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High-Performance Liquid Chromatographic Enantioseparation of Unusual Cyclic *β*-Amino Acids

Ph.D. Thesis

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CONTENTS

Publication list

Papers related to the thesis

I. **Zsanett Gecse**, István Ilisz, Melinda Nonn, Nóra Grecsó, Ferenc Fülöp, Rajalingam Agneeswari, Myung Ho Hyun and Antal Péter
High-performance liquid chromatographic enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on chiral ligand-exchange stationary phase.
Journal of Separation Science 36 (**2013**) 1335-1342. **i.f.: 2.594**

II. Zoltán Pataj, István Ilisz, **Zsanett Gecse**, Zsolt Szakonyi, Ferenc Fülöp, Wolfgang Lindner, Antal Péter
Effect of mobile phase composition on the liquid chromatographic enantioseparation of bulky monoterpene-based β-amino acids applying chiral stationary phases based on Cinchona alkaloid.
Journal of Separation Science 37 (**2014**) 1075-1082. **i.f.: 2.737**

III. István Ilisz, Zoltán Pataj, **Zsanett Gecse**, Zsolt Szakonyi, Ferenc Fülöp, Wolfgang Lindner, Antal Péter
Unusual temperature-induced retention behaviour of constrained ß-amino acid enantiomers on the zwitterionic chiral stationary phases ZWIX(+)^{TM} and ZWIX(-)^{TM}.
Chirality 26 (**2014**) 385-393. **i.f.: 1.886**

IV. István Ilisz, **Zsanett Gecse**, Gyula Lajkó, Melinda Nonn, Ferenc Fülöp, Wolfgang Lindner, Antal Péter
Investigation of the structure-selectivity relationships and van't Hoff analysis of chromatographic separations of unusual isoxazoline-fused 2-aminocyclopentanecarboxylic acids on Cinchona alkaloid-based chiral stationary phases.
Journal of Chromatography A 1384 (**2015**) 67-75. **i.f.: 3.926**

Total impact factor: 11.143

Other publications

V. László Sipos, István Ilisz, Anita Aranyi, **Zsanett Gecse**, Melinda Nonn, Ferenc Fülöp, Myung Ho Hyun and Antal Péter
High-performance liquid chromatographic enantioseparation of unusual isoxazoline-fused 2-aminocyclopentane-carboxylic acids on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phases.
Chirality 24 (**2012**) 817-824. **i.f.: 1.894**

VI. István Ilisz, **Zsanett Gecse**, István Szatmári, Ferenc Fülöp, Antal Péter
High-performance liquid chromatographic enatioseparation of naphthol substituted tetrahydroisoquinolines on polysaccharide-based chiral stationary phases.
Biomedical Chromatography 28 (**2014**) 142-151. **i.f.: 1.723**

VII. István Ilisz, **Zsanett Gecse**, Zoltán Pataj, Ferenc Fülöp, Géza Tóth, Wolfgang Lindner, Antal Péter
Direct high-performance liquid chromatographic enantioseparation of secondary amino acids on Cinchona alkaloid-based chiral zwitterionic stationary phases. Unusual temperature behavior.
Journal of Chromatography A 1363 (**2014**) 169-177. **i.f.: 4.169**

VIII. Nóra Grecsó, István Ilisz, **Zsanett Gecse**, László Schönstein, Ferenc Fülöp, Antal Péter
High-performance liquid chromatographic enantioseparation of amino alcohol analogues possessing 1,2,3,4-tetrahydroisoquinoline skeleton on polysaccharide-based chiral stationary phases.
Biomedical Chromatography 29 (**2015**) 788-796. **i.f.: 1.729**

IX. István Ilisz, **Zsanett Gecse**, Gyula Lajkó, Enikő Forró, Ferenc Fülöp, Wolfgang Lindner, Antal Péter
High-performance liquid chromatographic enatioseparation of cyclic β-amino acids on zwitterionic chiral stationary phases on Cinchona alkaloids.
Chirality, 27 (**2015**) 563-570. **i.f.: 2.025**

Commulative impact factor: **22.683**

Conference lectures

I. **Gecse Zsanett**, Nonn Melinda, Fülöp Ferenc, Péter Antal
Izoxazolin gyűrűvel kondenzált ciszpentacin származékok folyadékkromatográfiás vizsgálata koronaéter alapú királis állófázisokon
XXXV. Kémiai Előadói Napok
Szeged, Hungary, 29-31, Oktober, 2012, Abstr.: p. 61, oral presentation

II. Grecsó Nóra, **Gecse Zsanett**, Nonn Melinda, Fülöp Ferenc, Péter Antal
Izoxazolin gyűrűvel kondenzált 2-aminociklopentán karbonsav analógok sztereoizomerjeinek elválasztása ligandumcserés királis állófázison
XXXV. Kémiai Előadói Napok
Szeged, Hungary, 29-31, Oktober, 2012, Abstr.: p. 62, oral presentation

III. **Gecse Zsanett**, Grecsó Nóra, Nonn Melinda, Fülöp Ferenc, Péter Antal
Izoxazolin gyűrűvel kondenzált ciszpentacin származékok sztereoizomerjeinek elválasztása ligandumcsrés királis állófázison
Elválasztástudományi Vándorgyűlés 2012
Hajdúszoboszló, Hungary, 7-9, November 2012, Abstr.: P-41, poster presentation

IV. **Gecse, Zsanett**, Grecsó Nóra, Nonn Melinda, Fülöp Ferenc, Péter Antal
Izoxazolin gyűrűvel kondenzált 2-aminociklopentán karbonsav származékok sztereoizomerjeinek elválasztása ligandumcserés királis állófázison
Elválasztástudományi Ankét 2013
Budapest, Hungary, 13. May 2013, Hotel, Mercure Budapest Buda, oral presentation

V. Péter Antal, **Gecse Zsanett**, Ilisz, István, Szatmári István, Fülöp Ferenc
Tetrahidroizokinolin-vázas analógok királis kromatográfiája
Vegyészkonferencia 2013
Hajdúszoboszló, Hungary, 26-28, June 2013, poster presentation

VI. **Zsanett Gecse**, István Ilisz, István Szatmári, Ferenc Fülöp, Antal Péter
HPLC Enantioseparation of Naphthol-Substituted Tetrahydroisoquinolines on Polysaccharide-Based Chiral Stationary Phases
9th Balaton Symposium on High-Performance Separation Methods
Siófok, Hungary, 4-6, September 2013, Abstr.: P-44, poster presentation

VII. Ilisz I., Aranyi A, **Gecse Zs.,** Wernisch S., Lindner W., Péter A.
Enantioseparation of Amino-Acids on Newly Developed Zwitterionic Chiral Stationary Phases
9th Balaton Symposium on High-Performance Separation Methods
Siófok, Hungary, 4-6, September 2013, Abstr.: P-45, poster presentation

VIII. **Gecse Zsanett,** Ilisz, István, Fülöp Ferenc, Péter Antal
Naftol szubsztituált tetrahidroizokinolin analógok HPLC elválasztása poliszacharid alapú királis állófázisokon
XXXVI. Kémiai Előadói Napok
Szeged, Hungary, 28-30, Oktober, 2013, Abstr.: p. 37, oral presentation

IX. A. Péter, I. Ilisz, **Z. Gecse**, D. W. Armstrong, M.-H. Hyun, W. Lindner
High-performance liquid chromatographic enantioseparation of unusual amino acids
Advances in Chromatography and Electrophoresis & Chiranal 2014
Olomouc, Czech Republic, 10-14, February 2014, oral presentation

X. Antal Péter, István Ilisz, **Zsanett Gecse,** Wolfgang Lindner
High-performance liquid chromatographic enantioseparation of unusual amino acids
30th International Symposium on MicroScale Bioseparations
Pécs, Hungary, 27 April - 1 May 2014, oral presentation

XI. **Gecse Zsanett,** Ilisz, István, Nonn Melinda, Fülöp Ferenc, Péter Antal
Ikerionos típusú állófázisok alkalmazása izoxazolin gyűrűvel kondenzált 2-aminociklopentán karbonsav származékok sztereoizomerjeinek királis megkülönböztetésére
Elválasztástudományi Vándorgyűlés 2014
Egerszalók, Hungary, 12-14, November 2014, Abstr.: P-15, poster presentation

XII. Grecsó Nóra**,** Ilisz, István, **Gecse Zsanett,** Schönstein László, Fülöp Ferenc, Péter Antal
1,2,3,4-tetrahidroizokinolin vázú aminoalkoholok és származékaik enantiomerjeinek folyadékkromatográfiás elválasztása poliszacharid alapú állófázisokon
Elválasztástudományi Vándorgyűlés 2014
Egerszalók, Hungary, 12-14, November 2014, Abstr.: P-18, poster presentation

Abbreviations and symbols

AcOH	glacial acetic acid
BA	butylamine
CLE	chiral ligand-exchange
CLEC	chiral ligand-exchange chromatography
CSP	chiral stationary phase
Cu(II) ion	copper ion
$Cu(ClO_4)_2$	copper perchlorate
$Cu(NO_3)_2$	copper nitrate
$CuSO_4$	copper sulphate
DEA	diethylamine
EtOH	ethanol
EA	ethylamine
FA	formic acid
HILIC	hydrophilic interaction liquid chromatography
HPLC	high-performance liquid chromatography
Column IV	sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecilamino acetate-based CSP
MeCN	methyl cyanide
MeOH	methanol
NH_3	ammonia
NPM	normal-phase mode
PIM	polar-ionic mode
POM	polar-organic mode
PA	propylamine
PrOH	propan-1-ol
QN	quinine
QD	quinidine
RPM	reversed-phase mode
SCX	strong cation exchanger
SAs	selectands (analytes)
SOs	selectors
TEA	triethylamine
TPA	tripropylamine
TBA	tributylamine
(*v*/*v*)	volume to volume ratio
WAX	weak anion exchanger
ZWIX	zwitterionic exchanger
ZWIX(+)™	quinine-based zwitterionic stationary phase
ZWIX(-)™	quinidine-based zwitterionic stationary phase

k	retention factor: defined as $(t_R-t_0)/t_0$; t_R, retention time of analyte; t_0, column dead-time
α	separation factor: defined as k_2/k_1
R_S	resolution: defined as $2(t_{R2}-t_{R1})/(w_1+w_2)$; w, peak width measured on the baseline

1. INTRODUCTION AND AIMS

1.1. The importance of liquid chromatography

Chirality and the separation of chiral compounds have become increasingly important in recent decades in the pharmaceutical industry and modern analytical chemistry regarding both scientific and economic perspective.

Pharmaceutical properties of enantiomers of chiral drugs (*e.g*. metabolism, protein binding, receptor binding, absorption, excretion, activation–inhibition, *etc*.) may differ in the course of interaction with receptors [1]. Often only one of the enantiomers is responsible for the therapeutic effect (eutomer), while the other isomer (distomer) should ideally be harmless. The distomer, however, may trigger some undesirable side effects or even may be toxic [2]. Therefore, it is important to have enantiomers in chirally pure form [3]. Varied procedures, among others, crystallization, chiral derivatization, enzymatic resolution, *etc*. are available for the preparation of pure enantiomers. The most important methods, however, are (**a**) enantioselective synthesis and (**b**) separation of racemic mixtures. To check the chiral purity of the end product is always indispensable regardless the preparation method used. Pharmaceutical drug development and impurity control require highly reproducible techniques with high sensitivity and stereoselectivity. For this purpose, the most suitable and most frequently used method is high-performance liquid chromatography (HPLC) with the application of chiral columns.

1.2. The significance of chirality

As discussed above, the interest in the separation of chiral compounds, particularly in pharmaceutical research, has been increased nowadays. Chiral molecules in living organisms have paramount importance because amino acids in proteins, sugars, enzymes, *etc.* are chiral compounds.

The word ”*chirality*” origins from Greek – kheir – meaning ‘handness’ [4]. In the simplest type of chiral molecules four different atoms or groups are attached to the carbon atom (asymmetric center) (***Figure 1*****).**

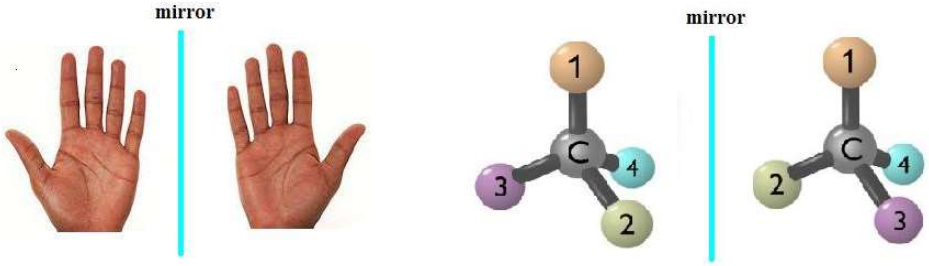

Figure 1. The concept of chirality

Chirality can be classified as central, axial, planar and helical systems. The chiral molecule and its mirror image are enantiomers to each other. The atoms of two enantiomer molecules have different spatial arrangements; therefore, the enantiomers are stereoisomers. The biological effects of chiral molecules can be significantly different even though their identical physical and chemical properties are alike except for the direction of the rotation of plane-polarized light [5]. A sample, which contains enantiomers in a 50:50 ratio, is called a racemate and it does not rotate the plane-polarized light. However, biological systems are able to distinguish enantiomers.

In the pharmaceutical and drug industries, the existence of chirality became particularly important after the thalidomide tragedy in the 1960s. Thalidomide, in its racemic form, was put on the market in the late 1950s as a sedative. The (+)-isomer was even therapeutic but the harmful (–)-form was shown to be responsible for the catastrophic malformations of embryos when thalidomide was administrated to women during pregnancy [6-8].

It is understandable, therefore, that chirality has become a very important issue in terms of the safety of drug production. Stereoisomeric mixtures as drug substances have been allowed to be marketed only in reasonable cases for a long time.

1.3. Aims of the work

The primary aim of this work was to develop simple, readily available chiral HPLC methods for the resolution of stereoisomers of β-amino acids, endowed both biological and pharmaceutical interest on newly developed chiral stationary phases (CSPs), such as chiral ligand-exchange-based (CLEC) and zwitterion-exchange-based (ZWIX) CSPs.

- The enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogues on chiral ligand-exchange column has been studied. The influence of

mobile phase composition and Cu(II) salts with different anions, the effect of $Cu(ClO_4)_2$ concentration, the structure of analytes, and temperature on the chiral recognition have been examined.

- The enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acid stereoisomers was investigated on new types of zwitterionic chiral stationary phases based on *Cinchona* alkaloids, with the aim of optimizing chromatographic conditions (mobile phase composition, bulk solvent composition, temperature, *etc*.).
- For the enantioseparation of monoterpene-based β-amino acids, zwitterionic chiral stationary phases based on *Cinchona* alkaloids were used, with the aim of developing new chromatographic methods for their chiral separation.
- The separation efficiencies of the two types of CSPs (CLEC and ZWIX) were compared on the basis of chromatographic data obtained for isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogues.

In all measurements, the chromatographic parameters (retention factor, selectivity factor, resolution) were determined. In the case of temperature dependence, thermodynamic parameters were calculated from plots of ln α versus 1/T.

2. LITERATURE REVIEW

Enantiomers have identical physical and chemical properties in an achiral environment, and their discrimination needs a chiral environment. The procedures used for the chromatographic separation of enantiomeric pairs can be classified into two methods: (1) indirect separations and (2) direct methods [9,10].

Indirect separation can be carried out on achiral stationary phases after the use of chiral derivatization agents to form diastereomeric derivatives.

Nowadays, the *direct method* is the most preferred separation method, which involves two techniques: either the application of a chiral mobile phase additive and enantioseparation on an achiral stationary phase or application of chiral stationary phases.

In this study, the direct method was preferred with the application of CSPs.

2.1. Direct separation with CSPs

The application of CSP is a convenient and widespread method for enatioseparation, where the chiral selector is covalently linked to or physically adsorbed on a silica support. It is the method of choice for both analytical and preparative applications.

When using CSP, the degree of separation is determined by the different interactions of enantiomers with the stationary phase (*e.g.* hydrogen bonding, π–π, dipole–dipole, ionic, electrostatic, hydrophobic or hydrophilic interactions, steric hindrance, *etc.*). According to *Dalgliesh*, at least a three-point interaction is necessary between the selectors (SOs) and selectands (SAs) in order to discriminate enantiomers [11]. *Pirkle* complemented this theory: at least one of these interactions must be stereochemically controlled [12]. The *three-point interaction model* (***Figure 2***) is the most reliable and still frequently utilized model to explain the process of chiral recognition.

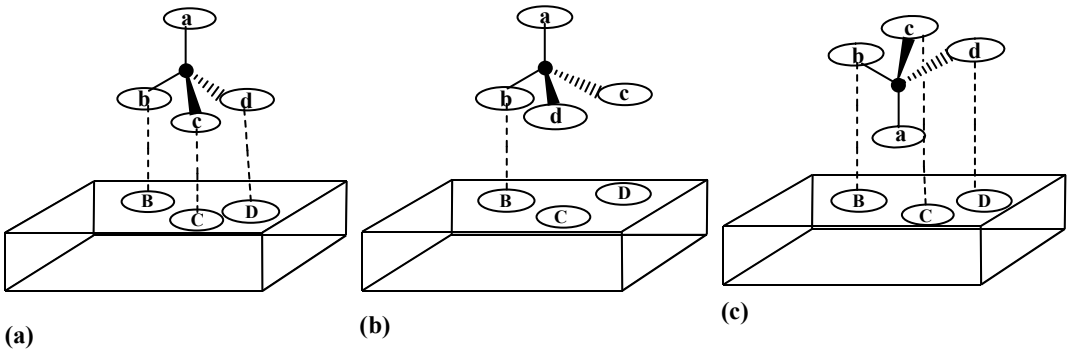

Figure 2. Explanation of the three-point model

CSPs can be grouped in several ways. Depending on their separation principles, the main classes are as follows: Pirkle-type, polymer- and protein-based, cavity-type, macrocyclic antibiotic-based, ligand-exchange, and molecularly imprinted CSPs [13] (***Table 1***).

Table 1. Groups of chiral stationary phases

Type of CSP		Selector	The main interactions
1.	Donor–acceptor (Pirkle-type CSP)	π-acidic – and π-basic groups	π–π and dipole
2.	Polymer	modified cellulose and amylose	polar and steric
3.	Protein	natural protein	ionic and hydrophobic
4.	Cavity	chiral crown ether	steric and complexation
		cyclodextrin, modified cyclodextrin	
5.	Macrocyclic antibiotic	macrocyclic glycopeptide	H-bonding, hydrophobic and polar
6.	Ligand exchange	amino acid–metal complex	complexation
7.	ion-exchanger	anion-, cation- and zwitter-ion-based selectors	ionic, polar, steric
8.	Molecular imprinted	selective sorbent (*e.g.* macromolecules, organic molecules)	steric

2.1.1. Macrocyclic antibiotic-based CSPs

The concept of using macrocyclic glycopeptides as chiral selectors was first introduced by *Armstrong* and co-workers [14,15]. Based on the number and impact of the publications dealing with enantioseparation on macrocyclic glycopeptides, it can be stated that these are still one of the most powerful CSPs available [16-27].

There are numerous macrocyclic antibiotic compounds, which are used as chiral selectors in HPLC including glycopeptides (vancomycin, teicoplanin, ristocetin A, avoparcin, and their analogues), ansamycins (rifamycins), and the polypeptide antibiotic thiostrepton. They have various structures with different atoms and groups along with basket-type moieties. Applying these CSPs, enantioseparation may be possible via numerous different mechanisms of interaction (*e.g.* hydrophobic, π–π complexation, hydrogen-bonding, dipole stacking, steric effects, electrostatic and short-distance van der Waals interactions or combinations thereof) between SO and SA, because of the variety of the functional groups [28-30]. These CSPs can be used efficiently in different chromatographic modes, such as normal- and reversed-phase mode (NPM and RPM), polar ionic mode (PIM), and polar organic mode (POM) [31-33,34]. The possibility of operating in different modes is one of the major advantages of these CSPs.

The macrocyclic glycopeptides-based CSPs have been commercialized under the trademark Chirobiotic^{TM} by Astec and very recently by Merck.

2.1.2. Chiral crown ether phases

The chiral crown ether-based CSPs group belongs to cavity-type stationary phases. Chiral separations with cavity-type CSPs are based on inclusion complexation and are achieved through a mechanism by which the guest molecule is accepted into the cavity of a host molecule. The host molecule possesses functional groups that act as barriers or interact with the guest molecule in a way ensuring enantioselectivity [35,36].

The name "*crown ether*" was proposed by *Pedersen*, when cations can be reversibly "crowned" [37]. Crown ethers possess useful properties in chromatography because various alkali metal and ammonium ions may form inclusion complexes. (*S,S*)-*bis*(Binaphthyl)-22-cown-6 ether, the first synthetic chiral macrocycle, was synthetized by *Cram et al.* [38]. Since then, numerous chiral crown ether-based CSP have been prepared (*e.g*. crown ethers containing binaphthyl unit, carbohydrate analogues, amino acids, amines and their derivatives, heterocyclic units, tartaric acid and its derivatives, and crown ethers containing phenol derivatives) [39-44]. These CSPs have been successfully applied for the separation of the enantiomers of primary amines [45-51].

The chiral recognition mechanism has not completely clarified yet. Nevertheless, the diastereoselective complexation of the primary ammonium group ($R\text{-}NH_3^+$) of the SAs within the chiral crown ether cavity of the CSP has been found to be essential [52].

2.1.3. Ligand-exchange CSPs

Thanks to the pioneering work of *Davankov* and *Rogozhin* [53-56], chiral ligand-exchange chromatography (CLEC) enjoys widespread applications, particularly for the separation of amino acids, amino alcohols, peptides, alkaloids, and hydroxy acids.

The basic principle of CLEC is the reversible coordination of immobilized SOs and SAs within the transition metal cation [Zn(II), Ni(II), and often Cu(II)] coordination sphere [57]. Regarding the mechanism of the ligand-exchange separation, the generally accepted view is that the Cu(II)–SO–SA complex forms a five-membered chelate ring and the ternary complex exhibits a square planar structure [58-60]. The separation is based on the differences in the strengths of complexes of the two enantiomers and, correspondingly, they spend different times on the stationary phase. When the Cu(II) ion and the polar groups form a planar structure through a coordination bond, the remaining part of the analyte is able to position above or below the plane. If the SA is on the same side of the fixed segment, then it has *cis* position. However, if they are on the opposite side, then the *trans* complex is

formed. Compounds, which are able to form such ternary complexes, are probably separable on a ligand-exchange CSP. The structure of such ligand-exchange stationary phase is depicted in ***Figure 3***. The mobile phase includes Cu(II) ions, which interact with the amino, carboxyl, and hydroxyl groups of SOs and with carboxyl and amino groups of the SAs (***Figure 3***).

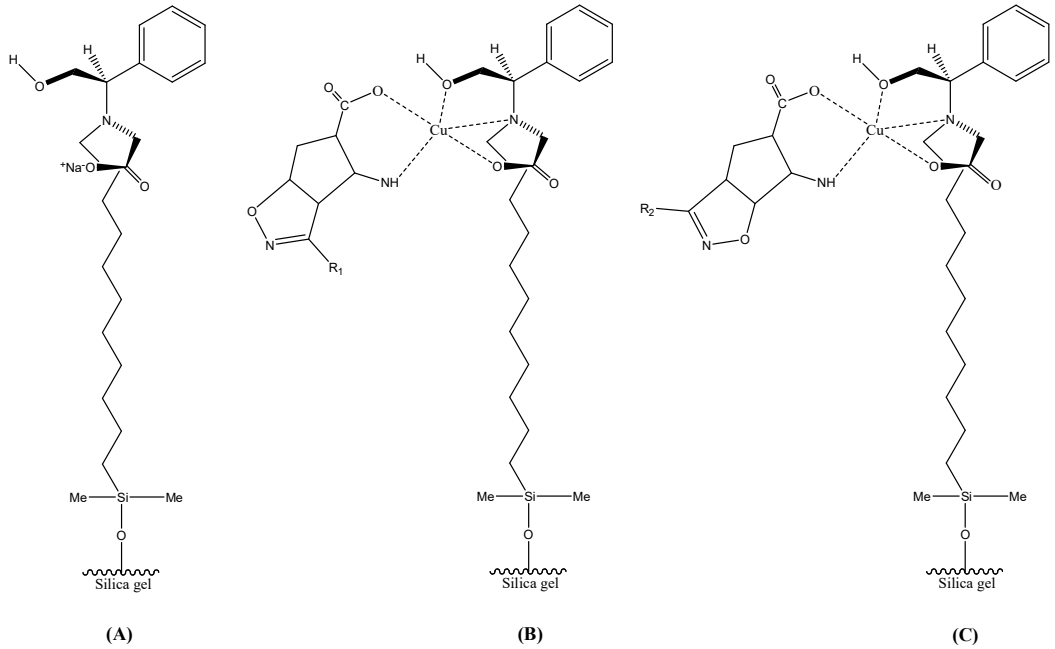

Figure 3. Structure of the selector of CLEC (A) and its terner complex with isoxazoline-fused 2-aminocyclopentanecarboxylic acids **1,2** (B) and **3,4** (C)

Considering the number of publications, CLEC has attracted significant research interest. α-Amino acids were separated by *Hare et al.* [61] and *Wagner et al.* [62] using their Cu(II) complexes as chiral mobile phase additives. *Davankov* and co-workers immobilized heptyl-, decyl-, and hexadecyl-L-hydroxyproline on silica gel. They successfully separated amino acid enantiomers and studied the effect of pH, Cu(II) and NH_4OAc concentration and the effect of organic modifier as well [63]. One year later, *N*-decyl-L-histidine and *N*-τ-*n*-decyl-L-histidine were dynamically bonded to the C18 stationary phase [64,65]. The separation of α-amino acid enantiomers was carried out in the presence of Cu(II) ions and the elution sequence was found to be L<D. *Karger et al.* **[**66,67**]** incorporated *n*-butyl and *n*-decyl spacer chains between the selector (α-amino acid derivative) and silica gel in order to improve selectivity. These columns were successfully applied in the presence of Cu(II) (as

mobile phase additive) for the separation of amino alcohols and dansyl-D,L-amino acids. *Gübitz et al.* [68,69] immobilized L-proline and L-hydroxyproline on silica gel via different spacer chains. They successfully separated α-amino acids, dansyl-amino acids, hydroxy acids, and various glycyl-dipeptides in the presence of $CuSO_4$. *Hyun et al.* [58,70-72] dynamically bonded new chiral selectors such as (1*S*,2*R*)-norephedrine, (*R*)-alaninol, (*S*)-*N*,*N*-carboxymethyl-undecyl leucinol sodium to the C18 stationary phase. These chiral selectors proved to be very efficient in the presence of Cu(II) for the separation of α-amino acids. (*R*)-*N*,*N*-Carboxymethyl-undecyl phenylglycinol sodium, a newer selector version, was covalently attached to silica gel. This CSP was applied for the separation of both α-amino and β-amino acids [73,74]. Nowadays, the commercially available ligand-exchange CSPs possess immobilized derivatives of L-proline [75], L-hydroxyproline [76,77] or L-histidine [78].

2.1.4. Donor–acceptor (Pirkle- or brush-type) CSPs

The first donor–acceptor CSPs were developed at the end of the 1970s. *Pirkle* and co-workers immobilized (*R*)-2,2,2-trifluoro-1-(9-anthryl)-ethanol on a silica support and separated the enantiomers of several π-acidic racemates [79-81].

Pirkle-type CSPs work according to the *Dalgliesh* concept [12,82-84]. In particular, π–π donor–acceptor interactions are essential for chiral recognition; therefore, most of these types of CSPs contain π-acidic and/or π-basic aromatic groups. These stationary phases are well suited for the separation of alcohols, aromatic acids, and their derivatives [85-88].

2.1.5. Zwitterionic-type CSPs

Chiral ion-exchangers may be regarded as Pirkle-type phases; however, they differ in utilization of ionizable groups. In the case of ion-exchange chromatography, the retention phenomenon primarily is based on interactions between the ions dissolved in the mobile phase and charged sites bonded to the stationary phase. Of *Cinchona* alkaloids, quinine has been successfully used for enantiomer separation in HPLC as a chiral ion-pairing agent in the mobile phase and as a ligand for chiral stationary phases [89-91]. In the 1990s, selectors based on *Cinchona* alkaloids were used as “brush”-type chiral stationary phases [92-94], which provide a weak anion exchange (WAX-type) group for ionic interactions. These *Cinchona* alkaloid-based columns exhibit broad applicability for enantiomer separation of

chiral acids comprising carboxylic, sulfonic, and phosphonic acids, preferentially in polar organic and reversed-phase modes.

Lindner *et al*. have applied quinine (QN) and quinidine (QD), two pseudo-enantiomeric *Cinchona* alkaloids with opposite configuration of two of their five stereocenters [95-98] (***Figure 4)***. The vinyl group (A) in QN and QD is often used for immobilization. The bulky quinuclidine system (B) contains a basic nitrogen atom (E), which is involved in protonation forming ionic interactions. The secondary hydroxyl group at C-9 (C) can serve as a site for modification. Carbamoyl modification significantly enhanced the enantiorecognition capabilities of the QN-based WAX-type stationary phase toward diverse chiral acids [92,99], in particular, when combined with a sterically demanding residue. The quinoline moiety (D) may establish π–π stacking and steric interactions. It contains a methoxy group, which may have a role in immobilization, too [100].

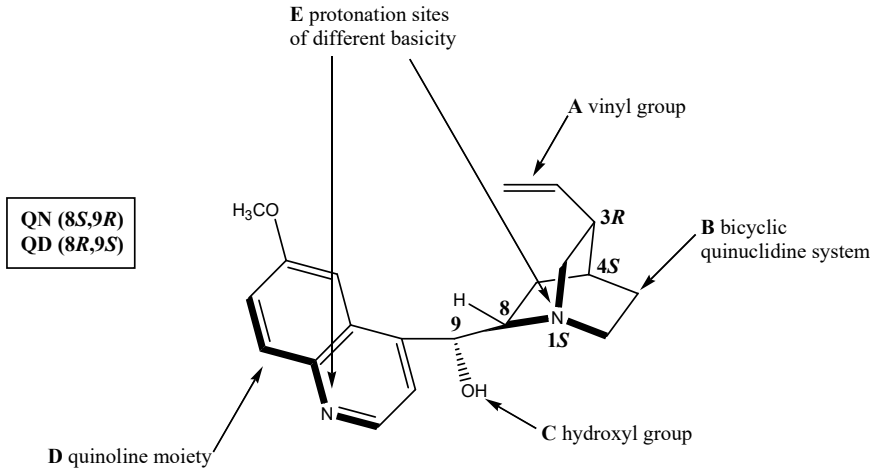

Figure 4. Structural properties of *Cinchona* alkaloids (Adapted from [95]).

Hoffmann et al. modified the structure of *Cinchona* alkaloids by incorporating a cyclohexane sulfonic acid group through a carbamoyl group at C-9 [95]. This modification ensured zwitterionic (ZWIX-type) anion and cation interactions between ampholytic analytes (amino acids, small peptides) and selectors (***Figure 5***).

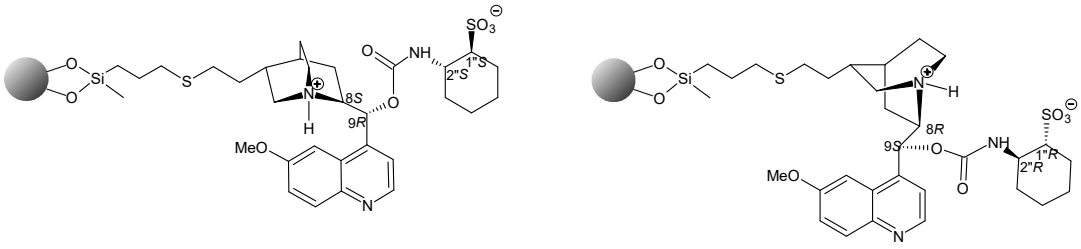

Figure 5. Structure of QN-based [ZWIX(+)^{TM}] and QD-based [ZWIX(-)^{TM}] columns

The application of these CSPs in non-aqueous polar–organic (POM) or polar–ionic (PIM) mode in the presence of low concentration of acid and base modifiers ensures zwitterionic interactions between ampholytic SAs and SOs.

2.2. Effect of temperature on retention of enantiomer separation

Enantioselective retention and separation are influenced by temperature [101-106]. For mechanistic considerations to be drawn, the differences in the changes in standard enthalpy $\Delta(\Delta H°)$ and entropy $\Delta(\Delta S°)$ of the enantiomers must be determined from a modified van't Hoff equation [*Eq.* (1)]:

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \phi \qquad (1)$$

$$\ln \alpha = -\frac{\Delta(\Delta H^\circ)}{RT} + \frac{\Delta(\Delta S^\circ)}{R} \qquad (2)$$

where k is the retention factor, $\Delta H°$ is the standard enthalpy of transfer of the solute from the mobile phase to the stationary phase, $\Delta S°$ is the standard entropy of transfer of the solute from the mobile phase to the stationary phase, R is the gas constant, T is temperature in Kelvin, ϕ is the phase ratio $\phi = V_S/V_M$ [the ratio of the volumes of the stationary phase (V_S) and the mobile phase (V_M)], $\Delta(\Delta H°)$ is the difference of the standard enthalpy of the two enantiomers, $\Delta(\Delta S°)$ is the difference of the standard entropy of the two enantiomers, and α is the selectivity factor ($\alpha = k_2/k_1$).

If $\Delta(\Delta H°)$ is invariant with temperature, then a linear van't Hoff plot is obtained. This expression shows that a plot of $R \ln \alpha$ *vs* $1/T$ has a slope of $-\Delta(\Delta H°)$ and an intercept of $\Delta(\Delta S°)$. If $\Delta(\Delta H)°$ and $\Delta(\Delta S)°$ are both negative, the enantioseparation is enthalpically driven, which is the most common situation.

2.3. The biological importance of β-amino acids

β-Amino acids have attracted considerable research interest during the past few decades due to their biological relevance. They play a central role in modern chemical research because of their important implication in synthetic and medicinal chemistry.

β-Amino acids are of minor significance compared to α-amino acids; however, some β-amino acids are found in more complex structures like alkaloids, lactones, and peptides [107]. In their free form, they show pharmacological effects. They have unique biological, neurological, and pharmaceutical activities. For example, they are components of some toxins and a special class of pore-forming lipopeptides, which exhibit antibacterial and antifungal activity [108].

On the other hand, unnatural β-amino acids are utilized as building blocks for the preparation of biologically active peptides, especially foldamers. The incorporation of conformationally constrained β-amino acids may provide peptides with a more rigid structure. This allows the investigation of receptor binding processes and preparation of peptide-based drug molecules with high biological potential [109-112].

Several representative compounds, such as oryzoxymycin and icofungipen, are bioactive compounds with antibacterial activities. For example, icofungipen disturbs the biosynthesis of proteins in *Candida albicans* and cispentacin exhibits antifungal properties. Isoxazolines are also valuable substances in medicinal chemistry, whereas various amino acids containing an isoxazoline moiety exhibit anti-influenza activity and antifungal properties. [113-116]. Amino acids bearing an isoxazoline ring can be used as precursors for the preparation of multifunctionalized derivatives with antiviral properties, such as peramivir and its analogues [117-120].

Commercially available enantiomerically pure monoterpenes and their analogues (α-pinene, 3-carene, and apopinene) can be used as building blocks for the synthesis of monoterpene-fused saturated 1,3-heterocycles or β-lactam-based peptidomimetics [121].

3. EXPERIMENTAL

3.1. Apparatus

Measurements were carried out on alternative HPLC systems.

System I: a Waters Breeze system consisting of a 1525 binary pump, a 2487 dual-channel absorbance detector, a 717 plus auto sampler, and Empower 2 data manager software (Waters Chromatography, Milford, MA, USA).

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

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal and reproducible retention factors were obtained for subsequent injections. This procedure was always followed when a new mobile phase or temperature was selected.

3.2. Investigated compounds

Isoxazoline-fused 2-aminocyclopentanecarboxylic acids (***Figure 6)*** [122,123] and monoterpene-based β-amino acids investigated (***Figure 7***) [124-127] were prepared at the Institute of Pharmaceutical Chemistry in Szeged.

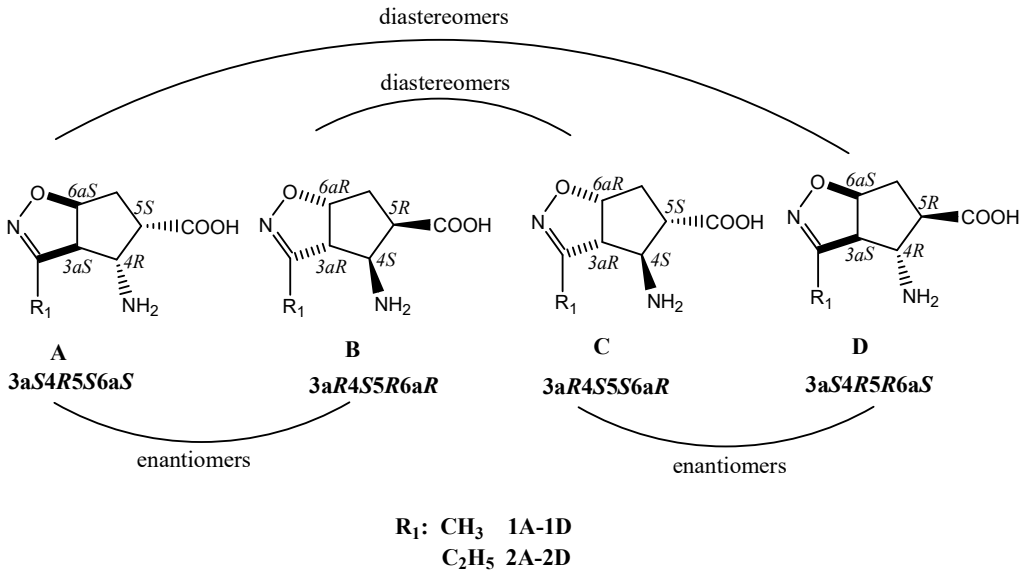

Figure 6. Structure of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogues

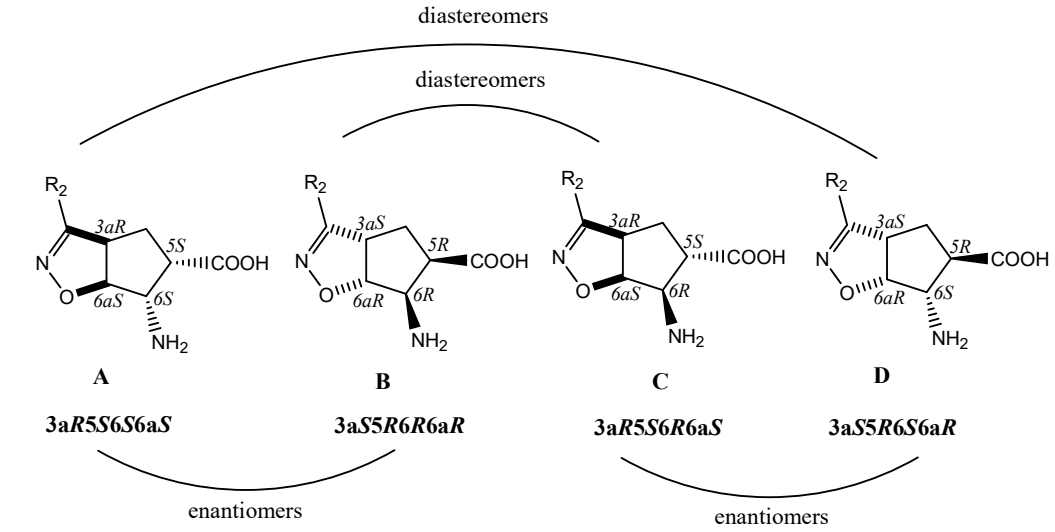

Figure 6. Structure of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogues

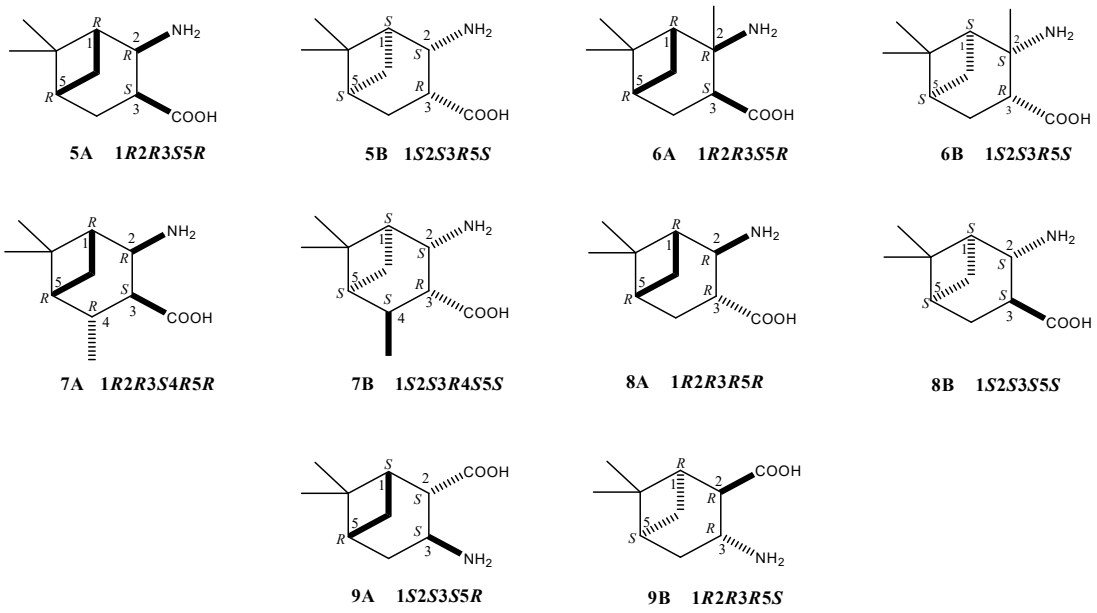

Figure 7. Structure of monoterpene-based *β*-amino acids

The preparations of the materials are discussed in the original papers [123-131]. Their IUPAC names are presented in the ***Appendix***.

3.3. Columns applied

Chiral ligand-exchange CSP [Column IV: sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based CSP, 150×4.6 mm I.D., 5 μm particle size] was prepared from (*R*)-phenylglycinol by covalently bonding (*R*)-*N,N*-carboxymethyl undecyl phenylglycinol monosodium salt, its derivative, to Kromasil silica gel [132].

Cinchona alkaloid-based zwitterionic stationary phases Chiralpak ZWIX(+)™ and ZWIX(-)™ columns were from Chiral Technologies Europe (Illkirch, France) (150 mm×3 mm I.D., 3 μm particle size for each column).

3.4. Chemicals and reagents

The applied methanol (MeOH), ethanol (EtOH), propanol (PrOH), 2-propanol (2-PrOH), and acetonitrile (MeCN) of HPLC grade, as well as ammonia (NH_3), ethylamine (EA), diethylamine (DEA), triethylamine (TEA), propylamine (PA), tripropylamine (TPA), butylamine (BA), tributylamine (TBA), formic acid (FA), and glacial acetic acid (AcOH) of analytical reagent grade were purchased from VWR International (Radnor, PA, USA). Cu(II) perchlorate, Cu(II) nitrate and Cu(II) sulphate and other reagents of analytical reagent grade were from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water was further purified by filtration on a 0.45 μm filter, type HV Millipore (Molsheim, France).

Mobile phases were prepared by mixing the indicated volumes of solvents. Stock solutions of analytes (0.2–1 mg mL^{-1}) were prepared by dissolution in the starting mobile phase.

4. RESULTS AND DISCUSSION

For an easier overview during the presentation of the results, abbreviations and a numbering system for the investigated compounds are introduced in the experimental part. All results have been disclosed in selected publications.

4.1. Separation of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogues on a chiral ligand-exchange stationary phase

All amino acids studied (***Figure 6***) possess an isoxazoline-fused cyclopentane skeleton. SAs **A**, **B** and **C**, **D** are *cis* and *trans* enantiomer pairs, respectively, while all other combinations are diastereomers. SAs **1** and **3** bear a methyl group at position 3, whereas SAs **2** and **4** have an ethyl group at position 3. These differences result in different physical properties, such as hydrophobicity, polarity, and rigidity of the molecules.

4.1.1. Effects of the mobile phase composition

First of all, the separation of the stereoisomers of isoxazoline-fused 2-aminocyclopentanecarboxylic acids (***Figure 6***) was evaluated on the chiral ligand-exchange stationary phase (***Figure 3***) with the following mobile phases: H_2O/MeOH (95/5 *v/v*), (85/15 *v/v*), (70/30 *v/v*), and (50/50 *v/v*) as well as H_2O/EtOH (95/5 *v/v*), (85/15 *v/v*), (70/30 *v/v*), and (50/50 *v/v*) containing 0.2 mM total concentration of $Cu(ClO_4)_2$ in the mobile phase (***Table 2***). For comparative purposes and to simplify the presentation, ***Table 2*** lists the chromatographic results obtained at all mobile phase compositions for SAs **1A**–**1D** and **4A**–**4D**. In contrast, only selected mobile phase compositions are given for SAs **2A**–**2D** and **3A**–**3D**.

Table 2. Chromatographic data, retention factors *(k),* separation factors (α), resolutions (R_S), and elution sequences of isoxazoline-fused 2-aminocyclopentane carboxylic acids

Analyte	Mobile phase H_2O/alcohol (*v/v*)+0.2 mM Cu(II)	k_1	k_2	α	R_S	Elution sequence
1A,1B	95/5, **a**	12.17	13.02	1.07	0.40	*B<A*
	85/15, **a**	2.15	2.37	1.10	0.15	-
	70/30, **a**	2.75	3.79	1.38	1.60	*B<A*
	50/50, **a**	4.74	7.92	1.67	2.60	*B<A*
	95/5, **b**	11.97	12.45	1.04	0.20	-
	85/15, **b**	4.00	4.48	1.12	1.75	*B<A*
	70/30, **b**	4.50	6.44	1.43	1.80	*B<A*
	50/50, **b**	7.93	13.88	1.75	2.60	*B<A*

Table 2 *(continued)*

Analyte	Mobile phase H_2O/alcohol (*v/v*)+0.2 mM Cu(II)	k_1	k_2	α	R_S	Elution sequence
1C,1D	95/5, **a**	16.64	16.64	1.00	0.00	-
	85/15, **a**	3.72	3.72	1.00	0.00	-
	70/30, **a**	4.15	4.52	1.09	0.20	-
	50/50, **a**	6.54	7.72	1.18	0.80	*D<C*
	95/5, **b**	15.10	15.10	1.00	0.00	-
	85/15, **b**	6.43	6.43	1.00	0.00	-
	70/30, **b**	5.98	6.52	1.09	0.20	-
	50/50, **b**	12.55	14.56	1.16	0.90	*D<C*
2A,2B	85/15, **a**	2.76	2.76	1.00	0.00	-
	50/50, **a**	5.86	8.85	1.51	2.30	*B<A*
	85/15, **b**	5.12	5.12	1.00	0.00	-
	50/50, **b**	8.90	13.71	1.54	2.25	*B<A*
2C,2D	85/15, **a**	4.66	5.22	1.12	0.45	-
	50/50, **a**	8.84	9.11	1.03	0.20	-
	85/15, **b**	7.99	9.11	1.14	1.00	*D<C*
	50/50, **b**	9.55	15.85	1.66	2.80	*D<C*
3A,3B	85/15, **a**	3.23	3.59	1.11	0.50	-
	50/50, **a**	7.50	11.48	1.53	2.55	*B<A*
	85/15, **b**	6.08	6.75	1.11	0.50	*B<A*
	50/50, **b**	11.32	16.53	1.46	2.20	*B<A*
3C,3D	85/15, **a**	4.65	8.23	1.77	2.40	*D<C*
	50/50, **a**	8.62	12.15	1.41	1.85	*D<C*
	85/15, **b**	7.14	12.92	1.81	3.55	*D<C*
	50/50, **b**	13.95	17.72	1.27	1.45	*D<C*
4A,4B	95/5, **a**	20.80	23.50	1.13	1.00	*B<A*
	85/15, **a**	4.27	4.91	1.15	0.45	*B<A*
	70/30, **a**	5.15	6.85	1.33	1.65	*B<A*
	50/50, **a**	8.15	12.88	1.58	3.23	*B<A*
	95/5, **b**	19.32	21.09	1.09	0.18	*B<A*
	85/15, **b**	7.33	8.50	1.16	0.75	*B<A*
	70/30, **b**	9.88	13.63	1.38	1.65	*B<A*
	50/50, **b**	11.28	17.15	1.52	2.10	*B<A*
4C,4D	95/5, **a**	20.28	38.73	1.91	3.90	*D<C*
	85/15, **a**	5.72	11.15	1.95	3.10	*D<C*
	70/30, **a**	6.64	11.95	1.80	2.95	*D<C*
	50/50, **a**	9.59	15.25	1.59	1.95	*D<C*
	95/5, **b**	13.52	26.80	1.98	2.50	*D<C*
	85/15, **b**	8.93	17.68	1.98	3.50	*D<C*
	70/30, **b**	11.29	20.32	1.80	1.55	*D<C*
	50/50, **b**	13.66	19.26	1.41	1.45	*D<C*

Chromatographic conditions: mobile phase, **a**, H_2O/MeOH (*v/v*), **b**, H_2O/EtOH (*v/v*), all containing 0.2 mM $Cu(ClO_4)_2$; flow rate, 0.8 mL min^{-1}; detection, 230 nm; temperature, ambient.

In reversed-phase liquid chromatography, we assume that the retention of analytes is controlled by the balance of two competing interactions. Namely, the hydrophilic interactions of analytes with the polar aqueous mobile phase and the hydrophobic interactions of analytes with the stationary phase. In the resolution of 2-aminocyclopentanecarboxylic acids both hydrophilic and hydrophobic interactions seem to

be involved. In most cases, a U-shaped retention curve was observed by changing the amount of organic solvent in the mobile phase. In the case of these types of β-amino acids, the retention factor (k_1) was the largest at both high and low water contents. This suggests that the enantioseparation was due to enhanced hydrophobic interactions between the SA and the SO in the water-rich eluents. In the RP mode, one of the most important interactions between the analyte and the CSP is the hydrophobic interaction, which is non-enantioselective in most cases. Upon increasing the EtOH or MeOH content from 5 to 15% of the mobile phase, the retention factor (k_1) decreased. However, it increased again with further increase in the alcohol concentration. Similar increases in k_1 were obtained by *Hyun et al.* [73,74] at high MeOH content and by *Wernicke et al.* [133] using 0–10% MeOH in an aqueous mobile phase with Cu(II)-Phe derivatives as selectors. An increase in the alcohol concentration reduces the polarity of mobile phase thereby reducing the solvation of polar amino acids and eventually increasing the hydrophilic interaction of SAs with the selector. In this event, the retention of SAs increases as the alcohol concentration in the mobile phase increases from 30 to 50%. A possible explanation for this phenomenon is that the separation may rather be controlled by the hydrophilic interaction liquid chromatography (HILIC) at high alcohol contents.

In this study, the inflection point and the slopes of the U-shaped curve at higher and lower alcohol contents differed somewhat for each compound, because of the different extents of solvation of the CSP and SAs in the applied mobile phases. As concerns the α and R_S values, both a decrease and an increase were observed with the change of the MeOH content. They exhibited minimum values at the H_2O/MeOH (85/15 *v/v*) mobile phase containing 0.2 mM $Cu(ClO_4)_2$. With increasing MeOH content both values increased with the exception of **2C,2D**, **3C,3D**, and **4C,4D**.

The sequence of elution of the enantiomers was identified by co-chromatography of racemic mixtures with an enantiomer of known configuration in most cases (***Table 2***) without the possibility to establish any general rule.

4.1.2. Effects of the nature of the alcohol modifier

The effect of the nature of alcohol (MeOH, EtOH, PrOH, 2-PrOH) content in the mobile phase was investigated for SAs **2A**–**2D** and **3A**–**3D**. The alcohol modifier influenced the retention in the H_2O/alcohol (85:15 *v/v*) mobile phase containing 0.2 mM $Cu(ClO_4)_2$ eluent at ambient temperature. In all cases, k_1 increased with increasing carbon number of the alcohol, as indicated in ***Figure 8***. The apolar character of the mobile phase increased in

the following sequence: MeOH < EtOH < PrOH < 2-PrOH. It seems that increasing the carbon number was unfavourable for polar interactions between the mobile phase and the SAs.

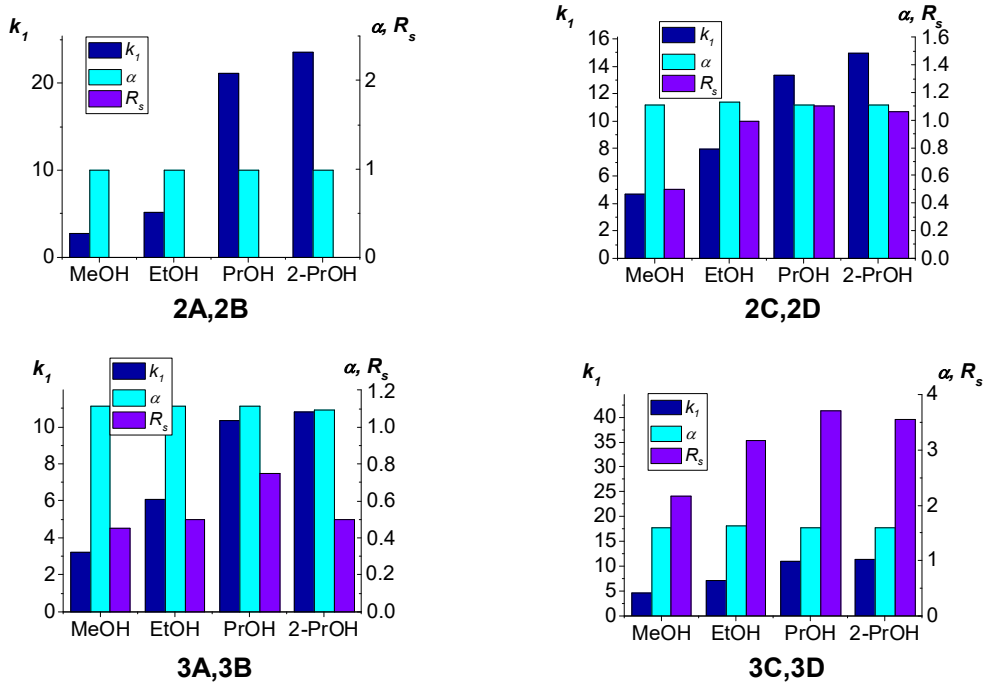

Figure 8. Influence of the nature of the alcohol modifier on the chromatographic parameters for SAs **2A**–**2D** and **3A**–**3D**.
Chromatographic conditions: mobile phase, H_2O/alcohol (85/15 *v/v*) containing 0.2 mM $Cu(ClO_4)_2$; flow rate, 0.8 mL/min; detection, 230 nm; temperature, ambient.

As concerns the effect of the nature of the alcohol modifier for enantioselectivity, no general trends could be observed: selectivity changed slightly for **2C,2D** and **3A,3B** and α varied between 1.09–1.14, and α ranged between 1.77–1.81 for **3C,3D**. In contrast, SAs **2A,2B** were not separable under these conditions. In all cases, resolution increased when MeOH was replaced by PrOH especially for SAs **3C,3D**.

4.1.3. Effects of the nature of the anion of Cu(II) salts

The influence of the $Cu(ClO_4)_2$ content was investigated in the separation of SAs **2A**–**2D** and **3A**–**3D**. An increase of the concentration of $Cu(ClO_4)_2$ in the mobile phase from 0.05 mM to 0.5 mM resulted in an unusual U-shaped retention curve (***Figure 9***). The retention factor (k_1) first decreased reaching a minimum at 0.2 mM Cu(II) and then increased again. However, α and R_S changed only slightly at a mobile phase composition of H_2O/MeOH (85/15 *v/v*) containing 0.05–0.5 mM $Cu(ClO_4)$.

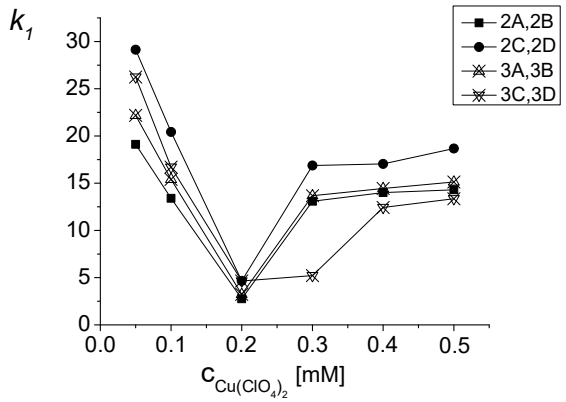

Figure 9. Effect of the $Cu(ClO_4)_2$ concentration on k_1 for SAs **2A**–**2D** and **3A**–**3D**. Chromatographic conditions: mobile phase, H_2O/MeOH (85/15 *v/v*) containing 0.05–0.5 mM $Cu(ClO_4)_2$; flow rate, 0.8 mL/min; detection, 230 nm; temperature, ambient.

Similar trends were observed by *Natalini et al*. applying *S*-benzyl-(*R*)-cysteine, *S*-trityl-(*R*)-cysteine [134], and *O*-benzyl-(*S*)-serine as selectors [135]. According to the assumption of *Hyun et al.* [73], the following equilibrium is established in the separation process (AA denotes the analytes):

$$[Cu(AA)(Fixed\ ligand)] + Cu^{2+} \rightleftharpoons [Cu(AA)]^{+} + [Cu(Fixed\ ligand)]^{+} \quad \textbf{(3)}$$

Increases in the concentration of Cu(II) in the mobile phase promoted the formation of more and more $[Cu(AA)]^+$ binary complexes and, therefore, retention decreases. Upon further increasing the Cu(II) concentration, Cu(II) acts as a competitor to both dative and hydrophobic/hydrophilic interactions between the SO and SA. According to *Kurganov* [55], stronger complexation events occur in the column at higher Cu(II) concentrations (0.3–0.5 mM). However, α and R_S do not show significant trends with the variation of Cu(II) concentration in the mobile phase.

In a further set of experiments, the effect of the nature of the Cu(II) salt anions on the separation efficiency was studied in the separation of SAs **2A**–**2D** and **3A**–**3D** in the aqueous mobile phase at a constant concentration of the anions. The variation of the Cu(II) salts exerts a significant effect on the retention parameters, as observed by *Natalini et al.* [134-136]. A comparison of the chromatographic data, obtained by using H_2O/MeOH (85:15 *v/v*) mobile phases containing 0.2 mM $Cu(ClO_4)_2$, $Cu(NO_3)_2$ or $CuSO_4$, demonstrated that k_1 changed significantly applying nitrate or sulphate counterions. The use of perchlorate counterion, in turn, resulted in much lower k_1 values (***Figure 10***). The ion-pairing ability of anions increase in the sequence of perchlorate<nitrate<sulphate. It is surmised that the apolar character of

the amino acid–selector–Cu(II)-anion complex increases resulting in an increase in retention. However, the nature of the anions showed a small effect on the enantioselectivity and resolution indicating that nature of anions exhibited small effect on enantioselective interactions.

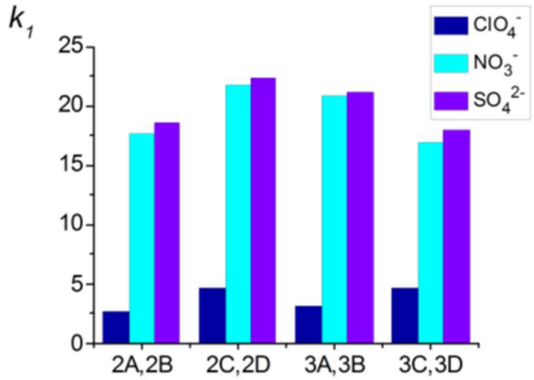

Figure 10. Effect of the nature of the Cu(II) salts on k_1 for SAs **2A**–**2D** and **3A**–**3D**. Chromatographic conditions: mobile phase, H_2O/MeOH (85/15 *v/v*) containing 0.2 mM $Cu(ClO_4)_2$, $Cu(NO_3)_2$ or $CuSO_4$; flow rate, 0.8 mL/min; detection, 230 nm.

According to *Natalini et al.* [134-136], Cu(II) counterions act as competitors in the complexation of the amino acids. The effect of these anions on the complexation process is dependent on the physico-chemical properties of the SAs and this may explain the resulting changes in the chromatographic parameters.

4.1.4. Influence of the structure of analytes

The structure of the SAs also influenced chiral recognition. In RP mode, ethyl-substituted analogues **2A**–**2D** and **4A**–**4D** afforded larger k_1 values (***Table 2***). However, these were accompanied by higher selectivity and resolution only in a few cases. As steric interactions are concerned, the position of methyl or ethyl group also affected the α and R_S values. For both α and R_S, enantiomers of **3A**–**3D** and **4A**–**4D** were better separated in most cases than enantiomers of **1A**–**1D** and **2A**–**2D**. The α and R_S values, in general, were better in the case of *trans*-isomers (**C,D**) than for *cis* compounds (**A,B**).

4.1.5. Effects of temperature on chiral recognition

To investigate the effects of temperature on chiral recognition, a variable-temperature study was carried out on Column IV CSP for all SAs over the temperature range 5–45 °C. Measurements were carried out with mobile phase H_2O/MeOH (95:5 *v/v*)

containing 0.2 mM $Cu(ClO_4)_2$ (***Table 3)***. As for chromatographic data, all recorded k_1 decreased with increasing temperature (***Table 3***). It is obvious, that selectivity decreases with increasing separation temperature. A transfer of the analyte from the mobile phase to the stationary phase, which can be described by the adsorption phenomenon, is generally an exothermic process and, consequently, k_1 and α decrease with increasing temperature. However, the change of α and R_S values was different: α decreased with increasing temperature, with the exception of **2A,2B**, **3A,3B**, and **4A,4B**, while R_S for **1A,1B** and **2C,2D** decreased whereas for **2A,2B**, **3A,3B**, **3C,3D**, **4A,4B**, and **4C,4D** increased with increasing the temperature (**1C,1D** was not separable under these condition). Since the effect of temperature on enantiomer separation is a complex phenomenon, an extensive study relating to the thermodynamics of this system was carried out. Accurate chromatographic data were collected to construct van't Hoff plots [*Eq.* (2)], and the thermodynamic parameters for the individual enantiomers were calculated from the slopes and intercepts of these plots.

Table 3. Temperature dependence of selected chromatographic parameters of enantiomers of isoxazoline-fused 2-aminocyclopentane carboxylic acids

Analyte	k_1, α,	Temperature (°C)					
	R_S	5	10	20	30	40	45
1A,1B	k_1	21.79	17.77	13.34	10.67	8.11	9.33
	α	1.11	1.10	1.08	1.07	1.06	1.00
	R_S	0.54	0.47	0.42	0.41	0.39	0.00
1C,1D	k_1	25.87	23.28	18.90	15.14	12.70	12.28
	α	1.00	1.00	1.00	1.00	1.00	1.00
	R_S	0.00	0.00	0.00	0.00	0.00	0.00
2A,2B	k_1	26.90	24.85	19.01	15.53	12.51	10.71
	α	1.00	1.00	1.07	1.09	1.11	1.11
	R_S	0.00	0.00	0.33	0.71	0.95	0.96
2C,2D	k_1	27.60	25.25	21.52	17.99	15.30	14.15
	α	1.21	1.20	1.18	1.16	1.14	1.13
	R_S	0.77	0.78	0.79	0.77	0.75	0.70
3A,3B	k_1	34.58	30.10	22.81	17.64	13.60	11.84
	α	1.00	1.06	1.08	1.08	1.09	1.10
	R_S	0.00	0.24	0.34	0.62	0.75	0.80
3C,3D	k_1	23.92	24.15	18.96	15.95	12.74	11.15
	α	2.01	1.83	1.86	1.77	1.73	1.70
	R_S	1.70	2.61	1.93	3.16	4.37	3.57

Table 3 *(continued)*

Analyte	k_1, α,	Temperature (°C)					
	R_S	**5**	**10**	**20**	**30**	**40**	**45**
4A,4B	k_1	44.41	35.96	26.46	19.30	14.73	14.70
	α	1.00	1.11	1.12	1.13	1.14	1.14
	R_S	0.00	0.44	0.66	0.98	1.05	1.19
4C,4D	k_1	27.93	23.73	22.64	18.78	16.10	13.90
	α	2.08	2.05	2.01	1.91	1.88	1.87
	R_S	1.90	2.38	3.74	3.87	5.27	5.10

Chromatographic conditions: mobile phase, H_2O/MeOH (95:5 *v/v*) containing 0.2 M $Cu(ClO_4)_2$; flow rate, 0.8 mL min^{-1}; detection, 230 nm

As a general trend, van't Hoff equation (ln α *vs.* $1/T$) gave linear plots, as indicated by correlation coefficients in ***Table 4***. It is widely accepted that both enantiomers undergo the same nonspecific interactions, whereas the more strongly retained one is subjected to additional stereospecific interactions. When selectivity decreased with increasing temperature, $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ were negative. In contrast, $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ were positive when selectivity increased with increasing temperature. In these cases, the change in the adsorption enthalpy with increasing temperature had a positive effect on enantioselectivity. On the other hand, when the positive $\Delta(\Delta S°)$ compensated the positive $\Delta(\Delta H°)$ and resulted in a negative $\Delta(\Delta G°)$, the selectivity increased with increasing temperature. Thermodynamically, this unusual behaviour may be attributed to positive $\Delta(\Delta S°)$ values indicating the importance of entropy contribution to chiral separation.

Table 4. Thermodynamic parameters, $\Delta(\Delta H°)$, $\Delta(\Delta S°)$, $\Delta(\Delta G°)$, and correlation coefficients (R^2) of isoxazoline-fused 2-aminocyclopentanecarboxylic acids (**1**–**4**)

Analyte	Corr. Coeff. (R^2)	$-\Delta(\Delta H°)$ (kJ mol^{-1})	$-\Delta(\Delta S°)$ (J mol^{-1} K^{-1})	$-\Delta(\Delta G°)_{298K}$ (kJ mol^{-1})
1A,1B	0.9963	0.8	2.4	0.1
1C,1D	-	0.00	0.00	0.0
2A,2B	0.9952	-2.2	-7.8	0.1
2C,2D	0.999	1.3	3.0	0.4
3A,3B	0.9992	-1.3	-4.8	0.2
3C,3D	0.9954	2.3	3.0	1.4
4A,4B	0.9953	-1.7	-6.4	0.2
4C,4D	0.9922	2.2	1.8	1.7

Chromatographic conditions: mobile phase, H_2O/MeOH (95/5 *v/v*) containing 0.2 mM $Cu(ClO_4)_2$; R^2, correlation coefficient of van't Hoff plot, ln α–$1/T$ curves

Under the conditions where $\Delta(\Delta H°)$ was negative, $\Delta(\Delta S°)$ was also negative, and positive $\Delta(\Delta H°)$ was accompanied by positive $\Delta(\Delta S°)$. $\Delta(\Delta S°)$ values are governed by the

difference in the number of degrees of freedom between the stereoisomers on the CSP, and mainly by the numbers of solvent molecules released from the chiral SO and the SA when the SA is associated with the CSP. Interactions of *trans*-isomers **2C,2D**, **3C,3D**, **4C,4D** as well as *cis*-isomer **1A,1B** were characterized by negative $\Delta(\Delta H°)$ values, while the other *cis*-isomers (**2A,2B**, **3A,3B**, **4A,4B**) exhibited positive $\Delta(\Delta H°)$ values. The trends in the change in $\Delta(\Delta S°)$ show that negative entropies were observed for SAs **1A,1B**, **2C,2D**, **3C,3D**, and **4C,4D** ***(Table 4)***. For **2A,2B**, **3A,3B**, and **4A,4B** above the T_{iso} temperature (the temperature at which the enantioselectivities balanced out), both $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ were positive indicating an entropically-driven separation. An example of entropically-driven separation is depicted in ***Figure 11***.

The thermodynamic parameter $-\Delta(\Delta G°)_{298}$ suggests that *trans* (**C,D**) analogues induce more efficient binding to the selector as reflected by the larger negative $\Delta(\Delta G°)$ values (analyte **1C,1D** was not separable).

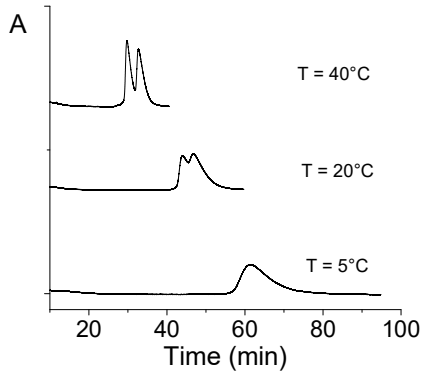

Figure 11. Entropically-driven separation on Column IV CSP for SAs **2A,2B**

4.2. Separation of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogues on Cinchona alkaloid-based chiral stationary phases

Stationary phases, which have a chiral ion-exchange type selector are a relatively new class of chiral columns. The quinine- and quinidine-based selectors and columns [ZWIX(+)™ and ZWIX(-)™] containing anion- and cation-exchange functional groups provide interaction between the charged functional groups of SOs and SAs (***Figure 5***).

4.2.1. Effect of composition of organic bulk solvents

The preferential mobile phases for the separation of SAs on the ZWIX CSPs are non-aqueous organic solvents in combination with acid and base additives (often referred to as

PIM) to ensure ionization of amino acids such as 2-amicocyclopentanecarboxylic acid analogues (***Figure 6***). MeOH as a protic solvent (which can suppress hydrogen-bonding interactions) and MeCN as an aprotic solvent supporting ionic interactions (but, at the same time, interfering with aromatic π–π-interactions) seemed to be the best choice as bulk solvent components [137,139,140].The influence of the composition of the bulk solvent on chromatographic parameters was investigated for 2-amicocyclopentanecarboxylic acid analogues on ZWIX(+)^{TM} and ZWIX(-)^{TM} in the MeOH/MeCN mobile phase system containing 25 mM TEA and 50 mM AcOH with increasing amounts of MeCN in MeOH from 25% to 75% (the acid-to-base ratio was kept constant at 2:1). By changing the bulk solvent composition, the acid–base equilibrium and proton activity may also change thereby affecting the chromatographic separation process. For comparative purposes and to simplify the presentation, ***Figure 12*** depicts the chromatographic results obtained for **1C,1D** and **4A,4B**.

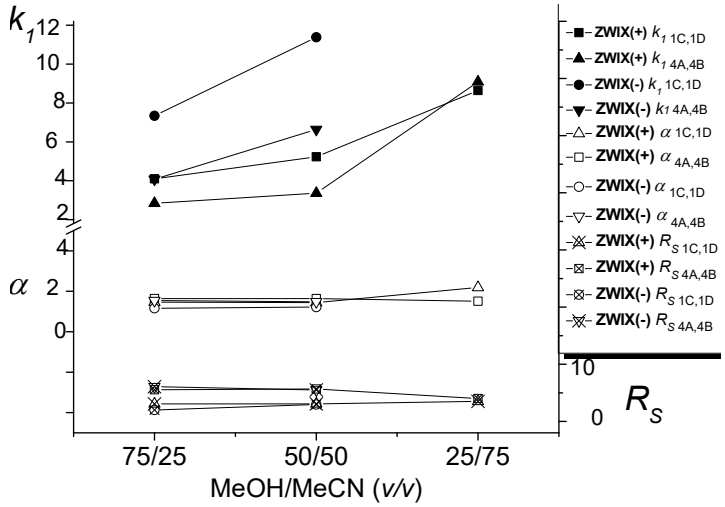

Figure 12. Effect of the composition of the bulk solvents on the chromatographic parameters for SAs **1C,1D** and **4A,4B** on ZWIX(+)^{TM} or ZWIX(-)^{TM} CSPs.

Retention, selectivity, and resolution of the SAs in most cases increased substantially with increasing MeCN content. In particular, on ZWIX(-)^{TM}, an increase of the MeCN content to 75 *v/v* resulted in extremely high *k* values (data not shown). A similar trend was observed by Hoffmann *et al.* for the separation of amino acids [138]. Mechanistically, the observed chromatographic behaviour can be summarized as follows: the interactions involved in a double ion-pairing process is less favoured in protic solvents, and higher MeCN content, therefore, supported the ionic interactions, which resulted in higher *k* values. A larger amount of MeCN most often enhanced α values and separation performance. With

increasing MeCN content in the mobile phase, solvation of the SAs probably decreases and stronger electrostatic interactions between the SAs and the SO take place.

4.2.2. Effects of the nature and concentration of acid and base as mobile phase additives

The anions and cations of acid and base additives affect the elution strength of the mobile phase, and a long-range ionic interaction between the zwitterionic SOs and SAs should be the primary interaction. The basis for enantiomer discrimination is the formation of transient diastereomeric non-covalent complexes. When ion pairing between SO and SA takes place, counterions in the mobile phase will competitively interfere with these interactions (***Figure 13***) [145]. Therefore, the variation of the nature and concentration of acid and base additives in the mobile phase is one of the most frequently used operation conditions for the optimization of the enantioseparation on *Cinchona* alkaloid-based CSPs [140-147].

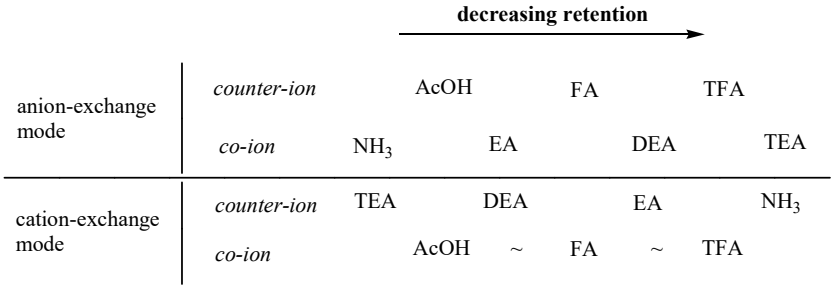

Figure 13. Effect of co- and counter-ion additives on retention on single ion-exchange-type CSPs [145-147]

The effects of the concentrations and ratios of the acid and base components as mobile phase additives were investigated for analytes **1A,1B** and **4C,4D** on ZWIX(+)^{TM} and ZWIX(-)^{TM} CSPs with a mobile phase of MeOH/MeCN (75/25 *v/v*) as bulk solvent (***Figure 14***). In one set of experiments, the concentration of AcOH was kept at a constant level (50 mM) and that of TEA was varied between 5–50 mM. In another set of experiments, in turn, the concentration of TEA was kept at a constant level (12.5 mM) and the AcOH concentration was varied between 3.125–50 mM.

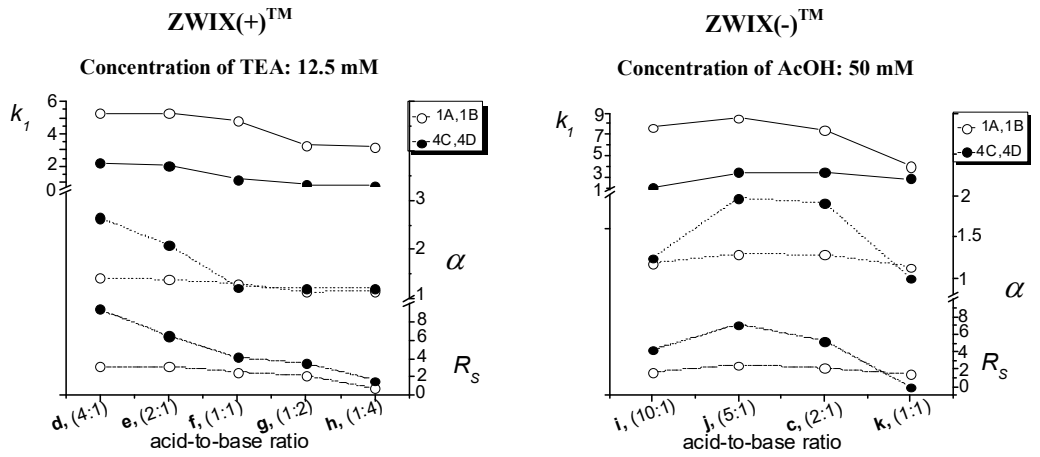

Figure 14. Effect of acid-to-base ratios on the chromatographic parameters for SAs **1A,1B** and **4C,4D** on ZWIX(+)^{TM} or ZWIX(-)^{TM} CSPs.

Chromatographic conditions: mobile phase composition, MeOH/MeCN (75/25 *v/v*) containing **d**, 50.0 mM AcOH and 12.5 mM TEA (4:1), **e**, 25.0 mM AcOH and 12.5 mM TEA (2:1), **f**, 12.5 mM AcOH and 12.5 mM TEA (1:1), **g**, 6.25 mM AcOH and 12.5 mM TEA (1:2), and **h**, 3.12 mM AcOH and 12.5 mM TEA (1:4); or MeOH/MeCN (75/25 *v/v*) containing **i**, 50.0 mM AcOH and 5.0 mM TEA (10:1), **j**, 50.0 mM AcOH and 10.0 mM TEA (5:1), **c**, 50.0 mM AcOH and 25.0 mM TEA (2:1), **k**, 50.0 mM AcOH and 50.0 mM TEA (1:1); flow rate, 0.6 mL min^{-1}; detection, 230 nm.

More favourable results were obtained at a slight acid excess (2:1), while at a base excess (1:4) a decrease in chromatographic parameters (k, α and R_S) was seen. Under a slight acid excess condition, the presence of negative and positive charges in both the SO and SA appears to favour double electrostatic interactions. The base excess condition seemed to be less preferential, because the amino groups of both the selector and the analytes are non-protonated. Consequently, the double electrostatic interaction concept between the SO and the SA is not favoured and k, α, and R_S decrease. Several literature data support our results indicating that the ion-exchange effect and the stoichiometric displacement model dominate the retention mechanism [96,97].

The nature of the acid and base additives in the mobile phase is a significant parameter in the optimization of the enantioseparation on zwitterionic CSPs. A number of studies demonstrated that the nature of co- and counter-ions has opposite effects on the anionic and cationic exchanger parts of the zwitterionic CSPs [145-147].

To compare the effects of the bases and acids as mobile phase additives, separations of analytes **1A,1B** and **4C,4D** were carried out with the same mobile phase composition on both ZWIX(+)™ and ZWIX(-)™ columns. The MeOH/MeCN content was kept constant (75/25 *v/v*), while the base (25 mM base) and the acid (50 mM) were varied (***Figure 15***). Seven different bases (EA, DEA, TEA, PA, TPA, BA, and TBA) and two acids (FA and AcOH) were chosen. The selected bases differ in the degree and nature of their alkyl

substitution on the *N* atom. The use of excess acid additives in the mobile phase ensured that the bases were present in their protonated "ammonium-ion" form.

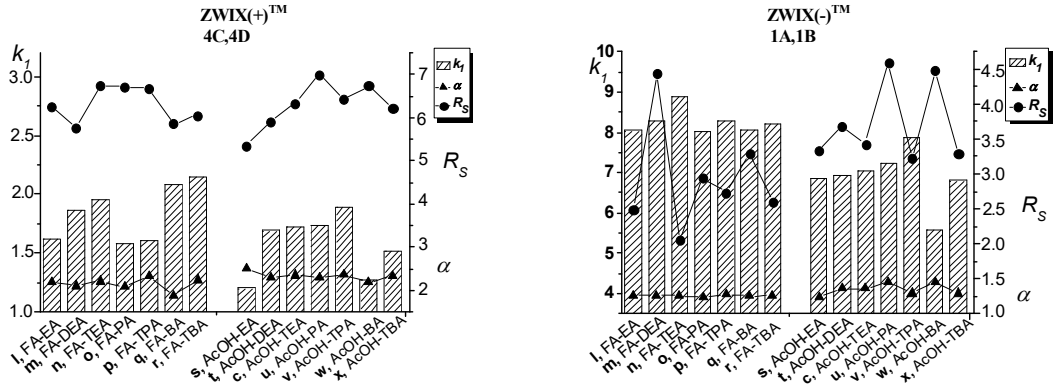

Figure 15. Effect of nature of acid and base additives on the chromatographic parameters for SAs **1A,1B** and **4C,4D** on ZWIX(+)^{TM} or ZWIX(-)^{TM} CSPs.

Chromatographic conditions: mobile phase, MeOH/MeCN (75/25 *v/v*) containing **l**, 50.0 mM FA and 25.0 mM EA, **m**, 50.0 mM FA and 25.0 mM DEA, **n**, 50.0 mM FA and 25.0 mM TEA, **o**, 50.0 mM FA and 25.0 mM PA, **p**, 50.0 mM FA and 25.0 mM TPA, **q**, 50.0 mM FA and 25.0 mM BA, and **r**, 50.0 mM FA and 25.0 mM TBA; or MeOH/MeCN (75/25 *v/v*) containing **s**, 50.0 mM AcOH and 25.0 mM EA, **t**, 50.0 mM AcOH and 25.0 mM DEA, **c**, 50.0 mM AcOH and 25.0 mM TEA, **u**, 50.0 mM AcOH and 25.0 mM PA, **v**, 50.0 mM AcOH and 25.0 mM TPA, **w**, 50.0 mM AcOH and 25.0 mM BA, **x**, 50.0 mM AcOH and 25.0 mM TBA; flow rate, 0.6 mL min^{-1}; detection, 230 nm.

Experimental results presented in ***Figure 15*** reveal that for a given SO and SA, k_1 values differ slightly when the base or acid components are varied. With a few exceptions, on both columns and with both acid additives (FA or AcOH), k_1 increased slightly as the degree of alkyl substitution of the *N* atom of the base increased. With increasing both the apolar character and bulkiness of amine additives (EA<DEA<TEA; PRA<TPA; BA<TBA), the solvation of polar amino acids in the mobile phase decreases and, therefore, k_1 increases.

The nature of the acids exerted a slight effect on retention. For the same SAs, the presence of FA resulted in slightly higher k_1 values compared to AcOH with the same base additive.

The nature of the base and acid modifier exerted a slight effect on the selectivity while for resolution by changing the base additives a larger change was observed without any general trends.

4.2.3. Influence of the structure of SAs and SOs on chromatographic parameters

The structures of the SAs (***Figure 6***) may influence the chromatographic behaviour. SAs **1** and **3**, besides the isoxazoline-fused cyclopentane skeleton, possess a 3-methyl group, while SAs **2** and **4** bear a 3-ethyl group. These structural differences lead to different steric effects and influence the hydrophobicity, bulkiness, and rigidity of the molecules and hence their interactions with SOs. As shown in ***Table 5***, the *cis* or *trans* position of the amino and carboxyl groups affected enantioseparation. On both CSPs, selectivities were larger for *trans* SAs [exceptions were analyte **1** and **3** on ZWIX(-)™] than for *cis* SAs.

Methyl or ethyl substitution also influenced retention parameters. On both SOs at a given mobile phase composition, the methyl-containing analogues (**1** and **3**) interacted more strongly with the SO than did the ethyl-substituted ones (**2** and **4**) resulting in higher retentions. As a result, the stronger interactions of methyl-substituted analogues provided larger α and R_S values indicating a larger difference in the interactions of the two enantiomers (**1** *vs*. **2** and **3** *vs*. **4**) with the CSP (exceptions were the ethyl-substituted **C,D** analogues). The position of the methyl or ethyl group on the isoxazoline ring influenced the chromatographic parameters, too. SAs **1** and **2** were more strongly retained than **3** and **4**, but α and R_S values generally were larger for **3** and **4** than for **1** and **2**. The R_2 positions of methyl and ethyl groups, despite the smaller retention, markedly influenced the overall chiral discrimination.

QN- and QD-based CSPs interact in different ways with SAs. Higher retention was generally observed on QD-based than on QN-based CSP, but in several cases ZWIX(+)™ differentiated the two enantiomers more efficiently resulting in larger selectivity.

Table 5. Chromatographic data retention factor (k), selectivity factor (α), resolution (R_S), and elution sequence of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on ZWIX(+)™ and ZWIX(-)™

Compound	Column	k_1	k_2	α	R_S	Elution
1A,1B	ZWIX(+)™	4.20	5.90	1.40	2.71	5*S*,4*R* < 5*R*,4*S*
	ZWIX(-)™	7.03	9.54	1.36	3.43	5*R*,4*S* < 5*S*,4*R*
1C,1D	ZWIX(+)™	5.42	7.76	1.46	3.00	5*S*,4*S* < 5*R*,4*R*
	ZWIX(-)™	7.34	8.52	1.16	2.32	5*R*,4*R* < 5*S*,4*S*
2A,2B	ZWIX(+)™	3.50	4.38	1.25	1.71	5*S*,4*R* < 5*R*,4*S*
	ZWIX(-)™	6.96	8.35	1.20	2.47	5*R*,4*S* < 5*S*,4*R*
2C,2D	ZWIX(+)™	4.74	7.72	1.63	2.73	5*S*,4*S* < 5*R*,4*R*
	ZWIX(-)™	5.36	6.16	1.55	1.92	5*R*,4*R* < 5*S*,4*S*
3A,3B	ZWIX(+)™	3.02	4.85	1.61	6.00	5*S*,6*S* < 5*R*,6*R*
	ZWIX(-)™	4.16	6.54	1.57	6.16	5*R*,6*R* < 5*S*,6*S*
3C,3D	ZWIX(+)™	1.93	4.50	2.33	4.62	5*S*,6*R* < 5*R*,6*S*
	ZWIX(-)™	4.57	4.89	1.25	1.55	5*R*,6*S* < 5*S*,6*R*
4A,4B	ZWIX(+)™	2.84	4.66	1.64	5.54	5*S*,6*S* < 5*R*,6*R*
	ZWIX(-)™	4.08	6.27	1.54	6.08	5*R*,6*R* < 5*S*,6*S*
4C,4D	ZWIX(+)™	1.80	4.36	2.42	6.00	5*S*,6*R* < 5*R*,6*S*
	ZWIX(-)™	2.77	5.54	2.00	5.66	5*R*,6*S* < 5*S*,6*R*

Chromatographic conditions: mobile phase, **c**, MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH; flow rate, 0.6 mL min^{-1}; detection, 215 or 230 nm or corona detector

4.2.4. Enantioseparation of the four stereoisomers of isoxazoline-fused 2-aminocyclopentane carboxylic acids

Since the biological activities of isoxazoline-fused 2-aminocyclopentanecarboxylic acids depend strongly on their configurations, it is essential to separate and identify not only their enantiomers, but also their diastereomers in a single chromatographic run. For the separation of four diastereomers in one chromatographic run the chromatographic conditions was optimized (see ***Appendix***). For the differentiation of diastereomers **1A**–**1D**, two different columns ZWIX(+)™ and ZWIX(-)™ and eluent systems had to be applied.

4.2.5. Effects of temperature on the chromatographic parameters

To investigate the effects of temperature on the chromatographic parameters, a variable-temperature study was carried out on ZWIX(+)™ and ZWIX(-)™ for **1A,1B**, **2A,2B**, **3C,3D**, and **4C,4D** in the temperature interval 5–50 °C. Experimental data for mobile phase **c** [MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH] and **n** [MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM FA] are collected in ***Table 6***. All recorded k_1 values decreased with increasing temperature except for **4C,4D** on ZWIX(-)™ in mobile phase **n** where k_1 increased slightly with increasing temperature. It is obvious that the separation factor decreases with increasing temperature. However,

exceptional results, namely, increasing α and R_S values with increasing temperature, were observed on ZWIX(+)™ CSP for **1A,1B** in mobile phase **n**, for **2A,2B** in mobile phase **c**, and on the ZWIX(-)™ for **3C,3D** in mobile phase **c**. Tabulated data indicate that the largest increase in α was observed with increasing temperature for analytes **2A,2B** in mobile phase **c** on ZWIX(+)™ (***Table 6***).

Table 6. Temperature dependence of retention factor of the first eluting enantiomer (k_1), separation factor (α), and resolution (R_S) of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on Chiralpak ZWIX(+)^{TM} and ZWIX(-)^{TM}

ZWIX(+)^{TM}							
Compound	**Mobile phase**	***k*, *α*, *R*$_S$**	**Temperature (°C)**				
			10	**20**	**30**	**40**	**50**
1A,1B	**c**	k_1	5.50	4.72	4.17	3.52	3.04
		α	1.49	1.41	1.37	1.31	1.25
		R_S	3.59	3.58	3.50	3.40	3.13
	n	k_1	7.46	6.46	5.64	4.92	4.31
		α	1.27	1.29	1.31	1.32	1.34
		R_S	2.56	2.65	2.93	2.86	2.96
2A,2B	**c**	k_1	5.59	3.82	2.48	1.64	1.14
		α	1.00	1.23	1.63	2.05	2.45
		R_S	0.00	1.48	2.57	4.86	6.60
3C,3D	**c**	k_1	2.20	2.01	1.79	1.64	1.49
		α	2.35	2.21	2.10	1.98	1.85
		R_S	4.00	5.09	4.08	2.60	2.73
4C,4D	**c**	k_1	2.14	1.93	1.70	1.49	1.29
		α	2.58	2.01	1.64	1.28	1.00
		R_S	6.25	6.22	4.27	1.71	0.00
	n	k_1	1.92	1.85	1.76	1.67	1.58
		α	2.28	2.20	2.12	2.04	1.96
		R_S	6.34	5.85	5.71	5.72	4.80
Compound	**Mobile phase**	***k*, *α*, *R*$_S$**	**Temperature (°C)**				
			5	**15**			
2A,2B	**c**	k_1	5.90	5.20			
		α	0.87	1.02			
		R_S	0.67	<0.20			

Table 6 *(continued)*

ZWIX(-)™							
Compound	**Mobile phase**	k, α, R_S	**Temperature (°C)**				
			10	**20**	**30**	**40**	**50**
1A,1B	**c**	k_1	8.46	7.77	7.08	6.29	5.45
		α	1.33	1.30	1.27	1.24	1.21
		R_S	2.71	2.72	2.60	2.43	2.38
	n	k_1	9.05	8.83	8.16	7.52	6.91
		α	1.28	1.24	1.24	1.23	1.21
		R_S	1.40	1.76	2.06	2.17	2.11
2A,2B	**c**	k_1	9.23	8.37	7.51	6.69	5.92
		α	1.23	1.20	1.18	1.16	1.13
		R_S	2.10	2.06	2.00	1.85	1.76
3C,3D	**c**	k_1	5.35	4.53	3.69	2.95	2.34
		α	1.06	1.19	1.34	1.49	1.66
		R_S	0.65	1.13	2.05	4.12	6.14
4C,4D	**c**	k_1	2.77	2.60	2.45	2.28	2.12
		α	2.04	1.94	1.86	1.77	1.68
		R_S	5.64	5.58	4.89	4.36	4.15
	n	k_1	1.98	2.04	2.07	2.11	2.14
		α	2.10	1.99	1.90	1.81	1.72
		R_S	4.12	5.56	4.97	5.21	4.88

Chromatographic conditions: mobile phase, **c**, MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH, **n**, MeOH/MeCN (75/25 *v/v)* containing 25 mM TEA and 50 mM FA; flow rate, 0.6 mL min^{-1}; detection, 230 nm

The effect of temperature on separation was complex—chromatographic data with the change of temperature changed in different ways. Values of k_1, α, and R_S decreased or increased with increasing temperature, which promoted us to carry out an extensive study dealing with the thermodynamics of enantiomer separation.

To calculate the thermodynamic parameters van't Hoff plots were constructed in all cases [*Eq.* (2)]. Data with respect to the changes in $\Delta(\Delta H°)$, $\Delta(\Delta S°)$, and $\Delta(\Delta G°)$ are depicted in ***Table 7***. The differences in the changes in standard enthalpy $\Delta(\Delta H°)$ and entropy $\Delta(\Delta S°)$ exhibited both positive and negative values. A negative $\Delta(\Delta H°)$ value indicates an exothermic transfer of the preferentially adsorbed enantiomer of analytes from the mobile to the stationary phase. Negative $\Delta(\Delta H°)$ values were found on ZWIX(+)™ for **1A,1B, 3C,3D**, and **4C,4D** in mobile phase **c** and **4C,4D** in eluent **n**, as well as on ZWIX(-)™ for **1A,1B, 2A,2B**, and **4C,4D** in mobile phase **c** and for **1A,1B** and **4C,4D** in eluent **n** (***Table 7***). When selectivity increased with increasing temperature, $\Delta(\Delta H°)$ values were positive and especially large positive $\Delta(\Delta H°)$ values were obtained in mobile phase **c** on ZWIX(+)™ for **2A,2B** and on ZWIX(-)™ for **3C,3D**. In these cases, the change in the adsorption enthalpy with increasing temperature had a positive effect on the enantioselectivity.

$\Delta(\Delta S°)$ is a measure for the change in the state of order induced by enantioselective SO–SA interactions. Under the conditions where $\Delta(\Delta H°)$ was negative, $\Delta(\Delta S°)$ was also negative, and positive $\Delta(\Delta H°)$ was accompanied with positive $\Delta(\Delta S°)$. Negative $\Delta(\Delta S°)$ values ranged from –3.0 to –55.1 J mol^{-1} K^{-1}, while the range of positive $\Delta(\Delta S°)$ was between 5.7–72.0 J mol^{-1} K^{-1}. The interactions of **2A,2B** with the selector of ZWIX(+)™ and **3C,3D** with the selector of ZWIX(-)™ were characterized by the highest positive $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values at eluent composition **c**.

Table 7. Thermodynamic parameters, $\Delta(\Delta H°)$, $\Delta(\Delta S°)$, Tx$\Delta(\Delta S°)$, $\Delta(\Delta G°)$, correlation coefficients, (R^2), T_{iso} temperature, and Q values of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on ZWIX(+)^{TM} and ZWIX(-)™

Analyte	Mobile phase	-Δ(Δ*H°*) (kJ mol^{-1})	-Δ(Δ*S°*) (J mol^{-1} K^{-1})	Corr. Coeff. (R^2)	-TxΔ(Δ*S°*)$_{298K}$ (kJ mol^{-1})	-Δ(Δ*G°*)$_{298K}$ (kJ mol^{-1})	T_{iso} (°C)	*Q*
ZWIX(+)™								
1A,1B	**c**	3.3	8.3	0.9950	2.6	0.7	123	1.3
	n	-1.0	-5.7	0.9959	-1.7	0.7	-88	0.6
2A,2B	**c**	-20.8	-72.0	0.9869	-21.5	0.7	14	0.9
3C,3D	**c**	4.6	8.9	0.9951	2.6	2.0	238	1.8
4C,4D	**c**	17.9	55.1	0.996	16.4	1.5	51	1.1
	n	2.8	3.0	0.9966	0.9	1.9	648	3.1
ZWIX(-)™								
1A,1B	**c**	1.9	4.2	0.9996	1.3	0.6	172	1.5
	n	3.3	8.3	0.9950	2.5	0.8	123	1.3
2A,2B	**c**	1.7	4.0	0.9993	1.2	0.5	152	1.4
3C,3D	**c**	-8.7	-30.8	0.999	-9.2	0.5	9	0.9
4C,4D	**c**	3.6	6.8	0.9982	2.1	1.5	255	1.7
	n	3.8	7.1	0.9974	2.1	1.7	254	1.8

Chromatographic conditions: mobile phase, **c**, MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH, **n**, MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM FA; flow rate, 0.6 mL min^{-1}; detection, 230 nm; R^2, correlation coefficient of van't Hoff plot, ln α–1/T curves; T_{iso},, temperature of ln k–1/T curves where enantioselectivity cancels; Q = $\Delta(\Delta H°)/\text{Tx}\Delta(\Delta S°)_{298K}$

The thermodynamic parameter $-\Delta(\Delta G°)_{298}$ suggests that ZWIX(+)™ induces more efficient binding of the SAs to the SO, as reflected by larger $-\Delta(\Delta G°)$ values. These $-\Delta(\Delta G°)_{298}$ values reflect the structural influence on separation. For analytes **1**–**4** on both columns, SO–SA complex formation proceeds via multiple intermolecular interactions and was generally exothermic with a corresponding negative entropic contribution. Exceptions are **1A,1B** in mobile phase **n** and **2A,2B** in mobile phase **c** on ZWIX(+)™, as well as **3C,3D** in mobile phase **c** on ZWIX(-)™. On ZWIX(+)™ for analyte **1A,1B** in mobile phase **n** and for **2A,2B** in mobile phase **c**, and on ZWIX(-)™ for **3C,3D** in mobile phase **c**, positive $\Delta(\Delta S°)$ compensated positive $\Delta(\Delta H°)$ and resulted in a negative $\Delta(\Delta G°)$.

In a separate investigation, thermodynamic data were used to calculate T_{iso} (isoenantioselective temperature) at which the enantioselectivity cancels out and the elution sequence would change. In most cases, T_{iso} was considerably higher than at room temperature and, consequently, enthalpically driven enantioseparations were obtained. For **2A,2B** on ZWIX(+)™ with MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH as mobile phase, with increasing the temperature from 5 °C to 50 °C, selectivity first decreased then, after a domain where no separation occurred, selectivity increased, and the elution sequence changed (***Figure 16*** and ***Table 6***).

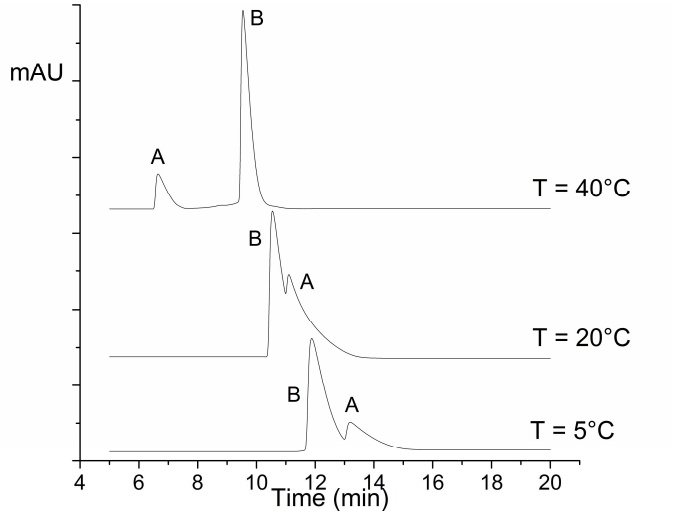

Figure 16. Effects of temperature for the separation of **2A,2B**

In a domain around the isoenantioselective temperature (T_{iso}), the separation of enantiomers could not be reached. In chiral recognition, this domain may be referred to as a "temperature-induced blind zone" [148]. Outside the blind zone, predominantly enthalpically- or entropically-driven enantioseparation can be observed. It should be mentioned that changes in the elution sequence with temperature changes were observed rarely [149-153].

4.3. Enantioseparation of monoterpene-based *β*-amino acids on *Cinchona* alkaloid-based chiral stationary phases

All investigated *β*-amino acids (***Figure 7***) possess a monoterpene-based skeleton. In the case of SAs **8** and **9**, the locations of the carboxyl and amino group are opposite, while SAs **6** and **7** possess one extra methyl group on position 2 or 4. Analogues **5** and **8** are

diastereomers, while **9** is a constitutional isomer of **5** and **8**. These differences result in different physical properties (*e.g.* steric effects, bulkiness, hydrophobicity) and these my influence the interactions with the chiral selector.

4.3.1. Effects of bulk solvent composition on chromatographic parameters

Cinchona alkaloid-based CSPs afforded the best results in PIM when a mixture of MeOH and MeCN was applied. To investigate the effects of the organic bulk solvent, chromatographic separation was carried out in MeOH/MeCN (75/25, 50/50, and 25/75 *v/v*) mobile phases containing 25 mM PA and 50 mM AcOH for all investigated SAs (***Figure 7***). To simplify the presentation and reduce the huge number of data, ***Figure 17*** depicts the chromatographic behaviour for analytes **5**, **7**, and **9** on ZWIX(+)™ and ZWIX(-)™.

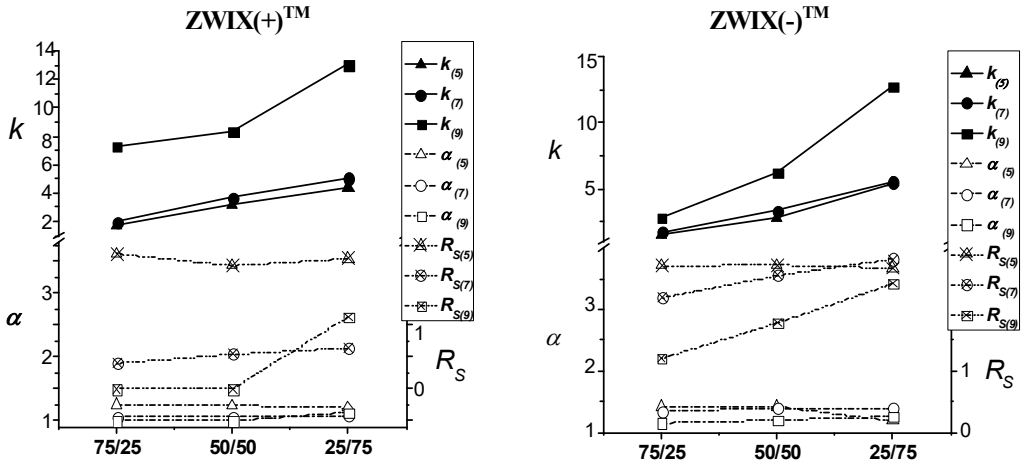

Figure 17. Effect of the compositions of the bulk solvents on the chromatographic parameters for SAs **5**, **7**, and **9** on ZWIX(+)^{TM} or ZWIX(-)^{TM} CSPs.
Chromatographic conditions: column, mobile phase composition, MeOH/MeCN (75/25, 50/50 and 25/75 *v/v*) containing 25.0 mM PA and 50 mM AcOH; flow rate, 0.6 mL min^{-1}; detection, 230 nm; temperature, 25 ºC; symbols, –▲–: k_1 for analyte **5**, –●–: for analyte **7**, and –■–: for analyte **9**; ···△···: α for analyte **5**, ···○···: for analyte **7**, and ···□···: for analyte **9**; ··-⊠-··: R_S for analyte **5** ··-⊠-··: for analyte **7**, and ··-⊠-·· : for analyte **9**.

The application of a higher MeCN content in the mobile phase increased the retention factor in all cases. The electrostatically driven interactions involved in the formation of the selector–analyte complexes are apparently weaker in protic solvents (*e.g.* MeOH) because of the larger solvation shell of the ionic sites. If the MeCN content is higher in the mobile phase, ionic interactions can be triggered as a consequence of the weaker solvation resulting

in longer retention [95,96,129,130]. A higher MeCN content did not provide a consistent separation performance, but it most often led to an enhanced enantioselectivity. However, on ZWIX(+)™ and ZWIX(-)™ for SAs **5A,5B**, slight decreases in α and R_S were observed, similar to the case of α-amino acids [97].

4.3.2. Effects of the nature of acid and base additives

Numerous studies and the results discussed above (see *4.2.2.*) demonstrated that co- and counter-ions have opposite impacts on the elution strength at the cationic and anionic exchanger parts of the zwitterionic CSPs [97,145]. An acid-to-base ratio of 2:1 was previously found to be the most effective for successful enantioseparation [97,140-144], with the concentration of the acid and base being kept at 50 mM and 25 mM, respectively. To explore the effects of the nature of the base and acid additives, separations were carried out on both ZWIX(+)™ and ZWIX(-)™ CSPs with the same bulk solvent composition [MeOH/MeCN (50/50 *v/v*) with 25 mM base and 50 mM acid]. NH_3, EA, DEA, TEA, and PA were selected as bases, and FA and AcOH were applied as acids.

The corresponding results are depicted in ***Figure 18***. Retention factors increased on both columns and with both acid modifiers as the degree of alkyl substitution on the nitrogen atom of the base increased. However, more apolar amine additives appear to exert disadvantageous influences on the solvation of ionic SAs resulting in increased retention.

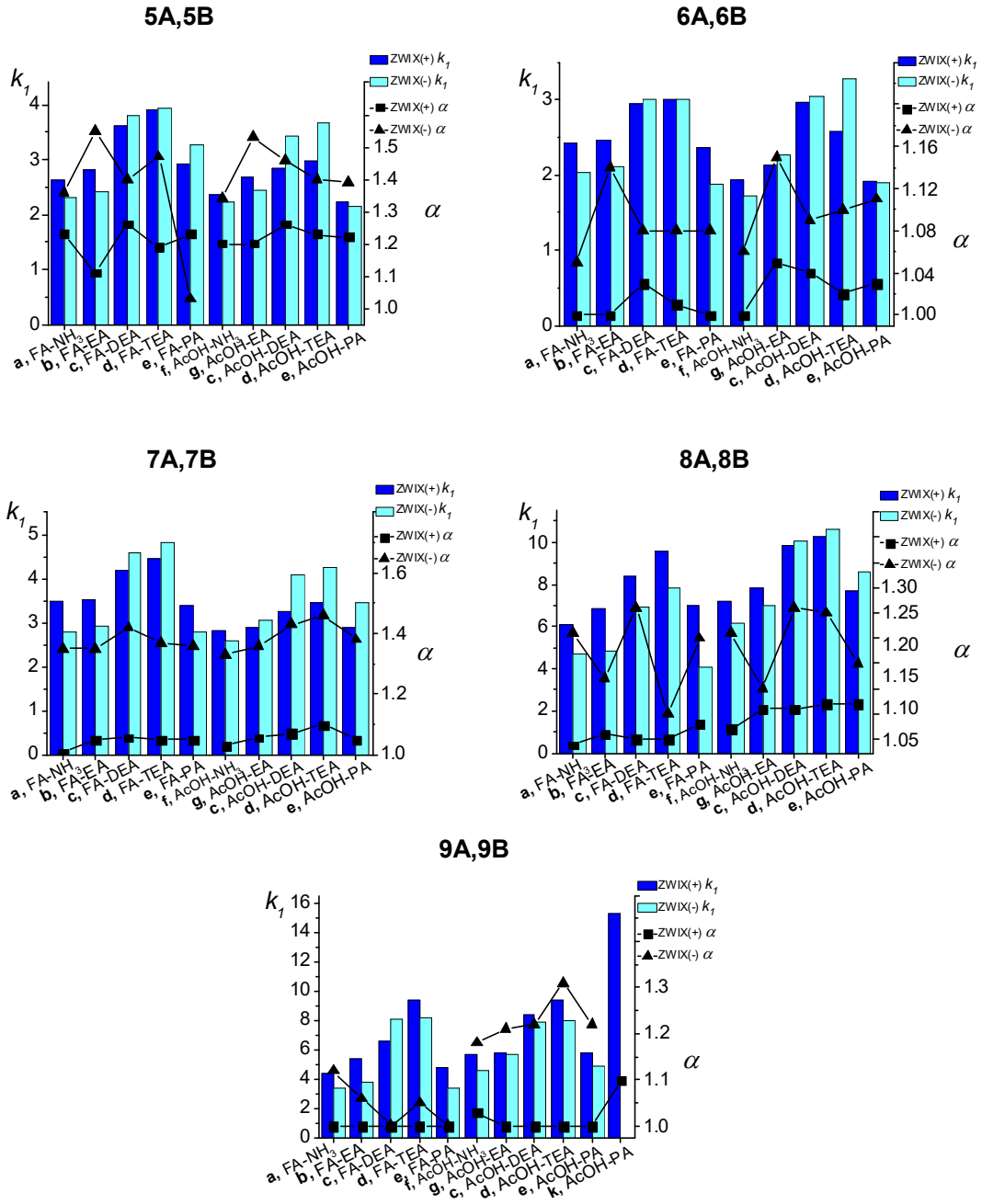

Figure 18. Effect of the acid and base additives on ZWIX(+)™ or ZWIX(-)™ CSPs Chromatographic conditions: column, Chiralpak ZWIX(+)™; mobile phase, MeOH/MeCN (50/50 *v/v*) containing **a**, 50 mM FA and 25 mM NH_3, **b**, 50 mM FA and 25 mM EA, **c**, 50 mM FA and 25 mM DEA, **d**, 50 mM FA and 25 mM TEA, **e**, 50 mM FA and 25 mM PA **f**, 50 mM AcOH and 25 mM NH_3, **g**, 50 mM AcOH and 25 mM EA, **h**, 50 mM AcOH and 25 mM DEA, **i**, 50 mM AcOH and 25 mM TEA, **j**, 50 mM AcOH and 25 mM PA, **k**, MeOH/MeCN (25/75 *v/v*) containing 25 mM PA and 50 mM AcOH; flow rate, 0.6 mL min^{-1}; detection, 230 nm; temperature, 25 ºC

According to the literature data [97,145] and previous experimental results (***Figure 15***), the effects of type of base on retention were in opposite direction on anion-exchange

and cation-exchange type selectors, depending on their role as co-or counterion (***Figure 13***). Base components work as displacers or counter-ions in the separation of these SAs.

The nature of the base exhibited a slight effect on selectivity without any general trends. α Changed in a narrow range on both columns. On ZWIX(+)™ α varied between 1.00–1.26, while this range was between 1.00–1.55 on ZWIX(-)™. The nature of applied bases influenced the resolution. As can be seen in ***Figure 18***, enantioseparations on ZWIX(-)™ were more effective than on ZWIX(+)™, but slightly higher α and R_S values were obtained on ZWIX(+)™ for SA **5** with the application of eluent **e**.

The type of acids used as mobile phase modifier exhibited only a slight effect on retention. Slightly smaller k_1 values were obtained with AcOH for SAs **5**, **6**, and **7**, and with FA for **8** and **9** using the same base additive. The effects of the nature of basis additives on the retention are complex. Similar to the base modifiers, the acids as mobile phase additives manifested opposite effects on the positively and negatively charged parts of the zwitterionic SO (***Figure 13***) [97,145]. A counterbalancing effect of organic acids on elution strength supports the co-ion effect of acids on separation.

4.3.3. Effects of the structure of analytes

The structure of amino acids affected both the retention and chromatographic behaviour. In SAs **5**–**7**, the amino and carboxyl groups are in *cis* position, while in **8** and **9** they possess the *trans* configuration. However, **6** and **7** have an extra methyl group at position 2 or 4, which ensures a more constrained structure for these SAs. These structural differences may influence the interactions between the chiral SO moiety and the SAs and, consequently, they affect both retention and chiral recognition. As data acquired on both CSPs show in ***Figure 18***, at a given mobile phase composition *trans* analogues (SAs **8** and **9**), in most cases, interacted more strongly with the SO than did the *cis* ones (SAs **5**–**7**) resulting in larger retentions on ZWIX(+)™ and ZWIX(-)™. As a consequence, higher retention, in most cases, resulted in larger α and R_S values indicating a larger difference in the interactions. Analogues **5** and **8** are diastereomers. Higher retention was observed for SAs **8** on both columns, while the structure differences of **5** and **8** exerted only a slight effect on selectivity and resolution. Regarding the chromatographic behaviour of SAs **6** and **7**, retention factors, selectivity, and resolution were lowest on both CSPs for SA **6**. The reason is that the extra methyl group at position 2 sterically hindered the main ionic interaction between the amino group of amino acid and the SO. Such steric hindrance was not observed

for SA **7** bearing a methyl group at position 4. On ZWIX(+)™, α values for SA **6** lay in the range 1.00–1.05 and for SA **7**, they are in the range 1.01–1.10. On ZWIX(-)™, in turn, α values for SA **6** lay in the range 1.05–1.14 and for SA **7** in the range 1.33–1.46. In contrast, a slightly better resolution was found for SA **7**.

4.3.4. Elution sequence of SAs

The *Cinchona* alkaloid-based chiral CSPs [ZWIX(+)™ and ZWIX(-)™] possess a QN and QD structure, respectively. These SOs behave like pseudo-enantiomeric CSPs and, therefore, the sequence of elution of the enantiomers of SAs, in principle, should be reversed. With the enantiomerically pure standards in hand, the elution sequences of SAs could be investigated (***Tables 8 and 9***). The configuration of the carboxyl group of A enantiomer of SAs **5**, **6**, and **7** was 3*S*. SA **9** exists as 2*S*, and because of the reversal of configuration, SA **8** is 3*R*. These differences in the configuration of the carboxyl group may explain the observed elution sequence. On ZWIX(+)™, the sequence of elution of SAs **5**, **6**, **7**, and **9** was found to be A < B, while it was B < A for SA **8**, whereas the opposite is the case for the ZWIX(-)™ CSP. Selected chromatograms for the separation of SAs are collected in ***Appendix***.

4.3.5. Effects of temperature and thermodynamic parameters

In most cases, the temperature influences stereoselective interactions. Accordingly, the column temperature often has to be optimized in enantioselective separations.

The effects of temperature on chiral recognition of monoterpene-based β-amino acid enantiomers on ZWIX(+)™ and ZWIX(-)™ CSPs were investigated. Experiments for all compounds studied were performed at different mobile phase compositions in the temperature range 10–50 °C. Experimental data for all SAs on both columns are listed in ***Tables 8 and 9***. Mobile phase compositions were as follows: MeOH/MeCN (50/50 *v/v*) containing 25 mM TEA and 50 mM FA (**d**), MeOH/MeCN (50/50 *v/v*) containing 25 mM TEA and 50 mM AcOH (**i**), MeOH/MeCN (50/50 *v/v*) containing 25 mM PA and 50 mM AcOH (**j**), MeOH/MeCN (25/75 *v/v*) containing 25 mM PA and 50 mM AcOH (**k**). Tabulated data reveals that, in most cases, k values on both columns decreased with increasing temperature. A transfer of the SA from the mobile phase to the stationary phase, which can be described by the adsorption phenomenon, is generally an exothermic process and, consequently, both k and α decrease with increasing temperature. However, on

ZWIX(+)™ for **6** and **7** in mobile phase **d** and **i**, and for **8** in mobile phase **d**, α increases with increasing temperature. On ZWIX(-)™ for **6**, **8**, and **9** with mobile phase **d**, both k_1 and α decrease with increasing temperature, while for **6** with eluent **i** increasing α values were found with increasing temperature.

Similar unexpected behaviour was previously observed for other amino acids, too [141-143].

Table 8. Temperature dependence of retention factors of the first eluting enantiomer (k_1), the second eluting enantiomer (k_2), separation factor (α), and resolution (R_S) of monoterpene-based 2-amino carboxylic acids on Chiralpak ZWIX(+) CSP

Compound	Mobile phase	k, α, R_S	Temperature (°C)					Elution sequence
			10	20	30	40	50	
5	**d**	k_1	4.32	4.08	3.84	3.55	3.33	*A<B*
		α	1.24	1.20	1.17	1.14	1.11	
		R_S	2.50	2.40	2.30	1.60	1.50	
6	**d**	k_1	3.24	3.07	2.93	2.81	2.71	*A<B*
		α	1.00	1.00	1.02	1.02	1.03	
		R_S	0.00	0.00	0.30	0.30	0.50	
	i	k_1	3.14	2.78	2.35	2.19	1.94	*A<B*
		α	1.00	1.00	1.05	1.05	1.08	
		R_S	0.00	0.00	0.30	0.40	0.60	
7	**d**	k_1	4.92	4.60	4.32	4.03	3.80	*A<B*
		α	1.03	1.04	1.05	1.06	1.06	
		R_S	0.50	0.60	0.60	0.70	0.80	
	i	k_1	4.20	3.74	3.26	2.73	2.41	*A<B*
		α	1.08	1.09	1.11	1.13	1.15	
		R_S	0.60	0.90	0.70	1.00	1.10	
8	**d**	k_1	11.08	9.88	9.03	8.40	7.79	*B<A*
		α	1.03	1.04	1.06	1.07	1.08	
		R_S	0.30	0.30	0.30	0.40	0.50	
	i	k_1	12.60	11.08	9.70	8.43	7.38	*B<A*
		α	1.14	1.12	1.11	1.10	1.10	
		R_S	1.60	1.60	1.50	1.40	1.00	
9	**d**	k_1	10.53	9.75	8.95	8.35	7.83	-
		α	1.04	1.00	1.00	1.00	1.00	
		R_S	0.40	0.00	0.00	0.00	0.00	
	k	k_1	18.05	16.11	14.54	13.13	11.98	*A<B*
		α	1.11	1.10	1.09	1.09	1.08	
		R_S	2.00	1.90	2.00	1.80	1.60	

Chromatographic conditions: mobile phase, **d**, MeOH/MeCN (50/50 *v/v*) containing 25 mM TEA and 50 mM FA, **i**, MeOH/ACN 50/50 (*v/v*) containing 25 mM TEA and 50 mM AcOH, **c**, MeOH/ACN 50/50 (*v/v*) containing 25 mM PA and 50 mM AcOH, **k**, MeOH/ACN 25/75 (*v/v*) containing 25 mM PA and 50 mM AcOH; flow rate, 0.6 mL min^{-1}; detection, 215 nm

Table 9. Temperature dependence of retention factors of the first eluting enantiomer (k_1), the second eluting enantiomer (k_2), separation factor (α), and resolution (R_S) of monoterpene-based 2-amino carboxylic acids on Chiralpak ZWIX(-) CSP

Compound	Mobile phase	k, α, R_S	Temperature (°C)					Elution sequence
			10	20	30	40	50	
5	**d**	k_1	4.23	4.02	3.84	3.67	3.51	*B<A*
		α	1.55	1.50	1.45	1.39	1.35	
		R_S	3.80	3.40	3.30	3.30	3.30	
6	**d**	k_1	2.83	2.91	2.96	3.01	2.99	*B<A*
		α	1.00	1.11	1.16	1.18	1.22	
		R_S	0.00	0.50	1.40	1.40	2.20	
	i	k_1	3.51	3.32	3.16	3.01	2.83	*B<A*
		α	1.11	1.12	1.13	1.14	1.15	
		R_S	0.60	1.00	1.60	1.40	1.80	
7	**d**	k_1	5.29	4.90	4.70	4.50	4.23	*B<A*
		α	1.41	1.38	1.34	1.32	1.30	
		R_S	2.80	2.70	2.60	2.50	2.60	
	i	k_1	4.58	4.35	4.12	3.92	3.76	*B<A*
		α	1.52	1.48	1.46	1.43	1.39	
		R_S	2.90	3.00	3.00	3.00	3.00	
8	**d**	k_1	7.64	7.77	7.91	7.99	8.10	*A<B*
		α	1.07	1.09	1.10	1.12	1.13	
		R_S	0.50	0.50	0.60	0.70	0.80	
	i	k_1	11.89	10.92	10.15	9.50	8.60	*A<B*
		α	1.37	1.33	1.31	1.29	1.28	
		R_S	2.60	2.40	2.10	2.10	2.00	
9	**d**	k_1	6.70	7.40	7.40	7.53	7.46	*B<A*
		α	1.00	1.00	1.06	1.13	1.17	
		R_S	0.00	0.00	0.60	0.90	1.20	
	i	k_1	9.11	8.24	7.68	7.21	6.79	*B<A*
		α	1.35	1.32	1.30	1.28	1.26	
		R_S	4.50	2.50	2.10	2.40	1.80	
	k	k_1	5.34	5.00	4.76	4.52	4.30	*B<A*
		α	1.24	1.22	1.20	1.19	1.18	
		R_S	1.70	1.70	1.70	1.60	1.60	

Chromatographic conditions: mobile phase, **d,** MeOH/MeCN (50/50 *v/v*) containing 25 mM TEA and 50 mM FA, **i**, MeOH/ACN 50/50 (*v/v*) containing 25 mM TEA and 50 mM AcOH, **k**, MeOH/ACN 25/75 (*v/v*) containing 25 mM PA and 50 mM AcOH; flow rate, 0.6 mL min^{-1}; detection, 215 nm

Changes of the temperature of the enantioseparation process usually result in changes in the conformation of the selector of the CSP and/or the analytes. The temperature-related conformation change may be reversible or irreversible and depends on mobile phase composition providing different retention characteristics. The changes in retention factors, selectivity, and resolution with temperature were not consistent, and an extensive study relating to the thermodynamics of this system was therefore carried out. On the basis of the chromatographic data, van't Hoff plots were constructed [*Eq.* (1) and (2)].

As a general trend, van't Hoff analysis of retention factors (ln *k vs 1/T*) gave pronounced linear plots. However, for SAs **6** and **9** on ZWIX(-)™ with mobile phase **d**, non-

linear plots were observed (***Figure 19***), while a linear plot was registered for ln α *vs 1/T* in the temperature range 20–50 °C.

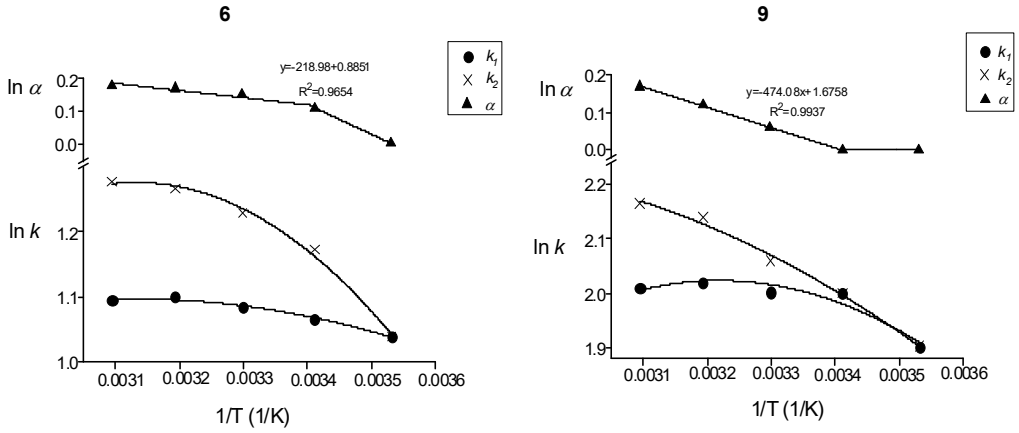

Figure 19. Non-linear van't Hoff plots of ln *k vs 1/T* and ln α *vs 1/T* for SAs **6** and **9** on ZWIX(-)™ CSP.
Chromatographic conditions: mobile phase, MeOH/MeCN (50/50 *v/v*) containing 25.0 mM TEA and 50 mM FA; flow rate, 0.6 mL min^{-1}; detection, 215 nm; temperature range, 10–50 ºC.

Differences in $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ are presented in ***Table 10***. $\Delta(\Delta H°)$ values on ZWIX(+)™ ranged from –2.1 to 1.5 kJ mol^{-1}, and on ZWIX(-)™ from –2.7 to 3.9 kJ mol^{-1}. The trends in the change in $\Delta(\Delta H°)$ showed similarity with the $\Delta(\Delta S°)$ values. Under the conditions where negative $\Delta(\Delta H°)$ values were measured, $\Delta(\Delta S°)$ values were also negative and the largest positive $\Delta(\Delta H°)$ was coupled with the largest positive $\Delta(\Delta S°)$. Generally, *k* and α decreased with increasing temperature resulting in negative $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values (enthalpically-driven separation). On the other hand, in entropically-driven separation positive $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values were observed, when *k* decreased and α increased with increasing temperature [148]. The largest positive $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ were obtained on ZWIX(-)™ for SAs **6**, **8**, and **9** in mobile phase system **d** indicating a larger entropic contribution to the chiral separation.

Table 10. Thermodynamic parameters [$\Delta(\Delta H°), \Delta(\Delta S°), \mathrm{Tx}\Delta(\Delta S°), \Delta(\Delta G°)$], correlation coefficients (R^2), and T_{iso} temperature of monoterpene-based 2-aminocarboxylic acids on ZWIX(+)™ and ZWIX(-)™ columns

ZWIX(+)							
Analyte	**Mobile phase**	**$-\Delta(\Delta H°)$ (kJ mol^{-1})**	**$-\Delta(\Delta S°)$ (J mol^{-1} K^{-1})**	**Correlation coefficients (R^2)**	**$-\mathrm{Tx}\Delta(\Delta S°)_{298K}$ (kJ mol^{-1})**	**$-\Delta(\Delta G°)_{298K}$ (kJ mol^{-1})**	**T_{iso} (°C)**
5	**d**	2.1	5.6	0.9929	1.7	0.4	105
6	**d**	-0.5	-1.9	0.9938	-0.6	0.1	-10
	i	-1.5	-5.2	0.9967	-1.5	0.1	15
7	**d**	-0.5	-2.0	0.9991	-0.6	0.1	-23
	i	-1.3	-5.1	0.9906	-1.5	0.2	-18
8	**d**	-0.9	-3.4	0.9950	-1.0	0.1	-8
	i	0.7	1.4	0.9974	0.4	0.2	210
9	**d**	0.7	2.2	0.9991	0.6	0.1	45
	k	0.4	0.7	0.9990	0.2	0.2	300
ZWIX(-)							
Analyte	**Mobile phase**	**$-\Delta(\Delta H°)$ (kJ mol^{-1})**	**$-\Delta(\Delta S°)$ (J mol^{-1} K^{-1})**	**Correlation coefficients (R^2)**	**$-\mathrm{Tx}\Delta(\Delta S°)_{298K}$ (kJ mol^{-1})**	**$-\Delta(\Delta G°)_{298K}$ (kJ mol^{-1})**	**T_{iso} (°C)**
5	**d**	2.7	5.6	0.9998	1.7	1.0	209
6	**d**	-2.2	-8.7	0.9895	-2.6	0.4	-
	i	-0.5	-2.9	0.9950	-0.9	0.4	-100
7	**d**	1.6	2.7	0,9906	0.8	0.8	319
	i	1.6	2.3	0.9996	0.7	0.9	420
8	**d**	-1.0	-4.3	0.9944	-1.3	0.3	-41
	i	1.3	2.1	0.9927	0.6	0.7	345
9	**d**	-3.9	-13.9	0.9937	-4.2	0.4	-
	i	1.2	1.8	0.9920	0.5	0.7	390
	k	1.0	1.8	0.9977	0.5	0.5	280

Chromatographic conditions: mobile phase, **d**, MeOH/MeCN (50/50 *v/v*) containing 25 mM TEA and 50 mM FA, **i**, MeOH/MeCN (50/50 *v/v*) containing 25 mM TEA and 50 mM AcOH, **k**, MeOH/MeCN (25/75 *v/v*) containing 25 mM PA and 50 mM AcOH; flow rate, 0.6 mL min^{-1}; detection, 215 nm; R^2, correlation coefficient of van't Hoff plot, ln α–1/T curves; T_{iso}, temperature of ln k–1/T curves where enantioselectivity cancels.

Calculated thermodynamic parameters suggest that mobile phase **i** induced a more efficient binding to the SO on both columns. It seems that the ZWIX(-)™ column is more suitable than ZWIX(+)™ for the enantioseparation of this type of SAs, as reflected by the larger $-\Delta(\Delta G°)_{298}$ values. ***Figure 20*** depicting an example for entropically-driven enantioseparation shows that α increases with increasing temperature.

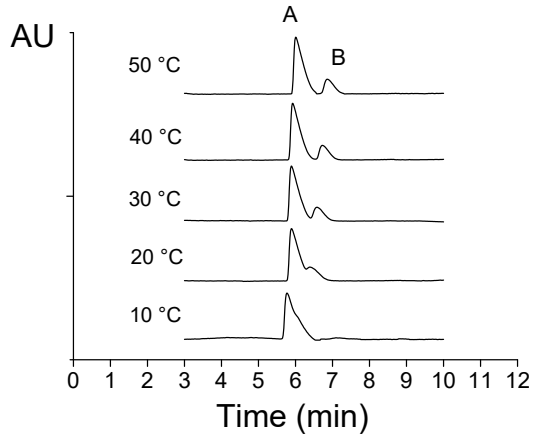

Figure 20. Temperature dependence for SA **6** on ZWIX(-)™ CSP.
Chromatographic conditions: mobile phase **d**, MeOH/MeCN (50/50 *v/v*) containing 25.0 mM TEA and 50 mM FA; flow rate, 0.6 mL min^{-1}; detection, 215 nm; temperature range 10–50 ºC.

In most cases, T_{iso} was higher than ambient temperature, and when T_{iso} was obtained at room temperature and selectivity increased with increasing temperature, entropically-driven enantioresolutions were observed.

4.4. Comparison of chiral ligand-exchange and Cinchona alkaloid-based stationary phases

The basic principle of CLEC is reversible coordination between the immobilized selector and analyte within the transition metal cation coordination sphere. Zwitterionic chiral ion-exchangers may be regarded as Pirkle-type CSPs but the ionizable groups participate in the retention process. It is essential for both columns that SA contains free amino and carboxyl groups. In the case of isoxazoline-fused cyclopentane analogues, the separation ability of these two types of CSPs can be compared.

A comparison of k_1 values indicates that isoxazoline-fused 2-aminocyclopentane carboxylic acids exhibit stronger interactions with CLEC CSP. Retention factors were generally lower on ZWIX(+)™ and ZWIX(-)™ CSPs than on the CLEC column.

Regarding the chromatographic efficiency of the two types of SOs, in most cases, α and R_S were higher on CLEC when k_1 values were lower on both ZWIX(+)™ and ZWIX(-)™ CSPs.

The effect of temperature on chiral recognition was investigated on both types of CSPs. A variable-temperature study was carried out on CLEC over the temperature range 5–45 °C and on ZWIX(+)™ and ZWIX(-)™ columns over the temperature range 5–50 °C. The

increase in temperature influenced the chromatographic parameters. In the studied temperature range, both enthalpically- and entropically-driven enantioseparations were observed on both types of CSPs.

A comparison of chromatographic data allows the conclusion that both types of columns are capable for the separation of these types of SAs.

SUMMARY

During the work of enantioseparation of two types of unusual *β*-amino acid analogues, isoxazoline-fused 2-aminocyclopentanecarboxylic acids and monoterpene-based *β*-amino acids were investigated. The chromatographic measurements were carried out on a chiral ligand-exchange and two *Cinchona* alkaloid-based stationary phases.

Enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on a chiral ligand-exchange stationary phase

The effects of the mobile phase composition [H_2O/MeOH and H_2O/EtOH containing 0.2 mM total concentration of $Cu(ClO_4)_2$] showed that, in most cases, U-shaped retention curves were observed. Both higher and lower water contents afforded the largest k_1 values. This was probably due to enhanced hydrophobic interactions between the analyte and the CSP. However, when the alcohol content of the mobile phase increased from 5 to 15%, k_1 first decreased, but further increases in the MeOH or EtOH content induced increases again. This suggests that the separation may be controlled by a HILIC mechanism at high alcohol contents.

The nature of the alcohol modifier (MeOH, EtOH, PrOH, and 2-PrOH) exerted major effects on the retention. Namely, k_1 increased with increasing carbon number of the alcohol. This phenomenon allowed the conclusion that increasing carbon number was disadvantageous for polar interactions between the mobile phase and the analytes.

The effects of the concentration of $Cu(ClO_4)_2$ were also investigated. On increasing concentration, Cu(II) gave a U-shaped k_1 curve. The increase in $Cu(ClO_4)_2$ concentration in the mobile phase promoted the formation of several SA complexes in the eluent thus shortening k_1. At higher Cu(II) concentrations more intricate complexation events occurring on the column may affect separation.

A comparison of the chromatographic data acquired with the use of $Cu(ClO_4)_2$, $Cu(NO_3)_2$ or $CuSO_4$ demonstrated that larger k_1 values were obtained by the application of nitrate or sulphate ions; perchlorate ions, in turn, resulted in lower k_1. The apolar character of the amino acid–Cu(II)-anion complex is suggested to induce an increase in retention when nitrate or sulphate ions are used.

The structure of the SAs also influenced chiral recognition. As concerns steric interactions, the position of the methyl or ethyl group affected k_1, α, and R_S values. In most cases, *trans*-isomers (**C,D**) exhibited larger enantioseparation than *cis*-isomers (**A,B**).

The effects of the temperature were investigated between 5–45 °C for all SAs. According to thermodynamic parameters determined in all cases, enantioseparations, in most cases, are enthalpically driven (except **2A,2B**, **3A3B**, and **4A,4B**).

Enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on Cinchona alkaloid-based CSPs

The *Cinchona* alkaloid-based zwitterionic CSPs afforded the best results in PIM when a mixture of MeOH and MeCN was applied. Retention, selectivity, and resolution of zwitterionic amino acids in most cases increased with increasing MeCN content. This was probably due to the decreased solvation effect of MeCN and the stronger electrostatic interaction of the SAs with the SO.

For comparison, the concentration of the acid and base components as mobile phase additives was investigated by varying the ratio of TEA and AcOH in polar ionic mobile phase. The results indicated that an acid excess (acid to base ratio 2:1) was favourable, while under basic conditions (1:4), a decrease in retention parameters was observed. The explanation for this phenomenon was that the ion-exchange effect and the stochiometric displacement model dominate the retention mechanism.

Seven different base modifiers and two acid modifiers were applied for the separation of analytes **1A,1B**, and **4C,4D**. As experimental results showed, k_1, α, and R_S values differ slightly when the base or acid component was varied. In most cases, k_1 increased as the degree of alkyl substitution or apolar character and bulkiness of aliphatic moiety on the *N* atom of base increased (EA<DEA<TEA; PA<TPA; BA<TBA). The nature of the applied acid also influenced the resolution. For the same analytes the presence of FA instead of AcOH, in most cases, resulted in slightly higher k_1 values when the same base additive was applied.

The differences of the structure of SAs influenced the chromatographic behaviour. CSPs based on *Cinchona* alkaloids interact in different ways with analytes. Higher k_1 values were generally observed on ZWIX(-)^{TM}; however, QN-based CSP distinguished the two enantiomers more efficiently in several cases.

For the differentiation of the four diastereomers of isoxazoline-fused 2-aminocyclopentanecarboxylic acid in a single chromatographic run, the chromatographic conditions were optimized. For the separation of **1A**–**1D,** however, two different columns and eluent systems had to be applied.

The effect of temperature was investigated between 5–50 °C on both CSPs. Chromatographic parameters decreased with increasing temperature, but an increase in α was registered in some cases. The thermodynamic parameters were determined in all cases, and enthalpy-controlled enantioselective discrimination was found in most cases. Entropy-controlled separations could also be observed with increasing temperature. On ZWIX(+)^{TM} for **2A,2B** with eluent c, T_{iso} was 14 °C; the change in elution sequence with changing temperature for **2A,2B** is illustrated in ***Figure 16***.

Enantioseparation of monoterpene-based 2-aminocyclopentanecarboxylic acids on Cinchona alkaloid-based CSPs

Non-aqueous polar ionic experimental conditions with MeOH and MeCN as bulk solvent were applied in this project. Similar to previous results, the retention of β-amino acids, in all cases, increased substantially with increasing MeCN content. Characteristically, a certain amount of MeCN on both columns enhanced selectivity and resolution with a few exceptions.

For a more detailed investigation of the effects of the nature of the acid and base components in the mobile phase, five different bases (NH_3, EA, DEA, TEA, and PA) and two acids (FA and AcOH) as mobile phase additives were selected. The nature of the applied acid and base modifiers exhibited only a slight effect on the chromatographic parameters. According to the experimental results, k_1 values increased on both columns with both acid modifiers as the degree of ethyl substitution on the nitrogen atom of the base increased. With a few exceptions, the application of TEA or NH_3 resulted in the largest and the smallest k_1 values, respectively. The effects of the nature of the acid additives on the retention are complex; however, it seems that there is a slight difference in the eluent strength when AcOH or FA was applied.

The effects of temperature were investigated on both CSPs between 10–50 °C and the thermodynamic parameters were determined in all cases. Usually, enantioselective discriminations were enthalpy controlled; however, for SAs **6**, **8**, and **9** on ZWIX(-)^{TM} CSP with mobile phase MeOH/MeCN (50/50 *v/v*) containing 25 mM TEA and 50 mM FA, separation was entropically driven.

The structures of β-amino acid analogues also influenced the chiral recognition. On both CSPs, at a given mobile phase composition, k_1 values for the *trans* analogues (SAs **8** and **9**) were larger than for the *cis* ones (SAs **5**–**7**) in most cases. For comparison of the diastereomers (SAs **5** and **8**), k_1 values were larger for SA **8** on both columns, while the

difference of the structure of analytes **5** and **8** exhibited only a slight effect on enantioselectivity. The extra methyl group at position 2 on SA **6** sterically hindered the main ionic interactions, resulting in lower k_1, α, and R_S values.

Elution sequences were determined in all cases. ZWIX(+)^{TM} and ZWIX(-)^{TM} CSPs behave like pseudo enantiomeric stationary phases, and the sequence of elution of SAs, in principle, should be reversed. On ZWIX(+)^{TM}, the elution sequence of SAs **5**, **6**, **7**, and **9** was found to be $A < B$, while it was $B < A$ for SA **8**. In contrast, the opposite trend was registered on the ZWIX(-)^{TM} CSP.

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APPENDIX

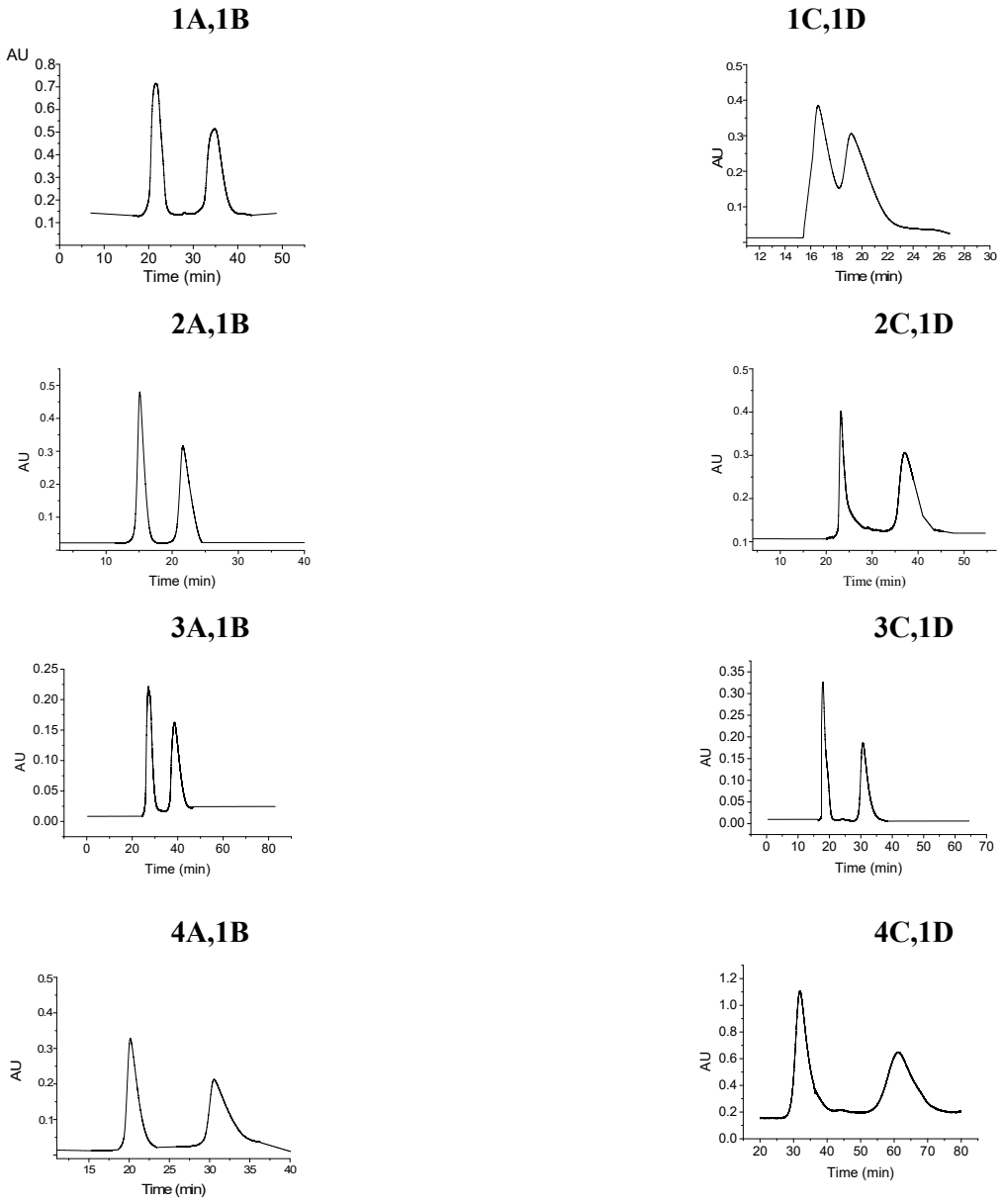
The IUPAC name of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogues (Figure 6)

(3a*S*,4*R*,5*S*,6a*S*)-4-amino-3-methyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**1A**), (3a*R*,4*S*,5*R*,6a*R*)-4-amino-3-methyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**1B**), (3a*S*,4*R*,5*R*,6a*S*)-4-amino-3-methyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**1C**), (3a*R*,4*S*,5*S*,6a*R*)-4-amino-3-methyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta-[d]isoxazole-5-carboxylic acid (**1D**), (3a*S*,4*R*,5*S*,6a*S*)-4-amino-3-ethyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**2A**), (3a*R*,4*S*,5*R*,6a*R*)-4-amino-3-ethyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**2B**), (3a*S*,4*R*,5*R*,6a*S*)-4-amino-3-ethyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**2C**), (3a*R*,4*S*,5*S*,6a*R*)-4-amino-3-ethyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**2D**), (3a*R*,5*S*,6*S*,6a*S*)-6-amino-3-methyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta-[d]isoxazole-5-carboxylic acid (**3A**), (3a*S*,5*R*,6*R*,6a*R*)-6-amino-3-methyl-4,5,6,6a-tetra-hydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**3B**), (3a*R*,5*S*,6*R*,6a*S*)-6-amino-3-methyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**3C**), (3a*S*,5*R*,6*S*,6a*R*)-6-amino-3-methyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**3D**), (3a*R*,5*S*,6*S*,6a*S*)-6-amino-3-ethyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta-[d]isoxazole-5-carboxylic acid (**4A**), (3a*S*,5*R*,6*R*,6a*R*)-6-amino-3-ethyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**4B**), (3a*R*,5*S*,6*R*,6a*S*)-6-amino-3-ethyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**4C**), (3a*S*,5*R*,6*S*,6a*R*)-6-amino-3-ethyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[d]isoxazole-5-carboxylic acid (**4D**).

The IUPAC name of monoterpene-based β-amino acids (Figure 7)

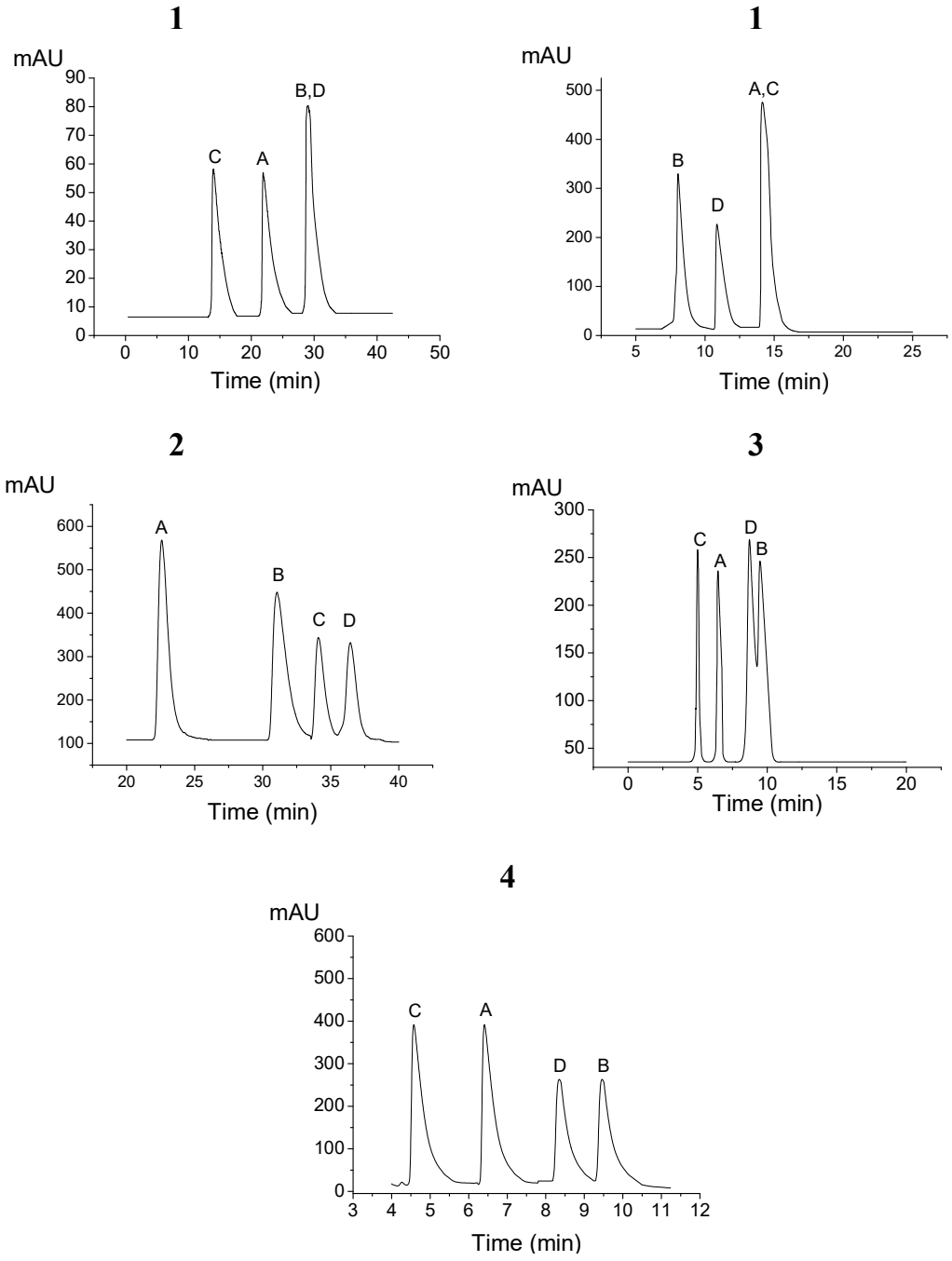
(1*R*,2*R*,3*S*,5*R*)-2-amino-6,6-dimethylbicyclo[3.1.1]heptane-3-carboxylic acid (**5A**), (1*S*,2*S*,3*R*,5*S*)-2-amino-6,6-dimethylbicyclo[3.1.1]heptane-3-carboxylic acid (**5B**), (1*R*,2*R*,3*S*,5*R*)-2-aminopinane-3-carboxylic acid (**6A**), (1*S*,2*S*,3*R*,5*S*)-2-aminopinane-3-carboxylic acid (**6B**), (1*R*,2*R*,3*S*,4*R*,5*R*)-2-amino-4,6,6-trimethylbicyclo[3.1.1]heptane-3-carboxylic acid (**7A**), (1*S*,2*S*,3*R*,4*S*,5*S*)-2-amino-4,6,6-trimethylbicyclo[3.1.1]heptane-3-carboxylic acid (**7B**), (1*R*,2*R*,3*R*,5*R*)-2-amino-6,6-dimethylbicyclo[3.1.1]heptane-3-carboxylic acid (**8A**), (1*S*,2*S*,3*S*,5*S*)-2-amino-6,6-dimethylbicyclo[3.1.1]heptane-3-carboxylic acid (**8B**), (1*S*,2*S*,3*S*,5*R*)-3-amino-6,6-dimethylbicyclo[3.1.1]heptane-2-carboxylic acid (**9A**), (1*R*,2*R*,3*R*,5*S*)-3-amino-6,6-dimethylbicyclo[3.1.1]heptane-2-carboxylic acid (**9B**).

Selected chromatograms of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on chiral ligand-exchange column



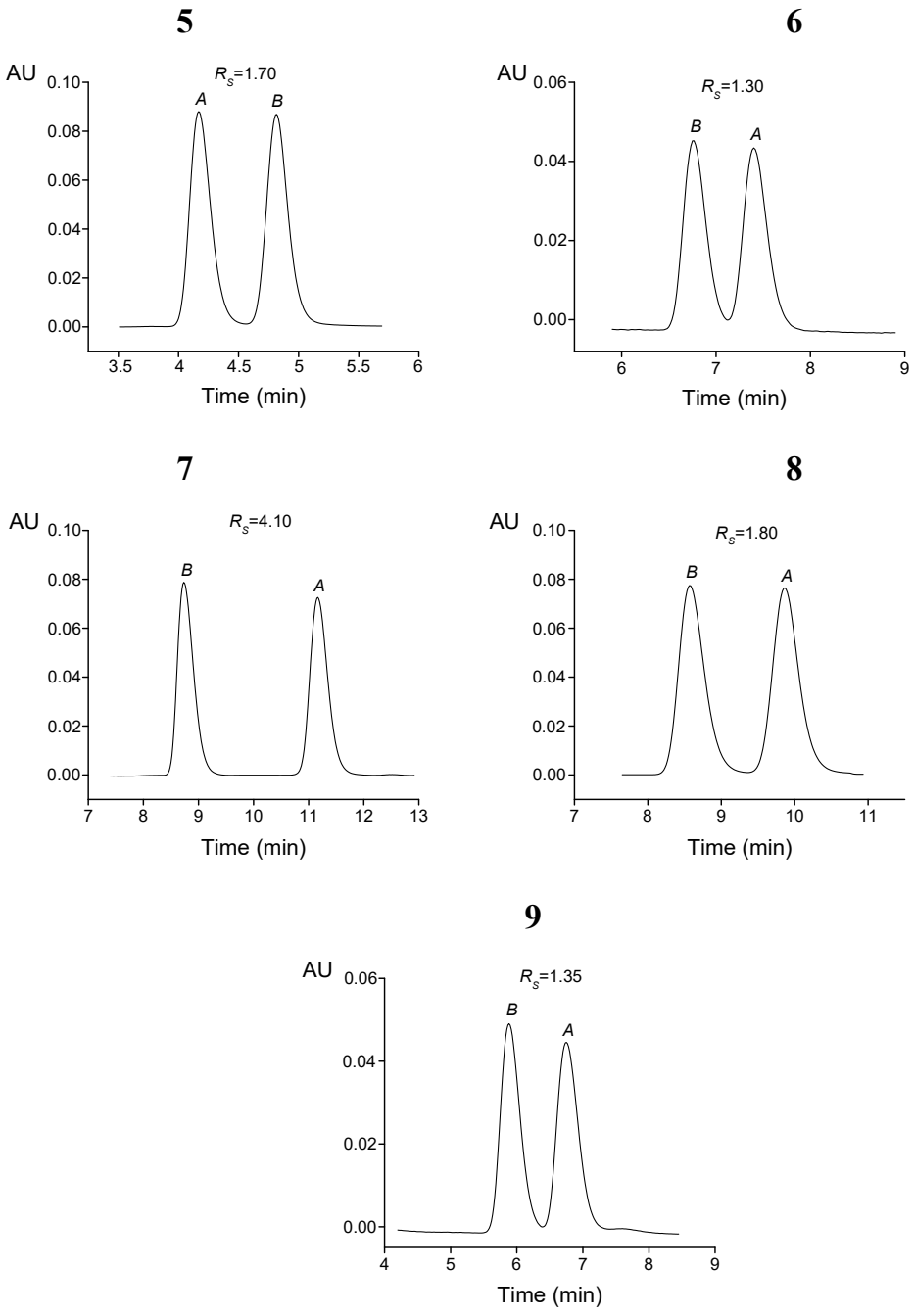
Chromatographic conditions: column: sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based CSP; mobile phase: for analytes **1A,1B, 2C,2D** and **3A,3B**, H_2O/EtOH (50/50 *v/v*) containing 0.2 mM $Cu(ClO_4)_2$, for analytes **1C,1D, 2A,2B** and **4A,4B** H_2O/MeOH (50/50 *v/v*) containing 0.2 mM $Cu(ClO_4)_2$, for analytes **3C,3D** and **4C,4D**, H_2O/EtOH (95/5 *v/v*) containing 0.2 mM $Cu(ClO_4)_2$; flow rate: 0.8 mL min^{-1}; detection, 230 nm; temperature: ambient.

Selected chromatograms of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on zwitterion-exchange CSP



Chromatographic conditions: columns, ZWIX(+)™ for **1A-1D** (left**)**, **2A-2D**, **3A-3D** and **4A-4D**, and ZWIX(-)™ for **1A-1D (**right**)**; mobile phase, **c,** MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH for **1A-1D** (left), **3A-3D** and **4A-4D**, MeOH/MeCN (25/75 *v/v*) containing 25 mM TEA and 50 mM AcOH for **1A-1D (**right**)** and **2A-2D**; flow rate, 0.6 mL min^{-1}; detection, 230 nm; temperature, 25 ºC.

Selected chromatograms of monoterpene-based β-amino acid analogues on zwitterion-exchange column



Chromatographic conditions: columns, ZWIX(+)™ for SAs **5** and **8**, and ZWIX(-)™ for SAs **6, 7,** and **9**; mobile phase, **c,** MeOH/MeCN (75/25 *v/v*) containing 25 mM PA and 50 mM AcOH for SAs **5, 8** and **9**, MeOH/MeCN (50/50 *v/v*) containing 25 mM TEA and 50 mM FA for SA **7,** and MeOH/MeCN (50/50 *v/v*) containing 25 mM TEA and 50 mM AcOH for SA **6**; flow rate, 0.6 mL min^{-1}; detection, 230 nm; temperature, 25 ºC.

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Research Article

High-performance liquid chromatographic enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on a chiral ligand-exchange stationary phase

The application of a chiral ligand-exchange column for the direct high-performance liquid chromatographic enantioseparation of unusual β-amino acids with a sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-Cu(II) complex as chiral selector is reported. The investigated amino acids were isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs. The chromatographic conditions were varied to achieve optimal separation. The effects of temperature were studied at constant mobile phase compositions in the temperature range 5–45°C, and thermodynamic parameters were calculated from plots of ln$k$ or lnα versus $1/T$. $\Delta(\Delta H^\circ)$ ranged from –2.3 to 2.2 kJ/mol, $\Delta(\Delta S^\circ)$ from –3.0 to 7.8 J mol^{-1} K^{-1} and $-\Delta(\Delta G^\circ)$ from 0.1 to 1.7 kJ/mol, and both enthalpy- and entropy-controlled enantioseparations were observed. The latter was advantageous with regard to the shorter retention and greater selectivity at high temperature. Some mechanistic aspects of the chiral recognition process are discussed with respect to the structures of the analytes. The sequence of elution of the enantiomers was determined in all cases.

Keywords: Chiral ligand-exchange chromatography / Isoxazoline-fused 2-aminocyclopentanecarboxylic acids / Sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based column
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1 Introduction

The importance of alicyclic β-amino acids in medicinal chemistry has increased considerably in recent decades because of their useful biological properties. They are present in natural products and several representative compounds such as cispentacin [(1*R*,2*S*)-2-aminocyclopentanecarboxylic acid] which exhibit antifungal properties. Oryzoxymycin [(*S*)-2-((5*R*,6*R*)-6-amino-5-hydroxycyclohexa-1,3-dienecarbonyloxy)-propanoic acid] and icofungipen ((1*R*,2*S*)-2-amino-4-methylenecyclopentanecarboxylic acid) are also bioactive compounds with antibacterial activities. The carbocyclic β-amino acid enantiomers can be used as building blocks for the preparation of modified biologically active peptide analogs, and as chiral auxiliaries in asymmetric transformations [1, 2]. Such compounds are widely applied as starting substances for the synthesis of heterocyclic compounds, potential pharmacons, and natural product analogs [3].

Isoxazoline ring-containing amino acids are also valuable substances in medicinal chemistry by virtue of their biological activities (anti-influenza, antifungal properties, and glutamatergic modulators) [4–7]. Isoxazoline-fused amino acids can be used as precursors for the preparation of multifunctionalized cyclopentane derivatives with antiviral properties, such as peramivir [(1*S*,2*S*,3*S*,4*R*)-3-[(1*S*)-1-acetamido-2-ethylbutyl]-4-(diaminomethylidene-amino)-2-hydroxy-cyclopentane-1-carboxylic acid] and its analogs [8–11].

The wide-ranging utility of these compounds requires analytical methods to check on the stereochemistry of the final product. One of the most frequently applied techniques is chiral HPLC. HPLC enantioseparations of β-amino acids have been performed by both indirect and direct methods. In the past decade, chiral derivatizing agents [12], chiral stationary phases (CSPs) such as macrocyclic glycopeptides [13–15], quinine derivatives [16], (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid derivatives [17–22] (also chiral NMR solvating agents) [23], and chiral ligand-exchange chromatography (CLEC) [24, 25] have been used for the analysis of the

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Abbreviations: CLEC, chiral ligand-exchange chromatography; **CSP**, chiral stationary phase

enantiomeric composition of β-amino acids including izoxazoline ring containing β-amino acids.

Since the early publications by Davankov et al. [26, 27], the value of CLEC in the separation of amino acid racemates has been amply demonstrated. Several chiral ligand-exchange stationary phases have been applied so far with different chiral selectors, e.g. proline [28, 29], norephedrine [30], a leucinol derivative [31], penicillamine [32, 33], *O*-benzyl-(*S*)-serine [24], *S*-benzyl-(*R*)-cysteine, *S*-trityl-(*R*)-cysteine [25, 34], and sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based materials [35, 36]. With Cu(II) as metal ion, the formation of a 5-membered chelate ring is normally expected and a square planar structure is usually proposed for the ternary complexes involving Cu(II) and chelating ligands [29, 30, 37].

Retention and separation are influenced by the concentration and nature of the mobile phase components, together with other variables, such as pH and temperature. In all CLEC systems, the eluent pH markedly defines the stability of the transient metal complexes and hence the chromatographic retention of analytes [24, 25, 38–42]. In chromatographic enantioseparations, the relationship between the chromatographic data and the column temperature is as follows:

$$\ln k = -\frac{\Delta H^{\circ}}{RT} + \frac{\Delta S^{\circ}}{R} + \ln\varphi \quad (1)$$

in which k is the retention factor, ΔH° is the enthalpy of transfer of the solute from the mobile phase to the stationary phase, ΔS° is the entropy of transfer of the solute from the mobile phase to the stationary phase, R is the gas constant, T is temperature, and φ is the reversal of the phase ratio (V_M/V_S) of the column.

Equation (1) reveals that a plot of $\ln k$ versus $1/T$ is linear, with slope $-\Delta H^{\circ}/R$ and intercept $\Delta S^{\circ}/R + \ln\varphi$, if ΔH° is invariant with temperature. Since the value of φ is often not known, the $\Delta S^{\circ *}$ values [$\Delta S^{\circ *} = \Delta S^{\circ} + R\ln\varphi$] calculated from the intercepts of the plots via Eq. (1) are generally used. Any uncertainty in the phase ratio affects all $\Delta S^{\circ *}$ values in the same manner.

The corresponding $\Delta(\Delta H^{\circ})$ and $\Delta(\Delta S^{\circ})$ values can be calculated as differences in ΔH° and ΔS°, respectively, for a given pair of enantiomers.

In chiral chromatography, however, the van't Hoff plots often deviate from linearity, possibly as a result of the inhomogeneity of the CSP surface, leading to a mixed retention mechanism [43]. Additionally, there are both achiral and chiral contributions to retention that can vary with a wide variety of experimental parameters [44–49].

In the present paper, direct HPLC methods are described for the separation of enantiomers of new cyclic isoxazoline ring containing β^3-amino acids (Fig. 1), with the application of a chiral ligand-exchange selector: sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate (Fig. 2). Besides enantioseparation, structure retention relationships were aimed to be investigated through the study of the chromatographic behavior and thermodynamic parameters in CLEC systems. All analytes were separated with different mobile phase compositions involving H_2O/alcohol + Cu(II) systems. The effects of the nature and concentration of the mobile phase additives (alcohol modifiers), Cu(II) salts, temperature, and the specific structural features of the analytes and selector on the separation are discussed on the basis of the experimental data.

2 Experimental

2.1 Chemicals and reagents

Racemic isoxazoline-fused 2-aminocyclopentanecarboxylates were synthetized by the 1,3-dipolar cycloaddition of nitrile oxides (ACN *N*-oxide and propionitrile *N*-oxide) to *N*-Boc-protected *cis*-2-aminocyclopent-3-enecarboxylate [50]. The major products were epimerized at C-5 with NaOEt in EtOH to give isoxazoline-fused *trans* amino esters. The 1,3-dipolar cycloaddition of nitrile oxides (ACN *N*-oxide and propionitrile *N*-oxide) to *N*-Boc-protected *trans*-2-aminocyclopent-3-enecarboxylate both regio- and stereoselectively furnished only one cycloadduct. These reactions were extended to the preparations of the enantiomerically pure forms, starting from the *cis*- and *trans*-2-aminocyclopent-3-enecarboxylate enantiomers [51]. The racemic and enantiomerically pure isoxazoline-fused ethyl 2-aminocyclopentanecarboxylates obtained were hydrolyzed in the presence of HCl/H_2O (2:1 v/v) in dioxane to give the corresponding amino acid derivatives **1–4** and their enantiomers **1a**, **1d**; **2a**, **2d**; **3a**, **3c**, and **4a**, **4c** (Fig. 1). Analytes **a**,**b** and **c**,**d** are enantiomer pairs; all other combinations are diastereomers.

Methanol (MeOH), ethanol (EtOH), propanol (PrOH), and 2-propanol (2-PrOH) of HPLC grade were purchased from Scharlau (Sentmenat, Spain). Cu(II) perchlorate, Cu(II) nitrate and Cu(II) sulfate and other reagents of analytical reagent grade were from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water was further purified by filtration on a 0.45-μm filter, type HV Millipore (Molsheim, France).

2.2 Apparatus and chromatography

The HPLC measurements were carried out on a Waters system consisting of a 1525 binary pump, a 487 dual-channel absorbance detector, a 717 plus auto sampler, and Empower 2 data manager software (Waters Chromatography, Milford, MA, USA). The chromatographic system was equipped with a Rheodyne Model 7125 injector (Cotati, CA, USA) with 20-μL loops.

Chromatographic separations were performed on a sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based column (150 × 4.0 mm, 5-μm particle size). The CSP was prepared from (*R*)-phenylglycinol by covalently bonding its derivative (*R*)-*N*,*N*-carboxymethyl undecyl phenylglycinol monosodium salt to Kromasil silica gel [32].

Stock solutions of amino acids (1 mg/mL) were prepared by dissolving them in the mobile phase.

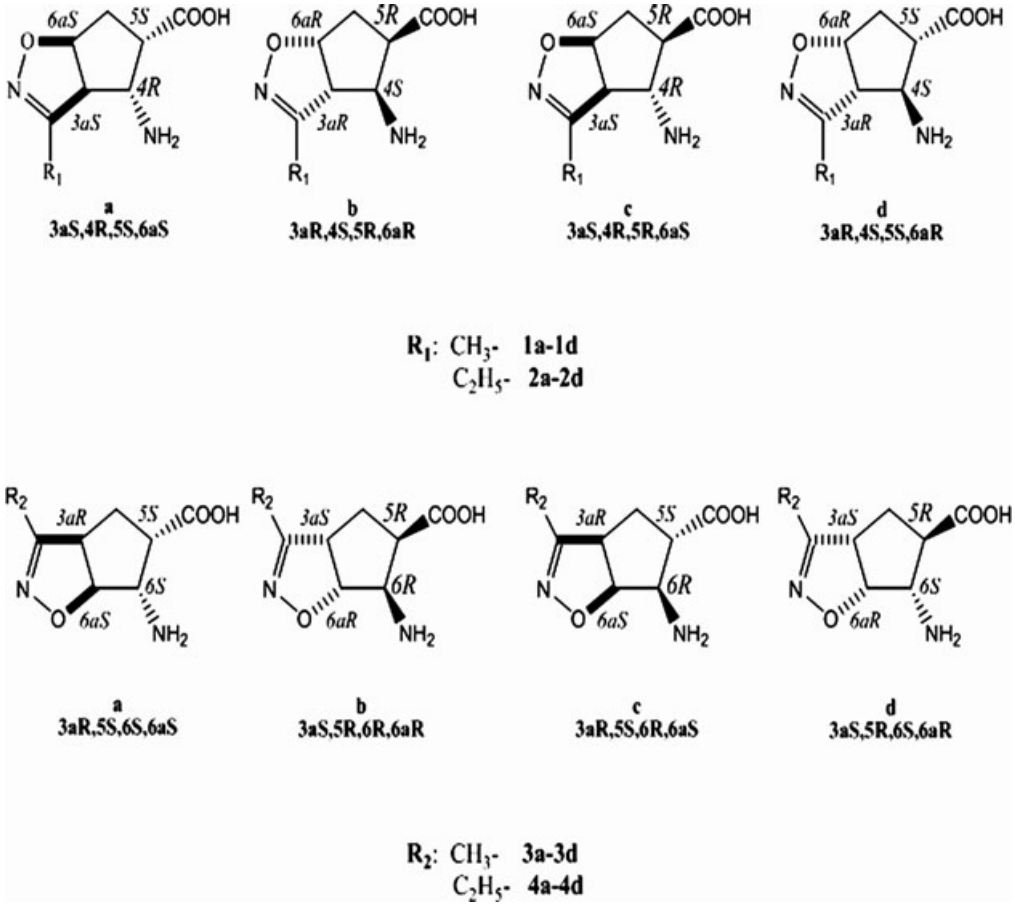

Figure 1. Structures of analytes.

3 Results and discussion

3.1 Effect of different parameters on chromatographic data

All compounds in Table 1 were evaluated by using the following mobile phases: H_2O/MeOH (95:5 v/v), (85:15 v/v), (70:30 v/v), and (50:50 v/v), and H_2O/EtOH (95:5 v/v), (85:15 v/v), (70:30 v/v), and (50:50 v/v), in all cases containing 0.2 mM total concentration of $Cu(ClO_4)_2$ in the eluent. To simplify the presentation, the chromatographic results for all mobile phase compositions are given for analytes **1a–1d** and **4a–4d**, while for analytes **2a–2d** and **3a–3d**, only the data for the mobile phase systems H_2O/MeOH (85:15 v/v), H_2O/MeOH (50:50 v/v), H_2O/EtOH (85:15 v/v), and H_2O/EtOH (50:50 v/v), all containing 0.2 mM $Cu(ClO_4)_2$ are given, since the trends in the results were the same for all analytes (Table 1). With these eluents, the stereoisomers of all analytes were retained and partial or baseline enantiomeric separations were achieved.

Chiral recognition in CLEC is explained as a result of the formation of mixed-ligand ternary complexes between the chiral selector and the enantiomers to be separated [52]. The formation and stability of these ternary complexes depend on numerous equilibria existing in the chromatographic column. The relative importance of a particular equilibrium depends on the separation conditions (mobile phase composition, quality and concentration of Cu(II) salts, pH, temperature, etc.) The retention of the amino acids is mainly determined by the equilibrium of ligand-exchange and by the hydrophobic/hydrophilic interactions [24, 25, 53]. In the case of isoxazoline ring containing β-amino acids the retention factor was largest at high and low water content. At high water content [H_2O/MeOH (95:5 v/v) and H_2O/EtOH (95:5 v/v)] this was probably due to the enhanced hydrophobic interactions between the analyte and the CSP in the water-rich mobile phases (Table 1). In the reversed-phase mode, one of the most important interactions between the analyte and the CSP is the hydrophobic interaction, which in most cases is non-enantioselective. Similar results were observed by Wernicke [53] using 0–10% MeOH in aqueous mobile phase and Cu(II)-Phe derivatives as selectors. On change of the MeOH and EtOH concentration from 5 to 15% k_1 decreases, but with further increase of the alcohol concentration it increases again (Table 1). An elevation of the alcohol concentration reduces the polarity of the mobile phase and eventually reduces the hydrophilic interaction of the analyte with the aqueous mobile phase. In this event, the retention of the analyte is enhanced as the alcohol concentration in the mobile phase is increased. With increasing MeOH content (from 10 to 50%)

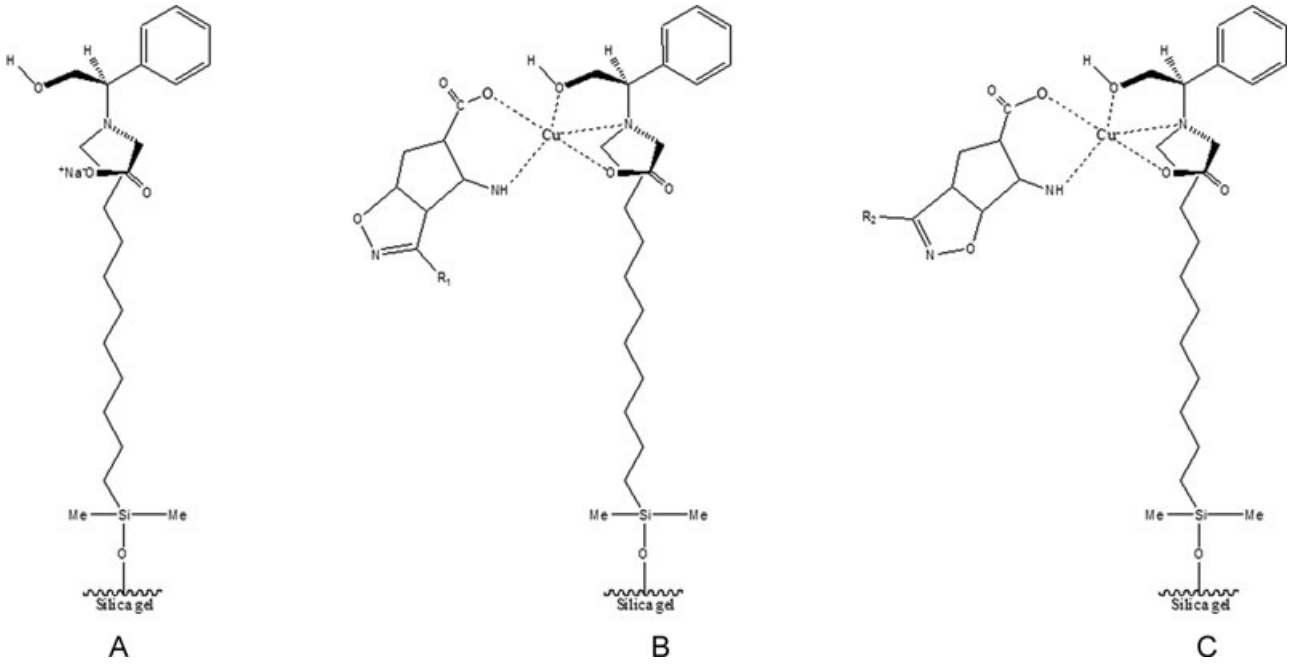

Figure 2. Structure of the sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based selector (A) and its terner complex with isoxazoline-fused 2-aminocyclopentanecarboxylic acids **1, 2** (B) and **3, 4** (C).

similar increase in k_1 was obtained by Hyun et al. for α- and β-amino acids on the same selector [35, 36]. This suggests that the separation may be controlled by hydrophilic interactions rather than by the reversed-phase (hydrophobic) mechanism at high alcohol contents [35, 36]. In the present study, the inflexion point and the slopes of the U-shaped curve at higher and lower alcohol concentrations differed somewhat for each compound. The different extents of solvation of the stationary phase under the applied conditions may explain the observed retention behavior. As regards the variations in the separation factors α and resolutions R_S with change of the MeOH content, both α and R_S often exhibited minimum values for the H_2O/MeOH (85:15 v/v) mobile phase system containing 0.2 mM $Cu(ClO_4)_2$, both values increasing if the MeOH content was raised. Exceptions were the *trans*-isomers of **2c**,**2d**, **3c**,**3d**, and **4c**,**4d**, where the α and R_S values continuously decreased with increasing MeOH content.

The nature of the alcohol modifier exerted major effects on the retention. Figure 3 demonstrates that, in the constant H_2O/alcohol (85:15 v/v) mobile phase systems containing 0.2 mM $Cu(ClO_4)_2$ at ambient temperature, the retention factor k_1 increased significantly with increasing carbon number of the alcohol (MeOH, EtOH, PrOH, and 2-PrOH), as indicated for analytes **2** and **3** (Fig. 3). The apolar character of the mobile phase increased in the sequence MeOH, EtOH, PrOH, 2-PrOH, i.e. with increasing carbon number of the alcohols, which is disadvantageous for polar interactions between the mobile phase and amino acids, as the retention factor increases. The nature of the alcohol modifier exhibited a small effect on the enantioselectivity: for **2c**,**2d** and **3a**,**3b** α varied in the range 1.09–1.14, and for **3c**,**3d** in the range 1.77–1.81 (**2a**,**2b** were not separable under these conditions). The highest difference in resolution was observed for analyte **3c**,**3d** when MeOH was replaced by PrOH.

In one set of experiments, the effect of the $Cu(ClO_4)_2$ concentration on the separation efficiency was investigated. On increase of the concentration of $Cu(ClO_4)_2$ from 0.05 mM to 0.5 mM, k_1 for analytes **2** and **3** first decreased significantly, reaching a minimum at 0.2 mM Cu(II) and then increased again (Fig. 4). A U-shaped curve describing the change in k_1 with increasing Cu(II) concentration is unusual. Similar behavior was observed by Natalini et al. applying *O*-benzyl-(*S*)-serine [24], *S*-benzyl-(*R*)-cysteine, and *S*-trityl-(*R*)-cysteine [25]. The increase in Cu(II) concentration in the eluent promoted the formation of several analyte complexes in the mobile phase. Consequently, the mass transfer equilibrium between the two phases is shifted toward the eluent thus shortening the retention time [25, 53]. As the Cu(II) concentration increases, Cu(II) acts more and more as the outstanding competitor to both the dative and hydrophobic/hydrophilic interactions between the analyte and selector. At higher Cu(II) concentration (0.3–05 mM) according to Kurganov [52], probably more intricate complexation events occurs in the column. Despite the large change in k_1, α and R_S changed only slightly: with the mobile phase H_2O/MeOH (85:15 v/v) containing 0.05–0.5 mM $Cu(ClO_4)_2$ α for the analytes **2c**,**2d**, **3a**,**3b**, and **3c**,**3d** was 1.12–1.14, 1.11–1.13, and 1.74–1.92, respectively, while R_S was 0.50–1.00, 0.45–0.85, and 2.45–4.00, respectively; the minimum was observed at 0.2 mM $Cu(ClO_4)_2$.

The anion of the Cu(II) salt exerts an appreciable effect on the chromatographic parameters, as was observed by Natalini et al. who studied the effect of copper counterions on the retention in details [24, 25, 34, 54]. The nature

Table 1. Retention factors (k), separation factors (α), resolutions (R_S), and elution sequences of isoxazoline-fused 2-aminocyclopentane carboxylic acids

Compound	Mobile phase H_2O/alcohol (v/v) + 0.2 mM Cu(II)	k_1	k_2	α	R_S	Elution sequence
1a,1b	**a**	12.17	13.02	1.07	0.40	*b* < *a*
	b	2.15	2.37	1.10	0.15	–
	c	2.75	3.79	1.38	1.60	*b* < *a*
	d	4.74	7.92	1.67	2.60	*b* < *a*
	e	11.97	12.45	1.04	0.20	–
	f	4.00	4.48	1.12	1.75	*b* < *a*
	g	4.50	6.44	1.43	1.80	*b* < *a*
	h	7.93	13.88	1.75	2.60	*b* < *a*
1c,1d	**a**	16.64	16.64	1.00	0.00	–
	b	3.72	3.72	1.00	0.00	–
	c	4.15	4.52	1.09	0.20	–
	d	6.54	7.72	1.18	0.80	*c* < *d*
	e	15.10	15.10	1.00	0.00	–
	f	6.43	6.43	1.00	0.00	–
	g	5.98	6.52	1.09	0.20	–
	h	12.55	14.56	1.16	0.90	*c* < *d*
2a,2b	**a**	2.76	2.76	1.00	0.00	–
	d	5.86	8.85	1.51	2.30	*b* < *a*
	e	5.12	5.12	1.00	0.00	–
	h	8.90	13.71	1.54	2.25	*b* < *a*
2c,2d	**a**	4.66	5.22	1.12	0.45	–
	d	8.84	9.11	1.03	0.20	–
	e	7.99	9.11	1.14	1.00	*c* < *d*
	h	9.55	15.85	1.66	2.80	*c* < *d*
3a,3b	**a**	3.23	3.59	1.11	0.50	–
	d	7.50	11.48	1.53	2.55	*b* < *a*
	e	6.08	6.75	1.11	0.50	*b* < *a*
	h	11.32	16.53	1.46	2.20	*b* < *a*
3c,3d	**a**	4.65	8.23	1.77	2.40	*c* < *d*
	d	8.62	12.15	1.41	1.85	*c* < *d*
	e	7.14	12.92	1.81	3.55	*c* < *d*
	h	13.95	17.72	1.27	1.45	*c* < *d*
4a,4b	**a**	20.80	23.50	1.13	1.00	*b* < *a*
	b	4.27	4.91	1.15	0.45	*b* < *a*
	c	5.15	6.85	1.33	1.65	*b* < *a*
	d	8.15	12.88	1.58	3.23	*b* < *a*
	e	19.32	21.09	1.09	0.18	*b* < *a*
	f	7.33	8.50	1.16	0.75	*b* < *a*
	g	9.88	13.63	1.38	1.65	*b* < *a*
	h	11.28	17.15	1.52	2.10	*b* < *a*
4c,4d	**a**	20.28	38.73	1.91	3.90	*c* < *d*
	b	5.72	11.15	1.95	3.10	*c* < *d*
	c	6.64	11.95	1.80	2.95	*c* < *d*
	d	9.59	15.25	1.59	1.95	*c* < *d*
	e	13.52	26.80	1.98	2.50	*c* < *d*
	f	8.93	17.68	1.98	3.50	*c* < *d*
	g	11.29	20.32	1.80	1.55	*c* < *d*
	h	13.66	19.26	1.41	1.45	*c* < *d*

Column, sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based CSP; mobile phase, **a**, H_2O/MeOH (95:5 v/v); **b**, H_2O/MeOH (85:15 v/v); **c**, H_2O/MeOH (70:30 v/v); **d**, H_2O/MeOH (50:50 v/v); **e**, H_2O/EtOH (95:5 v/v); **f**, H_2O/EtOH (85:15 v/v); **g**, H_2O/EtOH (70:30 v/v); **h**, H_2O/EtOH (50:50 v/v) all containing 0.2 mM $Cu(ClO_4)_2$; flow rate, 0.8 mL/min; detection, 230 nm; temperature, ambient.

of the anions exhibited only a small effect on the enantioselectivity: α remained practically constant. Depending on the nature of the anions in the mobile phase of H_2O/MeOH (85:15 v/v) containing 0.2 mM $Cu(ClO_4)_2$, $Cu(NO_3)_2$, or $CuSO_4$, the k_1 changed drastically (Table 2). Similarly, large changes in k_1 were obtained for aliphatic α-amino acids on *S*-trityl-(*R*)-cysteine selector [34], while with same selector investigating another set of amino acids, the same group reported that no noticeable variations on retention occurred [25]. Applying *O*-benzyl-(*S*)-serine selector [24, 54], the change in retention factors were negligible, while the resolution was highly influenced. Applying perchlorate, nitrate, sulfate counterions under the same conditions, we have found the same influence on the resolution that was observed by Natalini et al. [24]; the lowest resolution was found with perchlorate, while much higher but approximately the same resolution was obtained with nitrate and sulfate ions. Natalini et al. [24,25,34,54] found that each cupric anion type could act as competitor in the complexation of the amino acids, and their influence on the complexation process is strongly dependent on the peculiar physico-chemical properties of the analytes. This can be the reason in our case also that no general rule could be obtained for the explanation of the observed changes in the chromatographic parameters.

The structures of the analytes influenced the chiral recognition. At a given mobile phase composition in the reversed-phase mode, the ethyl-substituted analogs in most cases interacted more strongly with the selector than did the methyl-substituted ones, leading to slightly larger retentions (Tables 1 and 2). However, the stronger interactions resulted in larger α and R_S values in only a few cases, indicating almost the same interactions of the two enantiomers with the CSP. Through the steric interactions, the position of the methyl or ethyl group influenced the α and R_S values. In general, compounds **3** and **4** underwent better enantioseparation than did analytes **1** and **2**, respectively. In most cases, the *trans*-isomers (**c**,**d**) exhibited larger enantioseparation than did the *cis*-isomers (**a**,**b**).

The sequence of elution of the enantiomers was identified by co-chromatography of racemic mixtures with an enantiomer of known configuration in most cases (Table 1). For *cis*-isomers $b < a$ and for *trans*-isomers $c < d$ elution sequence was observed. Selected chromatograms for the separation of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs are depicted in Supporting Information Fig. S1.

3.2 Effects of temperature and thermodynamic parameters

In order to investigate the effects of temperature on the chromatographic parameters, a variable-temperature study was carried out for all analytes over the temperature range 5–45°C. Experimental data for the mobile phase H_2O/MeOH (95:5 v/v) containing 0.2 mM $Cu(ClO_4)_2$ are listed in Supporting Information Table S1. All of the recorded retention factors decreased with increasing temperature. The

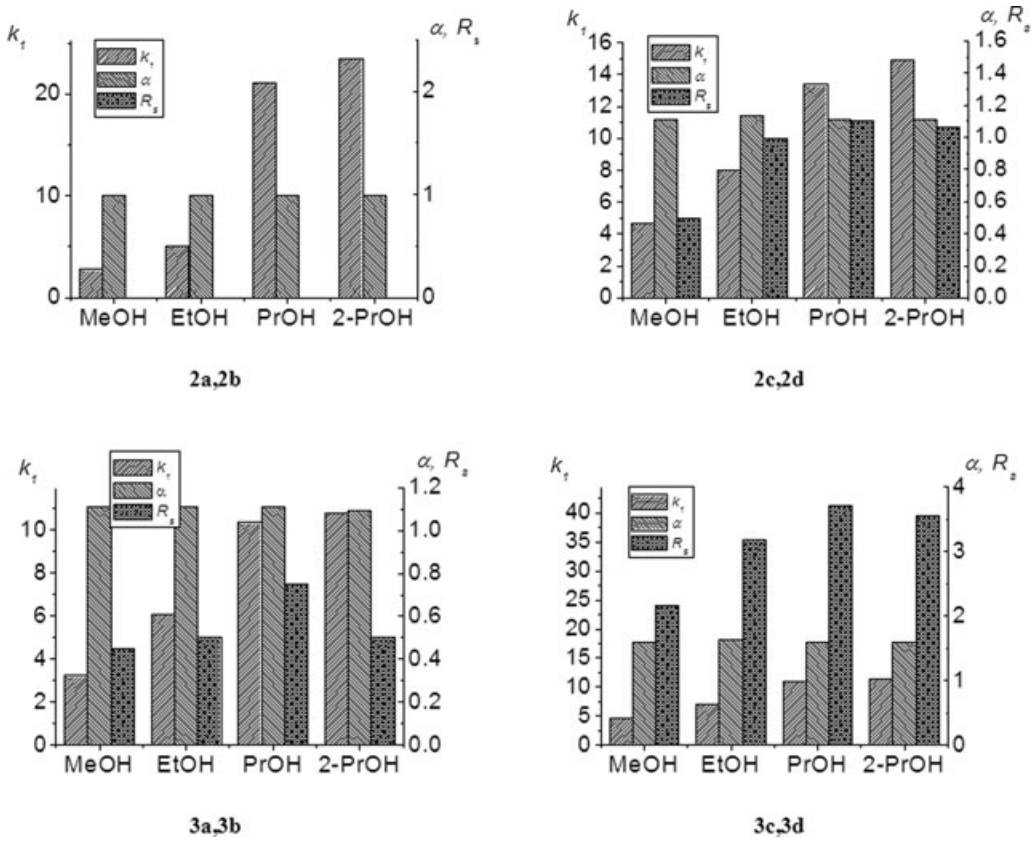

Figure 3. Effects of the nature of the alcohol modifier on the retention factor of the first-eluting enantiomer (k_1), the separation factor (α) and the resolution (R_S) for analytes **2** and **3**. Chromatographic conditions: column, sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based CSP; mobile phase, H_2O/alcohol (85:15 v/v) containing 0.2 mM $Cu(ClO_4)_2$; flow rate, 0.8 mL/min; detection, 230 nm; temperature, ambient.

separation factors α for analytes **1a**,**1b**, **2c**,**2d**, **3c**,**3d**, and **4c**,**4d** decreased with increasing temperature, whereas those for analytes **2a**,**2b**, **3a**,**3b**, and **4a**,**4b** increased with increasing temperature. R_S for analytes **1a**,**1b** and **2c**,**2d** decreased with

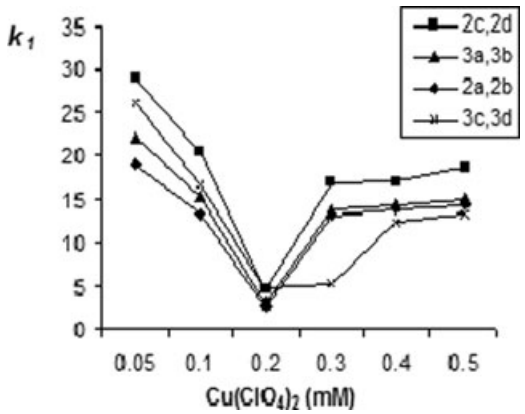

Figure 4. Effects of the $Cu(ClO_4)_2$ concentration on the retention factor of the first-eluting enantiomer (k_1) for analytes **2** and **3**. Chromatographic conditions: column, sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based CSP; mobile phase, H_2O/MeOH (85:15 v/v) containing 0.05–0.5 mM $Cu(ClO_4)_2$; flow rate, 0.8 mL/min; detection, 230 nm; temperature, ambient.

Table 2. Selected chromatographic parameters, k_1, α, and R_S, of enantiomers of isoxazoline-fused 2-aminocyclopentane carboxylic acids as a function of nature of anions

Analyte	k_1, α, R_S	Mobile phase H_2O/MeOH (85:15 v/v) containing 0.2 mM Cu(II)		
		ClO_4^-	NO_3^-	SO_4^-
2a,2b	k_1'	2.76	17.72	18.58
	α	1.00	1.00	1.00
	R_S	0.00	0.00	0.00
2c,2d	k_1'	4.66	21.74	22.42
	α	1.12	1.14	1.14
	R_S	0.50	1.00	1.05
3a,3b	k_1'	3.23	20.88	21.14
	α	1.11	1.11	1.12
	R_S	0.45	0.78	0.89
3c,3d	k_1'	4.65	16.87	17.96
	α	1.77	1.78	1.79
	R_S	2.41	3.68	3.77

Column, sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylamino acetate-based CSP; mobile phase, H_2O/MeOH (85:15 v/v) containing 0.2 mM Cu(II) ion; flow rate, 0.5 mL/min; detection, 205 nm; temperature, ambient.

Table 3. Thermodynamic parameters, ΔH°, $\Delta S^{\circ *}$, $\Delta(\Delta H^\circ)$, $\Delta(\Delta S^\circ)$, $\Delta(\Delta G^\circ)$, and correlation coefficients (R^2) of isoxazoline-fused 2-aminocyclopentane carboxylic acids (**1–4**)

Analyte	Enantiomer	$-\Delta H^\circ$ (kJ/mol)	$-\Delta S^{\circ *}$ (J mol^{-1} K^{-1})	Correlation coefficients (R^2)	$-\Delta(\Delta H^\circ)$ (kJ/mol)	$-\Delta(\Delta S^\circ)$ (J mol^{-1} K^{-1})	$-\Delta(\Delta G^\circ)_{298K}$ (kJ/mol)
1a,1b	1	19.9	46.0	0.9963	0.8	2.4	0.1
	2	20.7	48.4	0.9965			
1c,1d	1	14.2	24.0	0.9964	0.00	0.00	0.00
	2	14.2	24.0				
2a,2b	1	16.8	32.8	0.9952	−2.2	−7.8	0.1
	2	14.6	25.0	0.9921			
2c,2d	1	12.3	16.6	0.999	1.3	3.0	0.4
	2	13.6	19.6	0.9988			
3a,3b	1	19.6	40.9	0.9992	−1.3	−4.8	0.2
	2	18.3	36.1	0.9959			
3c,3d	1	15.7	28.9	0.9954	2.3	3.0	1.4
	2	18.0	31.9	0.9962			
4a,4b	1	19.6	39.2	0.9953	−1.7	−6.4	0.2
	2	17.9	32.8	0.9979			
4c,4d	1	15.7	27.4	0.9922	2.2	1.8	1.7
	2	17.9	29.2	0.9940			

Chromatographic conditions: column, sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based CSP; mobile phase, H_2O/MeOH (95:5 v/v) containing 0.2 mM $Cu(ClO_4)_2$; flow rate, 0.8 mL/min; detection, 230 nm; $\Delta S^{\circ *} = \Delta S^\circ + R \ln\varphi$, where φ is reversal of the phase ratio; R^2, correlation coefficient of van't Hoff plot, $\ln k - 1/T$ curves; $\Delta(\Delta H^\circ) = \Delta H_2{}^\circ - \Delta H_1{}^\circ$; $\Delta(\Delta S^\circ) = \Delta S_2{}^{\circ *} - \Delta S_1{}^{\circ *}$

increasing temperature, but for all other analytes increased with increasing temperature (analyte **1c**,**1d** was not separable under these conditions).

To shed light on the effects of temperature on the separation, accurate chromatographic data were used to construct van't Hoff plots (Eq. (1)). The ΔH° and $\Delta S^{\circ *}$ values were negative (Table 3).

$\Delta(\Delta H)^\circ$ and $\Delta(\Delta S)^\circ$ exhibited both negative and positive values. When $\Delta(\Delta H)^\circ$ and $\Delta(\Delta S)^\circ$ are both negative, the enantioseparation is enthalpically driven, as in the common case. The second-eluted enantiomer forms a more stable complex with the selector than does the first-eluted enantiomer, with more unfavorable entropy for enantioseparation. The separation factor decreases with increasing temperature. If the enantioseparation is entropically driven, the separation factor increases with increasing temperature, parallel with decreasing of retention (Supporting Information Table S1). These two advantages (retention time decreases and separation factor increases) make entropically driven enantioseparation especially attractive.

The $\Delta(\Delta H^\circ)$ values ranged from –2.3 to 2.2 kJ/mol (Table 3). The interactions of analytes **2c**,**2d**, **3c**,**3d**, and **4c**,**4d** (*trans*-isomers) were characterized by the highest negative $\Delta(\Delta H^\circ)$ values, while **2a**,**2b**, **3a**,**3b**, and **4a**,**4b** (*cis*-isomers) exhibited the lowest negative $\Delta(\Delta H^\circ)$. The trends in the change in $-\Delta(\Delta S^\circ)$ showed that the largest negative entropies were observed for analytes **1a**,**1b**, **2c**,**2d**, **3c**,**3d**, and **4c**,**4d** (2.4, 3.0, 3.0, and 1.8 J mol^{-1} K^{-1}, respectively). For analytes **2a**,**2b**, **3a**,**3b**, and **4a**,**4b**, both $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$ were positive in the investigated temperature range (Table 3). The $\Delta(\Delta S^\circ)$ values are governed by the difference in the number of degrees of freedom between the stereoisomers on the CSP, and mainly by the numbers of solvent molecules released from the chiral selector and the analyte when the analyte is associated with the CSP [55].

The thermodynamic parameter $-\Delta(\Delta G^\circ)_{298K}$ suggests that *trans* (**c**,**d**) analogs induce more efficient binding to the selector, as reflected by the larger negative $\Delta(\Delta G^\circ)$ values (analyte **1c**,**1d** was not separable under these conditions).

4 Concluding remarks

A chiral ligand-exchange column was applied for the direct separation of enantiomers of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs. As regards the chromatographic properties, almost baseline separation of the enantiomers of each analyte was achieved with $R_S > 0.9$. The chromatographic retention behavior and resolution proved to depend on the nature and concentration of the alcohol modifier, the nature and concentration of Cu(II) salt present, the temperature, and the nature and position of the alkyl substituent in the analytes. The values of thermodynamic parameters such as the changes in enthalpy, $\Delta(\Delta H^\circ)$, entropy, $\Delta(\Delta S^\circ)$, and Gibbs energy, $\Delta(\Delta G^\circ)$, depended on the structures of the analytes; in some cases, entropically driven separation was observed. The elution sequence was determined in all cases, and a general predictive rule could be found to describe the elution behavior of these compounds.

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The authors have declared no conflict of interest.

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Investigation of the structure–selectivity relationships and van't Hoff analysis of chromatographic stereoisomer separations of unusual isoxazoline-fused 2-aminocyclopentanecarboxylic acids on *Cinchona* alkaloid-based chiral stationary phases☆

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ABSTRACT

The enantiomers of four unusual, rather rigid isoxazoline-fused 2-aminocyclopentanecarboxylic acids were directly separated on a quinine- or a quinidine-based zwitterionic ion-exchanger as chiral selector. The effects of the mobile phase composition, the structures of the analytes and temperature on the separations were investigated. Experiments were performed at constant mobile phase composition in the temperature range 10–50 °C to study the effects of temperature, and thermodynamic parameters were calculated from plots of ln α versus $1/T$. Some mechanistic aspects of the chiral recognition process are discussed with respect to the structures of the analytes. It was found that the enantiomer separations were in most cases predominantly enthalpy-driven, but entropically-driven separations were also observed. The sequence of elution of the enantiomers was determined in all cases.

1. Introduction

Thanks to their pharmacological potential, cyclic β-amino acids have become an important research field in medicinal and organic chemistry. A number of biologically active compounds contain a cyclic β-amino acid unit, e.g. the antibiotics amipurimycin ((2*R*)-α-[[(2-aminocyclopentyl)carbonyl]amino]-2β-(2-amino-9*H*-purin-9-yl)-4α-(1,2-dihydroxyethyl)-3α,4β-dihydroxytetrahydro-2*H*-pyran-6β-acetic acid), or pitucamycin ((3*R*,4*R*,5*R*)-2-amino-3,4-dihydroxy-5-[[8-(hydroxymethyl)-3-oxophenoxazin-2-yl]amino]-6-oxocyclohexene-1-carboxamide). β-Amino acids can also be used as starting materials for the preparation of heterocyclic compounds, potential pharmacons and natural product analogs [1].

Isoxazolines are also important heterocyclic compounds in medicinal chemistry, various substituted isoxazolines exhibiting anti-influenza activity and antifungal properties [2–5]. Besides their biological properties, isoxazolines are additionally valuable compounds in synthetic chemistry, serving as important precursors for the preparation of multifunctionalized derivatives [6–8].

Since their stereochemistry determines the biological and physicochemical properties of amino acids, analytical methods are required to check on the enantiomeric excess of the final product. Among the possible methods, enantioselective high-performance liquid chromatography (HPLC) is routinely used for the discrimination of enantiomers. Despite the large number of chromatographic enantioseparations of β-amino acids presented in several review articles [9–13], relatively few papers have dealt with the enantioseparation of alicyclic β-amino acids. For the HPLC enantioseparations of alicyclic β-amino acids, chiral derivatizing agents [14] and different chiral stationary phases (CSPs), e.g. macrocyclic glycopeptides [15–18], (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid derivatives [19] and chiral ligand exchange columns [20], have been used. Zwitterionic *Cinchona*

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alkaloid-based CSPs were recently applied for the enantioseparation of alicyclic β-amino acids [21–23].

Temperature generally influences enantioselective retention and separation [24–28] to a great extent. In order to determine the enthalpic and entropic contributions to the retention, van't Hoff plots are generally applied [29], based on the equation

$$\ln k = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R} + \ln \phi \quad (1)$$

where k is the retention factor, ΔH° is the standard enthalpy of transfer of the solute from the mobile phase to the stationary phase, ΔS° is the standard entropy of transfer of the solute from the mobile phase to the stationary phase, R is the gas constant, T is temperature in Kelvin and ϕ is the phase ratio $\phi = V_S/V_M$ [the ratio of the volumes of the stationary phase (V_S) and the mobile phase (V_M)]. If both enantiomers have access to the same stationary phase volume, the $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$ values for the separated enantiomers can be determined through a modification of Eq. (1), from the relationship

$$\ln \alpha = -\frac{\Delta(\Delta H^o)}{RT} + \frac{\Delta(\Delta S^o)}{R} \quad (2)$$

where α is the selectivity factor ($\alpha = k_2/k_1$), $\Delta(\Delta H^\circ)$ is the difference in standard enthalpy change, and $\Delta(\Delta S^\circ)$ is the difference in standard entropy change for the two enantiomers. For the purposes of this study, the classical van't Hoff approach, assuming only one type of interaction site on the stationary phase, was used. For a more realistic approach, the contributions of enantioselective and nonselective (e.g. at the support material, achiral linkers, or achiral interaction sites of the selector) sites should be distinguished. This can be achieved through the application of nonlinear characterization methods [30,31].

The present paper describes direct HPLC methods for the separation of the stereoisomers of a set of constrained alicyclic β^3-amino acids (Fig. 1), with the application of zwitterionic *Cinchona* alkaloid-based CSPs (Fig. 2). For comparison purposes, most of the separations were carried out at constant mobile phase composition. On the basis of the experimental data, the effects of the mobile phase composition, specific structural features of the analytes and selectors and temperature on the retention are discussed. The elution sequence was determined in all cases.

2. Experimental

2.1. Preparation of the applied analytes

The preparation of racemic isoxazoline-fused cyclic β-amino acid derivatives was based on the 1,3-dipolar cycloaddition of nitrile oxides (generated *in situ* from nitroethane or 1-nitropropane) to *N*-Boc-protected ethyl *cis*-2-amino-3-cyclopentenecarboxylate or *N*-Boc-protected ethyl *trans*-2-amino-3-cyclopentenecarboxylate. The cycloaddition reactions were performed in THF at room temperature in the presence of Boc_2O and 4-dimethylaminopyridine. When *N*-Boc-protected ethyl *cis*-2-amino-3-cyclopentenecarboxylate was used as a dipolarophile, the

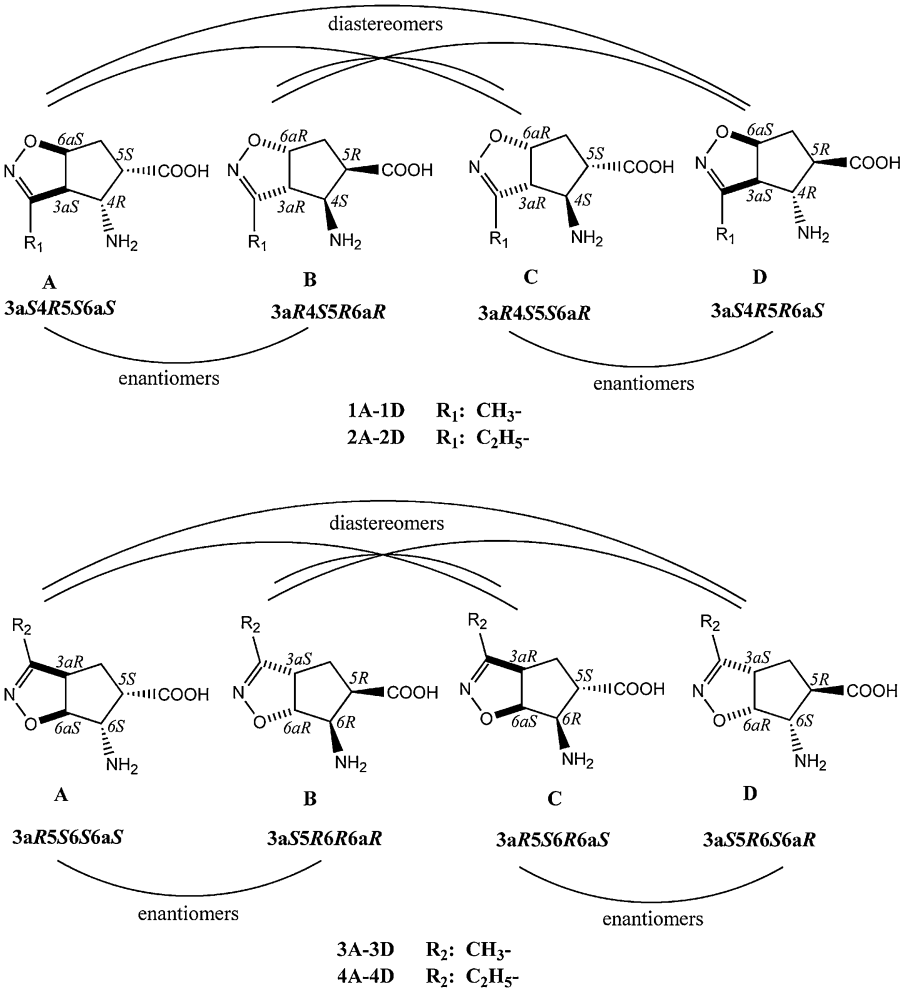

Fig. 1. Structures of investigated stereoisomers of isoxazoline-fused 2-aminocyclopentanecarboxylic acids.

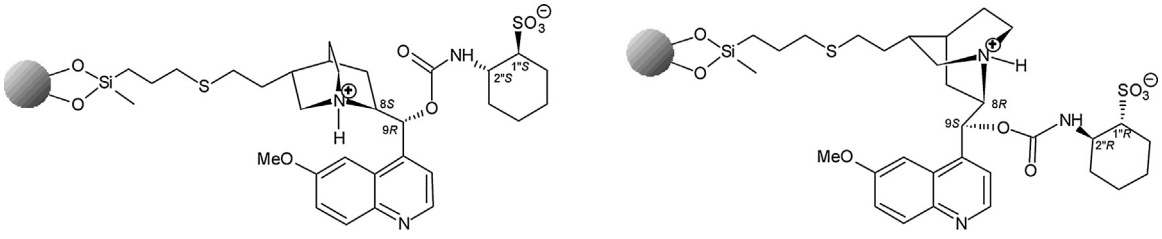

Fig. 2. Structure of *Cinchona* alkaloid-based chiral selectors and CSPs.

cycloaddition resulted in three isoxazoline-fused cyclic β-amino acid stereoisomers. The ratios of the stereoisomers were determined by ^{1}H NMR spectroscopy of the crude product (stereoisomeric ratio: 10:1:1). The products were purified by column chromatography [32]. (Yields: major product: 35–61%; minor product: 6–15%; very minor product: 6%). In the case of *N*-Boc-protected ethyl *trans*-2-amino-3-cyclopentenecarboxylate, the reaction was regio- and stereoselective and resulted in only one isoxazoline-fused derivative. The isolated yield was 27–46%. Epimerization of the cycloadducts at C-5 with NaOEt in EtOH gave the corresponding *trans* amino ester derivatives in 34–52% yields [33]. These reactions were also performed by starting from the pure *N*-Boc-protected ethyl-*cis*-2-amino-3-cyclopentenecarboxylate and *N*-Boc-protected ethyl *trans*-2-amino-3-cyclopentenecarboxylate enantiomers, prepared from racemic bicyclic β-lactam by enzymatic resolution [34,35]. The cycloadditions were performed similarly as for the racemic compounds. For the preparation of deprotected cyclic β-amino acid stereoisomers, hydrolysis was carried out in the presence of HCl/H_2O (2:1). The reactions in dioxane at room temperature resulted in amino acids **1A**, **1D**, **2A**, **2D**, **3A**, **3D**, **4A** and **4D** in quantitative yields. The purity of the products were analysed by NMR spectroscopy (higher than 98%) and elementary analyses (C,H,N, the error was less than 0.3%).

2.2. Chemicals and reagents

Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade, and ammonia, ethylamine (EA), diethylamine (DEA), triethylamine (TEA), propylamine (PRA) tripropylamine (TRP), butylamine (BA), tributylamine (TBA), formic acid (FA) and glacial acetic acid (AcOH) of analytical reagent grade were purchased from VWR International (Radnor, PA, USA). Milli-Q water was further purified by filtration on a 0.45-μm filter, type HV, Millipore (Molsheim, France).

2.3. Apparatus and chromatography

All analytical analyses were carried out on a 1100 Series HPLC system from Agilent Technologies (Waldbronn, Germany), consisting of a solvent degasser, a pump, an autosampler, a column thermostat and a multiwavelength UV–Vis detector and a corona charged aerosol detector from ESA Biosciences, Inc. (Chelmsford, MA, USA). Data acquisition and analysis were carried out with ChemStation chromatographic data software from Agilent Technologies.

The *Cinchona* alkaloid-based zwitterionic stationary phases used for analytical separations were Chiralpak ZWIX(+)^{TM} and ZWIX(−)^{TM} columns, 150 mm × 3 mm I.D., 3-μm particle size (for each column) (Chiral Technologies Europe, Illkirch, France).

Samples were prepared at approximately 0.2 mg ml^{-1} in the mobile phase. Flow rates were maintained at 0.6 ml min^{-1} and detection was performed at 215 and 230 nm or with the corona detector. Separations were achieved between 5 and 50 °C.

3. Results and discussion

3.1. Mobile phase selection

In the *Cinchona* alkaloid-based CSPs (Fig. 2), the weak tertiary amine group (pKa ∼ 9.5) on the quinuclidine site acts as an anion-exchanger in its protonated state, while the strong sulfonic acid site functions as a cation-exchanger moiety. The ion-exchanger parts of the selector promote electrostatic interactions providing retention, but secondary interactions, such as H-bonding, π–π, dipole–dipole and van der Waals forces, are required to the chiral recognition processes [36–38]. The *Cinchona*-derived CSPs have often been used in a non-aqueous polar ionic mode (PIM) with MeOH and MeCN as bulk solvent, and acid and base additives to ensure ionization of the analytes in the mobile phase [39].

The influence of the composition of the bulk solvent on the chromatographic parameters was investigated for all analytes on ZWIX(+)^{TM} and ZWIX(−)^{TM} in the presence of MeOH as protic solvent and MeCN as aprotic solvent. The mobile phase system consisted of MeOH/MeCN containing increasing amounts of MeCN (25%, 50% and 75%) and also 50 mM AcOH and 25 mM TEA, the acid-to-base ratio being kept constant at 2:1. The retention of zwitterionic amino acids in most cases increased substantially with increasing MeCN content [on ZWIX(−)^{TM}, increase of the MeCN level to 75% resulted in extremely high k values]. This trend was observed for all investigated analytes on both stationary phases and accords with the results obtained earlier for β-amino acids [21–23]. The increases in k on increase of the content of the aprotic polar MeCN were probably due to the decreased solvation effect of MeCN and the stronger electrostatic interactions of the selectands with the selector. The higher MeOH content in the bulk solvent promotes solvation together with an effect on the H-bond formation, and hence a decrease in the strength of interaction with the selector.

As concerns the selectivity, regardless of the nature and the position of the substituent, the separation factors (α) were similar, while the resolution in most cases exhibited maximum value at the MeOH/MeCN (50/50 *v/v*) mobile phase composition .Acid and base additives greatly influence the solvation of both selectors and selectands (analytes), and consequently variation of the ionic strength has a great effect on the overall chromatographic behavior. For comparisons of the effects of the concentrations and ratios of the acid and base components as mobile phase additives in one set of experiments the concentration of TEA was kept at constant level (12.5 mM) and the concentration of the AcOH was varied between 3.125 mM and 50 mM, while in another set of experiments the concentration of AcOH was kept at constant

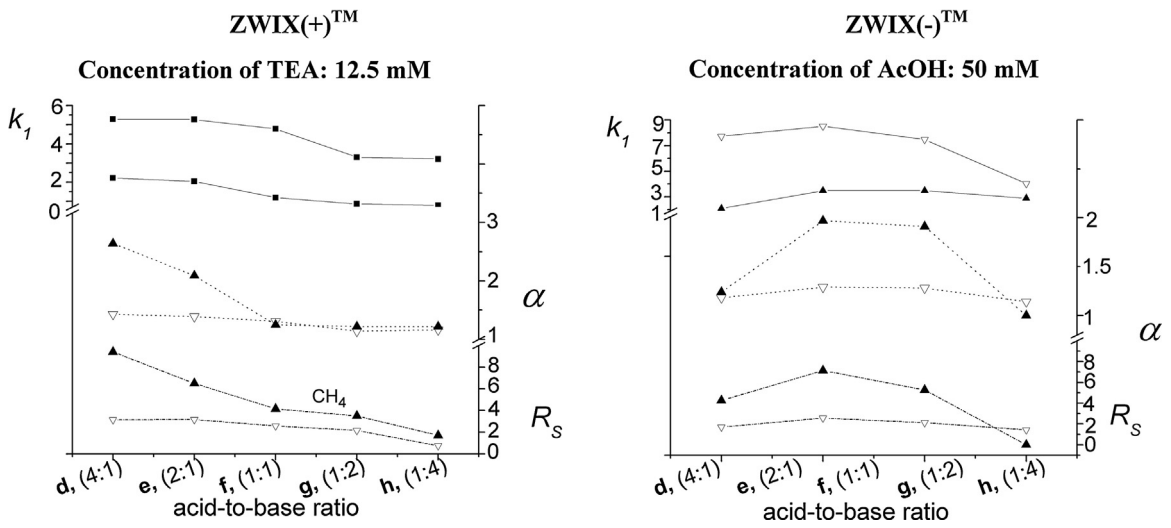

Fig. 3. Effects of acid-to-base ratio for **1A,1B,** and **4C,4D** on chromatographic parameters.
Chromatographic conditions: column, Chiralpak ZWIX(+)™ and ZWIX(−)™; mobile phase composition (including acid-to-base ratio)**,** MeOH/MeCN (75/25 *v/v*) containing **d,** 50.0 mM AcOH and 12.5 mM TEA (4:1), **e,** 25.0 mM AcOH and 12.5 mM TEA (2:1), **f,** 12.5 mM AcOH and 12.5 mM TEA (1:1), **g,** 6.25 mM AcOH and 12.5 mM TEA (1:2), and **h,** 3.12 mM AcOH and 12.5 mM TEA (1:4); or MeOH/MeCN (75/25 *v/v*) containing **i,** 50.0 mM AcOH and 5.0 mM TEA (10:1), **j,** 50.0 mM AcOH and 10.0 mM TEA (5:1), **c,** 50.0 mM AcOH and 25.0 mM TEA (2:1), **k,** 50.0 mM AcOH and 50.0 mM TEA (1:1); flow rate, 0.6 ml min^{-1}; detection, 230 nm; symbols, k_1: ▽ for **1A,1B,** ▲ for **4C,4D;** α: ▽: for **1A,1B** and ▲ for **4 C,4D;** R_S: ▽: for **1A,1B** and ▲ for **4 C,4D**

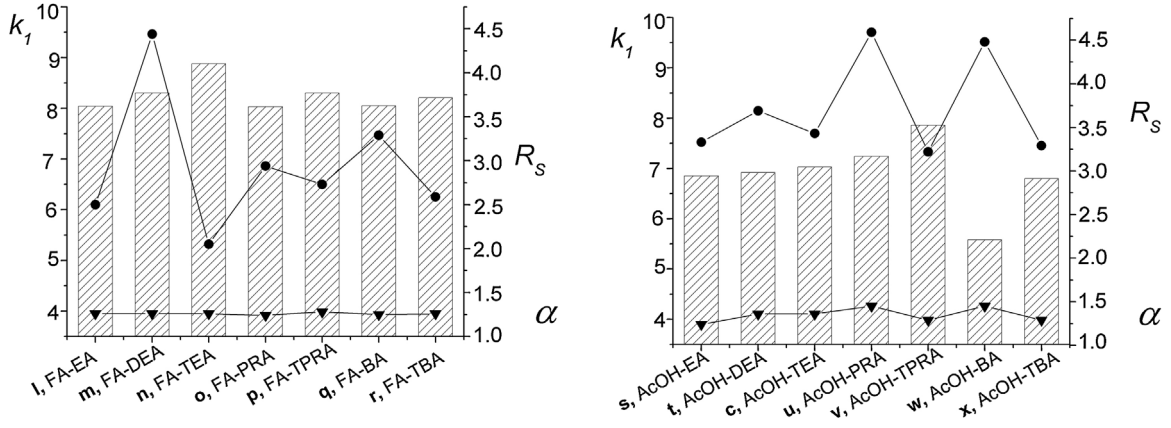

Fig. 4. Effects of nature of acid and base additives on chromatographic parameters of analyte **1A,1B** Chromatographic conditions: column, ZWIX(−)™; mobile phase, MeOH/MeCN (75/20 *v/v*) containing **l,** 50.0 mM FA and 25.0 mM EA, **m,** 50.0 mM FA and 25.0 mM DEA, **n,** 50.0 mM FA and 25.0 mM TEA, **o,** 50.0 mM FA and 25.0 mM PRA, **p,** 50.0 mM FA and 25.0 mM TPRA, **q,** 50.0 mM FA and 25.0 mM BA, **r,** 50.0 mM FA and 25.0 mM TBA, **s,** 50.0 mM AcOH and 25.0 mM EA, **t,** 50.0 mM AcOH and 25.0 mM DEA, **c,** 50.0 mM AcOH and 25.0 mM TEA**, u,** 50.0 mM AcOH and 25.0 mM PRA, **v,** 50.0 mM AcOH and 25.0 mM TPRA, **w,** 50.0 mM AcOH and 25.0 mM BA, **x,** 50.0 mM AcOH and 25.0 mM TBA; flow rate, 0.6 ml min^{-1}; detection, 230 nm; symbols, α: ▲; R_S: •.

Table 1
Chromatographic data retention factor (k), selectivity factor (α), resolution (R_S) and elution sequence of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on ZWIX(+)™ and ZWIX(−)™.

Compound	Column	k_1	k_2	α	R_S	Elution sequence
1A,1B	ZWIX(+)™	4.20	5.90	1.40	2.71	5S,4R < 5R,4S
	ZWIX(−)™	7.03	9.54	1.36	3.43	5R,4S < 5S,4R
1 C,1D	ZWIX(+)™	5.42	7.76	1.46	3.00	5S,4S < 5R,4R
	ZWIX(−)™	7.34	8.52	1.16	2.32	5R,4R < 5S,4S
2A,2B	ZWIX(+)™	3.50	4.38	1.25	1.71	5S,4R < 5R,4S
	ZWIX(−)™	6.96	8.35	1.20	2.47	5R,4S < 5S,4R
2 C,2D	ZWIX(+)™	4.74	7.72	1.63	2.73	5S,4S < 5R,4R
	ZWIX(−)™	5.36	6.16	1.55	1.92	5R,4R < 5S,4S
3A,3B	ZWIX(+)™	3.02	4.85	1.61	6.00	5S,6S < 5R,6R
	ZWIX(−)™	4.16	6.54	1.57	6.16	5R,6R < 5S,6S
3 C,3D	ZWIX(+)™	1.93	4.50	2.33	4.62	5S,6R < 5R,6S
	ZWIX(−)™	4.57	4.89	1.25	1.55	5R,6S < 5S,6R
4A,4B	ZWIX(+)™	2.84	4.66	1.64	5.54	5S,6S < 5R,6R
	ZWIX(−)™	4.08	6.27	1.54	6.08	5R,6R < 5S,6S
4 C,4D	ZWIX(+)™	1.80	4.36	2.42	6.00	5S,6R < 5R,6S
	ZWIX(−)™	2.77	5.54	2.00	5.66	5R,6S < 5S,6R

Chromatographic conditions: column, Chiralpak ZWIX(+)™ and ZWIX(-)™; mobile phase, **c,** MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 or 230 nm or corona detector.

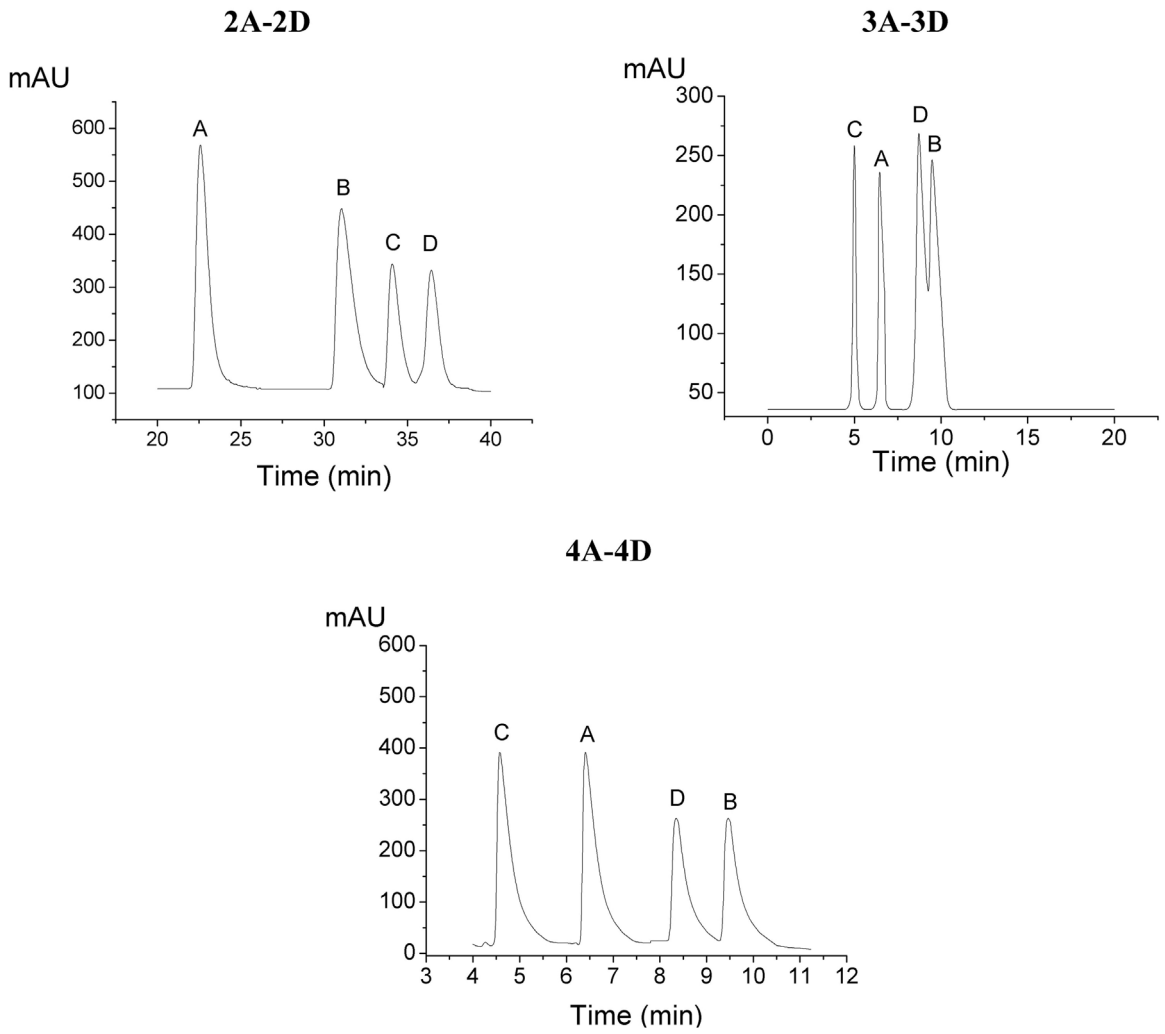

Fig. 5. Selected chromatograms Chromatographic conditions: column: ZWIX(+)^{TM}; mobile phase: MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH for **3A-3D** and **4A-4D**, MeOH/MeCN (25/75 *v/v*) containing 25 mM TEA and 50 mM AcOH for **2A-2D**; flow rate: 0.6 ml min^{-1}; detection: 230 nm; temperature: 25 °C.

level (50 mM) and the concentration of the TEA was varied between 5 mM and 50 mM. Fig. 3 depicts the chromatographic behavior of analytes **1A,1B** and **4 C,4D** on ZWIX(+)^{TM} and ZWIX(−)^{TM} columns with a mobile phase of MeOH/MeCN (75/25 *v/v*) as bulk solvent. The results indicated that an acid excess (2:1) was favorable, while under base excess conditions (1:4) a decrease in k_1 (and also α and R_S) was observed. Under acid excess conditions, the presence of positive and negative charges in both the selector and the analytes appeared to initiate double electrostatic interactions, but at a relatively high acid-to-base ratio (10:1) the ionization of the carboxyl groups of the analytes was suppressed, resulting in decreases in k_1, α and R_S. The base excess condition seemed to be less favorable, because of the lack of double electrostatic interactions since the amino groups of the selector and the analytes probably become non-protonated, and k_1, α and R_S decrease. These findings are in accordance with literature data, where the stoichiometric displacement model and the ion-exchange effects are assumed to dominate the retention mechanism. A more detailed description of the observed effect of the co- and counterions on the retention of ionized species on zwitterionic CSPs were published earlier [39,40].

For a more detailed investigation of the effects of the nature of the acid and base additives, separations of analytes **1A,1B** and **4C,4D** were carried out with the same bulk solvent composition, MeOH/MeCN (75/25 *v/v*) containing 25 mM base and 50 mM acid, on both ZWIX(+)^{TM} and ZWIX(−)^{TM} columns. As bases, EA, DEA, TEA, PRA, TPRA, BA and TBA were selected, which differ in the degree and nature of their alkyl substitution on the N atom, and FA and AcOH were applied as acids. An excess of the acid component in the mobile phase (acid-to-base concentration ratio ~2) ensured that the bases were present in their protonated "ammonium-ion" form. The experimental results (partially illustrated in Fig. 4) reveal that, in general, k_1 increased on both columns and with both acid additives as the degree of alkyl substitution on the N atom increased. (Similar results were obtained for **1A,1B** on ZWIX(+)^{TM}, and for **4C,4D** on both columns .) The presence of more apolar trisubstituted bases probably disadvantageously influences the solvation of ionic analytes, resulting in an increased retention. Ethyl, propyl and butyl substitution on the N atom exerted only a slight effect on the retention, and the presence of ethyl- and propyl-substituted bases was generally reflected in slightly higher k_1 values than for the butyl-substituted amines.

The nature of the base exerted a slight effect on the selectivity without any general trends. α varied in the range 1.22–2.50 on ZWIX(+)^{TM}, and 1.06–2.00 on ZWIX(−)^{TM} (data not shown). The nature of the applied bases influenced the resolution, but no general trend could be observed.

The nature of the acid applied as additive exhibited a slight effect on the retention. When the base was the same the use of FA instead of AcOH in most cases resulted in slightly higher retention.

3.2. Structure–retention (selectivity) relationship

The analytes in this study (Fig. 1) possess an isoxazoline-fused cyclopentane skeleton. Analogs **1** and **3** contain a 3-methyl group, and analogs **2** and **4** a 3-ethyl group, these structural differences leading to different steric effects and influencing the hydrophobicity, bulkiness, and rigidity of the molecules and hence their interactions with the chiral selectors.

On both CSPs, at a given mobile phase composition, the methyl-substituted analogs in most cases interacted more strongly with the selector than did the ethyl-substituted ones (**1** *vs.* **2** and **3** *vs.* **4**), resulting in larger retentions (Table 1). In parallel, the stronger interactions in most cases resulted in larger α and R_S values, indicating a larger difference in the interactions of the two enantiomers (**1** *vs.* **2** and **3** *vs.* **4**) with the CSP (exceptions were the ethyl substituted **C,D** analogs). Through the steric interactions, the positions of the methyl and ethyl groups influenced the k, α and R_S values. Analytes **1** and **2** were more strongly retained than **3** and **4**, but the α and R_S values usually were larger for **3** and **4** than for **1** and **2**. The R_2 position of the methyl and ethyl groups, despite the smaller retention, seemed to have a more marked influence in the overall chiral discrimination. The quinine- or quinidine-based CSPs interact in different ways with analytes. Higher retention was generally observed on ZWIX(−)^{TM} than on ZWIX(+)^{TM}, but in several cases ZWIX(+)^{TM} distinguished between the two enantiomers more efficiently, resulting in higher selectivity.

3.3. Separation of four stereoisomers of isoxazoline-fused 2-aminocyclopentanecarboxylic acids

Since the biological activities of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs depend strongly on their configurations, it is a basic task to separate and identify not only the enantiomers, but also the diastereomers in one chromatographic run. For the separation of the four stereoisomers in one chromatographic run, the separation was optimized by variation of the CSPs, the mobile phase composition and temperature. Fig. 5 illustrates few examples for the separation of the four stereoisomers.

3.4. Effects of mobile phase and temperature and thermodynamic parameters

In order to investigate the effects of temperature on the chromatographic parameters, a variable-temperature study was carried out on ZWIX(+)^{TM} and ZWIX(−)^{TM} columns over the temperature range 5–50 °C (in 10 °C increments). Experimental data for the mobile phase **c,** MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH, and for mobile phase **n,** MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM FA, are presented in Tables 2 and 3. A comparison of the retention factors in Tables 2 and 3 reveals that all of the recorded k_1 values decreased with increasing temperature (except for those of **4 C,4D** on ZWIX(−)^{TM} in mobile phase **n**). It is evident that an increase in the separation temperature lowers the separation factor, α. However, on the ZWIX(+)^{TM} in mobile phase **c,** for analytes **2A,2B,** in mobile phase **n,** for analyte **1A,1B** and on the ZWIX(−)^{TM} in mobile phase **c** for analyte **3 C,3D,** α (and R_S) increased with increasing temperature. Increasing temperature may improve the peak symmetry and efficiency, and therefore the resolution may also improve. (In several cases 10–15% of improvement could be observed in peak symmetry in the investigated temperature range, while for improved resolution see the example **1A,1B,** on ZWIX(−)^{TM} in mobile phase **n**.) An especially large increase in α was observed with increasing temperature on the ZWIX(+)^{TM} for analytes **2A,2B** in mobile phase **c** (Table 2).

Table 2
Temperature dependence of retention factor of first eluting enantiomer (k_1), separation factor (α) and resolution (R_S) of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on Chiralpak ZWIX(+)^{TM}.

Compound	Mobile phase	k, α, R_S	Temperature (°C)				
			10	20	30	40	50
1A,1B	c	k_1	5.50	4.72	4.17	3.52	3.04
		α	1.49	1.41	1.37	1.31	1.25
		R_S	3.59	3.58	3.50	3.40	3.13
	n	k_1	7.46	6.46	5.64	4.92	4.31
		α	1.27	1.29	1.31	1.32	1.34
		R_S	2.56	2.65	2.93	2.86	2.96
2A,2B	c	k_1	5.59	3.82	2.48	1.64	1.14
		α	1.00	1.23	1.63	2.05	2.45
		R_S	0.00	1.48	2.57	4.86	6.60
3 C,3D	c	k_1	2.20	2.01	1.79	1.64	1.49
		α	2.35	2.21	2.10	1.98	1.85
		R_S	4.00	5.09	4.08	2.60	2.73
4 C,4D	c	k_1	2.14	1.93	1.70	1.49	1.29
		α	2.58	2.01	1.64	1.28	1.00
		R_S	6.25	6.22	4.27	1.71	0.00
	n	k_1	1.92	1.85	1.76	1.67	1.58
		α	2.28	2.20	2.12	2.04	1.96
		R_S	6.34	5.85	5.71	5.72	4.80
Compound	Mobile phase	k, α, R_S	Temperature (°C)				
			5	15			
2A,2B	c	k_1	5.90	5.20			
		α	0.87	1.02			
		R_S	0.67	<0.20			

Chromatographic conditions: column, Chiralpak ZWIX(+)^{TM}; mobile phase; **c,** MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH; **n**, MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM FA; flow rate, 0.6 ml min^{-1}; detection, 230 nm

Table 3
Temperature dependence of retention factor of first eluting enantiomer (k_1), separation factor (α) and resolution (R_S) of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on Chiralpak ZWIX(−)^{TM}.

Compound	Mobile phase	k, α, R_S	Temperature (°C)				
			10	20	30	40	50
1A,1B	c	k_1	8.46	7.77	7.08	6.29	5.45
		α	1.33	1.30	1.27	1.24	1.21
		R_S	2.71	2.72	2.60	2.43	2.38
	n	k_1	9.05	8.83	8.16	7.52	6.91
		α	1.28	1.24	1.24	1.23	1.21
		R_S	1.40	1.76	2.06	2.17	2.11
2A,2B	c	k_1	9.23	8.37	7.51	6.69	5.92
		α	1.23	1.20	1.18	1.16	1.13
		R_S	2.10	2.06	2.00	1.85	1.76
3 C,3D	c	k_1	5.35	4.53	3.69	2.95	2.34
		α	1.06	1.19	1.34	1.49	1.66
		R_S	0.65	1.13	2.05	4.12	6.14
4 C,4D	c	k_1	2.77	2.60	2.45	2.28	2.12
		α	2.04	1.94	1.86	1.77	1.68
		R_S	5.64	5.58	4.89	4.36	4.15
	n	k_1	1.98	2.04	2.07	2.11	2.14
		α	2.10	1.99	1.90	1.81	1.72
		R_S	4.12	5.56	4.97	5.21	4.88

Chromatographic conditions: column, Chiralpak ZWIX(−)^{TM}; mobile phase, **c,** MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH, **n**, MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM FA; flow rate, 0.6 ml min^{-1}; detection, 230 nm

The differences in the changes in standard enthalpy, $\Delta(\Delta H^\circ)$, provide information on the relative ease of transfer of analytes from the mobile to the stationary phase. A negative $\Delta(\Delta H^\circ)$ value indicates an exothermic transfer of the preferentially adsorbed enantiomer of the analytes from the mobile to the stationary phase, indicating a favorable process. On ZWIX(-)^{TM} in most cases, while on ZWIX(+)^{TM} in several cases negative $\Delta(\Delta H^\circ)$ values

Table 4
Thermodynamic parameters, $\Delta(\Delta H^\circ)$, $\Delta(\Delta S^\circ)$, $Tx\Delta(\Delta S^\circ)$, $\Delta(\Delta G^\circ)$, correlation coefficients, (R^2) and T_{iso} temperature of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on ZWIX(+)™ and ZWIX(−)™.

Analyte	Mobile phase	$-\Delta(\Delta H^\circ)$ (kJ mol^{-1})	$-\Delta(\Delta S^\circ)$ (J mol^{-1} K^{-1})	Correlation coefficients (R^2)	$-Tx\Delta(\Delta S^\circ)_{298K}$ (kJ mol^{-1})	$-\Delta(\Delta G^\circ)_{298K}$ (kJ mol^{-1})	T_{iso} (°C)	Q
ZWIX(+)™								
1A,1B	c	3.3	8.3	0.9950	2.6	0.7	123	1.3
	n	−1.0	−5.7	0.9959	−1.7	0.7	−88	0.6
2A,2B	c	−20.8	−72.0	0.9869	−21.5	0.7	16	0.9
3C,3D	c	4.6	8.9	0.9951	2.6	2.0	238	1.8
4C,4D	c	17.9	55.1	0.996	16.4	1.5	51	1.1
	n	2.8	3.0	0.9966	0.9	1.9	648	3.1
ZWIX(−)™								
1A,1B	c	1.9	4.2	0.9996	1.3	0.6	172	1.5
	n	3.3	8.3	0.9950	2.5	0.8	123	1.3
2A,2B	c	1.7	4.0	0.9993	1.2	0.5	152	1.4
3C,3D	c	−8.7	−30.8	0.999	-9.2	0.5	9	0.9
4C,4D	c	3.6	6.8	0.9982	2.1	1.5	255	1.7
	n	3.8	7.1	0.9974	2.1	1.7	254	1.8

Chromatographic conditions: column, Chiralpak ZWIX(+)™ and ZWIX(−)™; mobile phase, **c,** MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH, **n,** MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM FA; flow rate, 0.6 ml min^{-1}; detection, 230 nm; R^2, correlation coefficient of van't Hoff plot, ln α–1/T curves; T_{iso}, temperature of ln k–1/T curves where enantioselectivity cancels; $Q = \Delta(\Delta H^\circ)/Tx\Delta(\Delta S^\circ)_{298K}$

were found (Table 4). The $\Delta(\Delta H^\circ)$ values ranged from -17.9 to 20.8 kJ mol^{-1}. The more negative $\Delta(\Delta H^\circ)$ values refer to the more efficient transfer from the mobile to the stationary phase and/or the stronger interaction of the enantiomers with the selector. When the selectivity increased with increasing temperature, the $\Delta(\Delta H^\circ)$ values on ZWIX(+)™ were positive; especially large positive $\Delta(\Delta H^\circ)$ values were obtained for **2A,2B** and **3C,3D** in mobile phase **c.** In these cases, the change in the adsorption enthalpy with increasing temperature had a positive effect on the enantioselectivity.

The entropy term $\Delta(\Delta S^\circ)$ is a measure of the change in the state of order induced by enantioselective selector–selectand interactions. Negative $\Delta(\Delta H^\circ)$ values for a pair of enantiomers are accompanied by a negative $\Delta(\Delta S^\circ)$. A negative $\Delta(\Delta S^\circ)$ can be attributed to an increase in order and/or a loss in the degrees of freedom of the adsorbed enantiomers. The formation of a highly ordered selector–selectand complex was manifested in a significant loss of freedom, reflecting a thermodynamically unfavorable process. The positive $\Delta(\Delta H^\circ)$ values for analytes **1A,1B, 2A,2B** on ZWIX(+)™ and for **3 C,3D** on ZWIX(−)™ were accompanied by positive $\Delta(\Delta S^\circ)$ (Table 4). The negative $\Delta(\Delta S^\circ)$ data ranged from −3.0 to −55.1 J mol^{-1} K^{-1}, and the positive $\Delta(\Delta S^\circ)$ data from 5.7 to 72.0 J mol^{-1} K^{-1}; especially large positive values were obtained for **2A,2B** on ZWIX(+)™ and **3 C,3D** on ZWIX(−)™**.**

The relative contributions of the enthalpic and entropic terms to the free energy of adsorption are difficult to visualize directly from the $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$ data, and the enthalpy/entropy ratios Q [$Q = \Delta(\Delta H^\circ)/[298 \times \Delta(\Delta S^\circ)]$] were therefore also calculated (Table 4). Comparison of the Q values for the individual analytes revealed that the enantioselective discrimination was in most cases enthalpically driven ($Q > 1.0$), but entropically driven ($Q < 1.0$) processes could also be observed. The enthalpy or entropy-controlled enantiomer separation depended on the mobile phase composition too, but no general rule could be established.

A comparison of the $-\Delta(\Delta G^\circ)$ values calculated for the two columns demonstrated that ZWIX(+)™ underwent more efficient binding with the analytes studied, as reflected by the slightly larger negative $\Delta(\Delta G^\circ)$ values. Under the conditions applied, in most cases the selector–selectand complex formation proceeded via multiple intermolecular interactions and was generally exothermic, with a corresponding negative entropic contribution. In the studied temperature range, the enantioresolution was entropically-driven, and the selectivity increased with increasing temperature (Table 2).

The data were used to calculate the isoenantioselective temperature T_{iso}, at which the enantioselectivity cancels and coelution appears (Table 4). In a temperature window around the T_{iso}, separation of the enantiomers could not be attained. This domain may be referred to as a "temperature induced blind zone" in chiral recognition [41]. In most cases, T_{iso} was considerably higher than room temperature; enthalpically-driven enantioseparation was observed. For **2A,2B** on ZWIX(+)™ with MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH as mobile phase, α first decreased on increase of the temperature from 5 °C to 50 °C; then, after a domain where no separation occurred, α slightly increased again, but the elution sequence reversed (Fig. 6 and Table 2). It should be mentioned that changes in elution sequence with temperature changes have generally been observed only rarely for separations with marginal enantioselectivity [42–47]. There have been rather limited observations where relatively good enantioselectivity was obtained in parallel with a change in elution sequence [47].

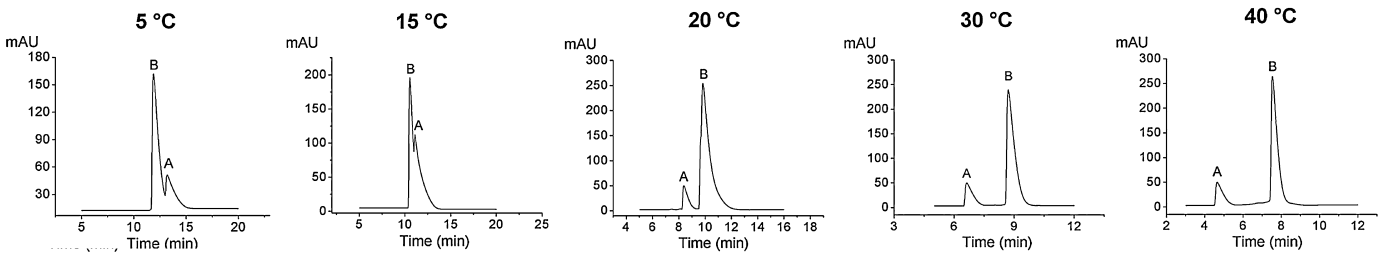

Fig. 6. Effects of temperature for **2A,2B**.
Chromatographic conditions: column: ZWIX(+)™, eluent: **c,** MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH; flow rate: 0.6 ml min^{-1}; detection: 230 nm; temperature: 5–50 °C.

4. Conclusions

Enantioselective separations of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs were investigated by using *Cinchona* alkaloid-based zwitterionic CSPs. The separations could be accomplished in PIM and the chromatographic retention behavior and resolution proved to depend on the nature and concentration of the bulk solvent and the acid and base modifiers, the temperature and the nature and positions of the substituents. Of the studied CSPs, ZWIX(+)™ appeared more suitable for the direct enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acids. The values of the thermodynamic parameters, such as the changes in $\Delta(\Delta H^\circ)$, $\Delta(\Delta S^\circ)$ and $\Delta(\Delta G^\circ)$ depended on the structures of the analytes and on the chiral selectors used. Most of the separations were predominantly enthalpically-driven, but entropically-driven separations were also observed. In the case of analyte **2A,2B**, relatively good enantioselectivity and a change in elution sequence were registered at low and high temperatures, this being a relatively rare example of such a phenomenon. However, it indicates that it is mandatory to investigate the elution sequence with regard to molecular recognition principles. The elution sequence was determined in all cases, and was found to be the opposite on ZWIX(+)™ and ZWIX(−)™ CSPs.

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Research Article

Effect of mobile phase composition on the liquid chromatographic enantioseparation of bulky monoterpene-based β-amino acids by applying chiral stationary phases based on *Cinchona* alkaloid†

Stereoselective HPLC separations of five sterically constrained monoterpene-based 2-aminocarboxylic acid enantiomers were carried out by using the newly developed zwitterionic chiral stationary phases Chiralpak ZWIX(+)™ and ZWIX(−)™ based on *Cinchona* alkaloid. In order to optimize the retention and enantioselectivity parameters, the ratio of the different organic solvents in the mobile phase and the nature of the acid and base additives (counter- and co-ions) were systematically varied. The effects of structure variants of the analytes on the resolution were investigated. The elution sequence was determined in all cases and observed to be opposite on ZWIX(+)™ and ZWIX(−)™.

Keywords: Aminocarboxylic acids / Enantiomeric separation / HPLC / Monoterpenes
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1 Introduction

Asymmetric syntheses of β-amino acids have recently gained importance because of their diverse applications in the synthesis of bioactive compounds that are currently at the focus of research interest [1–6]. β-Amino acid derivatives are excellent building blocks for versatile transformations, e.g. multicomponent reactions resulting in β-lactams, the syntheses of chiral catalysts, 1,3-heterocycles, and diaminopyrimidine derivatives, or the formation of self-assembling foldameric oligomers [7–10].

Commercially available monoterpenes and their analogs, such as α-pinene, 3-carene, and apopinene, are widely applied chiral sources for the preparation of sterically constrained β-amino acid derivatives [11–16], which are excellent building blocks for the syntheses of monoterpene-fused saturated 1,3-heterocycles, β-lactam-based peptidomimetics, or β-peptidic foldamers [10].

The synthesis of β-amino acids demands analytical methods to check the stereochemistry of the final products. One of the most frequently applied techniques is enantioselective (chiral) HPLC. HPLC enantioseparations of β-amino acids have been performed by both indirect and direct methods. In the past decade, new types of chiral derivatizing agents and chiral stationary phases (CSPs) have been introduced for the enantioseparation of β-amino acids and the results have been surveyed in several review papers [17–28]. The enantioselectivities of different macrocyclic glycopeptide-based CSPs have been compared by the application of monoterpene-based 2-aminocarboxylic acids [29].

Zwitterionic ion exchange type chiral selectors (SOs) based on *Cinchona* alkaloid operating in slightly acidic polar organic mobile phases allow three modes of ion exchange: the anion-exchange mode for the separation of chiral acids, cation-exchange mode for the resolution of chiral amines, and zwitterionic exchange for the enantioseparation of amphoteric compounds such as amino acids and small peptides [30–33].

This paper describes the enantioseparation of five monoterpene-based 2-aminocarboxylic acid enantiomers (selectands, SAs; Fig. 1) on *Cinchona* alkaloid based zwitterionic SOs (Fig. 2) in polar-ionic mode (PIM). The effects of the mobile phase composition, nature of the acid and base additives, and structural features of the SAs on the retention and enantioselectivity are discussed. The sequence of elution of the enantiomers was determined in all cases.

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Abbreviations: **AcOH**, glacial acetic acid; **CSPs**, chiral stationary phases; **DEA**, diethylamine; **EA**, ethylamine; **FA**, formic acid; **MeCN**, acetonitrile; **MeOH**, methanol; **PIM**, polar-ionic mode; **PRA**, propylamine; **SA**, selectand; **SO**, selector; **TEA**, triethylamine

2 Materials and methods

2.1 Chemicals and reagents

The enantiomers of monoterpene-based *cis*-β-amino acids were prepared in two steps by literature methods [10–15].

†This paper is included in the virtual special issue on Amino acids, proteins and peptides available at the Journal of Separation Science website.

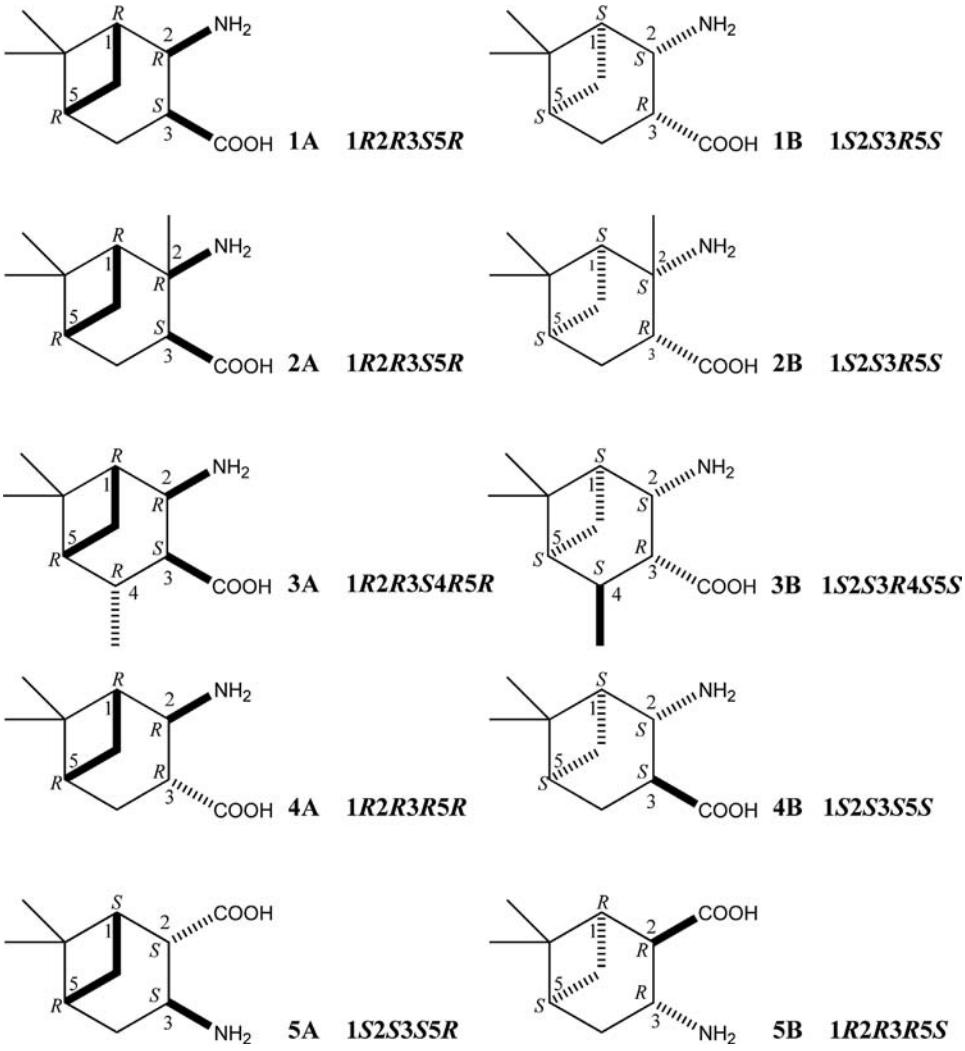

Figure 1. Chemical structures of SAs.

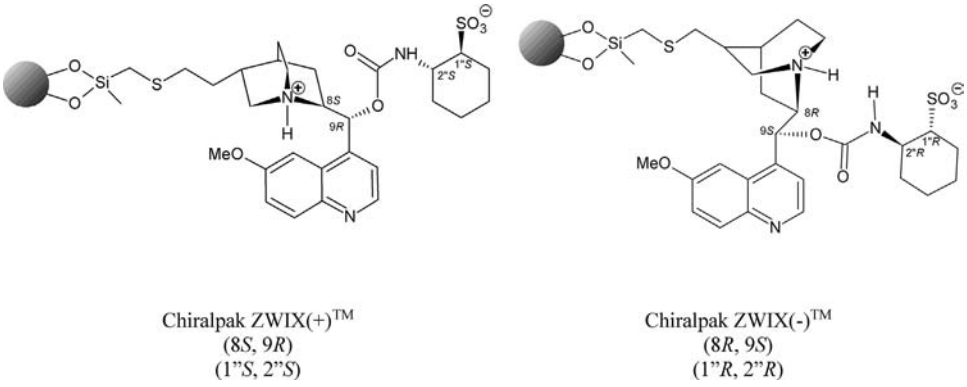

Figure 2. Structures of Chiralpak ZWIX(+)™ and ZWIX(−)™ CSPs.

Chlorosulfonyl isocyanate addition to the corresponding chiral monoterpene [(−)-apopinene for **1A**, (+)-apopinene for **1B**, (−)-α-pinene for **2A**, (+)-α-pinene for **2B**, (−)-δ-pinene for **3A**, and (+)-δ-pinene for **3B**] afforded β-lactams in highly regio- and stereospecific reactions, following treatment with hydrochloric acid resulting in the corresponding *cis*-β-amino acids [13, 15]. The acid-catalyzed ethanolysis of β-lactams derived from apopinene (**1A** and **1B**) led to *cis*-β-amino esters, which underwent base-catalyzed isomerization, followed by hydrolysis, to afford the corresponding *trans*-enantiomers **4A** and **4B** in excellent yields [14]. The regioisomeric *trans*-apopinane-based β-amino acids **5A** and **5B** were prepared by the stereoselective Michael addition of lithium dibenzylamide to (−)- and (+)-*tert*-butyl myrtenate, followed by catalytic debenzylation and hydrolysis of the resulting β-amino esters [16].

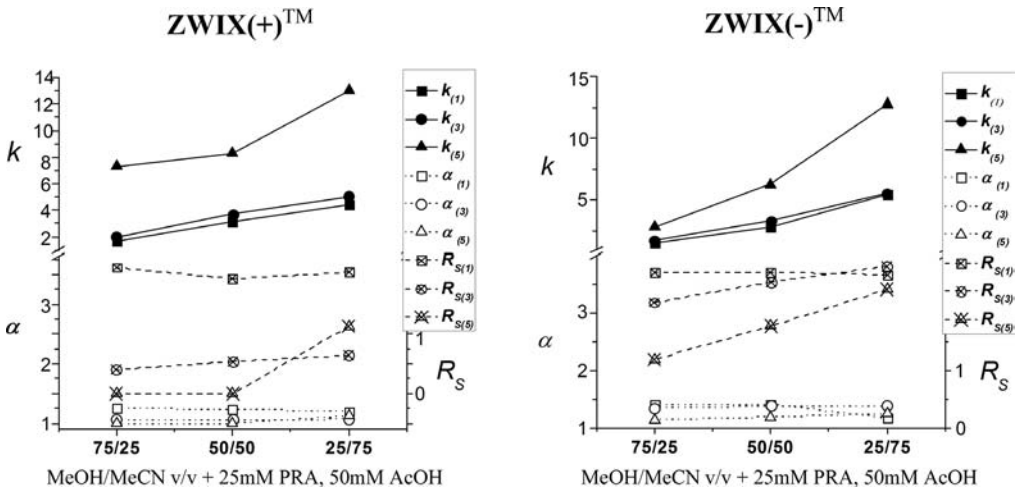

Figure 3. Effects of the compositions of the bulk solvents on the chromatographic parameters for SAs **1**, **3**, and **5** on ZWIX(+)^{TM} or ZWIX(−)^{TM} CSPs. Chromatographic conditions: column, Chiralpak ZWIX(+)^{TM} and ZWIX(−)^{TM}; mobile phase, MeOH/MeCN (75:25, 50:50, or 25:75 v/v) containing 25 mM PRA and 50 mM AcOH; flow rate, 0.6 mL/min; detection, 230 nm; temperature 25°C; symbols,–■–: k_1 for SA **1**,–●–: for SA **3**, and–▲–: for SA **5**;·□·: α for SA **1**,·○·: for SA **3**, and·△·: for SA **5**;-⊠-: R_S for SA **1**,-⊠-: for SA **3**, and-⊠-: for SA **5**.

Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were purchased from Merck (Darmstadt, Germany). Ammonia, ethylamine (EA), diethylamine (DEA), triethylamine (TEA), propylamine (PRA), formic acid (FA), and glacial acetic acid (AcOH) of analytical reagent grade were from Sigma–Aldrich (St. Louis, MO, USA).

2.2 Apparatus and chromatography

Chromatographic measurements were carried out on a 1100 Series HPLC system from Agilent Technologies (Waldbronn, Germany), consisting of a solvent degasser, pump, autosampler, column thermostat, and multiwavelength UV/Vis detector. Data acquisition and analysis were carried out with ChemStation chromatographic data software from Agilent Technologies. The Chiralpak ZWIX(+)^{TM} and ZWIX(−)^{TM} columns (150 × 3.0 mm id, 3 μm particle size for both columns) were from Chiral Technologies Europe (Illkirch, France).

Chromatography was performed in isocratic mode at a flow rate of 0.6 mL/min and a column temperature of 25°C (if not otherwise stated). UV detection was accomplished at selected wavelengths of 215 and 230 nm. The void volume of the columns (t_0) was determined by injecting a MeOH solution of acetone with detection at 280 nm. All SAs were dissolved in MeOH in the concentration range 0.5–1.0 mg/mL and further diluted with mobile phase.

3 Results and discussion

Through the protonated tertiary amine of the quinuclidine ring (Fig. 2), the *Cinchona* alkaloids quinine- and quinidine-based CSPs contain a weak anionic exchanger site and a strong sulfonic acid cationic exchanger site interlinked through several asymmetric carbon atoms (see Fig. 2). These SOs exhibited a zwitterionic character under the applied mobile phase conditions and are therefore capable of undergoing double ion pair formation with the ampholytic (zwitterionic) SAs. Besides the main ionic interactions, the retention mechanism and chiral recognition are supported by other types of secondary interactions, such as H-bonding, π–π, dipole–dipole, and/or van der Waals forces [30].

All of the investigated amino acids (Fig. 1) possess a monoterpene-based skeleton. The carboxy and primary amino groups provide the charged sites for the ionic interactions. SAs **1** and **4** are diastereomers, while **5** is a constitutional isomer of **1** and **4**. Besides carboxy and primary amino groups, analogs **2** and **3** possess one extra methyl group, in position 2 or 4, respectively. These differences result in different physical properties, such as the hydrophobicity, bulkiness, and rigidity of the molecules and these differences may strongly influence the interactions with the quinine- and quinidine-based chiral SOs.

3.1 Effect of composition of organic bulk solvents

Nonaqueous polar organic solvents in combination with acid and base additives (often referred to as PIM) proved to be the preferential mobile phase for the separation of zwitterionic SAs on the ZWIX CSPs [31]. MeOH was applied as protic solvent (which can suppress H-bonding interactions) with the additionally selected MeCN, an aprotic solvent known to support ionic interaction, but to interfere with aromatic (π–π) interactions [34]. To investigate the effects of the organic solvent, chromatographic separation was carried out in MeOH/MeCN (25:75, 50:50, and 75:25 v/v) mobile phases containing 25 mM PRA and 50 mM AcOH. Figure 3 depicts the chromatographic behavior of three representative SAs (**1**, **3**, and **5**) in the presence of different MeOH/MeCN

Table 1. Chromatographic data, separation factors (k), selectivity factor (α), resolution (R_S), and elution sequence of monoterpene-based 2-aminocarboxylic acids on ZWIX(+)™ column

Compound	Eluent	k_1	k_2	α	R_S	Elution sequence
1a,b	a	2.62	3.22	1.23	1.59	A < B
	b	2.82	3.14	1.11	1.52	A < B
	c	3.61	4.54	1.26	1.78	A < B
	d	3.91	4.64	1.19	2.00	A < B
	e	2.93	3.62	1.23	1.60	A < B
	f	2.37	2.85	1.20	1.55	A < B
	g	2.68	3.21	1.20	2.60	A < B
	h	2.84	3.58	1.26	3.00	A < B
	i	2.97	3.66	1.23	2.20	A < B
	j	2.24	2.73	1.22	2.00	A < B
2a,b	a	2.42	2.42	1.00	0.00	—
	b	2.46	2.46	1.00	0.00	—
	c	2.94	3.03	1.03	0.40	A < B
	d	3.00	3.03	1.01	0.30	A < B
	e	2.37	2.37	1.00	0.00	—
	f	1.93	1.93	1.00	0.00	—
	g	2.13	2.23	1.05	0.60	A < B
	h	2.36	2.45	1.04	0.40	A < B
	i	2.58	2.64	1.02	0.40	A < B
	j	1.91	1.97	1.03	0.40	A < B
3a,b	a	3.50	3.53	1.01	0.40	A < B
	b	3.52	3.70	1.05	0.71	A < B
	c	4.17	4.43	1.06	0.50	A < B
	d	4.44	4.64	1.05	0.50	A < B
	e	3.39	3.57	1.05	0.50	A < B
	f	2.81	2.89	1.03	0.40	A < B
	g	2.90	3.09	10.6	0.70	A < B
	h	3.24	3.47	1.07	0.60	A < B
	i	3.45	3.81	1.10	0.70	A < B
	j	2.88	3.02	1.05	0.55	A < B
4a,b	a	6.13	6.40	1.04	0.40	B < A
	b	6.88	7.28	1.06	1.19	B < A
	c	8.39	8.84	1.05	0.40	B < A
	d	9.55	9.98	1.05	0.40	B < A
	e	6.98	7.53	1.08	1.30	B < A
	f	7.20	7.70	1.07	0.40	B < A
	g	7.83	8.71	1.11	1.95	B < A
	h	9.85	10.97	1.11	1.40	B < A
	i	10.25	11.47	1.12	1.80	B < A
	j	7.69	8.64	1.12	1.40	B < A
5a,b	a	4.36	4.36	1.00	0.00	—
	b	5.38	5.38	1.00	0.00	—
	c	6.55	6.55	1.00	0.00	—
	d	9.33	9.33	1.00	0.00	—
	e	4.79	4.79	1.00	0.00	—
	f	5.66	5.82	1.03	0.40	A < B
	g	5.76	5.76	1.00	0.00	—
	h	8.41	8.41	1.00	0.00	—
	i	9.35	9.35	1.00	0.00	—
	j	5.77	5.77	1.00	0.00	—
	k	15.28	16.79	1.10	2.10	A < B

Chromatographic conditions: Column, Chiralpak ZWIX(+)™; mobile phase, MeOH/MeCN (50/50 v/v) containing (a) 50 mM FA and 25 mM NH_3; (b) 50 mM FA and 25 mM EA; (c) 50 mM FA and 25 mM DEA; (d) 50 mM FA and 25 mM TEA; (e) 50 mM FA and 25 mM PRA; (f) 50 mM AcOH and 25 mM NH_3; (g) 50 mM AcOH and 25 mM EA; (h) 50 mM AcOH and 25 mM DEA; (i) 50 mM AcOH and 25 mM TEA; (j) 50 mM AcOH and 25 mM PRA; (k) MeOH/MeCN (25/75 v/v) containing 25 mM PRA and 50 mM AcOH; flow rate, 0.6 mL/min; detection 230 nm; temperature, 25°C.

Table 2. Chromatographic data, separation factor (k), selectivity factor (α), resolution (R_S), and elution sequence of monoterpene-based 2-amino carboxylic acids on ZWIX(−)™ column

Compound	Eluent	k_1	k_2	α	R_S	Elution sequence
1a,b	a	2.30	3.13	1.36	2.35	B < A
	b	2.41	3.73	1.55	4.40	B < A
	c	3.79	5.31	1.40	2.90	B < A
	d	3.92	5.76	1.47	3.10	B < A
	e	3.28	3.40	1.03	0.40	B < A
	f	2.22	2.98	1.34	2.30	B < A
	g	2.44	3.74	1.53	4.42	B< A
	h	3.42	5.00	1.46	3.00	B < A
	i	3.68	5.17	1.40	3.10	B < A
	j	2.16	3.00	1.39	2.72	B < A
2a,b	a	2.03	2.12	1.05	0.40	B < A
	b	2.12	2.42	1.14	1.58	B < A
	c	3.00	3.24	1.08	0.55	B < A
	d	3.00	3.24	1.08	0.50	B < A
	e	1.87	2.02	1.08	0.70	B < A
	f	1.72	1.83	1.06	0.40	B < A
	g	2.27	2.62	1.15	1.81	B < A
	h	3.04	3.30	1.09	0.60	B < A
	i	3.28	3.62	1.10	1.30	B < A
	j	1.89	2.09	1.11	0.80	B < A
3a,b	a	2.77	3.75	1.35	2.15	B < A
	b	2.91	3.92	1.35	2.88	B < A
	c	4.58	6.51	1.42	2.55	B < A
	d	4.82	6.58	1.37	4.10	B < A
	e	2.80	3.80	1.36	3.20	B < A
	f	2.58	3.42	1.33	2.10	B < A
	g	3.06	4.14	1.36	3.27	B < A
	h	4.09	5.85	1.43	2.95	B < A
	i	4.25	6.20	1.46	3.00	B < A
	j	3.45	4.75	1.38	2.55	B < A
4a,b	a	4.73	5.96	1.26	2.89	A < B
	b	4.84	5.66	1.17	1.56	A < B
	c	6.90	9.04	1.31	2.40	A < B
	d	7.83	8.58	1.10	0.60	A < B
	e	4.08	5.12	1.25	2.90	A < B
	f	6.16	7.76	1.26	2.50	A < B
	g	6.99	8.01	1.15	1.07	A < B
	h	10.04	13.15	1.31	2.45	A < B
	i	10.59	13.80	1.30	2.25	A < B
	j	8.60	10.28	1.20	2.40	A < B
5a,b	a	3.40	3.81	1.12	1.00	B < A
	b	3.74	3.95	1.06	0.88	B < A
	c	8.07	8.07	1.00	0.00	—
	d	8.12	8.52	1.05	0.40	B < A
	e	3.39	3.39	1.00	0.00	—
	f	4.61	5.42	1.18	1.51	B < A
	g	5.68	6.87	1.21	2.44	B < A
	h	7.88	9.61	1.22	1.80	B < A
	i	8.01	10.53	1.31	1.05	B < A
	j	4.88	5.93	1.22	1.80	B < A

Chromatographic conditions: Column, Chiralpak ZWIX(−)™; mobile phase, MeOH/MeCN (50/50 v/v) containing (a) 50 mM FA and 25 mM NH_3; (b) 50 mM FA and 25 mM EA; (c) 50 mM FA and 25 mM DEA; (d) 50 mM FA and 25 mM TEA; (e) 50 mM FA and 25 mM PRA; (f) 50 mM AcOH and 25 mM NH_3; (g) 50 mM AcOH and 25 mM EA; (h) 50 mM AcOH and 25 mM DEA; (i) 50 mM AcOH and 25 mM TEA; (j) 50 mM AcOH and 25 mM PRA; flow rate, 0.6 mL/min; detection 230 nm; temperature, 25°C.

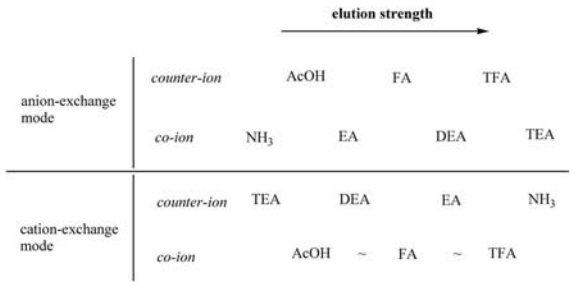

Figure 4. Elution strengths of co- and counterion additives on th retention on single ion exchanger type selectors, according to refs. [35–37].

mixtures as mobile phase bulk solvent on ZWIX(+)^{TM} and ZWIX(−)^{TM} CSPs. Application of higher MeCN content in the mobile phase in all cases substantially increased the retention (this is true for SAs **2** and **4**, too; data not shown). A similar trend was observed by Hoffmann et al. for the separation of amino acids [31]. In protic solvents, a proton transfer mechanism can be an integral part of the solvation. However, the interactions involved in double ion-pairing process (e.g. the formation of the SO–SA complex with zwitterionic CSP) is less favored in protic solvents, and lower MeOH content therefore supported the ionic interactions, which resulted in longer retention [31, 33]. However, a certain amount of MeCN most often enhanced the enantioselectivity and separation performance. The α and R_S values therefore increased with increasing MeCN content (exceptions were SAs **1** and **4** on ZWIX(+)^{TM} and **1** on ZWIX(−)^{TM}, where higher α and R_S values were observed in MeOH-rich mobile phases). In general, for β-amino acids of the present kind, a marked increase in separation performance was observed at higher MeCN content, in accordance with the results of Pell et al. [33]. However, in the case of α-amino acids, a higher MeCN content usually leads to subsequent decreases in enantioselectivity and resolution [31].

3.2 Effects of nature of base and acid as mobile phase additives

The nature of the various acid and base additives in the mobile phase is one of the most important parameters in the optimization of the enantioseparation on *Cinchona* alkaloid based CSPs. The different strengths of the ion-pairing and ion-exchange processes of the zwitterionic SAs and CSPs via the different competitive interactions of the particular additives exert a great influence on the efficiency of the enantioseparation. Moreover, the acid and base additives are able to change the physical properties of the solvent and thereby the solvation effects. Earlier studies demonstrated that the co- and counterions have opposite impacts on the elution strength at the cationic and anionic exchanger parts of the zwitterionic CSP [31, 35–37].

Mainly a base-to-acid ratio of 1:2 was previously applied for successful enantioseparation [33], the concentration of the base being kept at 25 mM, and that of the acid at 50 mM. Acid excess in the mobile phase ensured that the bases (amines) were present in their ammonium forms. For comparison of the effects of the nature of the base and acids as mobile phase additives, separations were carried out with the same mobile phase composition on both ZWIX(+)^{TM} and ZWIX(−)^{TM} columns MeOH/MeCN 50:50 v/v containing 25 mM base and 50 mM acid. Five different bases (NH_3, EA, DEA, TEA, and PRA) and two acids (FA and AcOH) were selected. The amines differed in the degree and nature of their alkyl substitution and possessed pK_a values of 9.25, 10.70, 10.84, 10.75, and 10.60, respectively, while the acids differed by one methyl group and had dissociation constants (pK_a values) of 3.75 and 4.76, respectively.

The experimental results (listed in Tables 1 and 2) revealed that the retention factors increased as the degree of ethyl substitution of NH_3 increased on both columns and with both acid additives. However, there were only slight differences between ethyl or propyl substitution of NH_3 on the retention; the k_1 values were in most cases very similar. On the basis of the k_1 values, the overall elution strength of the amine additives increased in the sequence TEA < DEA < EA ≤ PRA ≤ NH_3, i.e. the application of TEA or NH_3 resulted in the largest and smallest k_1 values, respectively. In accordance with the literature data, the effects of the nature of the base on the retention were opposite on anion-exchange and cation-exchange type CSPs, depending on their role as co- or counterions [31, 36, 37] (Fig. 4). Base components function as counterions in the separation of monoterpene-based 2-aminocarboxylic acids.

The nature of the base additives exerted only a slight effect on the selectivity on both columns, α changed in a narrow range. In terms of the different SA structure on the ZWIX(+)^{TM} CSP, α varied between 1.00 and 1.26, while on ZWIX(−)^{TM} it varied between 1.00 and 1.55 (Tables 1 and 2). The enantioseparation on ZWIX(−)^{TM} was more effective than that on ZWIX(+)^{TM} as α and R_S values were higher on ZWIX(−)^{TM} than on ZWIX(+)^{TM} (the only exceptions were SA **1** with eluent e, and SA **4** with mobile phase g). SA **5** on ZWIX(+)^{TM} could be baseline separated (R_S = 2.1) only with the application of a MeCN-rich mobile phase: MeOH/MeCN (25:75 v/v) containing 25 mM PRA and 50 mM AcOH.

The nature of the acids exerted a slight effect on the retention. For SAs **1**, **2**, and **3** FA, and for **4** and **5**, AcOH exhibited slightly higher k_1 values with the same base additive (an exception was SA **2** on ZWIX(−)^{TM}). The effects of the type of the acid additives on the retention are complex and their opposite impacts on the positively and negatively charged parts of the CSP have to be considered in the explanation [31, 35–37]. However, it seems that there is a slight difference in eluent strength when FA or AcOH is applied, supporting the co-ion effect of the acids on chiral separations.

The effects of the nature of the acid modifier (FA or AcOH) on the resolution were also investigated. The data presented in Tables 1 and 2 indicate that slightly higher R_S values were obtained when AcOH was applied. A significant

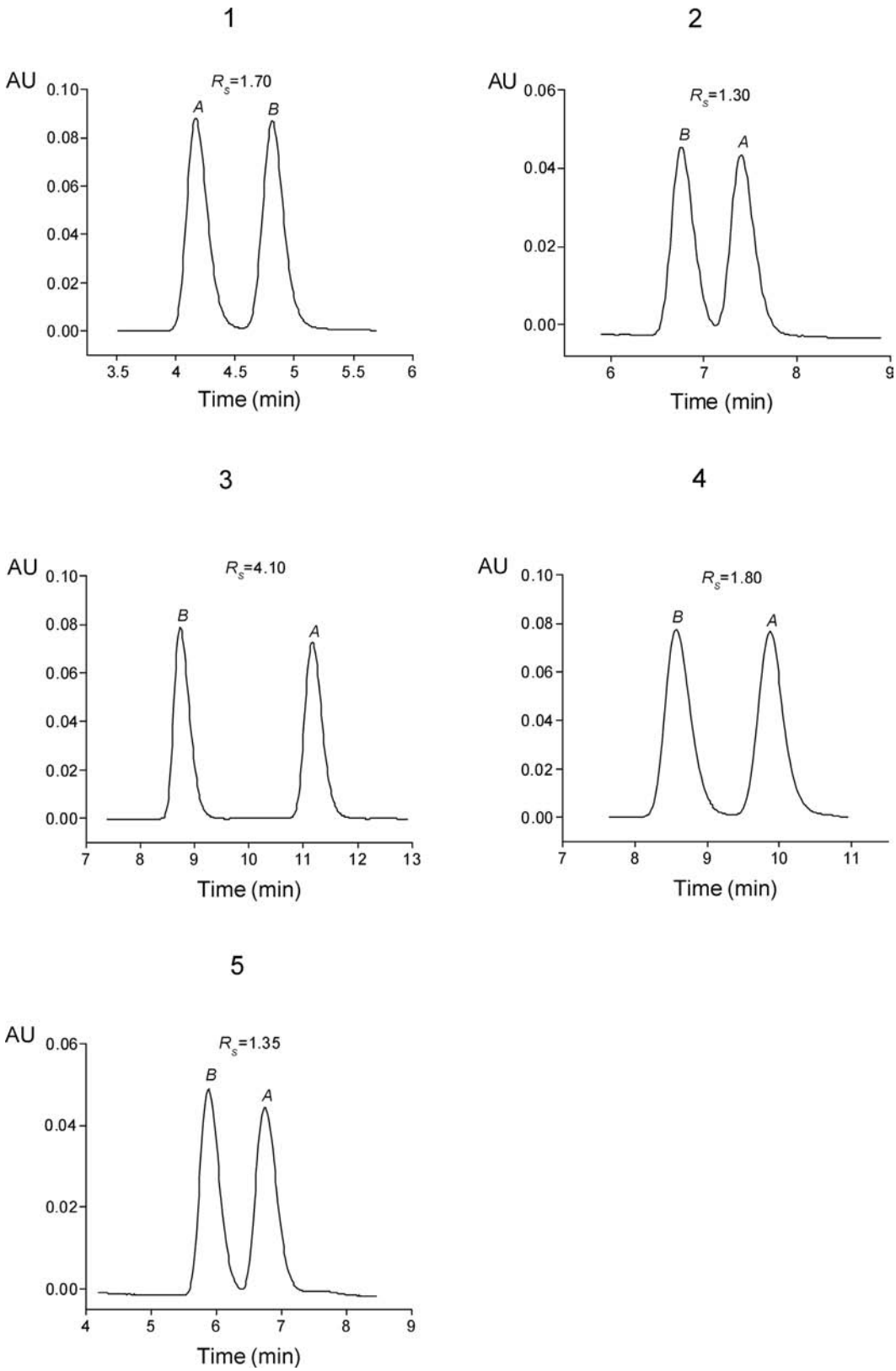

Figure 5. Selected chromatograms for monoterpene-based 2-aminocyclopentanecarboxylic acids. Chromatographic conditions: columns: ZWIX(+)^{TM} for SAs **1** and **4**, and ZWIX(−)^{TM} for SAs **2, 3,** and **5**; mobile phase: MeOH/MeCN (75:25 v/v) containing 25 mM PRA and 50 mM AcOH for SAs **1, 4,** and **5**, MeOH/MeCN (50:50 v/v) containing 25 mM TEA and 50 mM FA for SA **3**, and MeOH/MeCN (50:50 v/v) containing 25 mM TEA and 50 mM AcOH for SA **2**; flow rate: 0.6 mL/min; detection: 230 nm; temperature: 25°C.

difference in R_S values was found on ZWIX(+)^{TM} or ZWIX(−)^{TM}. On ZWIX(−)^{TM}, baseline separation was achieved in almost all cases when the mobile phase contained AcOH, whereas on ZWIX(+)^{TM} (with the exception of SA **1**, and in some cases SAs **4** and **5**) only partial resolution was observed with the application of either FA or AcOH.

3.3 Effects of the structure of the amino acids

The structures of the SAs may influence the retention and chiral recognition. The data presented in Tables 1 and 2 reveal that, for the same mobile phase composition, the retention factors of SAs **4** and **5** were the largest. It seems that the *trans*-position of the amino and carboxyl groups improves the SO–SA interactions by ion pairing. However, the high retention in the case of SA **5** on ZWIX(+)^{TM} and on ZWIX(−)^{TM} containing FA as acid modifier in the mobile phase is not necessarily accompanied by high enantioselectivity and resolution. The inverse positions of the carboxyl and amino groups in SA **4** as compared with SA **5** furnish evidence of an improved steric discrimination factor.

Comparison of the chromatographic behavior of the diastereomerically related SAs **1** and **4** showed significantly higher retention for SA **4** (*trans*-configuration), while only smaller differences in selectivity and resolution were registered. On ZWIX(+)^{TM}, α for SA **1** lay in the range 1.11–1.26, and for SA **4** in the range 1.04–1.12, while on ZWIX(−)^{TM} α for SA **1** lay in the range 1.03–1.55, and for SA **4** in the range 1.15–1.31. The resolution was in most cases slightly higher for SA **1**.

The retention factors were lowest on both columns for SA **2**. In parallel with the short retention, the chiral recognition was also small; on ZWIX(+)^{TM} and ZWIX(−)^{TM}, α varied in the interval 1.00–1.05 and 1.05–1.15, respectively. The extra methyl group at position 2, located on the same carbon atom as the amino group, sterically hindered the main ionic interaction between the protonated amino group of the amino acid and the sulfonic acid site of the SO on the CSP. Such steric hindrance was not observed to be caused by the methyl group at position 4 in SA **3**. These enantiomers were more retained than those of SA **2**, and the selectivity and resolution (especially on ZWIX(−)^{TM}) were also satisfactory.

3.4 Sequence of elution of monoterpene-based 2-aminocarboxylic acid enantiomers

The Chiralpak ZWIX(+)^{TM} and ZWIX(−)^{TM} CSPs possess a quinine and quinidine structure, respectively, coupled through a carbamoyl functionality with 2-aminocyclohexylsulfonic acid. These SOs are stereoisomers, but they behave as enantiomers. Thus, on change from the quinine- to the quinidine-based CSP, the sequence of elution of the enantiomers should also change. On ZWIX(+)^{TM}, the elution sequence for SAs **1**, **2**, **3**, and **5** was found to be A < B, while for SA **4** was B < A. The elution sequences were opposite on ZWIX(−)^{TM}, i.e. B < A for SAs **1**, **2**, **3**, and **5**, and A < B for SA **4**. The configuration of the carboxyl group of the **A** enantiomer of SA **4** was 3*R*, while that of SAs **1**, **2**, and **3** was 3*S* and that of **5** was 2*S* (the reverse configuration exists in the case of the B enantiomers). These differences in configuration of carboxyl group may explain the observed elution sequence. On ZWIX(+)^{TM}, the more retained enantiomers probably form more stable complex with quinuclidine ring having *S* configuration at position 8 and on ZWIX(−)^{TM} CSP the *R* configuration at position 8 was favorable. These findings corroborate our previously established hypothesis of the *Cinchona* alkaloid weak ion exchanger moiety playing the dominant role in the chiral recognition process [30]. Selected chromatograms for the separation of monoterpene-based 2-aminocarboxylic acids are depicted in Fig. 5.

4 Concluding remarks

HPLC methods were developed for the separation of the enantiomers of monoterpene-based 2-aminocarboxylic acids using *Cinchona*-based CSPs, i.e. Chiralpak ZWIX(+)^{TM} and ZWIX(−)^{TM} in PIM. By variation of the chromatographic parameters, the separations of the stereoisomers were optimized; as a result, baseline resolution was achieved for all the investigated SAs in at least one chromatographic system. The effects of the composition of the mobile phase, the nature of the co- and counterions, and the structures of the SAs are discussed. The elution sequence was determined in all cases and was found to be opposite on the ZWIX(+)^{TM} and ZWIX(−)^{TM} columns.

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IV.

Unusual Temperature-Induced Retention Behavior of Constrained β-Amino Acid Enantiomers on the Zwitterionic Chiral Stationary Phases ZWIX(+) and ZWIX(–)

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ABSTRACT The effects of temperature on the chiral recognition of cyclic β-amino acid enantiomers on zwitterionic [Chiralpak ZWIX(+) and ZWIX(–)] chiral stationary phases were investigated. Experiments were performed at different mobile phase compositions and under 10°C column temperature increments in the temperature range 10–50°C. Apparent thermodynamic parameters and T_{iso} values were calculated from plots of ln k and ln α versus 1/T, respectively. Unusual temperature behavior was observed, especially on the ZWIX(–) column, where the application of MeOH/MeCN (50/50 v/v) containing 25 mM triethylamine and 50 mM formic acid as mobile phase led to nonlinear van't Hoff plots and increasing retention time with increasing temperature. On both columns, both enthalpically and entropically driven separations were observed. *Chirality 26:385–393, 2014.* © 2014 Wiley Periodicals, Inc.

KEY WORDS: column liquid chromatography; enantiomer separation; monoterpene-based 2-aminocarboxylic acids; cinchona-alkaloid-based columns; temperature effect

INTRODUCTION

β-Amino acids and their foldameric oligomers are currently of significant interest,[1,2] since they are key building blocks of numerous bioactive molecules.[3–6] Icofungipen (PLD-118; (1*R*,2*S*)-2-amino-4-methylenecyclopentanecarboxylic acid), a β-amino acid, disturbs the biosynthesis of proteins in *Candida albicans*.[7] The monoterpene-based β-amino acids are excellent building blocks for syntheses of monoterpene-fused saturated 1,3-heterocycles, β-lactam-based peptidomimetics or β-peptidic foldamers.[8]

The synthesis of β-amino acids demands analytical methods, which can be used to elucidate the stereochemistry and enantiomeric excess of the final product. One of the most frequently applied techniques is enantioselective high-performance liquid chromatography (HPLC). For the enantioseparation of β-amino acids, new types of chiral derivatizing agents and chiral stationary phases (CSPs) have been applied, and the results of that work can be found in the literature.[9–18]

Enantiomer separations are inherently challenging, because enantiomers have exactly the same properties in an anisotropic environment. Enantioseparation is usually achieved through the formation of transitional diastereomeric complexes between the analytes (selectand enantiomers, SAs) and the chiral selector (SO) as part of the CSP. In most cases, the stereoselective interactions are sensitive to temperature. It has also been observed that there are both achiral and chiral contributions to retention that can vary with a wide variety of experimental parameters.[19–21] Accordingly, the column temperature often has to be optimized in enantioselective HPLC separations and kept well controlled.[22–25]

In investigations of the thermodynamic functions of enantioselective adsorption, van't Hoff plots may facilitate an interpretation of the mechanistic aspects of chiral recognition:

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \phi \quad (1)$$

where k is the retention factor, ΔH° is the standard enthalpy of transfer of the solute from the mobile phase to the stationary phase, ΔS° is the standard entropy of transfer of the solute from the mobile phase to the stationary phase, R is the universal gas constant, T is temperature (Kelvin) and ϕ is the ratio of the volumes of the stationary (V_S) and mobile (V_M) phases of the column. Since the value of ϕ is often not known, the $\Delta S^\circ *$ values [$\Delta S^\circ * = \Delta S^\circ + R \ln \phi$] calculated from the intercepts of the plots via Eq. (1) are generally used. Equation (1) reveals that a plot of ln k vs. 1/T is linear, with slope $-\Delta H^\circ/R$ and intercept $\Delta S^\circ/R + \ln \phi$, if ΔH° is invariant with temperature. In chiral chromatography, however, the van't Hoff plot often deviates from linearity; this is an indication of a mixed retention mechanism.

When solute molecules are transferred from the mobile phase to the stationary phase, the process is enthalpically favorable, but entropically unfavorable. If the solute is retained by the stationary phase, its freedom and entropy decrease; the entropy change, ΔS°, must be negative. On the other hand, when the molecule is adsorbed to the SO of the stationary phase, energy is used during the interaction, and the enthalpy ΔH^o must therefore also be negative.

Change of the temperature of the enantioseparation process usually results in variation of the conformation of the SO of the CSP and/or the SAs. The structural modification of the SAs resulting from the temperature change modifies the geometrical positions of the interaction sites within the SOs and the SAs. This also depends on the mobile phase composition, influencing the overall binding mechanism observed, which are manifested in different retention characteristics. The conformation change

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may be reversible or irreversible, depending on the characteristics of the SO of the CSP and the SAs. Changes in temperature may result in nonlinear variation of the pK_a values of chargeable SAs.[26] The temperature-related changes in conformation, together with the change in the enthalpy or entropy of adsorption, can generate a nonlinear van't Hoff plot.[26–31]

Chromatographic chiral separations are determined by the difference in free energy $\Delta(\Delta G°)$ of adsorption of the enantiomers:

$$\Delta(\Delta G°) = -RT \ln \alpha = \Delta(\Delta H°) - T\,\Delta(\Delta S°) \quad (2)$$

where α is the enantioselectivity factor. If $\Delta(\Delta H°)$ is constant within the given temperature range, the slope of a plot of $\ln \alpha$ vs. $1/T$ is $-\Delta(\Delta H°)/R$ and the intercept is $\Delta(\Delta S°)$.

Equation (2) clearly reveals that the stereoselectivity increases as the temperature decreases. At lower temperatures, the effectiveness of intramolecular interactions increases and therefore improves the enantioselectivity. Stronger interactions between the SA and the SO of the CSP are manifested in more negative $\Delta H°$ values, which is favorable because of the more exothermic process. The hypothesis is valid when conditions such as the structures of intermediate associates, the solvation of the SO and SA, and the configurations of the SA and SO are constant throughout the temperature range studied. On the other hand, the weaker intramolecular interactions that occur at higher temperature have an energetically positive effect on the $\Delta S°$ values. A less negative entropy indicates an increase in the number of degrees of freedom of the SAs in the mobile phase. In some cases, the gain in the change of entropy with increasing temperature is more significant than the loss in the change of enthalpy. From an enantioselectivity standpoint, this means that the selectivity increases with increasing temperature and a new set of $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ contributes to the overall observed enantioselectivity.

Newly developed *Cinchona* alkaloid-based zwitterionic ion-exchange-type chiral SOs and the related CSPs operate best under slightly acidic polar-ionic (PI) mobile phase conditions. Three modes of ion-exchange modes can be envisioned: an anion-exchange mode for the separation of chiral acids, a cation-exchange mode for the separation of chiral amines, and a zwitterionic ion-exchange mode for the enantioseparation of ampholytic compounds possessing both positive and negative charges.[32–35]

The present article describes the temperature-dependent enantioseparation of five monoterpene-based 2-aminocarboxylic acids representing bulky β-amino acid enantiomers (Fig. 1) in PI mode on *Cinchona* alkaloid-based zwitterionic CSPs (Fig. 2). The effects of temperature under different conditions on the retention, enantioselectivity, and thermodynamic parameters are discussed. The sequence of elution of the enantiomers was determined.

MATERIALS AND METHODS

Chemicals and Synthesis

The enantiomers of monoterpene-based β-amino acids were prepared by methods published previously.[36–42] Addition of chlorosulfonyl isocyanate to the corresponding chiral monoterpene afforded β-lactams in highly regio- and stereospecific reactions; this was followed by treatment with hydrochloric acid, which resulted in the corresponding *cis*-β-amino acids (**1a,b, 2a,b** and **3a,b**).[39,41] Acid-catalyzed ethanolysis of the β-lactams derived from apopinene (**1a** and **1b)** led to *cis*-β-amino esters, which underwent base-catalyzed isomerization, followed by hydrolysis,

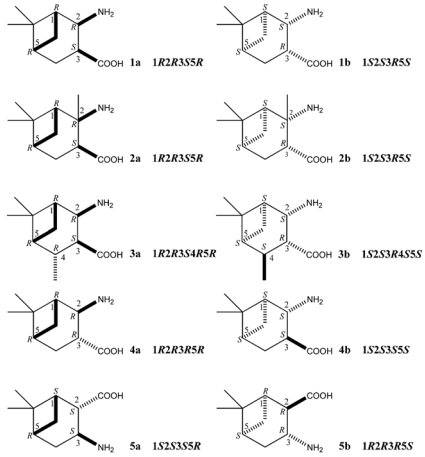


Fig. 1. Structures of the investigated analytes.

to afford the corresponding *trans* enantiomers **4a** and **4b** in excellent yields.[40] The regioisomeric *trans* apopinane-based β-amino acids **5a** and **5b** were prepared by stereoselective Michael addition of lithium dibenzylamide to (–)- and (+)-*tert*-butyl myrtenate, followed by catalytic debenzylation and hydrolysis of the resulting β-amino esters.[42]

Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were from Merck (Darmstadt, Germany). Triethylamine (TEA), propylamine (PRA), formic acid (FA), and glacial acetic acid (AcOH) of analytical reagent grade were purchased from Sigma-Aldrich (St. Louis, MO).

Instruments and Chromatography

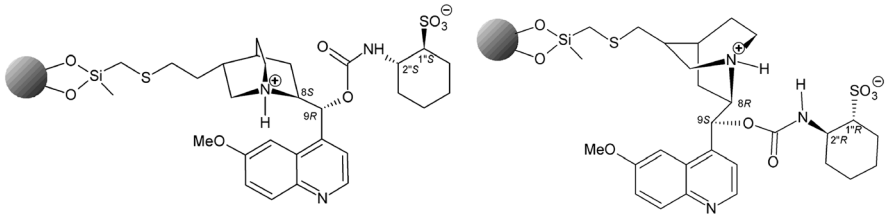
The apparatus for chromatography comprised a Waters Breeze system consisting of a 1525 binary pump, a 487 dual-channel absorbance detector, a 717 plus autosampler, and Empower 2 data manager software (Waters Chromatography, Milford, MA). The columns were thermostated in a Spark Mistral column thermostat (Spark Holland, Emmen, The Netherlands). The alternative 1100 Series HPLC system consisted of a solvent degasser, a pump, an autosampler, a column thermostat, a multiwavelength UV-VIS detector (all from Agilent Technologies, Waldbronn, Germany), and a corona charged aerosol detector from ESA Biosciences (Chelmsford, MA). Data acquisition and analysis were carried out with ChemStation chromatographic data manager software from Agilent Technologies. The precision of temperature adjustment was ±0.1°C.

The Chiralpak ZWIX(+) and ZWIX(–) columns (150×3.0 mm I.D., 3-μm particle size for both columns) were provided by Chiral Technologies Europe (Illkirch, France).

Chromatography was performed in isocratic mode at a flow rate of 0.6 ml min^{-1}; column temperature was varied in 10ºC increments between 10 and 50°C. Detection was accomplished by UV and corona discharge detection. The void volume of the columns (t_0) was determined by injecting a methanolic solution of acetone. Solutions of SAs were made in MeOH in the concentration range 0.5–1.0 mg ml^{-1}.

RESULTS AND DISCUSSION

In order to investigate the effects of temperature on the chromatographic parameters, a variable-temperature study was carried out on the *Cinchona* alkaloid-based ZWIX(+) and ZWIX(–) CSPs over the temperature range 10–50°C. Experimental data for all SAs on both columns in the mobile phases MeOH/MeCN (50/50 v/v) containing **(a)** 25 mM TEA and 50 mM FA, **(b)** MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM AcOH, **(c)** MeOH/MeCN (50/50 v/v) containing 25 mM PRA and 50 mM AcOH, and **(d)** MeOH/MeCN (25/75 v/v) containing 25 mM PRA and 50 mM AcOH are listed in Tables 1 and 2.



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

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

Fig. 2. Structures of Chiralpak ZWIX(+) and ZWIX(–) CSPs.

The tabulated data indicate that the retention, expressed as retention factor k, in most cases decreased with increasing temperature. Transfer of the SA from the mobile phase to the stationary phase is generally an exothermic process and consequently k (and α) decreases with increasing temperature. However, on the ZWIX(–) column with mobile phase **a**, MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM FA, the behavior of SAs **2**, **4,** and **5** was unusual; with increasing temperature, k (and α) increased. Such unusual behavior was recently observed for an entirely different chiral chromatographic system by Chankvetadze and colleagues[43] and for achiral separations by Adlof and Lis,[44] Wu et al.,[45] and Yogo et al.[46]

The unexpected behavior described here occurred only when the mobile phase contained FA and only for SAs **2, 4,** and **5** on ZWIX(–) CSP. In the studied temperature range, the conformation and solvation of the ZWIX(–) column SO probably do not change significantly. However, for SAs **2, 4,** and **5,** either the solvation process, or the structure of the intermediate associates, may be different. A possible explanation for this behavior may be the co-ion effect in the presence of FA. The extent of protonation of SAs **2, 4,** and **5** may decrease at higher temperature, and the interaction between the negatively charged carboxy group of the SA and the partially charged tertiary amino group of the quinuclidine ring may be strengthened, resulting in an increased level of retention. (The ion-pairing ability of the SO may also be affected by the temperature variance.) However, further studies are required for a better understanding of the mechanism of this behavior.

The changes observed in the selectivity and resolution with temperature were not consistent. In most cases, α and R_S decreased with increasing temperature. However, on ZWIX(+) for SAs **2** and **3** in mobile phases **a, b,** and **c**, and for SA **4** in mobile phase **a**, and on ZWIX(–) CSP for SA **2** in mobile phases **a, b,** and **c**, and for SAs **4** and **5** in mobile phase **a**, α and R_S increased with increasing temperature.

Since the effect of temperature on the enantiomer separation demands a complex interpretation, an extensive study relating to the thermodynamics of this system was carried out. Accurate chromatographic data were utilized to construct van't Hoff plots (Eqs. (1) and (2)), and the thermodynamic parameters for the individual enantiomers were calculated from the slopes and intercepts of these plots.

As a general trend, van't Hoff analysis of the retention factors (ln k vs. 1/T) gave linear plots, as indicated by the correlation coefficients in Tables 3 and 4. However, for SAs **2** and **5** on ZWIX(–) with mobile phase **a**, nonlinear plots were observed (Fig. 3). In contrast, the ln α vs. 1/T plots (Eq. (2)) exhibited linear behavior over the temperature range 20–50 °C, as depicted in Figure 3. These observations demonstrate that the factors causing nonlinearity for the relationship ln k vs. 1/T affect the retentions of the two enantiomers to nearly the same extent, suggesting that the origin of this phenomenon is relatively unrelated to the chiral recognition process. Below 20°C, the slope increased, i.e., ΔH° and ΔS° became more positive.

The ΔH° and $\Delta S^{\circ *}$ values for the enantiomers were negative on both ZWIX(+) and ZWIX(–) with the exception of SA **4** on ZWIX(–) with mobile phase **a** (Tables 3 and 4; due to the nonlinearity of the van't Hoff plots, ΔH° and $\Delta S^{\circ *}$ values for SAs **2** and **5** with eluent **a** were not calculated). These values indicate that solute transfer from the mobile phase to the stationary phase is enthalpically favorable but entropically unfavorable. Further, the absolute values of $-\Delta H^\circ$ and $-\Delta S^{\circ *}$ for the first-eluting enantiomer were usually smaller than those for the second-eluting enantiomer. Since the second-eluting enantiomers had larger $-\Delta S^{\circ *}$ values, they probably had fewer degrees of freedom on the CSP, i.e., the SO-SA associates were more stable due to a stronger intermolecular interaction or were less able to move or rotate. Multiple simultaneous interactions between the chiral SA and the SO appeared somewhat more likely for the second-eluting enantiomers than for the first-eluting enantiomers. This indicates that enantioselectivity is preferentially expressed by the behavior of the second peak, a combination with nonstereoselective interaction of both enantiomers. In other words, the retention of the first peak relates to SO-SA ion pair formation, without the onset of additional SO-SA interactions causing chiral discrimination.

On ZWIX(+) SAs, **2** and **3** at all mobile phase compositions, and SA **4** with mobile phase **a**, while on ZWIX(–), SA **2** with mobile phases **b** and **c** exhibited smaller $-\Delta H^\circ$ and $-S^{\circ *}$ values for the second-eluting enantiomers (SAs **2, 4,** and **5** exhibited unusual behavior with mobile phase **a**). The smaller $-\Delta H^\circ$ data for the enantiomers mean that the interactions between the SA and the SO are energetically less favorable with increasing temperature. The smaller $-\Delta S^{\circ *}$ indicated that the second enantiomer had more freedom in the stationary phase.

It was also observed that the absolute values of $-\Delta H^\circ_1$ and $-\Delta H^\circ_2$, and in parallel $-\Delta S^{\circ *}_1$ and $-\Delta S^{\circ *}_2$ for the ZWIX(–) column, were in most cases smaller than those for ZWIX(+); it seems that the interactions between the SAs and the SO were less favorable on ZWIX(–), but the difference in interaction of the two enantiomers with the SO does not follow this trend.

TABLE 1. Temperature dependence of retention factor of first eluting enantiomer (k_1), separation factor (α) and resolution (R_S) of monoterpene-based 2-amino carboxylic acids on Chiralpak ZWIX(+) CSP

			Temperature (°C)					
Compound	Mobile phase	k, α, R_S	10	20	30	40	50	Elution sequence
1	**a**	k_1	4.32	4.08	3.84	3.55	3.33	a<b
		α	1.24	1.20	1.17	1.14	1.11	
		R_S	2.50	2.40	2.30	1.60	1.50	
	b	k_1	3.66	3.27	2.76	2.38	2.15	a<b
		α	1.26	1.24	1.23	1.21	1.19	
		R_S	2.70	2.50	2.40	1.80	1.10	
	c	k_1	2.62	2.31	2.12	1.99	1.83	a<b
		α	1.27	1.23	1.21	1.18	1.14	
		R_S	2.30	2.30	2.20	2.10	2.00	
2	**a**	k_1	3.24	3.07	2.93	2.81	2.71	a<b
		α	1.00	1.00	1.02	1.02	1.03	
		R_S	0.00	0.00	0.30	0.30	0.50	
	b	k_1	3.14	2.78	2.35	2.19	1.94	a<b
		α	1.00	1.00	1.05	1.05	1.08	
		R_S	0.00	0.00	0.30	0.40	0.60	
	c	k_1	2.19	2.01	1.80	1.68	1.58	a<b
		α	1.00	1.00	1.06	1.07	1.07	
		R_S	0.00	0.00	0.30	0.40	0.50	
3	**a**	k_1	4.92	4.60	4.32	4.03	3.80	a<b
		α	1.03	1.04	1.05	1.06	1.06	
		R_S	0.50	0.60	0.60	0.70	0.80	
	b	k_1	4.20	3.74	3.26	2.73	2.41	a<b
		α	1.08	1.09	1.11	1.13	1.15	
		R_S	0.60	0.90	0.70	1.00	1.10	
	c	k_1	3.45	3.06	2.74	2.45	2.16	a<b
		α	1.03	1.05	1.06	1.06	1.07	
		R_S	0.40	0.60	0.70	0.80	1.00	
4	**a**	k_1	11.08	9.88	9.03	8.40	7.79	b<a
		α	1.03	1.04	1.06	1.07	1.08	
		R_S	0.30	0.30	0.30	0.40	0.50	
	b	k_1	12.60	11.08	9.70	8.43	7.38	b<a
		α	1.14	1.12	1.11	1.10	1.10	
		R_S	1.60	1.60	1.50	1.40	1.00	
	c	k_1	9.32	8.19	7.23	6.41	5.86	b<a
		α	1.14	1.13	1.12	1.11	1.10	
		R_S	1.80	1.90	1.70	1.70	1.20	
5	**a**	k_1	10.53	9.75	8.95	8.35	7.83	-
		α	1.04	1.00	1.00	1.00	1.00	
		R_S	0.40	0.00	0.00	0.00	0.00	
	b	k_1	11.25	10.10	8.99	7.71	6.94	-
		α	1.00	1.00	1.00	1.00	1.00	
		R_S	0.00	0.00	0.00	0.00	0.00	
	c	k_1	6.95	6.00	5.45	4.94	4.51	-
		α	1.00	1.00	1.00	1.00	1.00	
		R_S	0.00	0.00	0.00	0.00	0.00	
	d	k_1	18.05	16.11	14.54	13.13	11.98	a<b
		α	1.11	1.10	1.09	1.09	1.08	
		R_S	2.00	1.90	2.00	1.80	1.60	

Chromatographic conditions: column, Chiralpak ZWIX(+); mobile phase, **a,** MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM FA, **b**, MeOH/MeCN=50/50 (v/v) containing 25 mM TEA and 50 mM AcOH, **c,** MeOH/MeCN=50/50 (v/v) containing 25 mM PRA and 50 mM AcOH, **d,** MeOH/MeCN=25/75 (v/v) containing 25 mM PRA and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 nm.

The differences in the changes in enthalpy and entropy, -Δ(ΔH°) and -Δ(ΔS°), are also presented in Tables 3 and 4. The -Δ(ΔH°) values ranged from −1.6 to 2.1 kJ mol^{-1} on ZWIX(+), and from −1.9 to 2.7 kJ mol^{-1} on ZWIX(–). The trend in the change in -Δ(ΔS°) is similar to that in -Δ(ΔH°). Under the conditions where Δ(ΔH°) has negative values, Δ(ΔS°) was also negative and the largest positive Δ(ΔH°) was accompanied by the largest positive Δ(ΔS°). The interactions of **1** with ZWIX(+) and ZWIX(–) were characterized by the highest -Δ(ΔH°) and -Δ(ΔS°) values with eluent composition **a**.

When the selectivity increased with increasing temperature, Δ(ΔH°) and Δ(ΔS°) were positive. In these cases, the change in the adsorption enthalpy with increasing temperature had a positive effect on the enantioselectivity. On the other hand, the positive Δ(ΔS°) compensated the positive Δ(ΔH°) and

TABLE 2. Temperature dependence of retention factor of first eluting enantiomer (k_1), separation factor (α) and resolution (R_S) of monoterpene-based 2-amino carboxylic acids on Chiralpak ZWIX(-) CSP

			Temperature (°C)					
Compound	Mobile phase	k, α, R_S	10	20	30	40	50	Elution sequence
1	**a**	k_1	4.23	4.02	3.84	3.67	3.51	b<a
		α	1.55	1.50	1.45	1.39	1.35	
		R_S	3.80	3.40	3.30	3.30	3.30	
	b	k_1	3.89	3.72	3.58	3.43	3.28	b<a
		α	1.47	1.44	1.41	1.37	1.35	
		R_S	3.30	3.20	3.20	2.80	2.60	
	c	k_1	2.39	2.21	2.11	1.99	1.88	b<a
		α	1.44	1.40	1.37	1.35	1.33	
		R_S	2.90	2.80	2.60	2.50	2.50	
2	**a**	k_1	2.83	2.91	2.96	3.01	2.99	b<a
		α	1.00	1.11	1.16	1.18	1.22	
		R_S	0.00	0.50	1.40	1.40	2.20	
	b	k_1	3.51	3.32	3.16	3.01	2.83	b<a
		α	1.11	1.12	1.13	1.14	1.15	
		R_S	0.60	1.00	1.60	1.40	1.80	
	c	k_1	2.07	1.94	1.85	1.75	1.67	b<a
		α	1.08	1.10	1.11	1.13	1.13	
		R_S	0.50	0.80	1.00	1.20	1.30	
3	**a**	k_1	5.29	4.90	4.70	4.50	4.23	b<a
		α	1.41	1.38	1.34	1.32	1.30	
		R_S	2.80	2.70	2.60	2.50	2.60	
	b	k_1	4.58	4.35	4.12	3.92	3.76	b<a
		α	1.52	1.48	1.46	1.43	1.39	
		R_S	2.90	3.00	3.00	3.00	3.00	
	c	k_1	3.80	3.60	3.37	3.15	2.93	b<a
		α	1.42	1.38	1.36	1.34	1.32	
		R_S	3.90	3.70	3.70	3.50	3.30	
4	**a**	k_1	7.64	7.77	7.91	7.99	8.10	a<b
		α	1.07	1.09	1.10	1.12	1.13	
		R_S	0.50	0.50	0.60	0.70	0.80	
	b	k_1	11.89	10.92	10.15	9.50	8.60	a<b
		α	1.37	1.33	1.31	1.29	1.28	
		R_S	2.60	2.40	2.10	2.10	2.00	
	c	k_1	9.70	9.01	8.21	7.71	7.22	a<b
		α	1.22	1.20	1.19	1.18	1.17	
		R_S	2.10	2.10	2.10	2.00	2.00	
5	**a**	k_1	6.70	7.40	7.40	7.53	7.46	b<a
		α	1.00	1.00	1.06	1.13	1.17	
		R_S	0.00	0.00	0.60	0.90	1.20	
	b	k_1	9.11	8.24	7.68	7.21	6.79	b<a
		α	1.35	1.32	1.30	1.28	1.26	
		R_S	4.50	2.50	2.10	2.40	1.80	
	c	k_1	5.34	5.00	4.76	4.52	4.30	b<a
		α	1.24	1.22	1.20	1.19	1.18	
		R_S	1.70	1.70	1.70	1.60	1.60	

Chromatographic conditions: column, Chiralpak ZWIX(-)™; mobile phase, **a,** MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM FA, **b**, MeOH/MeCN=50/50 (v/v) containing 25 mM TEA and 50 mM AcOH, **c,** MeOH/MeCN=50/50 (v/v) containing 25 mM PRA and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 nm.

resulted in a negative $\Delta(\Delta G°)$. In eluent system **a,** containing FA as acid modifier, the largest positive $\Delta(\Delta H°)$ and especially $\Delta(\Delta S°)$ were obtained for SAs **2, 4,** and **5**; here, not only the selectivity, but also the retention increased with increasing temperature. However, for SAs **2** and **5** in eluent system **a**, the nonlinear van't Hoff plots over the entire temperature range were attributed to the change in retention mechanism. Thermodynamically, this unusual behavior may be attributed to the largest positive $\Delta(\Delta S°)$ values, indicating the importance of the entropy contribution to the chiral separation.

The thermodynamic parameter $-\Delta(\Delta G°)_{298}$ suggests that, both on ZWIX(+) and on ZWIX(–), mobile phase **b,** i.e., MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM AcOH, induced the binding to the SO more efficiently, as reflected by the larger $-\Delta(\Delta G°)$ values (SA **5** was not separable on ZWIX(+) under these conditions).

From the $-T\Delta(\Delta S°)$ data for some SAs, the positive $\Delta(\Delta S°)$ on both CSPs compensated for the positive $\Delta(\Delta H°)$ and resulted in a $-\Delta(\Delta G°)$ value (Tables 3 and 4). For these SAs in this temperature range, enantioresolution is entropically driven, and the selectivity increases with increasing temperature, as depicted for SA **2** in Figure 4.

The data were used to calculate the temperature T_{iso}, at which the enantioselectivity and the elution sequence change

TABLE 3. Thermodynamic parameters, ΔH°, ΔS°*, Δ(ΔH°), Δ(ΔS°), TxΔ(ΔS°), Δ(ΔG°), correlation coefficients (R^2) and T_{iso} temperature of monoterpene-based 2-aminocarboxylic acids on ZWIX(+) column

Analyte	Mobile phase	Stereo-isomer	-ΔH° (kJ mol^{-1})	-ΔS°* (J mol^{-1} K^{-1})	Correlation coefficients (R^2)	-Δ(ΔH°) (kJ mol^{-1})	-Δ(ΔS°) (J mol^{-1} K^{-1})	-TxΔ(ΔS°)$_{298K}$ (kJ mol^{-1})	-Δ(ΔG°)$_{298K}$ (kJ mol^{-1})	T_{iso} (°C)
1	**a**	1	5.0±0.2	5.5±0.8	0.9929	2.1	5.6	1.7	0.4	105
		2	7.1±0.2	10.9±0.8	0.9964					
	b	1	10.5±0.5	26.2±1.7	0.9930	1.0	1.3	0.4	0.6	395
		2	11.5±0.5	27.7±1.6	0.9956					
	c	1	6.7±0.3	15.6±1.1	0.9919	1.9	4.9	1.5	0.4	115
		2	8.6±0.4	20.5±1.2	0.9950					
2	**a**	1	3.4±0.1	2.3±0.3	0.9938	-0.5	-1.9	-0.6	0.1	-10
		2	2.9±0.1	0.4±0.1	0.9979					
	b	1	9.1±0.5	22.8±1.7	0.9967	-1.5	-5.2	-1.5	0.1	15
		2	7.6±0.3	17.6±1.1	0.9991					
	c	1	6.4±0.3	16.1±1.0	0.9929	-1.6	-5.7	-1.7	0.1	8
		2	4.8±0.2	10.4±0.6	0.9963					
3	**a**	1	4.9±0.1	4.1±0.3	0.9991	-0.5	-2.0	-0.6	0.1	-23
		2	4.4±0.1	2.1±0.3	0.9991					
	b	1	10.9±0.6	26.2±1.9	0.9906	-1.3	-5.1	-1.5	0.2	-18
		2	9.6±0.5	21.1±1.8	0.9910					
	c	1	8.8±0.2	20.7±0.8	0.9976	-0.7	-2.8	-0.8	0.1	-23
		2	8.1±0.3	17.9±0.9	0.9967					
4	**a**	1	6.6±0.2	3.5±0.8	0.9950	-0.9	-3.4	-1.0	0.1	-8
		2	5.7±0.1	0.1±0.1	0.9985					
	b	1	10.2±0.3	14.8±1.0	0.9974	0.7	1.4	0.4	0.2	210
		2	10.9±0.3	16.2±0.9	0.9981					
	c	1	8.9±0.2	13.1±0.6	0.9988	0.7	1.2	0.4	0.3	270
		2	9.6±0.2	14.3±0.6	0.9990					
5	**a**	1	5.7±0.1	0.5±0.1	0.9991	0.7	2.2	0.6*	0.1*	45
		2	6.4±0.2	2.7±0.8	0.9962					
	b	1	9.4±0.5	12.9±1.5	0.9928	-	-	-	-	-
		2	9.4±0.5	12.9±1.5	0.9928					
	c	1	8.1±0.3	12.6±1.0	0.9958	-	-	-	-	-
		2	8.1±0.3	12.6±1.0	0.9958					
	d	1	7.8±0.04	3.5±0.1	0.9990	0.4	0.7	0.2	0.2	300
		2	8.2±0.05	4.2±0.2	0.9989					

Chromatographic conditions: column, Chiralpak ZWIX(+)™; mobile phase, **a,** MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM FA, **b,** MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM AcOH, **c,** MeOH/MeCN (50/50 v/v) containing 25 mM PRA and 50 mM AcOH, **d,** MeOH/MeCN (25/75 v/v) containing 25 mM PRA and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 nm; $\Delta S^{\circ *} = \Delta S^{\circ} + R \ln \phi$, where ϕ is reversal of the phase ratio; R^2, correlation coefficient of van't Hoff plot, ln k – 1/T curves; T_{iso}, temperature of ln k – 1/T curves where enantioselectivity cancels;
*283 *K*; errors have been calculated as the standard errors of the slopes and intercepts of the fitted lines, applying the regression analysis of the Excel program.

TABLE 4. Thermodynamic parameters, ΔH°, ΔS°*, Δ(ΔH°), Δ(ΔS°), TxΔ(ΔS°), Δ(ΔG°), correlation coefficients (R^2) and T_{iso} temperature of monoterpene-based 2-aminocarboxylic acids on ZWIX(-) column

Analyte	Mobile phase	Stereo-isomer	-ΔH° (kJ mol^{-1})	-ΔS°* (J mol^{-1} K^{-1})	Correlation coefficients (R^2)	-Δ(ΔH°) (kJ mol^{-1})	-Δ(ΔS°) (J mol^{-1} K^{-1})	-TxΔ(ΔS°)$_{298K}$ (kJ mol^{-1})	-Δ(ΔG°)$_{298K}$ (kJ mol^{-1})	T_{iso} (°C)
1	**a**	1	3.5±0.03	0.5±0.1	0.9998	2.7	5.6	1.7	1.0	209
		2	6.2±0.1	6.1±0.4	0.9990					
	b	1	3.2±0.1	0.1±0.1	0.9971	1.7	2.6	0.8	0.9	380
		2	4.9±0.1	2.7±0.4	0.9979					
	c	1	4.4±0.1	8.3±0.5	0.9966	1.5	2.5	0.7	0.8	330
		2	5.9±0.2	10.8±0.7	0.9966					
2	**a**	1	-	-	0.9895**	-2.2*	-8.7*	-2.6*	0.4*	-
		2	-	-						
	b	1	4.0±0.2	3.8±0.5	0.9950	-0.5	-2.9	-0.9	0.4	-100
		2	3.5±0.1	0.9±0.2	0.9925					
	c	1	4.0±0.1	8.2±0.2	0.9976	-0.9	-4.0	-1.2	0.3	-48
		2	3.1±0.1	4.2±0.3	0.9986					
3	**a**	1	4.1±0.1	0.6±0.3	0.9906	1.6	2.7	0.8	0.8	319
		2	5.7±0.2	3.3±0.7	0.9954					
	b	1	3.8±0.04	0.6±0.1	0.9996	1.6	2.3	0.7	0.9	420
		2	5.4±0.1	2.9±0.2	0.9995					
	c	1	4.9±0.1	6.3±0.3	0.9974	1.5	2.1	0.6	0.9	440
		2	6.4±0.2	8.4±0.7	0.9969					
4	**a**	1	-1.1±0.05	-20.8±0.2	0.9944	-1.0	-4.3	-1.3	0.3	-41
		2	-2.1±0.03	-25.1±0.1	0.9994					
	b	1	6.0±0.1	0.5±0.2	0.9990	1.3	2.1	0.6	0.7	345
		2	7.3±0.2	2.6±0.5	0.9965					
	c	1	5.7±0.1	1.2±0.2	0.9996	0.8	1.1	0.3	0.5	450
		2	6.5±0.1	2.3±0.5	0.9986					
5	**a**	1	-	-	0.9937**	-3.9*	-13.9*	-4.2*	0.4*	-
		2	-	-						
	b	1	5.5±0.1	1.1±0.2	0.9920	1.2	1.8	0.5	0.7	390
		2	6.7±0.2	2.9±0.8	0.9990					
	c	1	4.1±0.1	0.4±0.2	0.9977	1.0	1.8	0.5	0.5	280
		2	5.1±0.1	2.2±0.4	0.9987					

Chromatographic conditions: column, Chiralpak ZWIX(-)™; mobile phase, **a,** MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM FA, **b,** MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM AcOH, **c,** MeOH/MeCN (50/50 v/v) containing 25 mM PRA and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 215 nm; ΔS°* = ΔS° +R ln ϕ, where ϕ is reversal of the phase ratio; R^2, correlation coefficient of van't Hoff plot, ln k – 1/T curves; T_{iso}, temperature of ln k – 1/T curves where enantioselectivity cancels,

*temperature range 20-50 °C;

**correlation coefficient of ln α vs. 1/T curves; errors have been calculated as the standard errors of the slopes and intercepts of the fitted lines, applying the regression analysis of the Excel program.

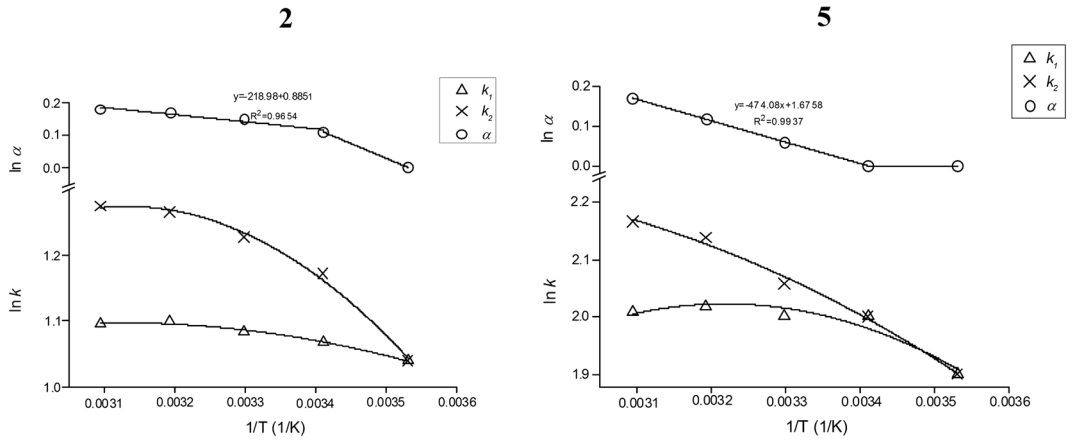


Fig. 3. Nonlinear van't Hoff plots of ln k vs. 1/T and ln α vs. 1/T for analytes 2 and 5 on ZWIX(–) CSP. Chromatographic conditions: column: ZWIX(–); mobile phase: MeOH/MeCN (50/50 v/v) containing 25 mM TEA and 50 mM FA; flow rate: 0.6 ml min^{-1}; detection: 215 nm; temperature range: 10–50°C.

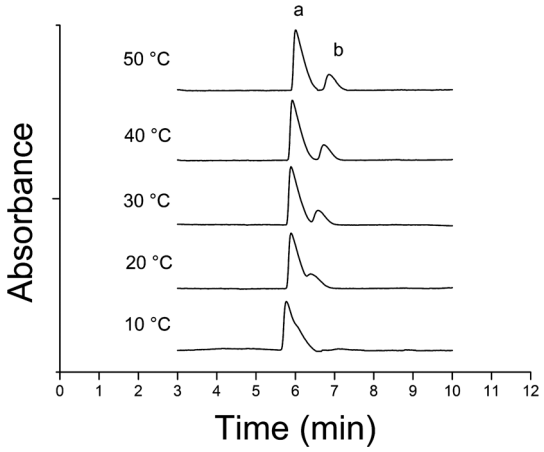


Fig. 4. Temperature dependence of the separation of analyte 2 (for chromatographic conditions, see Fig. 3).

(Tables 3 and 4). In most cases, T_{iso} was considerably higher than room temperature; enthalpically driven enantioseparation was obtained. When T_{iso} was obtained at lower than the ambient temperature, positive Δ(ΔH°) and Δ(ΔS°) were observed and the selectivity increased with increasing temperature. These enantioseparations were entropically driven.

Elution Sequence of Monoterpene-Based 2-Aminocarboxylic Acid Enantiomers

The Chiralpak ZWIX(+) and ZWIX(–) CSPs (see Fig. 2) are actually diastereomers but behave like pseudo-enantiomers.[32] As a consequence, on change from the quinine- to the quinidine-based CSP, reversal of the sequence of the elution of monoterpene-based 2-aminocarboxylic acids between ZWIX(+) and ZWIX(–) was observed in all cases.

CONCLUSIONS

The temperature effects for the separations of the enantiomers of monoterpene-based 2-aminocarboxylic acids (bulky β-amino acids) were investigated by using *Cinchona* alkaloid-based zwitterionic chiral SOs and CSPs: Chiralpak ZWIX(+) and ZWIX(–). In some cases, unexpected temperature behavior was observed: The selectivity, and in special cases, the retention time increased with increasing temperature. This unusual behavior was discussed on the basis of the thermodynamic data. The values of thermodynamic parameters such as the changes in enthalpy, Δ(ΔH°), entropy, Δ(ΔS°), and Gibbs energy, Δ(ΔG°), depended on the molecular structures of the SAs and the chiral SOs investigated.

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