA; flow rate, 0.6 ml min^{-1}; detection 295 nm; temperature, 25 °C